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DISSERTAÇÃO DE MESTRADO

BAIXA RAZÃO ÔMEGA-6 / ÔMEGA-3 EM UMA DIETA MATERNA MULTIDEFICIENTE PROMOVE ALTERAÇÕES EPIGENÉTICAS EM HISTONAS DE CÉLULAS NEURAIS DA PROLE QUE FAVORECEM A TRANSCRIÇÃO GÊNICA

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Orientadora: Profa. Dra. Belmira Lara da Silveira Andrade da Costa
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Recife, 16 de fevereiro de 2016

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota.”

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RESUMO

Ácidos graxos essenciais da família ômega-3 são cruciais durante o desenvolvimento cerebral atuando na transcrição gênica e sobre mecanismos epigenéticos, influenciando a gênese e diferenciação de neurônios e astrócitos. Por outro lado, evidências têm mostrado que a desnutrição materno-proteica pode modificar negativamente padrões epigenéticos do genoma fetal. O presente estudo investigou se uma razão ômega-6/ômega-3 (n-6/n-3) favorável na dieta materna pode influenciar alterações pós-traducionais em histonas de células neurais da prole, mesmo em uma condição de deficiência em proteínas e outros micronutrientes. Ratas *Wistar* foram mantidas em uma dieta com deficiência multifatorial (Dieta Básica Regional – DBR) possuindo, entretanto, uma baixa razão n-6/n-3 em relação a dieta controle, 30 dias antes do acasalamento e durante a gestação. Embriões de 16 dias de gestação e neonatos de 0 a 3 dias pós-natais foram utilizados para a realização de culturas corticais de neurônios e astrócitos, respectivamente. Acetilação de H3K9 e dimetilação de H3K4 e H3K27 foram avaliados por imunocitoquímica e citometria de fluxo. Análises por densitometria ótica mostraram aumento nos níveis de H3K4me2 nos astrócitos do grupo malnutrido (Mn) (27115±9892 $P < 0.005$) em relação ao grupo nutrido (N) (22583±6194) e nenhuma alteração nos níveis de H3K9Ac (Mn: 27401±10248 vs. N: 30034±10423) ou H3K27me2 (Mn: 16693±4842 vs. N: 16187 ±3378). Tratamento das culturas com um inibidor de histona acetiltransferase (HAT), promoveu uma redução da acetilação de maneira dose dependente menor nos astrócitos do grupo malnutrido em relação ao nutrido. Através de análises por citometria de fluxo não observaram-se diferenças entre as grupos (H3K9Ac - Mn: 13217± 6821 vs. N:14620± 6515 / H3K4me2 - Mn: 22917± 14938 vs. N: 25956± 14258). Por outro lado, o tratamento dos astrócitos por 24 horas com 100µM de DHA promoveu um aumento de 60% na acetilação de H3K9 do grupo malnutrido. Análise de western blot para GFAP mostrou que ambos os grupos apresentam a expressão predominante da isoforma fosforilada desta proteína com 50kDa. Imunocitoquímica para H3K9Ac em neurônios mostrou um aumento nos níveis de fluorescência no grupo malnutrido (Mn: 30107±6789 vs. N: 25257±7562, $P = 0.0075$), e nenhuma diferença entre os grupos em relação à H3K4me2 (Mn: 23203±7059 vs. N: 22654± 5376). Não houve ativação do fator de transcrição gênica NK-kB nestas células. O perfil de ácidos graxos do leite materno no estômago dos neonatos foi avaliado por cromatografia gasosa, mostrando que a razão ácido linoleico/ácido alfa-linolênico da DBR é mantida. H3K9Ac e H3K4me2 estão envolvidos no aumento da transcrição de genes relacionados à gênese e diferenciação de astrócitos e neurônios. Nossos resultados indicam uma possível relação entre a razão favorável de n-6/n-3 presente na DBR sobre a promoção da transcrição gênica, mesmo em um contexto de baixa proteína. Além disso, sugere-se que o DHA esteja atuando sobre a acetilação, mantendo-a estável, em função da sua influência sobre as enzimas que atuam nesse processo.

Palavras-chaves: Ômega-3. Alterações em histonas. Má-nutrição. Astrócitos. Neurônios.

ABSTRACT

Omega-3 essential fatty acids play key roles during brain development acting on gene transcription and epigenetic mechanisms influencing the genesis and differentiation of neurons and astrocytes. On the other hand, evidence has shown that maternal protein malnutrition may negatively change epigenetic patterns of the fetal genome. The present study investigated whether a low and favorable ômega-6/ômega-3 (n-6/n-3) ratio on maternal diet may induce histone post-translational changes in neural cells of the offspring, even in a condition of protein and micronutrients deficiency. Wistar rats were maintained on a diet with multifactorial deficiency (Regional Basic Diet-RBD) containing, however, a low n-6/n-3 ratio compared to the control diet, 30 days prior to mating and during gestation. Embryos of 16 days and newborns from 0 to 3 postnatal days were used to obtain cortical neuron and astrocyte primary cultures, respectively. Acetylation of H3K9 and dimethylation of H3K4 and H3K27 were evaluated by flow cytometry and immunocytochemistry. The results of optical density showed increased levels of H3K4me2 in astrocytes of the malnourished group (Mn) (27115 ± 9892; P <0.005) when compared to the nourished group (N) (22583 ± 6194). On the other hand, no change in the H3K9Ac (Mn: 27401 ± 10248 vs. N: 30034 ± 10423) or H3K27me2 (Mn: 16693 ± 4842 vs. N: 16187 ± 3378) were observed. Treatment of astrocyte cultures with an inhibitor of histone acetyltransferase (HAT), was able to decrease acetylation in a dose-dependent manner; however, this decay was less intense in astrocytes of the malnourished group compared to the nourished. Through analysis by flow cytometry no intergroup differences (H3K9Ac - Mn: 13217± 6821 vs. N: 14620 ± 6515 / H3K4me2 - Mn: 22917± 14938 vs. N: 25956± 14258). On the other hand, the treatment of astrocytes by 24 h with 100 µM DHA increased ~60% H3K9 acetylation levels of astrocytes in the malnourished group. Western blot analysis for GFAP showed in both groups the predominant expression of the phosphorylated isoform with 50 kDa. Immunocytochemistry for H3K9Ac and H3K4me2 in neurons indicated an increase in the levels of H3K9Ac fluorescence in the malnourished group (Mn: 30107 ± 6789 vs. N: 25257 ± 7562, P=0.0075) and no intergroup difference in the H3K4me2 (Mn: 23203 ± 7059 vs. N: 22654 ± 5376). No activation of the transcription factor NK-kB gene was found in these cells. The fatty acid profile of the breast milk in the stomach of newborns was evaluated by gas chromatography, showing that the linoleic/alpha-linolenic ratio present in the RBD is maintained. H3K9ac and H3K4me2 are involved in the increased transcription of genes related to the genesis and differentiation of astrocytes and neurons. In this way, the results obtained in this study points to a possible contribution of low n-6/n-3 present in the RBD on promotion of gene transcription, even in a context of low protein. In addition, it is suggested that DHA is acting on the acetylation, keeping it stable, probably due a potential influence on the enzymes involved in this process.

Keywords: Omega-3, histone 3. Malnutrition, Astrocytes, Neurons, epigenetics

LISTA DE FIGURAS E TABELAS

Figura 1. Alterações epigenéticas e efeitos sobre a transcrição gênica 18

Figura 2. Esquema do processo de metilação do DNA..... 19

Figura 3. Modificações pós-traducionais em histonas.....21

Figura 4. Fatores ambientais, alterações epigenéticas e repercussões fenotípicas...22

Figura 5. Regulação epigenética da expressão gênica e programação fetal.....23

Tabela 1. Composição percentual (g%) da dieta comercial padrão e da DBR.....15

Tabela 2. Composição percentual do total de ácidos graxos na dieta comercial padrão e na DBR.....15

LISTA DE ABREVIATURAS

AA	Ácido anacárdico
BDNF	Fator neurotrófico derivado do cérebro, do inglês *brain-derived neurotrophic factor*
BSA	Albumina de soro bovina, do inglês *bovine serum albumin*
CpG	Ligação fosfato - citosina-guanina
DBR	Dieta básica regional
DHA	Ácido docosahexaenóico, do inglês *docosahexaenoic acid*
DNMT	DNA metiltransferase
FBS	Soro fetal bovino, do inglês *fetal bovine serum*
GFAP	Proteína ácida fibrilar glial, do inglês *glial fibrilary acidic protein*
HAT	Histona acetiltransferase
HDAC	Histona desacetilase
HDM	Histona desmetilase
HMT	Histona metiltransferase
H3	Histona 3
K	Aminoácido Lisina
miRNA	MicroRNA
NPC	Célula precursora neural, do inglês *neural precursor cell*
n-3	Ômega-3
n-6	Ômega-6
PBS	Tampão fosfato salina, do inglês *phosphat buffer saline*
PB	Tampão fosfato, do inglês *phosphate buffer*
R	Aminoácido Arginina
SAM	S-adenosilmetionina
SNC	Sistema nervoso central
STAT3	Transdutor de sinal e ativador de transcrição 3, do inglês s*ignal transducer and activator of transcription 3*
TSA	Tricostatina
VPA	Ácido valpróico

SUMÁRIO

1 INTRODUÇÃO 12
2 FUNDAMENTAÇÃO TEÓRICA 13
2.1 Nutrição e Sistema Nervoso Central 13
2.2 Mecanismos de regulação epigenética 17
2.3 Nutrição e modificações epigenéticas no SNC 23
2.4 Mecanismos epigenéticos e diferenciação celular 26
3 JUSTIFICATIVA E HIPÓTESE 28
4 OBJETIVOS 30
4.1 Objetivo Geral 30
4.2 Objetivos Específicos 30
5 ARTIGO CIENTÍFICO 31
6 CONCLUSÕES E PERSPECTIVAS 61
7 REFERÊNCIAS BIBLIOGRÁFICAS 62
ANEXO 70

1. INTRODUÇÃO

Concentrações adequadas de diversos macro e micronutrientes na dieta estão relacionados ao correto desenvolvimento, funcionamento e maturação do sistema nervoso central (SNC). A presença de alterações dietéticas de curto ou longo prazo, especialmente quando impostas durante os períodos críticos do desenvolvimento cerebral, podem promover disfunções celulares e repercutir em desordens neurofisiológicas (ALAMY & BENGELLOUN, 2012; BELLUSCIO et al., 2014; HOEIJMAKERS et al., 2015). Tais repercussões podem resultar de modificações epigenéticas, caracterizadas por promover alterações na expressão gênica sem influenciar na sequência de nucleotídeos do DNA (HUIDOBRO et al., 2013; LAKER et al., 2013; LANDGRAVE-GÓMEZ et al., 2015). As modificações pós-traducionais em histonas, tais como a acetilação e metilação, estão entre os mecanismos relacionados com a regulação da expressão e silenciamento de genes, atuando fortemente nos processos de diferenciação e maturação das células nervosas (MURAO et al., 2016).

Ácidos graxos essenciais da família ômega-3, tais como o ácido docosahexanóico (DHA), são de grande importância durante o desenvolvimento e maturação do SNC (ELSHERBINY et al., 2015), atuando sobre a diferenciação de astrócitos e neurônios, e como modulador da comunicação intercelular e de circuitos de neurotransmissores (STROKIN et al., 2003; CHAMPEIL-POTOKAR et al., 2006; JOARDAR et al., 2006). Além disso, desempenham diversas funções antioxidantes, anti-inflamatórias, neuroprotetoras e na transcrição de vários genes (YAVIN, 2006). Por outro lado, aportes inadequados de DHA na dieta podem influenciar negativamente na neurogênese (SAKAYORI et al., 2011; TOKUDA et al., 2014), crescimento neurítico (MITA et al., 2016) síntese de neurotrofinas (MIYAZAWA et al., 2010) e em mecanismos de plasticidade sináptica (DE VELASCO et al., 2012; DE VELASCO et al., 2015).

Os crescentes índices de síndromes metabólicas representam um grande problema atualmente. Estas são favorecidas por alterações epigenéticas induzidas por dietas hiperlipídicas e hipercalóricas, onde existe um desbalanço entre os níveis de ômega-3 e ômega-6. Neste sentido, diversos trabalhos têm apontado os efeitos do ômega-3, em especial do DHA, modulando mecanismos epigenéticos e promovendo proteção ou reversão de danos em células e tecidos.

Apesar dos conhecimentos sobre a relação entre dieta e mecanismos epigenéticos, pouco se sabe sobre a influência de determinados modelos dietéticos sobre células diferenciadas do SNC, tais como astrócitos e neurônios, bem como do papel do ômega-3 neste contexto, em especial em relação a modificações pós-traducionais em histonas.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. NUTRIÇÃO E SISTEMA NERVOSO CENTRAL

Diversos macro e micronutrientes possuem um papel extremamente importante no correto desenvolvimento, funcionamento e maturação do sistema nervoso central (SNC). Desta forma, alterações dietéticas de curto ou longo prazo, especialmente quando impostas em períodos críticos do desenvolvimento cerebral, tais como durante a gestação e início da vida pós-natal, podem promover disfunções celulares e resultar em posteriores desordens neurofisiológicas (SOUZA et al., 2011; SABLE et al., 2011; ALAMY & BENGELLOUN, 2012; BELLUSCIO et al., 2014; HOEIJMAKERS et al., 2015). Considerando que o tempo de maturação das regiões encefálicas é distinto e envolve uma sequência de eventos temporalmente estabelecidos, a dieta pode influenciar diferentemente as áreas cerebrais e os processos de maturação e diferenciação celular, tais como mielinização e sinaptogênese (MORGANE et al., 2002; ALAMY & BENGELLOUN, 2012; BIRAN et al., 2012).

Modelos de má-nutrição, como de restrição proteica imposta em várias etapas do desenvolvimento e com diferentes graus de intensidade, vêm mostrando sua intrínseca relação com alterações no funcionamento normal do SNC (ALAMY & BENGELLOUN, 2012; FISCHER et al., 2015). Subnutrição por redução da injestão de alimento, bem como imposição de uma dieta com reduzidos níveis proteicos (7% de caseína), durante o período de gestação e aleitamento, foi capaz de promover aumento do dano oxidativo e alterações em defesas antioxidantes em diferentes regiões cerebrais da prole (PARTADIREDJA et al., 2005; FEOLI et al., 2006).

Diversos estudos usando um modelo de má-nutrição multifatorial, denominado de dieta básica regional (DBR) - caracterizado por níveis reduzidos de proteínas, tanto em quantidade como em qualidade, minerais, vitaminas e lipídios (TEODOSIO et al., 1990; MEDEIROS et al., 2008;) **(Tabela 1)** - revelaram aumento do estresse oxidativo

placentário, causando impacto no desenvolvimento da prole (VIEIRA-FILHO et al., 2009); maior excitabilidade do córtex cerebral (GUEDES et al., 1987); redução no número de astrócitos em regiões hipotalâmicas, como a área pré-óptica medial e o núcleo supraquiasmático (MENDONÇA et al., 2004); menor crescimento do tecido retiniano e redução na porcentagem de axônios mielinizados e na área de mielina do nervo óptico (ALMEIDA et al., 2005; VILELA et al., 2005).

Evidências obtidas no laboratório de Neurofisiologia da UFPE mostram que este modelo de má-nutrição, quando imposto às fêmeas trinta dias antes do acasalamento e durante toda a gestação, é capaz de promover alterações em parâmetros morfológicos e funcionais de astrócitos do córtex cerebral da prole (GONÇALVES-PIMENTEL *et al.,* manuscrito a ser submetido em anexo).

No entanto, apesar da DBR possuir um quantitativo inferior de lipídios quando comparado à dieta comercial padrão, esta dieta é rica em ácidos graxos da família ômega-3, derivados do ácido alfa-linolênico, e pobre nos derivados de alfa-linoleico, pertencentes à família ômega-6, promovendo uma razão ômega-6/ômega-3 (n-6/n-3) bem inferior ao encontrado na dieta controle **(Tabela 2)** (DE SOUZA et al., 2008). De Souza e colaboradores (2008) analisaram o perfil de ácidos graxos no córtex, hipocampo e cerebelo de animais mantidos com a DBR desde o período gestacional até os 70 dias de vida e observaram uma razão n-6/n-3 menor no hipocampo e cerebelo dos animais malnutridos em comparação ao grupo controle (DE SOUZA et al., 2008).

Augusto e colaboradores (2015) observaram que mesmo em um contexto de má-nutrição severa, imposta pela DBR trinta dias antes do acasalamento e mantida durante a gestação e aleitamento, a baixa razão n-6/n-3 presente na dieta favorece o equilíbrio redox do cerebelo em comparação ao córtex cerebral, mesmo havendo alterações estruturais nessa região (AUGUSTO et al., 2015, manuscrito submetido em anexo). Esses dados corroboram a maior incorporação de DHA observada no cerebelo da prole submetida à má-nutrição crônica (DE SOUZA et al., 2008).

Tabela 1. Composição percentual (g%) da dieta comercial padrão e da DBR

Composição	**Dieta comercial**	**DBR**
Proteínas	23,00	7,87
Carboidratos	52,00	69,24
Gorduras	4,00	0,80
Sais minerais	10,00	1,26
Fibras	8,00	7,21
Outros	3,00	13,62

Adaptado de TEODÓSIO *et al.*, 1990.

Tabela 2. Composição percentual do total de ácidos graxos na dieta comercial padrão e na DBR

Composição	**Dieta comercial**	**DBR**
Ácidos graxos saturados	27,41 ± 0,85	39,37 ± 0,78
Ácidos graxos monoinsaturados	22,52 ± 1,28	25,96 ± 0,78
Ácidos graxos poli-insaturados (Série linoleica)	46,39 ± 1,36	20,38 ± 0,90
Ácidos graxos poli-insaturados (Série linolênica)	3,44 ± 0,07	12,25 ± 0,57
Ômega-6/ômega-3	13,49 ± 0,19	1,69 ± 0,11

Adaptado DE SOUZA *et al*., 2008.

O equilíbrio entre os ácidos graxos ômega-3 e ômega-6 na dieta é de fundamental importância para o funcionamento do SNC. Derivados do seu metabolismo, tais como o ácido docosahexaenóico (DHA) e o ácido araquidônico (AA) podem atuar como segundos mensageiros lipídicos com características anti-inflamatórias e neuroprotetoras ou pró-inflamatórias, respetivamente, a partir da síntese de prostaglandinas, leucotrienos e tromboxanos.

Diversos trabalhos mostraram a importância do DHA para a manutenção de várias funções no SNC, tanto durante quanto após o período de desenvolvimento. Em razão disso, sua carência pode repercutir em inúmeras desordens neurológicas, tais como alterações comportamentais, locomotoras e cognitivas (FEDOROVA et al., 2006; BAZAN et al., 2011; MORANIS et al., 2012; BERNARDI et al., 2012; BRENNA

et al., 2014). Ao nível celular, foi demonstrado que o DHA atua modulando a relação entre astrócitos e neurônios em diversos circuitos dos sistemas de neurotransmissão, como serotoninérgico, dopaminérgico, e colinérgico (TAKEUCHI et al., 2002; STROKIN et al., 2003); auxilia na manutenção da comunicação intercelular mediada por junções comunicantes em membranas astrocitárias (CHAMPEIL-POTOKAR et al., 2006); participa na diferenciação morfológica de astrócitos e neurônios, aumentando o crescimento neurítico e promovendo sinaptogênese (JOARDAR et al., 2006; SAKAYORI et al., 2011; TOKUDA et al., 2014; DYALL, 2015); bem como na regulação da transmissão glutamatérgica mediada por astrócitos na região de CA1 do hipocampo (LATOUR et al., 2013).

Tem sido elucidado os papéis do ômega-3 sobre processos inflamatórios no SNC. Redução dietética de ômega-3 durante a gestação e lactação de ratos, foi capaz de aumentar a expressão de citocinas pró-inflamatórias, tais como IL-1β e IL-6, na prole aos 21 dias de vida, bem como promover alterações no fenótipo e motilidade da microglia (MADORE et al., 2014). Experimentos *in vitro*, com culturas de microglia, mostraram que o pré-tratamento com DHA reduz a expressão de óxido nítrico e TNFα induzidos por lipopolissacarídeo. Os mesmos autores viram ainda que *in vivo* a suplementação dietética com ômega-3 reduz a desmielinização induzida pela cuprizona, em um modelo de esclerose múltipla (CHEN et al., 2014).

Dados obtidos em nosso laboratório, utilizando um modelo de restrição de ácidos graxos essenciais durante duas gerações, mostram uma degeneração de neurônios dopaminérgicos e não dopaminérgicos na substância negra, um aumento nos níveis de peroxidação lipídica e redução da atividade de enzimas antioxidantes nesta região cerebral, em ratos jovens e adultos (CARDOSO et al., 2012). Adicionalmente, viu-se que este modelo de má nutrição é capaz de reduzir o número de neurônios que expressam o fator neurotrófico derivado do cérebro (BDNF) e induzir alterações em parâmetros morfofuncionais de astrócitos (SANTANA *et al.,* 2013; dados não publicados).

Lee e colaboradores (2015) viram que o DHA é capaz de prevenir a redução de neurônios dopaminérgicos da substância negra, provocada pelo tratamento com o pesticida paraquat, em um modelo de doença de Parkinson. Os mecanismos envolvidos nesse processo estão relacionados à um aumento da viabilidade celular, redução da produção de espécies reativas de oxigênio e aumento na expressão do fator de transcrição Nrf2, que atua regulando a expressão de proteínas antioxidantes

(LEE et al., 2015). Estes achados enfatizam o papel importante dos ácidos graxos da família ômega-3, e em especial do DHA, como um agente neuroprotetor.

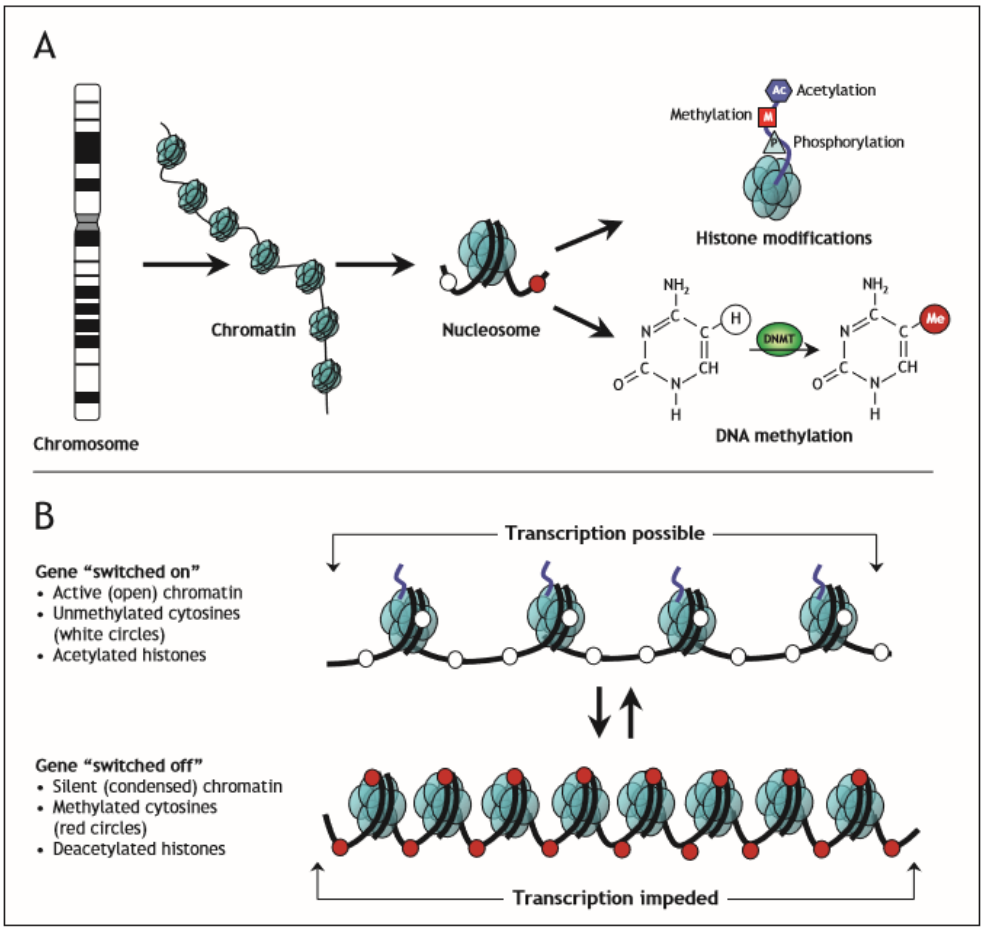
Tomados em conjunto, os dados apresentados mostram que a carência de determinados nutrientes na dieta pode influenciar profundamente o desenvolvimento do SNC e repercutir em diversas alterações ao longo da vida. Essas respostas têm sido associadas ao papel dos componentes da dieta sobre mecanismos de alterações epigenéticas, os quais estão intimamente envolvidas com a regulação da expressão gênica (ver 2.3).

2.2. MECANISMOS DE REGULAÇÃO EPIGENÉTICA

A primeira descrição de epigenética data da década de 40 pelo biólogo britânico Conrad Waddington. Ele utilizou esse termo para descrever a forma como um mesmo genótipo é capaz de promover a expressão de diferentes fenótipos durante o desenvolvimento dos organismos (WADDINGTON, 2012; LANDGRAVE-GÓMEZ et al., 2015). Ao longo dos anos, e com o avanço das tecnologias, esse conceito foi sendo complementado. Atualmente o termo epigenética é definido como alterações hereditárias na expressão gênica, susceptível a diversos fatores ambientais, que não envolvem alterações na sequência de nucleotídeos do DNA (CORTESSIS et al., 2012; HUIDOBRO et al., 2013; LAKER et al., 2013; LANDGRAVE-GÓMEZ et al., 2015).

Os mecanismos epigenéticos estão relacionados principalmente à alterações químicas do DNA e da cromatina, dentre estes os mais estudados são a metilação do DNA e as modificações pós traducionais em histonas **(Figura 1)**. Além destes, RNAs não-codificantes, como microRNAs, bem como alterações físicas em mecanismos não covalentes, representam também agentes de modulação epigenética (LILLYCROP et al., 2012; MILAGRO et al., 2013; TAMMEN et al., 2013).

Figura 1: Alterações epigenéticas e efeitos sobre a transcrição gênica

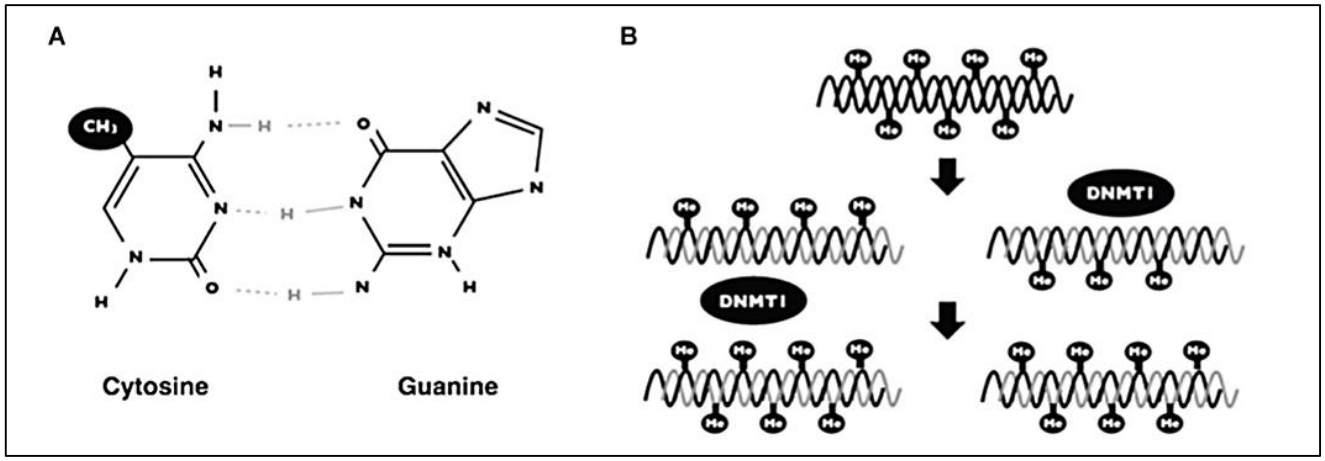


(A) Principais alterações epigenéticas, tais como modificações pós traducionais em histonas e metilação do DNA. (B) Esquema das alterações reversíveis da organização da cromatina que influencia na expressão de genes: os genes são expressos quando a cromatina está descondensada (ativa) e o contrário ocorre quando a cromatina está condensada (inativa). Círculos brancos= citosinas não metiladas, círculos vermelhos= citosinas metiladas. Fonte: RODENHISER & MANN, 2006.

Um dos mecanismos epigenéticos mais estudado é a metilação do DNA. Tal processo consiste na adição covalente de um radical metil (CH_3-) em uma base citosina, convertendo-a em 5-metilcitosina. As citosinas alvo de metilação encontram-se ligadas à guanina e localizam-se principalmente em regiões promotoras de transcrição gênica, constituindo as chamadas ilhas CpG (GOLDBERG et al., 2007; RODENHISER & MANN, 2006). Esta reação ocorre por meio de enzimas denominadas DNA metiltransferases (DNMT), destacando-se três tipos: DNMT1, DNMT3A e DNMT3B. A DNMT1 é responsável pela manutenção dos padrões de metilação, conservando-os durante o processo de divisão celular **(Figura 2)**; em contrapartida, a DNMT3A e a DNMT3B estão relacionadas à adição de novos padrões

de metilação, sendo por isso denominadas de metiltransferases *de novo* (RODENHISER & MANN, 2006; STASZEWSKI & PRINZ, 2014).

Figura 2: Metilação do DNA



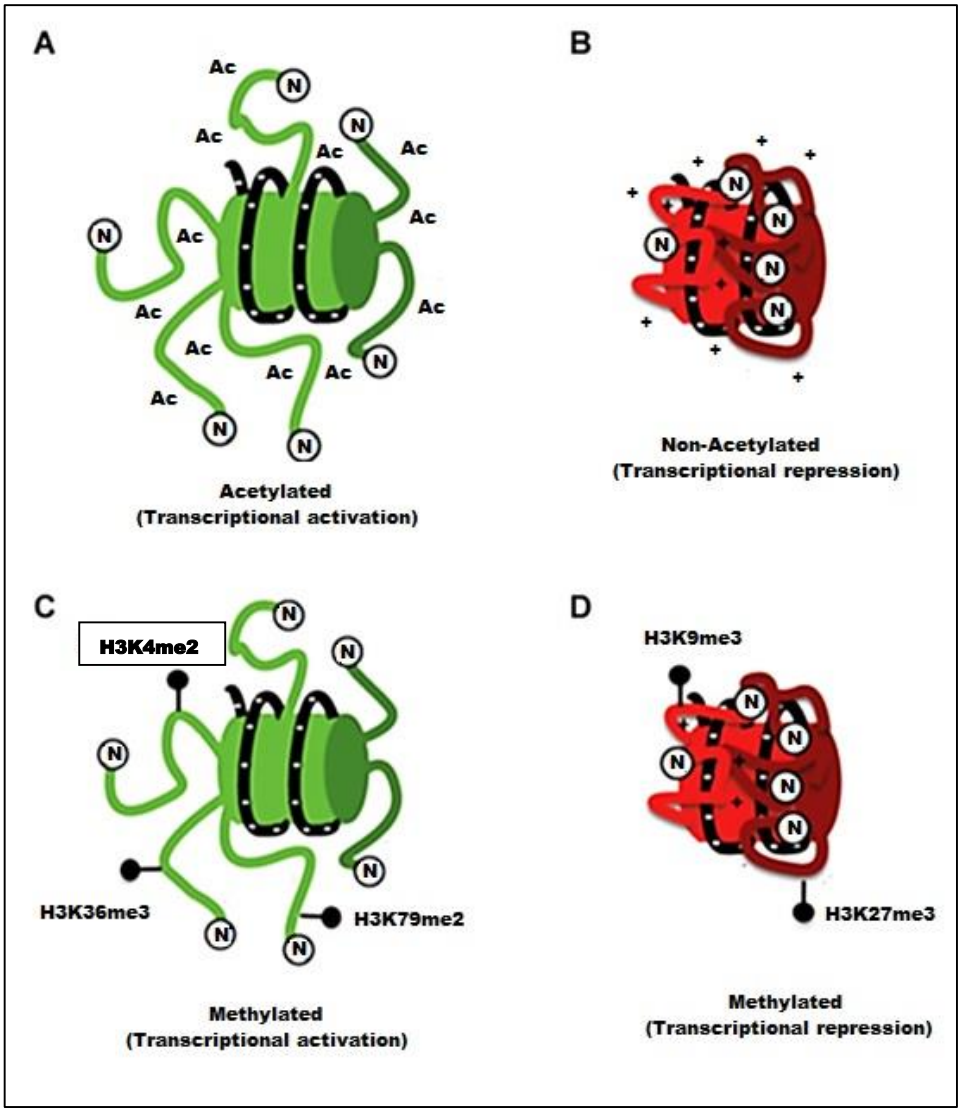
(A) Esquema mostrando a adição de um radical metil (CH_3-) no carbono 5 da citosina no sentido 5' - CpG-3'. (B) A manutenção da metilação do DNA é realizada através da DNA metiltransferase - DNMT1, quando ocorre a replicação do DNA. Fonte: LANDGRAVE-GÓMEZ *et al.,* 2015.

A remodelação da cromatina devido a modificações pós-traducionais de histonas também representa um importante mecanismos de regulação epigenética. As histonas são proteínas que possuem muitos aminoácidos com cargas positivas o que permite atrações com a molécula de DNA que, em contrapartida, possui carga negativa devido aos grupos fosfato. O enovelamento de cerca de 147 pares de base de DNA em um octâmero de histonas, compõe a unidade do nucleossomo que se repete para constituir a cromatina. Esse octâmero é formado por dímeros dos quatro tipos de histonas nucleares: H2A, H2B, H3 e H4 (CHAKRAVARTHY et al., 2005; COSTELLOE et al., 2006; LANDGRAVE-GÓMEZ et al., 2015). Um quinto tipo de histona, denominada de H1 ou H5, atua auxiliando a ligação entre os nucleossomos, além de estar envolvida na estruturação e estabilização da cromatina juntamente como outras proteínas não-histonas (CHAKRAVARTHY et al., 2005; LANDGRAVE-GÓMEZ et al., 2015). As histonas possuem um domínio globular, onde o filamento de DNA se envolve, e extremidades amino-terminais flexíveis (também denominadas de caudas) ricas nos aminoácidos lisina, arginina, serina e treonina. Estes são os

principais resíduos alvo das modificações pós-traducionais, das quais as mais conhecidas atualmente são a acetilação, metilação e fosforilação (JENUWEIN & ALLIS, 2001; COSTELLOE et al., 2006). Tais modificações são capazes de afetar a função dos cromossomos devido a alterações na carga eletrostática das histonas, provocando mudanças em sua estrutura e interação com a molécula de DNA; bem como da cromatina com fatores de transcrição e proteínas envolvidas na condensação e reparo do DNA. Dessa maneira, as histonas representam um importante fator de controle transcricional (JENUWEIN & ALLIS, 2001; MANOHAR et al., 2009).

A acetilação de histonas ocorre predominantemente em resíduos de lisina (K) das caudas amino-terminais e geralmente está associada com ativação transcricional. Em contrapartida, a metilação pode ocorrer em resíduos de lisina ou arginina (R) e está associada tanto à ativação quando inativação da transcrição, a depender do resíduo metilado e do grau de metilação (mono-, di-, ou trimetilado) **(Figura 3)** (HAKE et al., 2004; BANNISTER & KOUZARIDES, 2011; PATTARONI & JACOB, 2012; STASZEWSKI & PRINZ, 2014). A metilação de lisina 4 ou 36 em H3, por exemplo, está associada com a iniciação da transcrição, enquanto que a metilação da lisina 9 ou 27 em H3 está relacionada com a repressão da transcrição (HAKE et al., 2004; MAZE et al., 2012). O processo de acetilação e metilação, que consiste na adição de um radical acetil (-$\underline{COCH_3}$) ou metil (CH_3-), respectivamente, bem como a retirada desses grupos das lisinas/argininas é regulado por famílias de enzimas. As histona acetiltransferases (HAT) e as histonas desacetilases (HDAC) estão envolvidas na regulação da acetilação, enquanto que as histonas metiltranferases (HMT) e histonas desmetilases (HDM) atuam regulando a metilação (BANNISTER & KOUZARIDES, 2011).

Figura 3: Modificações pós-traducionais em histonas



Representação da interação dos domínios N-terminais das histonas com a molécula de DNA na presença (A) ou ausência (B) de acetilação. As histonas acetiladas apresentam uma menor interação com a molécula de DNA em comparação com as não acetiladas, cuja diferença de cargas aumenta a atração. Em contrapartida, diferentes marcas específicas de metilação da histona 3 estão associadas tanto com a ativação transcricional (C) quanto com a repressão (D). Fonte: LANDGRAVE-GÓMEZ *et al.,* 2015.

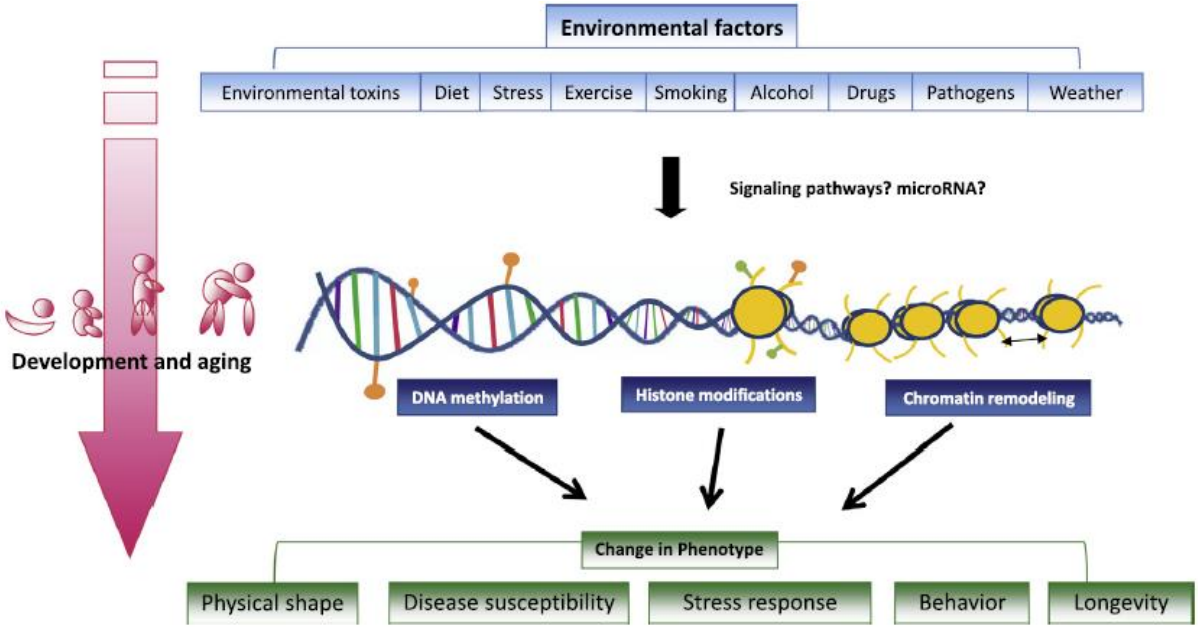
Um terceiro mecanismo que age como importante regulador pós-transcricional da expressão gênica são os microRNAs (miRNAs). Estes são moléculas de RNA de fita simples com cerca de 22 nucleotídeos, que não atuam como codificadores de proteínas. Seu mecanismo de ação se dá através da ligação a uma região 3' não traduzida de RNAs mensageiros (RNAm) alvo, causando a redução da síntese proteica (GOLDBERG et al., 2007; TAMMEN et al., 2013). Por possuírem sequências

pequenas e atuarem sem a necessidade de um pareamento completo, um único miRNA pode atuar na regulação de vários RNAm-alvo. Os miRNAs têm sido associados a diversas funções celulares, tais como proliferação, diferenciação e apoptose (GOUSTARD-LANGELIER et al., 2013).

Todos estes mecanismos descritos acima são sensíveis a fatores ambientais, que quando presentes em fases iniciais do desenvolvimento, podem causar alterações na transcrição de genes e repercutir a longo prazo em significativas mudanças na expressão fenotípica (KEVERNE et al., 2008; LILLYCROP et al., 2012).

Alterações nutricionais, uso do álcool e do cigarro, e poluentes atmosféricos são exemplos de elementos que podem causar efeitos negativos na saúde do indivíduo, tais como doenças metabólicas, desordens neurológicas e câncer, como produto de remodelações em mecanismos epigenéticos **(Figura 4)** (CORTESSIS et al., 2012; JIMÉNEZ-CHILLARÓN et al., 2012; HAGGARTY, 2013; TAMMEN et al., 2013; VAISERMAN, 2015).

Figura 4: Fatores ambientais que promovem ao longo da vida alterações epigenéticas e suas repercussões fenotipicamente



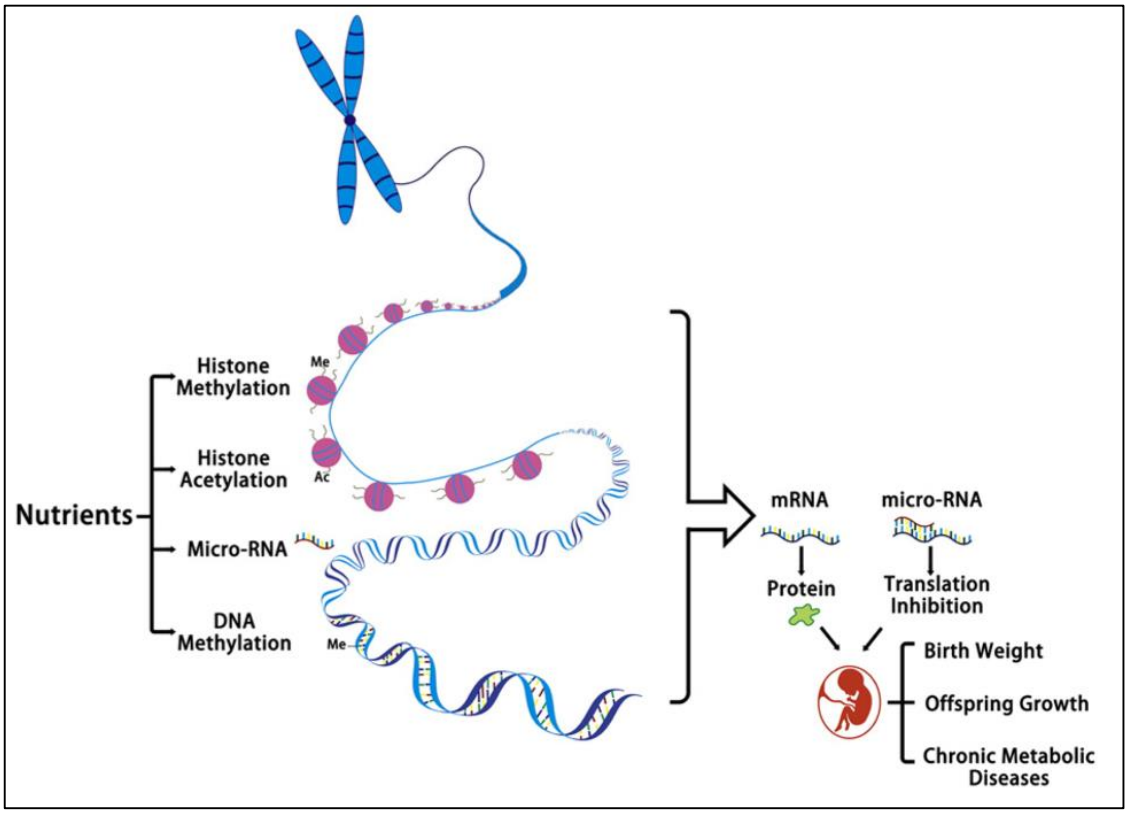
Fonte: TAMMEN, *et al.,* 2013.

2.3. NUTRIÇÃO E MODIFICAÇÕES EPIGENÉTICAS NO SNC

Dentre os fatores ambientais responsáveis por regular eventos epigenéticos, a dieta apresenta significativa relevância atuando em mecanismos moduladores da metilação do DNA, modificações pós traducionais de histonas e em miRNAs (VICKERS, 2014; VAISERMAN, 2015). O estado de nutrição materno pode afetar padrões epigenéticos do genoma fetal, promovendo alterações no estado nutricional e endócrino que resultam no desenvolvimento de adaptações levando à chamada “programação fetal” (JIMÉNEZ-CHILLARÓN et al., 2012; VICKERS, 2014; BOLTON et al., 2014; LIU et al., 2015; VAISERMAN, 2015; JI et al., 2016) **(Figura 5)**.

Modelos nutricionais envolvendo dietas com alto teor de gorduras, baixos níveis proteicos, bem como desnutrição intrauterina por ligação da artéria placentária, apontam alterações em diversos mecanismos epigenéticos, em diferentes tipos de tecido incluindo fígado, músculo esquelético, pulmão e algumas regiões encefálicas, tais como córtex, cerebelo, hipotálamo e hipocampo (JIMÉNEZ-CHILLARÓN et al., 2012; LANGIE et al., 2013; WENG et al., 2014).

Figura 5: Regulação epigenética da expressão gênica e programação fetal.



A má nutrição materna afeta o crescimento, desenvolvimento e a saúde da prole através de modificações epigenéticas. Os mecanismos subjacentes incluem a metilação do DNA, metilação e acetilação de histonas, e síntese de microRNA. Fonte: JI *et al.,* 2016.

Tanto macro quanto micronutrientes representam importantes moduladores epigenéticos. O processo de metilação do DNA, por exemplo, é catalisado pelas DNMTs, que fazem uso da S-adenosilmetionina (SAM) como doador de radical metil (CH_3). A SAM, por sua vez, é uma molécula gerada no ciclo da metionina que tem sua disponibilidade diretamente influenciada pela dieta (ZEISEL, 2009; AMARAL et al., 2011). O ácido fólico (ou folato), vitamina B12 e B6, bem como a colina e betaína, estão envolvidos no processo metabólico de formação da metionina e em sua conversão em SAM. Outros estudos mostram ainda que certos componentes da dieta podem afetar acetilação de histonas por meio de inibição da HDAC, incluindo o dissulfeto de dialila e sulforafanos, compostos presentes principalmente no alho e brócolis, respectivamente, e o ácido graxo de cadeia curta, butirato (DASHWOOD, 2006; MCGOWAN et al., 2008). Consequentemente, diversos mecanismos epigenéticos podem sofrer alterações consideráveis por meio da dieta e resultar em distúrbios no funcionamento normal do SNC (MCGOWAN et al., 2008; ZEISEL, 2009; NICULESCU et al., 2006; ANDERSON et al., 2012; LIU et al., 2015).

Mehedint e colaboradores (2010) viram que a submissão de fêmeas de camundongo C57BL/6 a uma dieta com deficiência em colina, a partir do 11º dia de gestação (período embrionário), promoveu redução da metilação de H3K9me1 e H3K9me2 em regiões específicas do hipocampo no cérebro fetal ao 17º dia gestacional. Em culturas de células progenitoras neurais (NPC) também submetidas a uma redução dos níveis de colina no meio, os mesmos autores observaram, além da redução da metilação de histonas, redução na proliferação e aumento da apoptose celular (MEHEDINT et al., 2010).

Ratas *Wistar* mantidas em uma dieta com excesso de ácido fólico e deficiência em vitamina B12, durante a gestação, apresentaram redução nos níveis de metilação global do DNA do tecido placentário; em contrapartida a suplementação dessa dieta com ômega-3 aumentou os níveis de metilação em relação ao grupo não suplementado (KULKARNI et al., 2011). O desbalanço na concentração desses micronutrientes na dieta materna também resultou em alterações no padrão de metilação global do DNA no cérebro de ratos neonatos e no hipocampo e córtex de animais adultos, mantidos sob a mesmas condições dietéticas. Aos três meses de

vida, viu-se ainda nesses animais a redução na expressão de RNAm para o BDNF e seu receptor TrkB, fator de crescimento neural (NGF), e proteína de ligação ao cAMP (CREB). Ambas as alterações foram normalizadas nos animais que tiveram a dieta materna suplementada com ômega-3 (SABLE et al., 2012; SABLE et al., 2013; SABLE et al., 2014,).

O papel dos ácidos graxos da família ômega-3, tais como o DHA, como modulador de mecanismos epigenéticos vem sendo cada vez mais elucidado. Em animais mantidos em uma dieta com reduzidos níveis de ômega-3 desde o período gestacional até os 80 dias de vida, que sofreram uma transição para uma dieta ocidental, com maiores níveis de açúcar e gordura, viu-se no córtex cerebral, um aumento da metilação do *Bdnf* e consequente redução da sua transcrição e tradução, bem como uma influência negativa em sensores metabólicos, tais como na razão NAD/NADH, SIRT1 e PGC1α. Em contrapartida, a exposição a uma dieta com elevados níveis de ômega-3, previne esses acontecimentos, mesmo no contexto de transição dietética (TYAGI et al., 2015). Em culturas de NPC de animais cujas mães foram submetidas a uma dieta suplementada com ácidos graxos ômega-3, observou-se uma maior diferenciação e proliferação celular associadas a um aumento da expressão de genes relacionados a estes processos, bem como de miRNA 210 (GOUSTARD-LANGELIER et al., 2013). Esses dados mostram que modificações induzidas ainda no útero podem persistir, construindo uma memória epigenética e plasticidade a longo prazo.

Influências do DHA face a um modelo neurodegenerativo promovido por elevadas concentrações de zinco em culturas de neuroblastoma humano também foram observadas por Suphioglu e colaboradores (2010) e Sadli e colaboradores (2012). Elevada concentração de zinco reduziu a acetilação da histona H3K9, aumentou os níveis de HDAC e alterou padrões de metilação e fosforilação de determinadas lisinas, elevando ainda os níveis de caspase-3; tais efeitos podem estar associados a uma maior susceptibilidade a processos apoptóticos. Em contrapartida, a suplementação do meio de cultura com DHA foi capaz de reverter este quadro. Tendo em vista que o excesso de zinco tem sido associado a doenças neurodegenerativas, tal como a doença de Alzheimer, estas evidências apontam para um dos mecanismos moleculares a partir dos quais a suplementação dietética com DHA pode exercer uma ação neuroprotetora (SUPHIOGLU et al., 2010; SADLI et al., 2012).

Tais dados reforçam a intrínseca relação entre a dieta e mecanismos epigenéticos, apontando ainda os possíveis efeitos modulatórios do DHA neste contexto.

2.4. MECANISMOS EPIGENÉTICOS E DIFERENCIAÇÃO CELULAR

Os processos de proliferação, diferenciação e maturação celular se dão em diferentes momentos do desenvolvimento cerebral sendo altamente coordenados por fatores de sinalização externos, como liberação de citocinas, bem como por mecanismos internos de regulação da expressão gênica e remodelações da cromatina (MORGANE et al., 2002; URAYAMA et al., 2013; GONZALES-ROYBAL & LIM, 2013; ROIDL & HACKER, 2014).

Os processos de metilação do DNA e modificações em histonas estão diretamente envolvidos com a neurogênese e gliogênese, e representam eventos críticos para a manutenção das funções do SNC de forma adequada (MITROUSIS et al., 2015; MURAO et al., 2016).

A atuação das HDACs, que reduzem o estado de acetilação das histonas por meio da remoção de radicais acetil, representam fatores chave para a diferenciação e maturação celulares desempenhando importante papel na formação de neuritos e desenvolvimento dentrítico (CHO & CAVALLI, 2014; VOLMAR et al., 2015). Kim e colaboradores (2009), observaram que o bloqueio da atuação da HDAC6 em culturas de neurônios cerebelares e hipocampais, é capaz de promover a redução do crescimento e arborização dendrítica, sugerindo a importância desta enzima sobre a dendritogênese (KIM et al., 2009). Em contrapartida, a inibição inespecífica de HDAC por ácido valpróico (VPA) em NPC do hipocampo é capaz de direcionar o destino de diferenciação celular, aumentando a neurogênese e inibindo a gliogênese através da indução de fatores de transcrição neurogênicos, incluindo o NEUROD1 (HSIEH et al., 2004). Através de diferentes mecanismos, as famílias de HDAC relacionam-se também com os processos de diferenciação e maturação de células de Schwann e oligodendrócitos, bem como na indução, regulação e manutenção da mielinização e regeneração axonal (MARIN-HUSSTEGE et al., 2002; JACOB et al., 2011).

A adição ou remoção de radicais metil às histonas, por meio das HMTs e HDMs, bem como a expressão de miRNAs, como miRNA-124 e miRNA-9, estão relacionados à relevantes funções ao longo da neurogênese, atuando na manutenção da identidade

neuronal em estágios iniciais do desenvolvimento de NPCs, bem como promovendo diferenciação e sobrevivência, aumentando a expressão de BDNF (JULIANDI et al., 2010; TAN et al., 2012; CASCANTE et al., 2014).

Modificações epigenéticas também tem sido propostas em relação à diferenciação de astrócitos. A alternância entre metilação e acetilação em regiões promotoras de genes específicos durante o desenvolvimento, refletem no aumento da expressão de GFAP, importante filamento intermediário do citoesqueleto de astrócitos e relacionado ao estado diferenciado destas células (URAYAMA et al., 2013). Durante a embriogênese o fator de transcrição STAT3 (Transdutor de sinal e ativador de transcrição 3), encontra-se altamente metilado o que impede sua ligação ao promotor de *Gfap*, desta forma, a acessibilidade da cromatina é extremamente importante para que esta ligação ocorra e promova a expressão da proteína GFAP durante a astrocitogênese (NAMIHIRA et al., 2004; URAYAMA et al., 2013). Asano e colaboradores (2009), evidenciaram que o aumento da acetilação da H3 em torno do sítio de ligação do STAT3 após tratamento de NPCs com ácido valpróico (VPA), um inibidor de HDAC, promove maior ligação deste ao promotor de GFAP (ASANO et al., 2009). Majumder e colaboradores (2013), também constataram que a redução da metilação do DNA, pela utilização do inibidor Aza-cytidina (Aza-C), e aumento da acetilação através da inibição da HDAC pela tricostatina (TSA) aumentaram a resposta de NPCs humanas a fatores de crescimento pró-astrocitários, como o BMP-2 e LIF. Tais dados sugerem que alterações epigenéticas nos promotores de genes relacionados a diferenciação de astrócitos poderiam estar influenciando o estado da cromatina (MAJUMDER et al., 2013). Além de importante para o controle da expressão de GFAP durante o processo de diferenciação, a acetilação de histonas também tem se mostrado crucial para a expressão de suas isoformas bem como na organização de seus filamentos (KANSKI et al., 2014).

É possível constatar que diversos mecanismos de regulação epigenética podem atuar de maneiras distintas, dependendo do estágio do desenvolvimento ou tipo celular, no entanto muitos destes processos são ainda pouco esclarecidos.

3. JUSTIFICATIVA E HIPÓTESE DO PRESENTE ESTUDO

Várias evidências descritas na literatura têm mostrado que os efeitos de uma má-nutrição durante o período crítico de desenvolvimento do SNC é capaz de induzir alterações permanentes no cérebro adulto. Uma das hipóteses para explicar tais alterações baseia-se em modificações epigenéticas capazes de induzir o silenciamento de alguns genes (JIMÉNEZ-CHILLARÓN et al., 2012). Apesar de vários estudos terem avaliado os efeitos da má-nutrição sobre parâmetros epigenéticos, pouco se sabe sobre esses efeitos no SNC, especialmente sobre tipos celulares específicos.

Alta razão ômega-6/ômega-3 em dietas ocidentais hiperlipídicas e hipercalóricas vem sendo associadas a modificações epigenéticas deletérias capazes de induzir síndrome metabólica e doenças neurodegenerativas (LAFOURCADE et al., 2011; JI et al., 2016). Assim, níveis desbalanceados de macro ou micronutrientes são capazes de induzir modificações permanentes no cérebro mas os mecanismos envolvidos ainda são pouco conhecidos. A importância relativa de cada um destes nutrientes é de grande interesse no tratamento dietético destas doenças para que possam ser melhor definidas estratégias de intervenção (VAHID et al., 2015). Desta forma, análise de alterações epigenéticas com suplementação dietética de nutrientes essenciais vem sendo indicada como um potencial método terapêutico (JI et al., 2016.)

Suplementação de ácidos graxos da família ômega-3 sobre respostas epigenéticas, atuando como agentes protetores em modelos de deficiência de vitaminas ou previamente a dietas hipercalóricas, têm sido relatadas na literatura (SABLE et al., 2014; TYAGI et al., 2015). Em linhagem de neuroblastoma humano viu-se que a suplementação do meio de cultura com DHA foi capaz de reverter diversas alterações sobre a acetilação, metilação e fosforilação de histonas promovidas por altas concentrações de zinco (SADLI et al., 2012).

O presente estudo visa analisar a importância relativa de uma condição favorável para síntese de ácidos graxos da família ômega-3 em uma dieta materna com deficiência proteica, sobre alterações pós-traducionais de histonas relacionadas à maturação de astrócitos e neurônios. Borba e colaboradores (2000) utilizando a Dieta Básica Regional (DBR) durante a gestação e lactação, encontraram uma adequada complexidade dendrítica e plasticidade em neurônios corticais de ratos jovens e adultos (BORBA et al., 2000). Usando esta mesma dieta, recente evidência

obtida em nosso laboratório indica modificações fenotípicas em astrócitos de neonatos e neurônios embrionários corticais sugestivas de um estado adaptativo favorável de diferenciação celular. Desta forma, o presente estudo visa testar a hipótese de que a despeito de uma deficiência proteica e em micronutrientes, a baixa razão ômega-6/ômega-3 nesta dieta materna pode promover modificações epigenéticas favoráveis a alterações neuroquímicas e morfológicas que ocorrem nestas células.

4. OBJETIVOS

4.1. OBJETIVO GERAL

Avaliar os efeitos de uma dieta que promove desnutrição multifatorial materno-fetal severa mas, que possui uma baixa razão ômega-6/ômega-3, sobre a acetilação e metilação da histona 3 de astrócitos e neurônios do córtex cerebral da prole de ratos.

4.2. OBJETIVOS ESPECÍFICOS

4.2.1. Investigar em culturas de astrócitos do córtex cerebral:

- Níveis de acetilação de H3K9 e dimetilação de H3K4 e H3K27 por imunocitoquímica e citometria de fluxo;
- Os efeitos da inibição da histona acetiltransferase (HAT) sobre os níveis de H3K9Ac;
- A influência da suplementação do ácido docosahexanóico (DHA) sobre os níveis de H3K9Ac
- As fases do ciclo celular e a expressão da proteína GFAP;

4.2.2. Investigar em culturas de neurônios do córtex cerebral os níveis de H3K9Ac e H3K4me2, e o estado do fator de transcrição NFkB por imunocitoquímica;

4.2.3. Avaliar o perfil de ácidos graxos do leite de neonatos por cromatografia gasosa.

5. ARTIGO CIENTÍFICO

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LOW OMEGA 6/OMEGA 3 RATIO IN A MATERNAL PROTEIN-DEFICIENT DIET PROMOTES HISTONE-3 CHANGES IN THE OFFSPRING`S NEURAL CELLS THAT FAVOR GENE TRANSCRIPTION

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Supplementary Figure: 1

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Abstract

Omega-3essential fatty acids play key roles during brain development, inducing gene transcription and epigenetic changes critical to genesis and differentiation of neurons and glial cells. Conversely, evidence has shown that maternal protein-malnutrition can negatively modify the epigenetic state of the fetal genome. The present study investigated whether a low omega-6/omega-3 ratio in a maternal diet could induce favorable post-translational histone modifications in the offspring`s neural cells even in the presence of protein deficiency. Adult female rats fed a control or deficient diet containing linoleic/alpha-linolenic fatty acid ratio of 1.69 since 30 days prior mating and during pregnancy. The offspring`s embryonic neurons and postnatal astrocytes of cerebral cortex were cultured for 48 h and 21 days, respectively. Acetylation and di-methylation levels in the histones H3K9Ac and H3K4Me2 were assessed by flow cytometry and immunocytochemistry. Cortical GFAP and histone protein levels were quantified by Western blot. The fatty acid profile of the neonate`s milk was analyzed by gas chromatography. The results showed similar intergroup expression of the phosphorylated GFAP isoform and increased immunoreactivity for f H3K4Me2 in astrocytes of malnourished animals, while no intergroup changes were detected for H3K9Ac. Treatment of astrocyte cultures with DHA increased H3K9 acetylation only in the malnourished group. Higher levels of H3K9Ac but not H3K4Me2 were found in the malnourished neurons. Altogether, the findings showed differential effects of malnutrition in H3 post-translational modifications in neurons and astrocytes, indicating a favorable condition for gene transcription. The data seems to reinforce the importance of omega-3 availability for these cells, even under protein-deficient condition.

Key words: omega-3 fatty acids, flow cytometry, histone post-translational modifications, malnutrition, astrocytes, neurons,

1. Introduction

A growing body of evidence has shown that the status of maternal undernutrition can modify the epigenetic state of the fetal genome, inducing developmental adaptations usually referred as fetal programming [1,2].

Epigenetic mechanisms are able to promote changes in the gene expression without modifying the sequence of DNA nucleotides [3–5]. Among these mechanisms, histone post-translational modifications such as acetylation and methylation are especially important given their role in chromatin remodeling during transcriptional activity regulation. It has been shown for example that low levels of histone acetylation and high levels of di- or tri-methylated H3K27 causes a reduction in gene transcription. On the other hand, increased acetylation and tri-methylation at residues H3K4, H3K9, H3K14 and H3K36 induce gene transcription [6]. Adequate levels of histone acetylation depend on a balanced activity of the enzymes histone acetyltransferase (HAT) and histone deacetylases (HDAC). On the other hand, histone lysine methyltransferases (HKMT) are responsible for methylation induction [7].

During early brain development, modifications in histone acetylation and methylation levels are necessary for the genesis and differentiation of neurons and glial cells. In fact, different classes of HAT, HDAC and HKMT present higher gene expression during development than in adulthood [8,9]. Some of these modifications in the offspring's epigenome can be induced by the intensity of maternal care [10]. The importance of the acetylation has been supported by studies demonstrating that some classes of HDAC (e.g. SIRT1) are necessary for cell fate determination of neural progenitor cells [11]. Furthermore acetylation of microtubules and tubulin molecules modulate their stability and interaction with other proteins [12]. In accordance with this fact, it has been reported that the acetylation of α-tubulin by the HAT elongator can control the migration and differentiation of cortical neurons [13].

Among the macronutrients able to modulate epigenetic mechanisms, recent evidence has indicated the relative importance of the omega-3 essential fatty acid alpha-linolenic as well as its derived long chain polyunsaturated acid (LC-PUFA), docosahexaenoic acid (DHA). The dietary availability of these fatty acids represents a theme of increasing interest in public health, especially due to their importance during brain development, maturation and aging [14]. DHA exerts crucial roles on the astrocyte and neuron differentiation, modulating calcium signaling, intercellular

communication and several neurotransmitter systems [15–18]. Furthermore, it has also been shown that inadequate amounts of DHA as a consequence of high omega-6/omega-3 ratio in the diet can modify the fate of neural stem cells in the brain reducing neurogenesis [19,20], can impair neurite outgrowth, synaptogenesis [21,22], neurotrophin synthesis [23,24], cell migration and synaptic plasticity [25–27].

Effects of zinc and DHA on epigenetic modifications were described in cultures of human neuroblastoma cell line [28]. According to this previous study, high zinc concentrations increased deacetylation, methylation and phosphorylation of H3 and decreased its acetylation. The supplementation of the culture medium with DHA reversed these deleterious effects of zinc on post-translational modification on the histones 3 and 4, and also decreased caspase-3 levels.

DHA supplementation in the maternal diet was also able to induce long-term effects in the offspring´s neural stem cells, kept in cultures for 25 days [29]. These effects corresponded to an increased expression of miRNA 210 and some trophic factors involved in neural cell proliferation, and reduced levels of HDAC. Furthermore, modifications in the dietary levels of folic acid, vitamin B12 and DHA have been related to changes in the DNA methylation pattern of the placenta in *Wistar* rats [30].

High omega-6/omega-3 ratio in hyperlipidic and hypercaloric western diets have been associated to epigenetic modifications able to induce metabolic syndrome and neurodegenerative diseases [2,31]. Unbalanced levels of macro or micronutrients are able to induce permanent modifications in the brain but the underlying mechanisms are still mostly unknown. The relative importance of each nutrient, in the context of neurodegenerative diseases, is of interest in order to better define intervention strategies for dietary treatment [32]. Therefore, targeting epigenetic changes with dietary supplementation of essential nutrients has been proposed as a potential therapeutic approach [2].

The present study aims to analyze the relative importance of a favorable condition for omega-3 synthesis in a protein deficient maternal diet, on histone post-translational changes related to astrocyte and neuron maturation. Using the Regional Basic Diet (RBD) during gestation and lactation, Borba et al. (2000) demonstrated an adequate dendritic complexity and plasticity in cortical neurons of weanling and adult rats [33]. The same maternal diet has induced phenotypic modifications in cortical astrocytes and neurons of neonates, suggestive of an adaptive and favorable state of

differentiation (our unpublished results). Therefore, we tested the hypothesis that despite a multinutrient deficiency, early and long term neurochemical and morphological changes in neural cells could be influenced by epigenetic mechanisms favored by a low and adequate omega-6/omega-3 ratio in this maternal diet.

2. Material and methods

2.1. Ethical procedures

All procedures adopted in the present study were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocol 23076.017529/2014-98), in accordance with the Brazilian College for Animal Care guidelines which follows the “Principles of Laboratory Animal Care” (NIH, Bethesda, USA).

2.2. Animals and diets

Adult nulliparous female rats (90 days old) weighing 200-250g were divided into two groups: a control nourished group, fed on a commercial rat chow diet containing 23% of a mixture of animal and soy protein, 50% carbohydrate, 3,5% lipid, 2% ash and 4% minerals (Labina® Purina of Brazil, Ltda); and a malnourished group fed on Regional Basic Diet (RBD) containing 8% protein in a 5:3 proportion of vegetal to animal protein, ~1% lipids and 1.3% minerals (table 1). The latter group was fed on RBD 30 days before mating and throughout gestation. Despite the reduced fat levels in RBD, this diet is characterized by a reduced proportion of the omega-6 linoleic fatty acid (20.38%) and increased levels of the omega-3 α-linolenic fatty acid (12.25%) when compared to the control diet (46.39% linoleic acid and 3.44% α-linolenic acid) [34]. The animals were maintained in a room at 22 ± 2 ◦C with 67% relative humidity at 12 h light / dark cycle (lights on at 06:00).

2.3. Astrocyte primary cultures

Astrocyte primary cultures from the cerebral cortex were prepared as previously described [35]. Briefly, pups up to postnatal day 3 from different litters per group were decapitated and the cerebral cortex was dissected in phosphate buffer containing 0.6% glucose (PBS-glucose), followed by mechanic dissociation. Cell suspension was diluted in Dulbecco's modified Eagle's medium containing nutrient mixture F-12 (DMEM-F-12, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 33mM glucose (Merck), 2mM glutamine (Calbiochem), 3mM sodium bicarbonate (Merck), 0.5mg/ml penicilin/streptomicin (Invitrogen), 2.5µg/ml amphotericin (Sigma) and then centrifuged for 5 minutes (1.500 rpm). Cells were plated in culture flasks (25cm^2) and kept at 37°C in a humidified 5% CO_2 95% air atmosphere. The medium was replaced every 2 days. After 10 days *in vitro*, the cells were trypsinized (0.25% trypsin + 0.2% EDTA; Sigma) for 5 min to obtain a more purified astrocyte culture. The cells were then replated either in culture flasks (25 cm^2, 7x10^5 cells) or 24-well plates (6x10^4 cells per well). Cells were fixed with 70% methanol for analysis by flow cytometry or 4% paraformaldehyde in phosphate buffer (PB, pH 7.4) for immunocytochemistry ten days after passage.

2.4. Neuron primary cultures

Primary neurons were obtained from embryos at the 16th day of gestation (E16). The first day of gestation was determined by the presence of sperm in the vaginal secretion of the dams. Pregnant females were anesthetized with isoflurane and submitted to a cesarean surgery to remove the embryos. After embryo removal, the dams were sacrificed by an overdose of isoflurane. Neurons were freshly dissociated from the cerebral cortex and mechanically dissociated in PBS-glucose and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the cells were re-suspended in Neurobasal medium (Gibco) supplemented with 2 mM glutamine (Calbiochem), 10µl/ml penicillin / streptomycin (Sigma) and 1% B-27 (Gibco). Cells were seeded onto 24-well plates (50,000 cels/well) where the wells had been coated with 1µg/ml poly-L-lysine (Sigma). The cells were incubated at 37°C in a humidified 5% CO_2 95% air atmosphere for 48h and fixed with 4% paraformaldehyde in PB (pH 7.4) for 20 min.

2.5. Immunocytochemistry assay

For immunocytochemistry analysis, cell cultures were plated onto glass coverslips coated with 1µg/ml poly-l-lysine (Sigma-Aldrich, USA), in 24-well plates. After fixation, cells were rinsed in phosphate buffer (PB) 0.1M and blocked with 3% BSA (Sigma-Aldrich, USA.) and 1% Triton X-100 (Riedel de Haen-Germany) in PB 0.1M for 30 min. To visualize epigenetic marks, the following antibodies were used: rabbit polyclonal antibody against Histone H3 [Ac Lys9] (Novus Biologicals USA, 1:500), Histone H3 [Di Methyl K4] (Bioss Antibodies USA, 1:500) and Histone H3 [Di methyl K27] (Merck-Millipore, USA, 1:500). After primary antibody incubation overnight at 4º C, cells were rinsed with PB 0.1M and incubated with the secondary antibody anti-rabbit Dylight 594 (Rockland, USA, 1:1000) for 3 h at room temperature. The coverslips were mounted on 40% glycerol in PB 0.1M and examined under a epifluorescent Leica, DM 5500-B microscope, (20 and 40x lens objective) coupled to a Leica, DFC 345 FX camera.

Quantitative analysis of fluorescence intensity in the astrocyte and neuronal nuclei positive for acetylated and methylated histones was carried out by optical densitometry using ImageJ software (version 1.45r, National Institutes of Health, Bethesda, MD, USA). For this analysis, 8 randomly selected microscope fields were assessed per coverslip and three coverslips were analysed for each of three independent cultures. An average number of 41.6 ± 7.1 neurons and 200 astrocytes per culture were set to be analyzed. The relative optical density measurements were independently performed by two observers and were blinded with respect to the experimental groups. As determined by using a stage micrometer, one field represented a culture area of 1.43 mm^2, where one coverslip had the total area of 132.6 mm^2. Thus, the total sampling area represents a value of approximately 8% of the total coverslip area.

In order to analyze metabolic damage in embryonic neurons of malnourished offspring, three independent primary cultures were analyzed regarding the cell location of the gene transcription factor NF-kB. Activation of the NF-κB is associated with nuclear translocation of the p65 component of the complex. Thus, cells were incubated with polyclonal rabbit antibody against NF-kB p65 (Rheabiotech, Campinas, Brazil) and visualized by DyLight-conjugated 594-labeled anti-rabbit IgG (1:1000, Rockland) for 3 h or biotinylated anti-rabbit (Jackson ImmunoResearch, USA; 1:1000) visualized

with ABC kit and diaminobenzidine (DAB). Fluorescent and bright field digital images of these cultures were obtained using the DM 5500-B microscope. Differential interferential contrast optics (100x objective lens, 1.2 aperture) was used to analyze the cells stained with DAB.

2.6. Flow Cytometry assay in astrocyte cultures

The protocol adopted for flow cytometry assay was similar to that previously described by [36]. The astrocytes cultured for 20 days (10 days after the first passage) were washed with serum free medium and trypsinized for 5 min. Then they were collected and fixed in 70% ice-cold methanol at 4 °C for 2 h. Following fixation, cells were washed twice in PBS containing 1% Tween 20 and then collected by centrifugation (1.500 rpm – 5 minutes). The cell pellet was re-suspended with primary antibodies rabbit Anti-Histone H3 [Ac Lys9] (Novus Biologicals USA, 1:500) or rabbit Anti-Histone H3 [Di Methyl K4] (Bioss Antibodies USA, 1:500) in PBS containing 1% bovine serum albumin (BSA) and incubated overnight at 4°C. The cells were then washed twice in PBS containing 1% Tween 20 and re-suspended in PBS containing 1% BSA and secondary antibody anti-rabbit Dylight 488 (Rockland, 1:1000) at room temperature and in the dark for 3 h. The cell cycle stage detection was obtained by staining with propidium iodide (10µg/mL) for 30 minutes. Analysis was done on a Accury C6 BD flow cytometer. Median channel fluorescence (MCF) values were calculated for each cell population following the subtraction of nonspecific labeling, analyzing only the secondary antibody (MCF) values. A total of 10,000 cells were quantified in each experiment. Six independent cultures/group were analyzed.

2.7. Treatment of astrocyte cultures with histone acetyltransferase inhibitor or DHA

The repercussion of malnutrition on H3K9 acetylation levels was also analyzed by treating the astrocyte cultures with the anacardic acid (AA) (Sigma Aldrich, USA), an inhibitor of HAT. In this case, 10 days after the first passage, the culture medium was supplemented with 5 µM or 10 µM AA for 30 min. After this, the cells were fixed with 4% paraformaldehyde in PB, (pH 7.4) and then submitted to immunocytochemical

staining and analysis of optical densitometry of fluorescence intensity in the astroglial nuclei. The quantitative analysis was performed as previously described in the section 2.5.

Astrocyte cultures were also supplemented with 100µM DHA in the presence of 2 µM α-tocopherol (Sigma Aldrich, USA) for 24 h. The cells were then fixed with 70% methanol and processed by flow cytometry to analyze H3K9 acetylation levels.

2.8. GFAP expression in the cortex of neonates

The cerebral cortex homogenates were obtained in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM MgCl2, 0,6 mM CaCl2, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% Triton x-100. Protein level was measured by Bradford assay.

Samples of cytoplasmic fractions were diluted in sample buffer (62.5 mM tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for 5 min. Thirty micrograms of protein per lane were electrophoretically separated in 15% sodium dodecyl sulphate–polyacrylamide gel at 120 mA as described by [37]. After separation, the proteins were transferred to Hybond-nitrocelullose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 2 h at 200 mA. Membranes were blocked for 1 h in Tris-buffered saline-Tween 20 (TBS-T) containing 5% of skim milk. Then, incubated in the primary antibody mouse anti-GFAP (Sigma-Aldrich EUA, 1:1000) diluted in TBS-T containing 5% of skim milk solution overnight at 4°C. After several washes in TBS-T, membrane was treated eith biotinylated secondary antibodie goat anti-mouse (Jackson ImmunoResearch, USA; 1:1000) diluted in TBS-T for 1 h. Thereafter, membranes were washed in TBS-T and stained by reaction with ECL detection reagent (Amersham ECL Plus) via chemiluminescence by ChemiDoc imaging system (Bio-Rad, USA).

2.9. Fatty acid profile in the milk ingested by the neonates

The fatty acid profile of the milk present in the stomach of the pups was assessed at the postnatal day 2. For this experiment, 6 liters per group were used. After anesthesia induced by a brief animal exposure to a low temperature (-20ºC), the pups were submitted to abdominal incision to remove the milk contained in the stomach

and then euthanized by decapitation. A pool of milk obtained from ~6-8 animals per litter was obtained to achieve a total of 50mg milk / litter. Each pup contributed with similar amount of milk. The samples were homogenized with 1 ml of 0.73% NaCl and mixed in 4 ml of chloroform/methanol solution (2:1 v/v) containing 0.005% according to [38] and then centrifuged at 3000 rpm for 10 min. The inferior phase containing the lipid fraction was removed and transferred to a tube previously washed with methanol, evaporated under nitrogen gas and stored at -20ºC. Thereafter, the fatty acids of the samples were transmethylated according to [39] and resuspended in hexane. The fatty acid profile was analyzed using a Shimadzu model GC14B gas chromatograph apparatus equipped with a flame ionization detector and HP-20 (carbowax 20 M) capillary column (25 m x 0.32 mm x 0.3 mm). The column temperature was initially 40 ºC for 1 min, then increased to 150 ºC by 55 ºC/min, and finally increased to 220 ºC by 1.7 ºC/min. The injector and detector temperatures were 200 and 220 ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.0 ml/min; injection was performed in split mode (1:20) and the injection volume was of 1.0 ml of sample isooctane extract. A standard fatty acid methyl ester mixture (Supelco, USA) was used to identify most of fatty acid methyl esters by their retention time. Fatty acid data were expressed as % of total peak area. Data are expressed as the mean ± standard error of the mean (SEM). Differences between groups were analyzed by Student's T test. Differences were considered significant at $P < 0.05$.

2.10. Statistical analysis

All data sets are expressed as means ± standard deviation (SD). All results were tested for normality using the Kolmogorov-Smirnov test. Statistical significance was evaluated with the unpaired *t*-test for parametric data or Mann-Whitney test for non-parametric data. Analysis of more than two variables was carried out using ANOVA followed by post hoc Newman-Keuls test. All data were analyzed with the GraphPad Prism software version 5.0 for Windows (San Diego, CA, USA). Differences were considered to be statistically significant when $p \leq 0.05$.

3. Results

3.1. Fatty acid profile in the milk ingested by the neonates

Figure 1 shows the mean percentage of milk fatty acid profile obtained from the stomach of neonate rats from mothers fed the control or RBD. The main fatty acids present in the milk were the capric (10:00), lauric (12:00), myristic (14:00) palmitic (16:00), palmitoleic (16:1n7), stearic (18:00), oleic (18:1 n-9), linoleic (18:2 n-6), α-linolenic (18:3 n-3), arachidonic (20:4 n-6), adrenic (22:4 n-6) and docosahexaenoic (22:6 n-3). Regarding the relative content of omega-6 essential fatty acids, lower amount of linoleic acid ($p<0.05$), but not its derived LC-PUFA arachidonic acid, was found in the milk of malnourished neonates, as compared to the control. On the other hand, no intergroup difference was detected for the relative proportion of α-linolenic or its derived docosahexaenoic acid in the milk. Increased levels of palmitic and oleic acid fatty acids were also detected in the milk of malnourished group when compared to the control.

Please, insert Figure 1 around here

3.2. Cell cycle and differentiation of astrocytes are not affected in malnourished group

To assess whether astrocyte cell cycle could be affected by the multideficient maternal diet, the astrocyte cultures were stained with propidium iodide, a DNA chain linker, and analyzed by flow cytometry. Most of cells in both groups were in the G0-G1 phase of the cell cycle. No difference in the percentage of cells in the phases S and G2-M were detected between astrocytes of the two groups (Fig.2A, B and C).

The GFAP expression profile was also not altered by RBD. The predominant GFAP isoform expressed in cerebral cortex homogenates of both groups was the phosphorylated isoform with 50 kDa (Fig. 2D). The results were normalized to β-actin levels as housekeeping cytoplasmic protein.

Please, insert Figure 2 around here

3.3. Analysis by optical densitometry of histone 3 acetylation and di-methylation levels in astrocytes primary cultures

Quantitative analysis of cortical astrocytes immunostained for H3K9 acetylated (H3K9Ac), H3K4 and H3K27 dimethylated are depicted in the Figure 3. The results obtained with three independent cultures, did not show difference in the average fluorescence of H3K9Ac present in astrocytes of the nourished (30034 ±10423 arbitrary units (au) as compared to the malnourished (27401 ± 10248 au) group (Fig. 3B). However, there was a significant increase in H3K4 dimethylation in astrocytes of the malnourished group (27115±9892 au $P < 0.005$) when compared to the control (22583±6194 au) (Fig. 3C).

H3K9Ac and H3K4Me2 have been related to increased gene transcription, so to assess whether the dietary model used in this study affected gene repression, dimethylation levels in the H3K27 was analyzed. The data presented in Figure 3D shows that the RBD imposed to the mothers before and during gestation was not able to provoke changes in the acetylation of this lysine (16693±4842 au) when compared to that observed in the nourished group (16187 ±3378 au).

Please, insert Figure 3 around here

3.4. Malnourished group seems to be less sensitive to inhibition of histone acetyltransferase (HAT)

The results of the 30 min treatment of astrocyte cultures with 5µM or 10µM AA, a known inhibitor of histone acetyltransferase (HAT) are shown in the Figure 4. As can be seen, the fluorescence intensity indicative of H3K9 acetylation levels in the nuclei of astrocytes is reduced in a dose-dependent manner in both groups (ANOVA followed by Student Newman Keuls test, $P<0.0001$). Nevertheless, the decay in the malnourished group is lower than in the control group in both AA concentrations used (ANOVA followed by Student Newman Keuls test, $P<0.0001$).

Please, insert Figure 4 around here

3.5. Analysis of post-translational changes in the histone 3 and effect of DHA supplementation in astrocyte cultures by flow cytometry

The results presented in Figure 5 and 6 show that no intergroup difference was detected in the fluorescence intensity of H3K9 acetylation (14620± 6515 au in the nourished *vs* 13217± 6821 au in the malnourished group). Similar result between the groups was obtained for the levels of H3K4 dimethylated (25956± 14258 au in the nourished vs 22917± 14938 au in the malnourished group).

The immunocytochemistry and flow cytometry data showed that the H3K9 acetylation levels in astrocytes did not differ between groups. To test the hypothesis that a favorable synthesis of omega-3 induced by RBD in the maternal diet would contribute to keeping H4K9Ac levels in malnourished neonates, cultures were treated with DHA 100 µM for 24 h and analyzed by flow cytometry. The results shown in Figure 5D illustrate that 100 µM DHA supplementation was able to significantly increase H3K9 acetylation (~60%) in astrocytes of malnourished group compared to the nourished one (Unpaired Student´s T test; $P < 0.05$).

Please, insert Figure 5 and 6 around here

3.6. Analysis of post-translational changes in the histone 3 acetylation and di-methylation of embryonic neurons

In cortical neuron cultures all the analysis was carried out by immunocytochemistry. The results obtained by optical densitometry showed higher levels of H3K9Ac in neurons of the malnourished group (30107±6789 au; n = 127 nuclei) compared to the control (25257±7562 au n = 127 nuclei, P = 0.0075) (Fig 7). On the other hand, no intergroup difference was found for H3K4Me2 levels (23203±7059 au; n= 112 nuclei in the malnourished vs 22654± 5376 au; n= 133 nuclei in the control group (Fig 8).

Please, insert Figures 7 and 8 around here

For analysis of the functional status primary neurons, embryonic cultures were immunostained for transcription factor NF-kB p65. The results presented in Figure 9

show that there was no translocation of NFkB to the nucleus indicating absence of gene transcription induced by this inflammatory marker.

Please, insert Figure 9 around here

4. Discussion

The present study investigated whether a maternal diet containing low and favorable omega-6/omega-3 ratio, even in the presence of protein deficiency could induce epigenetic mechanisms in the offspring, related to astrocyte and neuron differentiation. The results partially support our initial hypothesis, demonstrating a differential but not deleterious effect of this experimental diet on these cells during early stages of the cerebral cortex maturation.

4.1. *The fatty acid profile of the maternal milk*

The analysis of the fatty acid profile in the maternal milk provided to newborns showed a lower linoleic/α-linolenic fatty acid ratio in the malnourished group as compared to the control. This composition seems to reflect the relative contents of these fatty acids in the RBD. According to [34], the proportion of linoleic acid is 19.66 ± 0.85 % in RBD against 45.48 ± 1.15 % in the control while that of α-linolenic is 11.44 ± 0.57 % in RBD vs 2.87 ± 0.09 % in the control. It has been established that long term presence of polyunsaturated fatty acids in the maternal milk are provided by the diet and the fat storage of the maternal body, through endogenous synthesis in the liver, breast gland and other maternal tissues [40,41]. Therefore, the present findings reinforce that despite the low amount of lipids in the RBD, the levels of linoleic and α-linolenic acid are sufficient to maintain adequate levels of arachidonic acid and DHA in the milk of neonates. In addition to the beneficial effects of these fatty acids it should be noted that higher levels of oleic and palmitic fatty acid were also found in the milk of the malnourished group. Oleic acid also exert important role during development of the nervous tissue and its synthesis and release by astrocytes can be triggered by neuronal differentiation [42]. Under this condition, oleic acid released by astrocytes is used by neurons for the synthesis of phospholipids and is specifically incorporated into growth cones. Furthermore, this fatty acid promotes axonal growth, neuronal

clustering, and the expression of the axonal growth associated protein, GAP-43 [42]. Bourre and Dumont (2003) reported that an increase in the level of dietary oleic acid does not alter the sum of omega-3 fatty acids in either the liver or the nervous system [43]. Likewise, do not modify the sum of (n-6) fatty acids in the brain, myelin, and nerve endings. Regarding palmitic acid, pharmacological studies have shown that when this fatty acid is associated to linoleic via oral administration a benefic effect on cognitive disorders of senile dementia can be obtained [44]. On the other hand, when astrocytes in cultures are treated with this fatty acid alone, an inflammatory condition can be installed [45].

4.2. RBD maternal malnutrition did not change the cell cycle and GFAP expression

Prenatal malnutrition has usually been shown to either prolong the length of the cell cycle of neural cells or to alter the duration of specific phases of this cycle without changing its length (review in [46]). On the other hand, DHA supplementation in the maternal diet has been shown to induce long term changes in the rat neural stem cells, increasing the proliferation rate as well the phenotype differentiation [29]. The findings herein obtained using astrocyte primary cultures kept *in vitro* for 20 days did not show differences in the cell cycle phases between malnourished and control groups, indicating a similar astroglial proliferative profile. This data was reinforced by the comparable phosphorylated GFAP protein expression in homogenates of the cerebral cortex of neonates, another experimental read out for potential modifications in astrocyte proliferation and differentiation induced by RBD-induced maternal malnutrition. It has been well established that GFAP is involved in cellular remodeling during the mitotic, pathological and plastic processes [47]. GFAP phosphorylation is associated with mitotic division during cell proliferation, modulating the cytokinesis and astrocytic morphological differentiation [18,48,49]. In astrocyte primary cultures of the cerebral cortex, DHA supplementation increased the activity of the cyclic AMP-dependent protein kinase A which induces phosphorylation of several cellular substrates, including the GFAP molecule [18]. On the other hand, DHA deficiency in the astrocyte culture medium enhanced the expression of neutral and basic non-phosphorylated GFAP isoforms which impaired astrocyte morphological differentiation, keeping them in an immature phenotype [18]. Thus, the present findings show that, despite the relative low amount of lipids in the RBD, the low omega 6/omega 3 ratio

was sufficient to provide an adequate availability of DHA during offspring´s astroglial differentiation.

4.3. RBD- induced histone 3 post-translational modifications in cortical astrocytes

In the current work, the immunocytochemistry and flow cytometry assays showed that the H3K9 acetylation levels in the astroglia did not differ between groups. On the other hand, when these cells were treated with the AA, a known HAT inhibitor, the immunoreactivity for the H3K9 acetylated dose-dependently decreased less in the malnourished group than in the respective control. This finding suggests that this maternal nutritional condition could induce a differential balance between HAT and HDAC activities, favorable to H3K9 acetylation. In favor to this hypothesis, when DHA was supplemented in the culture medium, an impressive increase in the acetylation levels was detected in the astrocytes of the malnourished group. Although the mechanisms underlying these findings are not clear at the moment, these results are in agreement with those reported by [28] in human neuroblastoma cell line. According to this previous study, DHA supplementation in the culture medium was able to reverse the deleterious effects of zinc on histone post-translational changes, increasing H3K9 acetylation and decreasing HDAC1 protein levels. In neural stem cell primary cultures, it was also demonstrated that HDAC mRNA content was significantly reduced in the neonates whose mothers were fed an omega-3 supplemented diet during gestation [29]. An adequate level of H3K9 acetylation in cortical astrocytes of our malnourished neonates may indicate a benefic healthy state of these cells. Recent evidence has shown that when cortical astrocytes display an inflammatory status, increased levels of HDAC mRNA are also present in these cells [50]. In neuron-glia cell cultures obtained from the substantia nigra for example, H3 acetylation induced by HDAC inhibitors was able to upregulate the expression of the trophic factors GDNF and BDNF in astrocytes , promoting survival of dopaminergic neurons [51].

Even though no intergroup difference in the whole cell H3K4Me2 levels was herein detected by flow cytometry, the optical densitometry analysis of the astrocyte nuclei immunostained for H3K4Me2 revealed a low (~20%) but significant increase in the fluorescence intensity of this histone in the malnourished group, compared to the

control. Despite the differential sensitivity of these two approaches, some points deserve special attention in the discussion of this data. It has recently been suggested that H3K4 methylation induced chromatin remodeling has favorable neurophysiological effects in the central nervous system [45,52]. An interplay between histone acetylation and histone methylation has been recently reported [53]. According to this previous study, the inhibition of HDAC increased the levels of H3K4 dimethylation and trimethylation (H3K4Me2 and H3K4Me3) and such processes were linked to transcriptional activation of heat shock protein 70 (HSP70) in rat cortical neurons and astrocytes [53]. HSP70 has several cytoprotective properties in the brain, including anti-apoptotic and anti-inflammatory effects, contributing to neuroprotection against glutamate excitotoxicity [54]. Activation of HSP70 also induced anti-inflammatory actions by blocking NF-κB activation in the brain [55]. Thus, we cannot discard that increased H3K4 dimethylation in the astrocytes of our malnourished neonates represents an additional beneficial mechanism by which these cells can increase gene transcription and exert their function in adaptive condition of malnutrition. In favor to this hypothesis, it is noteworthy that no modifications were herein detected in these cells related to the expression of di-methylated H3K27, usually considered a marker of silenced chromatin.

4.4. RBD increased H3K4 acetylation levels in cortical embryonic neurons

Compared to astrocytes, cortical embryonic neurons showed a differential response to RBD-induced maternal malnutrition. Increased H3K9 acetylation occurred in the absence of modifications in the H3K4-di-methylation. H3 acetylation during brain development has been associated as a critical mechanism involved in the regulation of cell-type specific gene expression. During cerebral cortex development, histone acetylation affects the appropriate layer formation by regulating the fate propensity of neural stem cells from deep layer neurons to upper layer neurons [9]. Moreover, it was also recently reported that the multisubunit histone acetyltransferase Elongator, present in the neuronal cytoplasm, may regulate migration and branch formation of cortical neurons in the developing mouse brain by acetylating α-tubulin. These effects can induce microtubule stabilization [13]. In addition, acetylation of microtubules can modulate the recruitment of motor proteins and other cellular components involved in neuronal differentiation [12]. In the present study, although the analysis of H3K9Ac

was restricted to neuronal nuclei, immunorreactivity to this histone was also visualized in the cytoplasm and processes. In the primary cultures where neurons showed a more differentiated state, it was possible to detect H3K9Ac immunoreactivity in varicosities along the processes (Supplementary Figure 1). Preliminary analysis carried out in our laboratory has also shown that embryonic neurons of RBD malnourished group display a higher number of neurites emerging from the soma, indicating a differential state of cell differentiation when compared to the control. Complementary morphological analyses are in process in order to address this issue.

Considering the potential stimulus for gene transcription induced by increased H3K9Ac levels we analyze whether the embryonic neurons of malnourished offspring presents signs of a metabolic damage such as NF-kB activation. The results obtained in three independent cultures showed the absence of NF-kB p65 nuclear translocation indicating that inflammatory genes induced by this specific transcription factor were not activated. This finding suggests a favorable homeostatic state of these neurons and reinforces an adequate availability of DHA, considering that a deficiency in this fatty acid can induce NF-kB p65 translocation and neuroinflammatory status even in the presence of adequate levels of other macro and micronutrients [56].

In conclusion, the present findings demonstrate for the first time that low omega-6/omega-3 ratio in a maternal protein-deficient diet may contribute to positively modify epigenetic mechanisms during brain development. These effects were characterized by a differential histone post-translational modification in neurons and astrocytes. Altogether the findings indicate favorable conditions for gene transcription involved in the genesis and differentiation of these neural cells.

Conflict of interest

There is no conflict of interest in the work reported in the present paper.

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TABLE AND FIGURES

Table 1 Composition of the Regional Basic Diet (RBD)*

Ingredients	g	Protein	Carbohydrate	Fat	Ash	Fibers	Kcal%
Beans†	18.34	3.99	10.66	0.24	0.57	1.09	60.76
Manioc flour	64.81	0.84	48.59	0.12	0.43	5.64	198.80
Defatted, dried, salted meat†	3.74	2.74	------	0.06	0.06	----	11.50
Fat (from the meat)	0.35	-----	------	0.35	------	------	3.15
Sweet potato†	12.76	0.30	9.99	0.03	0.20	0.48	41.43
Total	**100.00**	**7.87**	**69.24**	**0.80**	**1.26**	**7.21**	**315.64**

*Expressed as g/100 g of diet according to Teodosio et al., 1990.
† Cooked, dried and ground.

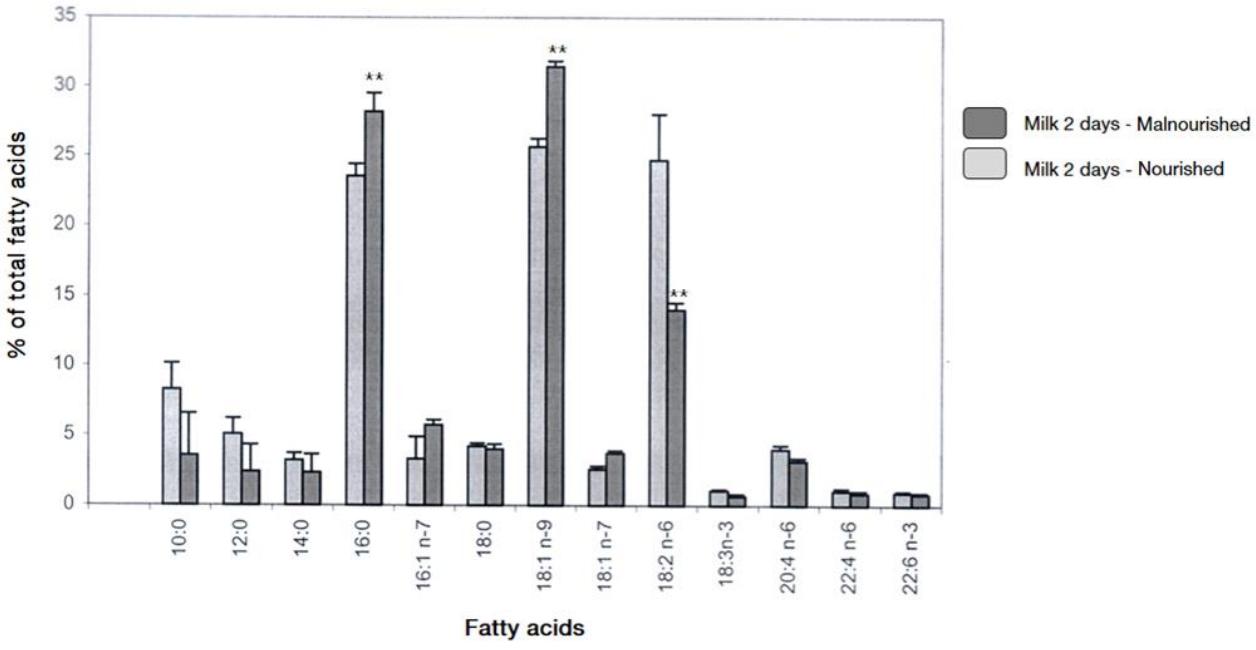


Figure 1: Fatty acid profile in the maternal milk obtained from the stomach of control and malnourished neonates at the postnatal day 2. Results are expressed as percentage of the total fatty acids. Capric (10:00), lauric (12:00), myristic (14:00), palmitic (16:00), palmitoleic (16:1n7), stearic (18:00), oleic (18:1 n-9),7-octadecenoic (18:1 n-7), linoleic (18:2 n-6), α-linolenic (18:3 n-3), arachidonic (20:4 n-6), adrenic (22:4 n-6) and docosahexaenoic (22:6 n-3).

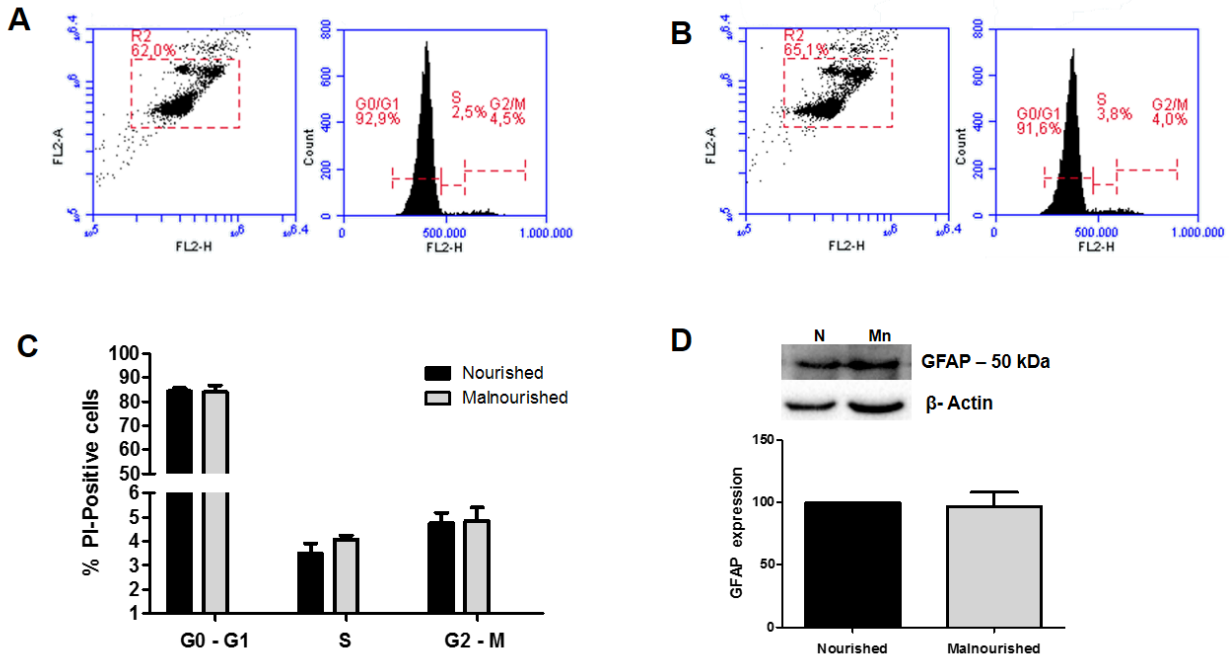


Figure 2: Cell cycle in astrocyte primary cultures and GFAP expression in cortical homogenates of neonates. A e B: marking the representation of the cell cycle and percentage of cells marked by propidium iodide at each stage in the nourished group **(A)** and malnourished **(B). C**: Percent of cells marked by propidium iodide at each stage of the cell cycle. **D**: GFAP protein expression assessed by Western blot in the nourished and malnourished groups. Data were normalized using β-actin as housekeeping protein. N: nourished. Mn: malnourished.

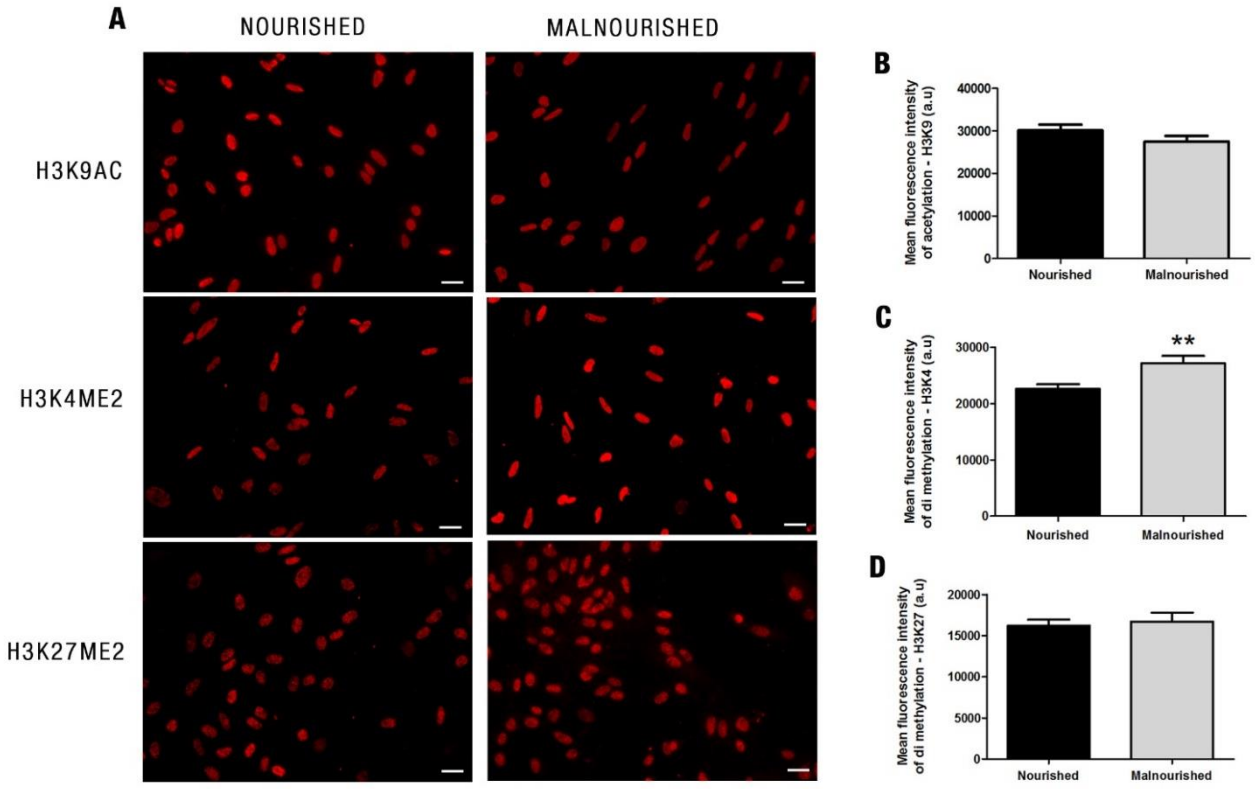


Figure 3: Fluorescence optical density of acetylated and methylated histone 3 in astrocyte primary cultures. A: Imunnoreactivity for H4K9Ac, H3K4Me2 e H3K27Me2 in nuclei of astrocytes from nourished and malnourished newborns. Average fluorescence intensity for H3K9Ac **(B)**; H3K4me2. **(C)**; H3k27me2 **(D)**. ** indicates P< 0.005 (Unpaired *t*-test). Data are presented as arbitrary units of fluorescence. Calibration bar:10 µm.

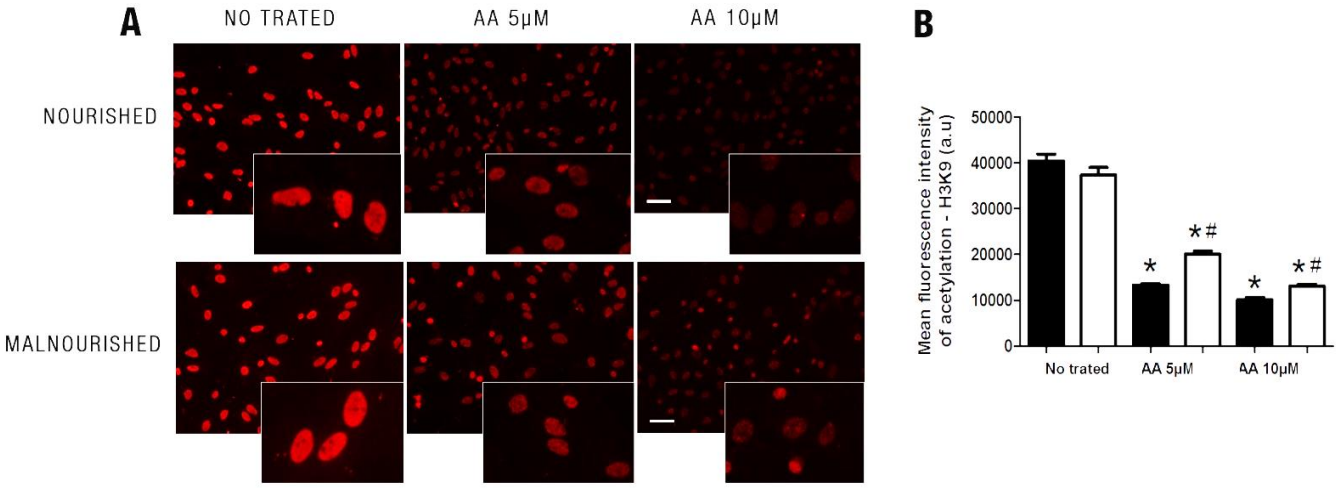


Figure 4: Treatment with histone acetyltransferase inhibitor differentially reduced H3K9Ac levels in astrocytes of nourished and malnourished groups. **A:** Low and high magnification images of representative astrocyte cultures of nourished and malnourished groups treated or not with anacardic acid (AA) at 5µM or 10µM for 30 min. **B:** Quantification of fluorescence intensity for H3K9Ac in nuclei of astrocytes after treatment with AA. * indicates P<0.0001 compared to respective control, # indicates P<0.0001 compared to the nourished group (ANOVA and post hoc Newman-Keuls test). Black bar, nourished group; white bar, malnourished group. Data are presented as arbitrary units of fluorescence. Calibration bar: 10 µm.

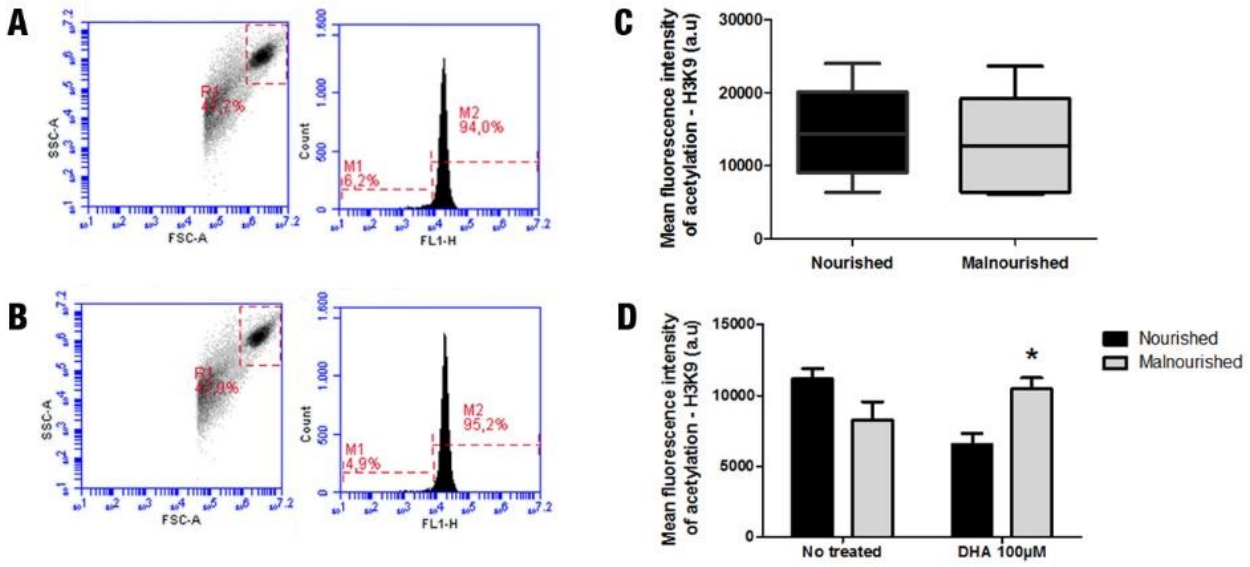


Figure 5: Flow cytometry analysis of H3K9Ac levels in astrocyte primary cultures **A** and **B**: Representation of cell population analyzed and the percentage of cells labelled for H3K9Ac in representative cultures of nourished **(A)** and malnourished **(B)** groups. **C:** Average of H3K9Ac fluorescence intensity. **D:** Average of H3K9Ac fluorescence intensity after treatment of astrocyte cultures with 100µM DHA for 24 h. * indicates P<0.05 (Mann-Whitney test). Data are presented as arbitrary units of fluorescence.

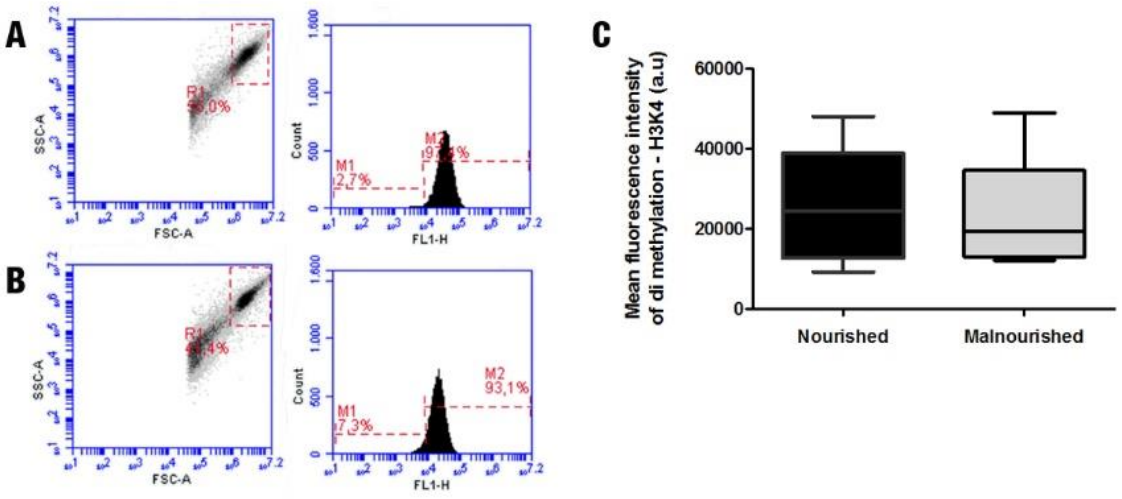


Figure 6: Flow cytometry analysis of H3K4Me2 levels in astrocyte primary cultures **A** and **B**: Representation of cell population analyzed and the percentage of cells labelled for H3K4Me2 in representative cultures of nourished **(A)** and malnourished **(B)** groups. **C:** Average of H3K4Me2 fluorescence intensity. Data are presented as arbitrary units of fluorescence.

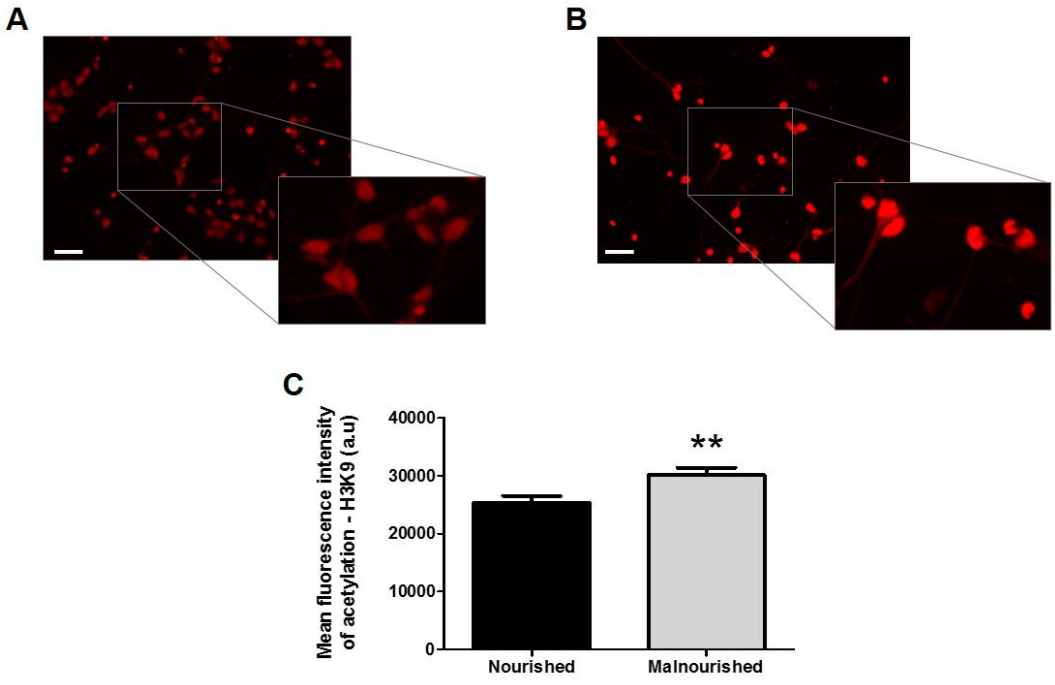


Figure 7: Fluorescence optical density of acetylated H3K9 in embryonic neuron primary cultures.. A e B: Imunnoreactivity for H4K9Ac in nuclei of neurons from nourished **(A)** and malnourished **(B)** embryonic animals. **C:** Average of H3K9Ac fluorescence intensity. ** indicates P =0.0075 (Unpaired *t*-test). Data are presented as arbitrary units of fluorescence. Calibration bar:10 µm.

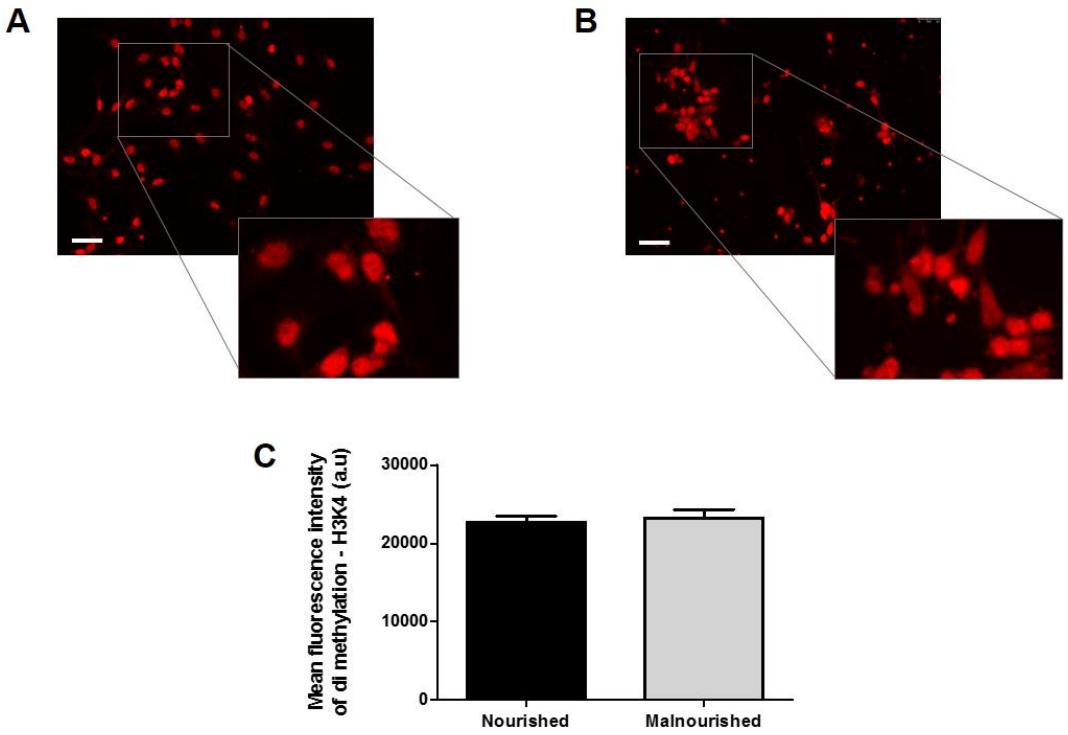


Figure 8: Fluorescence optical density of di-methylated H3K4 in embryonic neuron primary cultures. A e B: Imunnoreactivity for H3K4Me2 in nuclei of neurons from nourished **(A)** and malnourished **(B)** embryonic animals. **C:** Average of H3K4Me2 fluorescence intensity. Data are presented as arbitrary units of fluorescence. Calibration bar:10 µm.

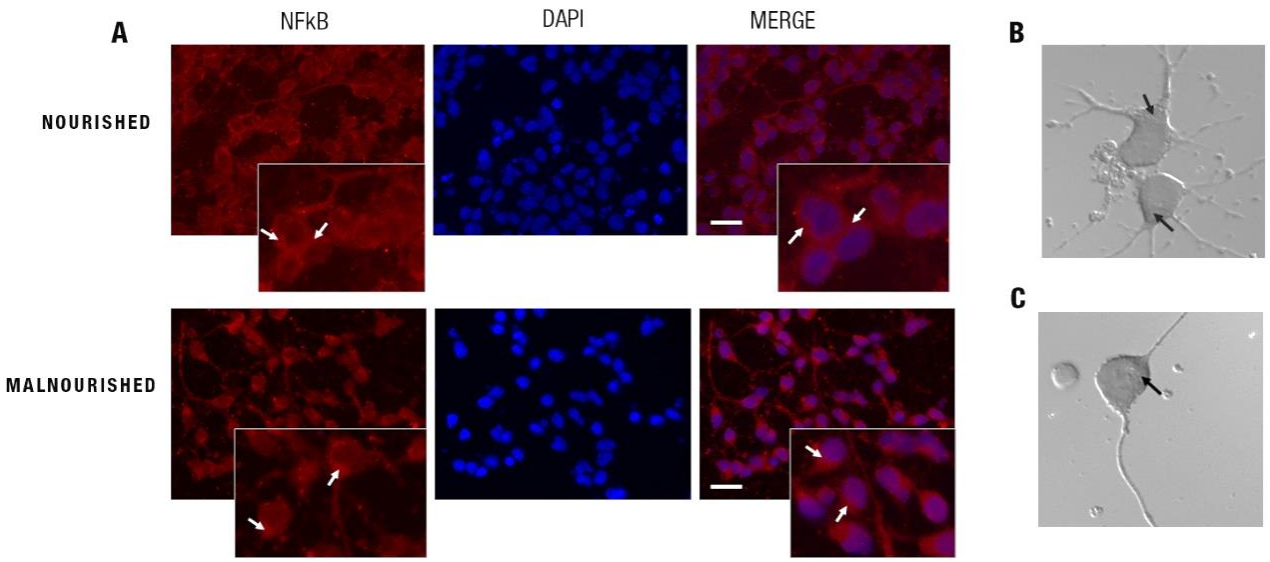
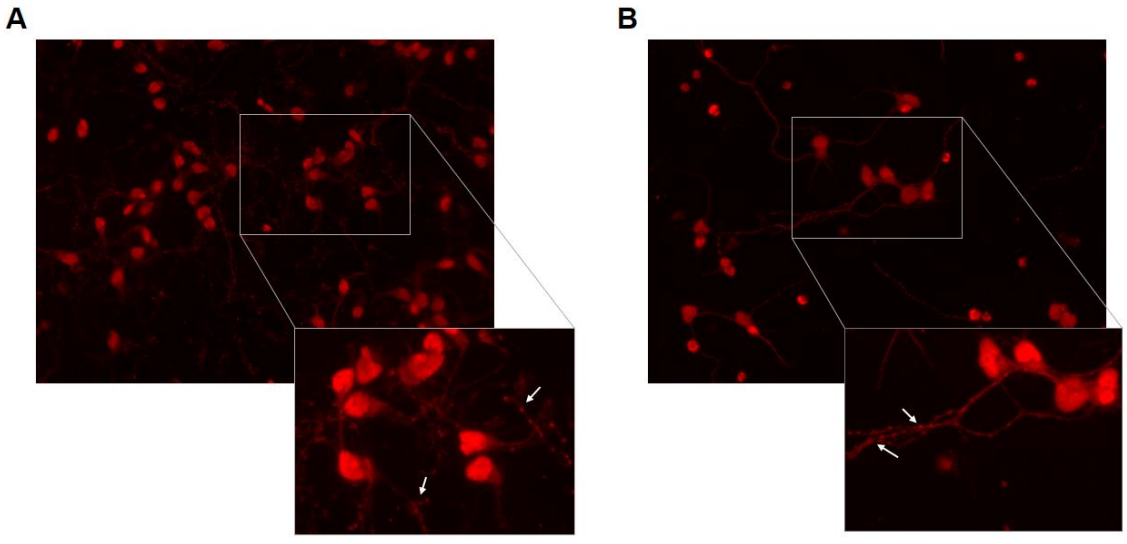


Figure 9: Low and high representative images of neuron primary cultures immnunoreacted for NF-kB and DAPI in the nourished and malnourished groups (A) showing the absence of translocation of this transcription factor to the nucleus. High magnification image of neurons immunoreacted for NF-kB, using Differential interferential optics microscopy from the nourished **(B)** and malnourished **(C)** groups. Arrows indicate NF-kB immunolabeled only in the cytoplasm. Calibration bar:10 µm



Supplementary Figure: Low and high representative images of neuron primary cultures immnunoreacted for H3K9Ac in the nourished **(A)** and malnourished groups **(B).** Arrows indicate that more differentiated neurons, were possible to detect H3K9acetilated immunoreactivity in varicosities along the processes.

6. CONCLUSÕES E PERSPECTIVAS

O aumento da dimetilação da H3K4, em astrócitos, e da acetilação da H3K9, em neurônios da prole de mães alimentadas com a DBR, indicam que este modelo dietético é capaz de promover a ativação da transcrição gênica, mesmo em uma condição de deficiência proteica. Adicionalmente, a menor sensibilidade da histona acetiltransferase (HAT) à inibição pelo ácido anacárdico, nos astrócitos do grupo experimental, pode se dever a razão favorável de ômega-6/ômega-3 na dieta. Possivelmente esta razão, favorecendo a síntese de DHA, exerce influência sobre a histona desacetilase (HDAC), mantendo um balanço adequando entre a adição e remoção dos radiais acetil. A influência do DHA sobre a acetilação de astrócitos foi reforçada pelo aumento nos níveis de H3K9Ac após suplementação deste ácido graxo nas culturas. Tidos em conjunto, nossos resultados sugerem o potencial efeito protetor do DHA sobre mecanismos epigenéticos em histonas de astrócitos e neurônios corticais, em um modelo de má-nutrição multifatorial severa.

Estudos adicionais serão realizados a fim de esclarecer a relação do DHA sobre a acetilação a partir do tratamento com diferentes doses nas culturas, bem como sua interação com a HDAC através da utilização de inibidores desta enzima, tais como o butirato de sódio. Além disso, análises de cromatografia gasosa em astrócitos encontram-se em andamento para avaliar o perfil de ácidos graxos mantidos nestas células.

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ANEXOS

ANEXO 1: Aprovação da Comissão de Ética no Uso de Animais (CEUA) da UFPE.

Universidade Federal de Pernambuco
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Recife, 09 de junho de 2014.

Ofício nº 22/14

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Belmira Lara da Silveira Andrade da Costa**
Departamento de Fisiologia e Farmacologia – CCB
Universidade Federal de Pernambuco
Processo nº 23076.017529/2014-98

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Repercussões da má nutrição severa e suplementação com DHA sobre alterações epigenéticas em astrócitos e neurônios corticais: uma abordagem *in vitro*".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério; Animais: ratos; Linhagem: *Wistar*; Idade: Progenitores adultos (entre 90 e 100 dias), neonatos (entre 0 e 3 dias de nascidos) e embriões (entre 15 e 18 dias de gestação); Peso: 250-300g (adultos) e 5-6g (neonatos); Número total de animais previsto no protocolo: 116 (38 progenitores, 30 neonatos e 48 embriões).

Atenciosamente,

Profª Marcia Vasconcelos
Vice-Presidente do CEUA/CCB-UFPE
SIAPE 2199635

CCB: Integrar para desenvolver

ANEXO 2: Manuscrito submetido à revista *The Cerebellum*

Fighting oxidative stress: increased resistance of rat cerebellum at weaning induced by low omega 6/omega 3 ratio in a protein-deficient diet

Ricielle Lopes **Augusto[1],** Alinny Rosendo **Isaac[1],** Ivanildo Inácio da **Silva Júnior[1],** David Filipe de **Santana[2],** Diorginis José Soares **Ferreira[2],** Claudia Jacques **Lagranha[2],** Catarina **Gonçalves-Pimentel[3],** Marcelo Cairrão Araujo **Rodrigues[1]** and Belmira Lara da Silveira **Andrade-da-Costa[1]***

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Abstract

The cerebellum is vulnerable to malnutrition effects. Notwithstanding, it is able to incorporate higher amount of docosahexanoic acid than cerebral cortex (Cx) when low n-6/n-3 fatty acid ratio is present in a multideficient diet. Considering DHA importance for brain redox balance, we hypothesize that this cerebellum feature improves its anti-oxidant status compared to the Cx. A chronic malnutrition status was induced before mating (on dams) and kept until weaning or adulthood (offspring). A group nutritionally rehabilitated from weaning was also analyzed. Morphometric parameters, total-superoxide dismutase (t-SOD) and catalase activities, lipoperoxidation (LP), nitric oxide (NO), reduced (GSH) and oxidized (GSSG) glutathione, reactive oxygen species (ROS) and reduced nicotinamide adenine dinucleotide/phosphate levels were assessed. Both ROS and LP levels (~53%) were increased in the Cx of malnourished young animals while the opposite was seen in the cerebellum (72% and 20% of the control, respectively). Consistently, lower (~35%) and higher t-SOD (~153%) and CAT (~38%) activities were respectively detected in these regions compared to control. In malnourished adult animals, redox balance was maintained in the cerebellum and recovered in the Cx (lower ROS and LP levels and higher GSH/GSSG ratio). NO production was impaired by malnutrition in either age, mainly in the cerebellum. The findings suggest that despite a multinutrient deficiency and a modified structural development, a low dietary n-6/n-3 ratio favor early antioxidant resource in the cerebellum and indicate an important role of astrocytes in the redox balance recovery of Cx in adulthood.

Keywords: Malnutrition; Oxidative stress; Cerebral cortex; Cerebellum; brain development

The Cerebellum

Fighting oxidative stress: increased resistance of rat cerebellum at weaning induced by low omega 6/omega 3 ratio in a protein-deficient diet

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	Instituto de Neurosciencia Translacional (INCT no. 573604/2008-8)	Dr. Belmira Lara da Silveira Andrade-daCosta

Abstract:	The cerebellum is vulnerable to malnutrition effects. Notwithstanding, it is able to incorporate higher amount of docosahexaenoic acid (DHA) than cerebral cortex (Cx) when low n-6/n-3 fatty acid ratio is present in a multideficient diet. Considering importance of DHA for brain redox balance, we hypothesize that this cerebellum feature improves its anti-oxidant status compared to the Cx. A chronic malnutrition status was induced on dams before mating and kept until weaning or adulthood (offspring). A group nutritionally rehabilitated from weaning was also analyzed. Morphometric parameters, total-superoxide dismutase (t-SOD) and catalase activities, lipoperoxidation (LP), nitric oxide (NO), reduced (GSH) and oxidized (GSSG) glutathione, reactive oxygen species (ROS) and reduced nicotinamide adenine dinucleotide/phosphate levels were assessed. Both ROS and LP levels (~53%) were increased in the Cx of malnourished young animals while the opposite was seen in the cerebellum (72% and 20% of the control, respectively). Consistently, lower (~35%) and higher t-SOD (~153%) and CAT (~38%) activities were respectively detected in the Cx
	and cerebellum compared to control. In malnourished adult animals, redox balance was maintained in the cerebellum and recovered in the Cx (lower ROS and LP levels and higher GSH/GSSG ratio). NO production was impaired by malnutrition at either age, mainly in the cerebellum. The findings suggest that despite a multinutrient deficiency and a modified structural development, a low dietary n-6/n-3 ratio favors early antioxidant resources in the cerebellum and indicates an important role of astrocytes in the redox balance recovery of Cx in adulthood.

Response to Reviewers:	Enclosed is our revised original paper entitled “Fighting oxidative stress: increased resistance of rat cerebellum at weaning induced by low omega 6/omega 3 ratio in a protein-deficient diet” that we are submitting to be considered for publication in The Cerebellum journal. We would like to thank the reviewers whose comments were valuable to the preparation of the present version of this manuscript. We took the challenging comments as an opportunity to improve this paper and described below the modifications and response to each of the suggestions. Reviewer 1 Specific comments: 1. The information provided in the Methods section" is confusing. The authors statethat only males offspring are included in the analysis, but just below state that both males and females were sampled. 2. The rational of the experimental approach is based on the observation that thecerebellum incorporates higher amount of DHA than cortex (de Souza et al., 2008). However, in the study by Souza' group only female offspring were used. Is there any difference between lipid peroxidation between males and females? Please, justify the choice of the males for your study. As marked in the section “Animals and diets” we clarified the methodology used to keep females and males during lactation period and justified why we used only males. All the changes are highlighted in red in this section. 3. In the discussion, the authors refer to the results on lipid peroxidation reported byBonatto (Bonatto et al., 2006), but fail to point out that the results were very much affected by methionine supplementation. According to the suggestion of the referee 1 we complemented this information in the third paragraph of discussion. 4. Some figures should be consolidated; ie. figures 3 and 7, and figures 4 and 8. We improved the quality of these figures according to this suggestion. .Reviewer #2: The authors focused on the enrichment in -3 fatty acid characteristic of RBD to see if it had any positive effect on the oxidative markers in the two brain regions during development and maturation. Unfortunately, the study lacks the essential morphological information on cerebellar development. With only biochemical data, it is difficult to assess how cerebellar development is affected (or not affected) by RBD. For example, impairments in granule cell proliferation, Purkinje cell development, as well as myelination are reported in the offspring when dams are maintained on PD during gestation and lactation (Ranade SC et al., 2012). Major points: 1. As shown by the significant reduction of tissue weight (Fig. 2), the cerebellum seems to be more severely affected by RBD than

	the cortex and does not seem to recover by switching to control diet after weaning (rehabilitated group). As the major part of cerebellar development takes place between birth and weaning, it is crucial to examine how the well-defined steps in cerebellar development are affected by RBD. According to these suggestions, we carried out additional experiments in order to analyze morphological parameters involved on cerebellar development in weanling rats. Analysis of percentual area occupied by molecular, granular, myelin layers was done in parasagittal brain sections stained by hematoxilin-eosin. In addition, we processed brain series for calbidin immunoreactivity in order to quantify Purkinje cell soma size and for Myelin Basic protein in order to quantify parameters of myelination such as the mean optical density in the core white matter as well as to analyze the MBP immunoreactivity pattern in both white and gray matters of cerebellar folia. The protocol for these new data are described in the end of Material and Methods section The results are shown in the text and illustrated in the table 3, Figures 3, 4 and 5. The discussion with respect to this new information was added in the first paragraph of this section. Minor point; 1. For comparison, include the composition of the control diet in Table 1. According to this suggestion we added a Table 2 with the composition of the control diet. We would like to reinforce that there is no conflict of interest in the work reported in the present revised manuscript. We confirm that the results are original and have not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. Also, a language correction was made to avoid excessive English mistakes. Finally, we would like to attest that all efforts were made and international guidelines were followed to minimize the number of animals used and their suffering in the additional experiments carried out for this new version of the manuscript. Thank you in advance, Dr. Belmira Lara da Silveira Andrade da Costa Departamento de Fisiologia e Farmacologia Centro de Ciências Biológicas, CCB Universidade Federal de Pernambuco, UFPE Cidade Universitária, Recife, PE, Brazil. Phone: 55-81- 21268530/FAX: 55-81-21268976 E-mail: bl@ufpe.br or belmira@gmail.com

ANEXO 3: Manuscrito a ser submetido

Maternal malnutrition induces adaptive neuron-astrocyte interaction which favors neurite outgrowth

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Introduction

It is well established that maternal malnutrition during pregnancy can modify the progeny`s brain competence to maintain homeostasis under stressful conditions[1–3]. Although some deleterious effects can be reduced by nutritional reposition starting from lactation period, others can persist until adult life as a consequence of epigenetic modifications[4–6]. Neurons and oligodendrocytes are especially vulnerable to different types of fetal and neonatal malnutrition while astrocytes are more resilient to lesions under those conditions[7].

Early and recent evidence *in vivo* has indicated that astrocyte phenotypic changes, delayed or precocious astrogliogenesis, can occur at birth in different brain regions as a result of low protein levels in the maternal diet[8]. Feoli *et al*. using a maternal diet containing only 7% protein (casein), during gestation and lactation periods, reported that increased levels of enzymatic and other molecular markers indicates a transient astrogliosis in the cerebral cortex but not in the hippocampus or cerebellum of malnourished pups at postnatal day 2[9]. Lower levels of glutathione and glutathione peroxidase were also found in the cerebral cortex and hippocampus of similar group of neonates, associated to lower total antioxidant reactivity[10]. However, several of these biochemical changes in astrocytes were completely recovered during brain maturation, even keeping the animals in the same protein restricted diet through adulthood[9,10].

Astrocytes, are implicated in the metabolic support of neurons, releasing neurotrophic factors, antioxidant molecules and components of extracellular matrix[11–13]. Early modifications in their metabolism or in their ability to react under condition of malnutrition may impair neuronal growth or survival as well as synapse function during brain development[14,15]. Recent evidence has indicated that maternal food restriction during pregnancy and lactation results in enhanced levels of glucose transport 1, glycogen and in the phosphorilated isoform of glycogen synthase kinase-3, mainly expressed in astrocytes, in the cerebral cortex of 10 days-old pups[16]. Although the repercussion of these effects were not still investigated, they may represent modifications in the neuron-astrocyte interaction, especially considering that brain glycogen is involved in several neuronal functions during development.

In vivo studies have indicated that pre and/or postnatal malnutrition induced by a diet containing 6% casein are able to promote permanent anomalies in the neuronal morphology in the cerebral cortex and other brain regions [17,18,6]. Parameters such as dendrite number and their complexity were not recovered after nutritional rehabilitation with adequate levels of protein (23% casein) even when glial cell density and synapse/neuron ratio is apparently restored [19].

On the other hand, using a maternal diet called Regional Basic Diet (RBD) that containing 8% protein mainly from vegetable sources and 1% of lipids, Borba *et al*., reported morphological phenotypic plasticity in cerebral cortex neurons of the progeny [20]. Interestingly, they observed that this type of diet reduced the soma size but did not impair neuronal dentritic development in the young progeny. Moreover, when a balanced diet was given to the animals from 42th postnatal day, the neuronal soma area increased at a greater extent than those of well-nourished group.

A peculiar feature present in the RBD diet is that despite its low quantity in lipids, it is relatively rich in n-3 α-linolenic fatty acid (12%) and contains reduced amount of n-6 linoleic fatty acid (~19.7%) which results in n-6/n-3 fatty acid ratio of 1.69 [21]. This ratio is lower than in casein-based diets mainly used as control containing ~55% linoleic and 6% α-linolenic fatty acid (n-6/n-3 ratio = 9.3; Passos et al., 2012) or the commercial diet Labina here used as control (n-6/n-3 ratio = 15; Souza et al., 2008). These essential fatty acids are precursors of the polyunsaturated long chain fatty acids (LC-PUFAs) docosahexaenoic acid (DHA) and arachidonic acid (AA), which usually play key roles during brain development[22–25]. DHA has been implicated in the neural stem cell proliferation, astrocyte and neuron differentiation and exerts neuroprotective actions[26]. AA is mainly involved in neuronal cell signaling and plasticity but in high levels can reduce DHA synthesis and induce an inflammatory condition[27]. Thus, a reduced n6/n3 ratio in the maternal diet is recommended during progeny's brain development favoring beneficial effects of DHA including those that involve neurite outgrowth and epigenetic modifications[28,25].

The present study investigated how a maternal dietary treatment with RDB could affect the proliferation and differentiation of astrocytes of neonate`s cerebral cortex as well as the ability of these cells to act on neuronal growth *in vitro*. We have tested the hypothesis that a low n6/n3 essential fatty acids ratio in this maternal diet

could minimize deleterious effects induced by its low protein and lipid levels on neuronal differentiation, favoring neurite outgrowth during neuron-astrocyte interaction. In order to investigate how each one of these cells could be affected by this type of malnutrition the axonal lenght and dendritic complexity were analyzed when neurons and astrocytes of neonates were provided from mothers with distinct nutritional condition.

Materials and Methods

Animals and diets

Sixty progenitor adult Wistar rats (40 females and 20 males) were used in this study. These animals were divided randomly into two groups according to the nutritional condition. Control rats were fed with a commercial balanced diet, comprising 22% protein content while malnourished rats were fed with RBD diet[29]. The composition of the experimental diets, both containing around 380 kcal/100g is shown in Table 1. The mothers started receiving the respective diets 30 days before mating and were maintained on those diets during gestation and first week of lactation. The offspring constituted the object of the present study. At parturition, litter size, total litter weight and mean birth weight of the pups were recorded. In the postnatal (P) days P1 to P3, pups from different litters (2-3 per litter/group) were grouped to obtain cerebral cortex primary cultures, as described below. All procedures were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocol #009428/200633), which complies with the “Principles of Laboratory Animal Care” (NIH, Bethesda, USA).

Commercial Balanced Diet		Regional Basic Diet (RBD)	
Nutrients	Quantity (%)	Nutrients	Quantity (%)
Proteins	22,58	Proteins	7,87
Lipids	8,62	Lipids	0,95
Carbohydrates	50,44	Carbohydrates	69,67
Fibers	2,00	Fibers	7,21
Minerals	4,09	Minerals	1,26

Table 1. Composition of diets.

Astrocyte primary cultures

Primary cultures of astrocytes were prepared as described by Moura-Neto *et al.*, 1983[30]. Pups from different litters per group were decapited and the cerebral cortex was dissected in phosphate buffer containing 0,6% glucose (PBS-glucose), followed by mechanic dissociation. Cell suspension was diluted in PBS-glucose plus Dulbecco's modified Eagle's medium and nutrient mixture F-12 (DMEM-F-12, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 33mM glucose (Merck), 2mM glutamina (Calbiochem), 3mM sodium bicarbonate (Merck), 0,5mg/ml penicilin/streptomicin (Invitrogen), 2,5µg/ml amphotericin (Sigma) and then centrifuged for 5 minutes (1,500 rpm). Cells were plated in plastic bottles (25cm^2) and incubated in DMEM-F-12 medium, at 37°C in a humidified 5% CO_2 95% air atmosphere. The medium was replaced every 2-3 days. After 10 days in vitro (div) it was carried out the first passage of cells (P1), using trypsin (0.25% + EDTA %; Sigma) for 5 minutes. Cells were kept on P1 for 10 div and two subsequent passages (P2 and P3) were done in order to investigate whether some features of astrocytes provided from malnourished pups could be reversed by the culture medium.

Immunocytochemistry

To analyze the expression profile of intermediate filaments and proliferation markers in astrocyte cultures was assessed. For immunocytochemistry, cell cultures were placed on coverslips coated with 1µg/ml poly-l-lysine (Sigma), in 24-well plates ($2x10^5$ cels/well), Three days after each passage, these cells were fixed with 4% paraformaldeyde for 20 minutes and washed in PBS 0,1M. To visualize the intermediate filaments, Vimentin and GFAP, immunofluorescence approach was adopted. Briefly, cells were firstly blocked with 3% BSA (Sigma Chemical Co.) and 1% Triton X-100 (Riedel de Haen-Germany) in PBS for 30 minutes and then incubated in the presence of rabbit anti-GFAP (Diag. Biosystems 1:200) and mouse anti-vimentin antibodies (Diag. Biosystems 1:200), overnight at 4ºC. After primary antibodies incubation, cells were rinsed with PBS and incubated simultaneously with the secondary antibodies anti-rabbit (Dylight 488, Rockland - 1:5000) and anti-mouse (Dylight 594, Rockland – 1:5000) for 3 hours at room temperature. After that, the cells were rinsed again in PBS and incubated with Hoechst 33342 (nuclear fluorescent

marker – 1:1000) for 5 minutes. The cultures were mounted on 40% glycerol diluted in PBS and examined under an epifluorescent Leica microscope.

To investigate the effect of maternal malnutrition on astrocyte proliferation in the cerebral cortex of neonates, double labeling for GFAP and the protein Ki67 was carried out in cells cultured for 3 div after the first passage (3dP1). In these experiments, cell cultures were first immunolabeled using a polyclonal mouse-anti Ki-67 (Novocastra, 1:200; overnight at 4°C) which was visualized using a biotinylated goat anti-mouse secondary antibody (Jackson, 1:1000) for 1 hour, followed by streptavidin (Sigma, 1:250) for more 1 hour and the cromogen diaminobenzidine (2.5 µg/ml + H_2O_2 0.03% in PBS 0.1M, for 10 minutes). Subsequently, cell cultures were rinsed in PBS and incubated in glycine buffer (0.02 M, pH 2.2) for 20 min. Then, they were washed in PBS and immunostained with rabbit anti-GFAP (Invitrogen; 1:200) and mouse-anti-vimentin (Biosystem; 1:200) for 18 h, at 4°C), which were visualized with the secondary antibodies goat anti-rabbit (Dylight 488, Rockland – 1:5000) and goat-anti-mouse (Dylight 594, Rockland – 1:5000) for 3h at room temperature.

Western blotting assay

Homogenates of astrocyte cultures 3 div after the first passage (3dP1) were obtained in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 0,6 mM $CaCl_2$, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% Triton x-100. An aliquot of each culture was taken to determine protein content by the BCA Protein Assay kit (Thermo Scientific). Samples of homogenates were diluted in sample buffer (62.5 mM tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for approximately 5 minutes. 30 micrograms of protein per lane were electrophoretically separated in 10% gradient sodium dodecyl sulphate–polyacrylamide gel at 120mA. After separation, the proteins were transferred to Hybond-nitrocelullose transfer membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 2 hours at 200mA. Membranes were blocked for 1 hour in Tris-buffered saline-Tween 20 (TBS-T) containing 5% of fat-free milk. Then, incubated in primary antibodies rabbit anti-GFAP (Dako; 1:4000) and mouse anti-vimentin (Diag. Biosystems 1:500) diluted in block solution overnight at 4°C. After several washes in TBS-T, biotinylated secondary antibodies (goat anti-rabbit

Jackson 1:1000 and goat anti-mouse Jackson 1:1000) diluted in TBS-T were added to the membrane and incubated for 2 hours, followed by streptavidin-HRP (Sigma, 1:500) for 1 hour. Proteins were visualized using the cromogen diaminobenzidine (Sigma, 2.5 µg/ml + H_2O_2 0.03% in PBS 0.1M). Digital images of the blots were obtained and the integrated optical density was estimated by using Image J 1.46 software (NIH, USA). The protein levels were normalized to that of the β-actin (Sigma, 1:2500) protein that was used as an internal standard.

Morphometry and quantification of expression profile of intermediate filaments in astrocyte monolayers and number of proliferating astrocytes

The analysis of proliferating astrocytes was achieved in three independent cultures/group. Three coverslips per culture were analyzed and digital images of 80 fields per culture were obtained using a DFC 345 FX camera coupled to a Leica, DM 5500-B automated upright microscope and 40x planachromatic objective. Bright field and fluorescent images were done for Ki-67 and GFAP/vimentin labeling respectively, at the same field. The mitotic index was measured by counting the percentage of positive ki-67 cells compared to the total number of astrocytes in the coverslips.

Co-cultures of neurons and astrocytes with similar or distinct nutritional condition

To investigate how intrinsic properties of cortical neurons or astrocytes of neonates delivered from malnourished mothers could affect neurite outgrowth, co-cultures of neurons onto astrocytes were carried out in four different combinations: 1) control neurons over control astrocytes; 2) malnourished neurons over control astrocytes; 3) control neurons over malnourished astrocytes and; 4) malnourished neurons over malnourished astrocytes. For these co-cultures pregnant females in the 16^{th} gestational day were anesthetized with isofluorane and then decapitated. The pre-anesthesia with volatile agents to prevent pain and reflexes was adopted as a recognized procedure by animal welfare regulatory agencies (e.g. CONCEA, Brazil; UK Animals Scientific Procedure, 1986). Four types Neurons of E16 embryos were freshly dissociated from the cerebral cortex in serum free DMEM-F12 medium. Confluent astrocyte monolayers that were 8 div in 24 well plates were washed three

times with serum free DMEM-F12 and subsequently, neurons freshly dissociated were plated over the astrocytes in a density of 50,000 cells/well. The co-cultures were kept at 37°C in a humidified 5%, CO_2 95% air atmosphere for 48 h and then fixed in paraformaldehyde 4% for 20 minutes.

Double immunostaining for neuronal and astrocyte markers was carried out in the co-cultures. In these experiments, cells were firstly incubated with 3% bovine serum albumin (BSA) in PBS + triton X-100 0.3% for 1 hour and then in a mixture of rabbit anti-GFAP (Invitrogen, 1:400) and mouse anti-β tubulin III in PBS + triton X-100 0.3% for 18 hours at 4°C. Neurons and astrocytes were respectively visualized with the secondary antibodies goat anti-mouse (Dylight 594, Rockland) and goat anti-rabbit (Dylight 488, Rockland) both diluted 1:5000 and incubated for 3 hours at room temperature. After that, the cells were rinsed in PBS and labeled with Hoechst 33342 (nuclear fluorescent marker – 1:5000) for 5 minutes. The cultures were mounted on 40% glycerol diluted in PBS and examined under an epifluorescent Leica microscope (Model DM 5500-B) coupled to a DFC 345 FX camera. Digital images of several fields per coverslips were taken to neuronal morphometric analysis, as described below.

Analysis of neuronal morphometry

Parameters of neurite outgrowth in neurons cultured onto astrocyte monolayers were measured using the Neuron J plug-in of Image J 1.46 software according to Meijering *et al*., (2004). Three coverslips per culture were analyzed and at least 25 fields were measured per coverslip. At least 100 neurons were measured per culture. All neurites emerged from neuronal soma were considered. The number of neurites emerging from the soma, the sum of all neurite measurements per neuron and the sum of all neurite measurements divided by the number of process per neuron was analyzed. Fractal dimension and branching area were assessed using Frac-Lab plug-in of Image J version 1.48 software according to protocol described by Barreto *et al*., (2014).

Statistical analysis

Statistical analyses were done using the following tests: Z-test for comparison between proportion of cells with distinct expression profile of intermediate filaments; Mann-Whitney for comparison of astrocyte soma size; Two-way ANOVA coupled to Tukey's Honestly-Significant Difference for comparison of multiple variables. All statistical analyses were performed using non-transformed data, except data of neuron axonal length obtained in co-cultures. In this case, data were transformed using the log (x+1) followed by two-way ANOVA, investigating the effects of neuron, astrocytes and the interaction between these factors in defining the axonal length in the co-cultures. It was used the SYSTAT 13, GraphPad Prism 4.0, and Origin Pro 8.0 softwares. $P< 0.05$ was considered statistically significant. The experiments were performed in triplicate, and each result represents the mean of independent experiments.

Results

Maternal RBD malnutrition induces long-lasting modifications on the intermediate filament expression in cortical astrocytes.

The quantitative analysis of number of cells expressing vimentin or GFAP only (Fig. 1A) or co-expressing both intermediate filaments (Fig. 1B) demonstrated that astrocyte cultures from malnourished newborns contain lower number of cells expressing vimentin+ (Control: 3dP1=23.6%, 3dP2=49.4%, 3dP3=24.3% *vs* Malnourished P1=12.8%, P2=29.3%, P3=7%) only as compared to control condition. These findings were seen despite the time of cell growth *in vitro*, being present in the early stages of cell growth 3dP1 (13 div) as well as 3dP3 (33 div). On the other hand, a greater proportion of cells co-expressing vimentin + GFAP (Control: 3dP1=13.4% and 3dP3=4.8% *vs* Malnourished: 3dP1=32.3% and 3dP3=15.1%), or expressing GFAP+ only, (Control: 3dP1=63%, 3dP2=27.6%, 3dP3=70.9% *vs* Malnourished: 3dP1=54.9%, 3dP2=47.75%, 3dP3=75.6%) were found in astrocyte cultures of malnourished animals when compared to that found in the control condition.

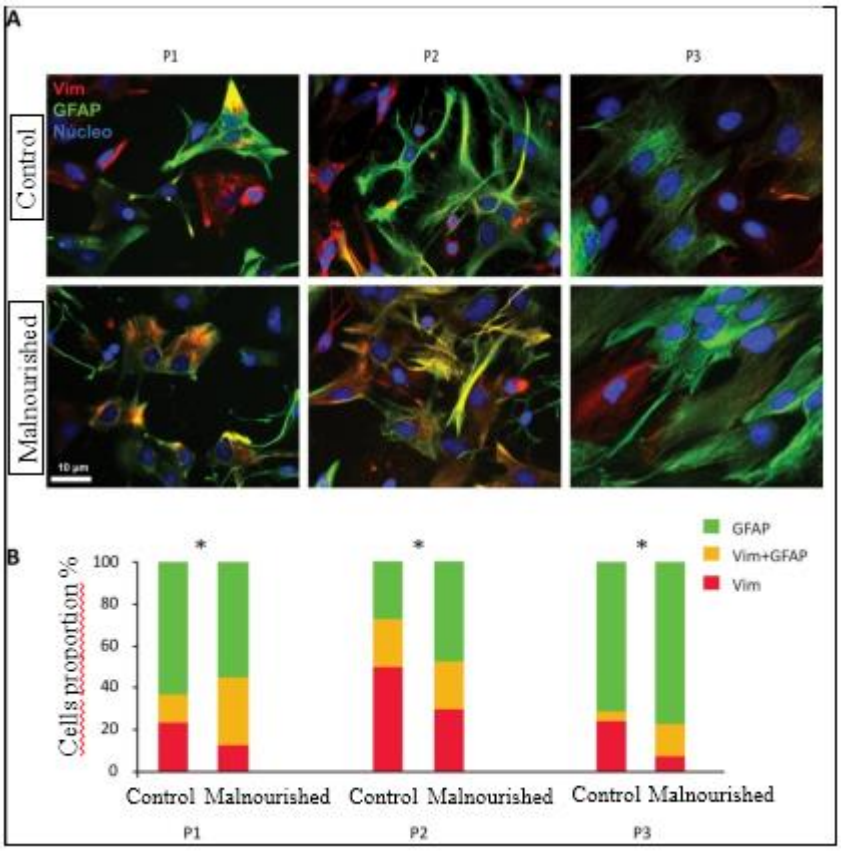


Figure 1. Effects of maternal malnutrition on regulation of intermediate filaments expression in primary cortical astrocytes cultures. (A) Representative photomicrographs of vimentin (red) and GFAP (green) expressions in astrocytes, over time. (B) Graphic shows proportion of three astrocyte populations in control and malnourished cultures, according vimentin expression (red bar), vimentin and GFAP co-expression (yellow bar) and GFAP expression (green bar), over time. Note similar patterns on regulation of intermediate filaments expression over time on control and malnourished groups. Total number of control cells countedP1=4446, P2=1691, P3=1105; Total number of malnourished cells counted P1=3299, P2=1775, P3=1599. Data were obtained from 2 cultures of each period of time. * $p<0.05$, Z test for difference between the proportions of both groups, for each period of time analyzed.

In order to evaluate if these effects could reflect modifications on protein levels of these intermediate filaments, western blotting experiments were carried out in homogenates of astrocyte cultures 3dP1. As shown in the Figure 2, RBD maternal malnutrition did not modify the total GFAP protein content in the astrocyte primary cultures.

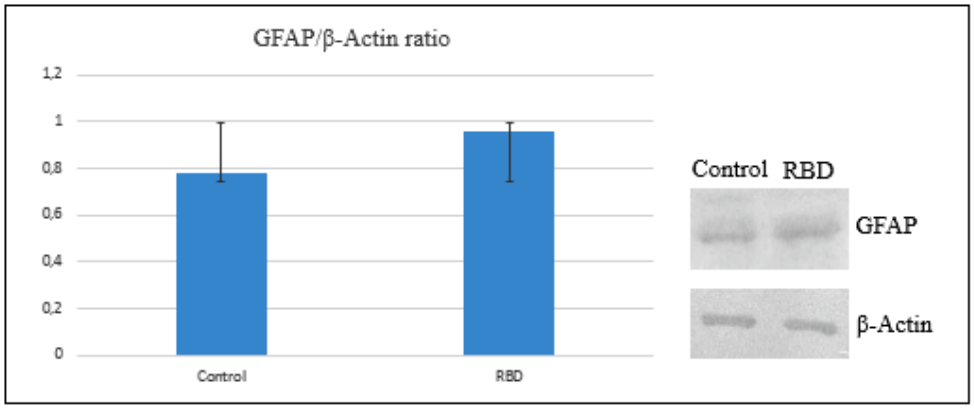


Figure 2. Western blotting to GFAP. Samples (30µg/lane) were separated by SDS-PAGE under reducing conditions followed by electrotransfer onto nitrocellulose membranes for immunodetection of GFAP (A) and Vimentin (B) in 13div.

Maternal malnutrition induced temporary effects in the soma size of astrocytes in different stages of development

Morphometric analysis of Vimentin+ or GFAP + astrocytes showed an effect of maternal malnutrition on the soma size. Vimenti + astrocytes have smaller soma size since early stages of pos-natal development in culture (23 days in vitro) while GFAP+ cells are smaller only at the second passage (23 days in vitro). This effect is reversed by the culture medium in the third passage (33 days in vitro) Figure 3 shows quantitative results obtained in this analysis.

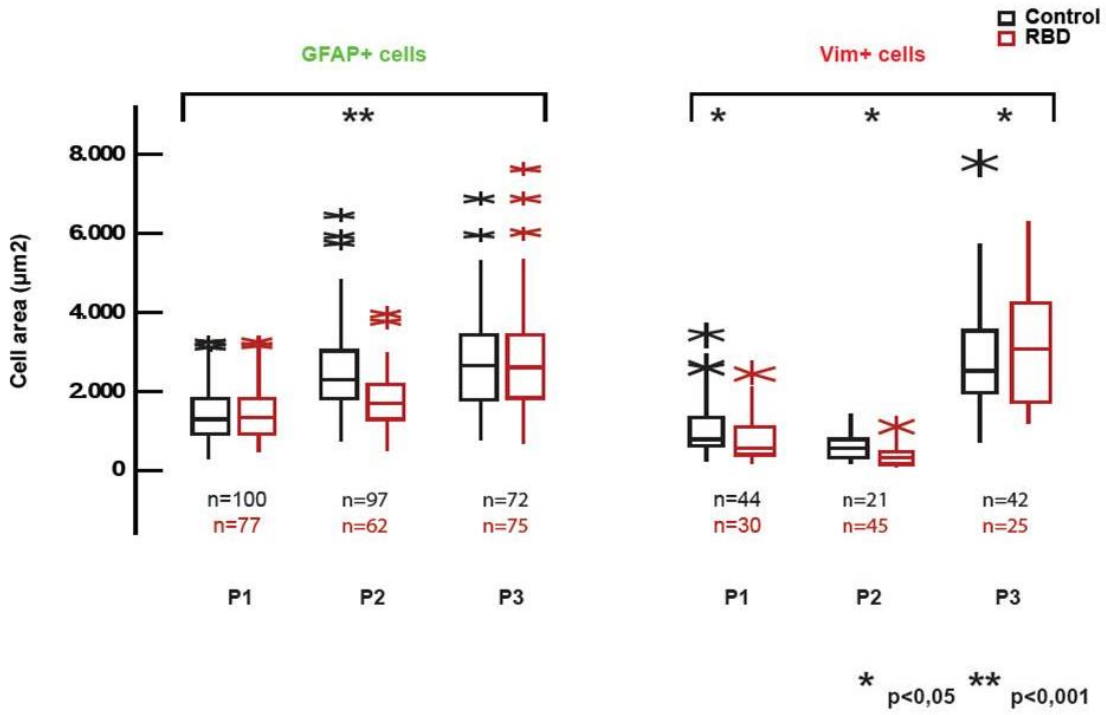
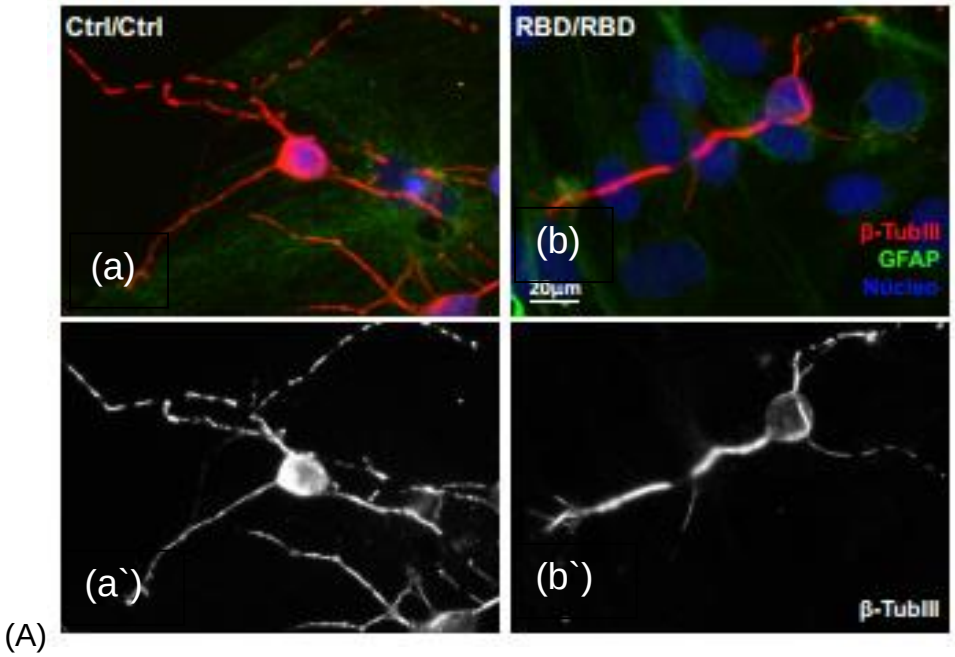


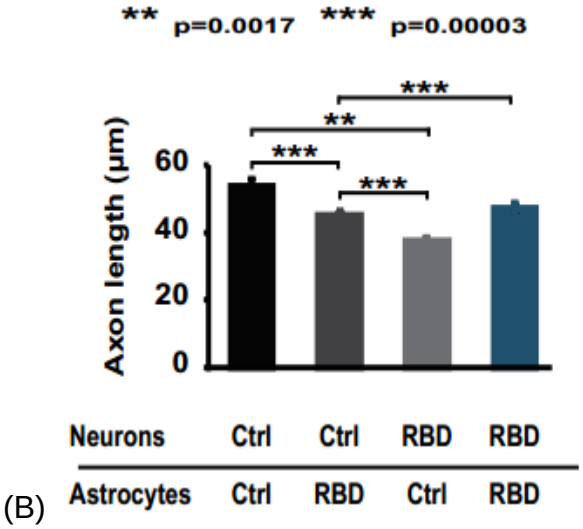
Figure 3: Morphometric analysis of astrocytes soma area immunoreactive to GFAP or Vimentin maintained in cultures for 13 (P1) 23 (P2) or 33 (P3) days in vitro.

Maternal malnutrition does not affect axonal outgrowth of neurons co-cultured over astrocytes of the same nutritional condition

Multiple comparisons between all the interactions demonstrated that axonal outgrowth was lower in co-cultures from distinct nutritional conditions, when compared to control co-culture. However, the axonal length is similar in co-cultures which cells are derived from the same nutritional context, suggesting that metabolic adaptations could be contributing to a better intercellular communication (Figure 4).



(A)



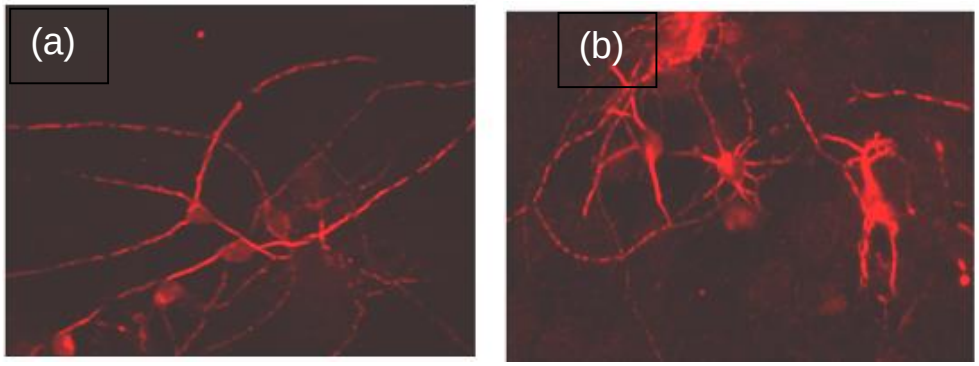
(B)

Fig. 4 Analysis of axonal length in co-cultures of cortical neurons and astrocytes. (A) Ilustrative photomicrographs of primary co-cultures of control and neurons astrocytes

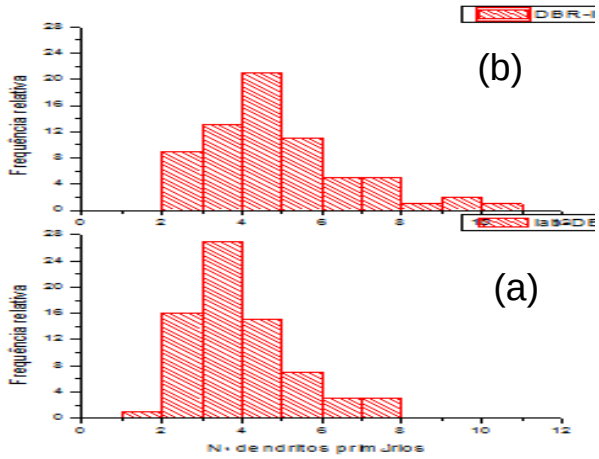
(a and a', Control/Control) as well as malnourished neurons and astrocytes (b and b', Malnour/Malnour). (a and b) show overlap from the different channels of immunostaining for neurons (β-tubulin III, red), astrocytes (GFAP, green) and nucleus cells (Hoechst, blue). Photomicrographs in gray scale (a' and b') show only β-tubulin III immunostaining used for axonal length measurement. (B) Box graphic show axonal length in co-cultures from different nutritional condition of neurons and astrocytes (Total number of cells analyzed: control neurons/control astrocytes = 327; malnourished neurons/control astrocytes = 438; control neurons/malnourished astrocytes = 373; and malnourished neurons/malnourished astrocytes = 362). Data were obtained from two distinct co-cultures.

Morphological phenotype plasticity of neurons co-cultures over astrocytes with different nutritional condition

Embrionary neurons of malnourished or control rats were co-cultured over astrocytes provided from pups under different nutritional background. Neurons of malnourished group display a phenotypic plasticity characterized by an increased number os neurites emerging from the soma in addition to smaller neurite length. **Figures 5A and 5B** show representative fields of two cocultures showing neurons immunolabelled for beta-tubulin III that were maintained for 48h over astrocytes with distinct nutritional condition. Index of fractal dimension of these cells is under analysis. Figure 5C and 5D shows the profile of number of neurites emerging from the soma in 50 neurons analyzed in each condition in a representative co-culture.



B



Analysis of astrocyte proliferation

Quantitative analysis in course of Ki67 positive astrocytes in the primary cultures suggest an increased number of proliferating astrocytes provided by cerebral cortex of malnourished neonates. Figure 6 shows representative fields of two astrocyte primary culture immunoreacted for ki-67 and the nuclear marker Hoechst

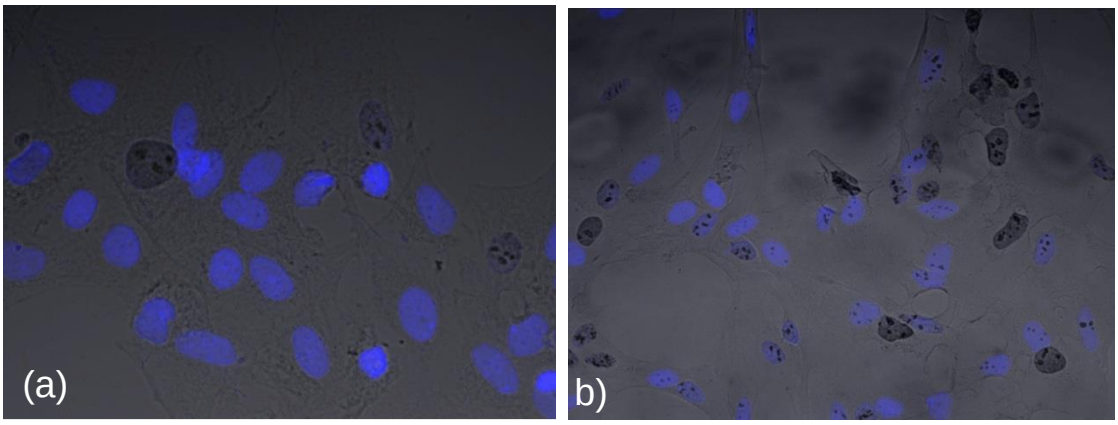


Figure 6. Cerebral cortex proliferating astrocytes identified by immunoreactivity to Ki67. (a) Representative field in astrocyte primary culture of control group (20X magnification) and (b) in culture of malnourished group (10X magnification) Arrowheads indicate Ki67 immunoreactive nuclei of proliferating cells. Blue nuclei are labeled by Hoechst.

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