Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós-Graduação em Neurociências

# EFEITOS DA SUPLEMENTAÇÃO COM ÁCIDO FÓLICO EM PRENHAS E SOBRE O DESENVOLVIMENTO DA PROLE SUBMETIDA AO MODELO DE HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

**Bruna Ferrary Deniz**

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Tese de Doutorado apresentada como requisito parcial à obtenção do título de Doutor em Neurociências pelo Programa de Pós-graduação em Neurociências da Universidade Federal do Rio Grande do Sul.

**Bruna Ferrary Deniz**

**Orientadora: Profa Dra Lenir Orlandi Pereira Silva**

Julho 2018

*“Educação não transforma o mundo.*

*Educação muda as pessoas.*

*Pessoas mudam o mundo.”*

(Paulo Freire)

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# SUMÁRIO

## RESUMO

O ácido fólico (AF) é uma vitamina do complexo B que participa da síntese de nucleotídeos e das reações de metilação do organismo, auxiliando nos processos epigenéticos. Sua deficiência durante a gestação foi relacionada com defeitos do fechamento do tubo neural, sendo indicada a suplementação com 0,4 mg/dia de AF. Contudo, no Brasil, o Sistema Único de Saúde (SUS) só fornece um suplemento com 5mg/dia de AF e já é visto um efeito dual dessa vitamina, especialmente dosagens acima do recomendado. Estudos anteriores já mostraram um efeito dual do tratamento com AF em animais submetidos a hipóxia-isquemia (HI) neonatal. Assim, o objetivo deste trabalho foi avaliar o efeito da suplementação com AF gestacional (uma dose similar a recomendada e uma considerada excessiva) no desenvolvimento da prole submetida a um modelo de HI e no desenvolvimento da gestação, no comportamento maternal e nas mães que foram suplementadas. Quando confirmado o acasalamento, as ratas Wistar prenhas foram separadas em 3 grupos de acordo com a dieta: 1) dieta padrão (PD), 2) suplementada com 2 mg/kg de AF (AF2) e 3) suplementada com 20 mg/kg de AF (AF20). Após o nascimento dos filhotes, todos os animais receberam a dieta PD. Aos 7 dias de vida pós-natal (DPN), os filhotes foram submetidos ao modelo de Levine-Vannucci para HI neonatal, gerando 6 grupos: 1) controle ração padrão (CTPD), 2) CT suplementado com 2 mg/kg de AF (CTAF2), 3) CTsuplementado com 20 mg/kg de AF (CTAF20), 4) hipóxia-isquemia ração padrão (HIPD), 5) HIsuplementado com 2 mg/kg de AF (HIAF2) e 6) HI suplementado com 20 mg/kg de AF (HIAF20). Nossos principais achados são que a suplementação com AF conseguiu prevenir os déficits cognitivos e o desbalanço do fator neurotrófico derivado do encéfalo (BDNF, do inglês) no hipocampo ipsilateral dos animais HI adultos. Somente a alta dose de AF conseguiu reverter o aumento da expressão de caspase-3 nos ratos HI adultos. A HI diminui a metilação da histona H3K4 e H3K27 no hipocampo ipsilateral dos animais adultos, sem efeito da suplementação. Ainda, a suplementação com AF e a lesão não alteraram o desenvolvimento da gestação, o comportamento maternal, o crescimento somático e os marcos do desenvolvimento da prole. A HI neonatal e a alta dose de AF (CTAF20) diminuíram a atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo ipsilateral dos animais aos 21 DPN. A mães AF20 também apresentaram déficits de memória após o desmame. Os resultados desse trabalho indicam que a suplementação com AF durante a gestação pode exercer efeito protetor contra um evento hipóxico-isquêmico neonatal, mas a alta dosagem da AF parece ter um efeito dual tempo e estrutura dependentes. Como a suplementação com essa vitamina já é realizada pelas gestantes, mais trabalhos são necessários para melhor compreender os mecanismos do AF e seu efeito dual.

**Palavras-chave:** Asfixia perinatal, vitamina, memória, plasticidade, epigenética, apoptose, reflexos neonatais.

**ABSTRACT**

Folic acid (FA) is a B-complex vitamin that participates in the nucleotides synthesis and in the methylation reactions of the body, aiding in the processes of epigenetics.Its deficiency during pregnancy leads to neural tube closure defects, and supplementation with 0.4 mg / day of FA is indicated.However, in Brazil, Public Healthy System only provides a supplement with 5mg / day of AF and a dual effect of this vitamin is already seen, especially in excess.Previous studies have shown this dual effect of AF treatment in animals submitted to neonatal hypoxia-ischemia (HI).Thus, the aim of this study was to evaluate the effects of FA supplementation (a recommended and an excessive dose) during pregnancy on the development of offspring submitted to an HI model and on the development of gestation, maternal behavior and on the dams that were supplemented.When the mating was confirmed, the pregnant Wistar rats were separated into 3 groups according to the diet: 1) standard diet (SD), 2) supplemented with 2 mg / kg of FA (FA2) and 3) supplemented with 20 mg / kg of FA (FA20).After the pups' birth, all animals received the SD diet. At the 7 PND, pups were submitted to the Levine-Vannucci model for neonatal HI, generating 6 groups: 1) SD control (CTSD), 2) CTFA2, 3) CTFA20, 4) SD hypoxia-ischemia (HISD) e 5) HIFA2 and 6) HIFA20.Our main findings are that FA supplementation was able to prevent cognitive deficits and BDNF imbalance in the ipsilateral hippocampus of adult HI animals. Only the high dose of FA was able to reverse the increased expression of caspase-3 in adult HI rats.HI decreases the methylation of histone H3K4 and H3K27 in the ipsilateral hippocampus of adult HI animals, with no effects of supplementation. Yet, neither supplementation nor injury altered the development of gestation, maternal behavior, somatic growth, and developmental milestones.Neonatal HI and high dose of FA (CTFA20) decreased $Na^{+}$, $K^{+}$ - ATPase activity in the ipsilateral hippocampus of the animals at 21 PND. FA20 dams also had memory deficits after weaning.The results of this work indicate that supplementation with gestational FA may exert a protective effect in a hypoxic-ischemic event, but the high dosage of FA appears to have a dual effect time and structure dependent. As supplementation with this vitamin, even in excess, is already used by pregnant women, more studies are needed to better understand the mechanisms of the FA and its dual effect.

**Key words:** Perinatal asphyxia, vitamin, memory, plasticity, epigenetics, apoptosis, neonatal reflexes.

## LISTA DE ABREVIATURAS

**5-MTHF**: 5-metilTHF

**AF**: Ácido fólico

**ATP**: adenosina trifosfato

**BDNF**: *brain-derived neurotrophic factor; fator neurotrófico derivado do encéfalo*

**DHFR**: dihidrofolato redutase

**DNA**: *deoxyribonucleic acid; ácido desoxirribonucleico*

**DNMT**: DNA metiltransferase

**FDA**: *Food and Drug Administration*

**HAT**: histonas acetiltransferases

**HDAC**: histonas deacetilases

**HDM**: histonas demetilases

**HI**: hipóxia-isquemia

**HIF-1α**: *Hypoxia-inducible factor 1*-alpha; *fator induzido por hipóxia1*-alpha

**HMT**: histonas metiltransferases

**MS**: metionina sintetase

**MTHFR**: metil tetrahidrofalato redutase

**PABA**: Ácido paraminobenzoico

**PSD-95**: *postsynaptic density protein 95*

**RDA**: R*ecommended Dietary Allowances*

**RNA**: *ribonucleic acid; ácido ribonucléico*

**SNC**: sistema nervoso central

**SUS**: Sistema Único de Saúde

**TDAH**: Transtorno do Déficit de Atenção/ Hiperatividade

**THF**: tetrahidrofolato

**TrKB**: *Tropomyosin receptor kinase B*

## LISTA DE FIGURAS



## ARTIGO 1.

**Fig. 1.** Experimental design – after mating confirmation, female Wistar rats were divided into 3 groups, according to diet: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). When the puppies were born, all animals received the SD diet. At the 7$^{th}$ PND, puppies were submitted to hypoxia-ischemia (HI), resulting in 6 groups of animals: Control with SD diet (CTSD), 2) Hypoxia-ischemia with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). From the 60$^{th}$ PND two set of animals were used for behavioral tasks or to assess the biochemical analysis

**Fig. 2.** Open Field task performed by the puppies at the 60$^{th}$ PND. A) Latency. B) Crossings. C) Rearings. D) Peripheral area. E) Central area. *different from the CTFA2 group. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 13-17 animals/ group

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**Fig. 8.** Synaptophysin optical densitometry of both hippocampus of the puppies after behavioral tasks. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 4-6 animals/ group

**Fig. 9.** BDNF concentration of both hippocampus of the puppies at the 60$^{th}$ PND. * different from CTSD, CTFA2 and HIFA20 groups. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 5-7 animals/ group

**ARTIGO 2.**

**Fig. 1**. Timeline of experimental procedures – After mating confirmation (E0), pregnant Wistar rats were divided into 3 groups, according to diet: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). At PND 1 (when pups where born) all animals started to receive the SD diet until the end of experiments. At the PND 7, the Levine-Vannucci model of HI was performed, resulting in 6 groups in the offspring: 1) Control with SD diet (CTSD), 2) Hypoxia-ischemia with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). At PND 60, puppies were euthanized, and plasticity and epigenetic assays were assessed

**Fig. 2**. Caspase-3 quantification – Protein expression of caspase-3 in the hippocampus (A) and representative bands of each protein (B). * Different from all CT groups and HIFA20 group. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group

**Fig. 3.** Synaptic proteins quantification – Synapsin (A) and PSD-95 (B) expression in the hippocampus and representative bands of each protein (C). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group

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**Fig. 6.** H3K27 methylation – Protein expression of H3K27me2 (A) and H3K27me3 (B) in the hippocampus and representative bands of each protein (C). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group

**Fig. 7**. Schematic representation of a proposal mechanism for H3 methylation and proteins expression in the ipsilateral hippocampus of neonatal HI animals in the adulthood. Neonatal HI cause hippocampal tissue damage and decrease methylation of histone H3 in both K 4 and 27. Methylation of H3K4 is related with gene expression and methylation of H3K27 is associated with gene repression. Considering these, it is possible to propose that the decreased H3K4me is blocking the synapsin expression and decreased H3K27me is increasing caspase-3 expression in the ipsilateral hippocampus. These alterations can partially explain the cognitive deficits observed after neonatal HI. FA supplementation during pregnancy was able to prevent the increase caspase-3 in HI animals, possibly indicating a neuroprotector effect.

**ARTIGO 3.**

**Fig. 1.** Experimental line – A) Mother rats (dams) experimental line. After pregnancy confirmation (E0), rats were divided into 3 groups, according to their experimental diets: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). After pups' birth (PND0), all rats

received the SD diet. Maternal behavior was assessed at PND 1, 4, 7 and 7. After weaning (PND21), some dams performed the Ox-maze task and others were euthanized to Na+, K+ - ATPase activity evaluation in the hippocampus. B) Offspring experimental line. After pregnancy confirmation (E0), rats were divided into 3 groups, according to their experimental diets: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). At the PND 7, pups were submitted to the HI. One day before the injury (PND6) and until PND19, neurobehavioral development of the offspring were evaluated. After weaning (PND21), pups were euthanized and the Na+, K+ - ATPase activitywas evaluated in the hippocampus

**Fig. 2.** Food consumption and Weight gain – Quantification of the food consumption (A) and the weight gain (B) of the dams during the pregnancy. Data are expressed as mean ± S.E.M. Repeated one-way ANOVA followed by Tukey's test, $p<0.05$. N= 12-14 rats/ group

**Fig. 3.** Maternal behavior – Evaluation of the maternal behavior at PND 1, 4, 7 and 10. Latency to initiate the nest (A), time far away from the pups (B), latency to get the first pup (C), time spend licking the pups (D) and time spend feeding the pups (E). Data are expressed as mean ± S.E.M. Repeated one-way ANOVA followed by Tukey's test, $p<0.05$. N= 6 rats/ group

**Fig. 4.** Ox-maze task – Memory evaluation of the dams by the ox-maze task. Latency to get the first reward (A and B), number of correct nose pokes (C and D), number of incorrect nose pokes (E and F) and time to complete the task (G and H). * difference from SD group. Data are expressed as mean ± S.E.M. Repeated one-way ANOVA (A, C, E and G) or one-way ANOVA (B, D, F and H) followed by Tukey's test, $p<0.05$. N= 6 rats/ group

**Fig. 5.** Na+, K+ - ATPase activity– Quantification of the Na+, K+ - ATPase activityin the hippocampus of the dams. Data are expressed as mean ± S.E.M. One-way ANOVA followed by Tukey's test, $p<0.05$. N= 6 rats/ group

**Fig. 6.** Somatic growth – Evaluation of the weight gain (A and B) and the body length (C and D) of the offspring since the birth until the weaning (PND21). Pups were evaluated before HI injury (A and C) and after HI injury (B and D). Data are expressed as mean ± S.E.M. Repeated two-way ANOVA followed by Tukey's test, $p<0.05$. N= 7-9 litters/ group

**Fig. 7.** Neurobehavioral development – Evaluation of neonatal reflexes of the pups at PND 6, 8, 10, 13, 16 and 19. Righting reflex (A), Cliff aversion (B), Negative geotaxis (C) and Gait (D). Data are expressed as mean ± S.E.M. Repeated two-way ANOVA followed by Tukey's test, $p<0.05$. N= 9-11 rats/ group

**Fig. 8.** Na+, K+ - ATPase Activity – Quantification of the Na+, K+ - ATPase activity in both hippocampus of the pups. * difference from CTSD group. Data are

expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 5-6 rats/ group

## LISTA DE TABELAS

**ARTIGO 3.**

# *1.INTRODUÇÃO*

## *1.1ÁCIDO FÓLICO*

Os folatos, folato e o ácido fólico (AF) são hidrossolúveis e fazem parte do complexo B, especificamente vitamina B9. Considerados nutrientes essenciais da dieta, não são produzidos pelo organismo e têm que ser adquiridos pela ingesta de alimentos como vegetais de folhas verdes, frutas e feijões ou de alimentos fortificados e suplementos. Para a forma natural é dado o nome de folato, enquanto AF é usado para designar a forma sintética. O AF é mais estável que o folato, não sofrendo clivagem pelo calor ou luz ultravioleta, por isso é a forma usada no preparo de alimentos (Patel &Sobczyńska-Malefora, 2017).

Os folatos são compostos por um anel pteridínico, PABA (ácido para amino benzoico) e uma cauda de ácido glutâmico (Figura 1). A forma natural possui até 6 moléculas do ácido glutâmico, enquanto a sintética possui apenas uma. Para ser absorvido, o folato tem que ser hidrolisado para apenas 1 molécula de ácido glutâmico. A enzima responsável por essa clivagem a monoglutamato, a *folylpoly-γ-glutamate carboxypeptidase* está presente na borda em escova das células do intestino delgado. Após ser clivado, o folato é absorvido e transportado pela veia porta (Blom & Smulders, 2011; Iyer & Tomar, 2009). Assim, quanto maior for a sua cauda, menor é a biodisponibilidade do folato pela necessidade de clivagem da cauda para a sua absorção. Sendo assim, a forma sintética AF possui uma melhor absorção que a forma natural, pois é monoglutâmica (Blom &Smulders, 2011;Mattson and Shea, 2003).

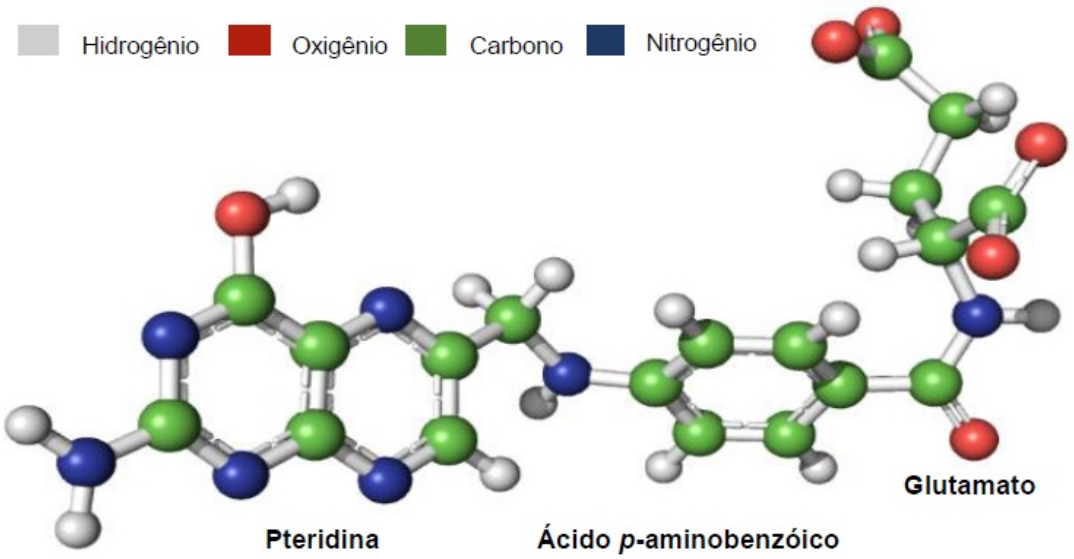


Figura 1 – Estrutura química 3D do Ácido fólico (PubChem).

Quando dentro das células, o AF recebe novamente a cauda de poliglutamatos e então é convertido nas formas biológicas ativas (Hyland et al., 2010). Numa reação de duas etapas pela dihidrofolato redutase (DHFR), o AF é reduzido a tetrahidrofolato (THF). Posteriormente, a metil tetrahidrofalato redutase (MTHFR), reduz o THF a 5-metilTHF (5-MTHF), que é a principal forma encontrada no plasma (Patel &Sobczyńska-Malefora, 2017). O 5-MTHF é transportado para o sistema nervoso central (SNC) principalmente através do plexo coróide para então chegar às células nervosas (Hyland et al., 2010). Além disso, o 5-MTHF participa da remetilação da homocisteína em metionina (Figura 2) pela metionina sintetase (MS), que é a principal doadora de grupo metil para as reações de metilação do nosso organismo, incluindo: DNA/RNA, histonas e proteínas (Patel &Sobczyńska-Malefora, 2017).

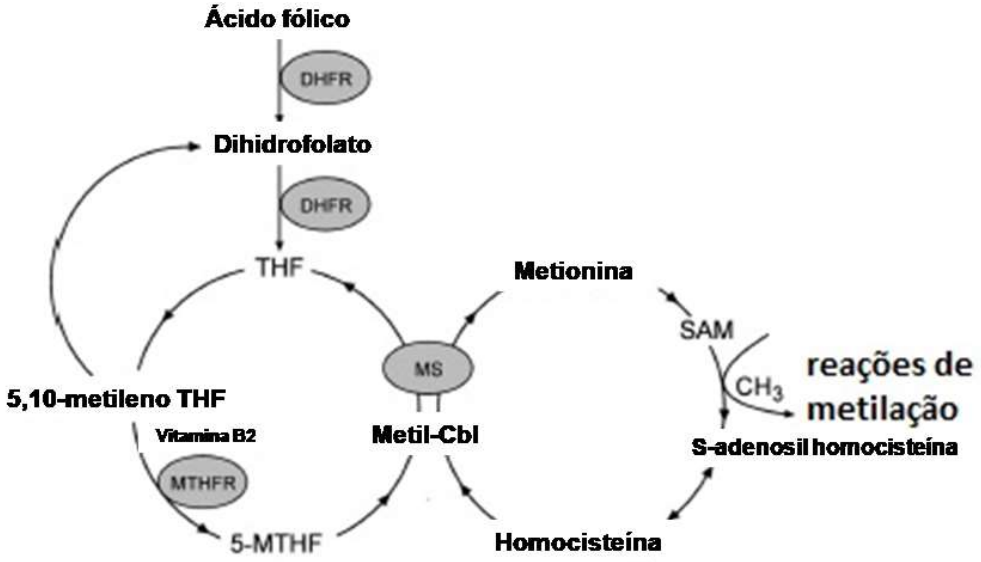


Figura 2 – Metabolismo do ácido fólico. DHFR: dihidrofolato redutase; THF: tetrahidrofolato; MTHFR: metil tetrahidrofolato redutase; 5-MTHF: 5-metiltetrahidrofolato; MS: metionina sintetase; SAM: S-adenosilmetionina. Adaptado de Patel &Sobczyńska-Malefora, 2017.

As principais funções do AF, além da remetilação da homoscisteína, incluem a participação no metabolismo de fragmentos de um-carbono, como a biossíntese de purinas e pirimidinas, e o metabolismo de aminoácidos (Iyer & Tomar, 2009). Já é bem reconhecida a relação da deficiência dessa vitamina com os defeitos do fechamento do tubo neural durante o período embrionário (Czeizel and Duda´s, 1992). Ainda, sabe-se que o desenvolvimento de aterosclerose e doença vascular cerebral e cardíaca está associado com a deficiência de folato (Barp, 2007). Por outro lado, em modelos animais, já foi evidenciado que a suplementação com AF pode prevenir eventos isquêmicos (Assaneli et al., 2004), doença de Alzheimer

(Mattson & Shea, 2003) e danos oxidativos causados pela homocisteinemia (Matté et al., 2007).

Os mecanismos que podem explicar os benefícios encontrados pela suplementação dessa vitamina podem estar relacionados com o potencial antioxidante do AF, não só por meio da participação do metabolismo da homocisteína, mas também na remoção de radicais livres (Joshi et al., 2001; Stanger & Wonisch, 2012). Seu possível papel antiapoptótico também já foi demonstrado em um modelo de cultura celular, onde foi observado diminuição da atividade da caspase-3 e redução na degeneração celular (Jia et al., 2008). Ainda, encontrou-se efeito do AF na diferenciação de células tronco embrionárias (Jia et al., 2008), bem como na neurogênese hipocampal após um modelo de isquemia cerebral (Zhang et al., 2012).

### *1.2 ÁCIDO FÓLICO NA GESTAÇÃO*

Como a deficiência de folato é fortemente relacionada com defeitos na formação do tubo neural, a suplementação com essa vitamina passou a ser recomendada pelo RDA (*Recommended Dietary Allowances*). Foi determinada a ingestão de 0,4 mg/dia de AF pelo menos 1 mês antes da gestação, até o final do primeiro trimestre (período de formação e fechamento do tubo neural). A prevenção pode ser feita através da dieta, suplementação com polivitamínicos e fortificação de alimentos (Ferreira, 2005; Oliveira, 2008). Considerando que muitas gestações não são planejadas e que possivelmente um bom número de mulheres não obtém o nível adequado de AF pela dieta, o FDA (*Food and Drug Administration*), nos

Estados Unidos, determinou o enriquecimento de cereais e de todas as farinhas com 0,14 mg de ácido fólico/ 100 g de farinha de trigo a partir de 1998. No Brasil, a fortificação de farinha de trigo e milho passou a ser obrigatória somente em 2002, através da resolução RDC 344. A partir dessa resolução, todos os fabricantes de farinhas foram obrigados a adicionar ferro e ácido fólico numa concentração mínima de 0,15 mg de ácido fólico por 100 g de farinha. E, ainda, definiu-se que os rótulos dos produtos devem conter a expressão: enriquecido, fortificado ou rico em AF (Ferreira, 2005). Além do Brasil, outros países como Canadá e China passaram a fortificar suas farinhas para diminuir a incidência de defeitos do fechamento do tubo neural (Gomes et al., 2016; Ray et al., 2002).

Recentemente, estudos têm apontado para um excesso na ingesta de AF tanto por suplementos, quanto pela dieta (Bailey et al, 2010; Patel& Sobczynska-Malefora, 2017; Selhub and Rosenberg, 2016). Uma pesquisa realizada nos Estados Unidos mostrou que numa população normonutrida, as gestantes ou outras pessoas que fazem suplementação com AF podem estar ingerindo até 200% da dose diária recomendada (Sittig et al., 2012).Ainda, no Brasil, o Sistema Único de Saúde (SUS) somente oferece gratuitamente um suplemento de AF com 5 mg/dia (Ministério da Saúde, 2014), que é mais de 10 vezes a dose recomendada.Porém, é importante ressaltar que a dose de 5 mg/kg é recomendada pelo Ministério da Saúde para mulheres com antecedentes de malformações congênitas, a fim de reduzir os riscos de uma nova gestação com malformaçõese também é indicada para pacientes com anemia.

A alta ingesta de AF pode apresentar os mais diversos efeitos. Alguns estudos com modelos animais apontam efeitos prejudiciais desse excesso de suplementação gestacional com AF, tais como: diminuição no peso e no tamanho dos embriões, atrasos no seu desenvolvimento, diminuição da espessura das paredes ventriculares do coração (Achón et al., 1999; Mikael et al., 2013), diminuição nos níveis e expressão do fator neurotrófico derivado do encéfalo (BDNF, do inglês) aos 22 dias e 3 meses de vida (Sable et al., 2012; Sable et al., 2014), aumento da glicose sanguínea e diminuição nos níveis de insulina em ratos adultos que tiveram suas mães suplementadas durante todo o período gestacional (Joshi et al., 2003) e diminuição de enzimas antioxidantes no nascimento (Roy et al., 2014). Contudo, efeitos benéficos também já foram relatados em estudos animais, como aumento da expressão da proteína básica de mielina no córtex cerebral durante a gestação (Lee et al., 2010), melhora nos reflexos neonatais, aprendizado espacial e déficits motores (Wang et al., 2017), melhora na memória espacial das mães e na resposta inflamatória de filhotes submetidos a um modelo de esquizofrenia (Canever et al., 2018), além da prevenção de defeitos do fechamento do tubo neural em humanos (Czeizel and Duda´s, 1992).

É importante ressaltar que poucos estudos avaliaram o efeito da suplementação com AF em diferentes doses nas mães. Canever e colaboradores (2018) demonstraram melhora na memória espacial, no estresse oxidativo e na resposta inflamatória nas ratas prenhes, principalmente com a suplementação de AF nas doses de 5 e 10 mg/kg na dieta. Contudo, a possível influência de uma dose acima do recomendado dessa vitamina no comportamento materno ainda não foi

investigada. Sabe-se que o comportamento materno é dependente da liberação de oxitocina e influencia o desenvolvimento e sobrevivência da prole (Tang et al., 2014; Yoshihara et al., 2017). Outros estudos com modelos animais já evidenciaram que alterações durante a gestação, como estresse (Gatta et al., 2018), dietas ricas em gorduras (Purcell et al., 2011) e uso de drogas, como antipsicóticos (Li, 2015), podem alterar o comportamento materno e, consequentemente, o desenvolvimento dos filhotes.

### *1.3 A HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL*

Sabe-se que o período perinatal e neonatal precoce é crítico para o desenvolvimentoe também muito suscetível a alterações do meio interno. Por exemplo, a asfixia perinatal é um fator que pode desencadear a encefalopatia hipóxico-isquêmica levando a profundas alterações do desenvolvimento. A hipóxia-isquemia (HI) encefálica neonataltem incidência na ordem de 1 a 3 para cada 1000 nascidos vivos em países desenvolvidos, sendo que 15 a 20% irá falecer no período pós-natal e em torno de 25% apresentará sequelas permanentes(Kurinczuk et al., 2010; Rumajogee et al., 2016). Os danos mais frequentes após a lesão hipóxico-isquêmica são: déficits cognitivos e de aprendizado, paralisia cerebral, problemas de coordenação motora e de motricidade ampla (Mwaniki et al., 2012).Em humanos, a HI ocorre frequentemente no período próximo ao nascimento, possuindo vários fatores etiológicos, como: prematuridade do neonato, dificuldades da expulsão e

sofrimento do feto, desnutrição, interrupção do fluxo sangüíneo umbilical e insuficiente troca de gases pela placenta (Procianoy & Silveira, 2001).

Em decorrência da HI é desencadeada uma série de eventos que vão culminar na morte celular em estruturas encefálicas. Os principais mecanismos envolvidos na patogênese da HI são: excitotoxicidade glutamatérgica, estresse oxidativo e inflamação (Figura 3) (McLean & Ferriero, 2004; Lai & Yang, 2011).

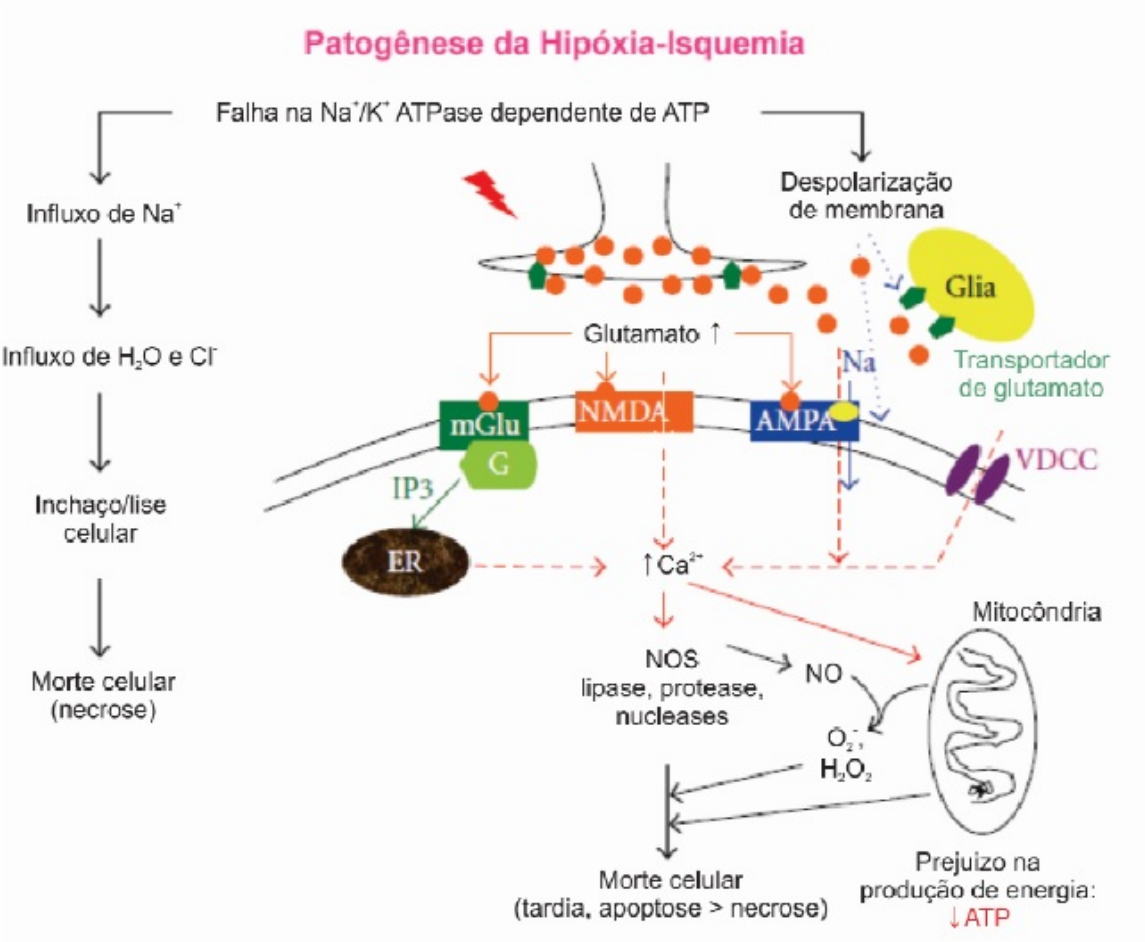


Figura 3 – Patogênese da hipóxia-isquemia encefálica neonatal. Adaptado de Lai & Yang, 2011.

Com a interrupção do fluxo sanguíneo, haverá uma diminuição da disponibilidade de glicose para o encéfalo e, consequentemente, uma diminuição de energia. Essa diminuição de energia vai levar a falência de enzimas como a $Na^+$, $K^+$ - ATPase, dependente de ATP. Essa enzima é responsável pela manutenção do gradiente iônico dos neurônios e quando sua atividade é diminuída pode ocorrer um aumento do influxo de íons sódio, com consequente influxo de água, inchaço da célula, lise e morte celular por necrose. Ainda, a despolarização celular vai aumentar a liberação de glutamato que vai ter sua captação pelos astrócitos prejudicada pela diminuição de ATP (Distefano & Pratico, 2010). Assim, ocorrerá um aumento desse neurotransmissor excitatório na fenda sináptica, superestimulando seus receptores pós-sinápticos e ocasionando maior influxo de íons cálcio na célula, ou seja, o evento chamado excitotoxicidade glutamatérgica. O aumento de cálcio intracelular por sua vez vai ativar diversas vias que vão gerar estresse oxidativo e morte celular por apoptose (McLean & Ferriero, 2004). Em conjunto, esses fatores patogênicos terão como consequência um extenso dano ao tecido nervoso encefálico, o qual é o fator determinante das alterações funcionais ao longo do desenvolvimento nos indivíduos acometidos pela HI (Miguel et al., 2015; Pereira et al., 2008; Rojas et al., 2015).

### *1.4 O MODELO DE LEVINE -VANNUCCI PARA A HI NEONATAL*

Os modelos animais são utilizados para estudar a patofisiologia e possíveis tratamentos de doenças em humanos. Um dos protocolos mais usados para o estudo da HI neonatal é o modelo de Levine (1960) modificado por Vannucci &

Vannucci (1997), que consiste na oclusão permanente unilateral da artéria carótida comum, associada à exposição à ambiente hipóxico (Figura 4) em animais com 7 dias de vida pós-natal. Estes procedimentos de isquemia e hipóxia têm como conseqüência lesões unilaterais encontradas no hipocampo, estriado e córtex cerebral, semelhantes àquelas encontradas em recém-nascidos humanos acometidos (Pereira et al., 2007; Miguel et al., 2015). A indução do modelo de HI é realizada em ratos com 7 dias de vida, pois o nível de desenvolvimento do encéfalo é considerado similar ao de recém-nascidos a termo ou prematuros (Sanders et al., 2005; Rumajogee et al., 2016). Tal estratégia permite que as alterações morfológicas, bioquímicas e cognitivas encontradas nos humanos sejam reproduzidas (Arteni et al., 2006; Ikeda et al., 2008).

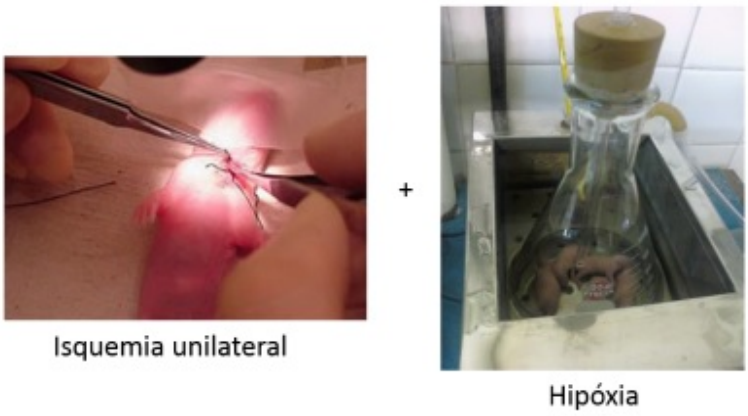


Figura 4 – Indução do modelo de HI de Levine-Vannucci através da oclusão permanente da artéria carótica comum direita e posterior exposição a ambiente hipóxico.

Diversos déficits cognitivos já foram evidenciados em animais submetidos à HI neonatal, como déficit na memória espacial no labirinto aquático de Morris em ratos adolescentes e adultos submetidos à HI neonatal (Pereira et al., 2007; Pereira et al., 2008), na memória aversiva no teste da esquiva inibitória (Arteni et al., 2003; Carletti et al., 2012), na memória de reconhecimento de objetos (Pereira et al., 2008) e em comportamentos característicos de déficit de atenção e hiperatividade (Miguel et al., 2015, 2017).

A avaliação dos reflexos neonatais é utilizada com um importante fator preditor de alterações comportamentais em adultos. Esses marcos indicam a maturação dos reflexos neurológicos e possíveis alterações motoras. (Allen & Alexander, 1997; Heyser, 2004). Assim como não há consenso quanto a déficits motores no modelo de Levine-Vannucci de HI, também não há quanto a alterações dos reflexos neonatais. Alguns estudos já evidenciaram algumas modificações nos marcos do desenvolvimento (Lubics et al., 2005; Sanches et al., 2012), porém um estudo do nosso grupo não encontrou diferença na maturação dos reflexos pela HI neonatal (Schuch et al., 2016).

Alterações bioquímicas e morfológicas no tecido nervoso são bem estabelecidas após a lesão hipóxico-isquêmica, principalmente no hipocampo que é uma região rica em sinapses glutamatérgicas e é uma das principais estruturas afetadas pela HI. Além disso, essa estrutura é responsável por boa parte dos déficits cognitivos observados nos ratos HI (Carletti et al., 2012; Miguel et al., 2015, 2017; Pereira et al., 2007; Rojas et al., 2013, 2016). Já foi ralatada diminuição da atividade da enzima Na+, K+-ATPase no estriado, córtex e hipocampo (Weis et al., 2011;

Carletti et al, 2012), aumento nos níveis de malondialdeído (MDA) e atividade da superóxido dismutase no hipocampo e córtex cerebral (Weis et al., 2011), bem como desequilíbrio nos níveis de BDNF no hipocampo (Pereira et al., 2009) e alteração na barreira hematoencefálica (Diaz et al., 2016). Ainda, quanto a alterações morfológicas, foi observado diminuição no volume hipocampal e na área estriatal ipsilateral a lesão (Pereira et al., 2008), diminuição da densidade de espinhos dendríticos no hipocampo ipsilateral à lesão (Rojas et al., 2013) e alterações na barreira hematoencefálica que podem desempenhar um importante papel na formação e desenvolvimento de edema após a HI neonatal (Yu et al., 2012, Diaz et al., 2016).

### *1.5 ALTERAÇÕES EPIGENÉTICAS NO DESENVOLVIMENTO*

As modificações epigenéticas participam do desenvolvimento e diferenciação celular e alterações nesses mecanismos durante o desenvolvimento podem ser responsáveis pelo aparecimento de algumas doenças (Tsankova et al., 2007; Gluckman et al., 2008). Os mecanismos epigenéticos são responsáveis por alterar o padrão da expressão de genes, de forma duradoura e/ ou hereditária, sem modificar a seqüência genética (Gluckman et al., 2008). A cromatina é composta por uma fita dupla de DNA que se enrola ao entorno de um octômero composto por quatro pares de histonas: H2A, H2B, H3 e H4 (Kouzarides, 2007). Assim, os principais mecanismos epigenéticos incluem: metilação/demetilação do DNA, modificações nas histonas e microRNAs (Figura 5) (Cantone & Fisher, 2013).

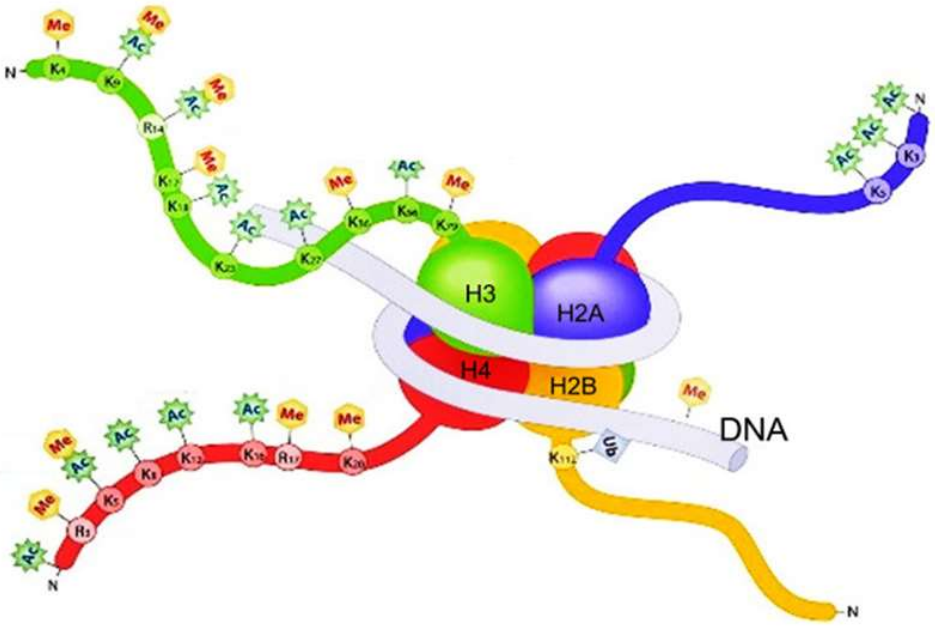


Figura 5 – Mecanismos epigenéticos – O DNA envolve o octômero de histonas, que é composto por quatro pares das histonas H2A, H2B, H3 e H4. Essa estrutura pode sofrer modificações que incluem a metilação e acetilação de histonas, além da metilação de DNA. Adaptado de Paschon et al., 2014

As enzimas DNA metiltransferases (DNMT) são as responsáveis pela metilação do DNA, que ocorre preferencialmente nas ilhas CpG localizadas na região promotora dos genes. Essa transferência de um grupo metila causa o silenciamento desses genes. Atualmente são conhecidas as enzimas DNMT3A, DNMT3B (responsáveis pela metilação de novo dessas ilhas, estabelecendo um padrão de metilação), DNMT1 (responsável pela manutenção do padrão de metilação no

desenvolvimento e divisão celular) e DNMT2 (metila pequenos grupos de RNA) (Goll and Bestor, 2005; Goll et al., 2006).

As histonas podem sofrer diversas modificações, sendo as mais conhecidas e estudadas a acetilação e a metilação (Karlic et al., 2010). A acetilação das histonas pelas enzimas histonas acetiltransferases (HAT) normalmente está associada com a ativação de genes. Já a sua deacetilação pelas histonas deacetilases (HDAC) está relacionada com o silenciamento gênico (Wang et al., 2008b). A metilação dessas proteínas é realizada pelas histonas metiltransferases (HMT) e é revertida pelas histonas demetilases (HDM). Diferentemente das outras modificações citadas, os efeitos da metilação das histonas depende da histona e do resíduo metilado (como a lisina, K), além do grau de metilação (podem ser mono, di ou trimetiladas). Normalmente, a metilação das histonas H3K4, H3K36 e H3K79 está relacionada com a ativação da expressão gênica, enquanto que a metilação de H3K9, H4K20 e H3K27 são associadas ao silenciamento gênico (Pedersen & Helin, 2010).

A participação do folato na formação da S- adenosilmetionina que é o principal doador de grupo metil no organismo, permite a participação dessa vitamina nos processos epigenéticos de metilação, tanto de DNA quanto de histonas (Mentch & Locasale, 2016). Sabe-se que o folato tem um papel importante na metilação de DNA por um mecanismo que envolve a atividade da DNA metiltransferase, mas que ainda não foi completamente elucidado (Burdge & Lillycrop, 2012). Já foi visto que a deficiência de folato na gestação leva à hipometilação encefálica de DNA nos filhotes (Van Mil et al., 2014). Por outro lado, não há consenso sobre os

efeitos do excesso de suplementação de folato. Estudos encontraram tanto hipermetilação de DNA quanto hipometilação no encéfalo da prole (Hoyo et al., 2011; Sable et al., 2013). Também foi observado que essas alterações encefálicas são duradouras ao longo da vida do filhote. Sable e colaboradores (2013) evidenciaram que a suplementação com AF em ratas prenhes alterou o padrão de metilação de DNA na prole, agravando com a idade. Os autores sugerem que a hipermetilação global de DNA pode alterar o desenvolvimento encefálico, já que esse aumento já foi mostrado em doenças como Alzheimer e bipolaridade.

O encéfalo em desenvolvimento é vulnerável a condições ambientais adversas, como a HI encefálica neonatal (Li et al., 2012), e mudanças epigenéticas acompanham o desenvolvimento da lesão ao tecido nervoso. Foi encontrada uma diminuição na metilação global de DNA, que persistiu ao longo do desenvolvimento em ratos submetidos à HI (Sable et al., 2013). Em um estudo que utilizou um inibidor da enzima DNA metiltransferase, foi observada uma diminuição da metilação de DNA e um aumento na expressão do HIF-1α (*Hypoxia-inducible factor 1-alpha*) (Ishida et al., 2007; Koslowski et al., 2011). Considerando que o aumento do HIF-1 α é um fator chave no desencadeamento do dano tecidual após a HI, propõe-se que a diminuição da metilação é um mecanismo responsável pelos achados pós-HI, bem como um potencial alvo terapêutico. Outro dado interessante é que quando há um aumento na acetilação de histonas, pela enzima HAT, foi observada uma co-ativação do HIF-1α (Kallio et al., 1998), indicando que as modificações nas histonas estão diretamente relacionadas com o dano gerado pela HI e também envolvidas com a expressão do HIF-1α**.**

### *1.6 O ÁCIDO FÓLICO E A HI NEONATAL*

Considerando o importante papel do AF no desenvolvimento encefálico e o seu potencial antioxidante (Joshi et al., 2001; Stanger & Wonisch, 2012), passamos a estudar seus possíveis efeitos na HI encefálica neonatal, já que se sabe que o estresse oxidativo é um dos mecanismos-chave na neuropatologia da HI. Em um recente estudo demonstramos que o tratamento com AF em ratos hipóxico-isquêmicos ocasionou melhora no déficit cognitivo no teste de esquiva inibitória, reversão do comportamento do tipo ansioso no campo aberto, bem como recuperação da atividade da enzima $Na^+,K^+$ - ATPase em estriado e córtex frontal (Carletti et al., 2012). Ainda, observou-se que o tratamento com AF preveniu/ reverteu a degeneração e morte neuronal 24 horas após a HI (dados não publicados). Porém, curiosamente, a administração de AF aumentou o déficit na memória espacial observado no Water maze e diminuiu a atividade da $Na^+,K^+$-ATPase em hipocampo de ratos adolescentes submetidos à HI (Carletti et al., 2016). Estes achados nos sugerem que a administração de AF a longo prazo pode ter resultados indesejados e que há que se considerar com mais cautela a dose a ser utilizada.

# 2. JUSTIFICATIVA E HIPÓTESE

A suplementação com AF já é realizada durante a gestação por conta dos seus efeitos benéficos na prevenção de defeitos de fechamento do tubo neural, mas ainda não há um consenso sobre os efeitos de uma alta dosagem dessa vitamina sobre o desenvolvimento do feto. Também pouco se sabe sobre os efeitos da suplementação com AF sobre a rata mãe e no comportamento maternal.

Ainda, estudos anteriores encontraram um efeito dual do tratamento com AF em animais submetidos à HI neonatal. Esse evento lesivo ocorre no período do periparto sendo, na maioria das vezes, imprevisível e acarreta a graves sequelas. É consenso que são poucas as opções de tratamentos efetivos para essa lesão; neste contexto o AF surge como opção a ser estudada, pois já suplementado durante a gestação e pode afetar o desenvolvimento de animais hipóxico-isquêmicos. Assim, decidimos avaliar os efeitos da suplementação com diferentes doses de AF no desenvolvimento da gestação, da prole submetida à HI neonatal, e nas mães e na sua relação mãe-filhote.

A hipótese do presente trabalho é que a dose recomendada será benéfica na prevenção de danos relacionados à HI neonatal e a dose excessiva será prejudicial para a prole, independente da lesão.

# *3. OBJETIVOS*

### *3.1 OBJETIVO GERAL*

O presente estudo tem como objetivo investigar os efeitos da suplementação com ácido fólico, tanto em uma dose equivalente a recomendada quanto em uma dosagem considerada excessiva, durante a gestação, no comportamento maternal e nas ratas mães após o desmame, bem como em parâmetros cognitivos, morfológicos e bioquímicos ao longo da vida da prole submetida a um modelo de hipóxia-isquemia neonatal.

### *3.2 OBJETIVOS ESPECÍFICOS*

**Avaliar nos filhotes submetidos à hipóxia-isquemia neonatal:**

- Parâmetros cognitivos, quantificação e plasticidade neuronal, e os níveis de BDNF aos 60 DPN;

- A expressão de proteínas relacionadas à apoptose e plasticidade, bem como os níveis de metilação das histonas H3K4 e H3K27 no hipocampo dos animais aos 60 DPN;

- O crescimento somático, a maturação e o aparecimento dos marcos do desenvolvimento e a atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo dos animais aos 21 DPN.

**Avaliar os efeitos da suplementação com ácido fólico durante a gestação:**

- Nos parâmentros descritos acima;

- No desenvolvimento da gestação e no comportamento maternal;

- Na memória e na atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo das ratas mães.

# 4. CAPÍTULO 1

"FOLIC ACID SUPPLEMENTATION DURING PREGANCY PREVENTS COGNITIVE IMPAIRMENTS AND BDNF IMBALANCE IN THE HIPPOCAMPUS OF THE OFFSPRING AFTER NEONATAL HYPOXIA-ISCHEMIA"

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# Folic acid supplementation during pregnancy prevents cognitive impairments and BDNF imbalance in the hippocampus of the offspring after neonatal hypoxia-ischemia

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**Abstract**

Folic acid (FA) supplementation (400 μg/day) has been recommended during pregnancy to prevent neural tube defects. However, in some countries, flours are required to be fortified with FA, possibly increasing the levels of this vitamin in pregnant women. Our previous studies have evidenced a dual effect of the FA treatment in a rat model of neonatal hypoxia-ischemia (HI). Aiming to better correlate with humans, this paper evaluated the effects of two different levels of FA supplementation during pregnancy on memory parameters and neuronal survival and plasticity in the hippocampus of rats submitted to the neonatal HI. During pregnancy, female Wistar rats received one of these diets: standard (SD), supplemented with 2 mg/kg of FA or with 20 mg/kg of FA. At the 7$^{th}$ PND, rats suffered the HI procedure. At the 60$^{th}$ PND rats were evaluated in the open field, Morris water maze, novel-object recognition and inhibitory avoidance tasks. Furthermore, neuronal density, synaptophysin densitometry and BDNF concentration were assessed in the hippocampus. Both doses of FA prevented the HI-induced memory impairments. The supplementation reversed the BDNF late increase in the hippocampus of the HI rats, but did not inhibit the neuronal death. In conclusion, FA supplementation during pregnancy prevented memory deficits and BDNF imbalance after neonatal HI. These findings are particularly relevant because neuroprotection was achieved even in the high level of FA supplementation during pregnancy, indicating that this intervention would be considered secure for the offspring development.
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*Keywords:* Perinatal asphyxia; Folate; Vitamin; Behavior; Brain damage

## 1. Introduction

In the 60s the first studies demonstrating that folic acid (FA) supplementation during pregnancy was effective in preventing neural tube defects (NTD) were published. Some years after, it was established that the daily ingestion of 0.4 mg of FA is the adequate dose to reach this protective effect [10,67]. Aiming to reach most women in their fertile period, some countries, such as USA, Canada and Brazil, adopted a policy of fortifying flour with FA [21,47]. The world consensus was that these actions have positive repercussion on the NTD cases, but recent studies have discussed that an excessive FA consumption may be occurring in people with adequate nutrition [6,40,55]. Sittig et al. [57] affirmed that, depending on their flours consumption, many people could consume more than 200% of the 0.4 mg recommended allowance. Additionally, in Brazil, the System of Public Health offers a 5 mg/day supplement [35], which is more than ten times the recommended dose by the WHO. Taking together, some women might have a high FA concentration, particularly during the gestational period, and the possible consequences, for themselves and the babies, are still being investigated.

Several studies using animal models have been conducted in order to better understand the effects of FA supplementation. FA supplementation in rodents has been associated with lower weight and size of the embryos, development delay and decreased neurotrophins

*Abbreviations:* FA, folic acid; NTD, neural tube defects; WHO, World Health Organization; BDNF, brain-derived neurotrophic factor; HI, hypoxia-ischemia; PND, post-natal day; DAB, 3,3'-diaminobenzidine; PBS, phosphate-buffered saline; CA, Cornu Amonis; AOI, area of interest; ANOVA, analysis of variance; ADHD, attention deficit hyperactivity disorder; TrkB, tropomiosin receptor kinase B; PI3K, phosphoinosite 3-kinase; mTOR, mechanistic target of rapamycin; HIF, hypoxia inducible factor; DNA, deoxyribonucleic acid

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levels, like brain-derived neurotrophic factor (BDNF; [1,34,52,53]). It was also identified that high levels of FA supplementation during pregnancy of mice altered gene expression in the pups' cerebral hemispheres, including many of them involved in the development [8]. Behavioral impairments have also been found: Sittig et al. [57] demonstrated that high FA dose affected memory and motivation in young rats. In addition, high-dose of FA supplementation during the period prior to conception resulted in alterations on hippocampal gene expression in offspring mice, suggesting epigenetic changes in oocytes can be induced by excess FA [24]. Conversely, recent studies in rats have demonstrated that high FA supplementation during pregnancy improved early reflexes, spatial learning and motor disability [66] and alleviated brain inflammatory response in an animal model of schizophrenia [12]. These results conflict with the negative findings described. Together, they seems to point to a dual effect of gestational FA supplementation.

Considering the positive effects of FA supplementation and the importance of this vitamin for the neurodevelopment, our group decided to investigate the potential of FA as a possible protector in a rat model of neonatal hypoxia-ischemia (HI). The incidence of HI is 2.5 out of 1,000 among live-born infants and 25% present severe and permanent sequelae such as cerebral palsy, cognitive and motor deficits [27,36]. In humans, HI frequently occurs in the peripartum period and can be triggered by a broad range of etiologic factors such as prematurity, malnutrition, interruption of umbilical blood flow and placental dysfunction [45]. Because of this multifactorial etiology, the neonatal HI is, in most of the cases, an unpredicted event. Then, it is still necessary to investigate the neuropathology of HI and to explore possible therapeutic strategies.

The Levine-Vannucci HI rat model has reproduced functional and neuropathological characteristics seen in humans such as cognitive impairment [32,33,43,44,50] and brain lesion, particularly in the hippocampus [33,43]. Such cerebral damage affects the synapses in the survival cells by decreasing dendritic arborization and dendritic spines density and altering the expression of synaptic proteins such as the synaptophysin and BDNF [22,42,50]. In our previous studies, we evaluated the effects of FA intraperitoneal (i.p.) administration after the neonatal HI insult in rats. The FA administration started after the HI injury in rat pups ($7^{th}$ postnatal day – PND) and continued daily until the end of the experiments. This treatment reversed the anxiogenic effect, the aversive memory impairment, and the $Na^+$, $K^+$ ATPase inhibition in frontal cortex and striatum consequent to HI [14]. Subsequently, FA treatment did not reverse the spatial memory deficit and, surprisingly, the FA impaired the performance of control animals and decreased $Na^+$, $K^+$ ATPase activity in the hippocampus of HI young rats. In contrast, in adults, FA reversed the $Na^+$, $K^+$ ATPase activity inhibition [13]. Such results clearly reveal a particular and unexpected effect of the FA on the hippocampus of young rats. These controversial findings indicate that FA administration on HI rats seems to result in contrasting effects depending on the brain structure and the developmental period; the direct administration (i.p.) during early development in the pups might not be adequate for the HI treatment.

Thus, the present study sought to delineate a more effective protocol of FA supplementation as a therapeutic strategy for neonatal HI encephalopathy. Here are some points supporting to this investigation: *first*, FA is a vitamin widely used and, undoubtedly, needs special attention because there its neurological effects are still unclear; *second*, FA supplementation is currently recommended to pregnant women and in some countries the flours are also fortified with FA, which can lead to above recommended levels; and, *finally*, we found conflicting data about the benefits of FA as a treatment directly administrated via i.p. in HI rat pups. Thus, the aim of this study was to evaluate the effects of FA supplementation in the dams' diets with two different doses of FA (one recommended by the WHO and the other representing the FA excess) on pups that suffered neonatal HI. We evaluated: cognitive parameters, neuronal density, synaptophysin density and BDNF concentration in the hippocampus. Our hypothesis is that FA supplementation in moderate level would prevent functional deficits as well as would alleviate the HI-induced hippocampal damage; in opposition, we believe that the excessive dose of FA would aggravate the consequences of the neonatal HI.

## 2. Experimental procedures

### 2.1. Animals

Male and female adult Wistar rats were obtained from a local breeding colony (Institute of Basic Health Science, Federal University of Rio Grande do Sul, Brazil). They were maintained in a temperature-controlled room (22±1°C) on a 12/12 light/dark cycle, with food and water available *ad libitum*. Daily vaginal smears were collected from female rats and when the proestrus phase was detected, they were put in a new cage with one male rat. After mating confirmation, the pregnant rats were randomly divided into three groups, according to the diet: 1) Standard diet (SD), 2) Supplemented with 2 mg/kg of FA (FA2) and 3) Supplemented with 20 mg/kg of FA (FA20). On the $7^{th}$ PND, pups of both genders were randomly separated between control and HI groups, resulting in six experimental groups: 1) Control with SD diet (CTSD), 2) HI with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). A subset of each group was used to perform the open field and the Morris Water Maze tasks (n: CTSD=14, CTFA2=15, CTFA20=17, HISD=13, HIFA2=16 and HIFA20=17) and 4–6 rats from each subset undergone cresyl violet staining afterwards. Another subset was used in the novel-object recognition and inhibitory avoidance tests (n: CTSD=8, CTFA2=13, CTFA20=12, HISD=10, HIFA2=12 and HIFA20=10) and, again, 4–6 rats per subset were used for synaptophysin and NeuN immunostaining. One more subset of animals, that did not undergo behavioral testing, was included for BDNF analysis (n: CTSD=5, CTFA2=6, CTFA20=6, HISD=5, HIFA2=4 and HIFA20=5). In all analyses, male and female rats were equally divided into the groups.

To carry out this study, the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the guide of the Federation of Brazilian Societies for Experimental Biology were used. This project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28136). The experimental design is presented in the Fig. 1.

### 2.2. Folic acid supplementation

Three different diets were administered: the standard diet, the diet supplemented with 2 mg/kg of FA (considered the appropriate level for Wistar pregnant rats; [3]) and the diet supplemented with 20 mg/kg of FA representing the excessive dose that can be obtained from supplements and in the diet itself [5]. All diets followed the AIN 93 Purified Diets for Laboratory Rodents (American Institute of Nutrition; [48]); protein level in the standard and treatment diets was 18% and all of them were isocaloric. The SD diet contained 2 mg/kg of FA needed for the proper animal maintenance and the supplemented diets had an addition of 2 or 20 mg/kg of FA (totalizing 4 and 22 mg/kg of FA, respectively). The supplementation occurred throughout the pregnancy. After birth, all rats received the SD diet until the end of the experiments.

### 2.3. Neonatal hypoxia-ischemia

Pups were submitted to the Levine-Vannucci HI model [28,61], at $7^{th}$ PND. While under anesthesia with halothane, a ventral neck incision was made, the right common carotid was isolated, and

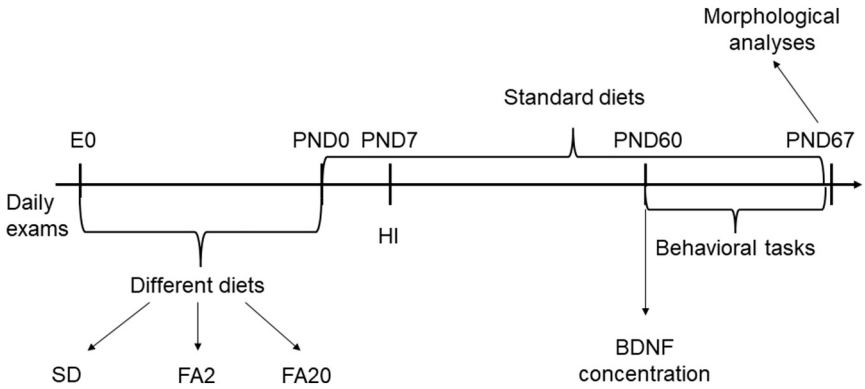


Fig. 1. Experimental design – after mating confirmation, female Wistar rats were divided into 3 groups, according to diet: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). When the puppies were born, all animals received the SD diet. At the 7$^{th}$ PND, puppies were submitted to hypoxia-ischemia (HI), resulting in 6 groups of animals: Control with SD diet (CTSD), 2) Hypoxia-ischemia with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). From the 60$^{th}$ PND two set of animals were used for behavioral tasks or to assess the biochemical analysis.

permanently occluded by a surgical silk thread. After a 2 h recovery period with their dams, animals were subjected to the hypoxic condition (8% oxygen and 92% nitrogen, during 90 minutes). Control pups received the sham surgery but did not have the arterial occlusion or hypoxia [44].

### 2.4. Behavioral tasks

The behavioral study intended to identify functional impairments consequent to the neonatal HI and the possible effects of the FA supplementation. To avoid interference in the results caused by several days of tests and because some tasks can cause anxiety and stress, one set of animals performed in the open field and the Morris Water Maze tests (n=13–17/group) and another set of animals were evaluated in the novel-object recognition and inhibitory avoidance tasks ($n$=8–13/group). The open field task was performed on the 60$^{th}$ PND and was followed by the Morris water maze from the 61$^{st}$ to the 66$^{th}$ PND. The novel-object recognition test was executed on the 60$^{th}$ and 61$^{st}$ PND in the other set. Inhibitory avoidance was followed on the 62$^{nd}$ and 63$^{rd}$ PND. Behavioral scoring was conducted by an experimenter blind to the experimental manipulations.

### 2.5. Open field

At the 60$^{th}$ PND, animals were submitted to the open field test. This task was used for assessing motor function, exploratory behavior, and anxiety, which are crucial for animals' survival and development [54]. The apparatus is a wood square arena divided into 12 equal quadrants of 50×50 cm, with 40 cm walls, as previously described [14]. The animals were placed facing the corner wall of the apparatus and their free exploration were recorded for 5 minutes and evaluated after. The latency to leave the first quadrant, the number of crossings, the number of rearings, and the time spent in the central and peripheral areas were analyzed.

### 2.6. Morris water maze

To evaluate spatial memory, one day after the open field, animals were submitted to the Morris Water maze task (from the 61$^{st}$ to the 66$^{th}$ PND). The apparatus is composed by a 117 cm diameter circular pool filled with water at 22°C. There were visual clues in the room and the pool was virtually divided into 4 quadrants. A circular platform was 2 cm submerged into the water. Animals were underwent to the Reference Memory protocol, which consisted of 5 training days (4 trials/day, 20 minutes of inter-trial interval) and a probe trial on the 6$^{th}$ day. In each training trial, animals were placed in the water from each quadrant in a random order. During the training the platform remained in the same position in all sessions. The latency to reach the platform with a maximum of 60 s was measured. If the rat did not reach the platform, it was gently moved through the water and placed in the platform for 10 s. In the probe trial (only 1 trial), the platform was removed, and the following variables were observed: the latency to reach the platform area, the number of crossings in the platform area and the time spent in the target and in the opposite quadrant [38,44].

### 2.7. Novel-object recognition

As the spatial memory function, object recognition memory is also considered an important rodent's innate behavior based on its natural propensity to explore the novelty. The novel-object recognition is widely used to investigate cognitive processes such as memory and learning, preference for novelty, and the influence of different brain regions in the process of recognition [4]. This task was performed on the open field apparatus described above, at the 60$^{th}$ and 61$^{st}$ PND. In the first session, a rat was placed on the apparatus with two similar objects and the time exploring each object was recorded for 5 minutes. To evaluate the short-term memory, the second session followed after a 5-minute interval, following previous methods [43,50,65]. Rats were placed back on the apparatus with one familiar and one new object. Their time exploring each object was again evaluated for 5 minutes. An object preference index was utilized to evaluate memory deficits in the second session (test). The index is calculated as the time difference between the exploration of the new object to the familiar object, divided by the sum of the time exploring both objects (B-A/B+A, being B the new and A the familiar object).

### 2.8. Inhibitory avoidance

During two days after performing the novel-object recognition test, the animals were used in the inhibitory avoidance task to evaluate aversive memory (62$^{nd}$ and 63$^{rd}$ PND). The apparatus consists of a 50 cm wide, 25 cm high and 25 cm deep acrylic box with a 7 cm high, and 9 cm wide platform. The floor of the apparatus is covered by parallel stainless-steel bars (EP 104R, INSIGHT, Brazil). The animals were placed on the platform facing a corner and the latency to step down with the four paws was recorded. On the first day, whenever the rat stepped down, it received two 0.6 mA footshocks for 2–3 s. The next day, the rat was again placed on the platform. It did not receive foot shocks when it stepped down. The latency to step down was recorded for the two sessions [14,26].

### 2.9. Morphological analysis

One day after the behavioral tasks, animals were profoundly anesthetized with thiopental and transcardiac perfused with 0.9% saline solution followed by 4% paraformaldehyde with phosphate buffer. Brains were removed and post-fixed for 4 hours, then were cryoprotected with 15% and 30% sucrose solution and frozen in liquid nitrogen. Using a cryostat, coronal serial sections (20 μm) were obtained of the dorsal hippocampus (Bregma -2.30 mm to -3.60mm, according to [41]).

For cresyl violet technique, sections were rehydrated and stained with 5% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) for 3 minutes, dehydrated in increasing concentrations of ethanol followed by xylene and coverslipped. For NeuN and synaptophysin immunohistochemistry, sections were washed in PBS solution and antigen retrieval (heating for 20 min in 0.01 M citrate buffer at 92°C) was realized only for NeuN. After cool down, endogen peroxidase was blocked with 3% hydrogen peroxide for 30 min. Sections were washed with PBS followed by PBS-Tx and were incubated with BSA and monoclonal mouse anti-NeuN antibody (1:1000) or monoclonal mouse anti-SYP antibody (1:200) for 48 h at 4°C. After, they were washed with PBS-Tx and incubated with secondary antibody rabbit anti-mouse IgG conjugated with peroxidase (1:500; Sigma-Aldrich) for 2 h at room temperature. DAB solution (0.06% 3,3 diaminobenzidine, Sigma-Aldrich, USA) was used as a reaction developer with 10% hydrogen peroxide for 5 min. Section were washed in PBS, dehydrated in ethanol, cleared with xylene and coverslipped [30].

### 2.10. Neuronal density

As previously mentioned, hippocampus has a particular vulnerability to ischemic events [33, 43] and here we are focused in this brain structure investigating whether the HI-induced neuronal injury could be altered following FA supplementation during pregnancy. To evaluate the neuronal density in the CA1 region of the dorsal hippocampus, cresyl violet technique and NeuN immunohistochemistry were utilized. The number of cells were counted bilaterally in 4 sections per animal, 4–6 animals/group (area: 374189.017 $\mu m^2$) using an Olympus BX40 microscope (magnification of 400×), for each technique. The average of cells counted per animal was used as an estimative of neuronal density [30].

### 2.11. Synaptophysin optical densitometry

*Synaptophysin is a* synaptic vesicle-associated protein involved in the synapse formation and function and it is considered an important indicator of neuroplasticity particularly in cases of neurological insults [22,31]. Images of both sides of the CA1 region of the dorsal hippocampus were obtained using an Image M2 Zeiss (Germany 400×) coupled to a camera. Two images per section (3–4 sections/animal) of each animal were captured (4–6 animals/group). Image Pro Plus Software 6.0 (Media Cybernetics, USA) was used, all images were converted to an 8-bit gray scale and optical density was measured in 3 AOI (area of interest, 600.3033 $\mu m^2$).

### 2.12. BDNF Concentration

This neurotrophic factor is considered essential for neuronal survival, synaptic plasticity and neurogenesis and some studies have shown association between BDNF function and the FA supplementation [7,20]. In the particular case of BDNF, it is well known that this molecule can be quickly altered by behavioral tasks, particularly those with long period of cognitive training or exercise [23,56]. Then, at 60$^{th}$ PDN, 5–7 animals/group that did not perform any behavioral task were euthanized by decapitation and the hippocampi were quickly dissected and placed in liquid nitrogen. The samples were stored at -80°C until the biochemical assay. BDNF concentration was assessed by the E-Max Elisa kit (Promega, USA) and was performed according to the manufacturer's instruction as briefly described by [42].

### 2.13. Statistical analysis

Two-way ANOVA followed by Tukey's *post-hoc* test, with *lesion* and *supplementation* as factors, was used for open field, Morris Water maze, novel-object recognition, neuronal density, Synaptophysin optical density and BDNF concentration. Repeated measures ANOVA followed by Tukey's test with lesion and supplementation as factors were only used to analyze the acquisition phase of the Morris Water maze over the days. Inhibitory avoidance was analyzed by Kruskal-Wallis followed by Mann-Whitney test. The effect size (partial eta-squared [η2P]) was also informed. The Statistic© software package was used, and differences were considered significant when $P<.05$.

## 3. Results

### 3.1. Open field

Two-way ANOVA identified on the latency a *lesion*supplementation* interaction effect ($F(1,86)=3.13$, $P<.05$, partial $\eta^2=0.06$). HIFA2 group had a lower latency to leave the first square when compared to CTFA2 group (Fig. 2A). No difference on the *lesion* ($F(1,86)=1.776$, $P=.18$, partial $\eta^2=0.02$) and on the *supplementation* ($F(1,86)=1.082$, $P=.343$, partial $\eta^2=0.02$) factor were found. Observing the crossings (Fig. 2B), it was identified a *lesion* effect, with higher number of crossings in the HI groups ($F(1,86)=3.99$, $P<.05$, partial $\eta^2=0.04$); there were no *supplementation* ($F(1,86)=0.66$, $P=.51$, partial $\eta^2=0.02$) or *supplementation*lesion* interaction effects ($F(1,86)=1.02$, $P=.36$, partial $\eta^2=0.02$). No further differences were observed in the other variables: rearings (*lesion* ($F(1,86)=0.22$, $P=.64$, partial $\eta^2=0.002$), *supplementation* ($F(1,86)=0.14$, $P=.87$, partial $\eta^2=0.002$), *lesion*supplementation* ($F(1,86)=0.32$, $P=.73$, partial $\eta^2=0.01$)), time in the peripheral (*lesion* ($F(1,86)=0.59$, $P=.443$, partial $\eta^2=0.002$), *supplementation* ($F(1,86)=2.63$, $P=.078$, partial $\eta^2=0.04$), *lesion*supplementation* ($F(1,86)=0.03$, $P=.97$, partial $\eta^2=0.002$)) and in the central area (*lesion* ($F(1,86)=0.59$, $P=.44$, partial $\eta^2=0.002$), *supplementation* ($F(1,86)=2.63$, $P=.08$, partial $\eta^2=0.04$), -*lesion*supplementation* ($F(1,86)=0.03$, $P=.97$, partial $\eta^2=0.002$)) (Fig. 2C-E).

### 3.2. Morris water maze

The latency to find the submerged platform in the acquisition phase in the Morris water maze was analyzed by Repeated-measures ANOVA: it was found significant effect in *lesion* ($F(1,86)=22.94$, $P<.05$, partial $\eta^2=0.20$) and *day* factors ($F(1,344)=54.51$, $P<.05$) with no effect on *supplementation* ($F(1,86)=0.31$, $P=.73$, partial $\eta^2=0.014$) and *lesion*supplementation*day* ($F(1,86)=0.83$, $P=.58$, partial $\eta^2=0.02$) factors. Firstly, the learning curve of each group was evaluated, compared with the first training day: CTFA2 and HIFA20 groups lowered their latency since the second day; CTSD, CTFA20 and HIFA2 groups took less time to find the platform starting in the third day. The worse performance was observed in the HISD group: these animals decreased the latency to find the platform only in the fifth day. Two-way ANOVA followed by Tukey's test was also performed to detect possible differences in each training day: in the days 1 and 5, significant *lesion* effect was identified ($F(1,86)=4.30$, $P<.05$ , partial $\eta^2=0.05$ and $F(1,86)=14.39$, $P<.05$, partial $\eta^2=0.14$ respectively) without differences on *supplementation* ($F(1,86)=1.38$, $P=.26$, partial $\eta^2=0.03$ and $F(1,86)=0.002$, $P=1.00$, partial $\eta^2=0.000$ respectively) and *lesion*supplementation* ($F(1,86)=0.19$, $P=.82$, partial $\eta^2=$

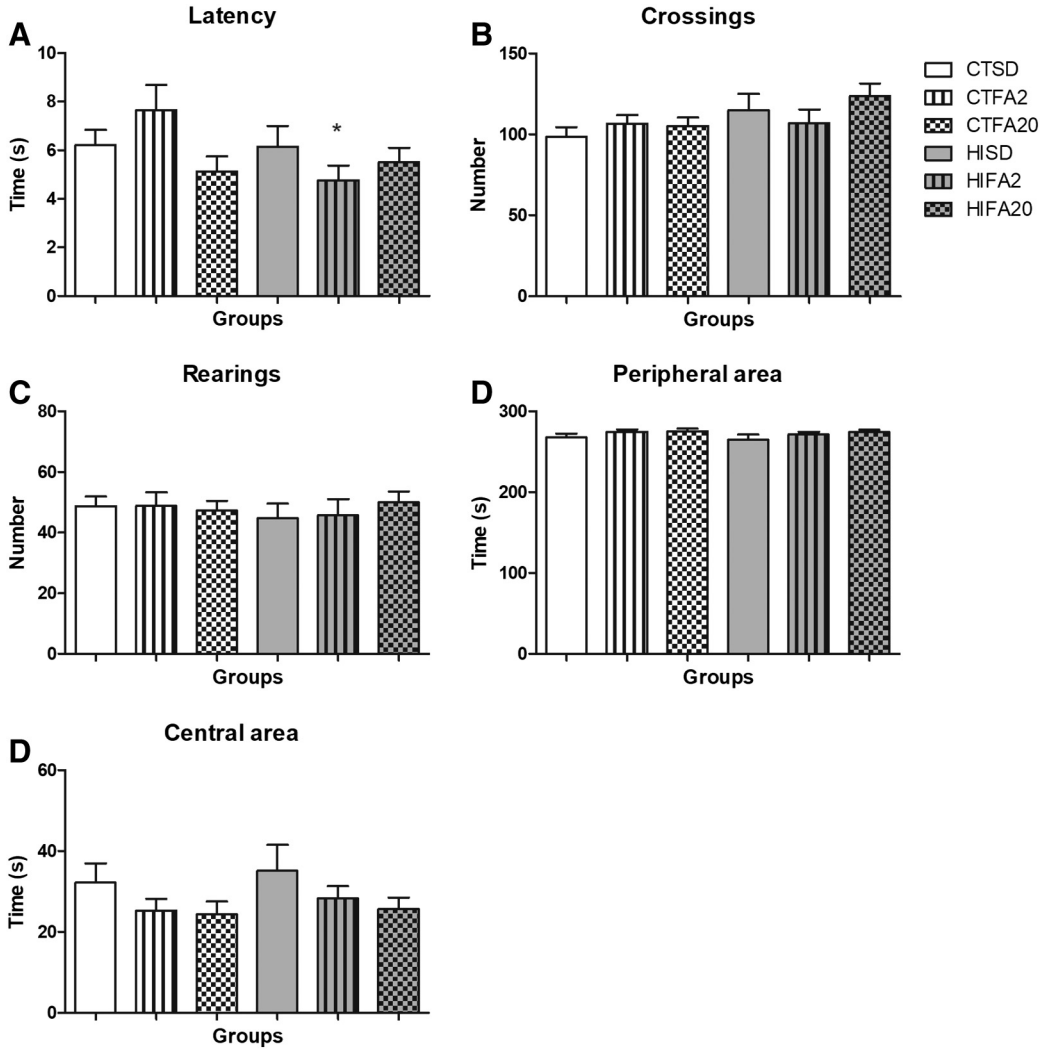


Fig. 2. Open Field task performed by the puppies at the 60th PND. A) Latency. B) Crossings. C) Rearings. D) Peripheral area. E) Central area. *different from the CTFA2 group. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$. $N=13–17$ animals/group.

0.005 and F(1,86)=0.51, $P=.60$, partial $\eta^2=0.01$ respectively). *Post-hoc* revealed that HISD group had higher latency compared to: all control groups in the second day (*lesion* (F(1,86)=12.45, $P<.05$, partial $\eta^2=0.13$), *supplementation* (F(1,86)=1.38, $P=.26$, partial $\eta^2=0.03$), *lesion*supplementation* (F(1,86)=2.47, $P=.09$, partial $\eta^2=0.05$)), CTSD and CTFA2 at the third day (*lesion* (F(1,86)=18.71, $P<.05$, partial $\eta^2=0.18$), *supplementation* (F(1,86)=0.08, $P=.92$, partial $\eta^2=0.002$), *lesion*supplementation* (F(1,86)=1.01, $P=.36$, partial $\eta^2=0.02$)) and CTSD at the day 4 (*lesion* (F(1,86)=14.24, $P<.05$, partial $\eta^2=0.14$), *supplementation* (F(1,86)=0.07, $P=.93$, partial $\eta^2=0.002$), *lesion*supplementation* (F(1,86)=1.29, $P=.28$, partial $\eta^2=0.03$)). No difference was identified between the HIFA2 and HIFA20, compared to the CT groups (Fig. 3). These results demonstrate spatial memory impairment consequent to HI with a recovery effect of both FA supplementation doses.

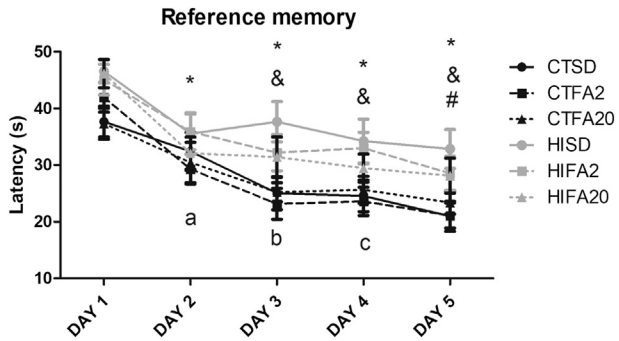


Fig. 3. Morris Water Maze – reference protocol performed by the puppies from the 61st PND. *CTFA2 and HIFA20 different from the day 1. & CTSD, CTFA20 and HIFA2 different from the day 1. # HISD different from the day 1. Repeated Two-way ANOVA followed by Tukey's test, $P<.05$. [a] HISD different from all CT groups. [b] HISD different from the CTSD and CTFA2 groups. [c] HISD different from the CTSD group. Two-way ANOVA followed by Tukey's test, $P<.05$. Data are expressed by mean±S.E.M. $N=13–17$ animals/group.

Two-way ANOVA and Tukey's test demonstrated that, in the probe trial, *lesion* effect in the latency was found (F(1,86)=6.34, $P<.05$, partial $\eta^2=0.07$): HI groups took more time to achieve the platform area (Fig. 4A) with no difference on *supplementation* (F(1,86)=0.23, $P=.8$, partial $\eta^2=.005$) and *lesion*supplementation* factors (F(1,86) =1.59, $P=.21$, partial $\eta^2=0.04$). Considering the crossings in the platform area, there was *lesion* (F(1,86)=15.93, $P<.05$, partial $\eta^2=0.16$) and *lesion*supplementation* interaction effect (F(1,86)=3.03, $P<.05$, partial $\eta^2=0.000$) with no *supplementation* effect (F(1,86)=0.002, $P=.99$, partial $\eta^2=0.07$). Tukey's test indicated that HISD group had fewer platform crossings in the target area when compared to CTSD and CTFA2 groups. Also, HIFA2 group also had fewer crossings than CTSD group (Fig. 4B). No effect was observed (Fig. 4C-D) on the

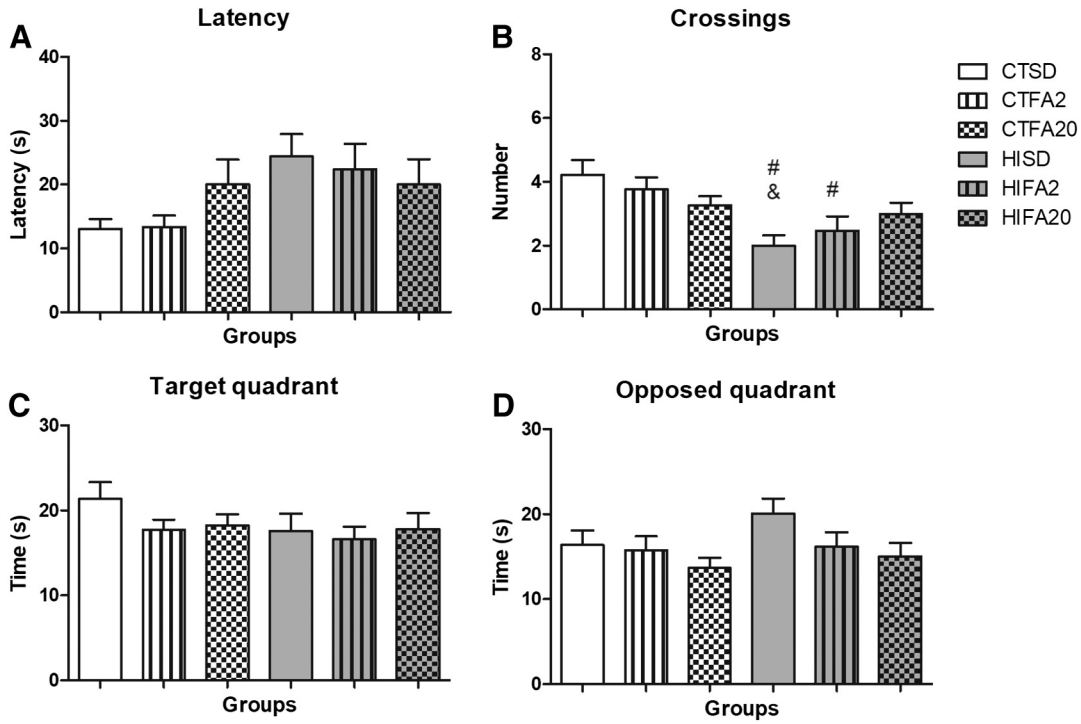


Fig. 4. Morris Water Maze - Probe trial performed by the puppies 24 hours after the end of the reference protocol. A) Latency. B) Crossings. C) Target quadrant. D) Opposed quadrant. # different from the CTSD group. & different from the CTFA2 group. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$. $N=13–17$ animals/group.

time spent in the target area (*lesion* ($F(1,86)=1.78$, $P=.19$, partial $\eta^2=0.02$), *supplementation* ($F(1,86)=0.98$, $P=.38$, partial $\eta^2=0.02$), *lesion*supplementation* ($F(1,86)=0.53$, $P=.59$, partial $\eta^2=0.012$)) and in the opposed quadrant (*lesion* ($F(1,86)=1.87$, $P=.18$, partial $\eta^2=0.02$), *supplementation* ($F(1,86)=2.69$, $P=.07$, partial $\eta^2=0.06$), *lesion*supplementation* ($F(1,86)=0.53$, $P=.59$, partial $\eta^2=0.01$)).

### 3.3. Novel-object recognition

Two-way ANOVA revealed significant effect of *lesion* factor ($F(1,59)=8.99$, $P<.05$, partial $\eta^2=0.13$), *supplementation* factor ($F(1,59)=4.98$, $P<.05$, partial $\eta^2=0.14$) and *lesion*supplementation* interaction effects ($F(1.59)=4.52$, $P<.05$, partial $\eta^2=0.13$). Tukey's *post hoc* evidenced that HISD group had lower novel-object preference index when compared to all control groups and HIFA20 group. Interestingly, HIFA2 and HIFA20 groups had similar preference index compared to controls. No differences were observed in the time exploring the two objects in the first session (Fig. 5).

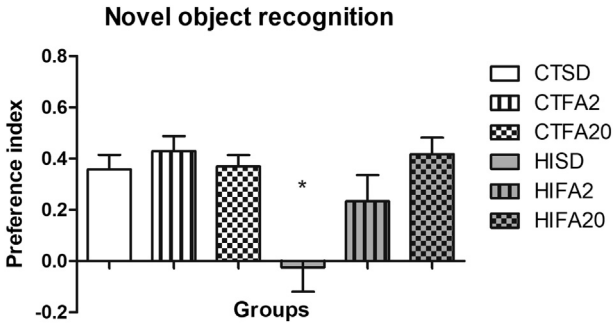


Fig. 5. Novel Object Recognition task performed by the puppies at the 60th and 61th PND. Data represent the preference index. *Different from all CT groups and HIFA20. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$. $N=8–13$ animals/group.

### 3.4. Inhibitory Avoidance

Kruskal-Wallis followed by Mann-Whitney test indicated HI effect in the test session ($H=11.88$, $P<.05$, effect size=0.17): HISD group had lower latency to step down the platform when compared to CTSD. Folic acid supplementation reversed this memory impairment: HIFA groups had the same pattern of avoidance compared to CT groups (Fig. 6).

### 3.5. Neuronal density

Two-way ANOVA for neuronal density (cresyl violet staining) revealed significant *lesion* effect ($F(1,22)=19.42$, $P<.05$, partial $\eta^2=0.68$) in the right hippocampus (ipsilateral). *Post-hoc* test indicated that all HI groups had fewer cells when compared to all CT groups. No effects on the *supplementation* ($F(1,22)=0.78$, $P=.49$, partial $\eta^2=0.07$) and interaction ($F(1,22)=0.18$, $P=.83$, partial $\eta^2=0.02$) were

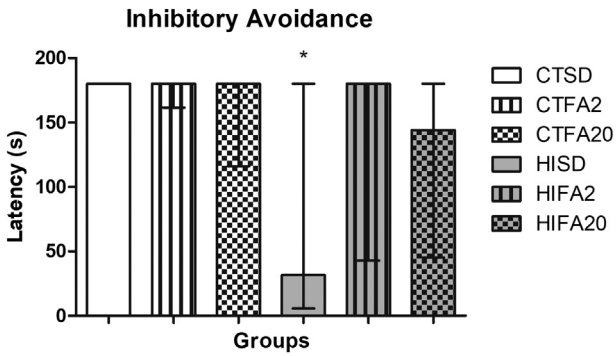


Fig. 6. Inhibitory avoidance task performed by the puppies one day after the novel object recognition task. *Different from CTSD group. Data are expressed by median± interquartile range. Kruskal-Wallis analysis followed by Mann-Whitney test, $P<.05$. $N=8–13$ animals/group.

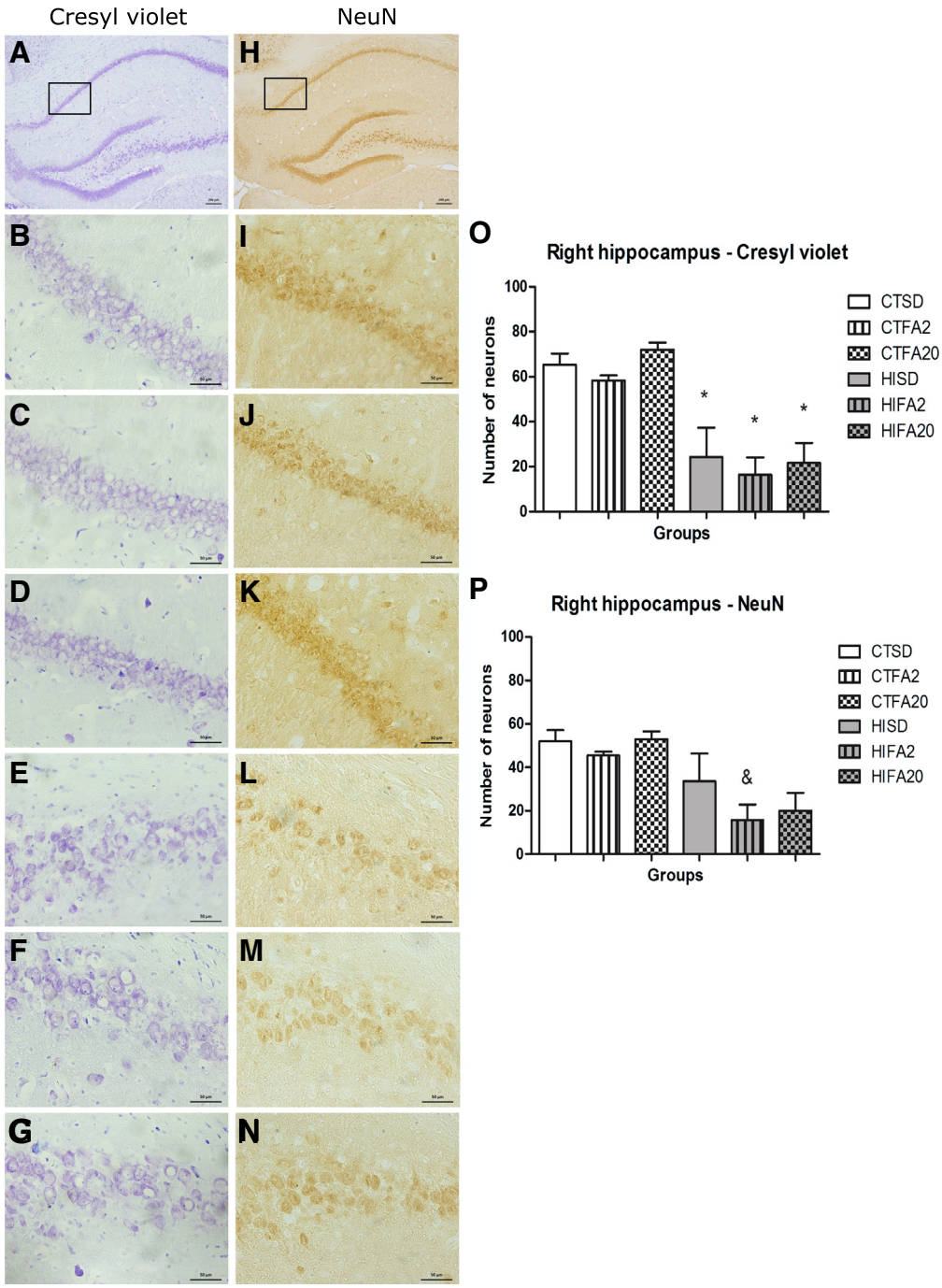


Fig. 7. Neuronal density in CA1 region of the right hippocampus of the puppies at the 60th PND. Cresyl violet staining showing the ipsilateral hippocampus (A) and the CA1 region analyzed (square). High magnification (400×) cresyl violet staining of the ipsilateral hippocampus of CTSD (B), CTFA2 (C), CTFA20 (D), HISD (E), HIFA2 (F) and HIFA20 (G) groups. O) Number of cresyl violet stained neurons in the ipsilateral hippocampus was quantified and statistically compared. NeuN immunohistochemistry showing the ipsilateral hippocampus (H) and the CA1 region analyzed (square). High magnification (400×) NeuN immunohistochemistry of the ipsilateral hippocampus of CTSD (I), CTFA2 (J), CTFA20 (K), HISD (L), HIFA2 (M) and HIFA20 (N) groups. P) Number of NeuN-positive neurons in the ipsilateral hippocampus was quantified and statistically compared. *Different from all CT groups. & different from CTFA20 group. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$, $N=4–6$ animals/group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

found (Fig. 7). In the left hemisphere, it was also observed a *lesion* effect ($F(1,22)=10.66$, $P<.05$, partial $\eta^2=0.03$) with HISD group different from CTFA20. One more time, no effects on the *supplementation* ($F(1,22)=1.99$, $P=.16$, partial $\eta^2=0.17$) and interaction ($F(1,22)=1.20$, $P=.32$, partial $\eta^2=0.11$) were found. Neuronal density was also evaluated by the NeuN immunohistochemistry and it was also confirmed the *lesion* effect ($F(1,24)=9.22$, $P<.05$, partial $\eta^2=0.39$), with lower number of neurons in the ipsilateral hippocampus of all HI groups and without effect of the FA supplementation ($F(1,24)=1.02$, $P=.38$, partial $\eta^2=0.08$) and interaction ($F(1,24)=0.36$, $P=.70$, partial $\eta^2=0.03$). Tukey's test only identified that the HIFA2 group presented a lower density when compared to CTFA20 group. No differences were observed in the contralateral hippocampus for NeuN density (*lesion* ($F(1,24)=0.03$, $P=.87$, partial $\eta^2=0.001$), *supplementation* ($F(1,24)=1.32$, $P=.28$, partial $\eta^2=0.10$) and interaction ($F(1,24)=0.90$, $P=.42$, partial $\eta^2=0.07$)).

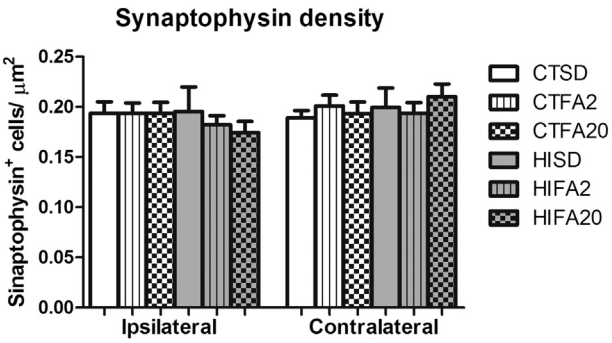


Fig. 8. Synaptophysin optical densitometry of both hippocampus of the puppies after behavioral tasks. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$. $N=4–6$ animals/group.

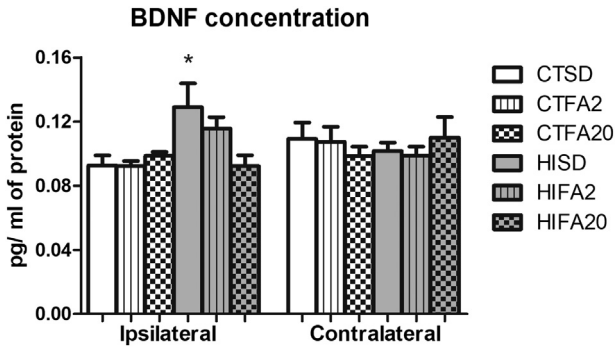


Fig. 9. BDNF concentration of both hippocampus of the puppies at the 60th PND. *Different from CTSD, CTFA2 and HIFA20 groups. Data are expressed by mean±S.E.M. Two-way ANOVA followed by Tukey's test, $P<.05$. $N=5–7$ animals/group.

### 3.6. Synaptophysin optical density

Statistical analysis didn't evidenced differences consequent to lesion (right ($F(1,24)=0.56$, $P=.45$, partial $\eta^2=0.03$; left ($F(1,34)=0.13$, $P=.72$, partial $\eta^2=0.01$)) or *supplementation* (right ($F(1,24)=0.31$, $P=.74$, partial $\eta^2=0.02$; left ($F(1,34)=0.14$, $P=.87$, partial $\eta^2=0.01$)) in both hemispheres for synaptophysin densitometry (Fig. 8).

### 3.7. BDNF concentration

Statistical analysis in the ipsilateral hippocampus indicated significant effect on *lesion* ($F(1,25)=8.42$, $P<.05$, partial $\eta^2=0.25$) and *lesion*supplementation* ($F(1,25)=4.10$, $P<.05$, partial $\eta^2=0.25$). Tukey's test evidenced that HISD group had a higher BDNF concentration when compared to CTSD, CTFA2 and HIFA20 groups. HIFA2 group was similar to CT groups and to HISD group, showing a partial recovery. The higher dose of FA was able to recover completely the BDNF levels in HI animals (Fig. 9). No effects were observed on the *supplementation* ($F(1,25)=2.04$, $P=.15$, partial $\eta^2=0.14$) and in the contralateral hippocampus (*lesion* ($F(1,31)=0.05$, $P=.82$, partial $\eta^2=0.002$), *supplementation* ($F(1,31)=0.04$, $P=.96$, partial $\eta^2=0.003$) and interaction ($F(1,31)=0.93$, $P=.40$, partial $\eta^2=0.06$)).

## 4. Discussion

This study was proposed to investigate how a diet supplementation with different doses of FA during pregnancy could affect pup rats that suffered a neonatal HI injury. This research is socially relevant due to its correlation with pregnant women who supplement with FA besides the quantity acquire by the diet. Our main findings revealed that both doses of FA (recommended by the WHO and excess of FA) can prevent memory deficits in adult animals caused by the HI insult. The supplementation did not prevent hippocampal neuronal death caused by the HI event but reversed the late increase of BDNF levels in this brain structure. These findings partially confirmed our initial hypothesis, since FA supplementation alleviated the HI-induced functional and some of neuronal consequences. Surprisingly, contrary to our proposition, even the excessive dose of FA achieved a protective role on this neonatal brain dysfunction.

Our group has been studying the behavioral deficits after neonatal HI in rats, which are well established in the literature. In the present study, we evaluated the animals in the following tasks: open field, water maze, novel-object recognition, and inhibitory avoidance. We selected these tasks based on these reasons: it is well established that animals' behavior in these tasks is changed by the neonatal HI; these tasks assess motor/cognitive/anxiety functions, which are important for neurodevelopment, particularly in the hippocampus; and finally we believe that these tasks could be helpful to identify possible functional effects of the FA supplementation.

### 4.1. Hyperactivity caused by neonatal hypoxia-ischemia in adult animals was not prevented by the FA supplementation

The open field task is used to evaluate anxiety and motor/exploratory function in rodents. In the present study, we found that the HI groups had more crossings than the CT groups. In addition, the HIFA2 group demonstrated a lower latency to leave the first square when compare to the CTFA2 group. These results indicated hyperactivity as a consequence of the injury, which was not prevented by the supplementation. The increased in motor and exploratory activities in rats affects their normal behavior and, consequently, enhances the probability of exposure to danger [39,51]. Hyperactivity was previously been observed in the HI model [50] and Miguel et al. [32,33] have proposed that the Levine-Vanucci model of HI can also be considered as a model for attention-deficit/hyperactivity disorder (ADHD). Few have studied the effects of FA supplementation and hyperactivity [8,19,63] but they have conflicting findings. In fact, our previous work did not show any effects of the HI lesion or the treatment with FA in the crossing assessment [14]. The current results suggest that FA supplementation does not beneficiate the brain structures related to hyperactivity behavior, such as the striatum and the frontal cortex. It is important to note that the exploratory function was evaluated in the present study due to its crucial role in cognitive processes, our focal point of investigation.

### 4.2. Folic acid supplementation during pregnancy prevented cognitive deficits caused by the HI

We have identified deficits in HI rats by assessing different memory protocols. We assessed the spatial memory in the Morris water maze. We observed that HI rats needed more days to learn the task and presented a poorer performance in the probe trial compared to controls. These results corroborate with previous studies that have demonstrated this spatial memory impairment due to neonatal HI [14,43,44]. It is well known the brain regions involved with Morris water maze performance: hippocampus, striatum and several cortical areas [15]. Importantly, these are the same regions affected by the neonatal HI injury [33,43,44], supporting poorer performance of HI rats in this task.

We also assessed the short-term memory of rats using the novel-object recognition task. As expected, the HI rats presented deficit in the preference index, not showing preference for the novel object. This

result suggests that HI rats did not differentiate the novel object from the familiar one, agreeing with previous studies that found the same impairment in HI animals in this task [43,50]. Damage to structures like the hippocampus and cortical areas affects the performance of rats in this paradigm [4] Additionally, we correlated the HI atrophy in these brain regions with the memory deficit observed in HI animals.

Finally, we assessed the emotional memory by the occurrence of an aversive event in the inhibitory avoidance task. Rats subjected to HI injury had a decreased latency to step-down the platform in the test day, corroborating with previous findings [14,50]. The hippocampus, amygdala and cortical areas are involved in the memory processes for the inhibitory avoidance behavior [25]. As discussed above, the HI injury affects these structures and, consequently, result in different types of memory deficits that are recognized in the literature. Since the hippocampus is one of the structures involved in all the tasks adopted and is the main area affected by the neonatal HI, it is reasonable to affirm that the HI cognitive deficits occur primarily due to this brain region atrophy.

Interestingly, both doses of FA were able to prevent these cognitive impairments. These results indicates that different levels of FA supplementation during pregnancy might exert a protective role in cases of neurological disturbance in the perinatal period. Given that insults that occur during the development of the fetus and in the peripartum period (like the neonatal HI) cannot be predicted, the findings of this study are promising, since the FA supplementation is already recommended during gestation. Contrary to the present findings, our previous results showed that direct FA administration caused spatial learning deficit in control rats without affecting the HI rats; additionally, FA resulted in hippocampal $Na^+$, $K^+$ ATPase dysfunction [13]. Using a similar treatment protocol, Sittig et al. [57] also found memory deficit in the Morris water maze task that was associated to reduced plasma concentration of thyroid hormones and their receptors in the rat hippocampus exposed to FA supplementation (8 mg/kg) during the adolescence. Considering these two works [13,57], it is reasonable to propose a common mechanism evolved in the negative results found. Our interpretation, in accordance with Sittig et al. [57], is that there is a particular vulnerability of the hippocampus to high FA levels in the early period of the development of rats. This proposition is based on some findings on the $Na^+$, $K^+$ ATPase activity: we identified recovery of the decreased $Na^+$, $K^+$ ATPase activity in the striatum and cortex in HI rats treated with FA [14] and, conversely, this enzyme activity is particularly impaired only in the hippocampus of animals that suffered neonatal HI and had FA administration [13]. Observing the present findings, it is reasonable to propose that FA acts in the hippocampus; this affirmation is based on the previous findings that suggested the particular vulnerability of this brain region to ischemic events and because the hippocampus is the central area for memory processes [33,50]. The protocol of FA treatment in the present study may be the main basis to justify our positive effects on the cognitive function; it is possible to assume that the FA supplementation during pregnancy have better outcomes than when FA supplementation was provided directly to the pups that suffered brain damage. Moreover, the supplementation during gestation in rats mimics the human condition better, having a more relevant social impact. Supporting this idea, a recent study supplemented the diet of mice with 20 mg/kg of FA before pregnancy until the end of lactation [5] and it was identified memory deficits and hippocampal atrophy in 3-week-old pups. These results supports our proposal that supplementation only in the gestation period, even in high doses, can be more effective in preventing memory impairments, as seen in the present study, and might not have a toxic effect on the hippocampal cells. Importantly, the cognitive benefit of FA supplementation in this study can still be observed in adulthood; it seems that FA supplementation during pregnancy provided additional support for the neurons, maybe mediating cellular survival after the HI injury, especially in structures like the hippocampus. Although we detected consistent functional deficits caused by HI and neuroprotective FA effects, future studies should increase the numbers of rats analyzed to confirm the potential of FA gestational supplementation. In the present study, small sample sizes were used for some groups due to the methodological limitations (i.e., mortality rate of ten percent in HI procedure or small litter size).

### *4.3. Neuronal death after HI in the ipsilateral hippocampus is not prevented by the FA supplementation*

To identify how the FA supplementation preserves the memory function in the HI rats, we analyzed the neuronal density by cresyl violet staining and NeuN immunohistochemistry. As expected, in the HI rats we found a diminished neuronal density in the right hippocampus (ipsilateral to arterial occlusion). This massive cellular death leads to hippocampal atrophy and, consequently, affects the memory parameters [33,43,44]. Contrary to our hypothesis, FA in both doses failed to prevent the hippocampal neuronal loss. FA supplementation during pregnancy is crucial to the brain development because this vitamin is a one-carbon donor. This unit is necessary for nucleotide synthesis and consequently for DNA replication and cell proliferation. Also, FA is directly involved in the remethylation of homocysteine to methionine, which is the primary methyl donor in most mammalian reactions [11]. In a rat model of folate deficiency during pregnancy, the FA supplementation protected the hippocampal ultrastructure of the adult offspring [66]. Therefore, it would be appropriate to assume that FA was neuroprotector, since a wide range of studies has suggested this possibility. This proposal is also founded on our previous results in which cognitive recovery with no effect on the hippocampal atrophy was identified, after a period of environmental enrichment in HI rats [43,44]; the protective effect was later identified on the spine dendritic density [50]. Thus, in the present study the FA intervention should be occurring in another target of action, probable involved in the neuroplasticity pathways.

### *4.4. Increased BDNF levels in the hippocampus after HI is prevented by the FA supplementation – effects on the neuroplasticity*

Intending to identify the possible role of the FA supplementation on the components involved on neuroplasticity, we evaluated the BDNF level and the synaptophysin expression in the hippocampus. The calcium-binding synaptophysin protein is located in the presynaptic vesicles being related to the synaptic function and neuroplasticity in hippocampal neurons [58]. In the present work we did not find any effect by the lesion or the supplementation in the synaptophysin densitometry, despite of the findings in the neuronal density. In a study using the same HI model, the authors also did not identify differences in the synaptophysin expression in the CA1 region of the hippocampus [64]. Given that HI rats had decreased neuronal density in the ipsilateral hippocampus, it is possible to propose that there was an upregulation of the expression of the synaptophysin as a compensatory mechanism. Since there is less number of neurons, the organism tries to increase the number of synapses in an attempt to avoid the functional deficits after the injury. From the present findings, we can propose for future studies to investigate other markers of neuronal plasticity such as synapsin-1, involved in the regulation of neurotransmitter release at synapses [60], and postsynaptic density protein (PSD-95), which is an important regulator of synaptic strength and has a central role in maintain the structural organization of the postsynaptic density [2]. In this context, our previous study demonstrated a decreased spine density in the hippocampus of HI rats [50]; maybe FA supplementation can be effective to establish a favorable environment to develop/maintain the synaptic function.

In agreement with our hypothesis, the FA reversed the HI-induced BDNF imbalance. High level of this neurotrophin was identified in HI rats in the ipsilateral hippocampus and the FA supplementation was able to maintain the BDNF levels compared to control rats. BDNF is a neurotrophic factor that exerts an important role in the cell survival and neuroplasticity [59]. Firstly, we can elucidate the effect of the increased BDNF levels in a chronic period after HI; we have demonstrated this finding in hippocampus of adult rats submitted to neonatal HI [42]. Interestingly, in such study the environmental enrichment also prevented this late increase in the BDNF which was associated with cognitive improvement. Thus, in that case as in the present study, it is reasonable to suppose that the late increased BDNF level can result in long term tissue vulnerability and, possibly, to a neurotoxic effect rather than neuroprotection. Increasing in the BDNF levels was also observed after a stroke model in rats [9]; and Vidaurre et al. [62] have proposed an excitotoxic effect of BDNF that might be related to an imbalance in the expression of different isoforms of the TrkB receptor. The BDNF binds to the TrkB receptor and activate different pathways that result in increased $Ca^{2+}$ release from the endoplasmic reticulum. This can lead to cell death or increase in protein transcription that can also exert neurotoxic effects to the neuron [17]. When the BDNF binds to their Trk receptors, the PI3K/AKT/mTOR pathway is activate and leads to the expression of the hypoxia inducible factor-1 alpha (HIF-1 alpha) [37,46]. This factor is a key mediator of the protein transcription that will stimulate the hypoxic phenotype expression [18]. This could explain why the late increase in the BDNF after the injury is not protective and can be related to the memory impairments observed in the HI animals.

Interestingly, both doses of FA supplementation reversed the increased levels of BDNF in HI animals indicating a possible mechanism of neuroprotection. The hypothesis of HIF-1 alpha is also supported by a recent study that demonstrated that FA decreased HIF-1 alpha in a hypoxia-induced injury model in cell culture [29]; decreasing this factor, the HI-induced brain damage can be partially prevented. Moreover, given that it is well established the important role of the FA on the methylation of several proteins, DNA and other molecules [11] and that the BDNF expression can be inhibited by the increased DNA methylation [16], it is possible to hypothesize that the high dose of FA is inhibiting the BDNF expression by enhancing the DNA methylation. We can then conclude that balancing the levels of this important neurotrophin, FA supplementation was able to alleviate the cognitive deficits caused by the neonatal injury.

## 5. Conclusions

In conclusion, our findings evidenced that FA supplementation during pregnancy was able to reverse cognitive impairments after the neonatal hypoxia-ischemia in rats. The supplementation did not prevent neuronal loss in the hippocampus but was effective in reversing the late BDNF imbalance, observed after the injury in the hippocampus. Such results demonstrated that FA during the gestation might have a potential protective effect in an unpredictable insult, such as the neonatal HI. Additionally, the present findings are particularly relevant due to the fact that FA neuroprotection was achieved even in high level of the FA supplementation during pregnancy, indicating that this intervention could be considered safe for the offspring development. However, in view of the fact that there are conflicting results in the literature about this issue, the clinical significance of these findings deserves careful discussion and further studies.

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## Compliance with ethical standards

To carry out this study, the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the guide of the Federation of Brazilian Societies for Experimental Biology were used. This project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28136).

## Conflict of Interest

The authors declare that they have no conflict of interest.

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# 5. CAPÍTULO 2

"FOLIC ACID SUPPLEMENTATION DID NOT IMPACT H3 METHYLATION LEVELS IN THE HIPPOCAMPUS OF RATS SUBJECTS TO NEONATAL HYPOXIA-ISCHEMIA"

(Submetido à Journal of Neurochemistry)

# FOLIC ACID SUPPLEMENTATION DID NOT IMPACT H3 METHYLATION LEVELS IN THE HIPPOCAMPUS OF RATS SUBJECTED TO NEONATAL HYPOXIA-ISCHEMIA

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## ABSTRACT

Neonatal hypoxia-ischemia (HI) affects, primarily, structures like hippocampus and striatum increasing neuronal death and decreasing proteins expression related to structural plasticity. Recently, it was demonstrated that epigenetics mechanisms, including histone methylation, play an important role in the HI pathophysiology and that folic acid (FA) supplementation during pregnancy can affect these epigenetics mechanisms and gene expression. However, we have identified a dual effect of FA treatment in neonatal HI animals. The aim of this study was to evaluate how neonatal HI affects: apoptosis (caspase-3) and synaptic (synapsin and PSD-95) proteins expression, and the methylation of histone H3 lysine (K) 4 and 27 in the hippocampus of adult animals. In addition, this study sought to investigate the effects of two different doses of FA supplementation during pregnancy on HI animals. Pregnant Wistar rats were divided according to their diets: standard (SD), supplemented with 2 mg/kg of FA or with 20 mg/kg of FA. HI procedure was performed at the 7$^{th}$ PND. Protein expression and H3 methylation were evaluated at the 60$^{th}$ PND in the rats' hippocampus.Neonatal HI resulted in increased caspase-3 expression, decreased synapsin expression and reduced H3K4me2, -me3 and H3K27me2, -me3 in the ipsilateral hippocampus. FA supplementation only prevented the augment in caspase-3 expression in the ipsilateral hippocampus. In conclusion, neonatal HI presented long-lasting effects in caspase-3-mediated cell death (prevented by the FA supplementation) and synaptic proteins in the hippocampus of adult rats. This is the first study to show that histone modifications may contribute to these chronic pathological findings in the hippocampus of HI animals.



**Keywords:**Perinatal asphyxia, Folate, Vitamin, programmed cell death, histone methylation


**Abreviations:** AIN: American Institute of Nutrition, BDNF: Brain-derived neurotrophic factor,CA: Cornu Amonis, FA: folic acid, H: histone, HIF: hypoxia-inducible factor,HI: hypoxia-ischemia, K: lysine, NTD: neural tube defects, PND: post-natal day, PSD: postsynaptic density.

## INTRODUCTION

Neonatal hypoxia-ischemia (HI) commonly occurs during the peripartum period and might cause permanent sequelae for the newborn and throughout the lifespan. The prevalence of neonatal HI in developed countries is around 3 per 1000 live births, whereas in developing countries can increase up to 22 per 1000 live births [1,2]. The etiologic factors can be placental abruption, prolonged labor, umbilical cord dysfunction and preterm labor [3,4]. As consequence of HI events, it has been found cerebral palsy, motor and cognitive disability, attention deficit/ hyperactivity disorder and epilepsy [5-7]. In neonatal HI, the brain damage, primarily in hippocampus and striatum, initiates right after the injury and continues during days, or even weeks, due to cellular death by apoptosis having the caspase-3 (cysteine-dependent, aspartate-specific peptidases) as a principal effector of this pathway [8-13]. Such nervous tissue injury involves massive cell death and structural neuroplasticity impairment by decreasing dendritic spines density and the expression of proteins involved in the synapses, like synaptophysin, PSD95 (postsynaptic density 95) and synapsin [14-16]. Consequently, such morphological damage will culminate in long-lasting cognitive deficits in rats [10-12, 14, 17-20].

It is well established that there are three main mechanisms involved in the HI pathophysiology: glutamatergic excitotoxicity, oxidative stress and inflammation [2,21,22]. Recently, studies have shown that epigenetic mechanisms, such as histone modifications, might be also implicated in the HI damage, possibly modifying the gene expression pattern of proteins responsible for the HI phenotype, like the hypoxia-inducible factor-1 α (HIF-1α) [23,24]. In this context, the use of inhibitors of histone deacetylase has shown promise results as a neuroprotection target after the neonatal HI [25,26]. Even though there are some promising results regarding the influence of epigenetic modifications on neurological diseases, there are few studies focusing on the HI neuropathology.

Epigenetic regulation modulates gene expression without mutation of the DNA sequence, which can have a crucial role in neonatal HI insults, particularly because these mechanisms are underlying the processes of developmental plasticity [27]. The main epigenetics modifications are DNA methylation/ demethylation, histone and microRNA modification by methylation, acetylation and/or phosphorylation [28]. Histones modifications have also been related with plasticity and memory, specially the H3 histone in the hippocampus

[29,30]. Methylation of the lysine (K) residues of this histone can have diverse effects on gene expression. Each residue can be mono-,di- or trimethylated and it is known that dimethylated H3K4 (H3K4me2) and trimethylated H3K4 (H3K4me3) are involved in increasing gene expression, while H3K27me2 and H3K27me3 has a role in repressing gene expression [31-33]. Notwithstanding the importance of histone methylation in cognitive processes, in health and disease, the influence of histone methylation on neonatal brain insults is poorly understood.

Playing an important role in methylation processes, the folic acid (FA), a B-complex vitamin, acts directly in the remethylation of the homocysteine to methionine, which is the principal methyl donor in organism to protein, DNA/RNA, histone and lipids [34]. Moreover, this vitamin acts in the prevention of neural tube defects (NTD), being recommended a 400 μg dose of FA per day one month before until the end of the first trimester of pregnancy [35,36]. Recently, data have pointed to the fact that normonutride women might have an excessive FA consumption [37,38] and the impact of this undue consumption, particularly on the offspring, is little researched. Studies using animal models have evidenced a dual effect of high dose of FA during pregnancy: increased DNA methylation, altered gene expression pattern in the hippocampus, cognitive deficits and decreased neurotrophins levels, like brain-derived neurotrophic factor (BDNF) [39-42]. Conversely, it was also observed improving in early reflexes, spatial learning, motor disability [43] and alleviating inflammatory parameters in an animal model of schizophrenia [44]. Our previous studies using the administration of FA (5 mg/kg) as a treatment to neonatal HI also identify dual effect of this vitamin. FA was able to reverse aversive memory impairment, anxiogenic effect and the inhibition of the Na+, K+ ATPase activity in frontal cortex and striatum after the HI injury in rats [17]. Surprisingly, the folate impaired the spatial memory in control animals and decreased the Na+, K+ ATPase activity in the hippocampus in HI animals [10]. In view of these conflicting findings, we can propose that the impact of the FA supplementation during pregnancy should be better investigated particularly in critical newborns, as in cases of neonatal asphyxia.

Taken that the neonatal HI is a severe injury that cause functional deficits from childhood until adulthood, it is important to study the neuropathological mechanisms and new therapeutic strategies. Since FA supplementation is already used during pregnancy, it is

possible that it might exert an effect in different pathways including epigenetics modifications in the HI insult. Also, it is important to elucidate the effects of an excessive dose of FA in these parameters. Therefore, this study proposes to investigate the effects of a recommended and an excessive dose of FA during pregnancy evaluating cellular death (caspase-3), plasticity (synapsin and PSD95) and epigenetics modifications (methylation of H3K4 and H3K27) in the hippocampus of adult rats submitted to neonatal HI.

## EXPERIMENTAL PROCEDURES

### Animals

Wistar rats from the local breeding colony (Institute of Basic Health Science, Federal University of Rio Grande do Sul, Brazil) were used to carry out this study. They were maintained in a room with controlled temperature (22 ± 1°C), light/dark cycle (12/12 hours) and received food and water *ad libitum*. Vaginal smears were collected daily from the female rats to detect the receptive phase (proestrus) of estrous cycle. Each receptive female was put in a different cage with one male rat to spend the night. When mating was confirmed it was considered the embrionary day (E) 0 and the pregnant rats were divided into 3 groups, according to diet: 1) Standard diet (SD), 2) Supplemented with 2 mg/kg of FA (FA2) and 3) Supplemented with 20 mg/kg of FA (FA20). At postnatal day (PND) 7, the day of HI procedure, puppies were then divided into 6 groups: 1) Control with SD diet (CTSD), 2) Hypoxia-ischemia with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). A timeline of experimental procedures is depicted in Fig. 1. All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the Arouca Law (Law nº 11.794/2008). Also, this project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28136).

### Folic acid supplementation

The supplementation with 2 mg/kg of FA was used because it is considered the equivalent for rats of the appropriate level of supplementation for pregnant women (400 µg per day)[45]. The higher dose of FA (20 mg/kg) was chosen to mimic the excessive consumption of FA that can occur by supplements intake and by the diet itself [46]. All

diets were prepared following the AIN-93 Purified Diets for Laboratory Rodents (American Institute of Nutrition) [47] with protein level in the standard and treatment diets was 18% and all of them were isocaloric. The SD diet contained 2 mg/kg of FA that is need for the proper animal maintenance and the supplemented diets had an addition of 2 or 20 mg/kg of FA. FA supplementation occurred after mating confirmation until the end of the gestation. After the pups birth, all animals received the SD diet until the end of the experiments.

**Neonatal hypoxia-ischemia**

At the 7$^{th}$ PND puppies were submitted to the Levine-Vanucci model of HI [48,49]. They were anesthetized with halothane and a ventral incision was made in the neck. The right commom carotid was identified, isolated and permanently occluded with surgical silk thread and, then, the puppies returned to their mothers for a 2h recovery period. After that, animals were placed in a hypoxic environment during 90 minutes (8% oxygen and 92% nitrogen). Control animals were submitted to anesthesia and incision, but did not have the arterial occlusion or the hypoxia exposure [12,20].

**Tissue preparation**

At the 60$^{th}$ PND animals were euthanized by decapitation and both hippocampi, ipsilateral and contralateral to arterial occlusion, were quickly dissected and frozen in liquid nitrogen. The samples were stored at -80°C until the biochemical assays.

**Elisa assay**

The EpiQuik$^{TM}$ Histone H3 Modification Multiplex Assay Kit (#P3100, Epigentek) was prior used as a screening of epigenetic modifications in the histone H3, since it evaluates the levels of: H3K4me1, me2 and me3; H3K9me1, me2 and me3; H3K27me1, me2 and m3; H3K36me1, me2 and me3; H3K79me1, me2 and m3; H3K9ac; H3K14ac; H3K18ac; H3K56ac; H3ser10P; H3ser28P. Global histone methylation of H3K4 (Kit #P-3017, Epigentek) and H3K27 (Kit #P-3020, Epigentek) was evaluated according manufacturer's instructions. Briefly, right (ipsilateral) hippocampus samples were homogenized and the histone fraction was extracted. Samples were placed into the assay wells and blocked at 37°C for 45 minutes; after they were incubated with the captured antibody (1:100) at room temperature for 60 minutes on an orbital shaker (100 rpm). Detection antibody (1:1000) was incubated at room temperature for 30 minutes and, then, developing solution was added to the wells at room temperature and protected from the light to allow the color

development. Absorbance was read at 450 nm. BCA protein assay (Thermo Scientific) was used to quantify total protein in the samples and aliquots were prepared with 400 ng of protein. Results are expressed in percentage of methylation in relation to control group CTSD.

**Western blotting analysis**

Caspase-3 (protein involved in the apoptosis pathway), synapsin (presynaptic protein related to the regulation of neurotransmitter release and neuroplasticity), PSD-95 (postsynaptic density protein), H3K4me2 and H3K4me3 (methylation of lysine 4 in Histone H3 is related to increased gene expression), H3K27me2 and H3K4me3 (methylation of lysine 27 of H3 is related to repression of gene expression) levels were evaluated by western blotting technique. The hippocampi samples were homogenized with citosolic extraction buffer (complete, Thermo Scientific) and then centrifuged at 3,000 rpm (4°C) for 10 minutes. BCA protein assay (Thermo Scientific) was used to quantify total protein in the samples and aliquots of 20 µg were prepared with DTT (Sigma-Aldrich) and NuPage 4x (Thermo Fischer). Protein denaturation occurred at 95°C for 15 minutes. Electrophoresis was performed on 4-12% polyacrylamide gradient gels (Invitrogen) and, subsequently, samples were transferred to a nitrocellulose membrane (Invitrogen) and blocked with BSA for 4 hours. The membranes were incubated overnight at 4°C with the following primary antibodies: anti-actin (Sigma-Aldrich, A4700, 1:10000), anti-caspase-3 (Cell Signaling, 9661S, 1:1000), anti-synapsin (Millipore, AB1543P, 1:2000), anti-PSD-95 (Abcam, 18258, 1:2000), anti-H3 (Epigentek, A-1112-100, 1:5000), anti-H3K4me2 (Epigentek, C10000, 1:10000), anti-H3K4me3 (Epigentek, C10000, 1:10000), anti-H3K27me2 (Epigentek, C10001, 1:10000), anti-H3K27me3 (Epigentek, C10001, 1:10000). After washing the membranes, they were incubated with the secondary antibodies anti-mouse 680 (Thermo Fischer, A28184, 1:5000) and anti-rabbit 800 (Thermo Fischer, A32735, 1:5000) at room temperature, protected from light, for 2 hours. The fluorescent signal was detected by the Oddisey Clx equipment (Li-Cor Biosciences, USA) and the intensity of bands was quantified using the ImageJ® software (National Institute of Health, USA) by densitometry. Caspase-3, synapsin, PSD-95 and H3 proteins were evaluated as a ratio with the actin expression. H3K4me2 and me3; and H3K27me2 and me3 were

analyzed as a ratio with the H3 expression. All results are presented as a percentage of the control group CTSD.

**Statistical analysis**

To all variables were performed two-way ANOVA followed by Tukey's post-hoc test, with *lesion* and *supplementation* as factors. The Statistic© software package was used, and differences were considered significant when $p<0.05$.

## RESULTS

### HI-induced Caspase-3 expression increase in the ipsilateral hippocampus was prevented by gestational FA supplementation

Two-way ANOVA revealed significant effect of lesion ($F(1,12)= 13.9$; $p<0.05$), supplementation ($F(2,12)= 3.9$; $p<0.05$) and interaction between factors ($F(2,12)= 4.6$; $p<0.05$), considering the caspase-3 expression in the ipsilateral (right) hippocampus. Tukey's test indicated that both HISD and HIFA2 groups had increased caspase-3 expression compared to all control groups and, additionally, supplementation with high dose FA prevented this effect. In the contralateral hippocampus the statistical analysis demonstrated lesion ($F(1,12)= 36.1$; $p<0.05$) and supplementation factors($F(2,12)= 6.2$; $p<0.05$), without significant interaction.Caspase-3 expression seems to be in low levels in all HI groups in the contralateral hippocampus (Fig. 2).

### Synaptic proteins were altered by the HI insult

Considering synapsin expression, significant main effect was observed on lesion factor in both ipsilateral ($F(1,12)= 5.1$; $p<0.05$) and contralateral hippocampus ($F(1,12)= 12.6$; $p<0.05$); decreased levels of this protein was observed in HI animals (Fig. 3A). No effect was achieved with FA supplementation. On PSD-95 expression, no difference was identified between groups (Fig. 3B).

### Epigenetic modifications

Based on the data collected with the EpiQuik$^{TM}$ Histone H3 Modification Multiplex Assay Kit, the H3K4 and the H3K27 were elected as the H3 targets for the present study (data not shown).

**Total H3K4 and H3K7 methylation was not change by HI or FA supplementation**

Using an ELISA assay, the total H3K4 and H3K7 methylation were assessed in the ipsilateral (right) hippocampus. Two-Way ANOVA did not identify significant effect on *lesion* or *supplementation* factors neither on the H3K4 nor on the H3K7 total methylation (Fig. 4).

The following results on dimethylated and trimethylated H3K4 and H3K7 are presented as a proportion in relation to total H3 immunocontent.

**Neonatal HI affected H3K4me2 and me3, and H3K7me2 and me3 expression**

On both relative H3K4me2 (Fig. 5A) and H3K4me3 (Fig. 5B) expression, two-way ANOVA showed significant effect of lesion factor in the ipsilateral (right) hippocampus: ($F(1,12)= 11.4$; $p<0.05$) and ($F(1,12)= 8.8$; $p<0.05$), respectively. The levels of proportion of H3K4 methylation/ H3 was lower in the hippocampus of HI rats, compared to controls. An apparent compensatory effect was observed in the contralateral hippocampus: ANOVA main effect indicated high relative H3K4me2 expression induced by the HI. Gestational FA supplementation did not result in significant changes on the H3K4me2 or me3 expression in the rat hippocampus. In harmony with the findings on H3K4 methylation, relative H3K7me2 (Fig. 6A) and me3 (Fig. 6B) in the ipsilateral hippocampus also resulted in significant main effect on lesion factor ($F= 27.0$ and 13.2, respectively; $p<0.05$) with lower expression in the HI groups. No differences were identified neither considering supplementation factor nor in relation to the contralateral hippocampal H3K7 methylation.

## DISCUSSION

This was the first study that investigated how a normal and an excessive dose of FA supplementation during pregnancy could affect the expression and methylation levels of different proteins in the hippocampus of adult rats that suffered neonatal HI. We observed that the HI lesion caused a late change in the expression of caspase-3 and Synapsin. Also, in adult rats, neonatal HI resulted in decreased hippocampal methylation levels in di-, trimethylated H3K4and H3K27, indicating long lasting effects on epigenetics mechanisms. Previous studies have evidenced differential gene expression after neonatal HI, some genes had increase and others presented decreased expression [50,51]. The findings of the present study suppsort the hypothesis that this altered gene expression observed after the HI injury

can be related to histone modifications, particularly on the H3 histone methylation levels. The higher dose of FA supplementation was able to prevent the increase in the caspase-3 levels without changing the epigenetic modifications in the ipsilateral hippocampus.

After the neonatal HI, data have already demonstrated that apoptosis continues for days, even weeks, [8,9] contributing to the enlargement of the HI brain damage. Caspase pathway is the main mechanism that can mediate this process of programmed cell death by apoptosis. Caspase family proteases are involved in the regulation of cell survival or death, inflammation, proliferation and differentiation. The apoptotic caspases can be divided into two groups: the initiators (caspase-2,-8,-9) and the executioners (caspase-3,-6,-7), being the latter responsible for the attack to structural and regulatory cell proteins [52,53]. The caspase-3 is the last to be activated in the apoptotic pathway, being the final executioner and one of the most studies. The results of the present study confirmed the increased caspase-3 expression in adult animals (60 DPN) that suffered neonatal HI, indicating that the cellular death continues after a long period, possibly contributing to the cognitive deficits previously observed [11,12,19]. Interestingly, in this study the high dose of FA was able to prevent the augment in the caspase-3 expression in the ipsilateral hippocampus. This result can indicate that FA prevented the advance of HI-induced cellular loss, caspase-dependent, in the hippocampus. Some mechanisms can be implicated in this protective effect. FA has been demonstrated to exert an antiapoptotic role, acting directly in the mitochondrial permeability transitions and decreasing pro-apoptotic proteins expression [54,55]. It has been also demonstrated the antioxidant properties of this vitamin, increasing the antioxidant enzymes activities and/or scavenging free radicals [56,57]. Additionally, it is also possible that FA can act as a regulator in the expression of cellular survival factors, like BDNF, allowing the survival and functioning of neurons in the hippocampus [39]. Moreover in [10], it was observed a recovery in the Na(+), K(+)-ATPase activity in the hippocampus of HI adult rats treated with FA. This data can be correlated with improvement in aversive memory impairment caused by the HI lesion [17], indicating that this enzyme is important to memory formation and may be a potential target of FA protection. In summary, we can suggest that FA exert a protective effect probably mediated by preventing the increased caspase-3 expression. Other mechanisms of action of the protective effect of this vitamin need to be further investigated.

It has been well established that neonatal HI affects cellular function by influencing the structural plasticity. Several changes occur during the synaptic plasticity process such as increase in presynaptic vesicles, neurotransmitter receptors, dendritic spines and its stabilization [58]. Expression of synaptic proteins considered markers of plasticity was evaluated in this study. The synapsins (four types) are peripheral membrane proteins present in the presynaptic vesicles [59], while the PSD-95 is a component of the postsynaptic density required to maintain its molecular organization [60]. Both are important to normal function of synapses and plasticity being related in learning and cognitive process [61]. In the present study, decreased expression in synapsin expression consequent to HI was observed, without change in PSD-95 levels, in the ipsilateral hippocampus of adult rats. Corroborating with our findings on synapsin expression, it was recently demonstrated that neonatal HI affects the expression of this protein [15,16]. In this context, in [13] it was found that HI injury decreased the dendritic spine density in the hippocampus and all these findings can be correlated with memory deficits observed after the HI [12,14]. Unexpected, FA supplementation during pregnancy did not prevent the synapsin decrease expression, indicating that this protein is not directly involved in the mechanisms of neuroprotective effect of this vitamin. As mentioned, FA participates in the methylation reactions, donating its methyl group to remethylate homocysteine in methionine, principal methyl donor in the cells [34]. This potential of FA motivated the present study since cognitive improvement by this vitamin was observed in HI animals [10,17] and altered gene expression was also found in this injury.

The starting point in which the mechanisms of neuropathological processes and neuroprotective effects are based, in most of the cases, is the modulation of gene expression. Epigenetic mechanisms can alter gene expression by DNA, microRNA and histone modifications. The DNA is wrapped in an octamer of histone that are subdivided in H2A, H2B, H3 and H4 histone; each one can be methylated, acetylated and phosphorilated in its K residues [27,28,62]. Recently it has been discussed that epigenetic processes are involved in the pathophysiology of neonatal HI. Altered gene expression and DNA methylation pattern were identified [23,24]; also, histone acetylation was related with HI damage [25,26]. Considering the memory deficits observed after the injury and that there are evidence of the histone H3 modifications in the learning and memory processes [29,30],

we decided to evaluate different levels of methylation in two important K residues (4 and 27) in this histone. To our knowledge, this is the first study to evaluate the methylation of histone H3 after neonatal HI. We observed that, even in adulthood, di- and trimethylation of H3K4 and H3K27 are decreased in the ipsilateral hippocampus after neonatal HI. To better interpret these data, it is important to consider that methylated H3K4 leads to activation of gene expression and, conversely, methylated H3K27 is related with silence gene expression [31-33]. In that regard, [50] showed a differential gene expression pattern after neonatal HI, with overexpression of genes related to transcription, stress and apoptosis and downregulation of ion transport and signal transduction (like G protein-related proteins) related genes. These results confirm the importance of gene expression modulation in disease pathophysiology. In the present study, the hippocampal histone modulation supports some possible interpretations, to understand the mechanism of HI pathology. Our data evidenced modification in both residues, K4 and K27, proposing that for the HI neuropathology some genes are more expressed (like pro-apoptotic proteins) and other are suppressed (like plasticity proteins).

We can suggest that HI event induced histone methylation changes, which were responsible, at least in part, for decreased expression of proteins involved. Given support for this proposition, [63] demonstrated that decreased Syn1 expression in the hippocampus and poor performance in the water maze was associated with increased DNA methylation in its promoter gene region in aged rat model. This data corroborates with ours since we observed a decreased expression of Syn and also decreased H3K4me, which is responsible for gene expression activation. Considering that increase in DNA methylation and demethylation of H3K4 histone in the promoter region are both associated with decreased expression of its genes, we can propose that this epigenetic modification is directly related with the decreased synapsin expression in the ipsilateral hippocampus. In addition, H3K4 methylation was also correlated with long-term memory plasticity and hippocampus-dependent memory function [64,65]. Considering this, it is possible to infer that the decreased expression of synapsin and H3K4me2,-me3 in the ipsilateral hippocampus of HI animals are inter-related and directly associated with the cognitive deficits previously observed after the HI injury [12,14]. It is well documented that neonatal HI affects plasticity parameters such as decreased expression of synaptic-related proteins

(synaptophysin, synapsin and PSD-95) and dendritic spine density [14-16]. Nevertheless, the molecular mechanisms involved in these alterations are not deeply understood. This study sought to contribute to comprehension of HI neuropathology by evaluating the methylation levels of histone H3 lysine 4 and 27; we have shown for the first time that these histone modifications are involved in lifelong manner in the HI pathology and might be, at least partially, responsible for the decreased Syn expression observed in the hippocampus of HI animals.

On the other hand, the histone modulation observed in the present study might also elucidate the increase in caspase-3 expression found in the ipsilateral hippocampus of HI animals. The relation of histone changes and apoptosis has been already described in a model of optic nerve crush, in which valproate treatment was able to alleviate neurodegeneration associated to reduced caspase-3 expression and H3K9 methylation [66]. It is important to consider that H3K9me is associated to gene repression, as H3K27me. The study [67] also found increase in H3K27 methylation correlated with increased caspase-3 expression in the hippocampus of rats submitted to a transient exposition to alcohol in the neonatal period. Controversially, we found a decreased H3K27 and H3K4 methylation and increased caspase-3 expression. In agreement with our findings, [68] observed that decreased H3K4me2 levels were associated with increased caspase-3 expression and cellular loss in auditory hair cells. Taking these findings together, we can propose that the decreased in H3 methylation in adults HI animals might be collaborating to the persistent increase in caspase-3 expression. We also identified that FA supplementation was able to reduce caspase-3 expression in the ipsilateral hippocampus without changing the methylation levels of H3K4 and H3K27. These data indicate that, besides its relation to methylation reactions, FA is preventing caspase-3-mediated cell death without modifying these epigenetic mechanisms. Alternatively, we cannot to reject the hypothesis that FA supplementation can be acting on histone modification, but its action can be transient, maybe particularly on early neurodevelopment.

It is necessary also to consider that besides of the targets described above, the changes in histone methylation pattern can have other consequences. For example, the modified methylation in histones H3K4 and H3K27 observed in this study might be causing a imbalance in BDNF expression. This neurotrophic factor has an important role in cell

survival and neuroplasticity [69] and it was already demonstrated that its expression is altered by epigenetics mechanisms [70-73]. The study [71] investigated the effects of a deficient iron diet during pregnancy in the development of the offspring. They found an increase in the H3K27me3 and a decrease in the H3K4me3 in the promoter region of BDNF in the hippocampus. These differences in methylated lysine residues in H3 were correlated with diminished BDNF expression in the hippocampus of the offspring. In [72] it was observed an increased in H3K27me3 in the promoter region of BDNF and a decreased expression of this factor in the ventral tegmental area induced by morphine. Taking these findings together, it is possible to infer that the altered histone methylation might also result in HI-induced BDNF imbalance, already observed in our previous studies [18].

In conclusion, this study evidenced that neonatal HI has lasting effects increasing caspase-3 and decreasing synapsin expression in the ipsilateral hippocampus in adult rats. Such alterations were associated with changes in H3K4 and H3K27 status in the hippocampal tissue. This study was the first to evaluate the histone modifications in the HI model, demonstrating that these epigenetic mechanisms are involved, at least in part, in chronic pathophysiology of this injury. However, future studies that correlate this epigenetics modification with specific promoter genes might collaborate with better understand of the HI injury pathway. High dose of FA supplementation was only able to prevent the late increase in the caspase-3 expression. In this aspect, another important contribution of this study is that FA supplementation did not intensify the brain damage consequent to neonatal HI or even alter parameters in control animals, indicating that this vitamin supplementation during pregnancy can be safe for the offspring development.

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**Compliance with ethical standards**To carry out this study, the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the guide of

the Federation of Brazilian Societies for Experimental Biology were used. This project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28136).

**Conflict of Interest**The authors declare that they have no conflict of interest.

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**LEGENDS AND FIGURES**

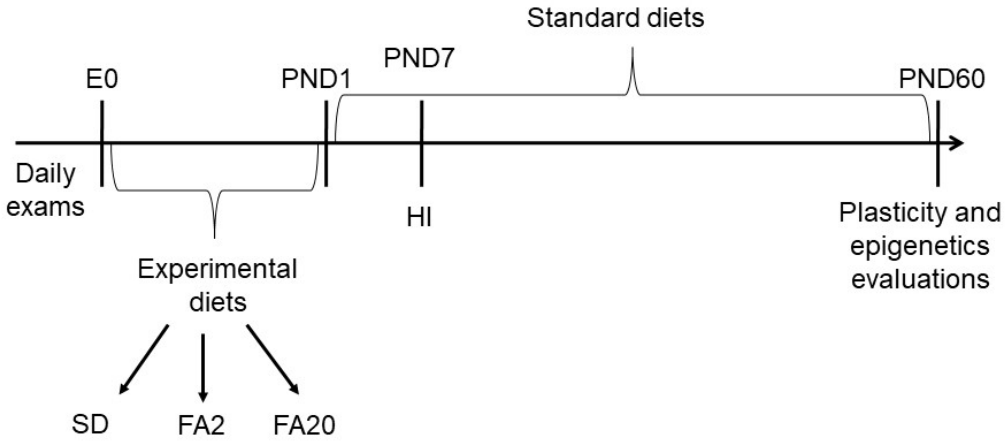


Fig. 1. Timeline of experimental procedures – After mating confirmation (E0), pregnant Wistar rats were divided into 3 groups, according to diet: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). At PND 1 (when puppies where born) all animals started to receive the SD diet until the end of experiments. At the PND 7, the Levine-Vannucci model of HI was performed, resulting in 6 groups in the offspring: 1) Control with SD diet (CTSD), 2) Hypoxia-ischemia with SD diet (HISD), 3) Control with FA2 diet (CTFA2), 4) HI with FA2 diet (HIFA2), 5) Control with FA20 diet (CTFA20) and 6) HI with FA20 diet (HIFA20). At PND 60, puppies were euthanized, and plasticity and epigenetic assays were assessed.

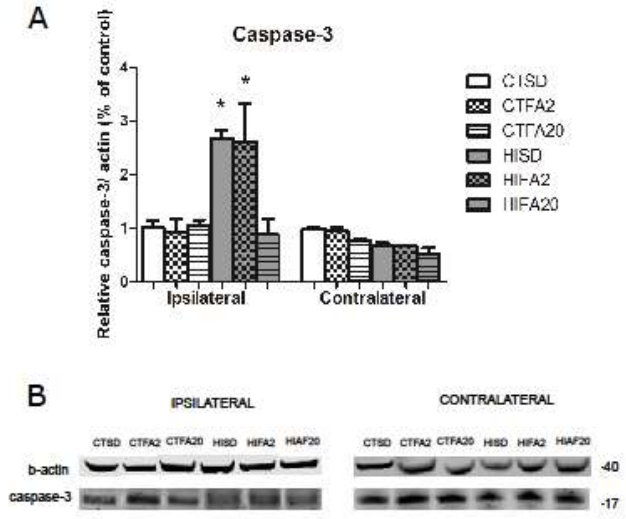


Fig. 2. Caspase-3 quantification – Protein expression of caspase-3 in the hippocampus (A) and representative bands of each protein (B). * Different from all CT groups and HIFA20 group. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group.

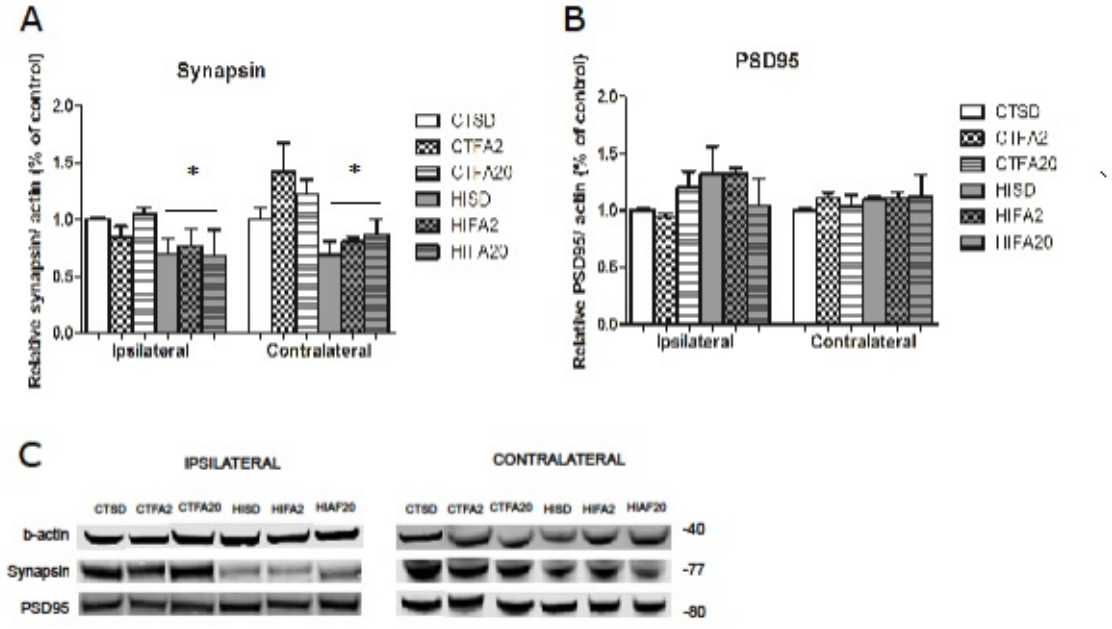


Fig. 3. Synaptic proteins quantification – Synapsin (A) and PSD-95 (B) expression in the hippocampus and representative bands of each protein (C). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group.

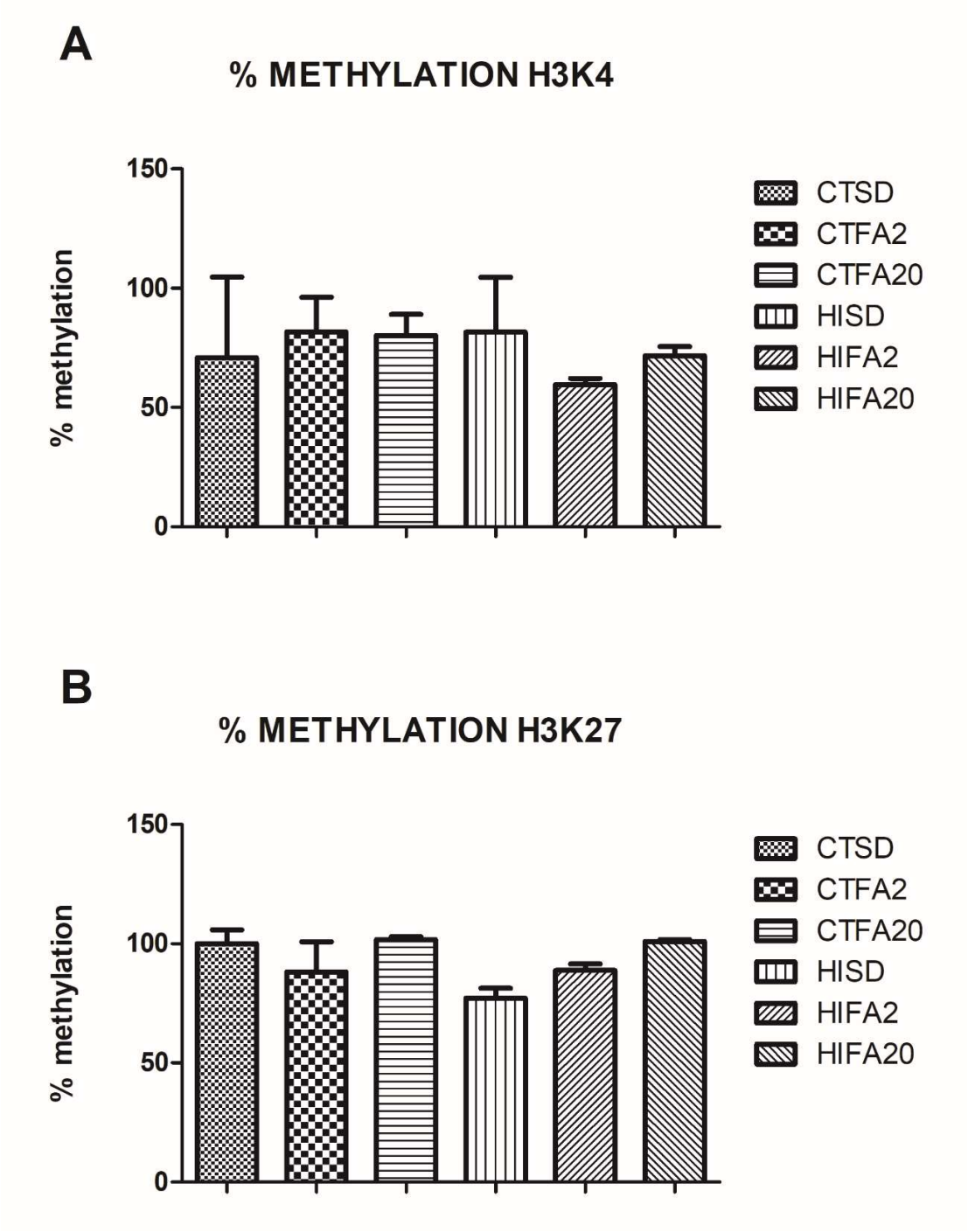


Fig. 4. Percentage of total methylation of H3K4 and H3K27 – Quantification of H3K4 (A) and H3K27 (B) methylation in the ipsilateral hippocampus. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 - 4 animals/ group.

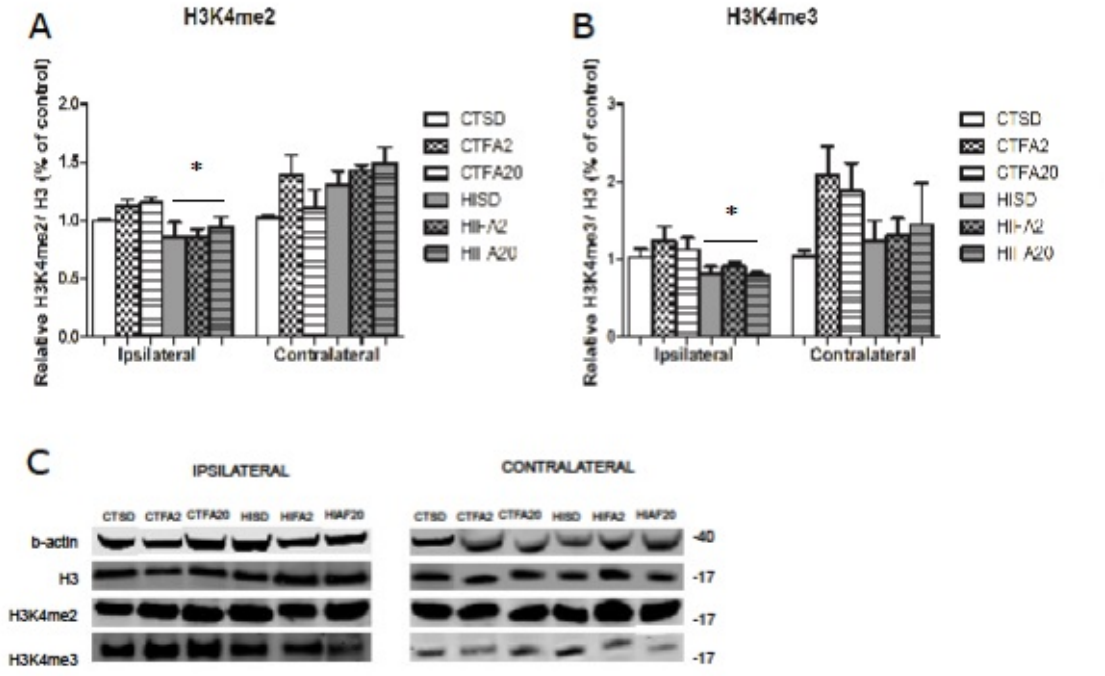


Fig. 5. H3K4 methylation – Protein expression of H3K4me2 (A) and H3K4me3 (B) in the hippocampus and representative bands of each protein (C). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group.

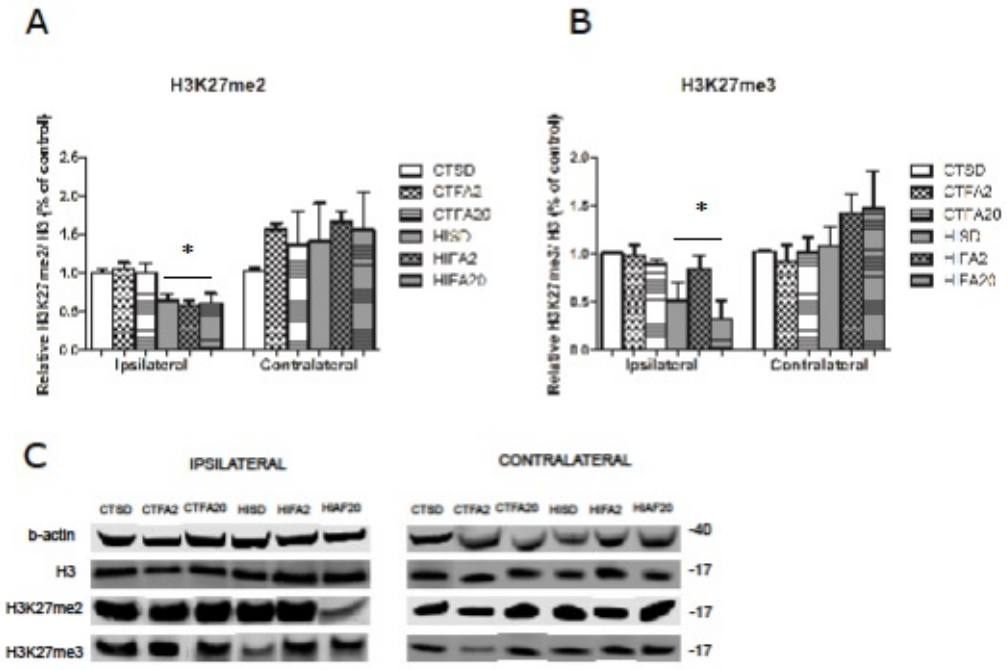


Fig. 6. H3K27 methylation – Protein expression of H3K27me2 (A) and H3K27me3 (B) in the hippocampus and representative bands of each protein (C). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 3 animals/ group.

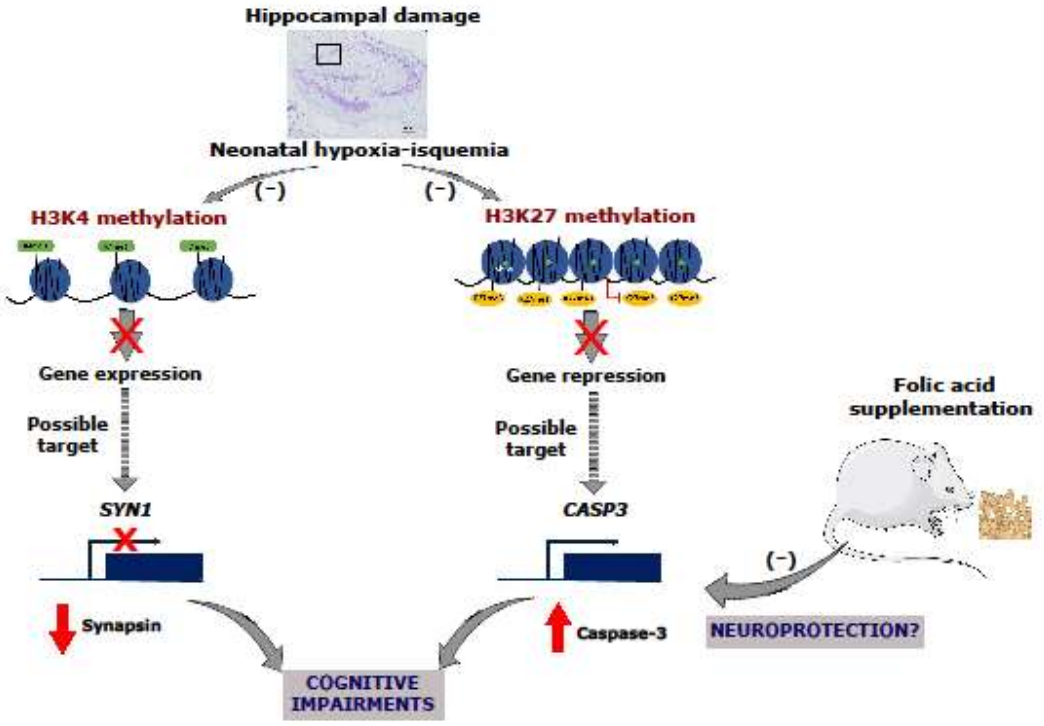


Fig. 7. Schematic representation of a possible mechanism for H3 methylation and proteins expression in the ipsilateral hippocampus of neonatal HI animals in the adulthood. Neonatal HI cause hippocampal tissue damage and decrease methylation of histone H3 in both K 4 and 27. Methylation of H3K4 is related with gene expression and methylation of H3K27 is associated with gene repression. Considering these, it is possible to propose that the decreased H3K4me is blocking the synapsin expression and decreased H3K27me is increasing caspase-3 expression in the ipsilateral hippocampus. These alterations can partially explain the cognitive deficits observed after neonatal HI. FA supplementation during pregnancy was able to prevent the increase caspase-3 in HI animals, possibly indicating a neuroprotector effect.

# 6. CAPÍTULO 3

"GESTATIONAL FOLIC ACID SUPPLEMENTATION DOES NOT AFFECTS THE MATERNAL BEHAVIOR AND THE EARLY DEVELOPMENT OF RATS SUBMITTED TO NEONATAL HYPOXIA-ISCHEMIA BUT THE HIGH SUPPLEMENTATION IMPAIRS THE DAM'S MEMORY AND THE $NA_+$, $K_+$ - ATPASE ACTIVITY IN THE PUP'S HIPPOCAMPUS."

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# Gestational folic acid supplementation does not affects the maternal behavior and the early development of rats submitted to neonatal hypoxia-ischemia but the high supplementation impairs the dam's memory and the $Na^+$, $K^+$ - ATPase activity in the pup's hippocampus

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ABSTRACT

Folic acid (FA) is a B-complex vitamin important to the development of the fetus, being supplemented during pregnancy. Our recent findings showed that gestation supplementation (normal and excess doses) prevented the cognitive deficits and BDNF imbalance in adult rats that were submitted to neonatal hypoxia-ischemia (HI). To better understand this protective effect, the present study aimed to evaluate whether FA supplementation could be related to (1) maternal behavior, memory and $Na^+$, $K^+$ - ATPase activity in the hippocampus of the dams; (2) on somatic growth, early neurobehavioral development and $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring; and (3) the effects of this supplementation in pups submitted to neonatal HI. Pregnant Wistar rats were divided into three groups, according to the diet they received during gestation: standard diet (SD), supplemented with 2 mg/kg of FA (FA2 – normal dose) and supplemented with 20 mg/kg of FA (FA20 –excessive dose). At the 7$^{th}$ PND pups were submitted to the Levine-Vannucci model of HI. During weaning the maternal behavior, the somatic growth and the neurobehavior development of pups were assessed. After weaning, the memory of the dams (by the Ox-maze task) and the $Na^+$, $K^+$ - ATPase activity in the hippocampus of both dams and offspring were evaluated. Considering the dams (1), both doses of FA did not alter the maternal behavior or the $Na^+$, $K^+$ - ATPase activity in the hippocampus, but a memory deficit was observed in the high FA-supplemented mothers. Considering the offspring (2), both FA doses did not affect the somatic growth or the neurobehavior development, but the FA20 pups had a decreased $Na^+$, $K^+$ - ATPase activity in the hippocampus. The FA supplementation did not change the parameters evaluated in the HI rats (3) and did not prevent the decreased $Na^+$, $K^+$ - ATPase activity in the hippocampus of the HI pups. These results indicate that normal FA supplementation dose does not influence the maternal behavior and memory and does not impact on the offspring early development in rats. Further studies are needed to confirm the effects of the high FA supplementation dose in the dams' memory and in the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring.

*Abbreviations:* AIN, American Institute of Nutrition; ATP, adenosine triphosphate; BDNF, Brain-derived neurotrophic factor; CA, Cornu Amonis; E, embrionary day; FA, folic acid; HI, hypoxia-ischemia; PND, post-natal day; WHO, World Health Organization

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## 1. Introduction

Folic Acid (FA) is a B-complex vitamin acquired by the diet that plays an important role in nucleotide biosynthesis and remethylation of homocysteine, participating in the methylation reactions of proteins, DNA/ RNA, and histone in our organism (Mentch and Locasale, 2016). This supplementation of this vitamin has been recommended (0.4 mg/ day) one month before and during the first trimester of the pregnancy to prevent neural tube closure defects. Some countries, such as Brazil, USA and Canada, decided to supplement the flours with FA to promote the necessary amount of this vitamin in the beginning of pregnancy (nervous system development period) in most women (Gomes et al., 2016; Ray et al., 2002). Positive effect of FA supplementation on the offspring neurodevelopment has been identified; in a large case-cohort study, including 45,300 children, Levine et al., (2018) demonstrated that maternal exposure to FA before or during pregnancy was significantly associated with a low likelihood of autism spectrum disorder in the offspring. Some findings have pointed that normonutride women might have an excess of FA by the supplements and diet itself (Bailey et al., 2010; Patel and Sobczyńska-Malefora, 2017; Selhub and Rosenberg, 2016). After the FA fortification regime in the US, it was identified an increase in FA intake that was approximately twice as large as previously projected (Choumenkovitch et al., 2002). Measurable concentrations of unmetabolized FA (UMFA) in blood, indicating a higher FA intake, were also observed in most of the individuals after the FA fortification (Pfeiffer et al., 2015; Kelly et al., 1997). Moreover, supraphysiologic concentrations of serum folate (> 45.3 nmol/L) was found after fortification in children aged less than 5 years (Pfeiffer et al., 2005). A prospective study with pregnant Canadian women demonstrated folate concentrations in serum and red blood cells in ranges generally considered to be high, in both early pregnancy and at delivery (Plumptre et al., 2015). Additionally, plasma UMFA was detectable in > 90% of maternal samples. The authors suggested that the higher folate concentrations were likely related to the higher proportion of women who consumed FA supplements during the first trimester −93% (Plumptre et al., 2015). Besides, in Brazil the System of Public Health only offers a 5 mg/day supplement (Ministério da Saúde, 2014), more than 10 times the recommended dose. However, the impact of a possible overdose of FA in humans has not been sufficiently investigated.

Currently, animal studies have focused on the offspring effects of an excessive amount of FA in the gestational period. It was observed lower weight and size of the embryos, development delay and decreased neurotrophins levels, such as brain-derived neurotrophic factor (BDNF) (Achón et al., 1999; Mikael et al., 2013; Sable et al., 2012; Sable et al., 2014). In contrast, it was evidenced improving in early reflexes, spatial learning and motor function (Wang et al., 2017). Fewer studies have focused on the effects of FA supplementation in pregnant rats, but Canever et al., (2018) reported that FA-supplemented dams presented an improvement in spatial memory. In contrast, Sittig et al., (2012) identified memory and motivation deficits in young rats supplemented with FA. In view of these contradictory results and also taken that there are few studies focusing on the effects of FA supplementation in the dams and in the offspring, this issue could be considered a relevant topic to be investigated.

Considering that FA supplementation is already adopted during pregnancy, our group decided to investigate the effects of this vitamin in an animal model of neonatal hypoxia-ischemia (HI). Interestingly we found that 2 different doses of FA supplementation during pregnancy (one recommended and one excessive) were able to prevent memory deficits and BDNF disbalance in adult HI rats (Deniz et al., 2018). Neonatal HI is a common cause of encephalopathy that occurs in consequence of decreased oxygen availability and/ or interruption of the blood flow in the peripartum period (McLean and Ferriero, 2004). Its prevalence is up to 22 per 1000 prematurely born babies (Rumajogee et al., 2016) and about 25% of the survivors will present neurological sequelae, such as cerebral palsy, epilepsy and attentional deficit/ hyperactivity disorder (Juul and Ferriero, 2014; Placha et al., 2016; Vannucci and Perlman, 1997). After the injury, there is a decrease in the energy available to the nervous tissue with consequently diminished ATP. This molecule is needed to the proper function of the $Na^+$, $K^+$ - ATPase enzyme which has been correlated with memory and learning process, being this enzyme localized contiguously with the N-methyl-D-aspartate (NMDA) receptor in the synaptic regions due to its important role in these processes (Brunelli et al., 1997; de Lores Arnaiz and Bersier, 2014). In addition, it is well known that this enzyme activity is decreased in the hippocampus of HI rats (Carletti et al., 2012, 2016; Rojas et al., 2015; Sanches et al., 2017; Weis et al., 2011) and our previous studies with FA treatment after HI injury found both an improvement or worsening in this enzyme activity (Carletti et al., 2012, 2016) by the FA. Since the hippocampus is a brain region rich in glutamatergic synapses, the excitotoxicity by this neurotransmitter can explain the cognitive deficits observed after the HI injury (Carletti et al., 2012; Miguel et al., 2015, 2017; Pereira et al., 2007; Rojas et al., 2013, 2016). Curiously, Schuch et al., (2016) did not find differences in the appearance and performance of early neurological reflexes in HI rats, despite the established cognitive deficits in adult HI rats. These findings indicate the importance of these reflexes, which are considered important predictor factors of behavioral deficits in adulthood, to the survival and development of rodents (Allen and Alexander, 1997; Heyser, 2004).

FA supplementation has been used during pregnancy in different doses (occasionally more than 10 times the recommended dose) and, based on the current evidences, it is reasonable to hypothesize that this could affect maternal behavior and the newborn's response to an unpredicted HI event. Thus, the present study aimed to investigate the effects of both recommended and excessive dose of FA supplementation during pregnancy on the maternal behavior, memory and $Na^+$, $K^+$ - ATPase activity in the hippocampus of dams. Another objective was to evaluate how these two doses of FA supplementation could affect the somatic growth, early neurobehavioral development and $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring submitted to neonatal HI.

## 2. Materials and methods

### 2.1. Animals

Male and female Wistar rats provided by the local breeding colony (Institute of Basic Health Science, Federal University of Rio Grande do Sul, Brazil) were maintained in a room with controlled temperature (22° ± 1), light/ dark cycle (12/12 h) and with food and water *ad libitum*. After a two-week adaptation, female rats were submitted to daily vaginal exams to detect their fertile period (proestrus) and, then, they were put in individual cages with one male during the dark period. When the pregnancy was confirmed (embrionary day E0), the rats were divided into 3 groups, according to the diet received during the gestational period: 1) standard diet (SD), 2) supplemented with 2 mg/kg of FA (FA2) and 3) supplemented with 20 mg/kg of FA (FA20). The Levine-Vannucci HI model was performed in the pups at the 7$^{th}$ postnatal day (PND), resulting in 6 experimental groups in the offspring: 1) control and SD diet (CTSD), 2) CT and FA2 diet (CTFA2), 3) CT and FA20 diet (CTFA20), 4) HI and SD diet (HISD), 5) HI and FA2 diet (HIFA2), and 6) HI and FA20 diet (HIFA20). The timeline of the experiments is represented in Fig. 1. The present study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28,136) and achieved in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the Arouca Law (Law no 11.794/2008).

### 2.2. Experimental diets

Pregnant rats received the experimental diets only during the

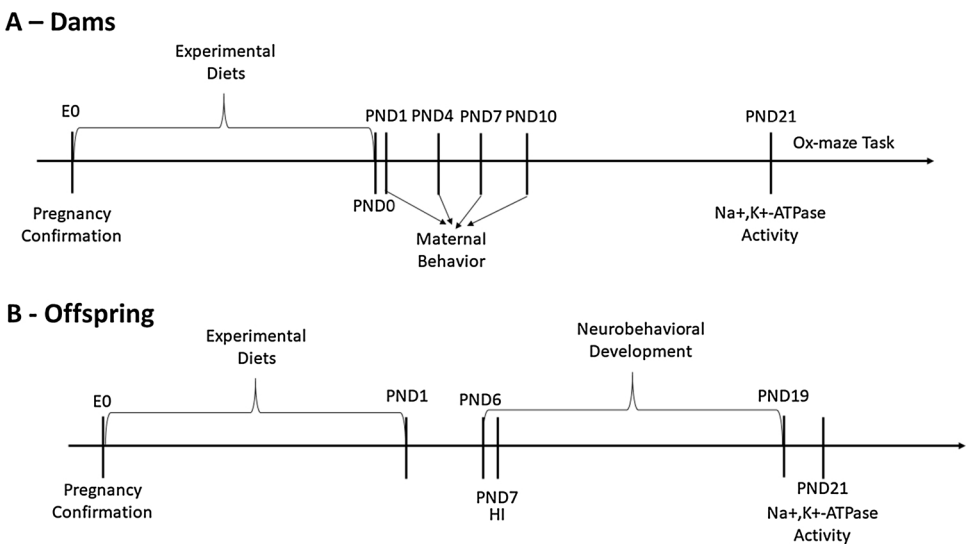


**Fig. 1.** Experimental line – A) Mother rats (dams) experimental line. After pregnancy confirmation (E0), rats were divided into 3 groups, according to their experimental diets: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). After pups' birth (PND0), all rats received the SD diet. Maternal behavior was assessed at PND 1, 4, 7 and 7. After weaning (PND21), some dams performed the Ox-maze task and others were euthanized to Na+, K+ - ATPase activity evaluation in the hippocampus. B) Offspring experimental line. After pregnancy confirmation (E0), rats were divided into 3 groups, according to their experimental diets: standard diet (SD), supplemented with 2 mg/kg of FA (FA2) and supplemented with 20 mg/kg of FA (FA20). At the PND 7, pups were submitted to the HI. One day before the injury (PND6) and until PND19, neurobehavioral development of the offspring were evaluated. After weaning (PND21), pups were euthanized and the Na+, K+ - ATPase activitywas evaluated in the hippocampus.

gestational period. After the birth of the offspring, all rats received the standard diet until the end of the experiments. All diets were isocaloric and isoproteic, according to the AIN-93 Purified Diets for Laboratory Rodents (American Institute of Nutrition; Reeves et al., 1993). Both supplemented diets had an addition of FA in its composition, besides the normal amount in the SD diet. The supplementation with 2 mg/kg of FA, for rats, is considered the equivalent quantity recommend by the WHO for pregnant women (400 µg/ day, Alonso-Aperte, 1997). The supplementation with 20 mg/kg of FA was chosen to mimic the excessive amount of FA that can be acquired by supplements and/or by the diet itself (Bahous et al., 2017; Deniz et al., 2018).

### 2.3. Neonatal hypoxia-ischemia

The Levine-Vannucci model of HI was performed at the 7$^{th}$ PND (Levine, 1960; Vannucci and Vannucci, 1997). Pups were anesthetized with halothane and an incision in the ventral surface was realized to allow the identification, isolation and occlusion of the right common carotid. The rats returned to their home cage with their mothers for a 2 h recovery period and, after that, they were submitted to a hypoxic atmosphere (8% $O_2$ and 92% $N_2$) for 90 min. Control animals were sham-operated, submitted to the anesthesia and the incision without the carotid occlusion or the hypoxia (Pereira et al., 2007; Miguel et al., 2017).

**Part 1: Mother Rats (Dams)**

The experimental line of the dams' evaluations is presented in the Fig. 1A.

### 2.4. Food consumption and weight gain

To detect possible differences in the palatability of the supplemented diets that could interfere in the course of the pregnancy, one day after the gestation confirmation and every 3 days until the birth of the pups (E 1, 4, 7, 10, 13, 16, 19, 22), the quantity of food ate in each day was measured (X = 200 g food available – food in the next day) and the differences in the dam weights in each day compared to the day before were verified (n = 12–14/group).

### 2.5. Number of pups per litter

One day after the birth, the number of pups per litter and their sex distribution was noted to identify possible changes by the FA supplementation during pregnancy.

### 2.6. Maternal behavior

To identify possible influences of FA supplementation in the maternal care, the rats in their home cages were recorded during 15 min after the somatic evaluations of the offspring in the PND 1, 4, 7 and 10. It was analyzed, by a blind observer, the latency to initiate the nest, the latency to get the first pup, the time feeding and licking the pups and the time far away from the offspring (n = 6/ group).

### 2.7. Ox-Maze task

Considering that previous studies have observed memory impairment after FA supplementation in control animals (Carletti et al., 2016; Sittig et al., 2012), we decided the evaluate the memory and the $Na^+$, $K^+$ - ATPase activity in the dams supplemented with FA. One day after weaning, mothers were evaluated in the Ox-maze task (n = 6–7/ group). This task evaluates learning and memory and was adapted from the original one of Wood et al., (2011). The apparatus is a black square acrylic box (60 cm x 60 cm x 30 cm high) divided in 12 cm squares. Four black acrylic boxes (10 cm x 10 cm x 10 cm high) with a hole (2 cm diameter x 2 cm deep) in each side were put in the apparatus. Each hole had one of the four symbols (O, X, =, II). Habituation with the Fruit loops (Kellogg's) and food restriction (reduction to, approximately, 90%) was realized for 2 days. The task was realized for 7 consecutive days and the blocks positions were changed daily. In all task days the reward (fruit loops) was in the hole with the O symbol. Rats were placed at the center of the apparatus and the latency to get the first reward, the incorrect and correct number of nose pokes, and the time to complete the task (maximum of 10 min) were recorded by a blind researcher. To minimize the olfactory cues, it was put bran of the reward in all holes and the apparatus and the blocks were cleaned with 30% ethyl alcohol between rats' tests (Rojas et al., 2015).

### 2.8. $Na^+$, $K^+$ - ATPase activity

One day after weaning rats were euthanized (n = 6/group) by decapitation and their hippocampi were dissected, frozen in liquid nitrogen and stored at −80 °C. Samples were homogenized and the $Na^+$, $K^+$ - ATPase activity assay was performed as described by Wyse et al., (1998). Briefly, the reaction initiates when ATP is added, and the activity is calculated by the difference of two assays and expressed by the released of inorganic phosphate (Pi) per min per mg of protein (Carletti et al., 2012, 2016; Rojas et al., 2015). Total protein quantification was

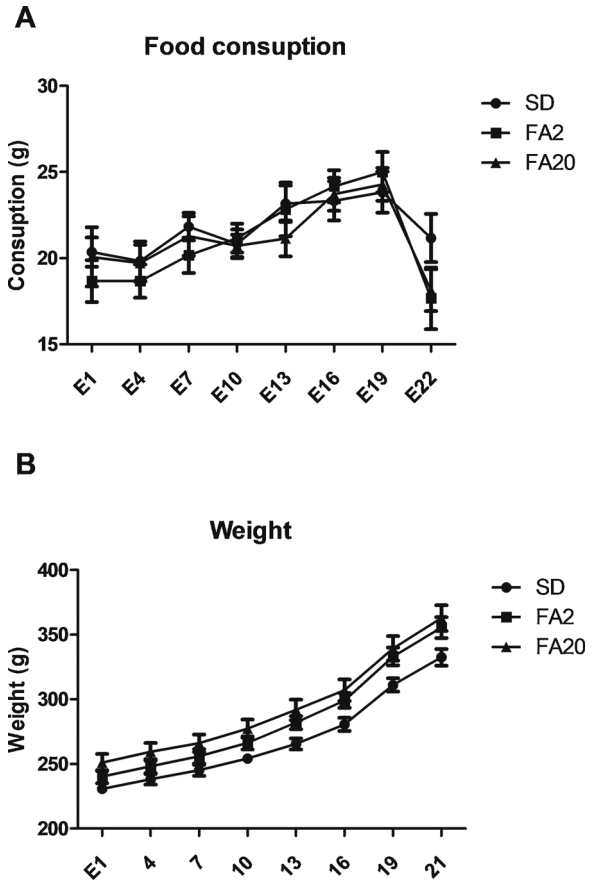


**Fig. 2.** Food consumption and Weight gain – Quantification of the food consumption (A) and the weight gain (B) of the dams during the pregnancy. Data are expressed as mean ± S.E.M. Repeated one-way ANOVA followed by Tukey's test, $p < 0.05$. N = 12–14 rats/ group.

measured by Bradford method.

Part II: Offspring

The experimental line of the offspring's evaluations is presented in the Fig. 1B.

### 2.9. Somatic growth

To identify changes in the somatic growth of the pups by the supplementation, their weight gain and body length were recorded every 3 days after birth until weaning. The weight of the pups was measured and an average per group per litter was analyzed. The body length was measured by the distance between the distal area of the head to the end of the tail. Once more, the body length of pups was measured and the average per group per litter was analyzed. Both variable was evaluated before (PND 1, 4 and 7) and after the HI injury (PND 10, 13, 16 and 19) separately (n = 7–9 litters/ group).

### 2.10. Early neurobehavioral development

Neurobehavioral development evaluations were assessed 1 day before and one day after the HI procedure (PND 6 and 8, respectively), and every 3 days until weaning (PND 10, 13, 16 and 19, n = 9–11/ group). The day of the first appearance of the following characteristics and reflexes were noted: eye opening, negative geotaxis, left hindlimb grasp, gait and cliff aversion reflex. It was also evaluated the following reflexes over the cited days: 1) Righting reflex: the time to turn from the supine to the prone position; 2) Cliff aversion reflex: rats were placed in the edge of a table with their forelimbs overhanging. It was recorded the time to turn 90° from the edge with a maximum of 60 s; 3) Negative geotaxis: pups were placed in a inclined board (45°) with their head down and the time to turn around and climb up the board was verified (maximum of 60 s); 4) Gait: rats were placed in the center of a circle (13 cm diameter) and the time to escape off the circle with both forelimbs was recorded (maximum of 30 s) (Schuch et al., 2016; Lubics et al., 2005).

### 2.11. $Na^+$, $K^+$ - ATPase activity

One day after weaning rats were euthanized (n = 6/group) by decapitation and both hippocampi were dissected, frozen in liquid nitrogen and stored at −80 °C. $Na^+$, $K^+$ - ATPase activity was performed as previous described.

### 2.12. Statistical analysis

One-way ANOVA with *supplementation* as factor was used to for the number and the sex distribution of pups per litter, the variable means of the Ox-maze task and the $Na^+$, $K^+$ - ATPase activity, all in the dams. Repeated one-way ANOVA with *supplementation* as factor was performed for the food consumption and weight gain during pregnancy, maternal behavior, Ox-maze task, and the somatic growth of the pups. Two-way ANOVA, with *lesion* and *supplementation* as factors, was used for the day of appearance of the neurobehavioral development and the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring. Repeated two-way ANOVA, with *lesion* and *supplementation* as factors, was performed for the pups' somatic growth after the HI injury and the neurobehavioral development. Tukey's *post-hoc* test was used when necessary. Differences were considered significant when $p < 0.05$. All analysis were performed in the Statistic© software package.

## 3. Results

### Part 1: Mother Rats (Dams)

### 3.1. Food consumption and weight gain

Repeated one-way ANOVA only evidenced a *day* effect ($F(7,245) = 10.87$, $p < 0.05$, partial $\eta^2 = 0.68$) on the food consumption during the gestation. No *supplementation* effect or differences between groups were observed. All groups had an increase of food ingested during the days, with exception of the last day, the birth day (Fig. 2A). In the PND 22, both supplemented groups ingested less chow than in the PND 16 and 19. Also, FA2 group ate more in the PND 19 when compared to PND 1 and 4. Considering the weight gain, it was also found a *day* effect ($F(7,245) = 24.99$, $p < 0.05$, partial $\eta^2 = 0.98$) with no differences by the supplementation or among groups were observed. All groups augmenting their weight during the gestation period, as expected (Fig. 2B).

### 3.2. Number of pups per litter

No differences in the number of pups per litter, or in the number of male or female pups were found between groups (Table 1).

**Table 1**
Number of pups per litter.

| | Total number of pups | Male pups | Female pups |
|---|---|---|---|
| SD | 11.4 ± 0.4 | 6.0 ± 0.7 | 5.3 ± 1.0 |
| FA2 | 11.3 ± 0.7 | 5.3 ± 0.6 | 6.0 ± 0.8 |
| FA20 | 11.9 ± 0.7 | 5.8 ± 0.6 | 5.9 ± 0.4 |

No differences were found. Data are expressed as mean ± S.E.M. One-way ANOVA, $p < 0.05$. N = 7–9 rats/ group.

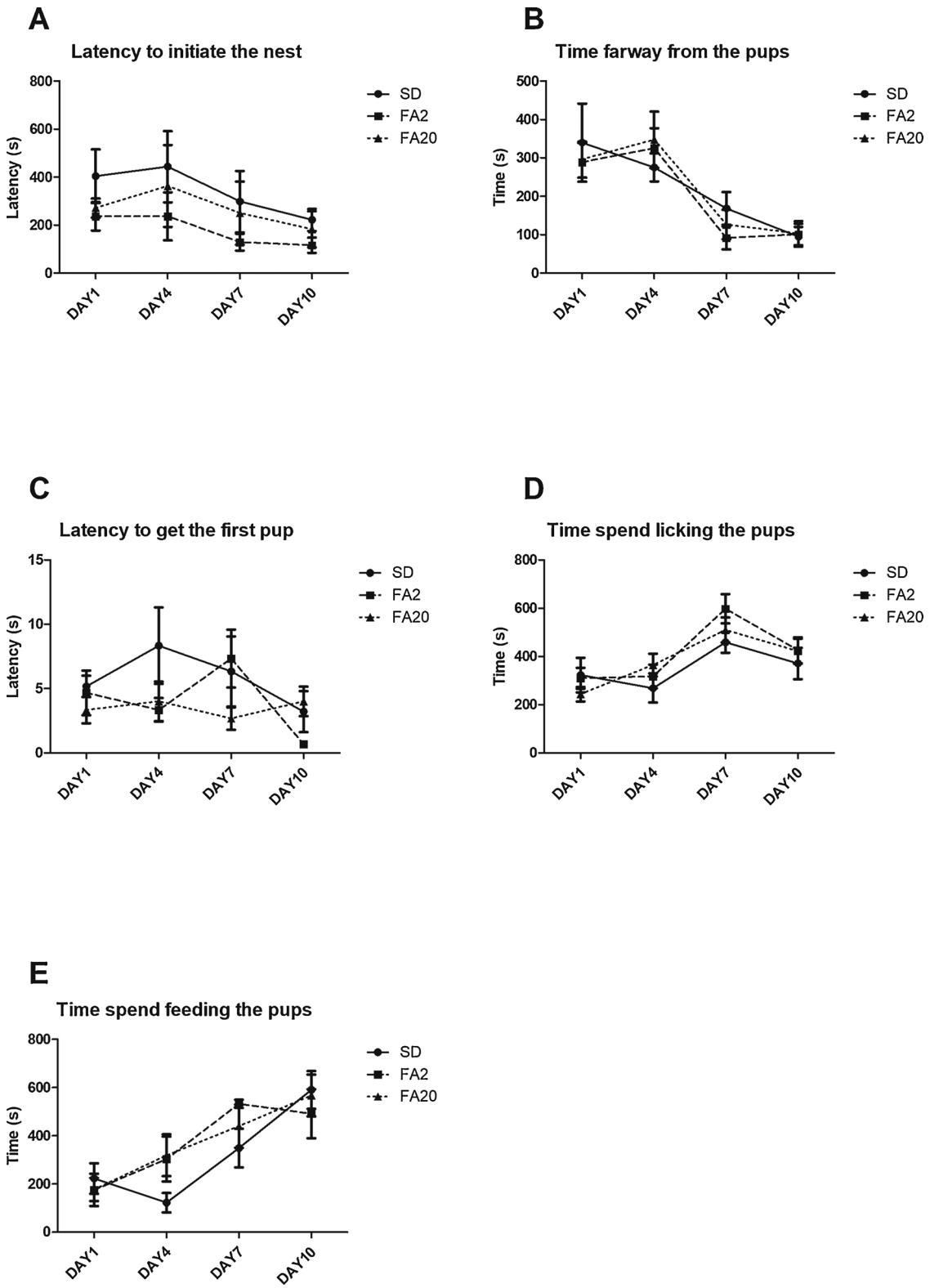


**Fig. 3.** Maternal behavior – Evaluation of the maternal behavior at PND 1, 4, 7 and 10. Latency to initiate the nest (A), time far away from the pups (B), latency to get the first pup (C), time spend licking the pups (D) and time spend feeding the pups (E). Data are expressed as mean ± S.E.M. Repeated one-way ANOVA followed by Tukey's test, $p < 0.05$. N = 6 rats/ group.

### 3.3. Maternal behavior

Repeated one-way ANOVA evidenced a *day* effect in the latency to initiate the nest building and in the time spend far away from the pups ($F(3,45) = 2.74$, $p < 0.05$, partial $\eta^2 = 0.49$; $F(3,45) = 18.53$, $p < 0.05$, partial $\eta^2 = 0.80$, respectively). No supplementation effect or differences between groups were observed. All groups decreased the time to initiate the nest and spend far away from the offspring during the observational days (Fig. 3A, B). A *day* effect ($F(3,45) = 21.96$, $p < 0.05$, partial $\eta^2 = 0.83$) was also observed in the time spent

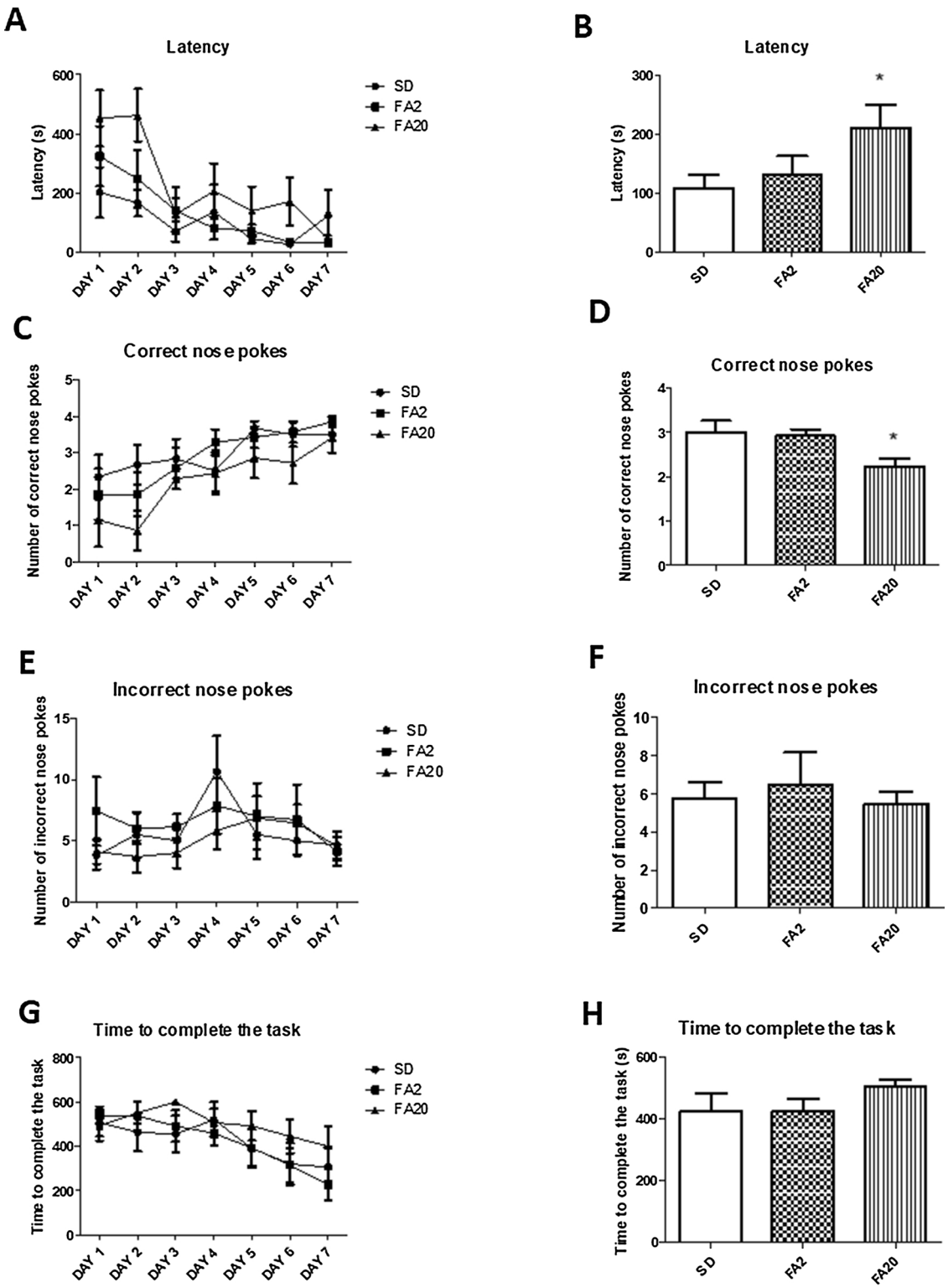


**Fig. 4.** Ox-maze task – Memory evaluation of the dams by the ox-maze task. Latency to get the first reward (A and B), number of correct nose pokes (C and D), number of incorrect nose pokes (E and F) and time to complete the task (G and H). * difference from SD group. Data are expressed as mean ± S.E.M. Repeated one-way ANOVA (A, C, E and G) or one-way ANOVA (B, D, F and H) followed by Tukey's test, $p < 0.05$. N = 6 rats/ group.

licking the pups (Fig. 3D), but it is important to note that no differences by the diet was observed again. All groups increased the time spent licking the pups in the PND 7 (the HI day) when compared to PND 1 (AF2 and AF20 groups) and to PND 4 (AF2 and SD groups). In the time feeding the offspring, a *day* ($F(3,45) = 14.24$, $p < 0.05$, partial $\eta^2 = 0.71$) effect was evidenced with no differences among the groups (Fig. 3E). SD and FA20 groups increased the time feeding the pups in the PND 10 when compared to PND 1. No differences were identified in the latency to get the first pup after they were put back to the cage (Fig. 3C).

### 3.4. Ox-maze task

Repeated one-way ANOVA indicated a *supplementation* ($F(2,17) = 3.65$, $p < 0.05$, partial $\eta^2 = 0.31$) and *day* ($F(6,102) = 8.08$, $p < 0.05$, partial $\eta^2 = 0.67$) effects in the latency to get the first reward (Fig. 4A); all groups decreased their latency during the task days without differences between groups. For the number of correct nose-pokes a *supplementation* ($F(2,17) = 4.26$, $p < 0.05$, partial $\eta^2 = 0.34$) and *day* ($F(6,102) = 6.38$, $p < 0.05$, partial $\eta^2 = 0.62$) effects was also observed with no differences among groups in the *pos-hoc* analysis (Fig. 4C). All groups augmented the correct answers during the days. And considering the mean, significant *supplementation* effect ($F(2,17) = 4.26$, $p < 0.05$, partial $\eta^2 = 0.36$) was observed for the number of correct nose-pokes, evidencing that the FA20 group had fewer correct answers when compared to the SD group (Fig. 4D). In the time to complete the task, a *day* ($F(6,102) = 5.47$, $p < 0.05$, partial $\eta^2 = 0.57$) effect was evidenced with no other differences, indicating that all groups decreased the time to complete the task (Fig. 4G). These findings indicated that FA20-supplemented dams had a poor performance in this task. No differences were detected for the number of incorrect nose-pokes (Fig. 4E–F) or for the means of time to complete the task (Fig. 4H).

### 3.5. $Na^+$, $K^+$ - ATPase activity

One-way ANOVA did not evidenced differences between groups in the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the dams (Fig. 5).

Part II: Offspring

### 3.6. Somatic growth

Before the HI injury (PND 1, 4 and 7): it was identified a growth curve in all groups for the weight gain variable (Fig. 6A), with *supplementation* ($F(2,47) = 7.66$, $p < 0.05$, partial $\eta^2 = 0.19$), *day* ($F(6,282) = 3626.82$, $p < 0.05$, partial $\eta^2 = 0.98$) and *supplementation*day* ($F(12,282) = 5.6$, $p < 0.05$, partial $\eta^2 = 0.17$) effects, without differences between groups. Similarly, the same pattern of growth curve was observed for the body length of the offspring (Fig. 6C), with *day* ($F(6,282) = 4151.89$, $p < 0.05$, partial $\eta^2 = 0.98$) and *supplementation*day* ($F(12,282) = 2.97$, $p < 0.05$, partial $\eta^2 = 0.14$) effects. The post-hoc test did not confirm differences between groups.

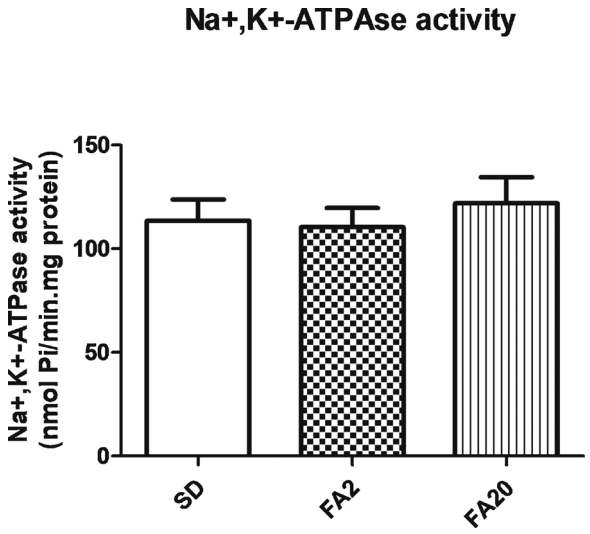


**Fig. 5.** Na+, K+ - ATPase activity– Quantification of the Na+, K+ - ATPase activityin the hippocampus of the dams. Data are expressed as mean ± S.E.M. One-way ANOVA followed by Tukey's test, $p < 0.05$. N = 6 rats/ group.

After HI injury (PND 10, 13, 16 and 19): Repeated two-way ANOVA was also used after the HI injury. For weight gain, a *supplementation* ($F(2,94) = 6.41$, $p < 0.05$, partial $\eta^2 = 0.12$), *lesion* ($F(1,94) = 4.51$, $p < 0.05$, partial $\eta^2 = 0.05$), *day* ($F(3,282) = 869.4$, $p < 0.05$, partial $\eta^2 = 0.95$) and *supplementation*day* ($F(6,282) = 2.58$, $p < 0.05$, partial $\eta^2 = 0.76$) effects were observed. All the groups had an increase in their weight over the days with no differences among them (Fig. 6B). The growth curve of body length was less pronounced in all groups, then no differences were identified. (Fig. 6D).

### 3.7. Early neurobehavioral development

Considering the day of appearance of the characteristics and reflexes analyzed, two-way ANOVA showed a *supplementation* effect for eye opening ($F(2,53) = 3.89$, $p < 0.05$, partial $\eta^2 = 0.13$), auditory startle ($F(2,53) = 6.34$, $p < 0.05$, partial $\eta^2 = 0.19$) and cliff aversion reflex ($F(2,53) = 3.9$, $p < 0.05$, partial $\eta^2 = 0.13$). Tukey's *pos-hoc* did not confirm any differences between groups (Table 2). No differences were observed in the negative geotaxis, left hindlimb grasp and gait.

For the analysis of the performance of the following reflexes during the days, repeated two-way ANOVA was used. In the righting reflex, a *day* effect ($F(5,265) = 177.24$, $p < 0.05$, partial $\eta^2 = 0.91$) was found. All groups decreased the time to perform such reflex during the days and no differences were observed between groups (Fig. 7A). A *day* effect ($F(5,265) = 24.04$, $p < 0.05$, partial $\eta^2 = 0.69$) was also observed in the cliff aversion reflex (Fig. 7B), indicating that all groups improved their performance. No differences by *lesion* or *supplementation* effects were observed. For negative geotaxis (Fig. 7C), repeated two-way ANOVA presented a *day* effect ($F(5,265) = 146.79$, $p < 0.05$, partial $\eta^2 = 0.93$). No differences among groups were observed and all groups diminished their time to realize the task during the days. Gait analysis showed a *supplementation* ($F(2,53) = 4.05$, $p < 0.05$, partial $\eta^2 = 0.13$) and a *day* ($F(5,265) = 145.93$, $p < 0.05$, partial $\eta^2 = 0.95$) effects (Fig. 7D). All groups took less time to escape the circle during the days and no differences between groups were observed.

### 3.8. $Na^+$, $K^+$ - ATPase Activity

Two-way ANOVA showed a *lesion* ($F(1,26) = 10.21$, $p < 0.05$, partial $\eta^2 = 0.09$) and *supplementation* ($F(2,26) = 3.6$, $p < 0.05$, partial $\eta^2 = 0.07$) effects in the ipsilateral hippocampus of the pups (Fig. 8). CTFA20 and all HI groups had a diminished $Na^+$, $K^+$ - ATPase activity when compared to CTSD group. This indicates a lesion impact on this enzyme activity that was not prevented by the FA supplementation. Also, the high dose of FA impaired the $Na^+$, $K^+$ - ATPase activity in control rats. No differences were observed in the contralateral hippocampus.

## 4. Discussion

The present study investigated the effects of FA supplementation during pregnancy on the maternal behavior, memory and $Na^+$, $K^+$ - ATPase activity in the hippocampus of the mother rats. Also, it was investigate whether this vitamin could affect the somatic growth, neurobehavioral development and $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring submitted to the neonatal HI. Our main findings are that FA supplementation, did not affect the gestation, the maternal behavior, the somatic growth or the neurobehavioral development in the offspring. These results have a social importance, since both doses of FA supplementation are already used in pregnant women and it seems that this supplementation is partially safe for the offspring. In contrast, we found that high FA dose impaired memory in the dams and the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the puppies.

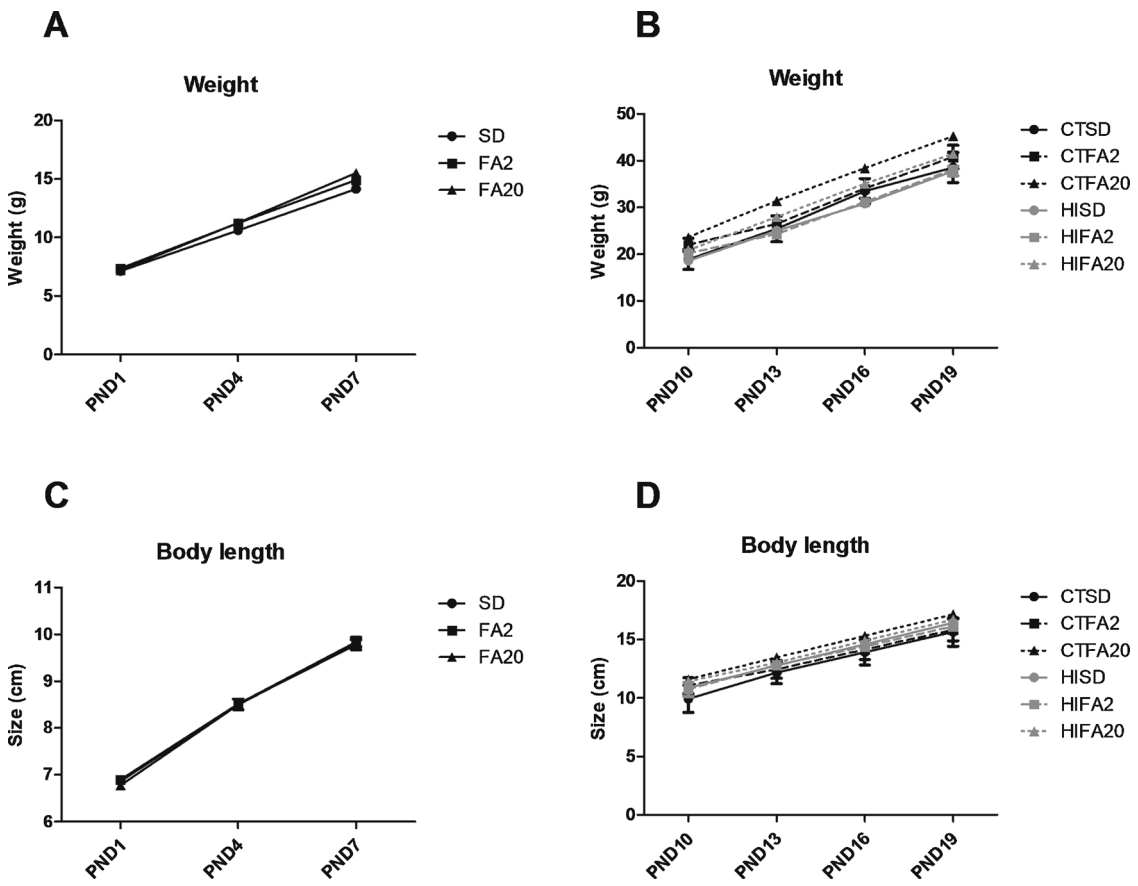


**Fig. 6.** Somatic growth – Evaluation of the weight gain (A and B) and the body length (C and D) of the offspring since the birth until the weaning (PND21). Pups were evaluated before HI injury (A and C) and after HI injury (B and D). Data are expressed as mean ± S.E.M. Repeated two-way ANOVA followed by Tukey's test, $p < 0.05$. N = 7–9 litters/ group.

**Table 2**
Days of appearance of neurobehavioral development.

| Group | Eye opening | Hindlimb grasp (left) | Auditory startle | Cliff aversion | Negative geotaxis | Gait |
|---|---|---|---|---|---|---|
| CTSD | 14.5 ± 0.5 | 6.4 ± 0.4 | 13.0 | 6.4 ± 0.3 | 7.6 ± 0.6 | 9.7 ± 0.9 |
| CTFA2 | 15.7 ± 0.3 | 6.2 ± 0.2 | 13.9 ± 0.4 | 6.6 ± 0.3 | 6.2 ± 0.2 | 11.2 ± 0.5 |
| CTFA20 | 14.3 ± 0.5 | 6.4 ± 0.3 | 13.0 | 6.0 | 7.1 ± 0.5 | 9.0 ± 0.7 |
| HISD | 14.5 ± 0.5 | 6.4 ± 0.3 | 13.0 | 6.2 ± 0.2 | 7.7 ± 0.7 | 9.5 ± 1.0 |
| HIFA2 | 15.0 ± 0.5 | 6.2 ± 0.2 | 13.7 ± 0.4 | 6.7 ± 0.3 | 7.1 ± 0.4 | 9.7 ± 0.5 |
| HIFA20 | 13.8 ± 0.4 | 6.5 ± 0.3 | 13.0 | 6.0 | 7.7 ± 0.6 | 8.9 ± 0.7 |

No differences were found. Data are expressed as mean ± S.E.M. Two-way ANOVA, $p < 0.05$. N = 9–11 rats/ group.

### *4.1. Folic acid supplementation caused memory impairment without affecting the pregnancy development or the maternal behavior*

The dams weight and food consumption were evaluated during pregnancy to reveal possible interferences of the supplementation with FA in the diets that could affect the gestational period. As expected, no differences between the FA-supplemented and the SD diets were observed, both for food consumption and weight. Canever et al. (2018) did not found differences in the weight of the pregnant rats during gestation that were supplemented with FA. Contrary to the present study, they found that FA-supplemented dams had an increase in the food intake when compare, especially, with FA-deficient, and this difference was associated with the vitamin supplementation itself. It is important to notice that the supplemented diets had different doses of FA from our study (5, 10 and 50 mg/kg of FA). Achón et al. 1999 were one of the first to evaluate the effects of FA excess (2 and 40 mg/kg of FA) in the gestational development and they, similarly to ours results, did not found differences in the weight or food consumption of the pregnant rats. Also, there was no difference in the number of live fetus per litter, also corroborating with our findings. One study that used the same doses of FA supplementation of ours (2 and 20 mg/kg of FA) investigated the embryo's development at the E14.5 (Mikael et al., 2013) and they found that FA supplementation decreased the number of implantation sites and increased the number of embryonic loss. These findings were in mice and evidenced the importance of understanding the effects of FA supplementation during pregnancy. The dual effect of FA is present in the literature and there is no consensus about the excess of this vitamin during gestation, but our results corroborated with the latest findings showing that, even in high doses, FA is safe for the pregnancy development.

Besides the pregnancy period, it is important to assess if the FA supplementation could affect the mothers' care of the offspring. The early maternal behavior is dependent of oxytocin and is highly important to the development and survival of the pups, since they are not fully mature at birth (Tang et al., 2014; Yoshihara et al., 2017). To our knowledge, this is the first study to evaluate the maternal behavior after

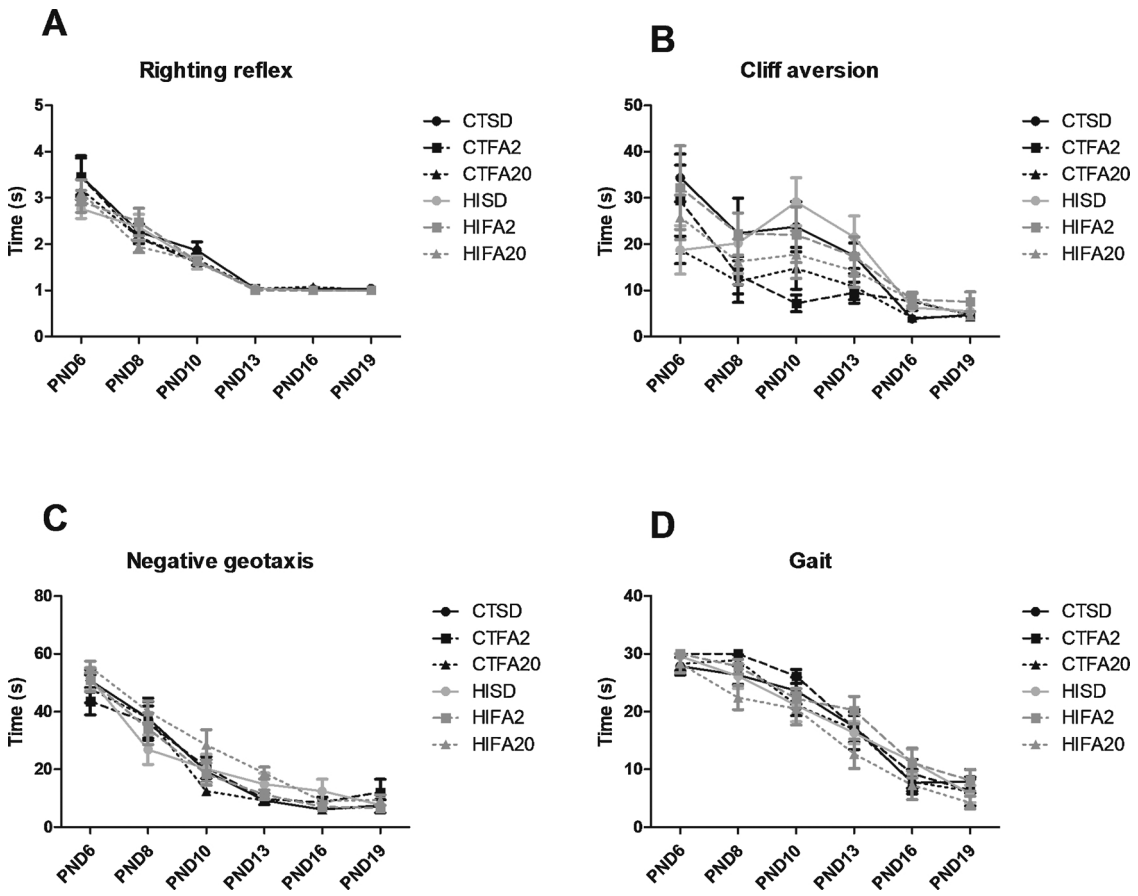


**Fig. 7.** Neurobehavioral development – Evaluation of neonatal reflexes of the pups at PND 6, 8, 10, 13, 16 and 19. Righting reflex (A), Cliff aversion (B), Negative geotaxis (C) and Gait (D). Data are expressed as mean ± S.E.M. Repeated two-way ANOVA followed by Tukey's test, $p < 0.05$. N = 9–11 rats/ group.

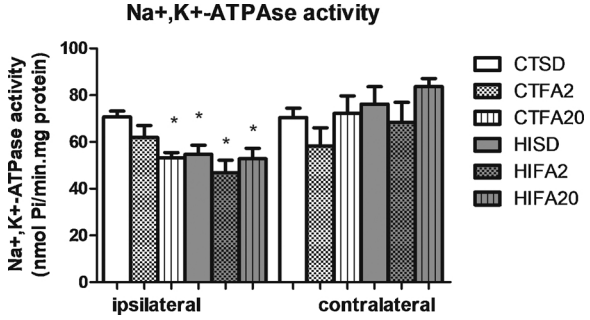


**Fig. 8.** Na+, K+ - ATPase Activity – Quantification of the Na+, K+ - ATPase activity in both hippocampus of the pups. * difference from CTSD group. Data are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p < 0.05$. N = 5–6 rats/ group.

FA supplementation during pregnancy. No differences were observed in the maternal care by both doses of the vitamin. We only identified that during the days of observation, the maternal behavior was improved. It has been indicated that this long-term maintenance of maternal care is hormone-independent (Bridges, 1975; Numan, 2015; Numan and Insel, 2003; Rosenblatt, 1975). In 1967, Rosenblatt demonstrated the hormonal stimulation is not the only responsible for maternal behavior in rats. Exposition to pups can induce maternal behavior. In fact, hormonal levels reduce significantly a few hours after the birth and this period (around 1 day after birth) is of special vulnerability of maternal care because it possibly corresponds to the transition of hormonal to non-hormonal regulation of maternal care (Bridges, 1975; Numan, 2015; Numan and Insel, 2003; Rosenblatt, 1975). This transition can, at least partially, explain the improvement in the maternal care observed in our findings. Also, somatosensory inputs from the pups during the days contribute to the augmenting expression of maternal behavior (Pedersen et al., 1995). Contrary to our findings, Gatta et al., (2018) observed a decreased in the activity maternal behavior (measured by feeding, licking, grooming and carrying the pups) during the first 5 days in dams that were submitted to a perinatal stress protocol. It is also known that treatment with antipsychotic during pregnancy disrupt the active components of maternal behavior by decreasing the time feeding the pups and increasing the latency to get the pups and start to build the nest (Li, 2015). In contrast, another study found that a high-fat diet during pregnancy and lactation increased the time spend nursing the pups and decreased the period that the mothers were resting, indicating that they spent more time with the offspring (Purcell et al., 2011). Then, our findings indicate that even in high doses, FA does not alter the mothers' care to the pups, probably because this vitamin supplementation does not present an aversive component or change the nutritional status of the female and due to the vital importance of this behavior to the proper development of the offspring.

Considering that the impact of FA supplementation on the mothers is rarely studied, we decided to evaluate the effect of this vitamin on the memory of the dams. We decided to use the ox-maze task because it requires visual discrimination and spatial memory, and it has no aversive component, being the reward the main stimulus of the rats to perform the task. Interestingly, the FA20 group presented a higher latency to find the first reward and less correct answers, indicating poor performance compared to the SD group. This is result is not completely unexpected since in our previous study (Carletti et al., 2016) rats that received FA intraperitoneally had a poor performance in the Morris water maze task, which also evaluates spatial memory. Similarly, Sittig

et al., (2012) found memory and motivation impairment in young rats that had received FA supplemented diets (5.3 mg/kg) and these data were associated to suppress thyroid hormones receptors function in the hippocampus. Canever et al., (2018) evaluated the FA supplementation during pregnancy and lactation and they found an improvement in the memory of the FA-supplemented dams (5 and 10 mg/kg of FA) in the Y-maze test. Despite the similarity, this study supplemented the mothers for a longer period and in different doses. Taking these findings together, it is possible to observe a time-dependent and dose-dependent effect of FA, revealing the importance of continuing to investigate this vitamin supplementation, observing its peculiarities In the present study, we observed a memory deficit by the high FA dose in the mothers even three weeks after the end of supplementation. This result indicated that the high dose of FA altered in a lasting manner the cognitive processes. It is alarming that the high dose used in this study is proportional to those provided by the Public Health System in Brazil. Then, it is extremely positive that the maternal behavior and the pregnancy period were not affected. It is important to consider that anxiety after pups removal in the weaning day (PND21) can affect the performance of the dams in the task, than further studies are needed to confirm this memory deficits, excluding the anxiety factor.

Trying to understand the neurobiology of the memory deficits observed in dams, we analyzed the $Na^+$, $K^+$ - ATPase activity in their hippocampus. Both doses of FA did not affect the activity of this enzyme. Few studies have evaluated the effects of FA supplementation in the mother and this was the first to analyze the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the dams. Our previous studies with FA administration (Carletti et al., 2012, 2016) in young rats also did not find changes in this enzyme activity in the hippocampus, striatum and cortex. As mentioned, Sittig et al., (2012) correlated the memory deficits after FA supplementation in young rats with decreased thyroid hormones function in the hippocampus. It is also known that FA can affect epigenetic process (Mentch and Locasale, 2016), influencing the methylation of DNA or histones. Since the memory impairment was observed after a long period of the end of the FA supplementation, is plausible to propose that this vitamin could be altering the expression of genes related to memory formation.

### *4.2. Folic acid and neonatal HI decreased $Na^+$, $K^+$ - ATPase activity without affecting the early neurodevelopment of the offspring*

Taken that FA supplementation is conducted during the development of the fetus, it is important to evaluate the growth of the offspring. The present study also proposed to investigate the effects of this supplementation in a HI model. Here, the Levine-Vannucci model of HI was performed at the PND 7 to mimic the injury in late-preterm infants (Smith et al., 2016). Recent studies have stated that PND 7 in rats can be comparable with a moderately preterm infant at birth (gestational week 34–36; Workman et al., 2013). This choice was defined by the increased prevalence of this injury in premature babies (Rumajogee et al., 2016). No differences in the weight or the body length of the pups were observed by the diets. Mikael et al., (2013) also did not found alterations in the weight or length of embryos (E14.5) by FA supplementation during pregnancy. It is known that different diets during pregnancy can impair the somatic growth of the offspring, such as: high fat diet (Mendes-da-Silva et al., 2014) and buritil oil (Medeiros et al., 2015). Then, the present findings indicate the safety of FA in the pups' early development. We also evaluated the somatic growth of the pups after the neonatal HI and no differences were found by the lesion or the supplementation. There are conflicting data about the somatic growth of the pups after the HI injury (Lubics et al., 2005; Sanches et al., 2012). Considering that the injury is progressive (Askalan et al., 2015; Northington et al., 2001) and mainly affect some brain regions, this could explain the reason that the somatic growth is not affect by the injury.

The early neurobehavioral development is another important variable of the evolution of the pups. It was well established that neurological reflexes and characteristics in neonates can be an important tool to predict behavioral modification in adults (Allen and Alexander, 1997; Heyser, 2004). Our findings did not evidence alteration in the maturation of the pups neither by the injury nor the supplementation. Our previous study (Schuch et al., 2016) also did not find modifications in the neonatal reflexes in HI rats. We can to propose that these rudimentary responses are preserved due the importance of sensorimotor responses to neonates' survival, probably involved in a spontaneous recovery after the HI injury (Farkas et al., 2009; Trollmann and Gassmann, 2009). Other point to be considered is that the Levine-Vannucci HI model is well-recognized by the cognitive deficits observed, especially, in adult rats (Carletti et al., 2012, 2016; Deniz et al., 2018; Miguel et al., 2015, 2017; Pereira et al., 2007; Rojas et al., 2013, 2015) but there is no consensus about motor deficits in this model (Lubics et al., 2005; Sanches et al., 2012; Van der Kooij et al., 2010) Taken that neonatal reflexes are almost all correlated to sensorimotor function it is possible that this HI model is not the best one to evaluate motor function. This limitation should be considered in this study that has its main focus on early development; future studies with adult rats can give further support for the impact of gestational FA supplementation on pup's development.

Our previous studies have already shown that neonatal HI impaired the $Na^+$, $K^+$ - ATPase activity in different brain structures in adult rats (Carletti et al., 2012, 2016). Considering the importance of this enzyme (McLean and Ferriero, 2004; Rumajogee et al., 2016; Rocha-Ferreira and Hristova, 2016) and that the hippocampus is primarily affect by the HI injury we decide to assess the $Na^+$, $K^+$ - ATPase activity in the hippocampus of HI rats. As expected, we observed decreased activity in the ipsilateral hippocampus in all HI rats, corroborating with our previous findings (Carletti et al., 2016). This finding can be correlated with cognitive deficits observed in HI animals. Interestingly, high FA supplementation also decreased $Na^+$, $K^+$ - ATPase activity in control rats, even long after the end of supplementation. Probably the FA supplementation in the period of neurodevelopment of the fetus has a significantly impact, changing the nervous cells in a more permanent way. Our previous study (Deniz et al., 2018) showed this lasting effect of FA in the prevention of cognitive deficits of adult HI and these was correlated with the inhibition of the late BDNF imbalance. Then, protective effects of FA are, probably, not dependent of $Na^+$, $K^+$ - ATPase activity. Considering that this vitamin seems to have a dual time-dependent effect, its excess need to be better understood during the pups' late development.

In conclusion, the present study showed that even high doses of FA supplementation during pregnancy are not able to impair the gestation and the offspring early development. Contrary to reported by Deniz et al., (2018), the effect of FA supplementation as a protective agent in neonatal HI could not be evidenced since the hypoxic-ischemic animals did not present early neurodevelopment disturbance. However, we can propose that the present findings in control group exposed to FA supplementation have indicated the safety of this vitamin during pregnancy even in high doses. Future studies need to focus in the effects of the high FA dose in the dams' memory and in the time-dependent effect in the $Na^+$, $K^+$ - ATPase activity in the hippocampus of the offspring.

## Compliance with ethical standards

To carry out this study, the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the guide of the Federation of Brazilian Societies for Experimental Biology were used. This project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (nº 28,136).

## Conflict of interest

The authors declare that they have no conflict of interest.

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# *7.DISCUSSÃO*

Este trabalho teve como objetivo avaliar os efeitos da suplementação gestacional de AF (dose recomendada e dose excessiva) no desenvolvimento da gestação, da prole submetida à HI neonatal e nas mães. Nossa hipótese foi parcialmente comprovada, pois foi observado que a suplementação com AF, em ambas as doses, na gestação foi capaz de prevenir os déficits cognitivos e o desbalanço de BDNF no hipocampo de ratos adultos submetidos à HI neonatal. Ainda, nos animais HI adultos, observamos que a alta dosagem de AF foi capaz de reverter o aumento da expressão de caspase-3 no hipocampo ipsilateral, sem reverter a diminuição da metilação da histona H3 lisinas 4 e 27. Não foram observadas diferenças no desenvolvimento da gestação ou da prole de ratas suplementadas. Porém, a alta dosagem de AF parece prejudicar a memória das ratas suplementadas e a atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo dos filhotes.

*A suplementação com AF na gestação previne os déficits cognitivos de animais HI adultos*

Trabalhos prévios do grupo já haviam evidenciado déficits cognitivos em animais adultos submetidos à HI neonatal (Rojas et al., 2013, 2015; Pereira et al., 2007; Miguel et al., 2015, 2017). Também já havíamos demonstrado o efeito dual do tratamento com AF, com injeção i.p. em animais HI, onde observamos melhora na ansiedade no teste do campo aberto e na memória aversiva no teste da esquiva inibitória (Carletti et al., 2012), porém prejuízo na memória espacial em animais controles tratados com AF (Carletti et al., 2016). Assim, como primeira etapa, este

projeto se propôs a investigar os efeitos da suplementação gestacional com AF nos déficits cognitivos de animais HI adultos. O teste do campo aberto foi usado inicialmente para avaliar a função motora e exploratória dos animais que é essencial para os testes cognitivos que seriam posteriormente realizados, além do seu uso como indicativo de ansiedade. Nossos resultados mostraram que os grupos HI apresentaram um maior número de cruzamentos quando comparado com os grupos controles, evidenciando o efeito da lesão e indicando hiperatividade. Rojas e colaboradores (2013) já haviam encontrado esse aumento no número de cruzamentos no teste do campo aberto nos animais submetidos à HI, corroborando os nossos achados. Posteriormente, nosso grupo passou a investigar se o modelo de Levine-Vannucci poderia servir como possível modelo para o Transtorno do Déficit de Atenção/ Hiperatividade (TDAH) e encontrou algumas características, como hiperatividade e inflexibilidade cognitiva, que confirmaram essa hipótese (Miguel et al., 2015, 2017). A suplementação com AF não teve efeito no aumento do número de cruzamentos e não há um consenso sobre o efeito dessa vitamina sobre a hiperatividade (Barua et al., 2014; Furuse et al., 2017; Virk et al., 2017). Provavelmente, após tanto tempo após o fim da suplementação, o AF não afetou as estruturas relacionadas com esse comportamento, como o estriado e o córtex pré-frontal. Assim, podemos concluir que a lesão hipóxico-isquêmica levou a uma maior hiperatividade nos animais que não foi prevenida pela suplementação com AF.

Como citado anteriormente, os déficits de memória já são bem estabelecidos no modelo de Levine-Vannucci de HI neonatal, sendo estes usados no presente

trabalho para avaliar os efeitos da suplementação com AF durante a gestação. No teste do labirinto aquático de Morris (para avaliar memória espacial), observamos que os animais HI precisaram de mais dias para aprender a tarefa e apresentaram uma piorperformance no dia do teste. Para a memória de curta duração, foi utilizado o teste de reconhecimento de objetos. Os animais HI tiveram um menor índice de exploração, indicando que eles não conseguiram diferenciar o objeto novo do familiar. No teste da esquiva inibitória (para avaliar a memória aversiva), os ratos HI apresentaram prejuízo ao levarem menos tempo para descer da plataforma no dia do teste. Esses resultados já eram esperados e corroboram a literatura (Carletti et al., 2012, 106; Rojas et al., 2013, 2015; Pereira et al., 2007; Miguel et al., 2015, 2017). Esses déficits são bem estabelecidos no modelo de HI neonatal utilizado, pois o hipocampo (que participa de todos os tipos de memória avaliados) é uma das principais estruturas afetadas.

Surpreendentemente, as duas doses de AF foram eficazes em prevenir os déficits em todos os testes utilizados. Esses resultados indicam que a suplementação com essa vitamina durante o desenvolvimento do feto tem efeito duradouro, visto em animais adultos, no hipocampo. Em nossos trabalhos anteriores observamos que o tratamento com AF (via intraperitoneal, 10 mg/kg) após a HI neonatal foi capaz de reverter os déficits na memória aversiva, mas afetou a aprendizado espacial em animais controles (Carletti et al., 2012, 2016). Sittig e colaboradores (2012) também trataram com AF (8 mg/kg) ratos adolescentes e observaram prejuízo no labirinto aquático de Morris associado a diminuição na função de hormônios da tireoide no hipocampo. Esses achados indicam que o tratamento com AF parece

afetar diretamente o hipocampo durante o desenvolvimento dos ratos, sendo que esta estrutura deve ser particularmente vulnerável a altas dosagens de AF. Assim, a partir dos nossos achados podemos concluir que a suplementação com o AF, mesmo em doses elevadas, durante a gestação parece exercer um efeito importante, principalmente no hipocampo, ao prevenir os déficits cognitivos observados nesse estudo. Possivelmente, durante o desenvolvimento do embrião, o uso dessa vitamina deve fornecer um suporte para o tecido nervoso em desenvolvimento, favorecendo a sobrevivência celular mesmo após um evento lesivo como a HI. Esses achados possuem uma alta relevância social, pois a suplementação com AF já ocorre durante a gestação (inclusive em doses elevadas como a fornecida pelo SUS) e eventos lesivos ao feto, como a HI, dificilmente conseguem ser previstos e geram danos permanentes à criança.

*A suplementação com AF impede o aumento tardio de BDNF, mas não diminui a morte neuronal no hipocampo de animais HI*

Para compreender os mecanismos moleculares envolvidos na prevenção dos déficits cognitivos pelo AF, focamos nossas investigações no hipocampo. Avaliamos a densidade neuronal pelas técnicas de cresil violeta e imunoistoquímica para NeuN e verificamos uma diminuição no número de neurônios em animais HI. Já é bem estabelecido na literatura que a HI leva a uma morte celular massiva nessa estrutura encefálica, gerando atrofia hipocampal e afetando os processos cognitivos relacionados (Miguel et al., 2015; Pereira et al., 2008, 2007). Curiosamente, as duas doses de AF não foram capazes de evitar a morte neuronal no hipocampo. Sabe-se que a suplementação com AF é realizada

durante a gestação para prevenir os defeitos do fechamento do tubo neural, pois essa vitamina atua diretamente no metabolismo de um-carbono, sendo necessária para a síntese de nucleotídeos e, consequentemente, para a replicação de DNA e proliferação celular. Nossos trabalhos anteriores verificaram o efeito neuroprotetor do enriquecimento ambiental em animais HI apesar de não haver diminuição na atrofia hipocampal (Pereira et al., 2007, 2008). Posteriormente, verificamos que esse efeito benéfico se dava pelo aumento da densidade de espinhos dendríticos nessa estrutura encefálica (Rojas et al., 2013). Assim, podemos propor que o efeito protetor do AF observado nesse trabalho pode estar relacionado a vias de neuroplasticidade.

Para confirmar essa hipótese, avaliamos a expressão de sinaptofisina e os níveis de BDNF no hipocampo dos animais adultos submetidos a HI neonatal. A sinaptofisina é uma proteína presente nas vesículas pré-sinápticas, sendo importante para a função sináptica e neuroplasticidade (Tarsa &Goda, 2002).Curiosamente, não foram observados efeitos nem da lesão nem da suplementação na densitometria da sinaptofisina. Zhao e colaboradores (2012) encontraram resultados que corroboram os nossos usando o mesmo modelo de HI. Considerando que há uma diminuição de células neuronais no hipocampo ipsilateral nos animais HI, podemos propor que há um aumento da expressão da sinaptofisina numa tentativa de preservar a função sináptica no lado mais afetado pela lesão. Ainda, outras proteínas, como a sinapsina e a PSD-95, podem ser avaliadas para a análise da plasticidade hipocampal.

O BDNF é um fator neurotrófico que exerce um papel importante na sobrevivência e plasticidade celular (Tajeda & Díaz-Guerra, 2017). Nossos resultados indicam um desequilíbrio tardio nos níveis desse fator no hipocampo ipsilateral dos animais HI, corroborando achados prévios do grupo (Pereira et al., 2009). Como proposto anteriormente, esse aumento tardio no BDNF também foi associado com déficits cognitivos e pode indicar uma vulnerabilidade a longo prazo do tecido e um efeito neurotóxico desse fator. Esse mecanismo neurotóxico pode estar relacionado a diferentes isoformas do receptor do fator, TrkB que ativariam a via levando a um maior influxo de íons cálcio e expressão de fatores relacionados ao fenótipo hipóxico (Duman & Voleti, 2012; Nakamura et al., 2011; Rathod et al., 2016; Vidaurre et al., 2012). Esse mecanismo poderia explicar a correlação do aumento tardio do BDNF no hipocampo com os déficits de memória encontrados em animais HI adultos.

Surpreendentemente, encontramos que as duas doses de suplementação com AF foram capazes de reverter esse aumento tardio do BDNF no hipocampo ipsilateral, indicando que esse pode ser um dos mecanismos de proteção dessa vitamina encontrado nesse trabalho. Liang e colaboradores (2017) encontraram uma diminuição da expressão de fatores relacionados ao fenótipo hipóxico pela suplementação com AF. Esse dado corrobora a nossa hipótese sofre o efeito neurotóxico do BDNF e poderia explicar a melhora cognitiva encontrada nos animais HI de mães suplementadas com AF. Ainda, é possível propor que o AF pode estar alterando padrões epigenéticos já que tem um papel relevante na

metilação de DNA/RNA e histonas (Bottiglieri, 1996) e seu efeito ainda é visto após um longo período do término da suplementação.

*A alta dose de AF previne o aumento da caspase-3, mas não altera a expressão de proteínas de plasticidade no hipocampo de animais HI*

Num segundo momento deste trabalho, tentamos investigar os possíveis mecanismos neuroprotetores do AF encontrado nos parâmetros cognitivos avaliados nos animais HI adultos. Inicialmente avaliamos a expressão da caspase-3 no hipocampo dos animais, que é uma das proteínas efetoras da via apoptótica de morte celular. Nossos resultados mostraram um aumento da expressão dessa proteína no hipocampo ipsilateral dos animais HI, indicando que a morte celular caspase-dependente segue ocorrendo por um longo período após a lesão. Essa continuidade do apoptose na HI já foi demonstrada (Askalan et al., 2015; Northington et al., 2001) e é responsável pelo alargamento da lesão e os déficits cognitivos evidenciados. Nossos resultados também evidenciaram que a alta dose de AF foi capaz de prevenir esse aumento na expressão de caspase-3 nos animais HI. Efeitos antiapoptóticos e antioxidantes já foram demonstrados por essa vitamina (Majumdar et al., 2012; Singh et al., 2011; Stanger & Wonisch, 2012; Quan et al., 2015) e podem estar correlacionados com a prevenção da morte tardia por apoptose na HI neonatal. Ainda, nossos resultados anteriores mostraram que o AF foi capaz de prevenir o desequilíbrio tardio do BDNF no hipocampo dos animais, o que poderia favorecer a sobrevivência das células.

Para avaliar se a neuroproteção do AF poderia estar relacionada com outras proteínas, investigamos a expressão da sinapsina e da PSD-95. A primeira é uma

proteína pré-sináptica presente nas vesículas (Thiel, 2004) enquanto a segunda compõe a densidade pós-sináptica, sendo necessária para a manutenção da organização molecular (Chen et al., 2011).Encontramos uma diminuição na expressão da sinapsina no hipocampo ipsilateral dos animais HI, sem modificações na expressão da PSD-95. Outros trabalhos também já encontraram diminuição da sinapsina após a lesão hipóxico-isquêmica (Griva et al., 2017; Xiong et al., 2018). Esses achados podem estar correlacionados coma diminuição da densidade de espinhos dendríticos após a HI que levariam aos déficits cognitivos já estabelecidos (Rojas et al., 2013). A suplementação com AF não foi capaz de prevenir essa diminuição da sinapsina, indicando que essa proteína não está relacionada com o mecanismo neuroprotetor da vitamina na idade avaliada.

*A suplementação com AF na gestação não inibe a diminuição da metilação da histona H3 no hipocampo de animais HI*

Considerando o importante papel do AF nas reações de metilação e mecanismos epigenéticos, decidimos avaliar como a suplementação e a HI poderiam influenciar esses mecanismos. Recentemente, estudos têm evidenciado o papel da epigenética na lesão da HI neonatal. Foram encontradas modificações no padrão de metilação de DNA, na expressão de genes e na acetilação de histonas (Ishida et al., 2007; Jaworska et al., 2017; Koslowski et al., 2011; Ziemka-Nalecz et al., 2017). Já foi demonstrado o papel da histona H3 nos processos de aprendizado e memória (Dias et al., 2015; Jarome & Lubin, 2014), que poderia ser correlacionado com os déficits cognitivos da HI. Considerando o papel do AF nas reações de metilação e a melhora cognitiva (dependente da H3) evidenciada nos filhotes de

mães suplementadas com essa vitamina, decidimos avaliar os efeitos da HI e do AF nos níveis de metilação da histona H3. Para o nosso conhecimento esse foi o primeiro trabalho a avaliar a metilação da H3 na lesão da HI neonatal. Nossos resultados indicaram diminuição da di e trimetilação da H3K4 e H3K27 no hipocampo ipsilateral dos animais HI. Relembrando, as histonas podem ser mono, di ou trimetiladas nos seus resíduos de lisina (K) e a metilação na H3K4 está relacionada com o aumento da expressão gênica, enquanto a metilação na H3K27 reprime a expressão gênica (Barski et al., 2007; Ferrari et al., 2014; Ruthenburg et al., 2007). Assim, nossos resultados indicam que há uma modulação diferencial da expressão de diversos genes, como demonstrado porHedtjärn e colaboradores (2004), sendo alguns genes mais expressos e outros reprimidos que levariam ao fenótipo encontrado nos animais HI.

Ainda, podemos sugerir mecanismos a partir das proteínas avaliadas no presente estudo e as modificações epigenéticas encontradas. A diminuição da expressão da sinapsina pode ocorrer pela diminuição da metilação da H3K4. Como a metilação desse resíduo é relacionado com o aumento da expressão gênica, a diminuição da sua metilação poderia levar a inibição da expressão gênica encontrada nesse trabalho. Ainda, a diminuição da metilação da H3K27, normalmente relacionada com a inibição gênica, poderia levar a uma maior expressão de alguns genes, como da caspase-3. Contrário à nossa hipótese inicial, a suplementação com AF não influenciou a metilação das histonas, nem mesmo nos animais HI. Porém, não podemos descartar que essas modificações epigenéticas podem ser transitórias, principalmente no período do

neurodesenvolvimento, além do envolvimento de outros marcadores epigenéticos não avaliados nesse estudo.

*A suplementação com AF não altera o desenvolvimento da gestação e nem o comportamento materno*

Num último momento, decidimos avaliar os efeitos da suplementação com AF no desenvolvimento da gestação, da prole e nas mães, já que não há consenso na literatura e quase nenhum estudo avaliando as mães. Inicialmente, avaliamos os possíveis efeitos das diferentes doses de AF no consumo das rações pelas prenhas que poderiam influenciar o desenvolvimento da gestação e, inclusive, o comportamento materno. Não observamos diferenças entre os grupos nem no consumo de ração, no peso ao longo da gestação e no número de filhotes por ninhada. Achón e coloboradores (1999) também não encontraram diferenças no peso, consumo de ração e no número de filhotes de prenhas suplementadas com altas dosagens de AF, corroborando os nossos achados. Contrariamente, Mikael e colaboradores (2012) encontraram diminuição no número de implantação e aumento no número de perda de embriões no E14.5 de ratas suplementadas com AF. Mais uma vez, podemos observar o efeito dual do AF e mostrar a importância de se continuar estudando seus efeitos uma vez que a suplementação com essa vitamina já é realizada na gestação.

Estudos anteriores já mostraram que interferências na gestação podem afetar o comportamento materno (Gatta et al., 2018; Li et al., 2015; Purcell et al., 2011), podendo influenciar o desenvolvimento e sobrevivência dos filhotes. Para nosso conhecimento, esse foi o primeiro trabalho a avaliar os efeitos de diferentes doses

de AF durante a gestação no comportamento materno. Nossos resultados não evidenciaram diferenças entre os grupos pela suplementação com AF. É provável que, por não apresentar um componente aversivo e nem alterar o status nutricional das mães, a suplementação com AF não interfere nesse comportamento tão importante para o desenvolvimento da prole.

*Alta dose de AF na gestação afeta a memória das ratas mães sem alterar a atividade da enzima Na+, K+ - ATPase no hipocampo*

Ainda, avaliamos a memória das mães e a atividade da $Na^+$, $K^+$ - ATPase no hipocampo.Encontramos que a alta dose de AF (20 mg/kg) causou déficit na memória das mães mesmo após um longo período do término da suplementação. Esse déficit não foi correlacionado com alteração na atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo. Sittig e colaboradores (2012) já haviam demonstrado que a suplementação com AF em alta dose prejudicou a memória de animais adolescentes e foi associada com a alteração da função dos receptores dos hormônios da tireoide no hipocampo dos animais. Ainda, em um estudo anterior do nosso grupo (Carletti et al., 2016) observamos que o tratamento com AF por um longo período causou déficit na memória de animais controles que também não foi associado com alteração na atividade da enzima $Na^+$, $K^+$ - ATPase. Contrariamente a esses achados, Canever e colaboradores (2018) encontraram uma melhora na memória de ratas suplementadas com AF, em diferentes doses, durante a gestação. Assim, mais uma vez, fica evidente o efeito dual dessa vitamina indicando a relevância do seu estudo.

*O AF na gestação e a HI neonatal não modificam o crescimento somático e os reflexos neonatais dos filhotes*

Também avaliamos os efeitos da suplementação com AF no desenvolvimento somático dos filhotes e não encontramos diferenças nem no tamanho corporal nem no peso da prole, entre os grupos, ao longo do desenvolvimento. Sabe-se que diferentes dietas durante a gestação podem afetar o desenvolvimento somático da prole (Medeiros et al., 2015; Mendes-da-Silva et al., 2014), provavelmente por alterar o status nutricional das mães, o que não é visto pela suplementação com AF. Além disso, também não encontramos diferenças no crescimento somático pela lesão. Não há consenso se a HI neonatal afeta o crescimento somático dos animais (Lubics et al., 2005; Sanches et al., 2012) e, considerando que o dano é progressivo e afeta regiões encefálicas específicas, é possível propor que a HI não afetaria o crescimento somático dos animais.

Os marcos do desenvolvimento possuem um papel importante na avaliação da maturação dos reflexos neonatais e servem como preditores de alteração do comportamento em adultos (Allen & Alexander, 1997; Heyser, 2004). Corroborando nosso trabalho anterior (Schuch et al., 2016), não encontramos alteração nos reflexos neonatais nem pela lesão, nem pela suplementação. Já foi proposto uma recuperação espontânea depois da HI (Farkas et al., 2009; Trollmann & Gassmann, 2009), considerando a importância dessas respostas sensoriomotoras para a sobrevivência dos filhotes. Ainda, é preciso discutir que esses reflexos possuem uma maior relação com a maturação da função sensoriomotora e o modelo de Levine-Vannucci parece não gerar alterações

motoras significativas (Lubics et al., 2005; Sanches et al., 2012; Van der Kooij et al., 2010), podendo explicar os resultados encontrados no presente trabalho.

*A HI neonatal e alta dose de AF na gestação diminuem a atividade da enzima Na+, K+ - ATPase no hipocampo dos filhotes*

Finalmente, avaliamos a atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo dos filhotes com 21 dias de vida pós-natal. Encontramos uma diminuição na atividade dessa enzima no hipocampo ipsilateral dos animais HI e dos animais controles de mães suplementadas com altas doses de AF. Estudos prévios do nosso grupo mostraram que a HI neonatal diminui a atividade da $Na^+$, $K^+$ - ATPase em diferentes estruturas encefálicas, como estriado, córtex e hipocampo (Carletti et al., 2012, 2016), corroborando os achados desse trabalho. Considerando o efeito dual e tempo-dependente encontrado por essa vitamina na literatura, é possível que nesse período do desenvolvimento a alta dose de AF seja prejudicial para a atividade da $Na^+$, $K^+$ - ATPase no hipocampo dos animais.

*Principais achados acerca da suplementação com AF durante o período gestacional no modelo de HI neonatal*

Diante do que foi exposto, podemos evidenciar que a suplementação com AF, independente da dose, durante a gestação foi benéfica por prevenir os déficits cognitivos a longo prazo causados pela HI neonatal. Ainda, não alterou o desenvolvimento da gestação, da prole e o comportamento materno. Esses resultados têm alta relevância social, pois a suplementação com AF já é realizada durante a gestação em humanos e mesmo em altas doses, como a fornecida pelo

SUS, parecem ser seguras. Eventos como a HI neonatal são dificilmente previstos e geram danos permanentes nas crianças, necessitando de cuidados e alto investimento em terapias ao longo da vida. Ainda não existem terapias efetivas para tratar lesões hipóxico-isquêmicas e os achados no presente trabalho indicam que a suplementação, já realizada, com AF durante toda a gestação pode ser neuroprotetora frente a lesões similares a HI. Apesar de todos os resultados positivos da suplementação com folato, encontramos que a alto dose de AF prejudicou a memória das mães e a atividade da $Na^+$, $K^+$ - ATPase no hipocampo dos animais jovens. Esses dados, evidenciam, mais uma vez, que o AF pode ser tanto protetor como tóxico, dependendo da dose, tempo de suplementação e estruturas avaliadas. Assim, mais estudos são necessários para investigar e entender os mecanismos envolvidos no uso dessa vitamina na gestação e frente a eventos lesivos ao SNC do feto, pois sua utilização já é realizada.

# *8. CONCLUSÕES*

Com base nos achados do presente trabalho podemos concluir que:

**A HI neonatal causou:**

- Déficits cognitivos, morte neuronal e desequilíbrio nos níveis de BDNF no hipocampo ipsilateral de ratos adultos;

- Aumento da expressão de caspase-3 e diminuição da expressão de sinapsina no hipocampo ipsilateral de animais adultos;

- Diminuição nos níveis de metilação da H3K4 e H3K27 no hipocampo ipsilateral de animais adultos;

- Diminuição da atividade da enzima $Na^+$, $K^+$ - ATPase no hipocampo ipsilateral de ratos com 21 DPN sem alterar o crescimento somático ou a maturação e o aparecimento dos marcos do desenvolvimento.

**A suplementação com AF durante a gestação:**

- Foi capaz de prevenir os déficits cognitivos e o desequilíbrio do BDNF causado pela HI sem inibir a morte neuronal no hipocampo ipsilateral dos animais adultos;

- Somente na alta dosagem foi capaz de prevenir o aumento da expressão de caspase-3 sem alterar a expressão de sinapsina no hipocampo ipsilateral dos animais adultos;

- Não foi capaz de reverter a diminuição da metilação na H3K4 e H3K27 no hipocampo ipsilateral dos ratos adultos;

- Não alterou o crescimento somático nem os marcos do desenvolvimento, mas causou diminuição da atividade da Na+, K+ - ATPase no hipocampo de animais

controles, não prevenindo a inibição da atividade dessa enzima nos animais HI aos 21 DPN;

- Não alterou o desenvolvimento da gestação nem o comportamento maternal, contudo a alta dosagem causou prejuízo na memória das mães, avaliadas no teste do Ox-maze.

# *9.PERSPECTIVAS*

Considerando os achados do presente trabalho, nossas perspectivas são:

- Avaliar as modificações epigenéticas em diferentes estruturas encefálicas de animais HI ao longo do desenvolvimento, bem como das suas mães suplementadas com diferentes níveis de AF;

- Avaliar outros parâmetros de plasticidade em diferentes estruturas encefálicas de animais HI ao longo do desenvolvimento e verificar os efeitos da suplementação gestacional com AF;

- Avaliar o estresse oxidativo em diferentes estruturas encefálicas de animais HI ao longo do desenvolvimento, bem como de suas mães suplementadas com AF;

- Avaliar parâmetros cognitivos das mães suplementadas com diferentes doses de AF durante a gestação.

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