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DIORGINIS JOSÉ SOARES FERREIRA

DISFUNÇÃO MITOCONDRIAL: Estudo sobre a interação entre a restrição proteica materna e o desequilíbrio oxidativo no tronco encefálico da prole

Recife – PE, Brasil

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DIORGINIS JOSÉ SOARES FERREIRA

DISFUNÇÃO MITOCONDRIAL: ESTUDO SOBRE A INTERAÇÃO ENTRE A RESTRIÇÃO PROTEICA MATERNA E O DESEQUILÍBRIO OXIDATIVO NO TRONCO ENCEFÁLICO

Tese apresentada ao Programa de Pós-Graduação em Neuropsiquiatria e Ciências do Comportamento da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Neurociências.

Aprovada em: 25/07/2017.

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Este trabalho foi desenvolvido no Laboratório de Bioquímica Geral, Molecular e do Exercício na Universidade Federal de Pernambuco no Centro Acadêmico de Vitória – (UFPE – CAV)

Dedico este trabalho às minhas tias, meu irmão e minha mãe, pois sem eles nada conseguiria.

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RESUMO

Extensivos estudos vêm mostrando a influência de estímulos ambientais durante as fases críticas de desenvolvimento do sistema nervoso central no surgimento/progressão de doenças na vida adulta, incluindo as cardiovasculares. Uma das hipóteses está relacionada a modificação de padrões moleculares e celulares na tentativa de preparar o indivíduo ao ambiente que será exposto posteriormente (resposta adaptativa preditiva). Nesse contexto, nosso laboratório tem voltado seus esforços em entender como a restrição proteica materna modula o equilíbrio oxidativo da prole em diferentes fases da vida. No sistema nervoso central, o tronco encefálico tem se destacado devido sua importância na regulação do controle central cardiovascular, onde o estresse oxidativo está intimamente relacionado à hipertensão neurogênica através da desregulação da resposta autonômica. O estresse oxidativo é um estado celular caracterizado pelo desequilíbrio entre produção e remoção de elementos oxidantes (espécies reativas), sendo a mitocôndria uma das principais fontes produtoras dessas espécies reativas. Diante disso, o presente estudo foi desenvolvido para avaliar os efeitos da restrição proteica nos períodos críticos do desenvolvimento (gestação e lactação) sobre a função mitocondrial e o equilíbrio oxidativo do tronco encefálico de forma imediata e tardia. Ratas gestantes da linhagem *Wistar* foram alimentadas *ad libitum* com dieta normoproteica (NP; caseína 17%) ou hipoproteica (LP; caseína 8%) durante toda gestação e lactação. Ao desmame e aos 128 dias depois, os filhotes machos foram eutanaziados e o tronco encefálico rapidamente removido para as análises da bioenergética mitocondrial, produção de espécies reativas, potencial elétrico de membrana mitocondrial ($\Delta\Psi m$), biomarcadores de estresse oxidativo e defesa antioxidante. Ao desmame, a restrição proteica materna induziu uma disfunção na bioenergética mitocondrial. Animais que sofreram o insulto nutricional apresentaram menor capacidade de consumo de oxigênio e produção de ATP. A esses, associam-se a menor oferta de cofatores reduzidos à cadeia transportadora de elétrons e a diminuição do $\Delta\Psi m$. Quanto ao equilíbrio oxidativo, a restrição proteica desencadeou um aumento na produção de espécies reativas e redução na capacidade antioxidante não enzimática e estado redox, fato que culminou na oxidação apenas de proteínas. Aos 150 dias, os animais que sofreram a restrição proteica materna ainda apresentavam disfunção na bioenergética mitocondrial. Entretanto, a menor capacidade fosforilativa não fora associada a menor oferta de cofatores reduzidos, mas ao desacoplamento mitocondrial desencadeado pela proteína desacopladora 2 (UCP2), o que gradualmente reduz o $\Delta\Psi m$ e compromete a conservação de energia. A produção de espécies

reativas mitocondriais manteve-se aumentada e, agora, somada ao aumento na produção de oxido nítrico e redução da dismutação do superóxido desencadearem grande oxidação a lipídeos. Em resumo, nossos resultados indicam que a disfunção mitocondrial se inicia imediatamente após o insulto nutricional e pode comprometer gradualmente a condução de prótons, conservação de energia, integridade de lipídios e levar à disfunção no tronco encefálico durante o envelhecimento.

Palavras Chave: Mitocôndria. Estresse oxidativo. Tronco encefálico. Restrição proteica.

ABSTRACT

Several studies have been demonstrating the relationship between enviromental stimulus during critical development periods of central nervous system on the rise and progression of diseases later in life, including the cardiovascular. One of the hypotheses is related to molecular and cellular changes in the attempt to prepare the organism to the following enviroment (predictive adaptive response). In this context, ou laboratory has focused on understand how the maternal protein restriction modulates the offspring oxidative balace in different life periods. In the central nervous system, the brainstem has standed out due its role in the regulation of the central cardiovascular control, wherein the oxidative stress is close related to the autonomic dysregulation-induced neurogenic hypertension. The oxidative stress is a cellular condition characterized by the imbalance between production and removal of oxidant elements (reactive species), being the mitochondria one of the major producers of those species. Thereby, the current study was performed to evaluate the immediate and extended effects of the protein restriction during critical development periods (gestation and lactation) upon the immediate and late consequences in the mitochondrial bioenergetics and the oxidative balance in the brainstem. Pregnant *Wistar* rats were fed *ad libitum* with normoprotein (NP; 17% protein-casein) or low-protein (LP; 8% protein-casein) diet throughout pregnancy and lactation periods. At weaning and 128 days later, the male offsprings were euthanized and the brainstem was quickly removed to assess the mitochondria bioenergetics, reactive species (RS) production, mitochondrial electric membrane potential ($\Delta\Psi m$), oxidative stress biomarkers and antioxidant defense. At weaning, maternal protein restriction induced a mitochondrial dysfunction. Animals that suffered the nutritional insult presented lower oxygen consumption capacity and ATP production. These results were associated to the reduction in the reduced cofactors supply to electrical transporter chain and to the $\Delta\Psi m$ reduction. Regarding oxidative balance, the protein restriction triggered an increase in the reactive species production and a decrease in the non-enzymatic antioxidant capacity and redox status, which together, culminated in the oxidative damage only to protein. At 150 days of life, the animals that suffered the maternal protein restriction still showed a dysfunction in the mitochondrial bioenergetics. At this age, the lower phosphorilation capacity was not associated to the reduced cofactors supply, but for the mitochondrial uncoupling by uncoupling protein 2 (UCP2) which gradually reduces the $\Delta\Psi m$ and compromises the energy conservation. The mitochondrial reactive species production was keept augmented, which added to the increase in the nitric oxide production and to the

superoxide dismutation reduction triggered a cogent lipid oxidation. In summary, our results indicate that the mitochondrial dysfunction begins immediatly after nutritional insult and may gradually compromise the proton conduction, energy conservation, lipid integrity, leading to brainstem dysfunction during ageing.

Keywords: Mitochondrion. Oxidative stress. Brainstem. Protein restriction.

LISTA DE ABREVIATURAS E SIGLAS

4-hidroxi-2-nonenal – 4-HNE

Ácido 2-etanossulfonico – HEPES

Ácido Desoxirribonucleico – DNA

Ácido Etileno Diamino Tetra-Acético – EDTA

Ácido graxo poliinsaturado – PUFA

Ácido Ribonucleico – RNA

Ácido Ribonucleico mensageiro – RNAm

Ácido Tricloroacético – TCA

Albumina de Soro Bovina Lipolizada – BSA

Ânion superóxido – $^{\bullet}O_2^-$

Cadeia Transportadora de Elétrons – ETC

Canais de Cálcio dependente de voltagem – VDAC

Canais de troca Ca^{2+}/Na^+

Carbonil Cianeto m-clorofenil Hidrazona – CCCP

Catalase – CAT

Citrato Sintase – CS

Controle Respiratório – RCR

Dialdeído malônico – MDA

Diclorofluoresceína – DCF

Difosfato de Adenosina – ADP

Dinucleotídeo de Flavina e Adenina na forma reduzida – $FADH_2$

Dinucleotídeo de Nicotinamida e Adenina (fosfato) na forma reduzida – NADPH

Dinucleotídeo de Nicotinamida e Adenina na forma oxidada – NAD^+

Dinucleotídeo de Nicotinamida e Adenina na forma reduzida – NADH

Doença de Alzheimer – AD

Doença de Huntington – HD

Doença de Parkson – PD

Doenças Não Comunicáveis – NCD

Erro padrão da média – SEM

Esclerose Lateral Amiotrófica – ALS

Esclorose Múltpla – MS

Espécies Reativas de Oxigênio – ROS

Etileno Bis-glicol. (ß-amino-ester-etil) N, N, N', N' – ácido tetra – EGTA

Flavoproteina-Q-oxidoredutase transferidor de elétrons – ETF-QOR

Glicerol-3-fosfato desidrogenase mitocondrial – mGPDH

Glicose-6-fosfato-desidrogenase – G6PDH

Glutationa na forma oxidada – GSSG

Glutationa na forma reduzida – GSH

Glutationa peroxidase – GPx

Glutationa redutase – GR

Glutationa-S-transferase – GST

Hipoproteico – LP

Mioglobina β2 – β2M

Monoamina oxidase – MAO

Mononucleotídeo de Flavina – FMN

N-metil D-Aspartato – NMDA

Normoproteico – NP

Núcleo do trato solitario – NTS

O-ftalaldeído de fluorescência – OPT

Peróxido de hidrogênio – H_2O_2

Piruvato Desidrogenase – PDH

Poro de transição de permeabilidade mitocondrial – MPTP

Potencial elétrico de membrana mitocondrial – $\Delta\Psi m$

Proteína beta-amilóide – Aβ

Proteína Desacopladora – UCP

Radical hidroxila – $OH^{\bullet}$

Rápido modo de captação de Cálcio – RaM

Reação de Cadeia de Polimerase – PCR

Reatividade Antioxidante Total – TAR

Receptor Ryanodina – RyR

Região Rostral ventrolateral do bulbo – RVLM

Restrição de Crescimento Intrauterino – IUGR

Retículo Sarcoplasmático/Endoplasmático – SER

Rotações por minuto – RPM

Sistema Nervoso Central – CNS

Superóxido dismustase citoplasmática – SOD1

Superoxido dismutase – SOD

Tampão de Respiração - RB

Tirosina – Tyr

Trifosfato de Adenosina – ATP

Triptofano – Try

Tris (hidroximetil) aminometano – TRIS

Ubiquinona - UQ

Ubiquinona na forma reduzida – UQH_2

Uniporta mitocondrial de Cálcio – MCU

SUMÁRIO

1 INTRODUÇÃO

É bem estabelecido que influências ambientais, especialmente as nutricionais, podem alterar inúmeras funções do organismo, podendo desencadear doenças crônico degenerativas, tais como diabetes, síndrome metabólica e doenças cardiovasculares. O aumento do risco a essas doenças, entretanto, não se limita às modificações fisiológicas do organismo adulto, mas também pode ser uma consequência de experiências negativas que aconteceram ainda na concepção.

Apesar dos mecanismos envolvidos neste processo não estarem totalmente esclarecidos, estudos sugerem que insultos nutricionais durante fases críticas do desenvolvimento possam modificar padrões moleculares e celulares na tentativa de preparar o individuo ao ambiente que será exposto (resposta adaptativa preditiva) (GLUCKMAN, HANSON e SPENCER, 2005; NETTLE, FRANKENHUIS e RICKARD, 2013). Entretanto, estudos sugerem que a incompatibilidade entre o ambiente previsto e o real, é o que de fato predispõe o organismo ao surgimento/progressão de doenças crônico degenerativas. Nos últimos anos, o estresse oxidativo tem sido apontado como um possível desencadeador molecular às doenças induzidas pela deficiência nutricional, incluindo a proteica (LUO *et al.*, 2006; MARTIN-GRONERT e OZANNE, 2012; FERREIRA, SELLITTI e LAGRANHA, 2016). Devido à relativa vulnerabilidade do Sistema Nervoso Central ao estresse oxidativo (HALLIWELL, 2006), estudos têm voltado sua atenção para a relação entre as desordens neurais e o equilíbrio oxidativo, onde a mitocôndria destaca-se devido a sua importância na produção de elementos pró-oxidantes e fornecimento energético.

Apesar da modulação da função mitocondrial e da produção de elementos pro-oxidantes serem amplamente utilizados em modelos experimentais de Alzheimer, Huntington, Parkinson e Esclerose Múltipla e Hipertensão (BUBBER *et al.*, 2005; CHAN *et al.*, 2006; DUTTA *et al.*, 2006; DAMIANO *et al.*, 2010), pouco se sabe a respeito dos efeitos da desnutrição proteica materna sobre a função mitocondrial e o equilíbrio oxidativo no tronco encefálico.

Na última década, o tronco encefálico tem sido fruto de estudos no nosso laboratório. Localizado entre o telencéfalo e a medula espinhal, o tronco encefálico é uma estrutura neural responsável pelo controle de circuitos neurais imprescindíveis para a vida, sendo descrito como uma das primeiras estruturas cerebrais estabelecidas durante o desenvolvimento

cerebral. Seus neurônios são responsáveis pela determinação do ritmo respiratório, regulação da pressão arterial, modulação de neurônio motores, modulação de respostas reflexas, integração de sinais sensoriais, programação motora sub-cortical, bem como processamento e integração de informações (NICHOLLS e PATON, 2009; DE NATALE *et al.*, 2015; VALLS-SOLE, 2015).

Nesse sentido, estudos têm demonstrado que o estresse oxidativo no tronco encefálico é capaz de modular a resposta autonômica, resultando em flutuações cardiovasculares (pressão arterial, resistência vascular, frequência cárdica) inapropriadas (CHAN *et al.*, 2006; CARDOSO *et al.*, 2009; CHAN *et al.*, 2009; HIROOKA *et al.*, 2011; CHAN e CHAN, 2014). Entretanto, no que se refere ao insulto nutricional e o estudo do estresse oxidativo no SNC, o tronco encefálico ainda é bastante negligenciado, e devido sua importância para inúmeras funções vitais do organismo, o presente estudo foi desenvolvido para entender os efeitos da restrição proteica nos períodos críticos do desenvolvimento sobre a bioenergética mitocondrial e o equilíbrio oxidativo de forma tardia e imediata.

2 HIPÓTESES

- A restrição proteica durante gestação e lactação desregula a bioenergética mitocondrial e o equilíbrio oxidativo no tronco encefálico, imediatamente após o insulto nutricional.
- A maior propensão a desajustes autonômicos na vida adulta é resultado de uma disfunção mitocondrial no tronco encefálico que se inicia após restrição proteica materna e se mantem durante toda a vida.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar os efeitos da restrição proteica materna na bioenergética mitocondrial e no equilíbrio oxidativo do tronco encefálico de ratos machos aos 22 e aos 150 dias de vida.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar em mitocôndrias do tronco encefálico de ratos machos com 22 e 150 dias cujas mães foram submetidas à dieta normo ou hipoproteica durante gestação e lactação:

- Consumo de oxigênio;
- Produção de Espécies Reativas de Oxigênio e Nitrogênio;
- Potencial elétrico de membrana mitocondrial;

Avaliar no tronco encefálico de ratos machos com 22 e 150 dias cujas mães foram submetidas à dieta normo ou hipoproteica durante a gestação e lactação:

- Biomarcadores de estresse oxidativo
    - Níveis de Substâncias Reativas ao Ácido Tiobarbitúrico;
    - Conteúdo de Carbonilas;
- Atividade enzimática:
    - Superóxido Dismutase (SOD);
    - Catalase (CAT);
    - Glutationa Peroxidase (GPx);
    - Glutationa-S-Transferase (GST);
    - Glicose-6-fosfato desidrogenase (G6PDH);
    - Citrato sintase;
- Estado Redox (GSH/GSSG);
- Níveis de cofatores reduzidos (NAD(P)H);
- Níveis de nitrato e nitrito de sódio;
- Expressão proteica de Óxido Nítrico Síntetase Neuronal (nNOS);
- Expressão gênica da Proteína desacopladora 2 (UCP2).

4 MATERIAIS E MÉTODOS

Foram utilizados ratos machos da linhagem Wistar provenientes do biotério localizado no Centro Acadêmico de Vitória de Santo Antão (CAV), mantidos em ciclo claro-escuro 12/12 horas com temperatura de (23 ± 2°C), umidade (50 a 70%) e mantidos em regime *ad libitum*

A manipulação e os cuidados com os animais seguiram as recomendações do Colégio Brasileiro de Experimentação Animal (COBEA) e aprovação do Comitê de Ética em Estudos com Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, processo nº: 23076.017807/2014-15.

4.1 OBTENÇÃO DOS GRUPOS EXPERIMENTAIS

Ratas virgens aos 80 dias de idade (n=12) foram acasaladas na proporção de 2 fêmeas para 1 macho. As ratas foram checadas diariamente e a visualização de espermatozóides em lâminas contendo secreção vaginal (esfregaço vaginal) foi considerado início da gestação. As ratas gestantes foram transferidas para gaiolas individuais e divididas em dois grupos experimentais de acordo com a dieta fornecida: grupo normoproteico (C, 17% de proteína, n=6) e grupo hipoproteico (D, 8% de proteína, n=6), ambos os grupos foram mantidos em regime *ad libitum* [Tabela 1].

Tabela 1: Composição de dieta experimental isocalórica com diferentes teores de proteína.

Ingredientes	Quantidade*	
	8 %	17%
Caseína (85%)	94,10 g	200,0 g
Mix Vitamínico**	10,0 g	10,0 g
Mix Mineral***	35,0 g	35,0 g
Celulose	50,0 g	50,0 g
Bitartarato de Colina	2,5 g	2,5 g
DL-Metionina	3,0 g	3,0 g
Óleo de Soja	70,0 mL	70,0 mL
Amido de Milho	503,4 g	397,5 g
Amido Dextrinizado	132,0 g	132,0 g
Sacarose	100,0 g	100,0 g

*Quantidade para 1 kg de dieta. Adaptado de: (REEVES, NIELSEN e FAHEY, 1993).

Os ratos foram observados todos os dias durante a gestação e constatado o nascimento só foram manipulados após 24h. Na 1ª manipulação, as ninhadas foram normalizadas para 9 filhotes por ninhada, os animais machos foram mantidos nas ninhadas e as fêmeas descartadas por meio de decapitação ou mantidas apenas para normatização da ninhada. Durante a lactação, os animais continuaram recebendo a dieta conforme seu grupo experimental até completarem 22 dias de vida, quando foram sacrificados e os tecidos coletados. Parte dos tecidos foi direcionada para o isolamento mitocondrial (ver tópico **5.4**) e a outra metade para demais analises bioquímicas (ver tópico **5.5**).

Os ratos da ninhada que não foram sacrificados foram alojados (4 animais por gaiola) e passaram a receber dieta labina ainda em regime *ad libitum* (água e ração). Aos 150 dias de idade, os animais foram sacrificados e os mesmos procedimentos que os animais aos 22 dias realizados (ver figura 3).

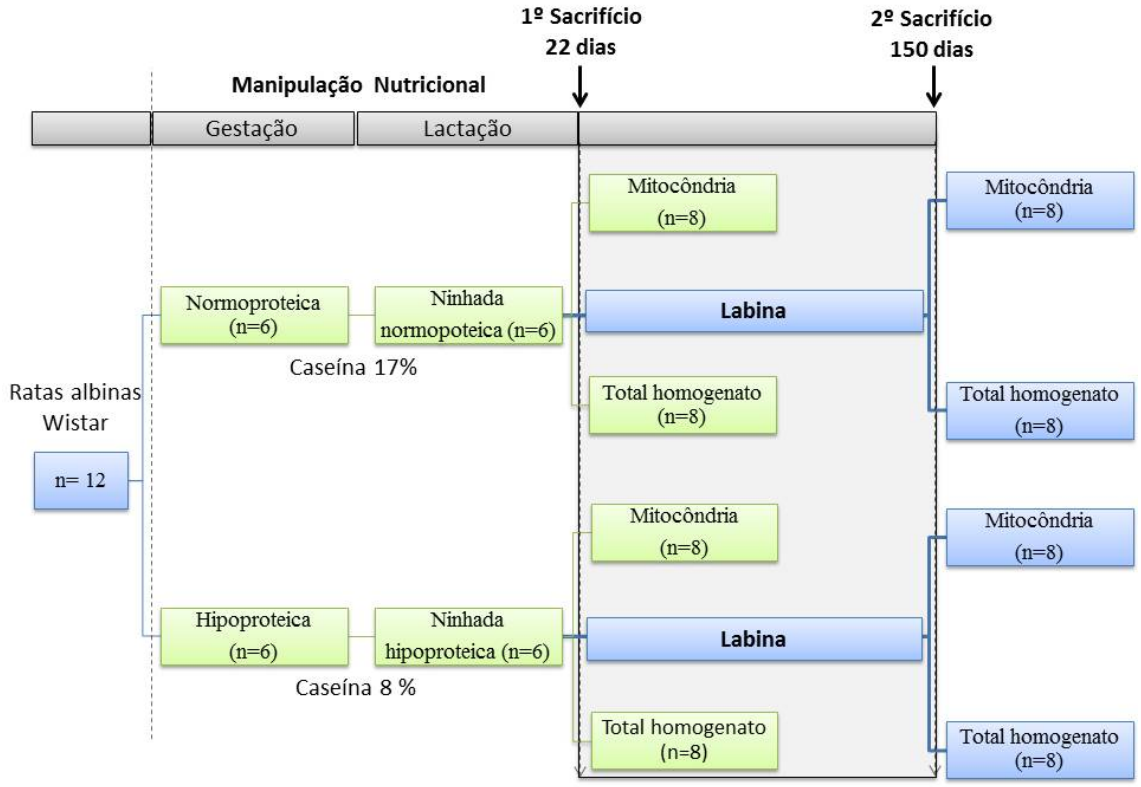

Figura 3: Desenho experimental do presente estudo

4.2 COLETA DOS TECIDOS

Completada a idade planejada, ambos os grupos foram sacrificados via decapitação e o tronco encefálico retirado em menos de 90 segundos. Parte dos tecidos foi direcionada para atividades referentes à mitocôndria e outra parte congelada e armazenada em freezer a uma temperatura de -80ºC e processados dentro de uma semana.

4.3 DOSAGEM DE PROTEÍNA

A concentração de proteína foi determinada pelo método de Bradford (1976), o qual se subsidia na determinação da concentração de ligações peptídicas através da medida da absorbância do complexo proteína-corante em comprimento de onda de 595nm. A absorbância é considerada diretamente proporcional à concentração de proteína na solução analisada, onde uma solução de Albumina de Soro Bovino - BSA a (2mg/mL) foi utilizada como padrão (figura 4).

Obs: A concentração de proteína foi utilizada previamente a todas as analises com o objetivo de normatizar a quantidade de proteína utilizada nos ensaios.

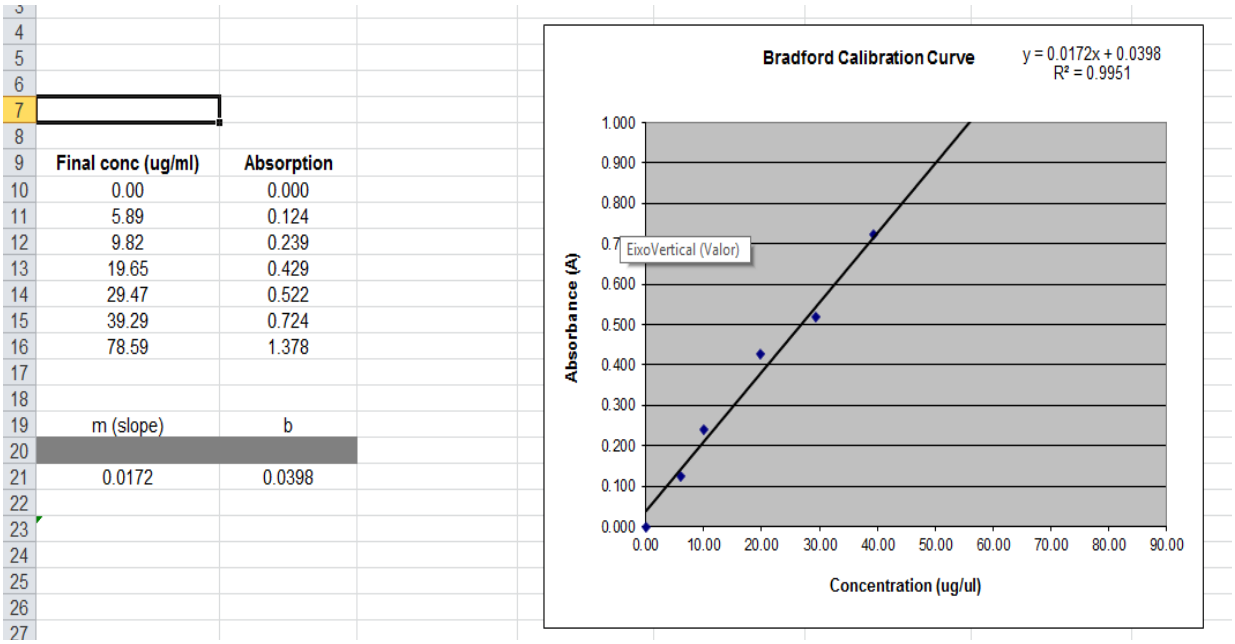

Figure 4: Curva padrão de proteína pelo método de Bradford

4.4 ISOLAMENTO MITOCONDRIAL

O isolamento mitocondrial foi adaptado de Lagranha et al., (2010). Resumidamente, após remoção do tronco encefálico, o tecido fresco foi imediatamente imerso em tampão gelado pH 7,4 contendo: Manitol 225 mM, Sacarose 75 mM, HEPES 4 mM, Taurina 2 mM e EGTA 0.5 mM. O tecido foi completamente homogeneizado usando um potter-Elvehjem (pistão de metal e um tubo de vidro) conectado a um homogeneizador digital IKA$^{@}$ RW 20 e para obtenção das mitocôndrias as seguintes etapas foram seguidas:

- Centrifugação do homogenato por 5 minutos a 1.180g numa temperatura de 4°C;
- Transferência dos sobrenadantes para novos tubos e nova centrifugação a 12.470g RPM por 10 minutos ainda na temperatura de 4°C;
- Descarte dos sobrenadantes e ressuspensão dos pellets (± 100µl) em tampão de respiração (TR), pH 7,4.

O TR foi composto por KCl 120 mM, HEPES 4 mM, K_2HPO_4 5 mM e 0,2% de BSA (p/v). Após a obtenção da mitocôndria, todos os experimentos foram realizados dentro de 2 horas com a mitocôndria mergulhada em gelo.

4.4.1 Avaliação da respiração mitocondrial (Consumo de Oxigênio)

As mitocôndrias foram incubadas em TR (1 mg protein/mL) numa câmara 600 SL conectada a um eletrodo de detecção de oxigênio (Clark-type) (Hansatech Instruments, Pentney King's Lynn, UK) a 28°C (LAGRANHA *et al.*, 2010). A respiração mitocondrial foi verificada através das seguintes etapas:

- Pipetagem do TR na câmara;
- Adição dos substratos do complexo I (10 mM de glutamato/0,4 mM de malato);
- Adição da mitocôndria, determinação do consumo de oxigênio em estado basal;
- Adição de 0.8 mM ADP ao meio, consumo de oxigênio em estado estimulado;
- Adição de Oligomicina 1µg/mL, consumo de oxigênio em estado inibido;
- Adição de Carbonilcianeto m-clorofenil-hidrazona CCCP 1µM, consumo de oxigênio em estado desacoplado.

4.4.2 Produção de espécies reativas totais

A produção total de espécies reativas foi avaliada utilizando o probe fluorescente 2',7' -diclorofluoresceina (H2DCF-DA) (VAN DER TOORN *et al.*, 2007; DA SILVA *et al.*, 2015a). Mitocôndrias, 100µg, foram incubadas em TR contendo substratos do complexo I nas mesmas concentrações descritas na sessão da respiração mitocondrial. A esse meio foram adicionados 5 µM de diclorofluoresceina (H2DCF-DA), composto químico que na presença de espécies reativas forma um produto fluorescente que pode ser monitorado em comprimentos de onda de excitação e emissão de 485 nm e 530 nm, respectivamente. A reação fora acompanhada em suave agitação no FLUOstar OMEGA (BMG Labtech, USA), por 8 minutos a uma temperatura de 28ºC (SRIRAM *et al.*, 1997).

4.4.3 Potencial elétrico de Membrana Mitocondrial (ΔΨm)

O potencial de membrana mitocondrial foi avaliado utilizando o reagente safranina-O (FELDKAMP *et al.*, 2007; PARK *et al.*, 2011). Esta análise baseia-se na afinidade elétrica da safanina-O a ânions, onde sua fluorescência pode ser monitorada com excitação de 485 nm e emissão de e 590 nm. O decaimento na fluorescência é um indicativo de adesão deste composto à membrana interna mitocondrial e está relacionado à diferença elétrica entre o espaço intermembrana e matriz mitocondrial. Cem microgramas de mitocôndria foram

incubados em TR contendo substratos do complexo I (**ver 4.4.1**). Após a adição de 5 µM de safranina-O, o decaimento da fluorescência a 28ºC foi acompanhado no FLUOstar OMEGA (BMG Labtech, USA) em suave agitação até alcançar um platô, momento em que foi adicionado 1 µM de CCCP para dissipar o potencial de membrana e novo monitoramento da fluorescência. Os resultados foram expressos através do percentual de Safranina-O captada (Delta da fluorescência).

4.5 PREPARO DO TECIDO PARA HOMOGENEIZAÇÃO

Os troncos encefálicos foram retirados do freezer e completamente homogeneizados em tampão de extração gelado (Tris base 50 mM, pH 7,4; EDTA 1mM; ortovanadato de sódio 1 mM; PMSF 200µg/mL e coquetel de inibidores de protease 10µl/mL). Após a homogeneização as amostras foram centrifugadas por 10 minutos a 1.180g a uma temperatura de 4ºC e os sobrenadantes utilizados posteriormente (5.5.1 - 5.5.12).

4.5.1 Medida do índice de lipoperoxidação das membranas

Para a dosagem da lipoperoxidação foi utilizada a técnica colorimétrica previamente publicada (BUEGE e AUST, 1978), que é uma técnica utilizada para avaliar oxidação de lipídios, pois o ácido tiobarbitúrico reage com os produtos da lipoperoxidação, entre eles o malondialdeído e outros aldeídos. À amostra foram adicionados, em volumes iguais, ácido tricloroacético 30% (p/v) e Tris-HCl 10mM (pH 7,4), os quais passaram por agitações sequenciais de 1 minuto. Após totalmente homogeneizadas, as amostras (300µg proteína) foram centrifugadas por 10 minutos a 2500xg a 4°C e ao seu sobrenadante uma alíquota de TBA 0,73% em igual volume adicionado. Esta mistura foi incubada por 15 minutos a 100ºC e imediatamente resfriada e transferidas para cubetas de quartzo, onde foram verificadas em espectrofotômetro a uma absorbância de 535nm. Os resultados foram expressos em nmoles de MDA por mg de proteína.

4.5.2 Medida do índice de oxidação de proteínas

A avaliação da oxidação de proteínas foi realizada através da técnica das carbonilas. Ao homogenato, mantido em gelo, foi adicionado TCA 30% e levado a centrifuga por 14 minutos a 1.180g. Após centrifugação o sobrenadante foi descartado e o pellet ressuspendido em 10mM de 2,4 dinitrofenilhidrazina (DNPH) e incubado em sala escura a temperatura ambiente por uma hora com agitações programadas a cada 15 minutos. Após período de

incubação, as amostras foram lavadas em tampão etil/acetato e centrifugadas três vezes e o pellet ressuspendido em 6M de hidrocloreto de guanidina, incubado por 30 minutos a 37ºC e a absorbância verificada a 370nm. Os resultados foram expressos em μM/mg proteina (REZNICK e PACKER, 1994).

Obs: Todas as enzimas foram expressas em U/mg de proteína (capacidade de catalisar a formação de 1μM de produto por minuto). A quantidade de proteína utilizada nas análises enzimáticas foi estabelecida na concentração de 0,3mg/mL.

4.5.3 Atividade enzimática: Superóxido dismutase (SOD)

A atividade da superóxido dismutase foi avaliada através do método da oxidação da adrenalina, o qual compete com a SOD podendo ser medido em espectrofotômetro a 480nm. Em uma cubeta de quartzo de 1mL, foi adicionado tampão carbonato 0,1M (pH10,2), EDTA 0,1mM, amostra e adrenalina 150mM. A diminuição na absorbância foi seguida por 90 segundos a 30ºC no comprimento de onda de 480nm e os resultados expressos em U/mg proteina a partir do coeficiente de extinção da adrenalina, 4020 M^{-1} cm^{-1} (MISRA e FRIDOVICH, 1972).

4.5.4 Atividade enzimática: Catalase (CAT)

A atividade da catalase (CAT) foi avaliada através da taxa de decomposição do peróxido de hidrogênio, verificada na absorção de 240 nm numa temperatura de 20°C. Em um meio de reação, contendo tampão fosfato 50mM (pH=7,0) amostra e H_2O_2 0,3mM. A absorbância foi monitorada durante 3min e os resultados expressos em U/mg de acordo com coeficiente de extinção molar (2,3 μM^{-1} cm^{-1}) (AEBI, 1984).

4.5.5 Atividade enzimática: Glutationa Peroxidase (GPx)

A atividade da Glutationa peroxidase foi verificada de acordo com Paglia e Valentine (1967). Foram combinados tampão fosfato 50mM (pH 7,0); EDTA 5mM; NADPH 8,4mM; glutationa redutase (100UL/mg proteína/mL); Azida sódica (NaN_3) 1,125M e GSH 0,15M. A reação enzimática foi iniciada pela adição de H_2O_2 2,2mM e a conversão do NADPH em

NADP fora acompanhada em espectrofotômetro a 340nm durante 4 minutos a 20°C. A unidade enzimática foi definida através da oxidação de 1µmol de NADPH por minuto por mg de proteína, sendo calculado com base na absortividade molar do NADPH a 340nm em cubeta de 1mL (6.22×10^{-6}).

4.5.6 Atividade enzimática: Glutationa-S-Transferase (GST)

Avaliou-se a atividade da Glutationa-S-Transferase (GST) de acordo com o previamente descrito por Habig (1974). O procedimento se constituiu da adição de tampão fosfato de potássio 0,1M (pH 6,5); EDTA 1mM; GSH 1mM; amostra e 1-cloro-2,4-dinitrobenzeno (CDNB) 1mM. A atividade da enzima foi avaliada a partir da formação de 2,4-dinitrofenil-s-glutationa (DNP-SG) por minuto a 30°C, o qual foi monitorado via espectrofotômetro em comprimento de onda igual a 340nm. A atividade da enzima foi expressa em U/mg proteina baseada no coeficiente de extinção do CDNB (9,6 mM^{-1} cm^{-1}).

4.5.7 Atividade enzimática: Glicose-6-fosfato desidrogenase (G6PDH)

A atividade da G6PDH foi realizada de acordo com Lohr & Waller (LÖHR e WALLER, 1974). Foi utilizado um tampão de ensaio contendo Tris 30mM; EDTA 1mM e $MgSO_47H_2O$ 5mM (pH 7,6). O meio de reação foi composto por Tampão de ensaio; $NADP^+$ 0,5mM; Triton X-100 a 0,5%; amostra e foi inicializada com a adição de Glicose-6-fosfato 4,8 mM. A atividade enzimática foi determinada de acordo com a produção de NADPH por minuto por miligramas de proteína, sendo calculado com base na absortividade molar do NADPH a 340nm em microplacas contendo 100µL (13,7462 M^{-1} cm^{-1}).

4.5.8 Atividade enzimática da citrato sintase (CS)

A CS é a primeira enzima do Ciclo de Krebs e é responsável por catalisar a condensação de actetil-CoA com oxalacetato para forma citrato, por isso considerada como uma das enzimas reguladoras da velocidade do ciclo (WIEGAND e REMINGTON, 1986). Sua atividade foi determinada de acordo com Newsholme (1976), utilizando uma mistura contendo Tris.HCl 100mM (pH 8,2), MgC_{l2} 1mM, EDTA 1 mM, 5,5-dithio-bis(2-nitrobenzoic acid) 0,2 mM, acetil-CoA 3 mM, oxalacetato 5 mM e amostra. A atividade

enzimática fora então determinada a partir da taxa de modificação da absorbância no comprimento 412nm durante 3 min a 25ºC (ALP, NEWSHOLME e ZAMMIT, 1976).

4.5.9 Estado Redox (GSH/GSSG)

O meio de ensaio foi composto por tampão fosfato 0,1M (pH 8,0) contendo EDTA 5mM e amostra, o qual foi incubado com o-Phthaldialdehyde (OPT) 1mg/mL em temperatura ambiente por 15 minutos. Depois de incubado, o ensaio teve sua fluorescência verificada em um comprimento de excitação e emissão de 350nm e 420nm, respectivamente. Para a determinação dos níveis de GSH, as amostras foram comparadas a uma curva padrão de GSH com conhecidas concentrações, através da equação da reta gerada (figura 5).

Para a determinação dos níveis de GSSG, as amostras foram primeiramente incubadas com N-ethylmaleimida (NEM) 0,04M por 30 minutos a temperatura ambiente seguido da adição do tampão NaOH 0.1M ao meio. Após esse procedimento, as amostras também foram incubadas com o-Phthaldialdehyde (OPT) 1mg/mL em temperatura ambiente por 15 minutos e tiveram sua fluorescência comparada a uma curva de concentrações conhecidas de GSSG. O estado redox foi determinado pela razão GSH/GSSG com previamente descrito (HISSIN e HILF, 1976).

[ ]µM	Fluorescência
0	0
4.06	83
8.12	154
16.24	383
32.48	697
64.96	1210

slope	
a	**b**
18.8	26.8

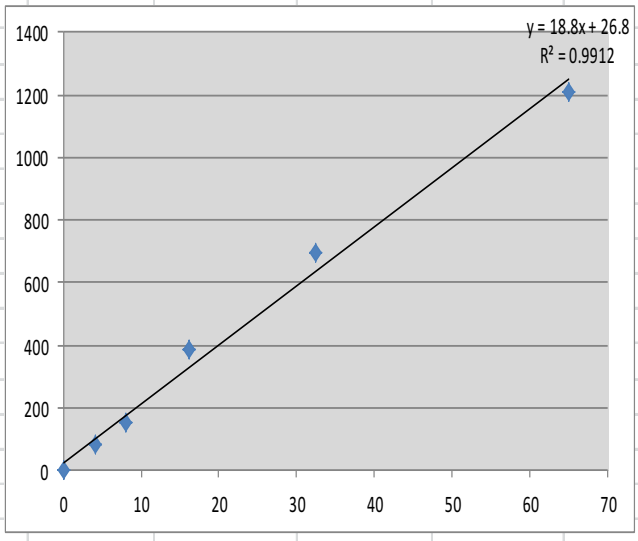

Figure 5: Curva padrão de GSH

4.5.10 Níveis de Nicotinamida adenina dinuclueotídeo (fosfato) reduzido (NAD(P)H)

O método se baseia na capacidade dos co-fatores reduzidos NADH e NADPH absorverem luz em um comprimento de 340nm, capacidade na qual esse método é baseado. Amostras foram incubadas em tampão (TRIS-HCl 50 mM; pH 7,4) em uma concentração de 0,5mg/mL, em seguida tiveram suas absorbâncias verificadas a 340nm e comparadas com uma curva padrão de NADH (figura 6) (YING, 2008).

[ ]µM	Abs
0	0
6.75	0.041
13.45	0.078
26.9	0.126
53.8	0.246
107.6	0.5

slope	
a	**b**
0.0046	0.0066

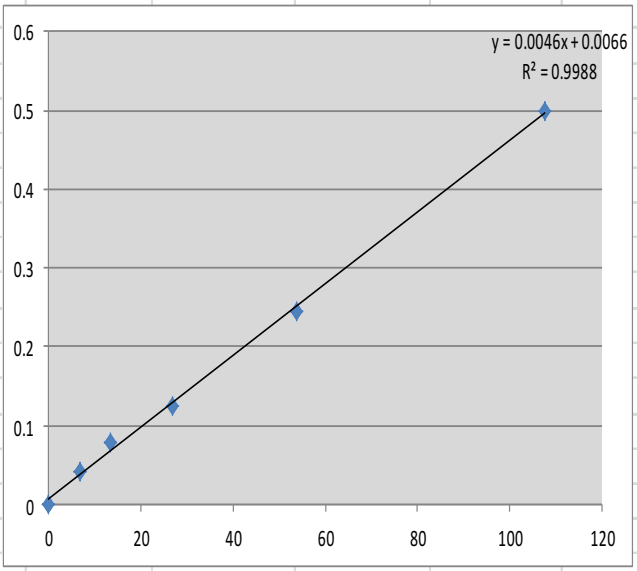

Figura 6: Curva padrão de NADH

4.5.11 Níveis de Nitrito de Sódio

Os níveis de nitrato e nitrito de sódio foram verificados e acordo com o método de Griess (GREEN *et al.*, 1982). Foram preparadas duas soluções: Reagente de Griess I (sulfanilamida 1% em ácido fosfórico 5% de concentração de 58,07mM) e Reagente de Griess II (Naftiletilenodiamina diidrocloreto 0,1% na concentração de 3,86mM). Quinhentos microgramas de amostra foram incubados às soluções I e II em iguais volumes por 10 minutos a 25ºC. A coloração rosa produzida foi então verificada no comprimento de 540 nm e comparadas a uma curva padrão produzida com Nitrito de Sódio (1.6 – 13.1µM) (figura 7).

[ ]µM	Absorb.
0	0
1,6425	0,093
3,285	0,211
6,57	0,374
13,14	0,631

slope	
a	b
0,0477	0,0269

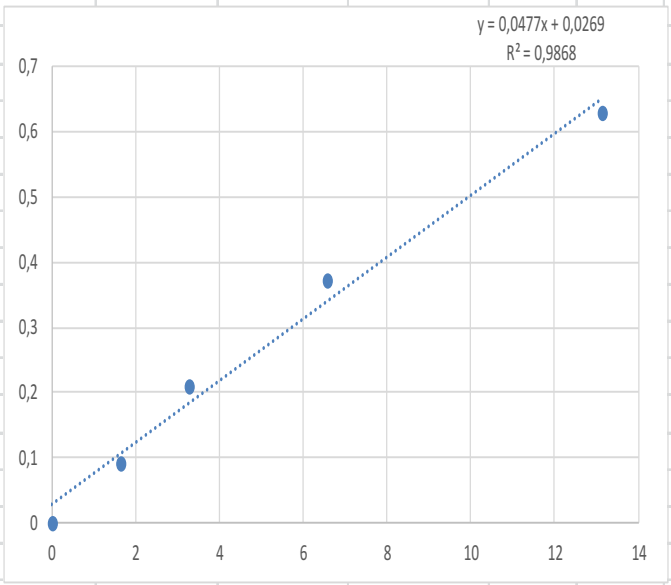

Figure 7: Curva padrão de Nitrito de Sódio

4.5.12 Expressão proteica da Óxido Nítrico Sintase Neuronal (nNOS)

Às amostras, foi adicionando tampão LAEMILLI 2x (pH 6,8: Glicerol 20%; TRIS 125mM; Ditiotreitol 100mM; Dodecil Sulfato de Sódio 4% e Azul de Bromofenol 0,002%) e armazenadas a -20ºC. Imediatamente antes da utilização, as amostras foram fervidas por 10 minutos e 10µg de proteína passaram por eletroforese (45 minutos a 30 volts em um gel de empacotamento 5%), seguido de (90 minutos a 100volts em gel de resolução 8%). A eletroforese foi realizada em um tampão pH 8,3-8,4 contendo: TRIS 25mM, Glicina 192mM e SDS 0,1%.

A transferência foi realizada no sistema de tanque a 15 volts por 10 horas a 4°C em um tampão de transferência (pH 8,3-9,3) contendo: TRIS 25mM, Glicina 192mM Metanol 20% e SDS 0,02%. Em suave agitação, as membranas (nitrocelulose) foram: lavadas com TBS-T (NaCL 160mM, TRIS 0,2M e Tween-20 0,1%) por 5 minutos; incubadas em 5% de leite livre de gordura preparado em TBS-T por 60 minutos; incubadas por 12h a 4ºC com Anticorpo Primário nNOS (rabbit polyclonal IgG, Santa Cruz Biotechnology, sc-1025 – 2,5:5000 em leite 5%); lavadas (3 x 5 minutos em TBS-T); incubadas a temperatura ambiente por 4 horas em Anticorpo Secundário (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology 1:5000 em leite 5%); lavadas com TBS-T (3 x 5 minutos); expostas a solução de revelação

ECL (Pierce® ECL Western Blotting substrate – Thermo Scientific) por 5 minutos e expostas e reveladas em filmes de raio-X em sala escura.

A transferência do gel foi verificada através da coloração reversível com Pounceau e as intensidades de banda verificadas por ImageJ 1.51f (NIH, Maryland, USA) (TOWBIN, STAEHELIN e GORDON, 1979; DA SILVA *et al.*, 2015b)

4.6 ISOLAMENTO DO RNA E REAÇÃO EM CADEIA DE POLIMERASE (PCR)

Tecidos congelados foram homogeneizados com 1mL TRIzol e deixados em repouso por 5 minutos em temperatura ambiente para a completa dissociação de complexos núcleo proteicos. Após esse período, 0,2 mL de clorofórmio foram adicionados e as amostras centrifugadas a 12.000 x g por 15 minutos a 4°C. A fase aquosa foi então transferida para um novo eppendorf em adição de 0,5mL de álcool isopropílico e acetato de amônia a 2M (CHOMCZYNSKI, 1993). Uma nova centrifugação foi realizada, e ao pellet, foi adicionado etanol 75% seguido de nova centrifugação por 5 minutos a 7.500 g a 4°C. Ao final, o RNA foi secado ao ar, suspendido em água livre de RNase e armazenado a -20°C. O RNA foi quantificado através da absorbância em comprimento de onda de 260nm e sua pureza acessada pela ração de 260/ 280nm (LAGRANHA *et al.*, 2004; LAGRANHA *et al.*, 2005).

A reação de PCR foi realizada de acordo com o protocolo SuperScript III One-Step RT-PCR System com Platinum Taq DNA Polymerase (Invitrogen, USA), contendo 200ng of RNA, 2x Reaction mix, 10 μM de cada primers, SuperScript® III RT/Platinum® Taq mix e DEPC água ultra pura. As amostras foram processadas em triplicata e normalizadas usando microglubulina β2 (β2M) (KUHN *et al.*, 2004; PEREZ *et al.*, 2007). O produto da amplificação do PCR foi verificado em Sistema pre-cast Ex-Gel® com SYBR®.

Tabela 2. Primers usados para análise de PCR

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
UCP2	TACTCTCCTGAAAGCCAACC	GCTGCTATAGGTGACAAAC
B2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG

4.7 ANÁLISE ESTATÍSTICA

Inicialmente foi utilizado um teste de Normalidade (Kolmogorov-Sminorv) e de homogeneidade de variância para determinar qual o tipo de análise a ser utilizada, paramétrico ou não paramétrico. Todos os valores foram expressos como médias e EPM, onde foi utilizado o teste *t student* não pareado e as análises foram realizadas no software GraphPad Prism 6.0® (GraphPad Software, Inbc. Jolla, CA, USA). O nível mínimo de significância foi considerado $p \leq 0.05$.

5 RESULTADOS

5.1 1ºARTIGO ORIGINAL

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Research report

Mitochondrial bioenergetics and oxidative status disruption in brainstem of weaned rats: Immediate response to maternal protein restriction

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ABSTRACT

Mitochondrial bioenergetics dysfunction has been postulated as an important mechanism associated to a number of cardiovascular diseases in adulthood. One of the hypotheses is that this is caused by the metabolic challenge generated by the mismatch between prenatal predicted and postnatal reality. Perinatal low-protein diet produces several effects that are manifested in the adult animal, including altered sympathetic tone, increased arterial blood pressure and oxidative stress in the brainstem. The majority of the studies related to nutritional programming postulates that the increased risk levels for non-communicable diseases are associated with the incompatibility between prenatal and postnatal environment. However, little is known about the immediate effects of maternal protein restriction on the offspring's brainstem. The present study aimed to test the hypothesis that a maternal low-protein diet causes tissue damage immediately after exposure to the nutritional insult that can be assessed in the brainstem of weaned offspring. In this regard, a series of assays was conducted to measure the mitochondrial bioenergetics and oxidative stress biomarkers in the brainstem, which is the brain structure responsible for the autonomic cardiovascular control. Pregnant *Wistar* rats were fed *ad libitum* with normoprotein (NP; 17% casein) or low-protein (LP; 8% casein) diet throughout pregnancy and lactation periods. At weaning, the male offsprings were euthanized and the brainstem was quickly removed to assess the mitochondria function, reactive oxygen species (ROS) production, mitochondrial membrane electric potential ($\Delta\Psi$m), oxidative biomarkers, antioxidant defense and redox status. Our data demonstrated that perinatal LP diet induces an immediate mitochondrial dysfunction. Furthermore, the protein restriction induced a marked increase in ROS production, with a decrease in antioxidant defense and redox status. Altogether, our findings suggest that LP-fed animals may be at a higher risk for oxidative metabolism impairment throughout life than NP-fed rats, due to the immediate disruption of the mitochondrial bioenergetics and oxidative status caused by the LP diet.

1. Introduction

It is well known that mitochondria play several functions in eukaryotic cells. Considered as the major energy producer, by means of ATP formation, mitochondria are also the main source of reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007). In physiologic conditions, mitochondrial ROS production is a tightly adjusted mechanism required for a plethora of cellular processes (e.g., cell signaling, gene expression, cellular growth and apoptosis) (Fukai and Ushio-Fukai, 2011). Since mitochondrion also has a high antioxidant capacity (Figueira et al., 2013), its dysfunction can

Abbreviations: EDTA, ethylene diamine tetra acetic acid; G6PDH, gucose-6 phosphate dehydrogenase; GSSG, oxidized glutathione; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione-S-transferase; LP, low-protein; NAD(P)H, nicotinamide adenine dinucleotide (phosphate) reduced; NP, normoprotein; RVLM, rostral ventrolateral medulla; SOD, superoxide dismutase; TRIS, tris (hydroxymethyl) aminomethane; TCA, trichloroacetic acid

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trigger an imbalance between ROS generation and removal, a disorder that has been associated with several diseases, including heart failure (Munzel et al., 2015), neurogenic diseases (Abramov et al., 2010; Kwon et al., 2004; Palomo and Manfredi, 2015) and hypertension (Chan, 2006).

Previous studies have shown that into the brainstem structure, specifically in the rostral ventrolateral medulla, mitochondrial impairment (e.g., disruption in complexes I and III of the electron transport chain) as well as decrease in the antioxidant capacity contributes to neurogenic hypertension via sympathoexcitatory overstimulation (Chan et al., 2009; Hirooka et al., 2011). Although a central oxidative imbalance has been described in several hypertension models (Chan et al., 2009; Nishihara et al., 2012; Oliveira-Sales et al., 2010), the cause of the imbalance is yet unclear.

Studies involving fetal programming have demonstrated a close relationship between nutritional insults in early life and later propensity for non-communicable diseases, including hypertension (Barker et al., 1989; Barros et al., 2015; de Brito Alves et al., 2014). One of the hypotheses to explain this relationship is the predictive adaptive response, which postulates that early adversities will provide to the organism under development a predictive adaptation in response to cues from the environment, resulting in anticipated phenotype adaptation for immediate survival and improved success in an adverse environment (Gluckman et al., 2005b; Nettle et al., 2013). However, the initial adaptation will be only temporary and the disparity between the prenatal prediction and postnatal reality will result detrimental to the organism, leading to an increased risk of non-communicable diseases in adulthood (Martin-Gronert and Ozanne, 2012; Wang et al., 2012; Warner and Ozanne, 2010).

Recently, studies on adult rats born from mothers fed a low-protein diet (LP) during gestation and lactation, showed that these animals have an increase in arterial blood pressure and in the cardiovascular sympathetic tone (Barros et al., 2015). Based on the fact that central oxidative stress might lead to those cardiovascular responses, we have previously studied the oxidative stress in the brainstem of animals using an experimental model published by Barros et al. We found a significant increase in oxidative damage to lipids in conjunction with a marked decrease in antioxidant defenses (Ferreira et al., 2015). Further investigations have shown that in animals at 30 days of age, born to an LP-fed mother, the arterial blood pressure was not affected, despite the higher sympathoexcitatory responses to peripheral chemoreceptor stimulation (de Brito Alves et al., 2014).

Due to the suggested relationship between oxidative imbalance and several pathologies, various studies have been conducted to clarify how the adult oxidative balance can be affected by early nutritional insult (Ferreira et al., 2015; Simmons, 2006; Tarry-Adkins et al., 2013). However, studies addressing the immediate adaptive response to nutritional insult are still scarce. Our present work was designed to test the hypothesis that a maternal LP diet during gestation and lactation periods affects negatively the mitochondrial bioenergetics and oxidative status in the brainstem of offsprings that this begins immediately after exposure to the maternal protein restriction.

2. Results

2.1. Mitochondrial function

Total mitochondrial protein content was not significantly different between the groups (NP 20.2 ± 6.4 μg/μl x LP 18.3 ± 3.4 μg/μl). However, the brainstem mitochondrial function was affected by the maternal LP diet. In the basal state of mitochondria respiration, LP-weaned rats showed a 66% higher O_2 consumption (10.75 ± 1.6 nmolO_2/mL) when compared to the NP-weaned rats (6.4 ± 1.07 nmolO_2/mL) (Fig. 1). In addition, when mitochondria were stimulated with ADP, the LP-weaned rats were unable to increase respiration to a rate equivalent to the NP-weaned rats, showing a 2.8-(30.6 ± 10.5 nmolO_2/mL) and 11.9-fold increase (77.12 ± 14.33 nmolO_2/mL), respectively (Fig. 1). Thus, LP-weaned animals showed a lower respiratory control ratio (5.83 ± 0.73) when compared to NP-weaned rats (8.30 ± 0.53) (Fig. 1 insert).

Assessing additional mitochondrial functions, we found that the LP-weaned rats produced 228% more ROS than NP-weaned rats (a.u. of fluorescence: LP, from 40.4 ± 1.8 to 95.2 ± 4.3; NP, from 27.4 ± 1.6 to 127 ± 7.5) (Fig. 2(A)), while the $\Delta\Psi_m$ decreased 33% in LP-weaned rats (a.u. of fluorescence: LP, from 2280.6 ± 380.6 to 1528.0 ± 255.0; NP, from 4317 ± 260 to 1683.6 ± 101.5) (Fig. 2(B)).

2.2. Oxidative stress biomarkers

Two oxidative stress biomarkers, malondialdehyde and carbonyl levels were measured. The results have shown that these biomarkers were affected differently by the perinatal LP diet in the weaned rats. While carbonyl levels were augmented by 123% (LP: 26.14 ± 3.4 x NP: 11.70 ± 1.27 μM/mg prot) in LP-weaned rats (Fig. 3(B)), no significant difference was observed in MDA levels between the LP and NP groups (Fig. 3(A)).

2.3. Enzymatic antioxidant system

Oxidative stress can be induced either by an increase in pro-oxidative agents or by a decrease in antioxidant defense. We measured several antioxidant enzymes and the majority showed no significant difference between LP and NP groups (SOD: NP 5.10 ± 0.77 *vs* LP 5.74 ± 0.18 U/mg protein, n=5–8; CAT: NP 1.17 ± 0.03 *vs* LP 1.11 ± 0.03 U/mg protein, n=5–8; GPx: NP 1.26 ± 0.03 *vs* LP 1.21 ± 0.09 U/mg protein, n=5; G6PDH: NP 0.44 ± 0.07 *vs* LP 0.33 ± 0.01 U/mg protein, n=5). Except for the GST activity, which decreased 47% in LP compared to NP (NP 13.9 ± 0.84 *vs* LP 7.3 ± 0.8 U/mg protein, n=5) (Fig. 4(E)).

2.4. Non-enzymatic antioxidant system

The antioxidant defense is constituted by enzymatic and non-enzymatic molecules. Therefore, we also measured the reduced glutathione (GSH), a major antioxidant non-enzymatic, molecule, which is responsible for counterbalancing excess pro-oxidative agents. Our data showed that LP-weaned rats had a 21% decrease

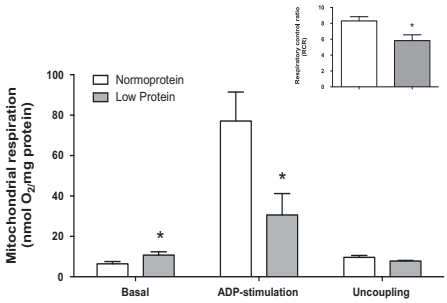

Fig. 1. Mitochondrial respiration capacity. Mitochondrial O_2 consumption in basal state, stimulated with ADP, uncoupled with carbonyl cyanide m-chlorophenyl hydrazone and the respiratory control ratio in brainstem of 22 day old male rats born to dams fed through perinatal period with either normo- (17% casein) or low-protein (8% casein) diet; n=4–8. Values are expressed as means ± SEM. (*$p \leq 0.05$ unpaired Student's *t* test).

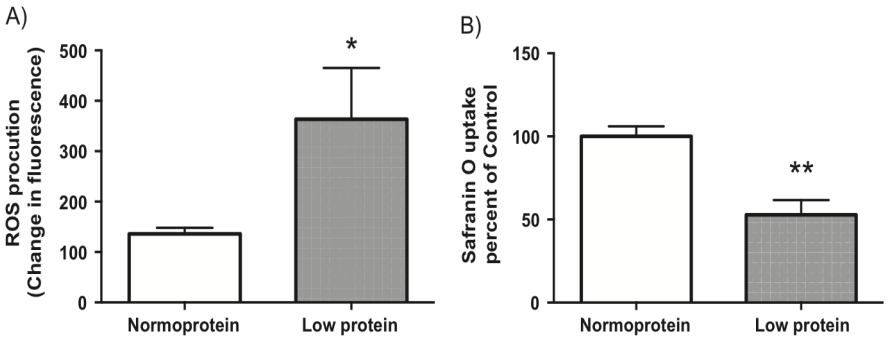

Fig. 2. Mitochondrial function. A) Mitochondrial ROS production and B) Mitochondrial membrane electric potential in the brainstems of 22 day-old male rats born to dams fed through perinatal period with either normo- (17% casein) or low-protein (8% casein) diet; n=4–8. Values are expressed as means ± SEM (*$p \leq 0.05$;**$p \leq 0.01$ unpaired Student's *t* test).

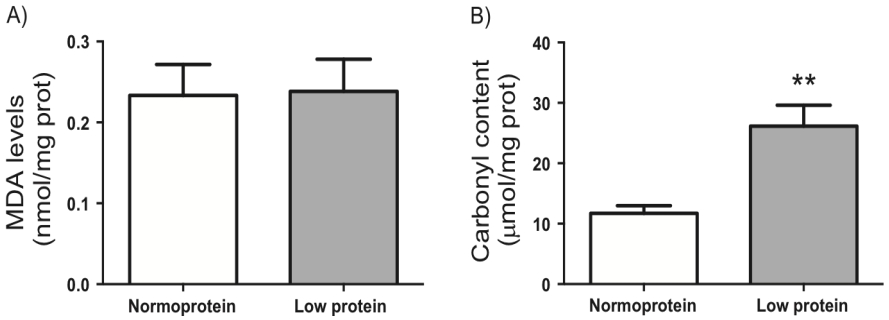

Fig. 3. Oxidative stress biomarkers. A) MDA levels and B) Carbonyl content in the brainstems of 22 day-old male rats born to dams fed throughout perinatal period with either normo- (17% casein) or low-protein (8% casein) diet; n=4–8. Values are expressed as means ± SEM (**$p \leq 0.01$; unpaired Student's *t* test).

in GSH levels (LP: 0.318 ± 0.013 *vs* NP: 0.257 ± 0.007 nmol/mg prot) (Fig. 5(A)), which in association with the increase in its oxidized form, the glutathione disulfide (GSSG) levels (LP: 0.491 ± 0.002 *vs* NP 0.416 ± 0.002), resulted in reduction of the redox state by 32%, (NP: 8.2 ± 0.3 *vs* LP: 5.5 ± 0.2) (Fig. 5(B)). In addition, LP-weaned rats showed a 25% reduction (80.7 ± 4.7 μmol/mg prot) compared to NP-weaned rats (108.9 ± 4.6 μmol/mg prot) in the main H^+ donor to resynthesis of GSH and maintenance of the redox mitochondrial state (Fig. 5(C)).

2.5. Citrate synthase activity

The citrate synthase is an enzyme from the Krebs cycle responsible for the condensation of acetyl-CoA with oxaloacetate forming citrate. Considered as a key step in the cycle, modifications in its activity can regulate the velocity of the cycle and limit the production of reduced co-factor for oxidative phosphorylation at electron transport chain. Our results showed that protein restriction during perinatal period down regulates the activity of this enzyme in 34% (NP: 38.1 ± 3.7 *vs* LP: 25.05 ± 3.1 U/mg prot) in brainstem (Fig. 6).

3. Discussion

The Developmental Origins of Health and Disease model asserts that nutritional insult within development and early ages leads to fetal adaptations in response to the signals in the intrauterine environment, resulting in permanent adjustments to support immediate survival and improve success in an adverse postnatal environment (Barker and Osmond, 1986a; Barker and Osmond, 1986b; Gluckman et al., 2005a; Hanson and Gluckman, 2008). However, inappropriate interpretations of prenatal signals and alterations in the postnatal environment may result in disparities between prenatal projections and postnatal reality (Nascimento et al., 2014; Nettle et al., 2013). In the present study, we examined the immediate effect of a perinatal LP diet on the brainstem mitochondrial bioenergetics and oxidative status, providing new data regarding the immediate response after a nutritional insult that begins in the gestational period. Our findings suggest that the mismatch between the predicted prenatal and the postnatal reality may act synergistically with the protein undernutrition.

Our findings have shown that a maternal LP diet early in development decreased mitochondrial respiratory activity by increasing basal respiration (state 4) and decreasing both the mitochondrial phosphorylation capacity (ADP like-mitochondrial state 3) and the respiratory control ratio, thus impairing overall mitochondrial function. Korshunov et al. (1997) demonstrated that an increase in state 4 is potentially dangerous for the cell due to an increased potential for superoxide formation (Korshunov et al., 1997). In accordance with this concept and supporting our results, are previous data demonstrating that newborn from mothers fed throughout the gestational period with LP diet showed lower phosphorylation capacity in brain (Muzzo et al., 1973), as well as a reduced cytochrome c oxidase activity (Gallagher et al., 2005). This reduction in phosphorylation capacity closely relates with the

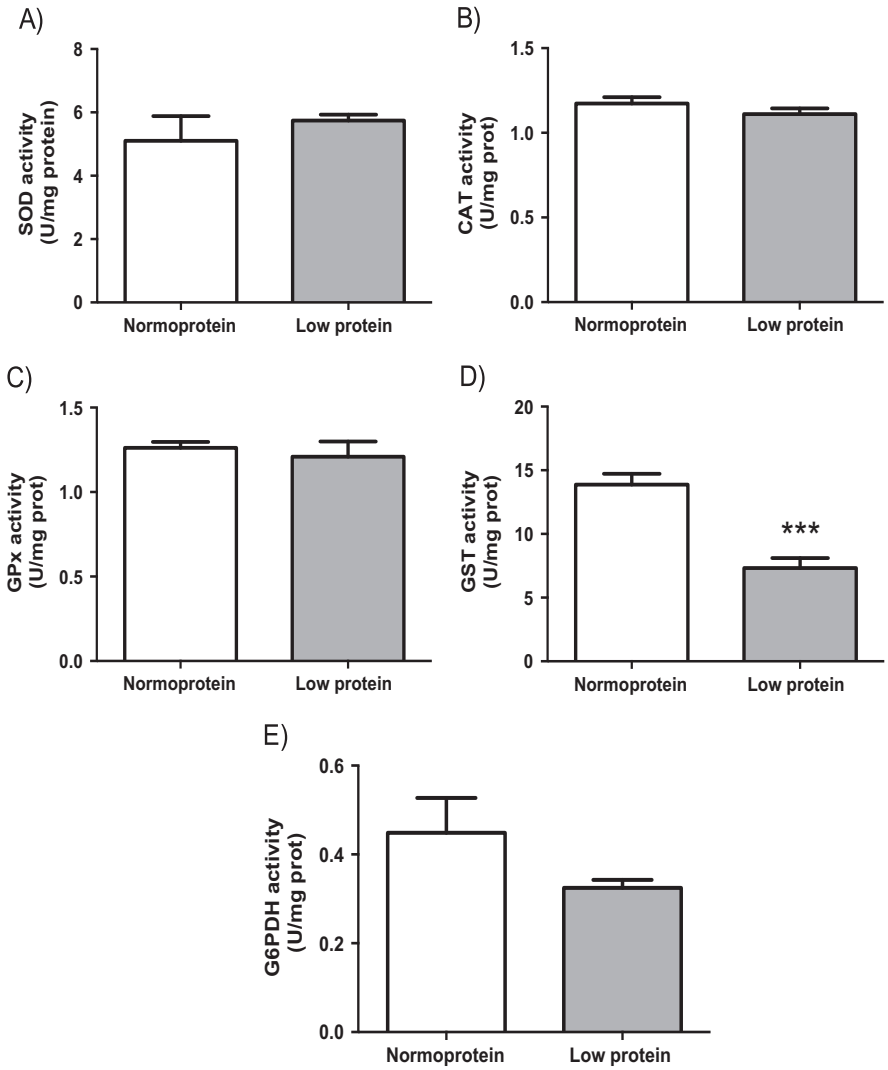

Fig. 4. Enzymatic antioxidant defense. A) Superoxide dismutase (SOD) activity, B) Catalase (CAT) activity, C) Glutathione peroxidase (GPx) activity, D) Glutathione-S-transferase (GST) activity, E) Glucose-6-phosphate dehydrogenase (G6PDH) activity in the brainstems of 22 day-old male rats born to dams fed throughout perinatal period with either normo- (17% casein) or low-protein (8% casein) diet; n=6. Values are means ± SEM (***$p \leq 0.001$ unpaired Student's *t* test).

lower citrate synthase activity found in our study. Since the citrate synthase is the first step in the Krebs cycle, it is expected that reduction in the activity results in diminished $FADH_2$ and NADH cofactors to maintain the electron flow in the ETC (Williamson and Cooper, 1980), fact corroborated by the low levels of NAD(P)H found in our LP animals. In addition, Olorunsogo (1989) reported that early protein restriction for 30 days down-regulated the activity of the succinate dehydrogenase, a complex II enzyme of the electron transport chain (ETC), leading to a lower responsiveness to ADP stimulation (Olorunsogo, 1989). Other studies have shown that defects in the mitochondrial respiratory chain induced either by loss of cytochrome c or ischemia resulted in a low respiration rate and increased superoxide formation (Kudin et al., 2004; Kushnareva et al., 2002; Kussmaul and Hirst, 2006).

With our finding that LP diet during development induced immediate disruption in mitochondrial function, we felt compelled to study ROS production and mitochondrial $\Delta\Psi$m, since the relationship between them and mitochondrial respiration controls the mitochondrion integrity (Starkov and Fiskum, 2003). Our data showed a marked increase in ROS production and mitochondrial depolarization. According to Starkov (2003), the decrease in mitochondrial potential membrane observed in LP-weaned animals, can induce a marked deceleration in the electron flow through mitochondrial complexes which may increase ROS generation (Starkov and Fiskum, 2003). In agreement with this hypothesis, Boveris et al. (1972) define as essential factors for ROS production O_2 viability and electron flow velocity.

The metabolism of ROS is tightly controlled in the cells. The rigid regulation of both production and removal of ROS makes fluctuations in their levels within cells transient and minimal

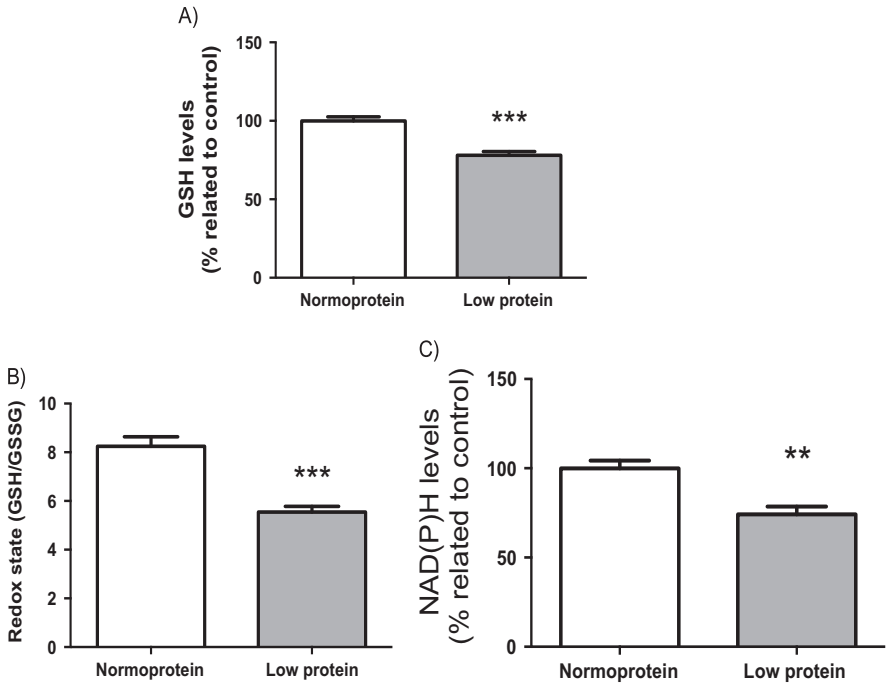

Fig. 5. Non-enzymatic antioxidant system. A) Reduced Glutathione (GSH) levels, B) Redox state and C) Nicotinamide adenine dinucleotide (phosphate) reduced levels in the brainstems of 22 day-old male rats born to dams fed throughout perinatal period with either normo- (17% casein) or low-protein (8% casein) diet; n=4–8. Values are means ± SEM (*$p \leq 0.05$;***$p \leq 0.001$ unpaired Student's *t* test).

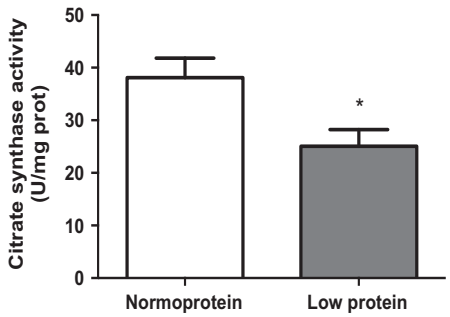

Fig. 6. Enzymatic activity of the citrate synthase in brainstems of 22 day-old male rats born to dams fed period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet) n=6. Values are means ± SEM (*$p \leq 0.05$ unpaired Student's *t* test).

under physiological conditions. However, in some pathological conditions increased levels of ROS production exceed the antioxidant defense system leading to oxidative stress (Halliwell, 1994). The role of these antioxidant systems is to convert ROS into harmless molecules, or at least into less reactive species (Halliwell, 1994). Our analysis of the enzymatic antioxidant system between the LP and NP groups showed no significant differences in 4 out of 5 enzymes studied. There is no consensus about the enzymatic changes in cerebral tissues from rats whose mother received a perinatal LP diet. Feoli et al. (2006) studying the cerebellum, cortex and hippocampus, found a difference in catalase (CAT) activity in rats at 60 days of age in the cerebellum, but not in the other brain areas. The effect on CAT activity present in only one area may be due to the different levels of susceptibility of each brain area to a nutritional insult. In a previous study in our laboratory, conducted with rats at 100 days of age whose mother received LP diet during the gestational period, we observed a significant decrease in the activity of all enzymes analyzed (SOD, CAT, GPx, GST, GR, G6PDH) (Ferreira et al., 2015). We speculate that the difference between these results and our present data is the age of the animals studied, since the present study was conducted with rats 22 days after birth and the previous with adult rats (78 days after the last day of the nutritional manipulation). In the glutathione system, only the GST activity was reduced in the LP-weaned rats, indicating a lower capacity to detox electrophilic compounds (Awasthi et al., 2005; Gajewska et al., 2015). It is noteworthy that GSTs can reduce lipid hydroperoxides through their selenium-independent glutathione-peroxidase activity and can also detoxify lipoperoxidation end-products such as 4-Hydroxynonenal (Singhal et al., 2015). Therefore, it is possible that the LP diet induces immediate mitochondrial disruption by increasing ROS generation through bioenergetics modulation and decreasing the enzymatic defense system, thus leading to cellular damage in the brain.

GSH is the major intracellular thiol involved in the non-enzymatic antioxidant process (Awasthi et al., 2005; Circu and Aw, 2012), and in our model this important component was decreased in the LP-diet group. This finding is in agreement with previous data from our laboratory showing a marked decrease in GSH levels in brainstem and heart of adult offsprings born to LP-treated mothers. It has been demonstrated that thiol groups are easily oxidized by ROS (Stadtman et al., 2005). Some studies suggest that GSH levels are important indicators of the cellular antioxidant capacity (Owen and Butterfield, 2010). Our data that a maternal LP diet decreases GSH level is in agreement with previous studies suggesting that cellular GSH concentrations are markedly reduced in response to protein malnutrition and oxidative stress (Griffith and Mulcahy, 1999; Lu, 2000). Both GSH and NAD(P)H are important small molecules responsible for the redox status in the

cell. As it occurred with GSH, LP diet also reduced the NAD(P)H levels, suggesting that the immediate response to maternal protein diet led to a significant impairment in the redox state, which contributed to the increased oxidative stress (Schafer and Buettner, 2001).

Our present studies showed an increased oxidation of proteins, but not of lipids. The reason why proteins may be preferentially targeted by ROS than lipids is unclear. A possible explanation is that certain proteins may be more susceptible to oxidative damage due to their specific amino-acid composition (Stadtman et al., 2005). Additional, studies with LP diet demonstrate that in animals at 60 days of age, tyrosine and tryptophan can also be affected by low protein diet in several regions of CNS (Feoli et al., 2006; Torres et al., 2010).

Taken together, our data suggest that exposure of the brainstem to the nutritional insult caused by the maternal protein restriction resulted in an immediate response manifested by an increase in oxidative stress. Previous studies with a low-protein diet have found similar results in the cortex (Bonatto et al., 2006) and hippocampus areas of the brain (Bonatto et al., 2005). In conclusion, this study provides new evidence that nutritional insult during the developmental period induces an immediate mitochondrial disruption and oxidative stress in brainstem in response to the maternal low protein insult. We believe that the combined effect of decreased respiration capacity, increased ROS production, decreased antioxidant capacity and redox impairment are the key mechanisms involved in the adverse effects observed in the brainstem of the LP-weaned rats. The damage sustained by the brainstem may interfere with the autonomic regulation, causing alterations in the respiratory frequency, and in the sympathetic and phrenic excitatory responses to peripheral chemoreflex activation, potentially leading to hypertension.

4. Methods and materials

4.1. Ethical standards

Experiments were carried out in accordance with the National Institutes of Health guide for animal experimentation (NIH Publications No. 80–23, revised 1978) and endorsed by the Ethical Committee of the Biological Science Center of the Federal University of Pernambuco (2,307601780/2014–15).

4.2. Animals, diet and experimental groups

Twelve female Albino *Wistar* rats obtained from our Academic Center (Federal University of Pernambuco), maintained at 22 ± 1 °C on a 12 h light-dark cycle (6:00–18:00) were mated in a ratio of 1 male to 2 females. The rats were checked daily and the day of conception was considered when spermatozoa were found in vaginal smear. Thereafter, the pregnant rats were divided into two groups according to their experimental diet. The diets were prepared at the Laboratory of Dietetic Techniques at the Federal University of Pernambuco, according to standards set by the American Institute of Nutrition-93 (Reeves et al., 1993) (Table 1). Although with different protein patterns, normoprotein (NP-17% casein) or low-protein (LP-8% casein), both diets had the same caloric value and were fed throughout the gestation and lactation periods. At 22 days of life, the rats were weaned and euthanized by decapitation. The brainstem was removed in less than one minute and immediately frozen for oxidative status analyses or immediately used for isolation of mitochondria and fresh analysis of mitochondrial bioenergetics (see mitochondria and tissue preparation).

Table 1
Nutritional composition of the diets (g/100 g diet).

Nutrients	Normal-protein (17% protein)	Low-protein (8% protein)
Casein (85%)[a]	20	9.41
Dextrin maize starch	13.2	13.2
Cellulose	5	5
Sucrose	10	10
Maize starch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0.25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

[a] Casein showed 85% purity.

4.3. Preparation of mitochondria

After the brainstems were removed, they were immediately minced and homogenized, using a potter-Elvehjem pestle and glass tube connected to a digital homogenizer IKA® RW 20, in an ice-cold mitochondrial isolation buffer containing 225 mM mannitol, 75 mM sucrose, 4 mM HEPES, 2 mM Taurine and 0.5 mM EGTA, pH 7.4. Subsequently, the samples were centrifuged at 4 °C for 5 min at 4000 RPM, the supernatants were collected and centrifuged once more at 4 °C for 10 min at 13,000 RPM. After the last centrifugation, the pellets were re-suspended in the respiration buffer (RB) consisting of 120 mM KCl, 4 mM HEPES, 5 mM K_2HPO_4 and 0.2% BSA (w/v), pH 7.4. The mitochondria were kept on ice during the assay. This protocol was adapted from Lagranha et al., (Lagranha et al., 2010).

4.4. Measurement of mitochondrial respiration

Mitochondria were incubated in RB (1 mg protein/mL) in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) at 28 °C. The mitochondrial respiration was assessed using Complex I (10 mM glutamate/0.4 mM malate) substrates and the mitochondrial phosphorylation started with 0.8 mM ADP (Nascimento et al., 2014).

4.5. Measurement of total ROS production

The ROS production was assessed by the dihydrodichlorofluorescein diacetate -H(2)DCF-DA method (da Silva et al., 2015; van der Toorn et al., 2007). Briefly, 0.1 mg of mitochondria was incubated in RB with complex I substrates, as described in the mitochondrial respiration section, followed by the addition of 5 μM DCF, which in the presence of reactive species forms a fluorescent product at 485 nm excitation and 530 nm emission. The reaction was followed by gentle shaking for 8 min in FLUOstar OMEGA (BMG Labtech, USA) at 28 °C.

4.6. Mitochondrial membrane potential ($\Delta\Psi m$)

The mitochondrial electrical membrane potential was performed as previously described (Feldkamp et al., 2007; Park et al., 2011). This assay relies on the electric affinity of safranin-O to anions, where the fluorescence decay is related to electrophoretic transportation into the mitochondria. The assay consisted of the addition of 5 μM dye to 0.1 mg mitochondria incubated in RB with complex I substrates, with the safranin fluorescence monitored at 485 nm excitation and 590 nm emission. After the fluorescence stabilized, 5 μM Carbonyl cyanide *m*-chlorophenyl hydrazone

(CCCP) was added to dissipate the $\Delta\Psi$. The analysis was performed at 28 °C in a FLUOstar OMEGA (BMG Labtech, USA) with gentle agitation.

4.7. Sample preparation for oxidative stress and antioxidant analyses

After frozen, brainstems from NP and LP rats were immersed in cold buffer containing 50 mM TRIS and 1 mM EDTA, pH 7.4, with the addition of 1 mM sodium orthovanadate and 200 μg/mL phenylmethanesulfonyl fluoride. Then they were homogenized with digital homogenizer IKA® RW 20 using a potter-Elvehjem pestle and glass tube on ice for no more than 1 min. Homogenates were centrifuged at 4000 RPM for 10 min at 4 °C and the protein used for oxidative status analyses after determination of protein concentration using the Bradford method (Bradford, 1976).

4.8. Evaluation of lipid peroxidation

Lipid peroxidation was analyzed using malondialdehyde (MDA) levels as previously published (Buege and Aust, 1978). Three hundred μg protein was sequentially mixed to 30% (w/v) Trichloroacetic acid (TCA) and 10 mM TRIS buffer, pH 7.4. This mixture was centrifuged at 2500x*g* for 10 min and the supernatant was boiled for 15 min with 0.73% (w/v) thiobarbituric acid. The pink pigment yielded then was measured at 535 nm absorption at RT and expressed as nmol/mg protein.

4.9. Determination of protein oxidation

The protein oxidation was assessed using the procedures highlighted by Reznick and Packer (1994). With the samples on ice, 30% (w/v) TCA was added to the sample and then centrifuged for 14 min at 4000 RPM. The pellet was re-suspended in 10 mM 2,4dinitrophenylhydrazine and immediately incubated in a dark-room for 1 h with agitation every 15 min Samples were washed and centrifuged three times in ethyl/acetate buffer and the final pellet was re-suspended in 6 M guanidine hydrochloride, incubated for 30 min at 37 °C and the absorbance read at 370 nm. The results were expressed as μM/mg protein.

4.10. Measurement of superoxide dismutase (SOD) activity

SOD determination was performed in accordance with the protocol developed by Misra and Fridovich (1972). In brief, 300 μg of protein were added to 0.05 M Carbonate buffer with 0.1 mM EDTA, pH 10.2. The reaction was started with 150 mM epinephrine and the SOD activity was determined by adrenaline auto-oxidation inhibition at 30 °C. The decrease in absorbance was followed for 1.5 min at 480 nm and the results expressed as U/mg protein (Misra and Fridovich, 1972).

4.11. Measurement of catalase (CAT) activity

The CAT activity was performed as previously described by Aebi (1984). Briefly, 0.3 M hydrogen peroxide and 300 μg protein were added to a 50 mM phosphate buffer, pH 7.0 at 20 °C and the absorption decay was monitored for 3 min at 240 nm with the results expressed as U/mg protein (Aebi, 1984).

4.12. Measurement of glutathione peroxidase (GPx) activity

GPx activity was performed in accordance with Paglia and Valentine (1967). Briefly, 300 μg of protein was added to a 50 mM phosphate buffer, pH 7.0 containing 5 mM EDTA; 0.28 mM NADPH; 3.75 mM sodium azide; 5 mM glutathione reduced (GSH) and glutathione reductase from Sigma (St. Louis, MO). The reaction was started with 2.2 mM H_2O_2. NADPH oxidation followed at 340 nm absorbance at 20 °C and its coefficient of extinction was used to determine the GPx activity as U/mg protein (Paglia and Valentine, 1967).

4.13. Measurement of glutathione-S-transferase (GST) activity

GST activity was measured as described previously by Habig, Pabst (1974) (Habig et al., 1974b). Two hundred μg of protein was incubated in a 0.1 M phosphate buffer, pH 6.5 containing 1 mM EDTA at 30 °C and the assay started with the addition of 1 mM 1 chloro-2.4-dinitrobenzene and 1 mM GSH. The formation of 2.4-dinitrophenyl-S-glutathione was monitored through the absorbance at 340 nm. One unit of enzymatic activity was defined as the amount of protein required to catalyze the formation of 1 μmol 2.4-dinitrophenyl-S-glutathione (Habig et al., 1974a).

4.14. Measurement of glucose-6-phosphate dehydrogenase (G6PDH) activity

G6PDH activity was measured as previously described by Löhr and Waller (1974). Briefly, 300 μg of protein was added to a 30 mM TRIS buffer, pH 7.6 containing 1 mM EDTA; 5 mM $MgSO_4 \cdot 7H_2O$; 0.5% Triton X-100 (v/v) and 0.5 mM $NADP^+$. The reaction was started with addition of glucose-6-phosphate and the NADPH formation rate was monitored for 5 min at 340 nm absorbance. The results were expressed as U/mg protein (Löhr and Waller, 1974).

4.15. Measurement of reduced glutathione (GSH) and oxidized glutathione (GSSG)

To assess GSH levels, the samples were firstly diluted in a 0.1 M phosphate buffer containing 5 mM EDTA, pH 8.0. Then, an aliquot from the diluted sample was incubated with o-Phthaldialdehyde at room temperature for exactly 15 min Fluorescence intensities measured at 420 nm and excitation at 350 nm were compared with a standard curve of known concentrations of GSH. To determine GSSG levels, the samples were incubated with 0.04 M N-ethylmaleimide for 30 min in RT followed by addition of 0.1 M NaOH buffer. When this mixture was ready, the same steps of the GSH assay were followed to determine the GSSG levels. The Redox State was determined by the ratio of GSH/GSSG as previously described (Hissin and Hilf, 1976).

4.16. Measurement of nicotinamide adenine dinucleotide (phosphate) reduced (NAD(P)H)

The levels of NAD(P)H were assessed based in their abilities to absorb light. Briefly, 100 μg of protein were added to a 50 mM Tris–HCl buffer and the absorbance at 340 nm compared with a standard curve with known concentrations of NADH (Boveris et al., 1972).

4.17. Measurement of citrate synthase activity

CS is the first enzyme in the Krebs cycle and catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate (Wiegand and Remington, 1986). Enzymatic activity was determined as described previously (Le Page et al., 2009). Briefly, the citrate synthase activity was in a reaction mixture containing (in mmol/L) 100 Tris. HCl (pH 8.2), 1 MgCl2, 1 EDTA, 0.2 5,5-dithio-bis (2-nitrobenzoic acid) ($\varepsilon = 13.6$ μmol/(mL cm^{-1}), 3 acetyl-CoA, 5 oxaloacetate and 0.3 mg/mL homogenate. The citrate synthase activity was measured by assessing the rate of change in absorbance at 412 nm over 3 min (30-s intervals)(da Silva et al., 2015).

Note: All reagents used were obtained from Sigma.

4.18. Statistics

All values are expressed as means ± SEM. Once the data were tested for normal distribution, a Student *t* test was used to assess the differences between groups. Comparisons were considered statistically significant at $p \leq 0.05$ and statistical analyses were performed using GraphPad Prism 6.0® software (GraphPad Software, Inbc.).

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5.2 2º ARTIGO ORIGINAL

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Mitochondrial dysfunction: Early nutritional insults as a trigger of late reactive species overproduction and brainstem energy failure

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Mitochondrial dysfunction: Early nutritional insults as a trigger of late reactive species overproduction and brainstem energy failure

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Abstract

Mitochondria are important organelles in eukaryotic organisms, wherein their capacity to produce energy vary among the tissues depending upon the amounts of oxygen consumed. Part of the oxygen consumed during ATP generation produces reactive oxygen species (ROS), which if not efficiently removed can trigger a systemic damage to molecular compounds characterized as oxidative stress. Several studies have demonstrated that mitochondrial dysfunction and oxidative stress in the central nervous system (CNS) are related to a plethora of neural disorders. In the brainstem, mitochondria impairment has been suggested to be involved with autonomic imbalance-induced hypertension, which has the most prevalence in low- and middle-income countries. Since the poor nutritional supply is a common issue in those countries, herein, we investigated the long-lasting effects of protein restriction during the critical period of brain development on several mitochondrial function parameters and oxidative stress hallmark in the brainstem of adult (i.e. 150 days of age) male rats. Maternal protein restriction induced an extended detrimental modulation in mitochondria function, decreasing the phosphorylation capacity with concomitant decrease in $\Delta\Psi m$, wherein the ROS overproduction-induced oxidative damage triggered a disruption in proton conductance, which may gradually compromise mitochondria energy conservation and likely culminate in brainstem dysfunction throughout ageing.

Keywords: Brainstem; Mitochondria bioenergetics; Oxidative stress; Protein restriction; Reactive Oxygen species

Introduction

Mitochondria are dynamic organelles that perform several interconnected actions (1). In eukaryotic cells, mitochondria represent the most important ATP producer, wherein the transfer of electrons from NADH and $FADH_2$ through the electron transport chain (ETC) is used to pump protons (complexes I, III and IV) across the inner mitochondrial membrane to create the mitochondrial membrane potential ($\Delta\Psi m$) (2), which is used to drive back protons through ATP-synthase while consume O_2 and condensate ADP + Pi (3, 4).

In addition, mitochondria are also the major source of Reactive Oxygen Species (ROS). Complexes I and III are thought to be the main producers, wherein the O_2 concentration and the electron flow velocity through the complexes can saturated ETC with electrons, which favors their direct transference to O_2 to generate ROS (9, 10). In normal conditions, the ROS produced are constantly quenched by the antioxidant system (enzymatic and non-enzymatic), however, the chronic imbalance between production and removal, is a metabolic status related to several pathologies and characterized by the oxidative damage to lipids, protein and DNA (11), known as oxidative stress. In the brain, the high O_2 consumption combined to its specialized characteristics, such as, prevalence of polyunsaturated fatty acid, amine content, ROS production by monoamine oxidase and the presence of excitotoxic amino acids, make this tissue particularly vulnerable to oxidative damage (12).

Numerous studies have demonstrated that mitochondria dysfunction is the hub of several neuronal disorders. In Alzheimer disease, mitochondrial complexes I and III from brain have been shown to have their activities down-regulated (13); in striatal neurons, dysfunction in the complexes I and II have been related to Parkinson and Huntington, respectively (14, 15); in multiple sclerosis, the axonal degeneration is commonly associated with reduction in the mitochondrial ATP production (16), which can be enhanced by further

mitochondrial damage (17). Central mitochondria dysfunction is also involved in cardiac impairments (18, 19), wherein the brainstem stands out in the regulation of cardiovascular homeostasis and systemic blood pressure control by modulating the sympathetic vasomotor tone (20, 21).

Studies from our department have demonstrated that nutritional impairment (protein restriction), in early life, can elevate the sympathetic activity and increase the blood pressure later in life (22, 23). Additionally, we also demonstrated that nutritional insult impairs the antioxidant capacity in the brainstem in adult animals (24), and recently, we have highlighted the association between hypertension and oxidative stress at transcriptional and functional levels in medulla oblongata from animals that received low protein diet during development (25). To better understand how nutritional insult target mitochondria bioenergetics, we studied male adult animals (i.e 150 days of age) that were protein restricted during their maternal life to access mitochondrial fundamental coupling states, proton circuit uncoupling as well as the RS production and removal.

Materials and methods

Animals, diet and experimental groups

Twelve pregnant *Wistar* rats were mated (1 male for 2 female) and the pregnant rats were divided into two groups of experimental diets, Normoprotein (17% of casein) or Low protein (8% of casein). Both diets had the same energetic value and were provided *ad libitum* throughout gestation and lactation period (26). At weaning, the male pups were housed, four per cage, and received commercial chow (Labina; Purina Agriband) until 150 days of life, when their brainstems were collected and the mitochondria function and oxidative parameters

evaluated. All the experiments were carried out in accordance with the National Institutes of Health guide for animal experimentation (NIH Publications No. 80-23, revised 1978) and endorsed by the Ethical Committee of the Biologic Science Center of Federal University of Pernambuco (2307601780/2014-15).

Mitochondrial preparations and bioenergetics assessment

The mitochondria brainstems were isolated by differential centrifugation, 1,180 g and 12,470 g for 5 and 10 minutes, respectively, as previously described (27). Briefly, mitochondria suspended in respiration buffer (RB) (pH 7.4) were analyzed at a 1 mg protein/mL concentration, at a constant 28ºC, and the rate of oxygen consumption was measured by using a high-resolution 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). Respiratory capacities were evaluated by using complex I (10 mM glutamate and 0.4 mM malate) and complex II (5 mM succinate + 4 µM rotenone) substrates followed by addition of 0.8mM ADP to measure the oxidative phosphorylation capacity. To assess the state 4 and the maximal non-phosphorylating respiration, it was sequentially added: 1.2 µM oligomycin and 5 µM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP).

Measurement of mitochondrial RS production

The ROS production was assessed by the Dichlorofluorescein diacetate (DCFDA) method (28). Mitochondria at 0.5 mg/mL were incubated in RB at 28ºC with complex I substrates and 5 µM DCFDA, then the fluorescence production was followed at 485 nm excitation and 530 nm emission for 8 min in FLUOstar OMEGA (BMG labtech, USA) with gentle shake.

Mitochondrial membrane potential (ΔΨ) determination

The mitochondrial membrane potential was performed as previously described (29). The assay was performed by adding 5 µM safranin-O dye to 0.5 mg/mL mitochondria incubated in RB with complex I substrates, wherein the safranin fluorescence was monitored at 485 nm excitation and 590 nm emission. Since the fluorescence achieved the state steady, 5 µM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added to dissipate the ΔΨ. The analysis was performed at 28ºC in FLUOstar OMEGA (BMG labtech, USA) with gentle shake.

RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

The RNA was obtained from brainstems by the guanidine isothiocyanate extraction method (30), using TRIzol Reagent, in accord to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Shortly, 1 mL of trizol was used to homogenized samples, after incubated 5 minutes at RT in order to allow the complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL) was added and the sample centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to another 1.5 ml tubes and was added 0.5 mL of cold isopropyl alcohol and ammonium acetate (2M) (31). A new centrifugation was performed and the RNA pellet washed with 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. At the end, the RNA pellet was air-dried, dissolved in DEPC RNase-free water and stored at -20°C. RNA was quantified by measuring absorbance at 260 nm and it is purity assessed by the 260/280 nm ratio (32, 33).

RT-PCR reactions were carried out using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase protocol (Invitrogen, USA). The reaction was performed

containing: 200ng of RNA, 2x Reaction mix, 10 µM of each primers, SuperScript® III RT/Platinum® Taq mix and DEPC ultrapure water. The samples were processed in triplicate and normalized using β2-microglobulin (β2M) gene as internal control (34-36). The visualization of the PCR amplification products, the amplification reactions were carried out in pre-cast Ex-Gel® with SYBR® Safe system and following manufacture's proceedings.

Table 1. Primers used for PCR analysis

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
UCP2	TACTCTCCTGAAAGCCAACC	GCTGCTATAGGTGACAAAC
B2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG

Homogenate preparation for biochemical assays

Frozen rat brainstems were homogenized in buffer containing 50 mM-TRIS and 1 mM EDTA, pH 7.4, with the addition of 1 mM sodium orthovanadate and 200 µg/mL phenylmethanesulfonyl fluoride, followed by the protein determination (37).

Measurement of citrate synthase activity

The citrate synthase (CS) is the first enzyme in the Krebs cycle and catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate (38). Its activity was accessed as previously described (39). Briefly, in a reaction mixture containing in (mM): 100 Tris-HCl (pH 8.2), 1 MgCl2, 1 EDTA, 0.2 5,5-dithio-bis(2-nitrobenzoic acid) (□=13.6 □mol/(mL.cm−1), 3 acetyl-CoA, 5 oxaloacetate and 0.3 mg/mL homogenate. The enzymatic activity was measured by assessing the rate of the absorbance change at 412 nm during 3 min with 30s intervals (40).

Measurement of Nicotinamide Adenine Dinucleotide (Phosphate) Reduced (NAD(P)H)

The levels of NAD(P)H were assessed based on their abilities to absorb light at 340 nm. Briefly, 100 µg of protein were added to 50 mM TRIS-HCl buffer and the absorbance produced compared with a standard curve with known concentrations of NADH (41)

Evaluation of Lipid Peroxidation

Lipid peroxidation was evaluated by assessing the malondialdehyde (MDA) levels as previously published (42). Three hundred µg of protein was sequentially mixed to 30% (w/v) Trichloroacetic acid (TCA) and 10 mM-TRIS buffer (pH 7.4). This mixture was centrifuged at 2,500 g for 10 min and the supernatant transferred to a glass tube with an equal volume of 0.73% (w/v) thiobarbituric acid. The mixture was boiled for 15 min and the pink pigment yielded, measured at 535 nm absorption and expressed at mmol/mg protein.

Measurement of nitrate and nitrite levels

The amounts of nitrate and nitrite were measured as described in Green (1982) (43). Briefly, the Griess method was performed by using Sulfanilamide (13.8 mM) and N-(1-Naphthyl) ethylenediamine dihydrochloride (0.9 mM) incubated for 10 minutes at 25ºC with 500µg of protein samples. The absorbance of the samples was read at 540 nm. The results were compared with a standard curve with known values of sodium nitrite (1.6 – 13.1 µM).

Measurement of superoxide dismutase (SOD) activity

SOD determination was performed in accordance to Misra and Fridovich (1972). In brief, 300 µg of protein were added to 0.05 M-Carbonate buffer with 0.1 mM-EDTA (pH 10.2). The reaction was started by addition of 150 mM-epinephrine and the SOD activity was determined by the epinephrine auto-oxidation inhibition at 30°C. The decrease in absorbance was followed for 1.5 min at 480 nm and the epinephrine extinction factor used to express the results as U/mg protein (44).

Measurement of Catalase (CAT) activity

The CAT activity was performed as previously described by Aebi (1984). Protein supernatants, 300 µg, were added to 50 mM-phosphate buffer (pH 7.0) and 0.3 M-hydrogen peroxide oxidation followed at 240 nm for 3 min at 20° C being the results expressed as U/mg protein (45).

Measurement of Glutathione peroxidase (GPx) activity

GPx activity was performed in accordance to Paglia and Valentine (1967). Briefly, 300 µg of protein was added to a medium containing: 50 mM-phosphate buffer (pH 7.0), 5 mM-EDTA, 0.28 mM-NADPH, 3.75 mM-sodium azide, 5 mM-glutathione reduced (GSH) and glutathione reductase from Sigma (St. Louis, MO). To start the reaction, 2.2 mM-H_2O_2 was added and the NADPH oxidation followed at 340 nm for 4 minutes at 20° C, wherein its molecular extinct factor was used to determine the GPx activity as U/mg protein (46).

Measurement of Redox State (GSH/GSSH)

We first analyzed each glutathione compound separately. To assess GSH levels, the samples were diluted 10 times in 0.1 M phosphate buffer containing 5 mM-EDTA (pH 8.0). Then, an aliquot from the diluted sample was incubated with 1mg/ml o-Phthaldialdehyde (OPT) at room temperature for 15 min and the fluorescence intensities assessed at 350 excitation and 420 nm emission.

To determine GSSG levels, the samples were incubated with 0.04 M N-ethylmaleimid for 30 min in RT followed by addition of 0.1 M NaOH buffer. Once this mixture was prepared, sample aliquots were incubated with OPT and had their fluorescence assessed by using the same parameters as in GSH. Both analyses were compared to a standard curve with known concentrations of GSH and GSSG and the Redox State determined by the ratio of GSH/GSSG (47).

Western blot analysis for nNOS protein

Protein expression was carried out as previously described (48). Briefly, after protein (15 µg), running in SDS poly-acrylamide gel at 8%, the membrane was incubated overnight with a rabbit polyclonal against nNOS (rabbit polyclonal IgG, Santa Cruz Biotechnology, sc-1025) in 5% defatted milk. Thereafter, the membrane was washed 3 times and incubated with secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology) at RT for 4 hours, washed three times again, exposed to ECL solution (Pierce® ECL Western Blotting substrate – Thermo Scientific) for 5 minutes and immediately exposed to X-ray film. Gel transfer efficiency and equal load was verified using reversible Ponceau S staining (da Silva 2015). The band intensities were measured in ImageJ 1.51f (NIH, Maryland, USA).

Statistical Analysis

All data were tested for the normal distribution and then a t-Student test was used to assess the differences between two groups. The statistical analyses were performed using GraphPad Prism 6.0® software (GraphPad Software, Inbc.) and comparisons were considered statistically significant at $p \leq 0.05$. All values are expressed as means ± SEM (n=4-6).

Results

Mitochondrial bioenergetics

To investigate the long-lasting effects of a maternal protein restriction on brainstem mitochondrial function, oxygen consumption was investigated with both complex I and complex II substrates (figure 1a and 1b, respectively). With NADH-related substrates, under basal condition (i.e. only in the presence of the glutamate and malate), mitochondria from LP animals consume more O_2 than NP animals (NP= 3.66 ± 0.42 vs LP= 5.52 ± 0.62 nmol O2/min/mg protein, p=0.03) as shown in the first panel of figure 1a. When we stimulated ATP synthase function by adding ADP, O_2 consumption in the NP group was now higher than in the LP group (NP= 15.67 ± 1.74 vs LP= 10.23 ± 1.48 nmol O2/min/mg protein, p= 0.05), which indicates an impairment in the ATP turnover and/or substrate oxidation. In state 4 (or resting state) and uncoupled state, no further differences were found between the groups (figure1a). In parallel, mitochondria incubated with complex II substrate and rotenone, showed no differences in their O_2 basal state consumption, while the state 3 presented similar patterns to those mitochondria fed with NADH substrates (NP= 21.20 ± 1.71 vs LP= 16.05 ± 1.28 nmol O2/min/mg protein, p=0.05) (figure1b). In addition, in contrast from the NADH

substrates incubation (figure 1b) LP mitochondria incubated with succinate had higher O_2 consumption in state 4 (NP= 5.24 ± 0.62 vs LP= 7.42 ± 0.36 nmol O2/min/mg protein, p= 0.02), and uncoupled (NP= 13.48 ± 1.28 vs LP= 18.73 ± 1.11nmol O2/min/mg protein, p= 0.02). Although there were some different behaviors with each substrate, the overall impairments triggered by protein restriction culminates in a decrease of the respiratory controls ratio in both conditions, 53% and 47% (figure 1a and 1b inserts).

Mitochondrial membrane potential (ΔΨm) and uncoupling protein

Several methods can be used to evaluate the membrane potential, herein we used the safranin-o uptake to estimate the electron difference between the mitochondrial spaces. In the assay, we evaluated the fluorescence decay, which has been correlated with the safranin attachment to internal mitochondria membrane in response to the increase in membrane potential. The animals that suffered protein restriction presented lower safranin-o uptake when compared to normoprotein animals (NP= 35.61 ± 4.8 vs LP= 19.32 ± 2.43 % safranin uptake, p= 0.02) (figure 2a). Because ΔΨm can be regulated by uncoupling protein, which consumes pmf without ATP formation, we evaluated the mRNA of UCP2. Our results demonstrated that UCP2 expression was expressed at higher levels in the brainstems of LP animals than in NP (NP= 0.063 ± 0.003 vs LP= 0.204 ± 0.043, p= 0.03) (figure 2b).

Substrate Oxidation

Since the oxidative capacity was reduced in the LP groups, we wondered if the mitochondrial ETC was dysfunctional. The electrons used in the ETC come from reduced co-factors, whose are mainly produced in Krebs' cycle. We performed assays that correlate with the Krebs

cycle flow and indicate the amounts of reduced NAD(P), as an important ETC electrons donor. Therefore, we evaluated the activity of citrate synthase and NAD(P)H levels. Both citrate synthase activity as well as NAD(P)H levels were not altered in the experimental groups (figures 2a and 2b).

RS production and Oxidative hallmarks

Having verified the oxidative capacity, ΔΨm and its uncoupling, we decided to evaluate the production of RS. It is well described that mitochondrial RS production is modulated by ΔΨm fluctuations and uncoupling parameters. Mitochondria isolated from LP animals presented higher production of RS than NP animals (NP=41.38 ±15.49 vs LP=156.6 ± 46.67 a.u., p= 0.05) (figure 4a), concomitantly, the LP animals also increased their nitrate and nitrite levels (NP=15.11 ± 1.09 vs LP=21.36 ± 0.21 µM/mg protein, p= 0.005) (figure 4b). In order to assess the source of the higher production of NO by LP animals, we evaluated the nNOS protein expression, which showed to be augmented almost 3 times (NP=0.0056 ± 0.003 vs LP=0.025 ± 0.006, p= 0.01) (figure 4c).

The production of reactive species (oxygen and nitric) as well as their interaction (superoxide and nitric oxide), culminated in oxidative damage, which was also demonstrated by lipids peroxidation assay from LP animals (NP=0.013 ± 0.0008 vs LP=0.024 ± 0.0003 mM/mg protein, p= 0.0003) (figure 4d).

Antioxidant analyses

In physiological conditions, the RS produced are constantly removed by the antioxidant system residing in both enzymatic and non-enzymatic capacity. The antioxidant system

quenches the RS by converting them to less harmful RS or water. The main enzymatic cascade involved in RS fighting was examined and we showed that maternal protein restriction promoted a decrease only in the activity of the superoxide dismutase (SOD) (NP=6.26 ± 0.24 vs LP= 4.83 ± 0.47 U/mg protein, p= 0.05) (figure 5a), without differences in CAT (p= 0.52) or GPx (p= 0.57). Additionally, we did not observe changes in the non-enzymatic antioxidant system (figure 5d, e).

Discussion

Studies have correlated mitochondria health with the proper function of several tissues, including the CNS. Due to the mitochondria involvement in a plethora of important cellular processes, different approaches have been used to study their dysfunction in order to further associations with illness causes or consequences. However, a variety of events can differently modulate the functions performed by mitochondria. Herein, we examined the two main mitochondrial functions (i.e mitochondrial respiratory control and oxidative balance) in order to understand how nutritional impairments in early life can affect adult organisms.

Mitochondrial dysfunction induced by maternal protein restriction

Oxygen consumption is a classical experiment to verify mitochondrial bioenergetics function. The respiratory control ratio represents an index of mitochondrial function (6). Herein, in both substrates used (complex I and complex II + rotenone), LP animals had their RCR reduced, indicating a decrease in the phosphorylate capacity.

The absolute respiration rate is a valuable tool to enhance understanding of mitochondrial bioenergetics. Once basal O_2 consumption was established, the state 3 is generated by the

addition of ADP, which accelerates the electron transport through the ETC complex while consumes O_2 and produces ATP (49). Our data demonstrated that mitochondria from LP animals were not capable to increase their O_2 consumption at the same rate as the NP animals, which in turn, does not allow LP mitochondria to reach higher energy demands when required. As described by Brand and Nicholls (6), the state 3 rate is strongly related to the ATP synthase function, being essentially modulated by the tissue-dependent ATP turnover and substrate oxidation. A previous study, in weaned rats, demonstrated that the protein restriction-induced phosphorylation impairment was related to a deceleration in Kreb's cycle and lower NAD(P)H supplies (27) after insult period, however at 150 days, none of these parameters were modulated, indicating an ATP synthase dysfunction.

Substrate oxidation also modulates O_2 consumption in the uncoupling state, wherein lower O_2 rates are associated to dysfunction in ETC elements, while its increase, compared to state 3, has been described in tissues with reduced ATP synthase activity (50). In mitochondria incubated with complex I substrates, no difference was found between groups, besides the increase trend in the LP (figure 1a). However, in complex II substrate incubation, the trend of modification became statistically significant, and the LP animals presented a higher O_2 consumption than NP. As highlighted by Nicholls (1997), mitochondrial substrates that donate electrons to the ubiquinone pool (e.g. succinate) have higher respiration rate than the NADH-substrates, in order to compensate the fewer protons pumped to intermembrane space (51), likely the difference was only verified in succinate substrate. Furthermore, succinate substrate also provided a higher O_2 consumption in LP resting mitochondria, which in turn is essentially modulated by the leak of protons, and indicates an inappropriate conductance of protons (6).

Our assumption regard the proton conductance impairment was confirmed by the lower $\Delta\Psi m$ in the LP animals (figure 2a), which indicates the hefty protein restriction-induced

mitochondrial uncoupling. In normal conditions, most protons reentry into mitochondrial matrix across the ATP synthase with concomitant ATP production and pmf consume, however, other components are also capable of consume pmf without ATP formation (56). Characterized as mitochondrial uncoupling, it relies on internal proteins such as adenine nucleotide translocase (ANT) and glutamate carrier, mitochondrial permeability transition pore and mitochondrial uncoupling protein (UCP) (56), which in turn, showed to be higher expressed in LP animals. The increase in UCP expression highlights the pmf disengagement, being a feasible mechanism involved in the mitochondrial bioenergetics impairment. Besides, the UCP isoform 2 is also related to the control of mitochondrial reactive oxygen species production and oxidative stress (57), wherein several studies have already suggested their relation with the maternal protein restriction-induced health disorders (25, 27, 58-60).

Oxidative stress and Mitochondrial Uncoupling

Beyond ATP production, the mitochondrion is also the main reactive species producer (61, 62). In the brain, the histological and physiological characteristics, such as, high O_2 consumption, large presence of excitatory neurotransmitters, high content of polyunsatured fatty acid (PUFA) and superoxide non-dependent hydrogen peroxide production, increase its vulnerability to oxidative damage (12). Studies have been demonstrated that RS overproduction and oxidative stress-induced mitochondrial impairment is related to several neuronal disorders (13, 14, 63). Superoxide and NO interaction-induced oxidative stress, in brainstem, have been associated also with baroreflex disruption (64), increase in the neuro-humoral activation (65), glutamate cytotoxicity (66), modulation of pressor responses (67) as well as hypertension and stroke (68, 69). Herein, the oxygen and nitric reactive overproduction combined with the inappropriate antioxidant fight (reduction in superoxide

dismutation) may be the feasible mechanism that culminated in the oxidative stress in the LP animals. Our data demonstrate that maternal protein restriction decreases the superoxide anion removal (Figure 5a), which in turn, may enhance the production of potent pro-oxidant elements (i.e. peroxynitrite and hydroxyl radical) by allowing longer interaction between $O_2^{\bullet-}$ and NO (11, 70).

Murphy et al. (2003) demonstrated that the superoxide can activate UCP (71). The increase in UCP activity diminishes the ΔΨm, which in turn, stimulates the electrical flux through ETC, thereby decreasing $O_2^{\bullet-}$ production (56). Furthermore, oxidative stress can also been involved in the UCP activation, wherein derivatives from lipid peroxidation increase UCP proton conductance in order to modulate ROS production (72). It seems that UCP function fluctuates according with redox environment. As described by Ryan (2011), ROS-dependent UCP activation might rely on reversible glutathionylation mechanisms, however, during prolonged periods of ROS overproduction, UCP uncoupling is kept deactivated to avoid a breakdown on the mitochondrial membrane potential (57). In parallel, Azzu (2008), proposed that products from lipid peroxidation (e.g. 4-hydroxy-2-nonenal) can activates UCP uncoupling irreversibly (73). In the brainstem, studies from our lab have been demonstrating that maternal protein restriction increases the mitochondrial RS production in young animals (e.g. 22 days of life) while decreases ΔΨm as well as the non-enzymatic antioxidant defense (27), and even though the RS overproduction, the oxidative damage to lipid is noticeable only later in life (e.g. older than 90) (24, 25).

Thereby, in the present study we propose a feasible mechanism related to the mitochondrial dysfunction induced by nutritional restriction in early life that the effects are still present in adulthood. We demonstrated here that the decrease in ΔΨm is a proton leaking-induced attempt to deal with the mitochondrial RS overproduction, however, the cogent $O_2^{\bullet-}$ and NO interaction-dependent oxidative damage reinforces proton uncoupling. The deregulation of

the proton circuit culminates in the mitochondria bioenergetics impairment, which may be the responsible for the brainstem pathogenesis induced by early nutritional restriction insults.

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Figure legends

Figure 1: Mitochondrial fundamental coupling states of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet, circles) or 8% casein (low-protein diet, squares). Mitochondrial O_2 consumption in basal state, stimulated with ADP, resting with oligomycin, uncoupled with carbonyl cyanide m-chlorophenyl hydrazone and respiratory control ratio in: (a) complex I and (b) complex II substrates. Data of Values are expressed as means ± SEM. (*p ≤ 0.05; **p ≤ 0.01 unpaired Student's *t* test), n=4-6.

Figure 2: Mitochondrial membrane potential and electrical uncoupling in the brainstems of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Percentage of Safranin O uptake; and (b) UCP2 mRNA expression. Values are expressed as means ± SEM (*p ≤ 0.05; **p ≤ 0.01 unpaired Student's *t* test), n=4-6.

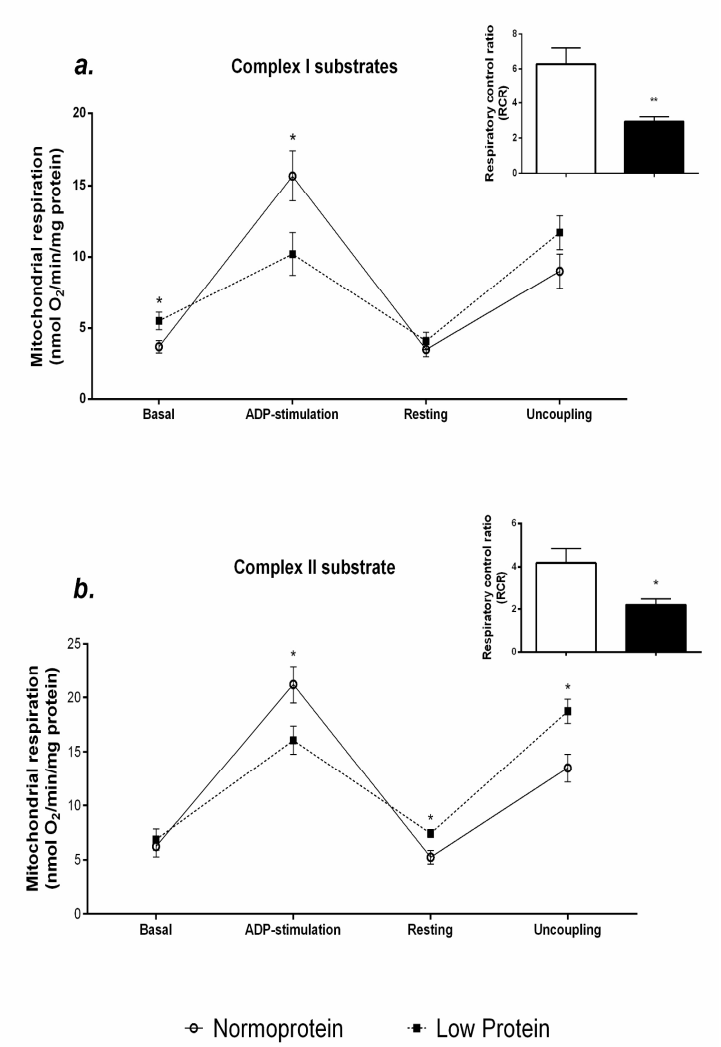
Figure 3: Mitochondrial substrate oxidation in the brainstems of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Citrate synthase activity and (b) content of NAD(P)H. Values are expressed as means ± SEM (*p ≤ 0.05) unpaired Student's *t* test), n=4-6.

Figure 4: Reactive Species productions and Oxidative stress in the brainstems of 150 days male rats born to dams fed throughout perinatal period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Mitochondrial RS production; (b) Indicative of nitric

oxide products; (c) Protein expression of neuronal nitric oxide synthase (nNOS) and (d) Indicative of lipid peroxidation (MDA levels). Values are means ± SEM (*p ≤ 0.05 unpaired Student's *t* test), n=4-6.

Figure 5: Antioxidant defense in the brainstems of 150 days male rats born to dams fed throughout perinatal period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Superoxide dismutase-SOD, (b) Catalase-CAT and (c) Glutathione peroxidase-GPx activities; (d) glutathione reduced levels-GSH; (e) redox state levels. Values are means ± SEM (*p ≤ 0.05 unpaired Student's *t* test), n=4-6.

Figure 6: Graphical abstract that summarizes the main findings in the current study



Mitochondrial fundamental coupling states of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet, circles) or 8% casein (low-protein diet, squares). Mitochondrial O2 consumption in basal state, stimulated with ADP, resting with oligomycin, uncoupled with carbonyl cyanide m-chlorophenyl hydrazone and respiratory control ratio in: (a) complex I and (b) complex II substrates. Data of Values are expressed as means ± SEM. (*p ≤ 0.05; **p ≤ 0.01 unpaired Student's t test), n=4-6

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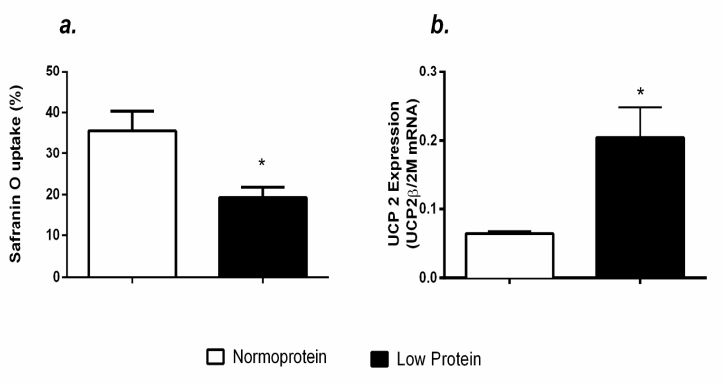
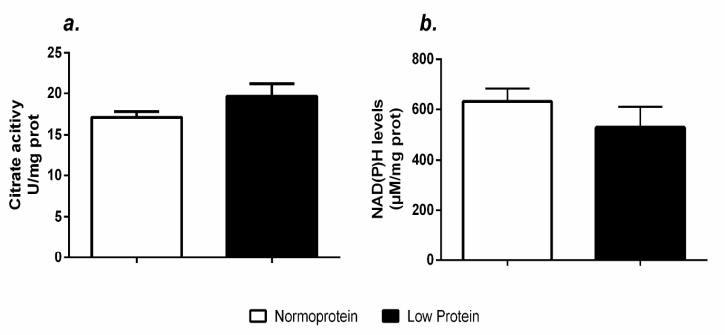


Mitochondrial membrane potential and electrical uncoupling in the brainstems of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Percentage of Safranin O uptake; and (b) UCP2 mRNA expression. Values are expressed as means ± SEM (*p ≤ 0.05; **p ≤ 0.01 unpaired Student's t test), n=4-6.

248x108mm (300 x 300 DPI)



Mitochondrial substrate oxidation in the brainstems of 150 days male rats born to dams fed throughout gestational and lactation period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Citrate synthase activity and (b) content of NAD(P)H. Values are expressed as means ± SEM (*p ≤ 0.05) unpaired Student's t test), n=4-6.

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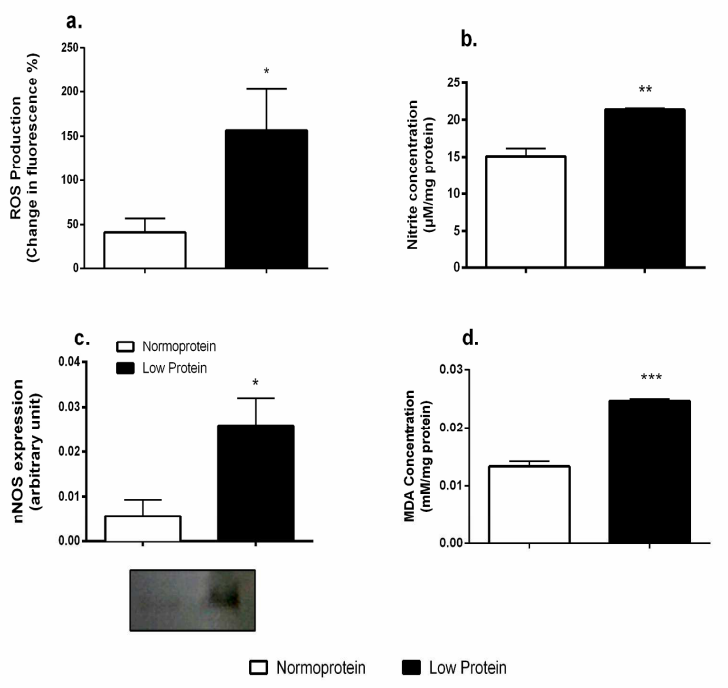

Reactive Species productions and Oxidative stress in the brainstems of 150 days male rats born to dams fed throughout perinatal period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Mitochondrial RS production; (b) Indicative of nitric oxide products; (c) Protein expression of neuronal nitric oxide synthase (nNOS) and (d) Indicative of lipid peroxidation (MDA levels). Values are means ± SEM (*p ≤ 0.05 unpaired Student's t test), n=4-6.

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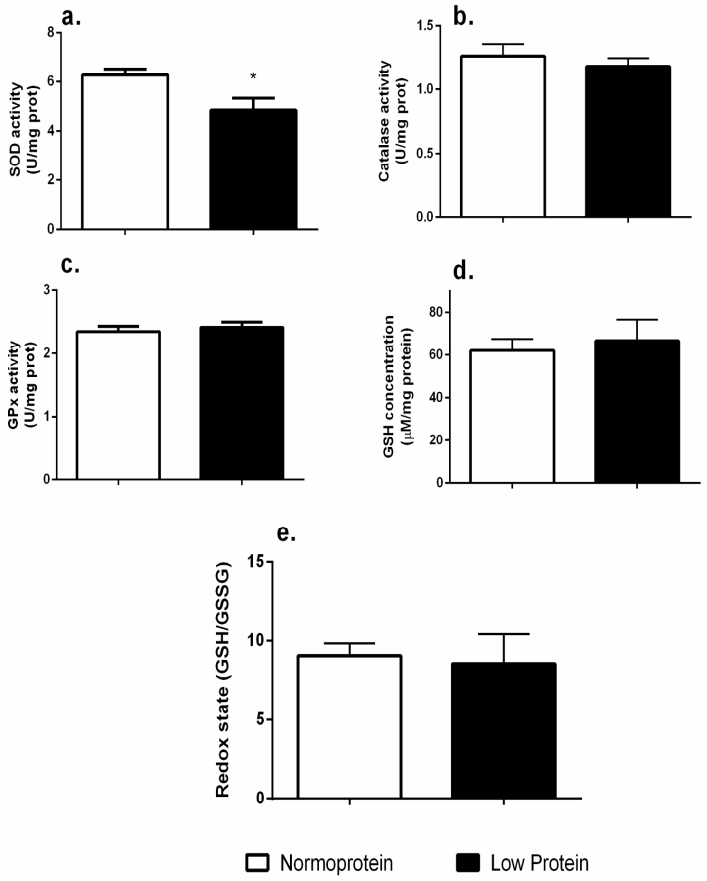

Antioxidant defense in the brainstems of 150 days male rats born to dams fed throughout perinatal period with either 17% casein (normoprotein diet) or 8% casein (low-protein diet). (a) Superoxide dismutase-SOD, (b) Catalase-CAT and (c) Glutathione peroxidase-GPx activities; (d) glutathione reduced levels-GSH; (e) redox state levels. Values are means ± SEM (*p ≤ 0.05 unpaired Student's t test), n=4-6.

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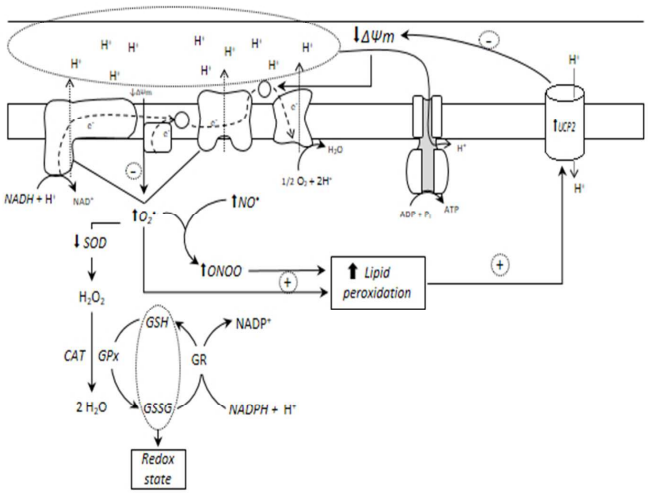

Graphical abstract that summarizes the main findings in the current study.

177x114mm (96 x 96 DPI)

6 CONSIDERAÇÕES FINAIS

Os resultados encontrados neste estudo confirmam nossa hipótese em relação à disfunção mitocondrial e o desequilíbrio oxidativo no tronco encefálico, desencadeado pela restrição proteica materna. Aqui, nós demonstramos que a restrição proteica durante a gestação e lactação modula negativamente a bioenergética mitocondrial mesmo quando não há incompatibilidade entre os ambientes. As disfunções desencadeadas pela restrição proteica nas idades estudadas podem modular negativamente as funções autonômicas desenvolvidas pelo tronco encefálico, fato que aumenta a propensão a desajustes cardiovasculares.

Apesar dos resultados na função mitocondrial (consumo de oxigênio, potencial de membrana e produção de espécies reativas) apresentarem semelhanças ao desmame e aos 150 dias, os animais apresentaram aumento no dano oxidativo a lipídios apenas tardiamente. Nossos dados sugerem que inicialmente, a produção excessiva de elementos pró-oxidantes tenha sido combatida pelo sistema antioxidante não enzimático, fato que justificaria a diminuição de moléculas do estado reduzido e estado redox.

Aos 150 dias, o funcionamento mitocondrial analisado em maior detalhe, nos mostra que as mitocôndrias não conseguem conservar a energia em seu circuito de prótons. A diminuição na taxa de remoção de superóxido, que não fora presente em animais novos, favorece a produção de espécies mais “danosas” aos componentes celulares, os quais reforçam o desacoplamento mitocondrial.

Juntos, nossos resultados indicam que a disfunção mitocondrial se inicia imediatamente após o estímulo nutricional de maneira independente da incompatibilidade de ambientes e que tais desajustes podem comprometer gradualmente a condução de prótons, modulando negativamente a capacidade de conservação de energia, a integridade de lipídios e levar a disfunção no tronco encefálico durante o envelhecimento.

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APÊNDICES

A- SÍTIOS MITOCONDRIAIS DE PRODUÇÃO DE ESPÉCIES REATIVAS DE OXIGÊNIO.

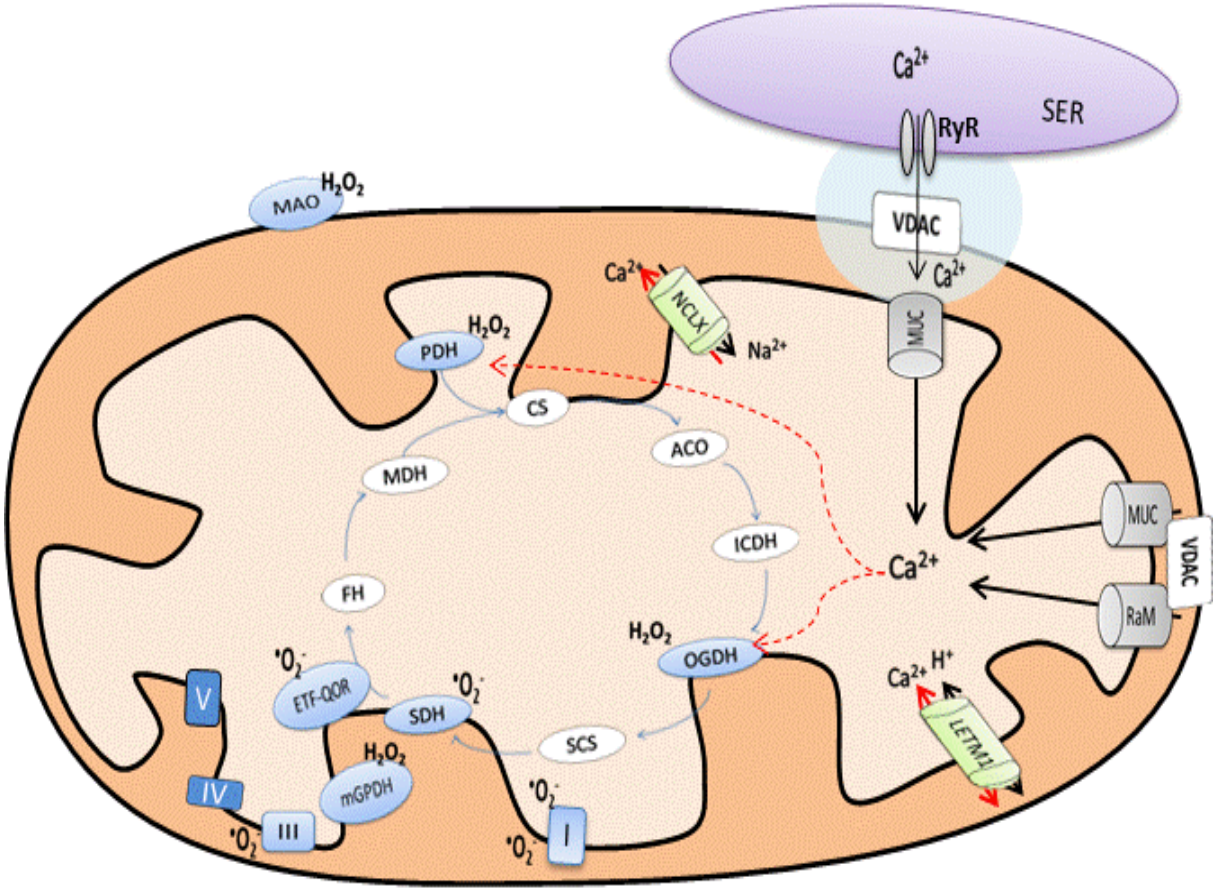

Figura 20: Representação gráfica dos sítios mitocondriais de produção de Espécies Reativas de Oxigênio

B- RESUMO DOS ACHADOS RELACIONANDO RESTRIÇÃO PROTEICA MATERNA E EQUILÍBRIO OXIDATIVO.

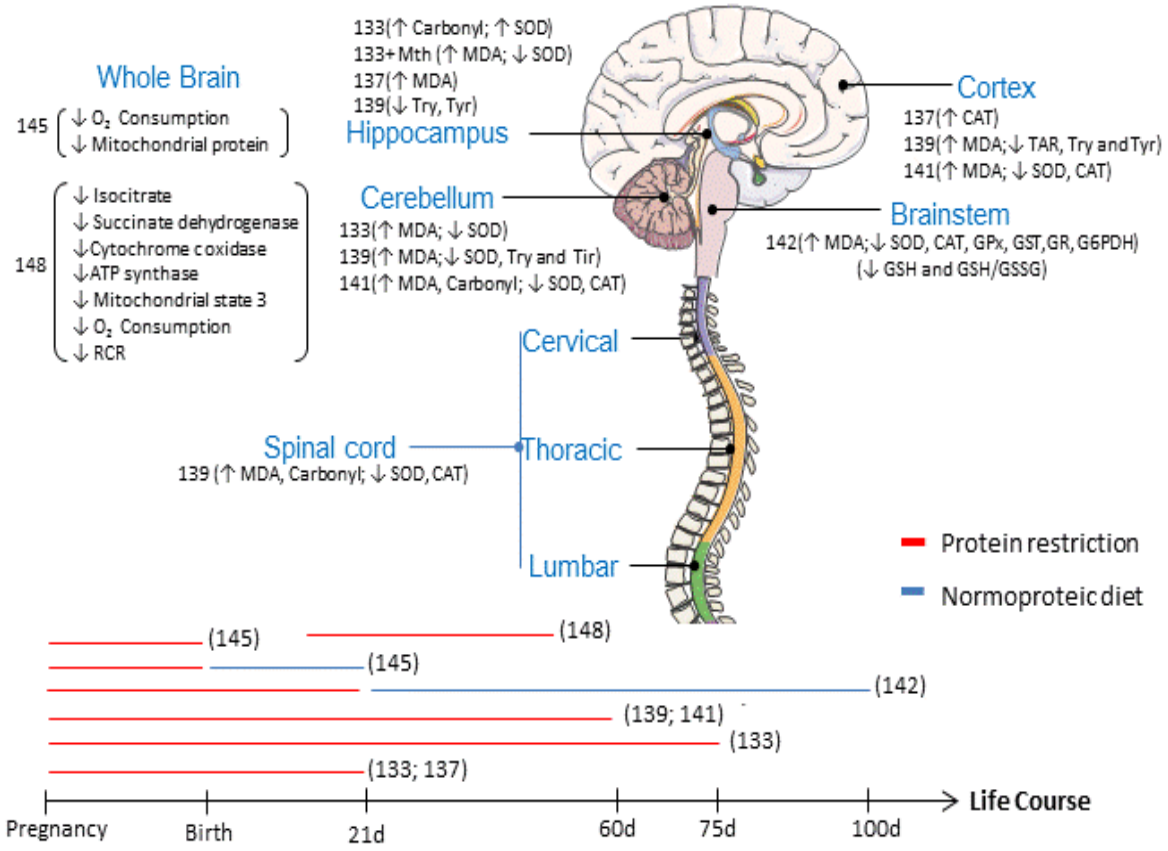

Figura 21: Esquema gráfico dos estudos produzidos com restrição proteica e equilíbrio oxidativo no SNC

C- RESPOSTAS INDUZIDAS PELA RESTRIÇÃO PROTEICA MATERNA NO TRONCO ENCEFÁLICO DE RATOS.

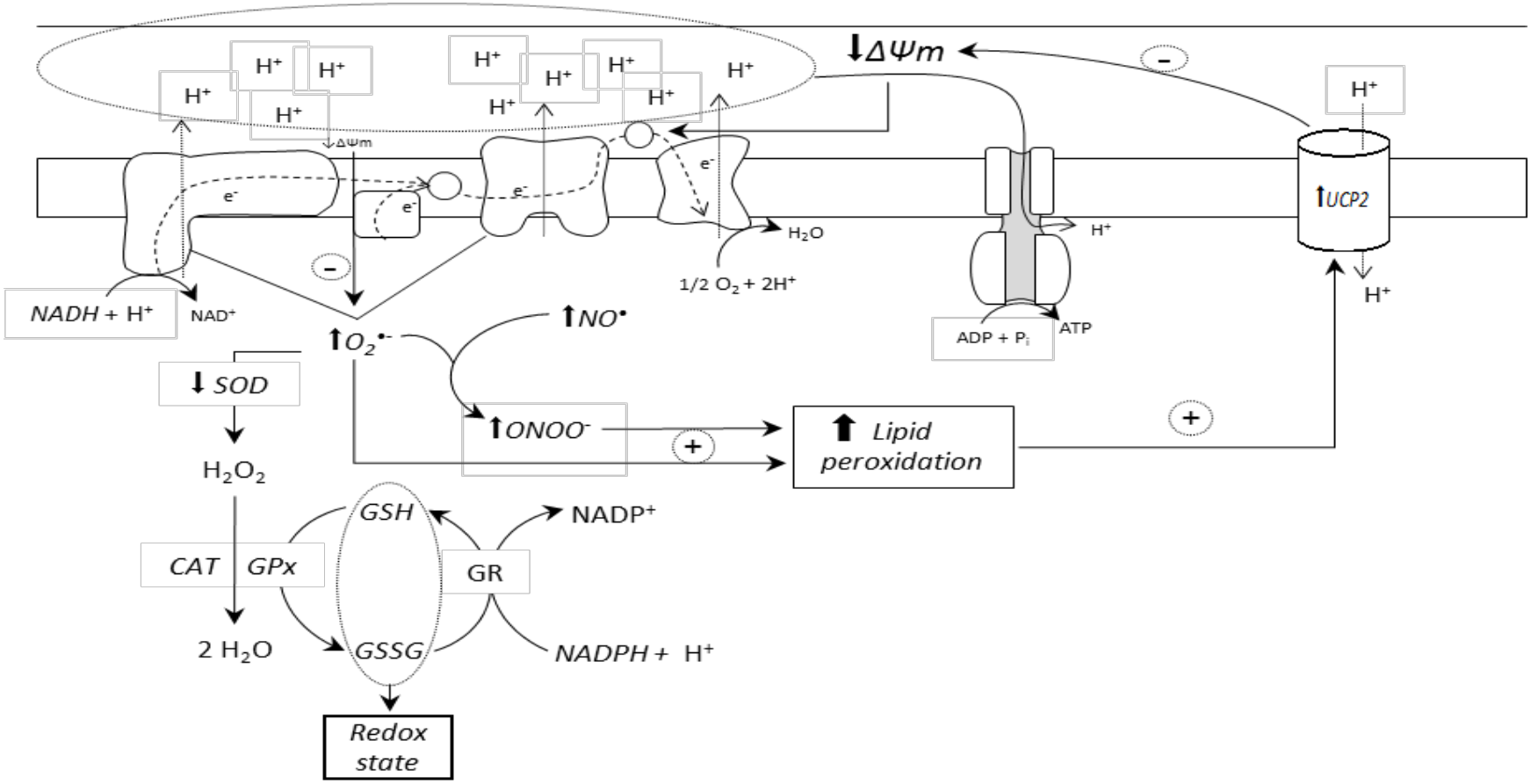

Figura 22: Resumo Gráfico. Efeitos da restrição proteica materna no tronco encefálico de ratos machos aos 150 dias de vida.

ANEXOS

A- PARECER DO COMITÊ DE ÉTICA

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
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Recife, 26 de setembro de 2014.

Ofício nº 54/14

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª. Claudia Jacques Lagranha**
Centro Acadêmico de Vitória - CAV
Universidade Federal de Pernambuco
Processo nº 23076.017807/2014-15

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, **"Equilíbrio Oxidativo: Estudo da desnutrição perinatal e predisposição a doenças cardíacas com origem no Sistema Nervoso Cental".**

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; Animal: rato; Linhagem: Wistar; Idade: Progenitores adultos e prole aos 22 e 150 dias; Peso: Aos 22 dias: 30g, adultos: 250-300g; Sexo: machos e fêmeas; Número total de animais previsto no protocolo: 126

Atenciosamente,

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

CCB: Integrar para desenvolver

B- SUBMISSÃO À FREE RADICAL RESEARCH

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Manuscript ID
GFRR-OM-2017-0149

Title
Mitochondrial dysfunction: Early nutritional insults as a trigger of late reactive species overproduction and brainstem energy failure

Authors
Ferreira, Diorginis
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Date Submitted
29-May-2017

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C- REVISÃO DE LITERATURA

Journal of Developmental Origins of Health and Disease, page 1 of 12.

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REVIEW

Protein undernutrition during development and oxidative impairment in the central nervous system (CNS): potential factors in the occurrence of metabolic syndrome and CNS disease

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Mitochondria play a regulatory role in several essential cell processes including cell metabolism, calcium balance and cell viability. In recent years, it has been postulated that mitochondria participate in the pathogenesis of a number of chronic diseases, including central nervous system disorders. Thus, the concept of mitochondrial function now extends far beyond the common view of this organelle as the 'powerhouse' of the cell to a new appreciation of the mitochondrion as a transducer of early metabolic insult into chronic disease in later life. In this review, we have attempted to describe some of the associations between nutritional status and mitochondrial function (and dysfunction) during embryonic development with the occurrence of neural oxidative imbalance and neurogenic disease in adulthood.

Received 30 December 2015; Revised 16 May 2016; Accepted 17 May 2016

Key words: developmental stage, mental health/illness, metabolic syndrome, molecular/cellular, outcome/system

Introduction

In most eukaryotic cells, oxidative phosphorylation is the main source of energy, wherein a complex of membrane proteins located in the inner mitochondrial membrane is able to generate large amounts of energy stored in the form of adenosine triphosphate (ATP).[1,2] Mitochondria are dynamic organelles whose roles in cell function originated in part from their prokaryotic ancestor (likely an α-proteobacterium) eons ago. The ability of those prokaryotes to provide energy to eukaryotes in an aerobic environment helped drive the evolution of those early one-celled eukaryotes into the multiplicity of multicellular forms that dominate life on earth today.[3–5]

In addition to their important function as the 'powerhouses' of eukaryotic organisms,[6–8] mitochondria play a role in the pathogenesis of certain chronic non-communicable diseases (NCD). In addition, many clinical and experimental studies have demonstrated a close relationship between nutritional status during embryonic development and the occurrence of metabolic impairment in the adult brain. The close correlation between poor early nutrition and subsequent metabolic dysfunction has led investigators to speculate that early insult to the mitochondrion is a key causative factor in the eventual occurrence of disease. This review focuses in particular on evidence of neural dysfunction associated with developmental undernutrition that results in damage to mitochondrial function. To place these subjects in proper context, we will first describe to the role of the mitochondrion in reactive oxygen species (ROS) production and oxidative stress, and then explore how ROS production (mitochondrial and non-mitochondrial) and nutrition-dependent mitochondrial damage specifically contribute to the development of neurogenic disease.

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Oxidative phosphorylation products: ATP and ROS

Energy production in mitochondria depends mainly upon a proton motive force generated by the electron transport chain (ETC), which transfers electrons through reduced cofactors, NADH and $FADH_2$, derived from either the oxidation of acetyl-CoA derived from the tricarboxylic acid (TCA) cycle or β-oxidation of fatty acids to molecular oxygen (O_2) as a final electron acceptor. The energy generated by the flow of electrons through the ETC is used to transport protons outward across the inner mitochondrial membrane, and the influx of those protons into the matrix through the ATP synthase complex is used to generate ATP from ADP + Pi. When the ETC becomes highly saturated with electrons, excess electrons can be directly transferred to O_2 to generate the superoxide anion ($^{\bullet}O_2^-$), which can be further reduced to a hydroxyl radical ($OH^{\bullet}$), an oxidizing agent even more damaging to cells than $^{\bullet}O_2^-$.[9] In animals living in an aerobic environment,

mitochondria are the major source of ROS, whose production depends essentially on O_2 concentration and the electron flow velocity.[10,11] Due to fluctuations in cellular respiration, the amounts of O_2 available to the ETC also fluctuate and consequently the generation of ROS can vary considerably among different tissues.[12–16]

Mitochondria, on the other hand, also have a high antioxidant capacity residing in both enzymatic and non-enzymatic systems. The role of these antioxidant systems is to convert the ROS into harmless molecules, or at least into less reactive species.[17] The enzymatic antioxidant system employs an enzymatic cascade in which each enzyme uses the product from the prior reaction as a substrate for use by the next enzyme [i.e. $^{\bullet}O_2^-$ conversion to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD); then H_2O_2 to H_2O by either catalase (CAT) or glutathione peroxidase (GPx)], while the non-enzymatic antioxidant system relies on molecules such as reduced glutathione (GSH) that are capable of donating H^+, to stabilize the reactive species.[18–20] Furthermore, H_2O_2 and organic peroxides can be reduced by thioredoxins and peroxiredoxins that use thiol compounds (SH) as their source of electrons.[21,22]

An imbalance between ROS production and removal in favor of retention of the oxidant compounds results in a condition of oxidative stress, characterized by oxidative damage to lipids, proteins, DNA that are causal to several clinical abnormalities.[23–26] In this review, we focus on altered mitochondrial ROS production and oxidative imbalance triggered by protein restriction during fetal development and its relation to the occurrence of specific neural disorders later in life.

Superoxide production sources

Superoxide, in most cases, is the first ROS produced, and it can be formed by auto-oxidizable reactions of non-radical molecules, both in mitochondrial enzymatic sites and non-mitochondrial enzymatic reactions.[27]

Two non-mitochondrial enzymatic reactions involve NADPH oxidase and xanthine oxidases. The first of these is a protein complex composed of membrane-associated cytochrome (b_{558}) containing the subunits ($gp91^{phox}$ and $p22^{phox}$), plus regulatory subunits localized in cytosol ($p47^{phox}$, $p40^{phox}$ e $p67^{phox}$) and a small G protein (Rac1 or Rac2). Although this enzyme complex is best recognized in phagocytic cells such as neutrophils, other cell types also produce $^{\bullet}O_2^-$ through NADPH oxidase activity.[28] Xanthine oxidase also results in the non-mitochondrial production of superoxide, and is often activated following ischemia reperfusion, wherein hypoxanthine and xanthine components are oxidized to urate with concomitant $^{\bullet}O_2^-$ production.[29] For more information about those sources of ROS, see the reviews by Cantu-Medellin and Kelly and Bedard and Krause.[30,31]

The mitochondrial monovalent reduction of O_2 to $^{\bullet}O_2^-$ is thermodynamically favored and is regulated by two factors. The first is the concentration of electron carrier in proteins in a redox form and the second is the proportion of these proteins that are able to react with O_2.[32] Although complexes I and III are the major sources of mitochondrial ROS,[13] there are additional mitochondrial sites that are able to produce ROS[32–34] (see Fig. 1). Some of these are described below:

(1) Pyruvate dehydrogenase (PDH) is a mitochondrial enzymatic complex with three main catalytic components[35] responsible for catalyzing the conversion of pyruvate to acetyl-CoA. It is proposed that the rate of ROS production from the PDH complex increased as the NAD(P)H/NAD(P)$^+$ pool reduce.[36,37]
(2) 2-oxoglutarate dehydrogenase is another important mitochondrial enzymatic complex present in the Krebs (TCA) cycle that is able to produce ROS through NADH oxidation.[38] The mechanism relies on a third enzymatic element, in which the flavin from dihydrolipoamide dehydrogenase can generate large amounts of ROS in the mitochondrial matrix as consequence of NADPH/NAD$^+$ ratio.[39]
(3) Mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) is a coenzyme located in the outer surface of the inner mitochondrial membrane that is able to transfer reduced cytosolic factors to the mitochondrial ETC.[40] In addition to the ROS generated from mGPDH, oxidation of glycerol 3-phosphate can drive electrons both to complex IV and to complex I, leading to additional ROS production from these mitochondrial sources/sites.[41]
(4) Electron transferring flavoprotein Q oxidoreductase (ETF-QOR). During fatty acid oxidation mitochondrial acyl-CoA dehydrogenase transfers electrons to ETF, which is then oxidized by ETF-QOR by donating electrons to the ubiquinone (UQ) pool.[42] Once the ratio of reduced ubiquinone (UQH_2) and UQ becomes elevated, the electron leak increases ROS generation.[43]
(5) Monoamine oxidase (MAO) is a flavoenzyme located in the outer mitochondrial membrane that deaminates biogenic amines in the central and peripheral nervous systems and blood[44,45] in two-step reactions. In the first reaction, the flavin prosthetic group is reduced and produces aldehyde and ammonium. In the second, the reduced flavin is oxidized to form H_2O_2.[46,47]
(6) Flavin site in complex II. Although the estimated $^{\bullet}O_2^-$ generation by this complex is ordinarily low, in a condition of low levels of succinate and diminished activities of complexes I and III, the flavin site complex can produce both superoxide and H_2O_2 at high rates.[13] The mechanism proposed for this is based on the electron leak achieved by flavin in the semi- or fully reduced state.[34]
(7) Flavin prosthetic group in complex I. This process relies on the flavin mononucleotide (FMN) binding site, whose full reduction during forward electron flow from NADH induces electron leak to O_2, producing $^{\bullet}O_2^-$.[48]
(8) Ubiquinone site in complex I. This source of $^{\bullet}O_2^-$ is associated with the reduction of UQ to UQH_2 by a substrate such as succinate, glycerol 3-phosphate or acyl-CoA. However, the electrons can also be driven

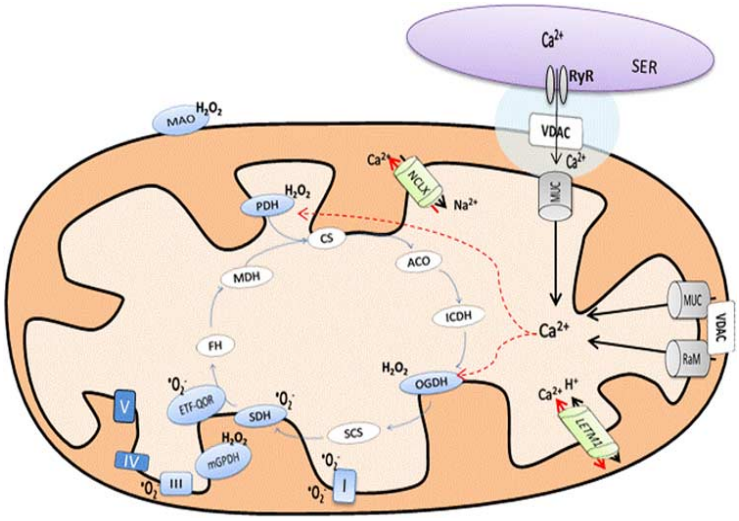

Fig. 1. Schematic representation of the mitochondrial sites of reactive oxygen species (ROS) production and Ca^{2+}-related ROS increase. In light blue, sites of ROS production: MAO, monoamine oxidase; PDH, pyruvate dehydrogenase; OGDH, oxoglutarate dehydrogenase; SDH, succinate dehydrogenase; ETF-QOR, electron transferring flavoprotein Q oxidoreductase; mGPDH, mitochondrial glycerol 3-phosphate dehydrogenase; electron transport chain complexes I and III. In gray, the proteins responsible for Ca^{2+} influx: RyR, ryanodine receptor; MCU, mitochondrial Ca^{2+} uniporter and RaM, rapid mode of calcium uptake. Dashed red lines indicate what enzymes have their ROS production stimulated by Ca^{2+} overload: OGDH and PDH. In light green, the proteins responsible for Ca^{2+} efflux: NCLX, Ca^{2+}/Na^{+} exchanger and LETM1, Ca^{2+}/H^{+} antiporter. Dark blue represents the other mitochondrial complexes; white ellipse; other enzymes from Krebs cycle; white rectangle, Voltage-dependent channels, VDAC; and purple, the sarcoplasmic reticulum.

reversely from UQH_2 to NAD^{+}, thereby generating $^{\bullet}O_2^{-}$ at high rates.[33,49]

(9) Outer ubiquinone site in complex III. The $^{\bullet}O_2^{-}$ production in this complex is based upon the electron transfer mechanism called Q cycle. Electron carriers into this complex gather the electrons from UQH_2 to water-soluble cytochrome *c* in a sequential process that results in the formation of an unstable semiquinone UQ^{-} that can reduce O_2 to superoxide.[14]

Calcium (Ca^{2+}) signaling and mitochondrial ROS overproduction

A compelling body of evidence shows that Ca^{2+} regulates numerous cellular functions, and that differences in Ca^{2+} concentration are controlled by complex membrane transport systems moving the cation between the extracellular environment, the cytosol and membrane de-limited intracellular organelles.[50] The mitochondrion stands out as a critically important organelle in Ca^{2+} homeostasis as this organelle can internalize cytoplasmic calcium derived from the extracellular environment as well as Ca^{2+} released from the smooth endoplasmic reticulum (SER) (syn. in muscle: 'sarcoplasmic' reticulum) (SR).[50,51]

Calcium crosstalk between mitochondria and the SER employs a ryanodine receptor (RyR)-mediated mechanism. Although recent evidences have described the expression of mitochondrial inner membrane RyR in cardiomyocytes and striatal neurons,[52] the RyR is better described as a channel protein located on the SER membrane that is sensitive to small changes in cytosolic Ca^{2+} concentration and to Ca^{2+} overload in the SER lumen.[53] In either situation, the RyR allows Ca^{2+} release from storage in the SER (or SR) into the cytosolic mitochondrial microdomains that facilitate Ca^{2+} uptake.[54,55] Voltage-dependent channels located in outer mitochondrial membrane allow the entry of Ca^{2+} into the intermembrane space, and then either of two different processes can mediate its influx into the mitochondrial matrix:

(1) The mitochondrial calcium uniporter (MCU), which relies on the negative mitochondrial membrane potential to take up Ca^{2+} into the matrix.[51]
(2) A rapid mode of calcium uptake (known as RaM), which is thought to respond to rapid changes in cytosolic Ca^{2+}.[56]

Calcium efflux, on the other hand, depends upon the Ca^{2+}/Na^{+} exchanger (NCLX), which is also able to switch the ion exchange flow (either forward or reverse) depending on cytosolic Na^{+} concentration and mitochondrial membrane potential.[57] Efflux is also dependent on the Ca^{2+}/H^{+} antiporter, which appears to be especially important in tissues that have low NCLX activity, such as liver, kidney and lung.[58]

Several reports have shown that Ca^{2+} can stimulate ROS production by different mechanisms:

(1) *Krebs cycle stimulation*. It has been proposed that Ca^{2+} can allosterically activate enzymes, such as PDH, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase in order to supply the ETC with reduced cofactors.[59] As discussed previously some of these enzymes can also produce ROS.[33]
(2) *A change in lipid organization in the inner mitochondrial membrane*. Studies in model membranes suggest that Ca^{2+} sequesters the cardiolipin attached to membrane carrier proteins, and that this membrane rearrangement in some way increases ROS production.[60]
(3) *Mobilization of intramitochondrial ferrous iron* (Fe^{2+}). Studies using isolated mitochondria have indicated that mitochondrial Ca^{2+} overload is associated with an increase in hydroxyl radical formation and oxidative damage. However, when the mitochondria were treated with a Fe^{2+} chelator, the oxidative damage was abolished.[61]
(4) *Opening of the mitochondrial permeability transition pore (MPTP)*. The outer mitochondrial membrane allows essentially a 'free' translocation of small molecules from the cytosol into the mitochondrion. However, selective transporters in the inner mitochondrial membrane are needed to assure homeostasis between cytosolic and matrix environments.[62] Mitochondrial Ca^{2+} overload combined with oxidative imbalance leads to the opening of the MPTP in the inner membrane, thereby allowing bi-directional traffic of small metabolites through the mitochondrial membrane[63] and a disruption of the normal electrolytic equilibrium. This disruption leads to mitochondrial swelling, a decrease in proton motive force, an increase of ROS production, and may also rupture the outer mitochondrial membrane with the consequent release of pro-apoptotic factors (e.g. cytochrome *c*, Smac/DIABLO, Omi/HtrA2 and others) into the cytosol.[64,65]

Oxidative impairment in the central nervous system (CNS): the mitochondrion as a trigger of neurogenic disease

Oxidative damage is innate to all eukaryotic cells. However, tissue types vary in their sensitivity to that damage, and by that measure the brain stands out as being particularly vulnerable to oxidative damage due to its morphologic and physiologic characteristics.[16] In normal resting conditions, the adult brain is responsible for over 15% of total O_2 consumption, an exceptionally large rate of oxygen use per unit mass compared with others tissues.[16] In addition to its heavy consumption of O_2, the brain is also vulnerable to oxidative damage due to the specialized characteristics of neural tissue as described below:

(1) The presence of excitotoxic amino acids, such as glutamate. Glutamate levels are tightly controlled in the brain, however, under conditions of stress, neurons undergoing apoptosis release a large amount of glutamate into the surrounding tissue. Furthermore, Mailly *et al.*[66] showed that neurons in the presence of excess hydrogen peroxide enter a prolonged excitatory state triggered by the continuous activation of *n*-methyl-D-aspartate (NMDA) receptors by glutamate.
(2) A high content of biologically important amines that are oxidized in the presence of O_2. Neurotransmitters such as dopamine, serotonin, adrenalin and noradrenalin react slowly with oxygen to produce superoxide, which in turn reacts with those neurotransmitters to form other ROS in a chain reaction.[67] Furthermore, several oxygenases possess tetrahydropteridine as a co-factor, which in elevated levels is able to induce ROS-dependent neuronal apoptosis.[68]
(3) SOD-independent H_2O_2 generation. Most ROS production occurs downstream from the dismutation of $^{\bullet}O_2^-$. However, the brain can generate large quantities of H_2O_2 independently of SOD activity. During the recycling of biogenic amines (e.g. serotonin, epinephrine, norepinephrine, dopamine), enzymes located in outer mitochondrial membranes of neurons and glia can form H_2O_2 through oxidative deamination of those amines.[69,70]
(4) Prevalence of polyunsaturated fatty acids (PUFA) in the CNS. PUFA are widespread in the CNS, and if antioxidant systems are not adequate to inhibit ROS formation, the ROS can remove hydrogen from PUFA or attach to it to initiate lipid peroxidation.[25] Once lipid peroxidation has been initiated, intermediate compounds react with oxygen to form lipid proxy radicals, which then react with PUFA in a cyclic reaction to generate isoprostanes as well as multiple α,β-unsaturated aldehyde products, such as acrolein, 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA).[71]

Due to the brain's particular vulnerability to oxidative stress, many studies have been designed to assess how varying relationships among mitochondria, oxidative imbalance and lipid peroxidation can predispose an individual to neurodegenerative diseases such as Alzheimer's (AD), Huntington's, Parkinson's diseases, multiple sclerosis and neurogenic hypertension.

In the case of AD, it was shown that oxidative stress as well as lipid peroxidation in the cerebral cortex and hippocampus exert a positive influence on disease progression by inducing amyloid-beta peptide (Aβ) accumulation,[72] wherein products of lipid oxidation impair energy production in the brain.[73] Moreover, AD patients exhibit lower cerebral activity of mitochondrial enzymes in the Krebs cycle[74] as well as an impairment in oxygen consumption via a decrease in complex I and III activities.[75] Such metabolic dysfunctions in neural tissue lead to an increase in ROS generation and a decrease in energy supply, thus enhancing the damage promoted by Aβ accumulation[75] and impairing many higher level brain functions, including judgment, memory and orientation.

Similarly a dysfunction in complex II may represent an important factor in Huntington's disease (HD), a disorder associated with cognitive deficits, psychiatric illness and

involuntary movements. Striatal degeneration induced by defective mitochondrial complex II function has been used as a common animal model of HD[76] and may reflect the disease process in humans Furthermore, disruptions in hippocampal calcium signaling, mitochondrial membrane potential, sensitivity of the MPTP, pyruvate dehydrogenase and complex IV activities, and an increase in lipid peroxidation have also been described in brains of patients with HD.[71,77]

Systemic inhibition of complex I has been used as an experimental model of Parkinson's disease (PD).[78] Complex I disruption in dopaminergic neurons, present mainly in the striate nucleus, results in decreased ATP production and increased mitochondrial ROS production, thereby stimulating pathways involved in MPTP activation as well as initiating the release of inflammatory and pro-apoptotic molecules to induce neuronal cell death.[79] Increased products from lipid peroxidation, such as F2-isoprostanes and 4-HNE can also contribute to neuronal death[25,80] in PD, which culminates in bradykinesia, rigidity and tremors induced by the striatal dopamine deficiency.[80]

Mitochondrial dysfunction has also been related to neuropathology of multiple sclerosis (MS). A decrease in the complexes I and III activities of 50% or more impairs the capacity of mitochondria to produce ATP.[81,82] The mismatch between energy requirements and ATP production in turn, contributes to axonal degeneration in upper motor neurons in MS patients.[81,83] The energy deficit is further enhanced by damage to mitochondrial DNA caused by nitric oxide or its products.[84] In amyotrophic lateral sclerosis (ALS), a neurologic disease characterized by motor neuron and neuromuscular junction degradation, oxidative stress is a major contributor to the etiology of the disease by impairing the machinery of transmitter release in the pre-synaptic motor nerve terminal of the neuromuscular junction.[85] In fact, a mutation in the gene coding for cytosolic SOD (SOD1) is responsible for 20% of ALS cases.[86] In addition, ALS patients exhibit decreased mitochondrial function and impairment in Ca^{2+} homeostasis, both of which contribute to oxidative damage in the lumbar and thoracic spinal cord.[87] It is proposed that the downstream oxidative damage in ALS patients depends largely on the capacity of the defective SOD1 to increase ROS production both in mitochondria and in plasma membrane bound NADPH oxidase.[88,89]

Central redox balance also plays a key role in cardiac diseases arising from CNS defects.[90] Nuclei located in the brainstem, including the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitaries (NTS) play key roles in neurogenic hypertension,[91] wherein the imbalance of ROS and nitric oxide in neurons within these nuclei can alter the peripheral vascular system by increasing sympathetic vasomotor tone.[23,92] Chan *et al*. demonstrated that in spontaneously hypertensive rats, the increase in blood pressure is directly related to lower expression and activity of mitochondrial superoxide dismutase and catalase in the RVLM.[93] Additional studies found that the elevation of $^{\bullet}O_2^-$ and H_2O_2 in brainstem sites such as RVLM and NTS originate from activation of NADPH oxidase via protein kinase C and phosphatidylinositol 3-kinase, as well as via an increase in intracellular Ca^{2+}, and down regulation of mitochondrial uncoupling protein 2 (UCP2) and reduction in ETC capacity[94–97] contributes to the increase in arterial blood pressure.

Early oxidative stress as a developmental determinant of health and disease in later life

It is well known that environmental influences can alter numerous internal body functions and as a result can trigger such NCD as diabetes, metabolic syndrome, and cardiac disorders.[98] An increase in NCD risk is not limited to physiologic changes occurring in adulthood but may also result from adverse events that occur much earlier in life. Thus, endogenous and exogenous signals[99] if occurring within certain critical developmental windows within the embryonic period can permanently affect physiologic processes within the mature individual.[100]

The first suggestion that this phenomenon exists came from observations of the occurrence of impaired glucose tolerance and the development non-insulin-dependent diabetes in a 64-year old who had exhibited a significantly reduced growth rate in early life.[101] The data suggested that poor nutrition during periods of fetal life and infancy induced diabetes via changes in B-cell function. The researchers further hypothesized that permanent adaptations to the early nutritional deficit provided survival benefits by shunting glucose to critical organs and away from those organs considered as secondary for survival.[101] Ironically the permanent adaptations so necessary for survival in fetal life/infancy may be the same physiologic alterations that predispose and individual to chronic disease in later life.

The ability to express different phenotypes following a physiologic challenge is known as phenotypic plasticity, and in development is dependent upon specific temporal windows during which the organism is especially prone to change its developmental pattern in order to survive.[102] Further investigation has shown that the post-developmental environment helps to determine whether the initial exposure will or will not be harmful.[103,104] This suggested that the early influences acted as environmental cues that led to adaptive responses providing survival advantages to the individual in later life. However, if the postnatal environment does not 'match' or coordinate with the prenatal environment, the adaptations that occurred during development would no longer be advantageous to the individual and would predispose him or her to the occurrence of adult diseases.[105]

As human mothers are able to quench small environmental perturbations of short duration,[106] it would be expected that environmental changes during life generate a mother's phenotype and this phenotype will shape the offspring's adaptations, by generating a variability in metabolic capacity.[107,108] Wells[107], suggested that the maternal phenotype is responsible for the adaptations in her offspring, and that her phenotype might depend, in turn, on the environmental history of close ancestors, as has been reviewed in detail.[109]

Martin-Gronert and Ozanne[110] highlighted three proposed mechanisms of how events in the perinatal period of development can produce life-long effects in the individual. The first mechanism involves the occurrence of permanent structural changes in key organs such as the brain, pancreas and kidney. The second mechanism involves changes in gene expression resulting from epigenetic modification, as has been described elsewhere[111,112] (e.g. DNA methylation, histone modification and microRNA action on mRNA).[111] The third mechanism for the long-lasting effects of a developmental insult is dependent upon the process of cellular ageing. An example of this is the induction of cellular senescence secondary to mitochondrial dysfunction and increased oxidative stress, as suggested by Luo *et al.*[113]

In the past several years, oxidative stress has been studied as a molecular trigger for the effect of maternal nutritional deficiency on NCD occurring later in life in her offspring. In animal models of protein restriction, researchers have found mitochondrial dysfunction in the early mouse embryo[114] and heart; oxidative stress in pancreatic islets as a consequence of a mismatch among antioxidant enzymes (i.e. increase in SOD with concomitant decrease in CAT and GPx);[115] an increase in pancreatic oxidative stress and heart rate associated with aging[116] and an increase in the protein expression of enzymes related to ROS production as well as oxidative stress and DNA damage.[117]

Early low-protein diet and cerebral oxidative impairment:

Nutrition is often considered as the greatest exogenous influence in early life. Classified as the main non-genetic contributor to changes in brain development, nutritional inadequacy has been known to have several deleterious effects on the fetal brain.[118–122] Early studies of restrictive diets on offspring had described oxidative impairment, such as decreased GSH levels in the forebrain;[123] lower mRNA expression of SOD and CAT in the brain;[124] increased vascular $^{\bullet}O_2^-$ production[125] and a decrease in SOD activity,[126] and in newborn infants, an increase in oxidative damage as a consequence of the reduction in serum antioxidant capacity.[127,128]

Although all categories of nutrient are important in brain development, protein has the greatest effect on neural function.[129] Early protein deficiency affects the brain in several ways that vary with the period of exposure, the type of protein deficiency and its severity, and also with the specific cerebral region.[130] The critical period for brain development is marked by several specific temporal windows in which the processes of neurogenesis, neuron migration, and neuron alignment and orientation are quickly increased then either decreased or ceased.[131]

As proteins do not readily cross the placenta into the fetal circulation, nutritional deficits in the mother are generally transmitted to the fetus the level of the amino acid composition of the ingested protein. Consequently, the lack of any one of the essential amino acids in the maternal protein diet can lead to a complete protein deficiency in the fetus.[131] Nutritional protein restriction may reduce antioxidant capacity by inhibiting the synthesis of antioxidant enzymes,[132] and the resulting oxidative stress on fetal cells could alter gene expression and further damage the cells with oxidized proteins and lipids.[113]

Several studies have demonstrated the effects of protein restriction in oxidative balance and mitochondrial function in the CNS (see Fig. 2). Bonatto *et al.*[133] evaluated these parameters in the hippocampus of rats exposed to moderate protein restriction from the 1st day of the gestational period until 75 days of postnatal life and found an increase in protein oxidation but a decrease in lipid oxidation. The investigators suggested that the opposing effects of a protein-restricted diet on proteins *v.* lipids resulted from an overall increase in SOD activity in the protein-restricted group, wherein the elevated SOD protected lipid, but not protein, from oxidation. As the activity of CAT did not change with restricted protein, it was suggested that the higher activity of SOD without a concomitant up-regulation of CAT drives the accumulation of H_2O_2, followed by formation of the hydroxyl radical ($^{\bullet}OH$),[134] considered among the most reactive of ROS. Thus hydroxyl radical formation could be the responsible for the increased protein oxidation observed in the face of increased SOD activity.[135,136] As evidence of this, when the protein-restricted animals were supplemented with methionine, SOD activity was reduced and the animals exhibited increased oxidative damage to their lipids.

Further investigations in 21-day-old rats,[133,137] showed that low-protein diet increases oxidative damage to lipids in the cerebellum and hippocampus but has no influence on oxidation, in the cortex. Evaluation of SOD and CAT activities in those brain regions, moreover, showed that SOD activity was reduced only in cerebellum with low-protein diet, and not in either the cortex or hippocampus. A possible explanation for the cerebellar damage, is that low-protein induced decrease in SOD activity enhances interaction between $^{\bullet}O_2^-$ and $NO^{\bullet}$ to form peroxynitrite, which is capable of oxidizing lipids, proteins and thiol compounds as well as DNA.[138]

Feoli *et al.*,[139] on the other hand, showed no difference in ROS production in either cerebellum, cortex or hippocampus of animals fed a low-protein diet (casein 7%) from the first gestational day until 60 days of life. Although ROS levels did not change, an increase in lipid peroxidation in the cerebellum and cortex occurred due to a decrease in SOD activity in the cerebellum, and the reduction in total antioxidant reactivity in the cortex. When the authors evaluated the content of tryptophan and tyrosine, (important neurotransmitter precursors) as a measure of damage, all three brain regions were negatively affected by low-protein diet, and the damage was closely related to the lower serotoninergic and catecholaminergic neurotransmitter concentrations.[140]

Tatli *et al.*,[141] by evaluating three types of induced CNS damage: (1) intrauterine growth restriction (IUGR), (2) moderate and (3) severe protein restriction in five different CNS regions (cortex, cerebellum, cervical, thoracic and lumbar cord) showed that only severe undernutrition triggered lipid

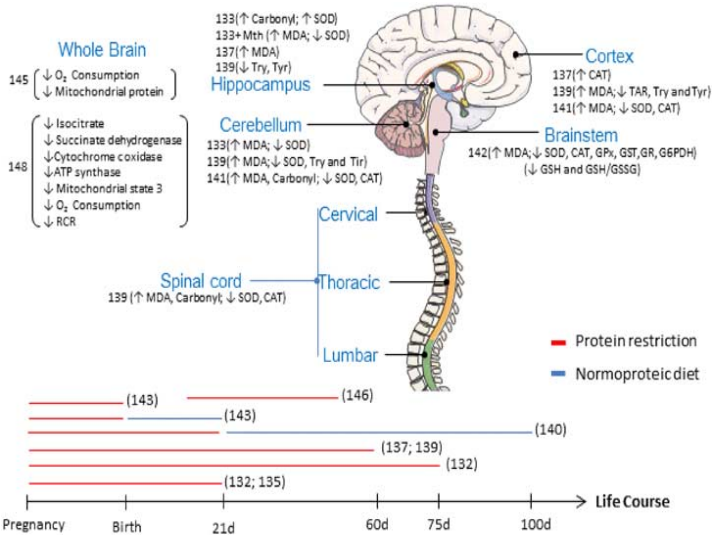

Fig. 2. Main findings on early protein restriction and central nervous system oxidative balance. Red lines represent protein restriction, blue lines, normoprotein diet, and the length of each approximates the time during which either feeding state was applied for each study referred to in the text. Numbers in parentheses refer to the references cited in the text. MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; TAR, total antioxidant reactivity; Try, tryptophan; Tyr, tyrosine; and RCR respiratory control ratio.

oxidation in all five CNS structures at 60 days of life. Protein oxidation was shown to vary in proportion to the degree of undernutrition, with the cerebellum showing greater sensitivity to protein oxidation than the other CNS regions. In addition to increasing oxidative biomarkers, early nutritional adversity also decreased the activity of SOD and CAT in all regions analyzed.

In our laboratory, we have evaluated offspring through 100 days of age from mothers fed a low-protein diet throughout the perinatal period (gestation and lactation) and found that low-protein animals had increased oxidative damage in the brainstem. The data showed a marked decrease in antioxidant capacity, wherein enzymatic activities of SOD, CAT, GPx and glutathione-*S*-transferase were decreased by over 15%. The redox state was also affected by the maternal low-protein diet through a reduction in glutathione concentrations as a consequence of lower glutathione resynthesis and a decrease in NADPH supply.[142] Oxidative imbalance in certain brain regions is directly related to the occurrence of cardiovascular impairments,[143] mainly hypertension.[23,90,91,144] Thus, it is feasible that early nutritional insult is a central trigger for the development of hypertension in adulthood.

Assessing brain O_2 consumption, Muzzo *et al.*[145] found that newborns from mothers fed throughout the gestational period with a diet containing only 4% protein, exhibited decreases in both brain mitochondrial protein and in O_2 consumption. Animals that were reefed from the 1st until 16th postnatal day also showed a reduction in O_2 consumption and phosphorylation capacity. Although the gestational period encompasses most of the period during which neurogenesis occurs,[146] in rats, the duration of maternal lactation represents the most important period for brain development.[131] Thus, several studies have described effects of protein restriction during other periods of brain development that may lead to lasting changes even after refeeding due to the impairment in the metabolic activity.[118,147]

In rats experiencing severe protein restriction during a period of 30 days (from the 18th to the 48th postnatal day),[148] brain mitochondria were shown to have alterations in Krebs cycle, isocitrate and succinate dehydrogenase, as well as in ETC enzymes, cytochrome *c* oxidase and ATP synthase. In addition, the mitochondrial ETC exhibited an impairment in function, such that low-protein animals were less responsive to ADP stimulation by complexes I and II substrates.

Despite the important role of the mitochondrion in several neurogenic diseases (i.e. PD, AD, ALS, etc.), very few studies have investigated the role of mitochondrial dysfunction in the central oxidative imbalance induced in protein restriction models. The antioxidant system, however, has been shown to be significantly affected by a low-protein diet, which also contributes to the disruption of mitochondrial capacity and may compromise the overall brain function.

Conclusion

In this review, we discuss the importance of mitochondrial dysfunction and oxidative stress in the development of neural disorders, and show how such diseases could be induced by nutritional insult during development. Although decreases in mitochondrial content and/or activity have been demonstrated

in several studies employing nutritional manipulation either during the gestational and/or locational period, further investigations will be necessary to determine the specific mechanism causing mitochondrial dysfunction due to a protein-poor diet within a restricted developmental window. Compelling evidence has already shown that several neurogenic diseases are associated with mitochondrial disruption accompanying high-fat intake. Therefore it seems likely that a diet low in protein could also disrupt mitochondrial function sufficiently to induce neurogenic disease. Many studies to date have shown that protein restriction deeply affects central oxidative balance by decreasing antioxidant capacity. Further studies must be conducted, however, to assess the contribution of ROS generators in this oxidative disruption.

The underlying mechanisms responsible for impaired mitochondrial function in metabolic disorders induced by low-protein diet during embryonic development have not yet been elucidated. It is hoped, however, that prospective clinical investigations of mitochondrial function in healthy and diseased humans will begin to provide insight into precisely how early nutritional deficit and the occurrence of metabolic disease in adulthood are linked.

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