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RESISTÊNCIA DO CEREBELO DA PROLE DURANTE A LACTAÇÃO AO INSULTO OXIDATIVO INDUZIDO POR UMA DEFICIÊNCIA MATERNA MULTIFATORIAL

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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.

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“Estamos na situação de uma criancinha que entra em uma imensa biblioteca, repleta de livros em muitas línguas. A criança sabe que alguém deve ter escrito aqueles livros, mas não sabe como. Não compreende as línguas em que foram escritos. Tem uma pálida suspeita de que a disposição dos livros obedece a uma ordem misteriosa, mas não sabe qual ela é.”

(Albert Einstein)

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RESUMO

Evidências recentes têm indicado que a Dieta Básica Regional (DBR), usada como modelo de má-nutrição severa multifatorial, contém elevada proporção de ácido linolênico e baixa de ácido linoleico, sendo capaz de aumentar a incorporação do ácido docosahexaenóico (DHA) no cerebelo e hipocampo, mas não no córtex cerebral de ratos adultos. Neste trabalho investigamos se a má-nutrição induzida pela DBR é capaz de alterar a capacidade antioxidante do cerebelo de forma diferenciada em relação ao córtex cerebral, em ratos jovens e adultos. A má-nutrição materna foi induzida 30 dias antes do acasalamento, durante a gestação e aleitamento pela DBR. Três grupos experimentais foram analisados: grupo controle, grupo desnutrido até 90 dias e grupo desnutrido até o desmame e então submetido à reposição nutricional até a vida adulta. Parâmetros relacionados ao status oxidativo, como, níveis de lipoperoxidação (LP), níveis de glutationa reduzida (GSH) e oxidada (GSSG) e atividade das enzimas antioxidantes, catalase (CAT) e superóxido dismutase total (t-SOD), estimação de espécies reativas de oxigênio (ROS), nos níveis de óxido nítrico (NO) e nicotinamida adenina dinucleótido reduzida (NADH) e/ou fosfato reduzida (NADPH), foram analisados em homogenados de córtex cerebral e cerebelo. Nos animais jovens desnutridos houve maiores níveis de ROS e LP no córtex cerebral (~53%) e menores níveis no cerebelo (72% e 20%) em relação ao grupo controle. Consistentemente, menor (~35%) e maior (~153%) atividade da t-SOD foi detectada respectivamente nessas regiões em relação ao controle. A atividade da CAT não foi diferente no córtex cerebral, mas foi maior (~38) no cerebelo dos animais desnutridos comparados ao grupo controle. Em animais adultos desnutridos, o equilíbrio redox foi mantido no cerebelo e recuperado no córtex cerebral (níveis mais baixos de ROS e LP e maior razão GSH/GSSG). Produção NO foi prejudicada pela desnutrição em qualquer idade, principalmente no cerebelo. Os resultados evidenciam uma menor vulnerabilidade do cerebelo ao insulto oxidativo induzido pela DBR quando comparado ao córtex cerebral. Embora as diferenças no estágio de desenvolvimento e o gasto metabólico das duas regiões devam ser considerados, os dados sugerem que mesmo diante de uma deficiência nutricional multifatorial, uma baixa razão nos níveis de ômega 6/ômega 3 na dieta e uma menor produção de NO pode favorecer a reserva antioxidante em regiões encefálicas onde a incorporação de DHA é favorecida.

Palavras-chaves: Desnutrição. Estresse oxidativo. Antioxidante. Ômega-3.

ABSTRACT

Recent evidence has indicated that the Regional Basic Diet (RBD), used as multifactorial severe malnutrition model, contains a high proportion of alpha-linolenic acid and low proportion of linoleic acid, being able to increase the incorporation of docosahexanoic acid in the cerebellum and hippocampus, but not in cerebral cortex of adult rats. In this work we investigated whether malnutrition induced by RBD can differentially alter the antioxidant capacity of the cerebellum and cerebral cortex in young and adult rats. Maternal malnutrition was induced 30 days before mating, during pregnancy and lactation by RBD. Three experimental groups were analyzed: control group, malnourished group up to 90 days and malnourished group until weaning and then submitted to nutritional supplementation to adulthood. Parameters related to oxidative status, such as lipid peroxidation levels (LP), reduced (GSH) and oxidized (GSSG) glutathione levels and the activity of antioxidant enzymes, catalase (CAT) and superoxide dismutase total (T-SOD), estimation of reactive oxygen species (ROS), nitric oxide levels (NO) and reduced nicotinamide adenine dinucleotide (NADH) and / or phosphate reduced (NADPH) were analyzed in homogenates of cerebral cortex and cerebellum. In young animals malnourished there were higher levels of ROS and LP in the cerebral cortex (~ 53%) and lower levels in the cerebellum (72% and 20%) compared to the control group. Consistently lower (~ 35%) and higher (~ 153%) activity of t-SOD was detected respectively in these regions compared to the control. The CAT activity was not different in the cerebral cortex, but was higher (~ 38) in the cerebellum of the malnourished animals compared to the control group. In malnourished animals adults, the redox balance was maintained in the cerebellum and the cerebral cortex recovered (lower levels of LP and increased ROS and GSH / GSSG ratio). NO production was impaired by malnutrition at any age, especially in the cerebellum. The results show a lower vulnerability of the cerebellum to oxidative insult induced by DBR compared to the cerebral cortex. Although the differences in the development stage and the metabolic cost of the two regions must be considered, the data suggest that even taken into account the multifactorial nutritional deficiency, a low ratio in the levels of omega 6/omega 3 in the diet and a smaller production of NO, can favor antioxidant resource in brain regions where the DHA incorporation is favored.

Keywords: Malnutrition. Oxidative stress. Antioxidant. Omega-3.

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LISTA DE ABREVIATURAS

ATP	Trifosfato de adenosina, do inglês adenosine triphosphate
ANOVA	Análise de variância
BSA	Albumina de soro bovino, do inglês bovine serum albumin
CAT	Catalase
DAB	Diaminobenzidina
DBR	Dieta básica regional
DCFH-DA	Diclorofluoresceína diacetato
DHA	Ácido docosahexaenóico, do inglês *docosahexaenoic acid*
ERs	Espécies reativas
EROs	Espécies reativas de oxigênio
ERNs	Espécies reativas de nitrogênio
GFAP	Proteína ácida fibrilar glial, do inglês *Glial fibrilary acidic protein*
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
HNE	4-hidroxi-2-*trans*-nonenal
$HO^{\bullet}$	Hidroxila
H_2O_2	Peróxido de hidrogênio
MDA	Malondialdeído
O_2	Oxigênio
PBS	Tampão fosfato salina, do inglês *phosphat buffer saline*
PMSF	Fenilmetil-sulfonil fluoreto, do inglês *Phenylmethyl- sulfonyl fluoride*
RL	Radicais livres
SFB	Soro fetal bovino
SN	Sistema nervoso
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico, do inglês *thiobarbituric acid reactive substances*
TF	Tampão fosfato

SUMÁRIO

1 INTRODUÇÃO

O cérebro é um órgão extremamente suscetível ao estresse oxidativo. Isto se deve, entre outras causas, ao seu baixo conteúdo de defesas antioxidantes, ao alto conteúdo lipídico, ao alto consumo de oxigênio por unidade de massa de tecido e ao alto conteúdo de ferro em algumas áreas particulares (NAZARI et al., 2012).

Apesar da existência de um sistema de defesa antioxidante endógeno, muitos antioxidantes devem ser adquiridos através da dieta. A suplementação nutracêutica é apontada como alternativa para a prevenção e tratamento de doenças por fornecer benefícios à saúde. Alguns trabalhos utilizando suplementação nutracêutica de micro ou macronutrientes específicos apontam seus benefícios sobre a proteção antioxidante. ZAIDI & BANU, 2004, por exemplo, ao utilizarem suplementação de vitamina E em ratos com estresse oxidativo cerebral, observaram um aumento nos níveis de glutationa, na atividade da superóxido dismutase e da catalase, e diminuição da lipoperoxidação, caracterizando a eficácia no restabelecimento do sistema antioxidante inerente. Um outro exemplo de alimento nutracêutico é o ácido graxo polinsaturado ácido linolênico ou seu derivado ácido docosahexaenoico (DHA) da família ômega-3. Quando incorporados à alimentação, ambos tornam-se importantes aliados na prevenção do dano oxidativo, principalmente se os níveis de ácidos graxos da família ômega 6 (ácido linoleico ou o ácido araquidônico) estiverem baixos (YAVIN, 2006). Por isso, seus níveis adequados na dieta, favorecendo uma baixa razão ômega-6/ômega-3 são fundamentais para a proteção contra o estresse oxidativo em algumas regiões encefálicas (AVRAMOVIC et al., 2012; ZUGNO et al., 2014; CHUA, et al., 2014; LIU et al., 2014).

Tendo em vista, que alguns nutrientes adquiridos através da dieta são essenciais para o funcionamento eficaz das defesas antioxidantes, um contexto de desnutrição é, potencialmente, capaz de afetar este sistema de defesa. FEOLI *et al*., 2006a verificaram déficit da defesa antioxidante no córtex cerebral de animais neonatos (2 dias pós-natal) submetidos a uma restrição proteica de 7% de caseína durante a gestação e o aleitamento, sugerindo que alterações no metabolismo dos astrócitos poderiam ser induzidas pela deficiência proteica nas fases iniciais de desenvolvimento encefálico. No entanto, os mesmos autores também verificaram que a capacidade antioxidante do córtex foi restaurada na idade adulta mesmo mantendo o quadro de deficiência proteica na dieta. Em animais alimentados

com dieta deficiente em zinco foi observado dano oxidativo ao DNA, levando a mutações celulares o que contribui para um maior risco de câncer (ALAM & KELLEHER, 2012).

Assim, considerando a importância de uma nutrição adequada para o funcionamento satisfatório do sistema nervoso central (ALAMY& BENGELLOUN, 2012; DJUKIC M. et al., 2014) e para a formação eficiente do sistema de defesa contra agentes oxidantes (VALENZUELA et al., 2012), o presente estudo tem como objetivo avaliar se a deficiência nutricional multifatorial, induzida pela Dieta Básica Regional (DBR), mas também caracterizada por conter uma baixa razão nos níveis de ômega-6/ômega-3 pode alterar distintamente a capacidade antioxidante do córtex cerebral e cerebelo.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 ESTRESSE OXIDATIVO

O estresse oxidativo é o desequilíbrio entre a formação e remoção de agentes oxidantes no organismo, decorrente da geração excessiva de radicais livres (RLs) e/ou diminuição de agentes antioxidantes (EL-BASSYOUNI et al., 2012; NORAZIT et al., 2012). Os RLs são quaisquer espécies químicas que contenham um ou mais elétrons não emparelhados em seu orbital eletrônico mais externo, ao passo que as espécies reativas (ERs) são quaisquer espécies oxidantes altamente reativas, incluindo os RLs (DROGE, 2002; NAZARI, et al., 2012). Devido a esta característica são moléculas reativas, ou seja, instáveis e por esta razão reagem com outras moléculas para se tornarem estáveis. Moléculas oxidantes retiram os elétrons de biomoléculas importantes tais como: lipídios, proteínas, carboidratos e os ácidos nucléicos. Ao perder um elétron as biomoléculas sofrem modificações em sua forma e função (DROGE, 2002; CHAMI & CHECLER, 2012). A oxidação dessas moléculas biológicas pode afetar a integridade dos processos fisiológicos. A lipoperoxidação, por exemplo, pode alterar a fluidez, a permeabilidade e até mesmo levar à ruptura da membrana plasmática (BUTTERFIELDA et al., 2006; WONG-EKKABUT et al., 2007; NICOLSON & ASH, 2014). Já o dano oxidativo em proteínas pode levar a alterações ou inativação de enzimas, inativar canais iônicos ou receptores presentes na membrana plasmática das células levando à distúrbios celulares (GRIMSRUD et al., 2008; PANIERI et al., 2013).

Dentre as ERs destacam-se as de oxigênio (EROs) e de nitrogênio (ERNs) que se caracterizam por serem espécies oxidantes altamente reativas (DROGE, 2002; NAZARI, et al., 2012). As EROs e ERNs são parte integrante do metabolismo humano e são observadas em diversas condições fisiológicas, como, por exemplo, na degradação de ácidos graxos (EL-BASSYOUNI et al., 2012), na atividade de células do sistema imunitário (CHAMI & CHECLER, 2012) e na transdução de sinais celulares (RIBEIRO et al., 2005). Além disso, a formação dessas ERs faz parte do metabolismo celular normal podendo ser produzidas nas membranas celulares, no citoplasma e nas mitocôndrias. Um dos mecanismos mais relevantes para produção de EROs em seres humanos é o metabolismo energético, isto é, a respiração mitocondrial (RANDOLPH-GIPS & SRINIVASAN, 2012). Normalmente o organismo está em equilíbrio entre a produção e a degradação ou remoção ou conversão de ERs, que existem em baixas concentrações em todos os tecidos (DROGE, 2002).

Devido a capacidade de dano que as moléculas oxidantes podem causar ao organismo, o estresse oxidativo, que é justamente o desequilíbrio entre agentes oxidantes/agentes antioxidantes em favor dos agentes oxidantes, está relacionado com uma variedade de doenças, incluindo câncer, aterosclerose, síndrome metabólica, doenças hepáticas e neurodegenerativas, e além disso, uma maior produção de ERs também estão relacionadas com envelhecimento (WANG et al., 2012; 2014; ORSUCCI et al., 2013; BLEIN et al., 2014; LI et al., 2013, 2014; ALMAIDA-PAGÁN et al., 2014; XIE et al, 2014; GENTRIC et al., 2015).

2.2 SISTEMA DE DEFESA ANTIOXIDANTE

A produção contínua de ERs durante os processos metabólicos levou ao desenvolvimento de mecanismos de defesa antioxidante pelo organismo. Sua finalidade é limitar os níveis intracelulares de ERs e, desta maneira, controlar a ocorrência de danos (SHAMI & MOREIRA, 2004; FREDERICO et al., 2012; YAN et al., 2013). Qualquer substância que, presente em menores concentrações que as do substrato oxidável seja capaz de atrasar ou inibir a oxidação deste de maneira eficaz é denominada antioxidante (HALLIWEL, 2001; MEHLA et al., 2010).

As defesas antioxidantes endógenas podem ser divididas em enzimáticas e não enzimáticas e têm como objetivo remover ou impedir a geração de ERs (HALLIWELL et al., 2004). Entre as principais enzimas antioxidantes estão a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) **(Figura 1)** (DRASTICHOVA et al., 2012; BYSTROM et al., 2014; URBANSKA et al., 2014). Já os compostos não enzimáticos sintetizados pelo organismo na defesa antioxidante são, por exemplo, a bilirrubina, o ácido úrico, a melatonina e a glutationa, que agem a fim de manter o estado de equilíbrio redox celular (SINGH et al., 2004; GORDON, 2012).

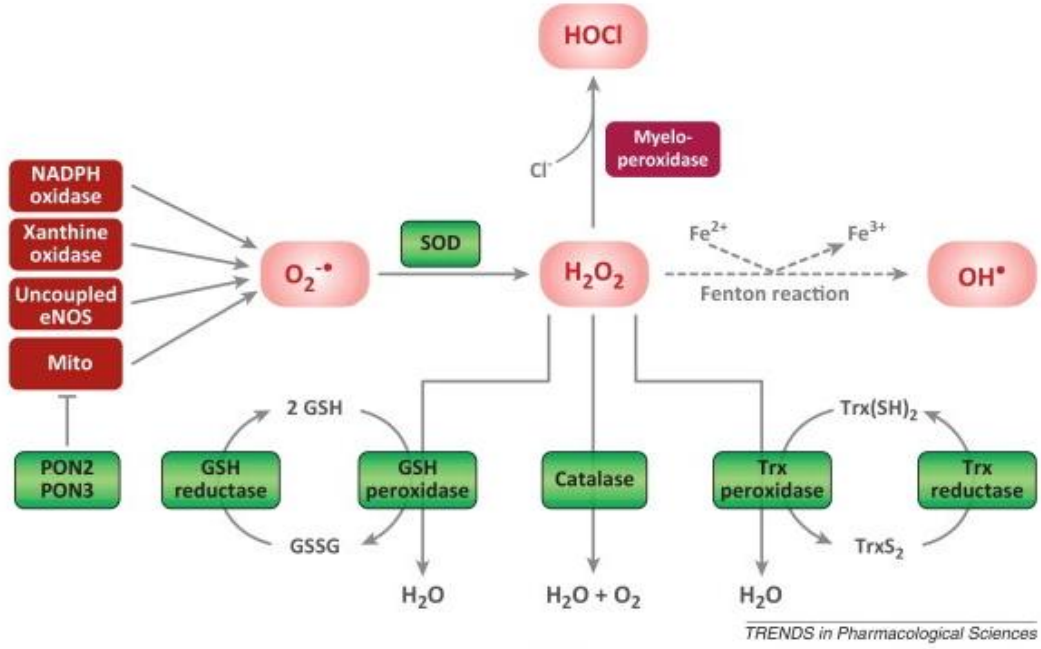

Figura 1. Sistema enzimático de defesa antioxidante. *NADPH* – nicotinamida adenina dinucleótido fosfato reduzida. *SOD* – superóxido dismutase. *TRX* – tioredoxina. *GSH* – gluationa reduzida. *GSSG* – gluationa oxidada. *PON* – paraoxonase. Fonte: LI et al., 2013.

Há antioxidantes que não são sintetizados pelo organismo, mas que podem ser adquiridos através da dieta **(Figura 2)**. Visto que há cada vez mais fatores ambientais que contribuem para um aumento da produção de ERs no organismo, antioxidantes exógenos são importantes, pois servem como um complemento da defesa antioxidante endógena contribuindo para eliminação eficiente do excesso dessas substâncias deletérias. Como exemplo de alguns fatores externos que aumentam a formação de ERs temos a radiação ultravioleta, a poluição do ar e o estresse. Para garantir esta proteção antioxidante advinda dos alimentos, faz-se necessário inserir diversos nutrientes à dieta, dentre os quais se destacam o selênio, o zinco, o cobre, o ferro, o manganês, os flavonoides além das vitaminas A, C, E (BIANCHI & ANTUNES, 1999; DIAO et al, 2012; WRZOSEK et al, 2013; YU & SCHELLHORN, 2013; HAZELL et al, 2013). No entanto, é essencial o equilíbrio destes nutrientes na dieta, pois o excesso de ferro, cobre e vitamina C, por exemplo, ao invés de favorecer uma defesa antioxidante eficiente, favorecem o aumento de EROs (WELCH et al., 2002; MENDES DA SILVA et al., 2014).

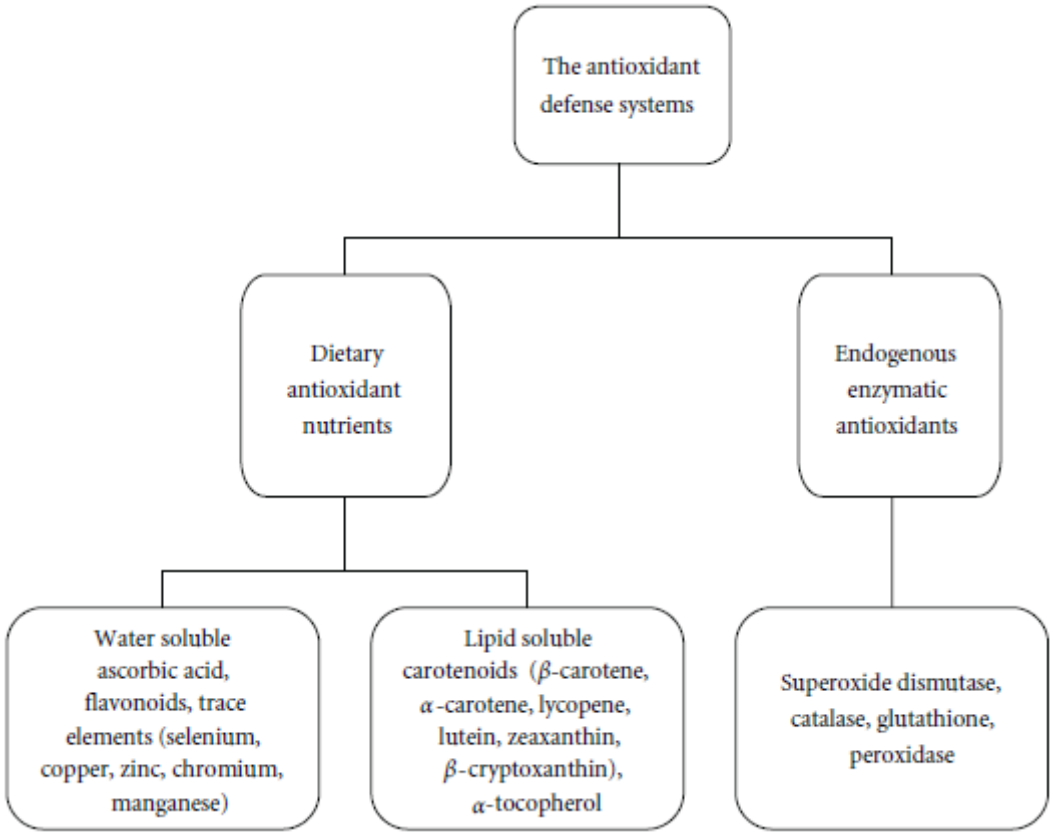

Figura 2. O sistema de defesa antioxidante compreende mecanismos enzimáticos endógenos e não enzimáticos exógenos adquiridos através da dieta. Fonte: ALISSA & FERNS, 2012.

2.3 SISTEMA NERVOSO CENTRAL E ESTRESSE OXIDATIVO

O sistema nervoso central (SNC) é notadamente sensível a lesões oxidativas (RÃO et al., 2012). Isso se deve a características intrínsecas do SNC e de seu metabolismo que o tornam mais propenso a danos causados por espécies oxidantes. O consumo elevado de oxigênio (O_2) pelo cérebro é um exemplo disso. O cérebro sozinho é responsável por 20% do O_2 consumido pelo organismo, porém ele representa apenas 2% do peso corporal, esta proporção indica a alta atividade oxidativa deste órgão. A grande demanda por O_2 deve-se principalmente ao alto consumo de trifosfato de adenosina (ATP) pelos neurônios, para manter o potencial de membrana e o fluxo de neurotransmissores. Portanto, os neurônios dependem muito da eficiência das mitocôndrias. A mitocôndria pode gerar agentes oxidantes, pois elétrons podem vazar da cadeia transportadora de elétrons e gerar EROs que, por sua vez, podem danificar a mitocôndria. Em contrapartida, a mitocôndria lesada apresenta maior vazamento de elétrons e, consequentemente, produz mais EROs, gerando um ciclo vicioso (RAMSDEN et al., 2012; SALMINEN & PAUL, 2014; ABDUL-MUNEER et al., 2014; ŞENOL et al., 2014).

Um atributo relevante da predisposição do SNC ao dano oxidativo, é o fato de que muitos neurotransmissores são oxidáveis. A oxidação de neurotransmissores como dopamina, serotonina e noradrenalina por monoamina oxidases é um dos principais mecanismos de geração de peróxido de hidrogênio (H_2O_2) no cérebro (HALLIWELL, 2001; BISAGLIA et al., 2014; CHIANG et al., 2014).

Vale salientar que muitas áreas do cérebro possuem grande quantidade de íons de ferro e, como foi referido anteriormente, esses íons podem favorecer o aparecimento de ERs, pois catalisam reações de formação de agentes oxidantes. A maior parte destes de íons de ferro está estocada em forma de ferritina e uma parte menor no sítio ativo de enzimas. No líquido cefalorraquidiano, os íons de ferro estão complexados a transferrina, a qual se encontra totalmente saturada. Lesões no cérebro prontamente resultam na liberação de íons de ferro capazes de catalisar a formação do radical hidroxila ($HO^{\bullet}$), a peroxidação lipídica e a oxidação de neurotransmissores (FERNANDEZ et al., 2007; DU et al., 2014; WEIDONG et al., 2014).

As membranas neuronais são potentes geradores de RLs, graças aos ácidos graxos altamente insaturados presentes em sua constituição lipídica. Estes ácidos graxos poli-insaturados são extremamente susceptíveis à peroxidação lipídica, a qual resulta no acúmulo de hidroperóxidos de lipídio (LOOH). Estes podem ser degradados na presença de íons de ferro ou cobre gerando radicais alcoxila ($LO^{\bullet}$) e peroxila ($LOO^{\bullet}$). Os radicais $LO^{\bullet}$ e $LOO^{\bullet}$ podem danificar proteínas de membrana e também atacar outras moléculas de lipídio, propagando a peroxidação lipídica. Os produtos finais da decomposição de LOOH são inúmeros e incluem aldeídos altamente citotóxicos, como o malondialdeído (MDA), acroleína e 4-hidroxi-2-*trans*-nonenal (HNE). HNE parece especialmente citotóxico para células neuronais, já que em alguns modelos animais, a formação de HNE precede a morte destas células (HALLIWELL, 2001; UTTARA et al., 2009; RAUCHOVÁ et al., 2012; SHICHIRI 2014; LOUBOUTIN & STRAYER, 2014; HALL et al., 2015).

Mesmo com toda capacidade intrínseca do SNC em favorecer o aparecimento de EROs ele apresenta mecanismos de defesa antioxidante eficientes, porém menores em quantidade quando comparados aos de outros sistemas do organismo. Os níveis de SOD, CAT e GPx por exemplo, são baixos quando comparados com outros órgãos (COOPER & KRISTAL, 1997; RAUCHOVÁ et al., 2012; PICCO et al., 2014). No cérebro, a catalase é encontrada em microperoxissomos e, provavelmente, não interage eficientemente com o H_2O_2

gerado em outros compartimentos celulares, como por exemplo, na mitocôndria (HALLIWELL, 2001). Os níveis baixos de catalase podem não afetar expressivamente os mecanismos de defesa antioxidantes do cérebro, já que uma gama de outros sistemas de defesa antioxidante está presente. Todas as áreas do cérebro contêm antioxidantes enzimáticos como, a SOD, GPx, a glutationa redutase (Gr) e a tiorredoxina redutase (Tr). Antioxidantes não enzimáticos de baixo peso molecular, como o ascorbato e o α-tocoferol também são encontrados no cérebro. Neurônios e glia possuem um sistema de transporte ativo que concentra o ascorbato intracelular na ordem dos milimolares. O α-tocoferol, por ser lipossolúvel, aloja-se nas membranas, atuando como um poderoso inibidor da peroxidação lipídica (HALLIWELL, 2001). A glutationa é o principal antioxidante não enzimático encontrado nas células. É um fator importante, pois é a principal defesa antioxidante extra-mitocondrial presente nas células (RANDOLPH-GIPS & SRINIVASAN, 2012). No cérebro os astrócitos representam a principal fonte de glutationa e a interação metabólica neurônio-astrócito é responsável pela sustentação de glutationa.

Algumas doenças neurodegenerativas podem ser tanto resultantes como geradoras de um desequilíbrio redox na produção e consumo de ERs (DALLE-DONNE et al., 2006; ORSUCCI et al., 2013; XIE et al, 2014). Muitos estudos apontam que alternativas nutracêuticas podem ser eficazes em controlar o dano oxidativo não só no SNC como também em outros sistemas. VALENZUELA et al., 2012 utilizando-se da suplementação dietética em ácidos graxos poli-insaturados em ratos que apresentavam esteatose hepática, observaram desencadeamento de uma resposta antioxidante eficaz em controlar os danos causados pela doença. ZAIDI & BANU, 2004 observaram uma melhora efetiva nos níveis de glutationa, na atividade da SOD e da CAT, e decréscimo de lipoperoxidação em cérebros de ratos submetidos a suplementação nutricional com vitamina E.

2.4 DESNUTRIÇÃO E DESENVOLVIMENTO CEREBRAL

No Brasil, a desnutrição ainda é, infelizmente, um dos grandes entraves sociais, mas que vem diminuindo paulatinamente. Sua diminuição está relacionada com período de transição nutricional existente hoje no país. Este período é caracterizado por modificações nos padrões nutricionais relacionados com mudanças econômicas, sociais, demográficas e relacionadas à saúde (POPKIN et al., 1993; Disponível em: <portalsaude.saude.gov.br>). Estratégias de saúde pública voltadas a segurança nutricional, com o a finalidade de prevenir a

desnutrição ou a obesidade que são doenças resultantes da inadequação alimentar vem sendo desenvolvidas pelo estabelecimento de políticas públicas. Uma das diretrizes da Política Nacional de Alimentação e Nutrição estabelecida em 2011, por exemplo, é o desenvolvimento e o apoio à pesquisa, à inovação e à tecnologia em temas de Nutrição (Disponível em: <portalsaude.saude.gov.br>).

Tanto o desenvolvimento cognitivo quanto a maturação do SNC dependem das características genéticas de cada indivíduo, de estímulos ambientais e de uma adequada nutrição. Alterações em algum desses fatores, principalmente durante o período crítico **(Figura 3)** de desenvolvimento do SN, podem aumentar a predisposição a doenças crônicas e levar ao retardo no desenvolvimento cognitivo (MORGANE et al., 1993; JOSHI et al., 2003; MARQUES et al, 2014; BERROCAL-ZARAGOZA et al., 2014; FAA et al., 2014; RICHETTO & RIVA, 2014; McCADDON, 2013; MHILLAJ et al., 2015). Este período crítico representa uma etapa importante e única no desenvolvimento, que não pode ser revertida ou repetida posteriormente. Logo, a carência nutricional durante a gestação e/ou no início da vida pós-natal, mesmo que transitória, pode levar a vários graus de disfunção cerebral a longo prazo possivelmente por influenciar a diferenciação e/ou a função celulares (SPARRE et al., 2003; MARTINS et al., 2011; SOUZA et al., 2011; ALAMY & BENGELLOUN, 2012; BELLUSCIO et al., 2014; HOEIJMAKERS et al., 2015). Por esta razão, a desnutrição materno-fetal pode levar à adaptação e/ou programação metabólica que é definida como um processo resultante de um insulto, ocorrido no período crítico de desenvolvimento, que induz a alterações permanentes na estrutura e/ou função de um órgão (PETRY et al., 2001; LUO, et al 2006). Geralmente o cérebro é o principal órgão que o organismo tenta resguardar durante um insulto. Por exemplo, na programação metabólica ocorre redistribuição do fluxo sanguíneo fetal para o cérebro como forma de preserva-lo em detrimento aos demais órgãos (CHARRIAUT-MARLANGUE et al., 2013; POUDEL et al., 2015).

Tanto em roedores como em seres humanos o SNC, ao nascimento, difere de outros tecidos, pois este se encontra ainda imaturo. É durante o desenvolvimento pós-natal que o cérebro vai maturando através de substanciais mudanças quantitativas e qualitativas. As mudanças qualitativas como, por exemplo, divisão celular, diferenciação e formação de redes neuronais, são as mais significativas, pois ocorrem principalmente e/ou exclusivamente durante o desenvolvimento. O desenvolvimento cerebral não é caracterizado apenas pelo aumento de tamanho e peso do cérebro, mas também pela síntese de diversos componentes

celulares, em paralelo à neurogênese e à gliogênese, migração de neurônios e células gliais e diferenciação celular com aumento no tamanho da célula (MORGANE et al., 2002).

As diferentes regiões do cérebro apresentam tempo distinto de maturação. Algumas apresentam desenvolvimento mais precoce e ainda intrauterino, como, por exemplo, o córtex cerebral, outras apresentam desenvolvimento mais tardio e pós-natal, como o cerebelo. Isso se deve ao fato de cada estrutura do cérebro apresentar diferentes tipos celulares com características típicas de divisão. Assim, a maturidade do SNC envolve uma série de estágios sobrepostos temporalmente que seguem uma sequência precisa, diferindo de região para região cerebral (MORGANE et al., 2002). Diferente do que ocorre em outras regiões do SNC, os neurônios do cerebelo são gerados em dois neuroepitélios germinativos, ocorrendo em duas ondas temporais de processos de proliferação e migração. Tal desenvolvimento ocorre em uma sequência similar embora com diferente proporção entre roedores e primatas (BIRAN et al., 2012). As figuras 3 e 4 abaixo comparam a sequência temporal de eventos que ocorrem no desenvolvimento do córtex cerebral e cerebelo de roedores e humanos.

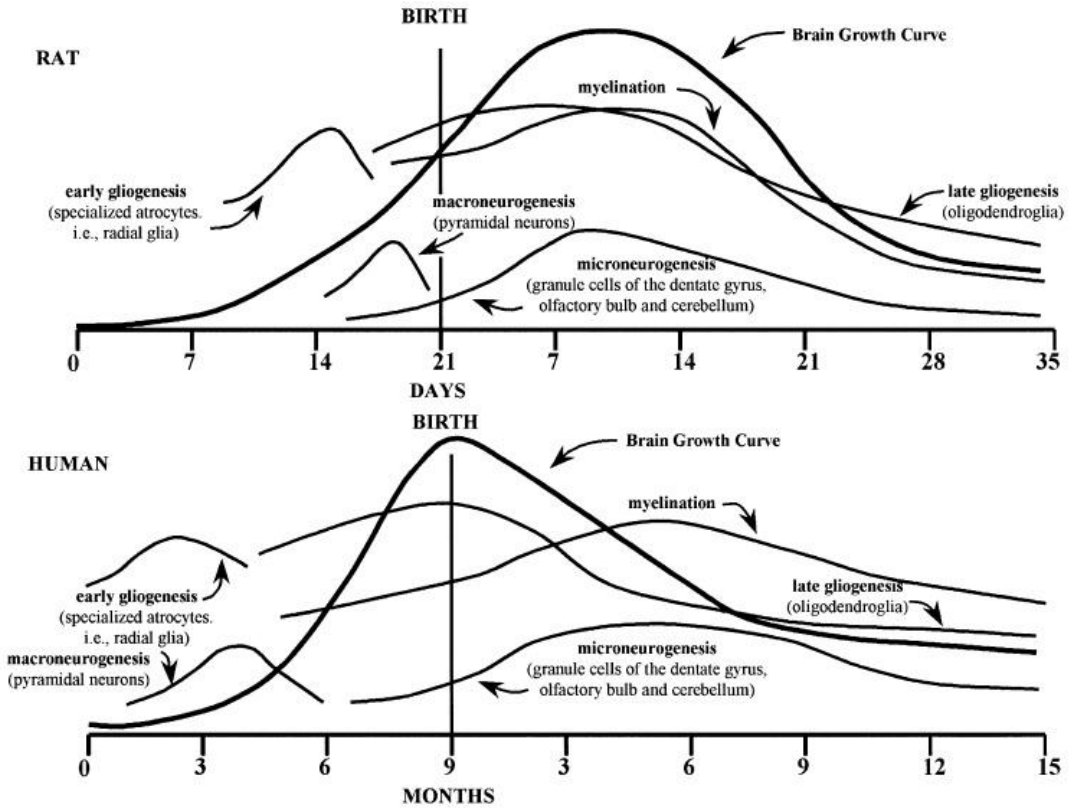

Figura 3. Comparação entre o período crítico do desenvolvimento do córtex cerebral em ratos e humanos. Fonte: MORGANE et al., 1993**.**

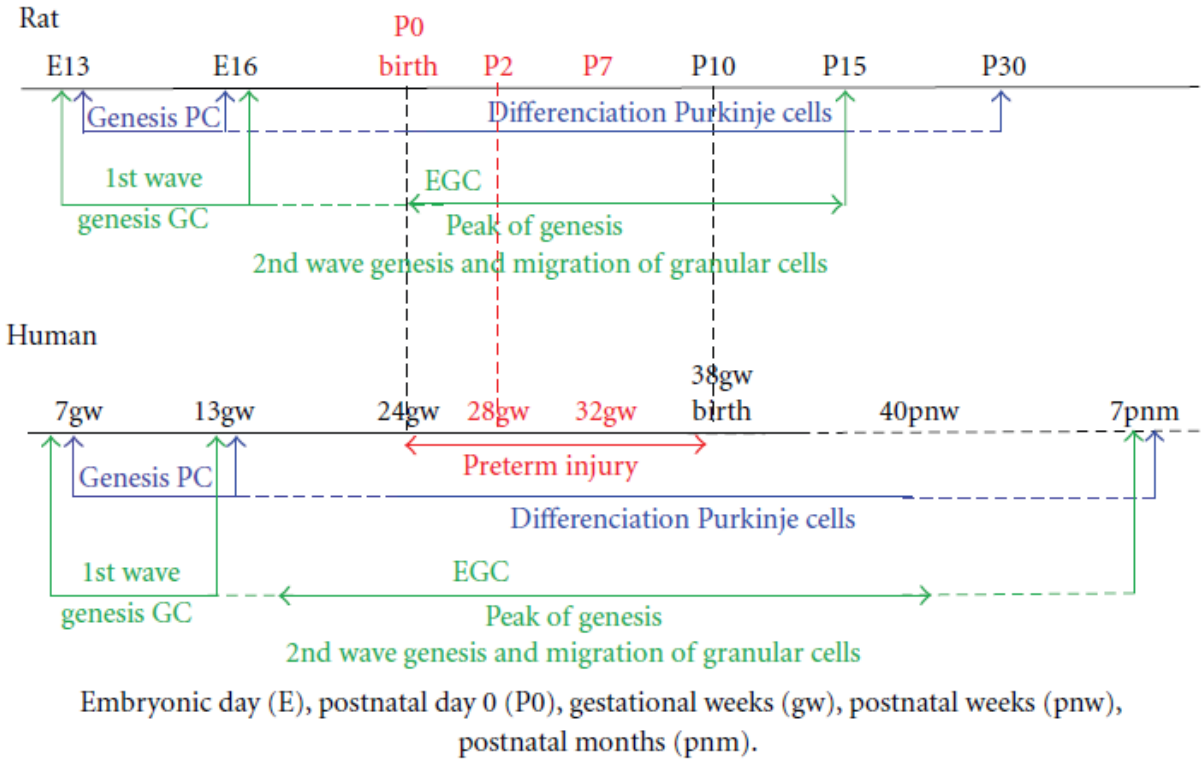

Figura 4. Comparação entre o período crítico do desenvolvimento do cerebelo em ratos e humanos. PC – Células de Purkinje. GC – Celulas granulares. EGC- camada granular externa. Fonte: BIRAN et al., 2012.

A Dieta Básica Regional (DBR) utilizada como um modelo experimental de desnutrição severa **(Tabela 1)** é caracterizada por níveis reduzidos de minerais, vitaminas e lipídios, e possui uma grande deficiência proteica tanto em quantidade como em qualidade (TEODOSIO et al., 1990; MEDEIROS et al., 2008). De acordo com TEODÓSIO et al., 1990 o quadro de desnutrição provocado pela DBR em roedores se assemelha aos sinais clínicos de Marasmo observado em crianças. Uma desnutrição materno-fetal induzida pela DBR aumentou o estresse oxidativo placentário causando impacto no desenvolvimento da prole (VIEIRA-FILHO et al., 2009). Por outro lado, mesmo quando a desnutrição provocada pela DBR ocorre apenas durante o período de aleitamento, algumas alterações permanentes na excitabilidade do córtex cerebral, foram detectadas (GUEDES et al., 1987). Um menor número de astrócitos em regiões hipotalâmicas como a área pré-óptica medial e o núcleo supraquiasmático foram encontradas em animais jovens e adultos submetidos a esta dieta a partir da gestação ou aleitamento (MENDONÇA et al., 2004). Dentre outros estudos com a DBR no SN, VILELA et al., 2005 observaram um menor crescimento do tecido retiniano e uma maior densidade de células ganglionares em animais jovens desnutridos pela DBR desde a gestação, mas não detectaram mudanças na distribuição de neurônios imunorreativos ao neuropeptídio Y no folheto intergeniculado do tálamo nem nos terminais destes neurônios no

núcleo supraquiasmático, apesar de uma menor área citoarquitetônica ter sido observada neste ultimo núcleo. Em 2005, ALMEIDA et al., detectaram que a DBR durante o aleitamento foi capaz de induzir uma menor porcentagem de axônios mielinizados, bem como diminuiu significativamente a área de mielina no nervo óptico. Alterações na ontogenia de alguns reflexos relacionados ao nível de mielinização foram também descritas em animais desnutridos pela DBR durante o aleitamento (BARROS et al., 2006)

Enquanto alguns efeitos da DBR sobre a excitabilidade cortical parecem ser devidos principalmente à sua deficiência proteica (ANDRADE et al., 1990), alguns efeitos desta dieta sobre a diferenciação e reatividade neuronais são surpreendentes. BORBA et al., 2000 observaram notável plasticidade fenotípica morfológica em neurônios do córtex cerebral da progênie de mães alimentadas com a DBR desde a gestação. Interessantemente, estes autores observaram que esta dieta comprometeu a área do soma, mas não afetou o desenvolvimento dendrítico neuronal em células GABAérgicas e nitrérgicas do córtex cerebral. Além disto, observaram que após reposição nutricional a partir do 42º dia, os neurônios dos animais previamente desnutridos apresentaram uma considerável recuperação tornando-se maiores que os do grupo controle

DE SOUZA et al., 2008 analisaram o perfil de ácidos graxos na DBR e no córtex frontal, cerebelo e hipocampo da prole cronicamente submetida a esta dieta até 70 dias de vida. Estes autores observaram proporções significativamente mais elevadas de ácido alfa linolênico e menos elevadas de ácido linoleico na DBR quando comparada à dieta comercial utilizada como controle **(Tabela 2)**. No hipocampo e cerebelo, mas não no córtex cerebral dos animais adultos desnutridos pela DBR foram observados níveis mais elevados de DHA, comparados aos do grupo controle. Uma baixa razão nos níveis de ácido araquidônico/DHA foi observada nestas regiões encefálicas quando comparado à mesma razão no grupo controle. DE SOUZA et al., 2008 também relataram que a reposição dietética a partir do aleitamento com a dieta comercial labina, reduziu os níveis de DHA encefálicos em relação aos que se mantiveram na dieta DBR até a idade adulta.(SOUZA et al., 2008).

Um recente trabalho detectou que animais jovens (30-40 dias) desnutridos pela DBR a partir do nascimento até o desmame apresentaram maiores níveis de lipoperoxidação em homogenados do córtex cerebral apenas quando doses pró-oxidantes de vitamina C foram administradas durante o aleitamento, mas nenhum efeito foi detectado como devido à desnutrição per se (MENDES-SILVA et al. 2014). Recente evidência obtida em nosso laboratório tem indicado que parâmetros morfológicos e funcionais dos astrócitos do córtex cerebral podem ser alterados em ratos neonatos cujas mães foram desnutridas pela DBR a

partir de 30 dias antes do acasalamento e durante toda a gestação (PIMENTEL et al., manuscrito a ser submetidos em anexo).

Tabela 1. Composição percentual da dieta controle (LABINA) e experimental (DBR)

Composição	LABINA	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 controle (comercial) e na experimental (DBR)

Composição	Ração comercial	DBR
Ácidos graxos saturados	27,41	39,37
Ácidos graxos monoinsaturados	22,52	25,96
Ácidos graxos poli-insaturados (Serie linoleica)	46,39	20,38
Ácidos graxos poli-insaturados (Serie linolênica)	3,44	12,25
Ômega6/ômega3	13,49	1,69

Adaptado DE SOUZA et al., 2008.

2.5 EFEITOS DA DESNUTRIÇÃO SOBRE O ESTADO REDOX DO SN

O grau e a natureza das defesas antioxidantes dependem do estado nutricional e da ingestão de nutrientes. Isto porque, os aminoácidos, minerais e vitaminas, por exemplo, obtidos através da dieta, contribuem tanto para formação quanto para manutenção deste sistema de defesa (DIAO et al., 2012; WRZOSEK et al., 2013; YU & SCHELLHORN, 2013; HAZELL et al., 2013 SCHULZE et al., 2014). FEOLI et al. 2006a, mostram que a desnutrição proteica (7% caseína) a partir do acasalamento diminui a reserva antioxidante total e a atividade da glutationa peroxidase sem afetar os níveis de peroxidação lipídica e atividade da SOD e CAT no córtex cerebral de neonatos (2 dias), mas estes parâmetros são

revertidos na idade adulta mesmo mantendo o quadro de má nutrição proteica. Alguns estudos relacionam o estado oxidativo encontrado no tecido cerebral de ratos submetidos a deficiência de micro ou macro nutrientes (BONATTO et al., 2005, 2006; EHRENBRINK et al., 2006; FEOLI et al., 2006a; ADEBAYO et al., 2014), à particularidade que este tecido apresenta à ação dos RLs, como relatado anteriormente. No entanto, vem sendo discutido que a resposta neuronal ao estresse oxidativo não é uniforme entre as várias regiões encefálicas. Esta vulnerabilidade diferencial depende de um número de fatores incluindo uma alta produção intrínseca e elevada demanda para sinalização celular envolvendo EROS, baixa produção de ATP, disfunção mitocondrial e alta resposta inflamatória local (WANG et al., 2010, 2014). O desenvolvimento diferenciado de cada região ao longo da vida pos-natal também parece influenciar o estado oxidativo quando insultos nutricionais são impostos durante a gestação ou aleitamento. PARTADIREDJA et al., 2005 e 2008 evidenciaram que a restrição proteica moderada (12%) durante o período pré-desmame em camundongos é capaz de aumentar a transcrição do RNAm para a SOD no cerebelo, mas não em outras regiões encefálicas analisadas. Estes autores também discutem que alterações oxidativas que ocorrem no início da vida pós-natal diante de quadros de desnutrição não comprometem todos os sistemas antioxidantes.

A vulnerabilidade do córtex cerebral e cerebelo ao estresse oxidativo vem sendo estudadas utilizando vários modelos de má-nutrição proteica, e resultados controversos vem sendo descritos (BONATTO et al., 2005; FEOLI et al., 2006). O presente estudo, considerando as evidências prévias de uma maior incorporação de DHA no cerebelo de animais submetidos a DBR (DE SOUZA et al., 2008) e o metabolismo e desenvolvimento diferenciado pós-natal desta região encefálica, comparado ao córtex cerebral (BIRAN et al. 2012), visa testar a hipótese de que o mesmo poderá apresentar uma maior resiliência ao insulto oxidativo induzido por esta dieta multifatorial.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o impacto de uma desnutrição multifatorial materno-fetal severa sobre a defesa antioxidante do córtex cerebral e cerebelo de ratos jovens recém-desmamados e de ratos adultos cronicamente malnutridos ou cuja transição para uma dieta controle foi feita após o desmame.

3.2 OBJETIVOS ESPECÍFICOS

Investigar no córtex cerebral e cerebelo de ratos albinos jovens e adultos alguns biomarcadores de estresse oxidativo que incluem:

- Níveis de peroxidação lipídica;
- Níveis de espécies reativas de oxigênio;
- Níveis de nitrito;
- Atividade das enzimas antioxidantes superóxido dismutase e catalase;
- Níveis de glutationa reduzida e oxidada;
- Níveis de nicotinamida adenina dinucleótido reduzida e/ou nicotinamida adenina dinucleótido fosfato reduzida.

4 ARTIGO CIENTÍFICO

Artigo na forma de manuscrito submetido à revista ***Cerebellum***.

Fighting oxidative stress: increased resistance of rat cerebellum at weaning upon maternal multinutrient deficiency

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Abstract

Regional Basic Diet is a model of multifactorial malnutrition. Notwithstanding, it contains a low alpha-linolenic/linoleic acid ratio, what favors docosahexanoic acid (DHA) incorporation in the brain, especially in the cerebellum. Considering DHA importance for brain redox balance, we hypothesize in this study that this diet improves the anti-oxidant status of the rat cerebellum compared to the cerebral cortex (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. 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 (~153%) t-SOD activities were respectively detected in these regions compared to control. Moreover, increased CAT activity (~38%) was seen in the cerebellum of malnourished group. 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, a low dietary omega 6/omega 3 ratio can improve 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; Antioxidant; Cerebral cortex; Cerebellum.

Introduction

Maternal protein deficiency imposed on rats a month prior to conception, and during gestation and lactation, has been related to delayed and impaired brain development of the progeny at weaning [1-3]. This type of nutritional insult is also able to induce permanent physiologic modifications in adulthood increasing the probability for developing metabolic and neurodegenerative diseases [2, 4-8]. It has been hypothesized that reduced development in malnourished young animals may be related to the effects of malnutrition on the anti-oxidant defense system [9-11] and epigenetic modifications may be related to long term changes in the life span [12].

In addition to protein contents in the diet, the synthesis and maintenance of several antioxidant systems depends on the quality of lipids, minerals and vitamins that need to be obtained from the diet [13-17]. The vulnerability of the brain to nutritional insults also depends on the developmental stage of each region during the early postnatal days [5]. The natural protracted postnatal development of the cerebellum, is an important feature that makes this region particularly vulnerable to the effects of protein malnutrition [18, 19]. Despite the vulnerability of the cerebellum to deficiency in specific nutrients such as thiamine, copper and vitamin E [20-22], adaptive mechanisms in protein or lipid synthesis and amino acids metabolism have been described in this region during the lactation period or in adulthood [23-25]. Post-mortem analysis of malnourished children with clinical signs of marasmus showed that the phospholipid/DNA ratio in the forebrain and cerebellum was higher than that observed in healthy children [26]. On the other hand, among the different phospholipids, sphingomyelin was found to be selectively decreased in these regions, indicating a special vulnerability of the myelination process [26].

The repercussion of restricted feeding or protein deficient diet on the redox balance of the cerebral cortex (Cx) and cerebellum has been reported [9, 27-31]. Although controversial results have been found in different experimental models, most of these studies demonstrate that several antioxidant systems recover their efficiency in adulthood despite the maintenance of the malnourished status [10, 27-29, 32, 33].

In the present study, we investigated whether the oxidative status of Cx and cerebellum can be differentially affected by an experimental model of severe malnutrition, the Regional Basic Diet (RBD) [34]. This diet produces in the rat a similar condition to that found in clinical signs of marasmus [34]. The RBD is based on data from food consumption surveys from low-income human populations and has been used in several previously published

studies on neural, inflammatory and immune effects of malnutrition [35-39]. In spite of the multinutrient deficiency, including low contents of lipids, a peculiarity of this diet is its low ratio (1.69) of n-6/n-3 essential fatty acids [40]. This ratio favors an appreciable incorporation of the n-3 polyunsaturated docosahexaenoic acid (DHA) in brain regions, especially in the cerebellum and hippocampus when compared to the Cx [40]. Recent evidence has also indicated that RBD–induced malnutrition imposed on mothers during the lactation period is not sufficient to affect lipoperoxidation (LP) levels in the Cx of the offspring at 30-40 days [41]. Therefore, considering that the low n-6/n-3 ratio in the diet and adequate DHA incorporation are able to reduce brain oxidative stress [42], we hypothesized that despite a dietary protein deficiency the cerebellum of young and adult animals would be able to develop a better anti-oxidant response under this nutritional condition than the Cx.

Experimental Procedure

Animals and diets

Adult nulliparous female rats weighing 250–300 g (90 days of age) were divided into two groups according to the diet: a control nourished group (C) fed on a commercial rat chow diet containing 22% of a mixture of animal and soy protein, 50% carbohydrate, 3.5% lipid, 2% ash and 4% minerals (Presence® Purina, Brazil) and a malnourished group (M) fed on a Regional Basic Diet (RBD) containing only 8% protein in a 5:3 proportion of vegetal to animal protein [34] starting 30 days before mating. The composition of RBD is shown in the *Table 1*. According to [40], even containing 1 % lipids, the fatty acid profile of this diet is characterized by higher and lower amount of alpha-linolenic acid (12%) and linoleic acid (8%) respectively, compared to the control diet (3,5% alpha linolenic acid and 15% linoleic acid). Dams were maintained on the control or RBD diets throughout their gestation and lactation periods. At parturition, litter size, total litter weight and mean birth weight of the pups were recorded. Litters containing 10- 13 pups were then reduced to 6 pups each, keeping males and females in a similar proportion and keeping the median weighted animals.

Rat offspring (n = 110) were the object of the present study and only males were used for the experimental assays. All analyses were performed with young rats at weaning (P21; being P0 = day of birth; P21C and P21M for control and malnourished groups respectively) and adult rats at 90 days. After weaning, part of the control group continued to be fed with the commercial chow (P90C) and the malnourished group was subdivided into chronic

malnourished group, continuously fed with RBD (P90M), and the rehabilitated group transferred to commercial chow (P90R). At weaning, male and female pups of both control and malnourished dams animals were sampled randomly from different litters and housed, three per cage, in a room maintained at 22±2 ◦C with 67% relative air humidity. The rats were kept on a 12 h light/dark cycle (lights on 6:00 h). The malnourished groups were separated and kept receiving the same deficient diet *ad libitum*. All procedures were approved by the Ethics Committee in the Use of Animals of Federal University of Pernambuco (UFPE) (process number 23076.010332/2013-47), complying with international standards established by "National Institute of Health Guide for Care and Use of Laboratory Animals".

Tissue processing

On the day of the experiment, the animals were sacrificed by decapitation under isofluorane anesthesia. Pre anesthesia with volatile agents to prevent pain and reflexes is recognized by animal welfare regulatory agency (National Council for the Control and Animal Experimentation, CONCEA). We previously have compared experiments conducted with or without the use of a volatile anesthetic. The biochemical results were not affected [59].

The brains were removed rapidly and carefully, rinsed in ice-cold saline, and dried on filter paper. Each experimental day, cortical and cerebellar tissue of 3 young (P21) and 2 adult (P90) animals was homogenized in a 0.9% (w/v) NaCl solution (1:10) at 4ºC, centrifuged for 10 min at 1000 g at 4ºC. Analysis of lipid peroxidation, estimation of reactive species of oxygen and estimation of nitrite concentration were carried out immediately after centrifugation and the remaining supernatants were stored at -80^{0}C for further biochemical analyses.

Lipoperoxidation (LP) quantification

LP was measured by estimating malondialdehyde (MDA) levels using a thiobarbituric acid (TBA) reaction (TBARS method) according to [60]. In the TBARS test reaction, MDA or MDA- like substances and TBA react to produce a pink pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 80 µL of 8.1% sodium dodecyl sulfate, 600 µL of 20% acetic acid pH 3.5, and 600 µL of 0.8% TBA solutions boiled in water for 60 min to 200 µL of Cx and cerebellum supernatants. After tap water cooling, 600 µL of n-buthanol was added to the sample, centrifuged at 2500g for 10 min and the

organic phase was read at 532 nm using a plate reader (Thermo Scientific, varioskan flash spectral scanning multimode reader). Experiments were carried out in triplicate. The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations of a 1,1,3,3-tetramethoxy propane solution. As the positive control, Cx and cerebellum of control group samples were incubated in a 30 μM sodium nitroprusside (SNP) solution for 45 min before the assay.

Reactive oxygen species (ROS) quantification

The relative levels of ROS generated were monitored by a fluorometric assay using dichlorofluorescein diacetate (DCFH-DA). This non-fluorescent molecule can be passively loaded into whole tissue since it is freely diffusible across cell membranes. Once internalized, esterases cleave the diacetate group. The product is then converted into a highly fluorescent DCF in the presence of ROS [61]. Equal volumes (100 μL) of supernatant and DCFDA reagent were placed in 96 well plates and incubated for 30 min at 37ºC. The fluorescence was measured at 504 nm excitation and 529 nm emission. The values were presented as fluorescence units per milligram of protein.

Estimation of nitrite concentration

Nitrite levels were estimated using the Griess reagent which serves as an indicator of nitric oxide (NO) production as described by [62]. Equal volumes (100 μL) of supernatant and the Griess reagent were placed in 96 well plates and reacted for 10 min at room temperature (~22ºC). The absorbance of the diazonium compound was measured at a wavelength of 540 nm. Samples of control Cx and cerebellum samples were incubated in a 300 μM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for nitrite production. The results were expressed as nmol per mg of protein with reference to a standard curve built with known sodium nitrite concentrations.

Total superoxide dismutase (t-SOD) activity

Assessment of total SOD (t-SOD) enzymatic activity was performed according to [63] at 25◦C. Triplicates of Cx and cerebellum supernatants (60 μL) were previously incubated in a water bath at 37◦C and then added to 920 μL of 0.05% sodium carbonate buffer pH 10.2 in 0.1mM EDTA. The reaction was developed by adding 20 μL of 150 mM epinephrine in 0.05% acetic acid. Absorbance changes per 15 seconds for a total of 2 min, at 480 nm, were measured. One unit of t-SOD was defined as the enzyme amount causing 50% inhibition of

epinephrine oxidation. Tissue t-SOD enzymatic activity was expressed as units per milligram of protein (U/mg protein).

Catalase (CAT) activity

CAT activity was measured according to [64]. Triplicates of Cx and cerebellum supernatants (60 μL) were added to 905μL of sodium phosphate buffer pH 7.0. The reaction was developed by adding 35 μL of 300 mM hydrogen peroxide in sodium phosphate buffer. The rate constant k of H2O2 decomposition under our experimental conditions of temperature (22◦C) and pH (7.0) was determined to be 2.3 by measuring the absorbance changes per 10 sec, for 1.5 min at 240 nm. Enzymatic activity was also expressed as units per milligram of protein (U/mg protein).

Reduced glutathione levels (GSH)

GSH levels were analyzed according to [65]. 450 μL of phosphate buffer 100 mM with EDTA (5 mM) (pH 8.0) were added to 50 μL of the supernatant. 50 μL of this mixture plus 140 μL of phosphate buffer 100 mM plus 10 μL orthophtaldehyde solution (OPT) were placed in 96 well plates and incubated for 20 min at room temperature, protected from light. Absorbance was recorded in a spectrofluorimeter using a wavelength of 350 nm. The results were expressed as μmol per mg of protein with reference to a standard curve built with known GSH concentrations.

Oxidized glutathione levels (GSSG)

GSH levels were analyzed according to [65]. Beforehand, 50 μL of the supernatant were incubated at room temperature with 20 μL of N-ethylmaleimide (NEM) 0.04 M for 30 minutes to interact with GSH present in the tissue. To this mixture, 430 μL of sodium hydroxide (NaOH) 0.1 M was added. 50 μL of this mixture plus 140 μL of NaOH 0,1 M plus 10 μL OPT were placed in the wells of a 96 well plate. This mixture was incubated for 15 min at room temperature and protected from light. The reading was made in a spectrofluorimeter using a wavelength of 350 nm for issue. The results were expressed as μmol per mg of protein with reference to a standard curve built with known GSSG concentrations.

Estimation of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) concentration

A Tris-HCl buffer at pH 7.4 was added to the Cx and cerebellum supernatants. A final volume of 300 μL with a fixed amount of protein (0.2 mg) was used in 96 well plates at room temperature (~22ºC). The reading was made in a spectrofluorimeter using a wavelength of 340 nm where both NADH and NAD(P)H are sensitive. The results were expressed as μmol per mg of protein with reference to a standard curve built with known NADH concentrations.

Statistical analysis

All data sets are expressed as means ± standard deviation (SD). All groups were tested for normality using the Kolmogorov-Smirnov test. All groups showed a normal distribution. Accordingly, statistical significance was evaluated with the unpaired *t*-test or ANOVA followed by post hoc Newman Keuls test, using GraphPad Prism software version 5.0 for Windows (San Diego, CA, USA). Differences were considered to be statistically significant when $p \leq 0.05$.

Results

Body weight

Body weights of young rats during lactation are presented in *Fig. 1A*. During this period, body weights of the malnourished group were significantly smaller when compared to the control group ($p < 0.0001$). Body weights of post weaning animals are presented in *Fig. 1B.* From the 30th to 90th day of life, body weights of the malnourished and rehabilitated groups were still lesser than the control group ($p < 0.0001$). However, average body weight of the rehabilitated group was significantly higher when compared to the malnourished group ($p < 0.0001$).

Please, insert Figure 1 around here

Cerebral cortex and cerebellum weights

Cortical and cerebellar tissue weights of weanling animals at the 21st day were significantly smaller in the malnourished group when compared to the control group ($p = 0.0089$ and $p < 0.0001$, respectively *Fig. 2A and B*). Cortical tissue weight in the adult malnourished group was significantly lesser than the in the control group ($p < 0.0001$), but this weight was recovered in the rehabilitated group when compared to the control group ($p <$

0.01) (*Fig. 2C*)**.** Cerebellar tissue weight in both adult malnourished and rehabilitated groups was still significantly smaller than that found in the control animals ($p < 0.0001$), although a partial recovery was detected in the rehabilitated group when compared to the malnourished one ($p < 0.0001$), *Fig. 2D.*

Please, insert Figure 2 around here

Oxidative status in weanling animals

Lipoperoxidation and reactive oxygen species levels

In young animals, distinct effects were induced by the experimental diet in the two analyzed regions. Figure 3A demonstrates increased (~53%) lipid peroxidation levels, assessed by MDA levels, in the Cx of the malnourished compared to the control group ($p = 0.0369$). However, lower MDA levels (~20% of the control) were detected in the cerebellum of the malnourished group ($p < 0.0001$) as shown in *Fig. 3B.*

Consistent with these results, distinct effects on ROS production was induced by the RBD diet in the two regions analyzed. As seen in *Fig. 3C*, DCF fluorescence was significantly higher in the Cx of the malnourished (~53%) compared to the control group ($p < 0.0001$). An opposite effect was found in the cerebellum **(***Fig. 3D***)**, where a significant decreased DCFDA fluorescence was found in the malnourished group (~72% of the control one; $p < 0.0001$).

Please, insert Figure 3 around here

Nitric oxide and NAD(P)H levels

As shown in *Fig. 4A*, the production of NO, assessed by nitrite levels, was about 37% lower in the Cx of the malnourished group than in the control one ($p = 0.0009$). A more expressive reduction (~76% difference) was observed in the cerebellum of malnourished animals versus the respective control ($p < 0.0001$), *Fig. 4B.*

Lesser NAD(P)H levels, compared to control, were also found in both Cx (20%; $p = 0.0136$) and cerebellum (50%, $p = 0.004$) of young malnourished animals (*Fig. 4D*).

Please, insert Figure 4 around here

Total SOD and CAT enzymatic activities

A significant decrease in t-SOD activity (~35%) was found in the Cx of young malnourished animals when compared to the control group ($p = 0.0013$) **(***Fig. 5A***)**. An opposite effect was detected in the cerebellum **(***Fig. 5B***)**, where an expressive increase (~150 %) in the activity of this enzyme was induced by malnutrition ($p < 0.0001$).

The CAT activity was not modified by malnutrition in the Cx of young animals **(***Fig. 5C***)**. However, in the cerebellum a significant increase in the activity of this enzyme was found in the malnourished group (~38%) compared to the control one ($p = 0.0167$) **(***Fig. 5D***).**

Please, insert Figure 5 around here

GSH and GSSG levels

In the Cx of the young animals, lower GSH levels (~20%) were found in the malnourished versus control group ($p = 0.0063$) but no different was detected for GSSG content nor the GSH/GSSG ratio *(Fig. 6A)*. RBD malnutrition not only modified GSH (~30% lower; $p = 0.0217$) but also the GSSG (~60% lower; $p = 0.0001$) levels in the cerebellum **(***Fig. 6B***)**. Such modifications induced an increased GSH/GSSG ratio in this cerebral region of the malnourished group, ($p = 0.006$).

Please, insert Figure 6 around here

Oxidative status in adult animals

Lipoperoxidation and reactive oxygen species levels

As shown in the *Fig. 7A*, a significant decrease in MDA levels was detected in the Cx of the adult malnourished group when compared to both the control ($p < 0.01$) and the rehabilitated groups ($p < 0.05$). In the cerebellum, MDA levels of the malnourished group were similar to the control. However, lower levels of MDA were found in the rehabilitated group ($p < 0.0001$) compared to both control and malnourished groups *(Fig. 7B).* In agreement with LP findings above described, lower levels of ROS (~45%) were detected in the Cx of malnourished and rehabilitated groups in comparison to the control animals ($p <$

0.0001) (*Fig. 7C*)**.** In the cerebellum, lower levels of ROS (~70% difference) were found in the malnourished group than in the control one ($p < 0.0001$). The rehabilitated group also showed significant lower ROS levels when compared to both control ($p < 0.0001$) and malnourished groups ($p < 0.05$).

NO production, GSH and GSSG levels

The deleterious effect of RBD-induced malnutrition on NO production was maintained in the Cx of adult animals. As can be seen in *Fig. 8A*, nitrite levels were significantly lower (~50%) in the Cx of the malnourished and rehabilitated groups compared to the control one ($p < 0.0001$). Similar findings were obtained in the cerebellum of both malnourished (~44% less) and rehabilitated groups (~60% less) compared to the control group ($p < 0.0001$). In the cerebellum of the rehabilitated group, even lower NO levels were found when compared to the malnourished group *(*$p < 0.05$) **(***Fig. 8B***)**.

Regarding GSH levels in the Cx of adult animals, an increase of ~72% was found in the malnourished group compared to the control one ($p < 0.0001$). Nevertheless, in the rehabilitated group lower GSH levels **(***Fig. 8C***)** were detected, compared to both malnourished ($p < 0.0001$) and control ($p < 0.05$) groups. GSSG levels in the Cx did not differ between malnourished and control groups but were significantly decreased in the rehabilitated group ($p < 0.05$) **(***Fig. 8C***)**. Due to increased GSH levels in the malnourished group, the GSH/GSSG ratio was significantly increased when compared to that obtained in the control and rehabilitated groups ($p < 0.0001$) **(***Fig. 8C***)**.

In the cerebellum of adult animals the increase induced by RBD malnutrition on GSH levels was more expressive (~100 %) when compared to that found in the Cx. This also occurred in the rehabilitated (~200 %) compared to the control group ($p < 0.0001$) **(***Fig. 8D***)**. No difference was detected for GSSG levels in the malnourished when compared to the control group **(***Fig. 8D***).** However, GSSG levels in the rehabilitated group were significantly increased in comparison to the control one ($p < 0.05$) **(***Fig. 8D***)**. Taken together, such modifications provide an increased GSH/GSSH ratio in both malnourished ($p < 0.0001$) and rehabilitated ($p < 0.01$) groups as compared to the control one **(***Fig. 8D***)**.

Discussion

According to previously published data, maternal low-protein or feeding restriction malnutrition can modify the oxidative status of cerebellum and Cx especially in young

progeny [9, 11, 28, 31, 43, 44]. In the present study we hypothesized that chronically multideficient RBD feeding containing low n-6/n-3 ratio could differentially affect the cerebellum redox balance. We created a chronical environment of multideficiency on dams (since 30 days before gestation) and on offspring (until the end of lactation or adulthood). This hypothesis was based on previous evidence demonstrating that under chronic dietary treatment with RBD, the cerebellum of the progeny was able to incorporate a higher amount of DHA in the membrane phospholipids than the Cx of the matched or control groups [40].

Malnutrition-induced changes on the somatic growth

Regarding the animal somatic growth, the results reported in this study support the assumption that RBD induces severe malnutrition in the experimental subjects, especially affecting body growth when compared to brain growth as previously demonstrated [34]. The more expressive weight deficit observed in the cerebellum as compared to the Cx of young malnourished animals is in agreement with previous data from the literature and seems to reinforce the influence of the developmental time scale of this region as being protracted compared to the Cx [18, 23]. Moreover, in the cerebellum, the nutritional rehabilitation was not able to reverse the deleterious effect on the tissue growth, similarly to what has been previously described by using a protein restricted diet or reduced feeding [19, 23]. It has been widely discussed in epidemiological and experimental studies that such permanent effects on the cerebellum structure are potential mechanisms involved in motor and cognitive disabilities detected in adulthood [18, 19]. In the present study the findings demonstrated that permanent modifications in the cerebellum weight of adult RBD malnourished animals did not affect biochemical parameters involved in the anti-oxidative status of this region.

Resilience of cerebellum enzymatic antioxidant systems in malnourished weanling rats

In weanling animals at 21 days, lower levels of ROS and LP were detected in the cerebellum of malnourished group compared to the control, while the opposite effect was found in the Cx. These findings were in part due to a compensatory increase of t-SOD and CAT enzymatic activities observed in the cerebellum of young rats. In the Cx the oxidative insult induced by severe malnutrition was able to impair t-SOD activity and did not change the reactive state of CAT. These findings contrast with what has been reported in neonates when a maternal protein deficiency diet (6% casein) containing adequate lipid levels but n6/n3 ratio ~ 9 was given from 4 weeks before conception until birth [11]. According to these authors, higher LP levels were detected in the cerebellum, Cx and hippocampus of

malnourished neonates. The levels of LP were also increased in the cerebellum of malnourished rat offspring when similar maternal low protein diet (8% casein) was given from conception until the weaning [28]. Although differences between the present study and these previous reports can be influenced by animal strain or period of malnutrition, our present findings for the cerebellum seem to reinforce the hypothesis that a low n6/n3 ratio can favor its antioxidant resource even under a condition of protein deficiency.

NO production was differentially but permanently impaired by RBD-induced malnutrition in the cerebral cortex and cerebellum

Another important point to be considered in the present study is that lower LP levels detected in the cerebellum of weanling malnourished animals may also be associated with the expressive reduction of NO production in this region (~80% difference) relative to the control group or to the modifications found in the Cx (~38% difference). A lower amount of NO in malnourished young animals would reduce the LP status considering the lower probability of this volatile gas to react with free radicals and form peroxynitrite [45]. NO is implicated in numerous brain functions including learning, memory, feeding behavior and synaptic plasticity [46] and has a crucial role in cerebellum development as well as in motor and cognitive functions [47]. In addition, other study has shown that neuronal NOS is quantitatively more abundantly expressed in the cerebellum than in the Cx [48].

Feeding an arginine-, protein- or thiamine-deficient diet during brain growth spurt period impairs constitutive and inducible NO synthesis in a number of neural regions and especially in the cerebellum and thalamus [49-51]. Our findings agree with these previous studies, in particular due to the fact that RBD combines reduced amounts of protein, arginine and thiamine [34]. Nevertheless, the mechanisms involved in the more expressive reduction of NO production in the malnourished cerebellum, compared to the Cx of young animals, are not yet clear. Taken into account that two steps of the NO synthesis require NAD(P)H [52], one could speculate that this differential reduction could be in part related to a greater depletion of this coenzyme that was detected in the RBD-malnourished cerebellum (~50%) compared to that found in the Cx (~20%) of young animals.

The mechanisms involved in the lower levels of NAD(P)H in homogenates of the cerebellum of malnourished weanling animals was not investigated in the present study. However, we cannot discard the possibility of a developmental effect considering the evidence that pre and postnatal malnutrition can induce a delayed expression of these coenzymes in the progeny between postnatal days 8 and 22 [53]. According to this latter

study, retarded enzymatic development seemed to parallel the retarded morphological development of cerebellum.

It is noteworthy that lower levels of NO in both Cx and cerebellum of malnourished young animals were not reversed by nutritional rehabilitation from weaning until 90 days of life, indicating permanent effects of this dietary treatment during the brain growth spurt period. These findings suggest potential epigenetic effects induced by RBD. Multinutrient deficiency has been associated with dysfunctional immune responses involving NO production [54]. Future studies could show how the immune response of cerebellum and Cx can be affected by this nutritional condition.

Relative importance of GSH/GSSG ratio in the redox balance of cerebral cortex and cerebellum at weaning and especially in adulthood

Another important finding detected in the present study is related to the redox balance defined by GSH/ GSSG ratio in the Cx and cerebellum of young and adult animals. Lesser GSH concentration was found in both regions at weaning but these changes did not modify the cortical GSH/GSSG. In the cerebellum, a higher GSH/GSSG ratio was found especially due to a more expressive reduction (-60%) in the GSSG content. Analyzing the concentration of GSH in the cerebral cortex and cerebellum during the ontogeny of nourished rats, [55] demonstrated that a peak occurs at the beginning of postnatal week 2, coincident with a period of intense synaptogenesis. [32], reported a similar ontogenetic profile of GSH concentration during the two first postnatal weeks in the Cx of both malnourished and control rats, although lower GSH levels were induced by malnutrition on postnatal days 2 and 15. According to these authors, by the 60^{th} postnatal day a full recovery in GSH content had occurred in the same rats, despite the maintenance of the animals on a protein deficient diet. Thus, even considering differences in the nutritional approach adopted in our study, the findings regarding GSH/GSSG levels in the Cx and cerebellum of our malnourished young group add new information that despite a lesser GSH synthesis at weaning, the redox balance involving this antioxidant system was not impaired in these regions.

In the central nervous system, astrocytes play a central role in the synthesis, storage and release of glutathione [56, 57] while neurons need to take up extracellular cysteine from astrocytes for GSH synthesis [57]. Therefore, it is possible that a reactive state of astrocytes involving GSH synthesis can be involved in the positive redox balance observed in the Cx of malnourished adult rats characterized by an meaningful increase in the GSH/GSSG ratio (~75%) when compared to the control and rehabilitated animals. A stronger increase in GSH

levels was observed in the cerebellum of malnourished (~100%) and rehabilitated adult animals (~200%) when compared to the control ones. Usually GSH levels can be negatively influenced by NO production [58]. Considering that NO synthesis was especially impaired in the cerebellum of young and adult malnourished and rehabilitated animals, we are tempted to speculate that these changes may influence the compensatory mechanisms involving GSH synthesis.

Conclusion

The present findings show for the first time that a maternal multideficient diet containing a low n3/n6 ratio can differentially affect the oxidative status of Cx and cerebellum of weanling progeny. The relative resilience of the cerebellum is in part due to the ability of its enzymatic and non-enzymatic antioxidant defense systems to keep an adequate redox balance early in life and during adulthood. On the other hand, RBD induced malnutrition was able to permanently impair NO production in both brain regions. The data also suggested that an astroglial reactivity can be a potential mechanism involved in the redox balance recovery detected in the cerebral cortex of adult animals.

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LEGENDS

Figure 1. Body weights of control and malnourished rats on 2, 6, 12, 16 and 21 postnatal days, during lactation (A). Body weights of control, malnourished and rehabilitated adult rats on 30, 60, and 90 days of life (B). * indicates $p < 0.0001$ compared to control group, # indicates $p < 0.0001$ compared to malnourished group (Paried *t*-test or ANOVA and post hoc Newman-Keuls test).

Figure 2. Cortical and cerebellar tissue weights of young rats (A and B) and adult rats (C and D). Values represent mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared to control group, # $p < 0.05$, ## $P < 0.01$, ### $P < 0.0001$ compared to malnourished group (Paried *t*-test or ANOVA and post hoc Newman-Keuls test). n is the number of animals analyzed.

Figure 3. MDA levels in the pool of cerebral cortex (A) and cerebellum (B) of young rats. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control. ROS levels in the pool of cerebral cortex (C) and cerebellum (D) of young rats. Values represent mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ compared to control group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.0001$ compared to malnourished group (Unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

Figure 4. Nitrite concentration as an indicator of NO production in the pool of cerebral cortex (A) and cerebellum (B) from young rats. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control. NAD(P)H levels in the pool of cerebral cortex (C) and cerebellum (D) of young rats Values represent mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ compared to control group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.0001$ compared to malnourished group (Unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

Figure 5. t-SOD activities in the cerebral cortex (A) and cerebellum (B) of young rats. CAT activities in the cerebral cortex (C) and cerebellum (D) of the same animals Values represent mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** indicates $P < 0.0001$ compared to

control group (unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

Figure 6. GSH, GSSG levels and GSH/GSSG ratio in cerebral cortex (A) and cerebellum (B) of young rats. The left ordinate scale indicates GSH or GSSG concentrations, and the right ordinate scale indicate the GSH/GSSG ratio. Values represent mean ± standard deviation. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001 compared to control group, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.0001 compared to malnourished group (Unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

Figure 7. MDA levels in the cerebral cortex (A) and cerebellum (B) of adult rats. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control. ROS levels in the cerebral cortex (C) and cerebellum (D) of adult rats. Values represent mean ± standard deviation. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001 compared to control group, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.0001 compared to malnourished group (Unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

Figure 8. Nitrite concentration as an indicator of NO production in the cerebral cortex (A) and cerebellum (B) of adult rats. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control. GSH, GSSG levels and GSH/GSSG ratio in cerebral cortex (C) and cerebellum (D) of adult rats. The left ordinate scale indicates GSH or GSSG concentrations, and the right ordinate scale indicates the GSH/GSSG ratio. Values represent mean ± standard deviation. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001 compared to control group, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.0001 compared to malnourished group (Unparied *t*-test or ANOVA followed by Newman Keuls test as post hoc). n is the number of animals analyzed.

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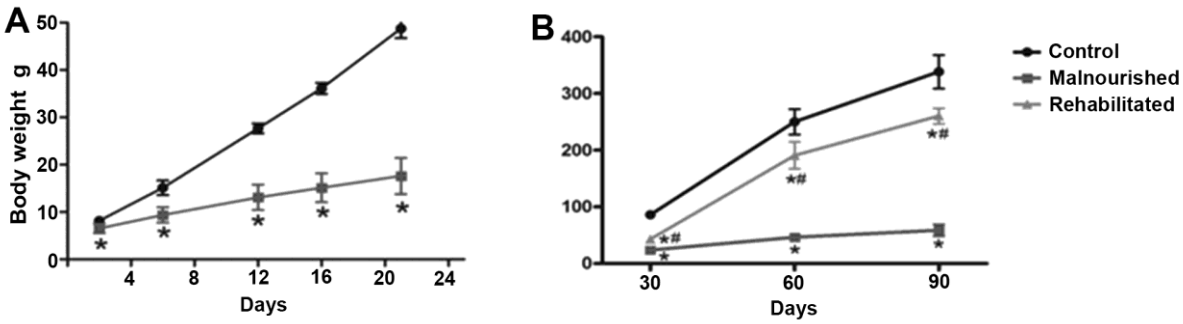
Figures

Figure 1



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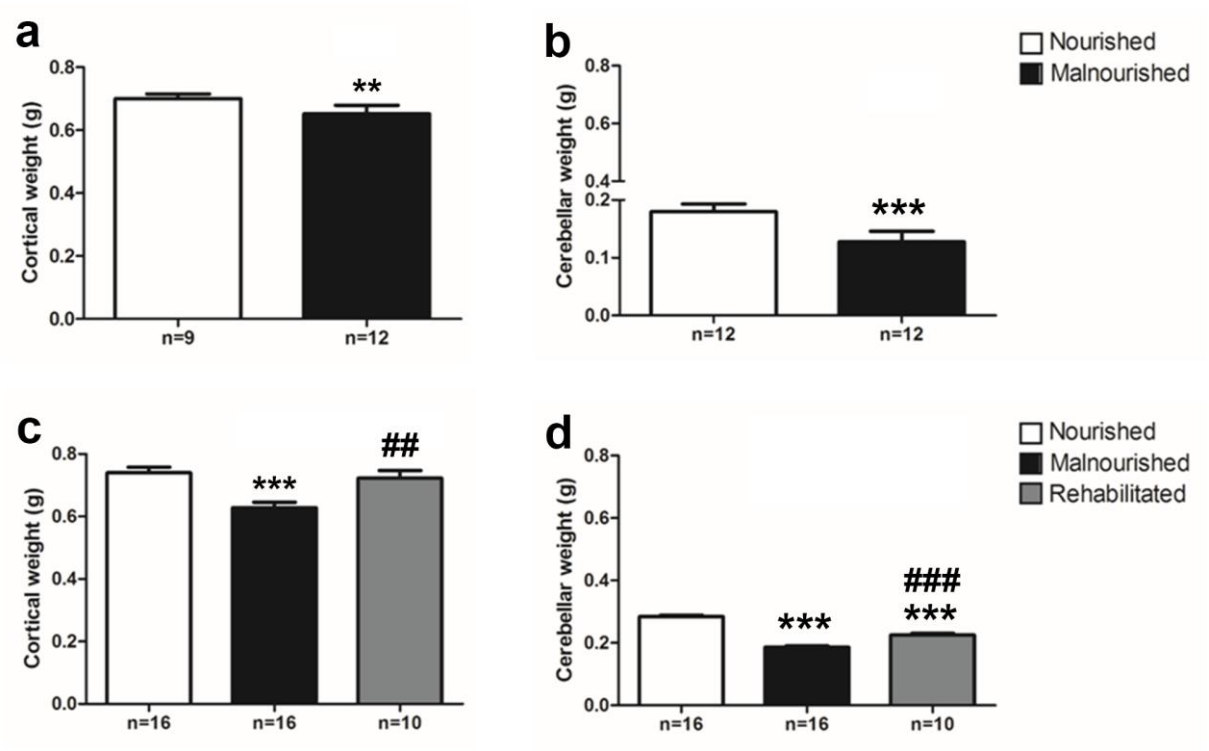

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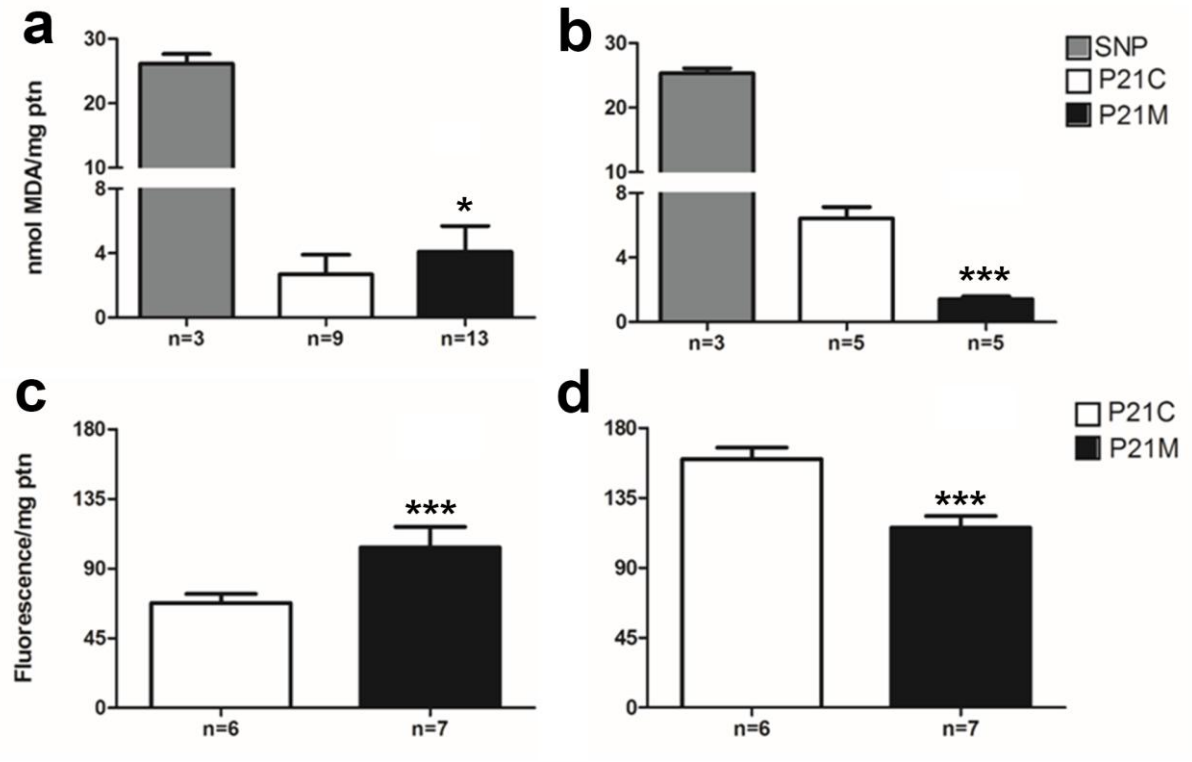

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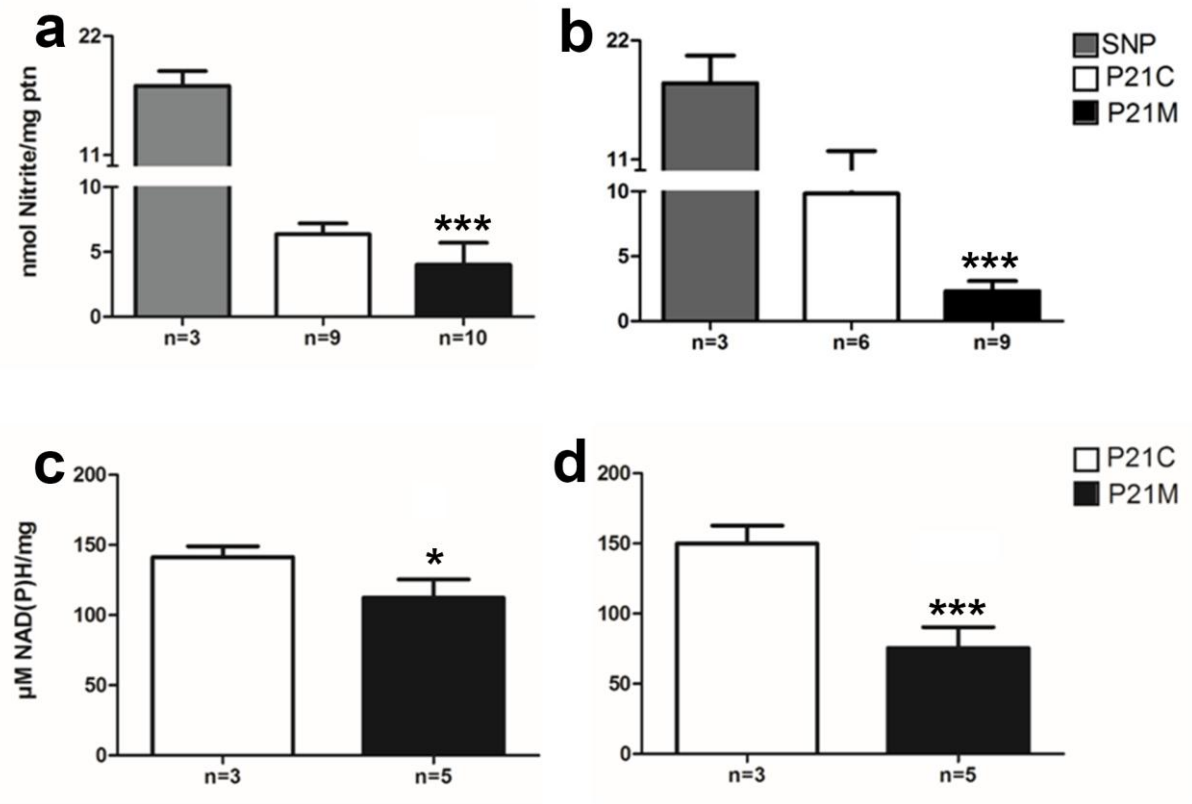

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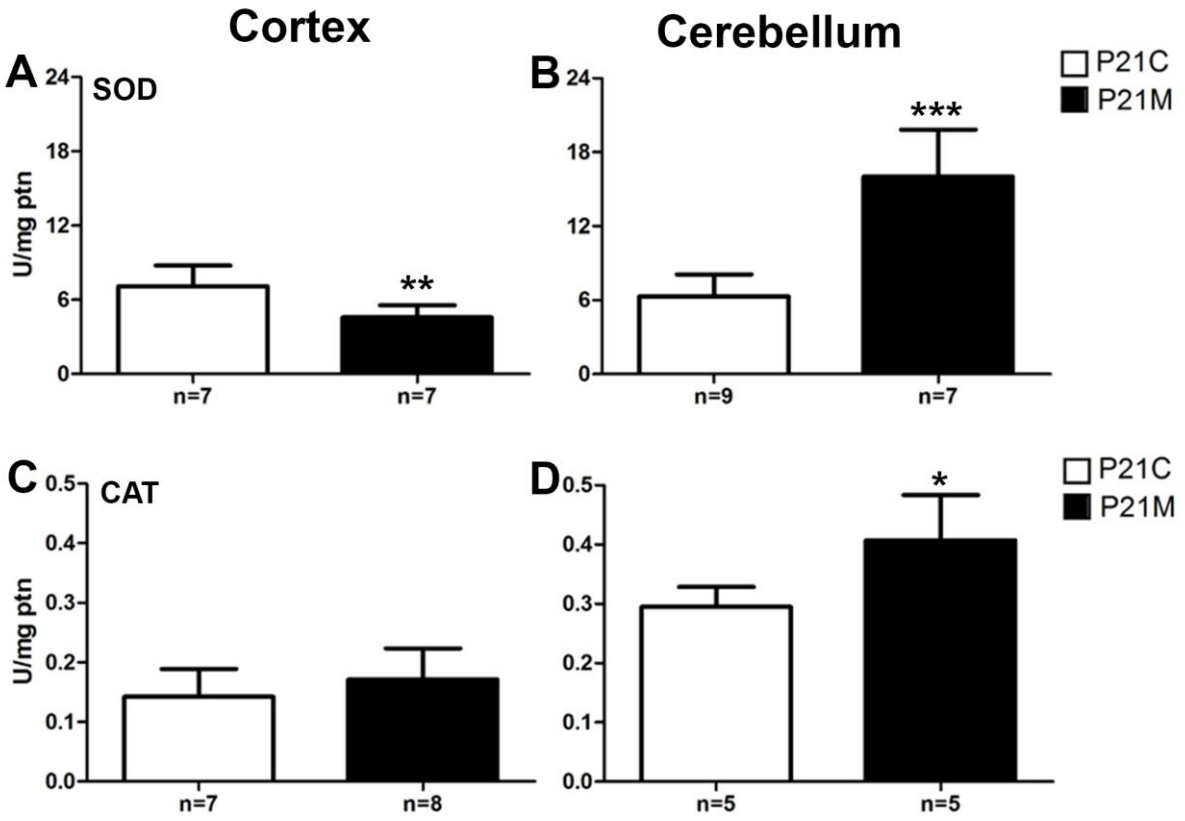

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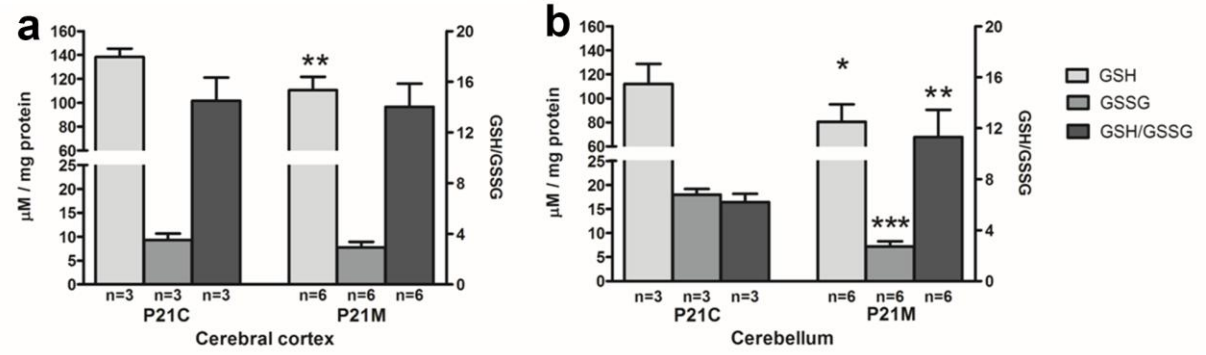

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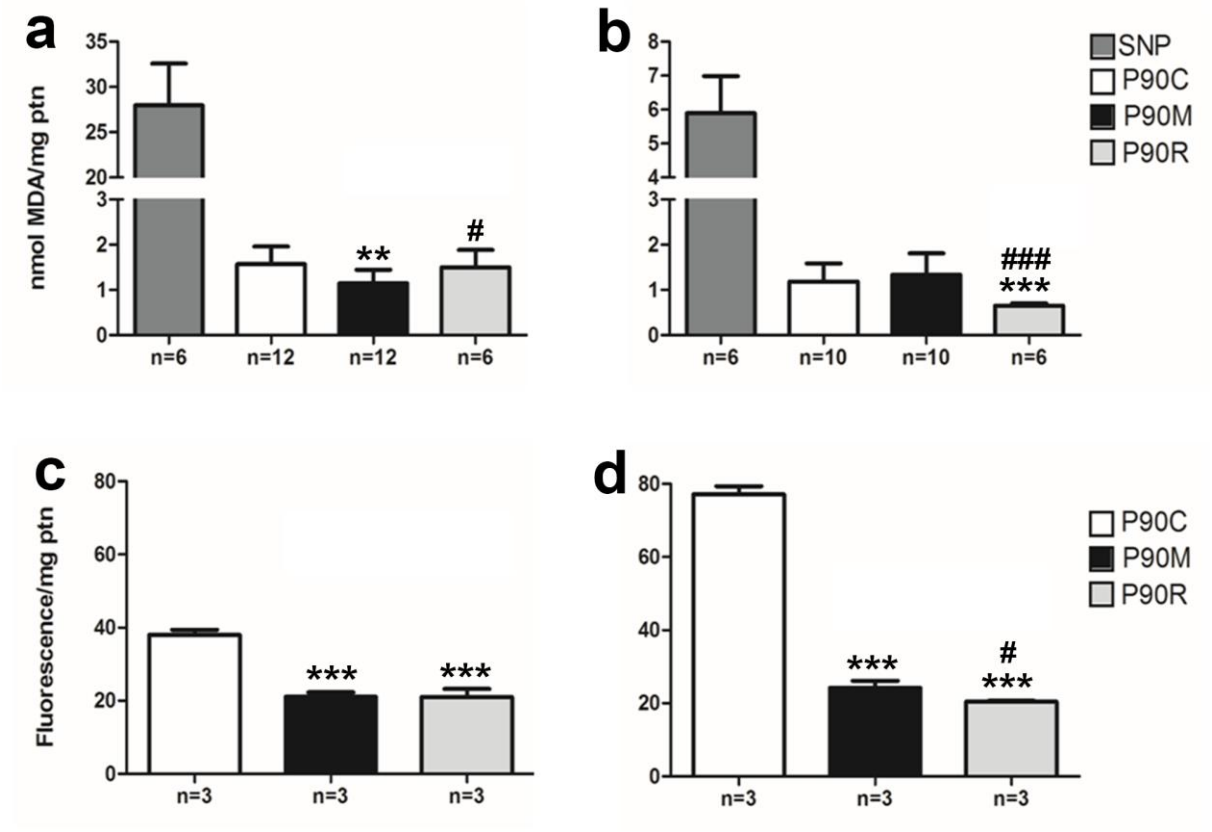

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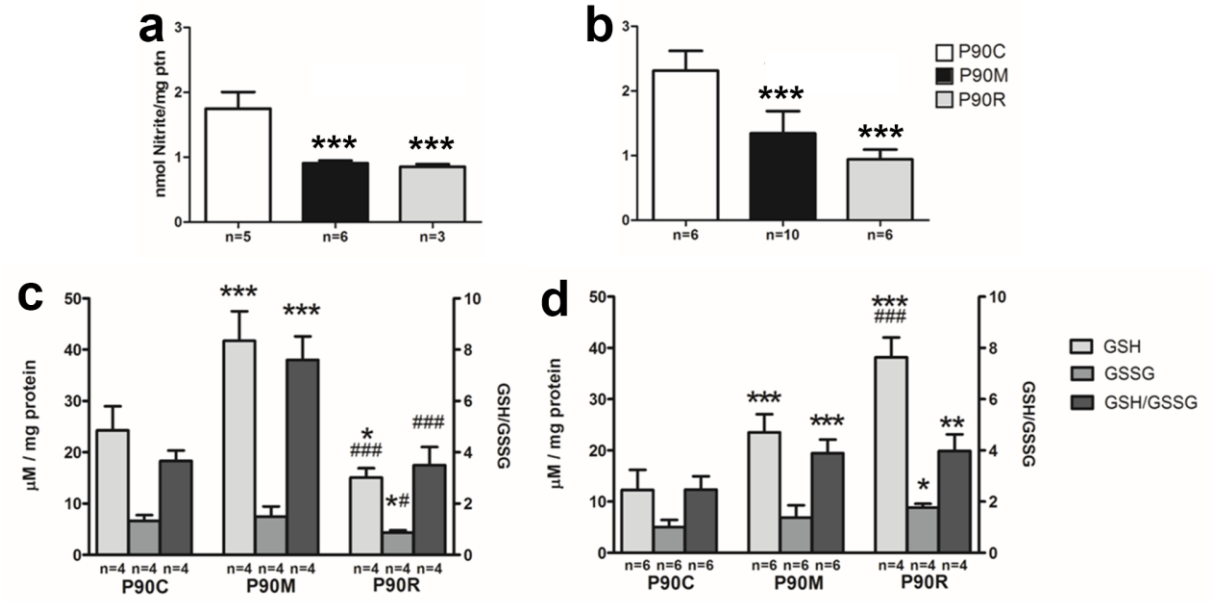

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ANEXOS

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

ANEXO 2. Trabalho apresentado na forma de pôster ao congresso da Sociedade Brasileira de Neurociências e Comportamento (SBNeC 2014).

ANEXO 3. Manuscrito a ser submetido.

ANEXO 4. Capítulo de livro submetido. (Rede Glial)

ANEXO 5. Manuscrito em colaboração a ser submetido**.**

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

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Recife, 06 de junho de 2013.

Ofício nº 576/13

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª Belmira Lara da Silveira Andrade Costa**
Universidade Federal de Pernambuco
Departamento de Fisiologia e Farmacologia
Processo nº 23076.010332/2013-47

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 multifatorial sobre a capacidade antioxidante do córtex cerebral em ratos jovens e adultos".**

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 e prole neonata e adulta; Peso: 250-300g(adultos) e 8 a 62g (neonatos); sexo: machos (progenitores: 4; prole: 72) e fêmeas (progenitoras: 10; prole: 72); nº total de animais: 158 ratos (14 progenitores e prole 144)

Atenciosamente,

Profª Tania Rieger
Presidente do CEUA/CCB-UFPE
SIAPE 2306924

CCB: Integrar para desenvolver

SBNeC

XXXVIII Reunião Anual da SBNeC

10 a 13 de Setembro de 2014, Búzios - RJ

Certificamos que

O resumo 03.023, DIETA MATERNA COM ELEVADOS NÍVEIS DE ÔMEGA-3 E DEFICIÊNCIA PROTÉICA RETARDA RESPOSTA ANTIOXIDANTE DO CÓRTEX CEREBRAL MAS NÃO DO CEREBELO. e RICIELLE LOPES AUGUSTO, ALINNY ROSENDO ISAAC, RENATA VIRGINIA CAVALCANTI SANTOS, CATARINA GONÇALVES PIMENTEL, EMERSON ALEXANDRE NEVES DA SILVA, VIVALDO MOURA NETO, BELMIRA LARA DA SILVEIRA ANDRADE DA COSTA foi apresentado em forma de painel na

XXXVIII Reunião Anual da Sociedade Brasileira de Neurociências e Comportamento - SBNeC, realizada no Centro de Convenções do Hotel Atlântico Búzios em Armação de Búzios - Rio de Janeiro, de 10 a 13 de setembro de 2014.

Cecília Hedin Pereira
Presidente da SBNeC

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].

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 contains 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) 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 RBD 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 (process number #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 were 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.

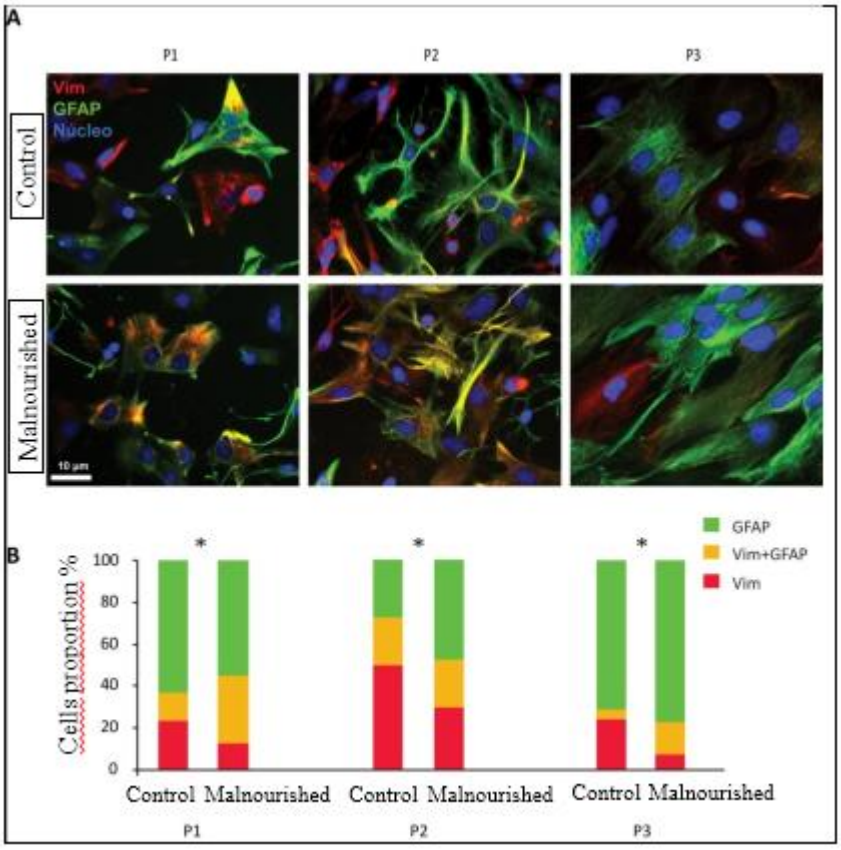


Figure 1. Effects of maternal malnutrition on regulation of intermediate filaments expression in primary cortical astrocytes cultures. (A) Representative photomicrographs of vimentin (red) and GFAP (green) expressions in astrocytes, over time. (B) Graphic shows proportion of three astrocyte populations 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.

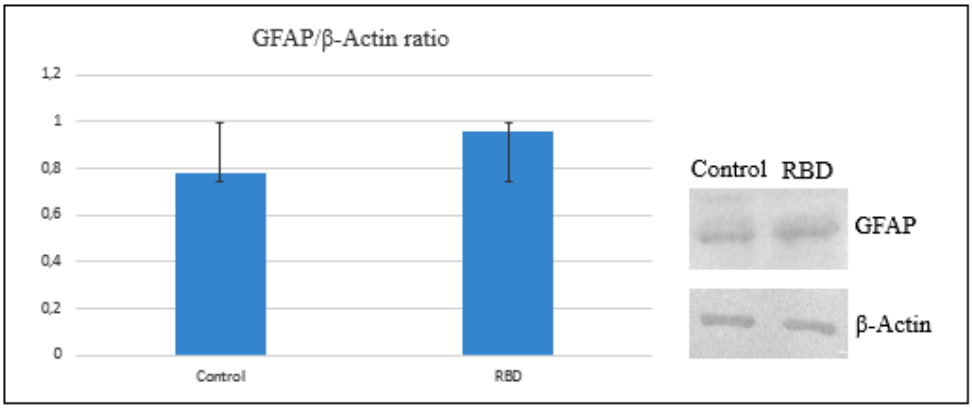


Figure 2. Western blotting to GFAP. Samples (30µg/lane) were separated by SDS-PAGE under reducing conditions followed by electrotransfer onto nitrocellulose membranes for immunodetection of GFAP (A) and Vimentin (B) in 13div.

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.

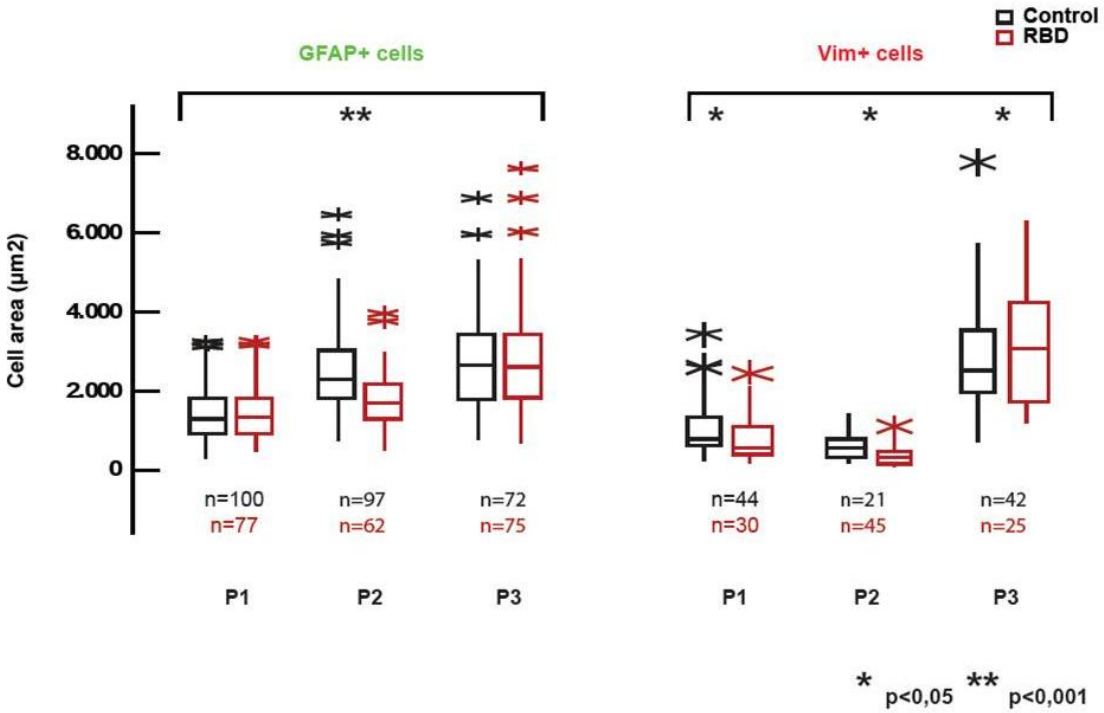


Figure 3: Morphometric analysis of astrocytes soma area immunoreactive to GFAP or Vimentin maintained in cultures for 13 (P1) 23 (P2) or 33 (P3) days in vitro.

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).

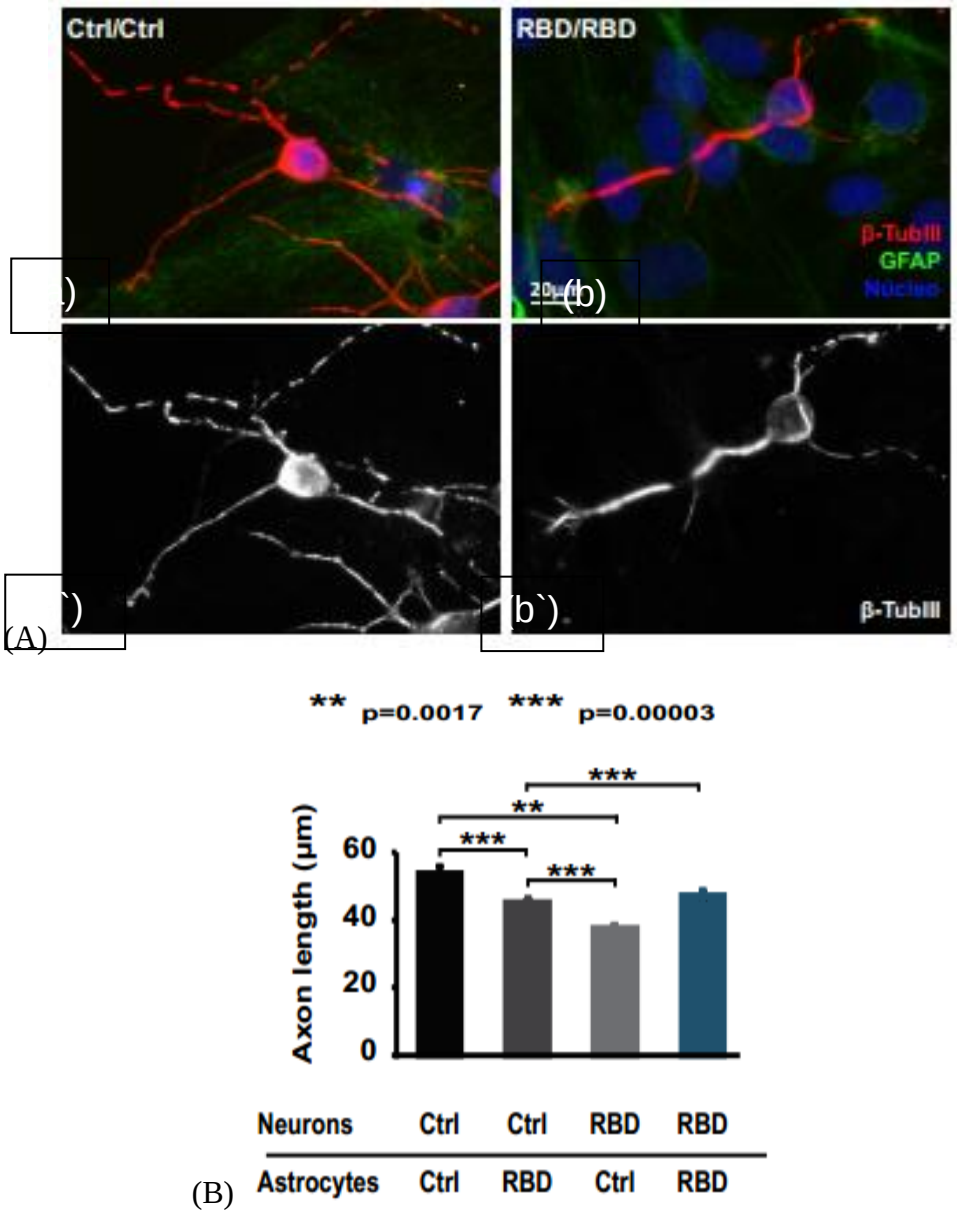
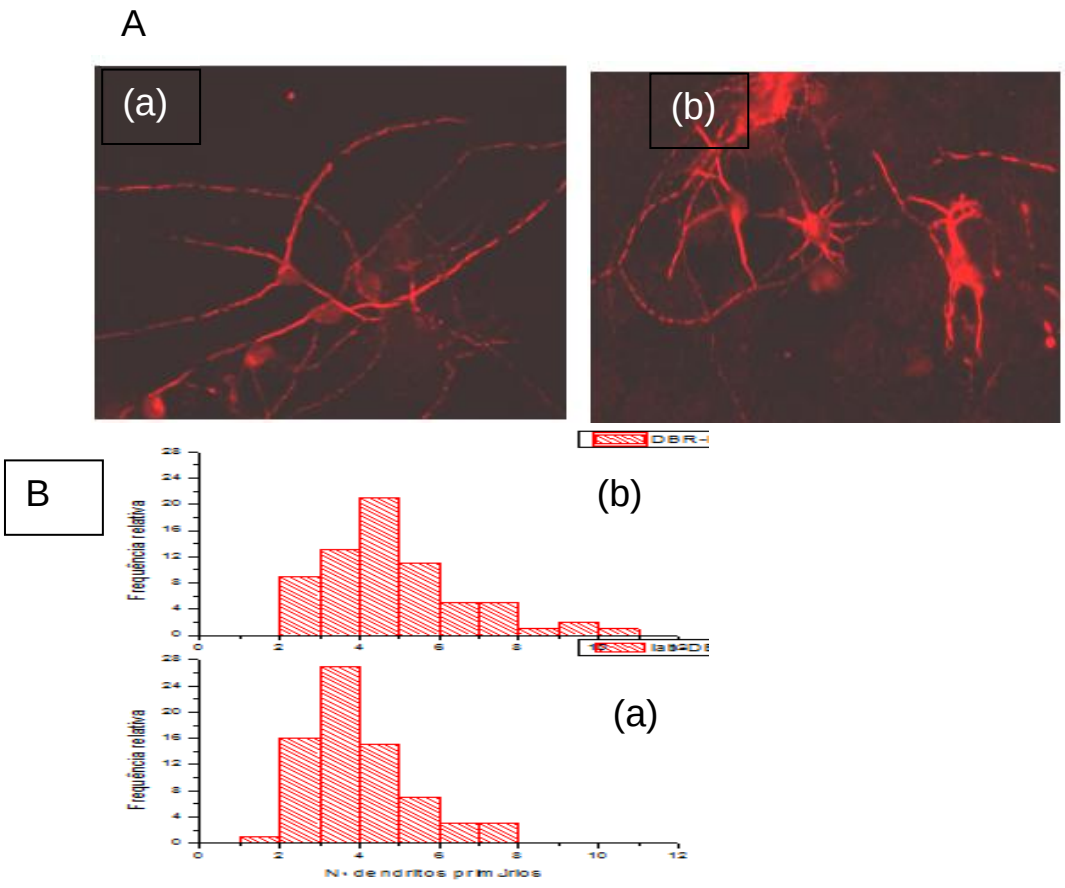


Fig. 4 Analysis of axonal length in co-cultures of cortical neurons and astrocytes. (A) Ilustrative photomicrographs of primary co-cultures of control and neurons astrocytes (a and a', Control/Control) as well as malnourished neurons and astrocytes (b and b', Malnour/Malnour). (a and b) show overlap 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 of the 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.



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

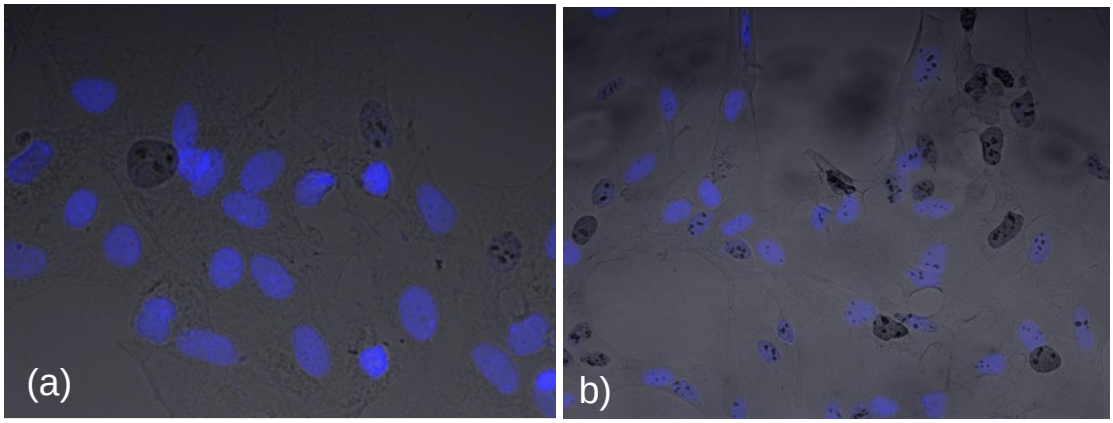


Figure 6. Cerebral cortex proliferating astrocytes identified by immunoreactivity to Ki67. (a) Representative field in astrocyte primary culture of control group (20X magnification) and (b) in culture of malnourished group (10X magnification). Arrowheads indicate Ki67 immunoreactive nuclei of proliferating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

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ANEXO 4.

Capítulo de livro submetido à Rede Glial Luso Brasileira e que será editado pela Universidade de Coimbra em 2015

RESTRIÇÃO DIETÉTICA EM ÁCIDOS GRAXOS ÔMEGA-3 INDUZ REATIVIDADE FENOTÍPICA NA MICROGLIA DA *SUBSTANTIA NIGRA* DE RATOS

TITLE: Nutritional restriction of omega-3 fatty acids induces phenotypic plasticity in the microglia of rat *substantia nigra*

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Resumo

Deficiência crônica em ácido docosahexaenóico (DHA) induz neurodegeneração no sistema nigro-estriatal de ratos. Para investigar se atividade microglial contribui para este efeito, o presente estudo analisou alterações fenotípicas indicativas de seu estado reativo na *substantia nigra*. Ratos foram mantidos a partir da concepção em dietas contendo níveis adequados ou deficientes em ácido alfa-linolenico e analisados na idade adulta. Os resultados evidenciaram alterações morfológicas e marcação para a lectina obtida do Lycopersicum esculentum na microglia do grupo deficiente em DHA em comparação ao controle. Os achados sugerem que reatividade microglial pode contribuir para a neurodegeneração induzida pela deficiência em DHA na *substantia nigra*.

Abstract

Chronic docosahexanoic acid (DHA) deficiency induces neurodegeneration in the rat nigrostriatal system. To investigate whether microglia activity in the *substantia nigra* contributes to this effect, this study analyzed phenotypic changes indicative of their reactive state. Rats were raised from conception in diets containing adequate or deficient levels of alpha-linolenic acid and analyzed in adulthood. The results showed morphological changes and labeling with lectin obtained from *Lycopersicum esculentum* in the microglia of deficient group in comparison to the control. The findings suggest that microglial reactivity can contribute to *substantia nigra* neurodegeneration induced by DHA deficiency.

INTRODUÇÃO

O ácido graxo polinsaturado docosahexaenoico (DHA) da família ômega-3 e seus metabólitos desempenham funções essenciais durante o desenvolvimento do sistema nervoso (Innis, 2007). Além de constituinte estrutural das membranas, atua como sinalizador de eventos moleculares capazes de modular mecanismos epigenéticos (Goustard-Langelier *et al*., 2013), interações intra e inter-celulares e a transcrição gênica (Yavin, 2006). Níveis balanceados de DHA e do ácido araquidônico (AA) da família ômega-6, são necessários ao adequado metabolismo cerebral, em parte pelas ações opostas que estes podem exercer sobre mecanismos inflamatórios (Schimidtz *et al.,* 2008) envolvidos em algumas doenças degenerativas (Bousquet *et al.,* 2011). Na doença de Parkinson, por exemplo, a neurodegeneração da substantia nigra (SN) tem sido associada, dentre outros fatores, ao estado inflamatório decorrente da reatividade microglial (Croisier et al., 2005; Sanchez-Guajardo et al., 2013). Neste contexto a suplementação dietética do DHA tem sido indicada como uma alternativa nutracêutica por aumentar a síntese de eicosanóides anti-inflamatórios (Calder, 2012) e por exercer ações inibitórias sobre a expressão de fatores de transcrição gênica envolvidos na síntese de citocinas pro-inflamatórias (Ji *et al*, 2012).

Evidências recentes indicam que desequilíbrio nos níveis de ômega-3 e 6 na dieta materna induzem reatividade microglial no estriado (Kuperstein *et al.,* 2008; razão ω6/ω3=173) e alteram fenótipo e motilidade da microglia no hipocampo, durante o aleitamento (Madore *et al.,* 2014; razão ω6/ω3=280). Tais resultados, no entanto, não refletem apenas o efeito de uma deficiência de DHA uma vez que níveis de AA foram aumentados. Utilizando um modelo de restrição dietética crônica em ácidos graxos essenciais, capaz de induzir depleção de DHA (~65%) sem afetar o AA encefálico, evidenciamos neurodegeneração na *substantia nigra* (SN) associada a elevados níveis de óxido nítrico e lipoperoxidação, além de redução na expressão de BDNF em animais adultos (Cardoso et al., 2012, 2013).

Neste estudo, considerando as evidências de modulação do DHA na sinalização neuroinflamatória, hipotetizamos que a deficiência em DHA por si só, na ausência de elevados níveis de AA induz reatividade fenotípica na microglia da SN como um dos mecanismos envolvidos na neurodegeneração observada neste núcleo (Cardoso et al., 2013).

MÉTODOS UTILIZADOS

Ratos Wistar foram subdivididos em dois grupos de acordo com as dietas, as quais diferiram quanto à composição adequada (controle) ou reduzida (deficiente) em ácidos linoleico e alfa-linolênico (Cardoso et al., 2013). Tal dieta foi mantida por duas gerações e os encéfalos de animais machos (90-100 dias) da segunda geração foram analisados. Após perfusão transcardíaca dos animais, secções parassagitais (45 µm) dos encéfalos foram obtidas em criostato (Leica). Para visualização da microglia foram utilizados o anticorpo

monoclonal anti-IBA-1 feito em coelho (Wako Chemical) e a lectina do tomate *Lycopersicum esculentum (*Sigma-Aldrich*)* sendo a imunomarcação obtida pelo complexo avidina-biotina-peroxidase (Vector Labs) e o cromógeno diaminobenzidina. Análise do fenótipo morfológico da microglia foi realizada em 3 animais por grupo, nos limites citoarquitetônicos da SN (nível medial). Três secções parassagitais por animal, foram analisadas. Imagens digitalizadas foram obtidas (microscópio Nikon DS-Fi1; objetiva 100x/1.25) e o programa Image J, 1.45s, NIH, USA utilizado para análise morfométrica.

RESULTADOS

O painel apresentado na figura 1 ilustra imagens de regiões da SN de 2 animais representativos dos grupos controle ou deficiente em ômega-3. Em ambos os grupos as células da microglia imunorreativas à proteína IBA-1, encontram-se uniformemente distribuídas ao longo deste núcleo. Porém, é possível detectar que no grupo deficiente várias células da microglia apresentam uma imunorreatividade mais intensa caracterizada pelo espessamento do corpo celular e prolongamentos, (Fig 1C e 1D) quando comparadas às observadas no grupo controle (fig 1A e 1B). Análise quantitativa de parâmetros morfométricos realizada ao nível medial da SN evidenciou um aumento significativo ($p< 0.0001$; Mann Whitney test) na área do soma (mediana 62.12 μm^2; min=24.16; max:125.33) e na forma elipsoidal dada pela diferença entre o diâmetro máximo e mínimo (mediana 5.281 µm; min=1.03; Max=8.51) da microglia do grupo deficiente (n=318) comparado ao grupo controle (medianas 44.27 μm^2 [17.63-86.50 μm^2] e 3.92 µm [1.06-8.70 µm] n= 300).

Além das alterações morfológicas observadas nas células do grupo deficiente foi também detectada imunomarcação para a lectina do tomate *lycopersicum esculentum*, que detecta microglia em diferentes estágios de reatividade e neuroinflamação (Pelaez et al., 1999; Borner et. Al., 2011). As figuras 1E e 1F ilustram respectivamente, imagens representativas da SN de animais dos dois grupos, evidenciando marcação da lectina em vasos, no grupo controle e em vasos e células da microglia no grupo deficiente em DHA.

DISCUSSÃO E CONCLUSÕES GERAIS

Modificações na forma e dimensão do corpo celular da microglia têm sido correlacionadas com os diferentes estágios de atividade que a mesma apresenta em condições de neuroinflamação (Torres-Platas et al 2014). Neste estudo evidenciamos que uma deficiência específica em DHA, sem elevação dos níveis de AA, é capaz de promover alterações fenotípicas na microglia da SN indicativas de um estado reativo. Os resultados reforçam a idéia de um efeito modulatório do DHA sobre a função da microglia (Ji et al 2012; Pettit et al 2013) e enfatizam a importância do aporte adequado deste ácido graxo para minimizar condições neurodegenerativas.

***Uma a Três Conclusões em bullet point**

Deficiência em ômega-3 aumenta a vulnerabilidade da substantia nigra à neuroinflamação

Deficiência em ômega-3 per se induz reatividade microglial na substantia nigra

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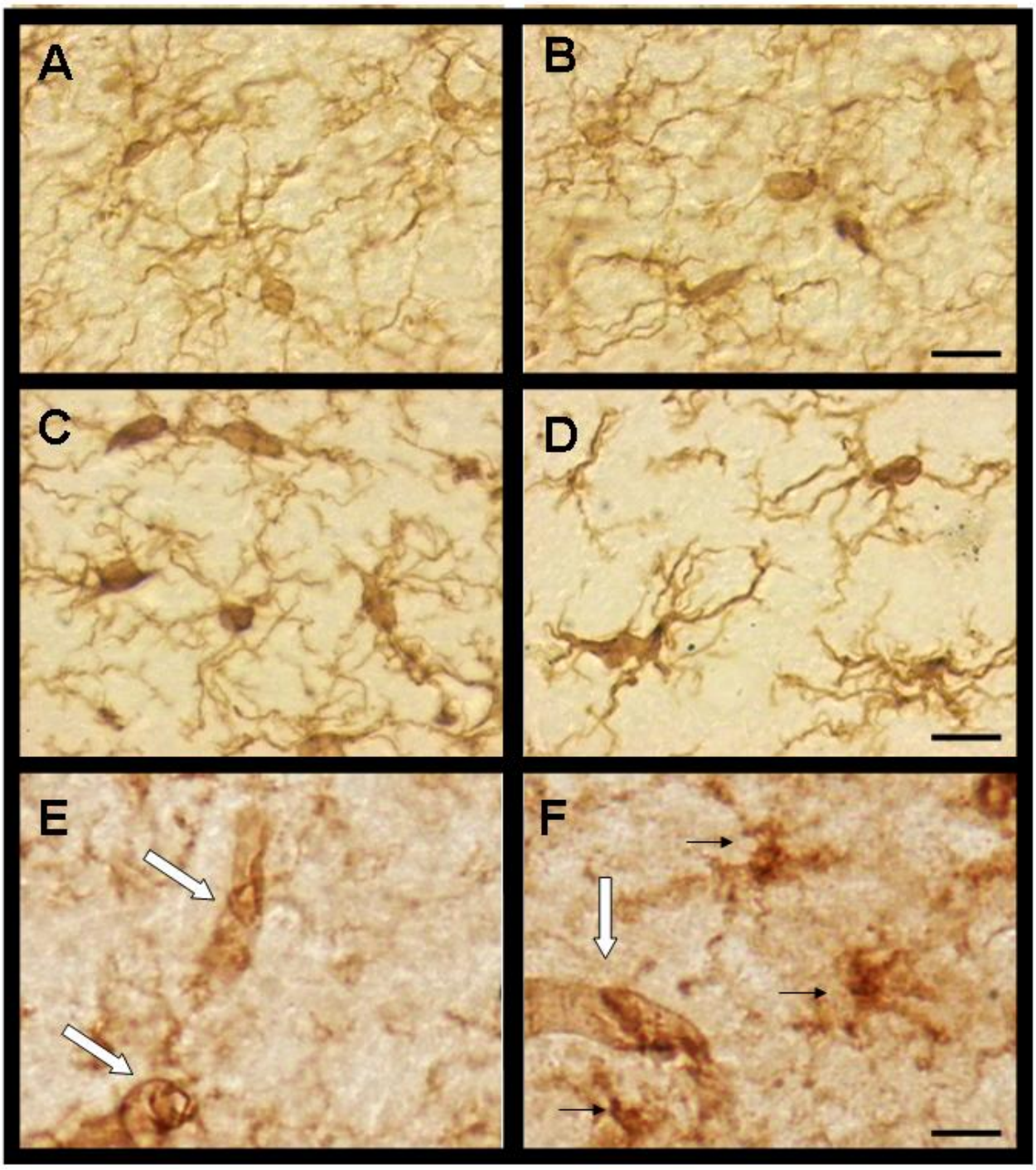
Legenda da Figura

Figura 1: Alterações fenotípicas induzidas pela deficiência em DHA na microglia da substância negra, evidenciada pela imunorreatividade à proteína IBA-1(A e B no grupo controle) (C e D no grupo deficiente) e à lectina do tomate *Lycopersicum esculentum (E*, controle e F, deficiente*)*. Observar alterações na dimensão e forma do corpo e prolongamentos celulares. Setas brancas em E e F indicam marcação de vasos e setas pretas marcação de microglia reativa. Escala = 10 µm.


A
B
C
D
E
F

ANEXO 5.

Artigo em colaboração a ser submetido à Brain Behavior Research

NEUROPROTECTIVE EFFECT OF ANACARDIC ACIDS ON OXIDATIVE STRESS INDUCED BY PARKINSON´S DISEASE MODEL

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Running title: Anacardic acid induces neuroprotection in Parkinson´s disease model

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ABSTRACT

BACKGROUND:

Anacardic acids (AA) are obtained from cashew nut liquid of *Anacardium occidentale*. Evidence *in vitro* and *in vivo* has shown its low toxicity, anti-inflammatory and antioxidant effects, suggesting its potential therapeutic use. Nevertheless, none of these studies were carried out in order to test the antioxidant effects of AA systemic administration on the central nervous system. The present study hypothesized that AA can exert neuroprotection on the cerebral cortex and nigrostriatal system against oxidative stress induced by rotenone.

METHODS: Adult rats were treated by gavage with AA (1-100 mg/kg) or vehicle 1h before rotenone (3 mg/kg; s.c.) for 5 consecutive days. Behavioral parameters were analyzed using open field, rotarod tests and elevated T-maze tests. Signals of neurodegeneration in the substantia nigra (SN) as well as lipoperoxidation, in the nigrostriatal system and cerebral cortex were also evaluated.

RESULTS: In the group treated with AA the rotenone-induced lipoperoxidation was reversed starting with the lowest dose used (1 mg/kg/day). Increased activity of total superoxide dismutase enzyme was detected using 25-100 mg/kg AA in all regions analyzed. The impairment in behavioral parameters induced by rotenone was reversed in animals treated with AA from 10 mg/kg. This flavonoid also blunted SN neurodegeneration signals provoked by rotenone.

CONCLUSION: Oral administration of AA prevented rotenone-induced oxidative stress in the brain, in part due to its modulatory action on superoxide dismutase enzyme, reducing neurodegeneration in the nigrostriatal system.

GENERAL SIGNIFICANCE: Antioxidant effect and low toxicity of AA open new perspectives on potential systemic therapeutic use of this flavonoid in Parkinson´s disease.

Keywords: Neuroprotection, rotenone, lipid peroxidation, superoxide dismutase, substantia nigra, fluorojade C.