UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO ACADÊMICO DE VITÓRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO, ATIVIDADE FÍSICA E PLASTICIDADE FENOTÍPICA – PPGNAFPF

IDELFONSO BELTRÃO DE BARROS JÚNIOR

EFEITOS DE DIETA HIPERLIPÍDICA PÓS-DESMAME SOBRE A BIOENERGÉTICA MITOCONDRIAL E METABOLISMO OXIDATIVO DE FÍGADO DE RATOS SUBMETIDOS À DIETA HIPOPROTEICA PERINATAL

Vitória de Santo Antão
2019

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Área de concentração: Bases experimentais e Clínicas da Plasticidade Fenotípica

Orientadora: Mariana Fernandes Pinheiro
Coorientador: Diogo Antonio Alves de Vasconcelos

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Aprovado em: _27_ / _02_ / _2019_.

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Aos meus pais, irmãos e irmãs, amigos, e minha esposa Taiane que, com muito carinho e apoio, não mediram esforços para que eu chegasse até esta etapa de minha vida.

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“Saber muito não lhe torna inteligente. A inteligência se traduz na forma que você recolhe, julga, maneja e, sobretudo, onde e como aplica esta informação."

(Sagan, Carl. 1995, pp. 102)

RESUMO

Nas últimas décadas, configurou-se um novo paradigma nutricional, no qual a desnutrição foi substituída pela supernutrição, caracterizando uma transição nutricional. Isso tem sido associado ao consumo de dietas ocidentalizadas enriquecidas com ácidos graxos saturados (AGS), levando ao aumento do fator de risco para doenças crônico-metabólicas na vida adulta. Neste trabalho, avaliou-se uma dieta rica em ácidos graxos saturados (HL) após o desmame, na prole de ratas submetidas à restrição proteica materna sobre a bioenergética mitocondrial hepática. Ratos Wistar foram acasalados e durante a gestação e lactação, as mães receberam dietas controle (NP, proteína normal 17%) ou com baixa proteína (LP, 8% proteína). Após o desmame, os ratos receberam dietas normolipídica (NL) ou HL (+59% AGS) até 90 dias de vida. O controle respiratório das mitocôndrias de fígado foi maior em LP-NL comparado ao grupo NP-NL (36,81%, $p<0,05$) e menor entre LP-HL e LP-NL (25,71%, $p<0,01$). Observou-se que o grupo LP-HL apresentou inchamento mitocondrial maior do que NP-HL (49,21%, $p<0,05$) e LP-NL (67,07%, $p<0,01$). Este efeito foi potencializado após adição de Ca^{2+} (20 μM) no grupo LP-HL (272,06%, $p<0,01$) e totalmente prevenido na presença de ciclosporina A (inibidor do poro de transição de permeabilidade mitocondrial) e EGTA (quelante de cálcio). Os níveis de malondialdeído (MDA) aumentaram 42,53% ($p<0,01$) comparando LP-NL com LP-HL e NP-HL com LP-HL (54,35%, $p<0,001$). Os níveis de carbonila aumentaram 72,23% ($p<0,01$) entre LP-NL e LP-HL, assim como entre NP-HL e LP-HL (60,07%, $p<0,05$). A avaliação da resposta antioxidante enzimática revelou que as manipulações nutricionais não alteraram significativamente a atividade da SOD. No entanto, observou-se que CAT (48,14%, $p<0,05$) e GPx (21,02%, $p <0,05$) apresentaram menor atividade entre os grupos LP-NL e LP-HL. A atividade da GST apresentou aumento quando comparado NP-NL com LP-NL (38,12%, $p <0,05$) e NP-NL com NP-HL (44,55%, $p <0,01$). Dieta com maior quantidade de AGL levou a uma diminuição dos níveis de GSH no grupo NP-HL (29,74%, $p<0,05$) em relação ao controle (NP-NL). Não houve alteração significativa nos níveis de tióis totais. A análise por RT-PCR mostrou que a expressão de PGC-1α foi maior em LP-HL quando comparado com os grupos LP-NL (178,10%, $p <0,0001$) e LPHL (95,87%, $p <0,001$). Por outro lado, Tfam mostrou uma diminuição entre LP-HL e LP-NL (22,55%, $p <0,001$), como também entre LP-HL e LP-NL (18,63%, $p <0,01$). A expressão gênica de MFN2, CypD e GRP75 não mostrou diferenças entre os grupos. Na análise da expressão de VDAC, houve aumento comparando os grupos NP-HL e NP-NL (16,03%, $p <0,05$), LP-NL com LP-HL (17,97%, $p <0,01$), como também entre LP-HL e NP - HL (15,10%, $p <0,05$). Este estudo mostrou que dieta hiperlipídica enriquecida com AGS induz a disfunção mitocondrial no fígado de ratos jovens e este efeito foi potencializado quando os animais foram alimentados com dieta de baixo teor de proteínas durante períodos críticos de desenvolvimento. Nossos resultados sugerem que esta disfunção mitocondrial está associada com estresse oxidativo, maior expressão de VDAC que potencializa a abertura do poro de transição de permeabilidade e menor biogênese mitocondrial.

Palavras chave: Mitocôndria de fígado. Dieta com baixa proteína. Estresse oxidativo. Dieta ocidentalizada.

ABSTRACT

In the last decades, a new nutritional paradigm has been configured, in which undernutrition has been replaced by over nutrition, characterizing a nutritional transition. This has been associated to the consumption of westernized diets enriched in saturated fatty acids (SFA), leading to increased risk factor for chronic metabolic diseases in adulthood. In this work, we evaluated a diet rich in saturated fatty acids (HL) after weaning of the offspring rats submitted to maternal protein restriction on the hepatic mitochondrial bioenergetics. Wistar rats were mated and during gestation and lactation, mothers received control diets (NP, normal protein content 17%) or low protein (LP, 8% protein). After weaning, rats received either normolipidic (NL) or HL (+59% SFA) diets up to 90 days of life. The respiratory control of liver mitochondria was higher in the LP-NL compared to NP-NL (36.81%, $p<0.05$) and lower between LP-HL compared with LP-NL group (25.71%, $p<0.01$). It was observed that LP-HL group showed higher mitochondrial swelling than NP-HL (49.21%, $p<0.05$) and LP-NL (67.07%, $p<0.01$). This effect was potentiated after 20 μM Ca^{2+} addition on the LP-HL group (272.06%, $p<0.01$) and totally prevented in the presence of cyclosporine A (mitochondrial permeability transition pore inhibitor) and EGTA (calcium chelator). Malondialdehyde (MDA) levels increased 42.53% ($p<0.01$) comparing LP-NL with LP-HL and NP-HL with LP-HL groups (54.35%, $p<0.001$). Carbonyl levels increased 72.23% ($p<0.01$) between LP-NL and LP-HL, as also comparing NP-HL with LP-HL groups (60.07%, $p<0.05$). The evaluation of the enzymatic antioxidant response revealed that the nutritional manipulations did not change significantly the SOD activity. However it was observed that CAT (48.14%, $p<0.05$) and GPx (21.02%, $p<0.05$) were found reduced between LP-NL and LP-HL groups. GST activity showed increase when compared NP-NL with LP-NL (38.12%, $p<0.05$) and NP-NL with NP-HL (44.55%, $p<0.01$). SFA diet led a decrease of GSH levels in NP-HL (29.74%, $p<0.05$) related to control group (NP-NL). There was no significant change in the total thiols levels. The RT-PCR analysis showed that PGC-1α expression was higher in LP-HL when compared to LP-NL (178.10%, $p<0.0001$) and LPHL (95.87%, $p<0.001$) groups. Conversely, Tfam showed a decrease between LP-HL and LP-NL (22.55%, $p<0.001$) as also between LP-HL and LP-NL (18.63%, $p<0.01$). Gene expression of MFN2, CypD and GRP75 did not show differences among groups. In the VDAC analysis, there were increases comparing NP-HL and NP-NL groups (16.03%, $p<0.05$), LP-NL with LP-HL (17.97%, $p<0.01$), as also between LP-HL and NP-HL (15.10%, $p<0.05$). This study showed that SFA-enriched high fat diet induces mitochondrial dysfunction in liver of young rats and this effect was potentiated when the animals were fed with low protein diet during critical periods of development. Our results suggest that this mitochondrial dysfunction is associated with oxidative stress, greater VDAC expression that potentiates the opening of the mitochondrial permeability transition pore and lower mitochondrial biogenesis.

Key-words: Liver mitochondria. Low protein diet. Oxidative stress. Westernized diet.

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FIGURAS DO ARTIGO ORIGINAL

LISTA DE SÍMBOLOS E ABREVIATURAS

Ag - Prata

AT(D)P – adenosina tri (di) – fosfato

Ca^{2+} – Cálcio

CDNB - 2,4-dinitroclorobenzeno

CO_2 – Gás carbônico

Cu^{+} - Íon cobre

CCCP – Cianeto de carbonila m-clorofenil hidrazona

CsA – Ciclosporina A

CTE – Cadeia de transporte de elétrons

DNA – Ácido desoxirribonucleico

DNP-SG - 2,4-Dinitrofenil-S-glutationa

mtDNA – DNA mitocondrial

DOHaD - Developmental Origins of Health and Disease

EGTA – Etileno glico – bis(b-aminoetil éter)-N,N,N´,N´-ácido tetraacético

EROs – Espécies reativas de oxigênio

$FADH_2$ – Flavina adenina dinucleotídeo reduzido

Fe^{2+} – Ferro

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

H_2DCF-DA - Diacetato de diclorodihidrofluoresceína

H_2O_2 – Peróxido de hidrogênio

HEPES – (N-[2-Hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid])

K^{+} – Potássio

KCl - Cloreto de potássio

LPO - Lipoperoxidação

MFR – Mitocôndria de fígado de rato

Mg – Magnésio

Mfn2 – Mitofusina 2

MnSOD – Superóxido dismutase dependente de manganês

NAD^+ – Nicotinamida adenina dinucleotideo (estado oxidado)

NADH – Nicotinamida adenina dinucleotideo (estado reduzido)

$NADP^+$ – Nicotinamida adenina dinucleotideo fosfato (estado oxidado)

NADPH – Nicotinamida adenina dinucleotideo fosfato (estado reduzido)

$NO^{\bullet}$ – Óxido nítrico

NOS – Óxido nítrico sintase

O_2 – Gás oxigênio

$O_2^{\bullet -}$ – ânion superóxido

$OH^{\bullet}$ – Radical hidroxil

OMS – Organização Mundial da Saúde

PGC1-α - Coativador do receptor ativado por proliferador de peroxissomo gama 1-alpha

Pi – Fosfato inorgânico

PMSF - Fluoreto de fenilmetano sulfonil

PTPM – Poro de transição de permeabilidade mitocondrial

RNA – Ácido ribonucleico

SH – Sulfidrilas

TBARS - Substância Reativa ao Ácido Tiobarbitúrico

TPM – Transição de permeabilidade mitocondrial

TFam – Fator de transcrição mitocondrial A

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SUMÁRIO

1 INTRODUÇÃO

1.1 Plasticidade fenotípica e DOHaD

Devido ao ritmo das mudanças alimentares na população em geral, a Organização Mundial da Saúde (OMS) concentra esforços para combater as doenças crônico-metabólicas que estão aumentando sua prevalência. Dados recentes mostram que as estratégias para prevenir estas doenças incluem mudanças no estilo de vida e dieta. Essas mudanças se mostram mais efetivos se iniciadas em períodos críticos do desenvolvimento, como gestação, lactação e primeira infância (WHO-OMS, 2011; NASCIMENTO *et al.*, 2014).

Os seres humanos, como outros seres vivos, precisam se adaptar ao ambiente ao qual estão expostos, necessitando de uma "plasticidade" que os ajude diante desse novo desafio. Como mostrado por West-Eberhard (2003), o desenvolvimento dessa plasticidade tem sua essência no período crítico em que o sistema é plástico e sensível ao meio ambiente, seguido de uma perda dessa plasticidade e de uma melhor capacidade funcional. Isso permitiria, em outras palavras, a produção de fenótipos que se ajustassem melhor ao ambiente, chamado de “plasticidade fenotípica” (WEST-EBERHARD, 1989; 2005; MOCZEK, 2015).

Em 1976, Ravelli *et al.*, em um estudo epidemiológico, analisaram uma população de 300.000 homens, filhos de mulheres expostas a um período de escassez de alimentos durante o cerco nazista da Alemanha na Segunda Guerra Mundial, chamado de "fome holandesa". Os homens, na idade adulta, tinham diferentes padrões de composição corporal, dependendo da idade em que haviam sido expostos à desnutrição materna durante a vida intrauterina. Os resultados mostraram que, se a mãe sofreu desnutrição no primeiro semestre de gravidez, a incidência de obesidade aumentou significativamente em seus filhos; mas se a desnutrição ocorreu no último trimestre gestacional, as crianças apresentaram baixa incidência de obesidade (RAVELLI *et al.*, 1976).

Barker *et al.* seguiu essa hipótese de que condições adversas, tanto na fase intrauterina quanto na vida infantil, aumentavam o risco de doenças cardiovasculares. Em um artigo clássico publicado em 1989, correlacionou o peso ao nascer de indivíduos adultos nascidos no início do século XX no Reino Unido. Como resultado, as pessoas nascidas com baixo peso tiveram um crescimento diferente, comparado ao grupo de pessoas nascidas com peso normal (Barker *et al.*, 1989). Assim, o feto responderia a esses sinais com adaptações, como um metabolismo mais adequado a esse excesso nutricional ou tamanho corporal reduzido, o que ajudaria a sobreviver a essa privação nutricional (BARKER *et al.*, 1989; BARKER, 2006; HANSON; GLUCKMAN, 2011; BATESON *et al.*, 2014).

A hipótese de Origem Desenvolvimentista da Saúde e da Doença (sigla em inglês *DOHaD*) propõe que doenças metabólicas como hipertensão e diabetes na idade adulta são uma resposta à desnutrição durante a vida fetal e a infância (BARKER, 2006). O *DOHaD* é uma hipótese que agrega um conjunto de informações, abrangendo epidemiologia, estudos clínicos e translacionais, demonstrando a ligação entre fatores maternos, perinatais e da primeira infância e o risco de desenvolver doenças crônicas não-transmissíveis na vida adulta (FLEMING *et al.*, 2015). Alguns fatores envolvidos são conhecidos, como um excesso nutricional, uso de cigarros e bebidas alcoólicas, estresse e poluentes (GLUCKMAN *et al.*, 2008; DRAKE; REYNOLDS, 2010; HANSON; GLUCKMAN, 2011). Mesmo sendo uma hipótese, diversos trabalhos mostram que a plasticidade fenotípica e o *DOHaD* estão em consonância com a transição nutricional que a população brasileira vem passando nas últimas décadas, evidenciados pelos altos índices de problemas crônico-metabólicos já citados.

1.2 Epigenética

Em 1940, Conrad Waddington formulou o termo epigenética, para tentar definir "o ramo da biologia que estuda as relações de causa e efeito entre genes e seus produtos, apresentando o fenótipo" (WADDINGTON, 1940; JABLONKA; LAMB, 2002). A regulação epigenética desempenha um papel importante na transdução funcional da informação contida no genoma. Os processos epigenéticos incluem a metilação do DNA, que é a adição de um grupamento metil nos resíduos da base nitrogenada citosina, que pode inibir a expressão de genes; modificações pós-traducionais de histonas, onde as histonas podem sofrer modificações que convergem em uma mudança na conformação da cromatina, o que pode levar a um aumento ou uma diminuição na expressão gênica; e RNAs não-codificantes, moléculas de RNA que são transcritas para o DNA mas não em proteínas, dentre elas o microRNA (JABLONKA; LAMB, 2002; HAIG, 2004; GODFREY *et al.*, 2007; BIANCO-MIOTTO *et al.*, 2017).

As modificações epigenéticas são evidenciadas em dados experimentais, mostrando que diversas vias podem ser afetados em vários órgãos, incluindo o fígado (Feinberg, 2007). Essas modificações epigenéticas podem ser a gênese de alterações bioquímicas e fisiológicas nesse órgão, incluindo modificações no metabolismo lipídico, como altos níveis de colesterol no sangue, e em enzimas antioxidantes hepáticas, como a catalase (CHAPMAN *et al.*, 2000). Tais alterações são importantes, visto que a mitocôndria está envolvida em processos bioquímicos essenciais a nível celular, como a respiração celular, e sua disfunção tem sido

associada nos últimos tempos a uma grande variedade de doenças degenerativas e metabólicas.

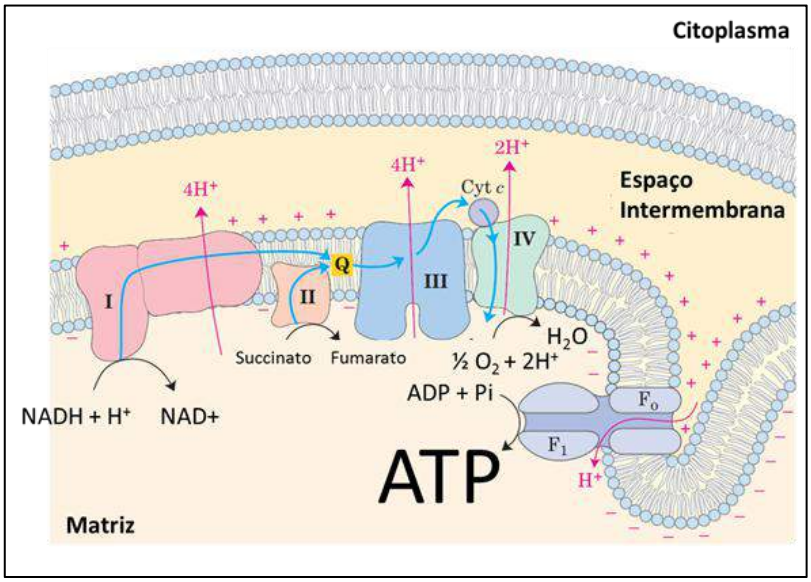
1.3 Bioenergética mitocondrial

Os níveis de gás oxigênio (O_2) foram aumentando gradativamente na atmosfera ao longo dos anos, e os seres vivos tiveram que se adaptar para o utilizar como parte importante na síntese de energia. As plantas fazem a fotossíntese, que como a respiração, pode usar diferentes fontes de elétrons (TAVERNE *et al.*, 2018). A atividade fotossintética é o processo pelo qual, além das plantas, algas e bactérias utilizam a luz solar mais o CO_2 para produzir matéria orgânica, estando esses seres como base da vida em geral no nosso planeta, configurando como o principal meio de produção de energia dos seres autotróficos (LAMBERS *et al.*, 2008).

Em seres heterotróficos, como os mamíferos nos quais os seres humanos estão inclusos, o gás oxigênio é essencial. Os seres aeróbicos precisam de O_2 para realizar uma produção eficiente de energia, e eliminando CO_2 que será utilizado pelos seres autotróficos. No nosso organismo, o O_2 é imprescindível na produção de energia na mitocôndria (HALLIWELL; GUTTERIDGE, 2007). Entretanto, a necessidade de O_2 acaba escondendo o fato de que é uma molécula reativa na sua forma monoeletrônica, e esses seres aeróbicos sobrevivem porque possuem defesas antioxidantes que tentam anular um possível efeito maléfico.

Esse O_2 é essencial para a mitocôndria, e a falta do mesmo pode levar a consequências fisiopatológicas, desde problemas nas funções aeróbicas à injúria isquêmica, podendo levar à morte celular (GNAIGER *et al.*, 1995). Nesta organela ocorre a maior produção de energia para a célula, porque utiliza substratos, predominantemente vindos da dieta, para formação de ATP através de reações que utilizam elétrons e cofatores, tanto no ciclo de *Krebs*, via anfibólica central na produção energética; quanto na fosforilação oxidativa, etapa metabólica da respiração celular. Tanto a respiração celular quanto a fosforilação oxidativa normalmente são eventos acoplados para que ocorra liberação de ATP (PORTER; BERDANIER, 2002) (Figura 1). A mitocôndria é tão importante que problemas na sua função podem resultar numa sinalização para a morte celular (SHAUGHNESSY *et al.*, 2014), além disso, a mitocôndria tem papel na homeostase lipídica, na sinalização metabólica celular, e nos níveis de íons cálcio (Ca^{2+}) (SUEN *et al.*, 2008; TAIT; GREEN, 2012; CHENG; RISTOW, 2013). A atividade mitocondrial é importante, portanto, para o funcionamento celular, que pode ser alterado por um desbalanço oxidativo indesejado.

Figura 1– Cadeia transportadora de elétrons e fosforilação oxidativa



Fonte: Nelson; Cox (2014).

1.4 Espécies reativas de oxigênio (EROs), sistemas antioxidantes e estresse oxidativo

Na mitocôndria, de 2 a 5% do gás oxigênio para a produção de energia acabam se transformando em espécies reativas de oxigênio (EROs) e/ou radicais livres (TURRENS, 2003) (Figura 2). A enzima citocromo oxidase participa da formação de H_2O a partir desse gás, justamente no fim da cadeia transportadora de elétrons (BARBOSA *et al.*, 2010), e a ação do citocromo *c* tenta evitar a formação dessas EROs que em baixas concentrações, possuem importantes papeis fisiológicos, como em vias de sinalização, homeostase celular, crescimento e diferenciação celular, e apoptose (HALLIWELL, 1991; BAYR, 2005; MARTELLI; NUNES, 2014).

Radicais livres podem ser definidos como as "moléculas, ou fragmentos de moléculas, que contém um ou mais elétrons desemparelhados em seus orbitais" (HALLIWELL; GUTTERIDGE, 2007; VALKO *et al.*, 2007). A descoberta de que o gás oxigênio gerando radicais livres é relativamente recente na história científica. Inicialmente, as causas das propriedades maléficas do oxigênio foi primeiramente associada com os radicais livres na publicação do trabalho de Gerschman *et al.* (1954), que propuseram a teoria dos radicais livres em detrimento à toxicidade do O_2, devido à sua forma reduzida. Depois disso, foi proposto que os radicais livres têm papel no processo do envelhecimento humano (HARMAN, 1956). Em 1969, McCord e Fridovich descreveram a enzima superóxido

dismutase e forneceu evidências da importância dos radicais livres no organismo (Mccord e Fridovich, 1969). Desde então, vários achados de como os seres vivos se adaptaram à produção de radicais livres, desenvolvendo mecanismos de defesa antioxidante, vêm sendo estudados.

As espécies reativas de oxigênio envolvem radicais livres e outra molécula, o peróxido de hidrogênio. Essas moléculas incluem:

- Ânion superóxido (O_2^-), considerado a ERO primária porque é formada a partir do oxigênio molecular, é um radical pouco reativo, mas que tem a propriedade química de penetrar membranas, agindo somente no local onde é produzido, mas mesmo assim tem um potencial para gerar danos nos sistemas biológicos (BABIOR, 1997; NORDBERG; ARNER, 2001);
- Peróxido de hidrogênio (H_2O_2), que mesmo não sendo um radical livre, é um intermediário da reação que forma o radical hidroxil, sendo bastante importante porque pode ultrapassar membranas celulares (Halliwell e Gutteridge, 2007);
- Radical hidroxil (OH^-), que é formado a partir da reação catalisada pelos íons metálicos Fe^{2+} e Cu^+, chamada reação de Fenton, onde foi observada em 1876 pela primeira vez a forte propriedade oxidante de uma solução de peróxido de hidrogênio e íons Fe^{2+} (FENTON, 1894; NORDBERG; ARNER, 2001), sendo uma molécula muito reativa, capaz de provocar mais danos que qualquer outra EROs (ROVER JÚNIOR *et al.*, 2001; HALLIWELL; GUTTERIDGE, 2007; ANDRADE *et al.*, 2010).

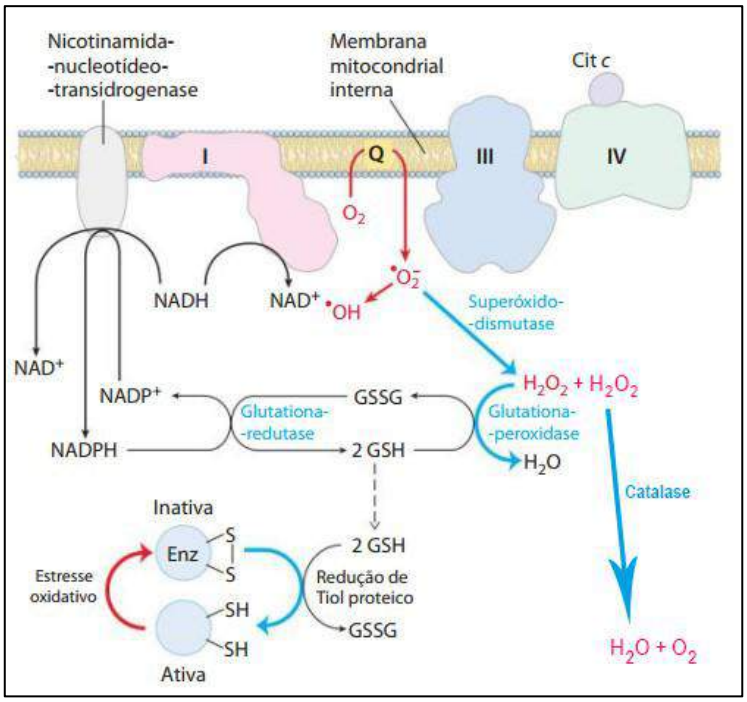
A definição de estresse oxidativo é bastante variada e vem evoluindo junto com as descobertas dos seus mecanismos. Sies e Cadenas (1985) propuseram que o estresse oxidativo corresponde à uma perturbação do balanço entre pró-oxidantes e antioxidantes, em detrimento da formação, resultando na oxidação de biomoléculas. Allen e Tresini (2000) redefiniram o estresse oxidativo para uma desordem na sinalização e no controle celular. Halliwell e Gutteridge (2007), em seu clássico livro “Free Radicals in Biology and Medicine”, definiram como um dano biomolecular causado por um ataque de espécies reativas nos constituintes dos organismos vivos.

O estresse oxidativo é importante no envelhecimento e em muitos processos bioquímicos. Sabe-se hoje que a homeostase redox, como controle do pH, é central para a vida. Em bioenergética e funções vitais, os processos de oxidação-redução estão praticamente envolvidas em todos os processos fundamentais, incluindo a ativação e a desativação de ciclos e a modulação, ou integração, da atividade dos sistemas orgânicos (SIES *et al.*, 2017).

Em altas concentrações, as EROs podem ser mediadores importantes de danos à estruturas celulares, ácidos nucleicos, lipídeos e proteínas (VALKO *et al.*, 2007). O DNA mitocondrial (mtDNA) e proteínas mitocondriais também são vulneráveis ao dano oxidativo por que ocorre uma alteração da integridade do mtDNA, inibindo complexos na cadeia transportadora de elétrons, afetando o transporte de Ca^{2+} e ativando a sinalização pro-apoptótica, porque são produzidos durante a geração de energia normal pela fosforilação oxidativa e geração de ATP promovida por essa cadeia (SHAUGHNESSY *et al.*, 2014).

O sistema de defesa antioxidante pode ser dividido em não-enzimático e enzimático. O sistema não-enzimático é integrado por compostos de origem dietética, como a vitamina E, vitamina C, carotenoides, entre outros (BIANCHI; ANTUNES, 1999). Disfunção mitocondrial, uma das consequências da produção em excesso de EROs, está associada à inflamação (DE MELLO *et al.*, 2017). O sistema enzimático, por sua vez, trabalha em conjunto. A enzima superóxido dismutase (SOD) é encontrada na mitocôndria e no citosol, e converte o ânion superóxido para peróxido de hidrogênio em uma reação que envolve íons hidrogênio e libera água (HALLIWELL, 1994; BABIOR, 1997). Outra enzima é a catalase (CAT), que catalisa duas moléculas de peróxido de hidrogênio para formar duas moléculas de água, e uma de gás oxigênio (Jacob, 1995). A glutationa peroxidase (GPx) utiliza duas moléculas de glutationa reduzida (GSH) e uma de peróxido de hidrogênio, formando duas moléculas de água e uma de glutationa oxidada (GSSG) (Figura 2) (HUBER *et al.*, 2008; RAZA, 2011).

Figura 2 – Formação de EROs e defesas antioxidantes



Fonte: Adaptada de Nelson e Cox (2014).

A produção de EROs, em excesso, é danosa e pode trazer problemas para muitas moléculas, como em uma reação em cadeia (HALLIWELL, 1994). O estresse oxidativo ocorre quando a produção de EROs é maior que a capacidade de as defesas antioxidantes reverterem essa situação, ou quando não tem defesas antioxidantes para evitar a produção desses EROs. Alguns fatores podem modular o estresse oxidativo, como a dieta, estilo de vida, uso de cigarro, atividade física, uso de bebidas alcoólicas, entre outros (BARBOSA *et al.*, 2010). Algumas doenças estão sendo relacionadas com estresse oxidativo, como aterosclerose, diabetes, doenças cardíacas, câncer e doenças do sistema imune, além do processo de envelhecimento (BIANCHI; ANTUNES, 1999). Uma das formas de analisar o impacto negativo do desbalanço oxidativo é ofertando uma dieta experimental que provoque um excesso nutricional.

1.5 Dietas experimentais

O uso de dietas experimentais é bastante empregado para analisar o impacto de excesso nutricional em algum órgão ou tecido específico durante a vida do animal, alguns em períodos críticos de desenvolvimento. Duas dietas têm impactos bem conhecidos na gestação e na lactação: as dietas hipoproteica e hiperlipídica.

No caso de dietas hipoproteicas, pouca proteína é ofertada durante o

desenvolvimento tecidual, e também podem estar associadas a compostos pró e antioxidantes, em diferentes órgãos e tecidos, como testículos (RODRÍGUEZ-GONZÁLEZ *et al.*, 2014), tecido sanguíneo (TAKEMORI *et al.*, 2013), músculo esquelético (Tarry-Adkins *et al.*, 2016), pâncreas (THEYS *et al.*, 2009), rins (TARRY-ADKINS *et al.*, 2007; BEM *et al.*, 2014; LIU *et al.*, 2018), coração (TARRY-ADKINS *et al.*, 2013; NASCIMENTO *et al.*, 2014; BRAZ *et al.*, 2017; CARTHAGENES *et al.*, 2017), cerebelo (AUGUSTO *et al.*, 2017), e tronco encefálico (BRITO ALVES *et al.*, 2016; FERREIRA, D. J. S. *et al.*, 2016; FERREIRA, D. S. *et al.*, 2016; DE SOUSA *et al.*, 2017).

Em análises experimentais envolvendo dietas hipoproteicas, realizadas no Centro Acadêmico de Vitória, já foi mostrado que uma restrição nutricional pode diminuir a capacidade de respiração mitocondrial, e maior produção de EROs no coração (NASCIMENTO *et al.*, 2014), e que pode ser revertido e/ou minimizado pelo hormônio estradiol (BRAZ *et al.*, 2017). Também o exercício físico moderado pode aliviar o impacto negativo da dieta hipoproteica nesse mesmo órgão (CARTHAGENES *et al.*, 2017). No tronco encefálico, o trabalho de Ferreira, D. S. *et al.* (2016) mostrou que houve também menor atividade antioxidante em ratos machos; no entanto, esse efeito foi minimizado em ratas fêmeas em função do período reprodutivo, possivelmente relacionado aos níveis de estradiol (DE SOUSA *et al.*, 2017).

No fígado não é diferente. Ficam bastante evidenciados alguns efeitos relacionados à restrição nutricional, uma vez que os danos teciduais trazem problemas metabólicos importantes ao organismo (MADDINENI *et al.*, 2013; VEGA *et al.*, 2016), incluindo modificações morfológicas e redução de leptina circulante (MITCHELL *et al.*, 2015), esteatose hepática (LÍVERO *et al.*, 2014), além de fibrose hepática e inflamação (TARRY-ADKINS, *et al.*, 2015).

As dietas hiperlipídicas são empregadas, em sua maioria, devido ao alto teor energético, ou em relação à composição de gordura presente, avaliando os diferentes possíveis impactos de cada tipo de lipídeo presente; ou acompanhadas por algum composto que possa reverter os efeitos negativos associados ao estresse oxidativo gerado pela dieta, funcionando como pró ou antioxidantes. Assim como as dietas hipoproteicas, essas dietas são estudadas em diferentes tecidos, como: cérebro (CHE *et al.*, 2018), hipocampo (ALZOUBI *et al.*, 2018), baço (CUI *et al.*, 2012), tecido adiposo branco (AMENGUAL-CLADERA *et al.*, 2012), coração (RINDLER *et al.*, 2013), e músculo esquelético (BODEN *et al.*, 2012; Liu *et al.*,

2015).

No fígado, modelos experimentais com dietas hiperlipídica têm mostrado resultados bastante adversos e importantes (DE ASSIS *et al.*, 2012; MACEDO *et al.*, 2013; ZHOU *et al.*, 2018), incluindo esteatohepatite não-alcoólica (ZHONG; LIU, 2018), esteatose hepática (VALENZUELA *et al.*, 2012; Hou *et al.*, 2016), resistência à insulina (RAFFAELLA *et al.*, 2008; SATAPATI *et al.*, 2012; ZHANG *et al.*, 2013; FRANKO *et al.*, 2014), produção de citocinas pró-inflamatórias (MAHMOUD *et al.*, 2012), menor biogênese mitocondrial (NADAL-CASELLAS *et al.*, 2010), dano ao DNA mitocondrial, degradação proteica e apoptose (YUZEFOVYCH *et al.*, 2013), além da obesidade (ENOS *et al.*, 2014; CRESCENZO *Et Al.*, 2015).

Pouco se conhece na literatura trabalhos que utilizaram duas dietas experimentais em diferentes fases do desenvolvimento na bioenergética mitocondrial. Por isso, analisar os possíveis efeitos negativos de alterações nutricionais na mitocôndria é essencial, uma vez que disfunção nessa organela está associada ao surgimento e/ou progressão de doenças crônico-metabólicas. Dessa maneira, a proposta deste trabalho foi avaliar a bioenergética mitocondrial hepática de ratos submetidos à dieta hipoprotéica durante a gestação e lactação e à dieta hiperlipídica (rica em ácidos graxos saturados) do período pós-desmame à idade adulta.

1.6 Hipótese

Exposição a uma dieta com redução de proteína, durante a gestação e a lactação, seguida por uma dieta hiperlipídica pós desmame à idade adulta, induz desbalanço oxidativo hepático e alterações na bioenergética mitocondrial em fígado de ratos.

2 OBJETIVOS

2.1 Geral:

Avaliar as alterações bioenergéticas mitocondriais e do metabolismo oxidativo hepático de ratos submetidos à dieta hipoprotéica durante a gestação e lactação e à dieta hiperlipídica do período pós-desmame à idade adulta (90 dias de vida).

2.2 Específicos:

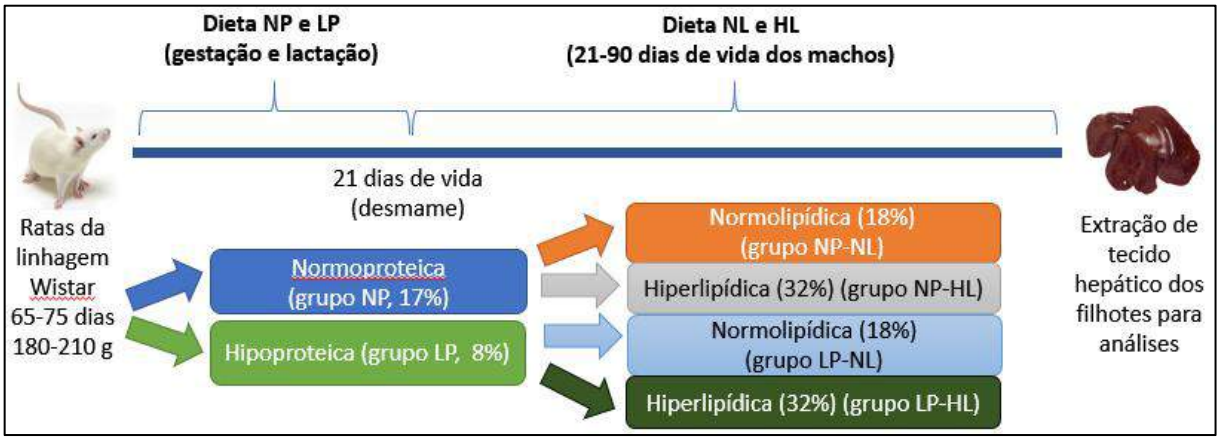
Avaliar os efeitos de dietas hipoproteica durante a gestação e lactação e hiperlipídica pós-desmame na(o):

- Capacidade funcional da cadeia transportadora de elétrons das mitocôndrias de fígado de ratos;
- Indução da transição de permeabilidade mitocondrial (TPM) em mitocôndrias de fígado de ratos;
- Avaliar biomarcadores de estresse oxidativo: peroxidação lipídica (TBARS), oxidação proteica (Carbonilas), oxidação de grupos sulfidrilas (SH) de proteínas;
- Níveis de glutationa reduzida (GSH);
- Atividade das enzimas antioxidantes como a superóxido dismutase, catalase, glutationa-S-transferase e glutationa peroxidase no tecido hepático de ratos;
- Expressão de genes relacionados a biogênese mitocondrial (PGC1-α e TFam) e bioenergética mitocondrial (MFN2, CyPD, GRP75 e VDAC).

3 MATERIAL E MÉTODOS

3.1 Animais e Dieta

Figura 3 – Desenho experimental



Fonte: BARROS JÚNIOR, I. B. de, 2019.

Ratas virgens da linhagem *Wistar (Rattus norvegicus)* foram acasaladas e após a confirmação da prenhez foi ofertada uma dieta normoprotéica (grupo C; 17% de proteína) ou hipoproteica (grupo HP; 8% de proteína) até o final da lactação. Após o nascimento dos filhotes, a prole foi reduzida para oito filhotes por rata. Após o desmame, os filhotes dos grupos C e HP receberam: a dieta C, normolipídica, formando os grupos C-C e NL-C (grupo NL; 18% de lipídeos) (Tabela 1); ou a dieta hiperlipídica (grupo HL; 32% de lipídios) (Tabela 2), formando os grupos C-HL e HP-HL. As dietas foram baseadas de acordo com o *American Institute of Nutrition* – AIN-93 (Reeves *et al.*, 1993) e a Pesquisa de Orçamento Familiar (para preparo da dieta hiperlipídica; IBGE/Brasil, SFB 2002/2003) (CARVALHO *et al.*, 2013; FERRO CAVALCANTE *et al.*, 2013). Todos os quatro grupos, compostos por filhotes machos, permaneceram em suas respectivas dietas até os 90 dias de vida (Figura 3). Os animais foram mantidos em biotério de experimentação com temperatura de 22°C ± 2, ciclo claro-escuro de 12/12 horas. A manipulação e os cuidados com os animais seguiram as recomendações do COBEA (o que significa COBEA). O presente projeto foi aprovado pelo Comitê de Ética para Uso Animal (CEUA) da UFPE (processo: nº 23076.044287/2015-96, Anexo A).

Tabela 1- Composição da dieta Low-protein (g/100 g de dieta)

Ingredientes	Dieta com teor normal de proteína (17 %)	Dieta com baixo teor de proteína (8 %)
Caseína (85 %) (g)	20	9,41
Amido de milho dextrinizado (g)	13,2	13,2
Celulose (g)	5	5
Sacarose (g)	10	10
Amido de milho (g)	39,74	50,34
Óleo de soja (g)	7,6	7,6
Bitartarato de colina (g)	0,25	0,25
Metionina (g)	0,3	0,3
Mix Vitamínico* (g)	1	1
Mix Mineral (g)**	3,5	3,5
Densidade energética (kJ/g)	16,26	16,26

*O Mix Vitamínico é composto pelos seguintes reagentes (em mg/Kg de dieta): retinol, 12; colecalciferol, 0,125; tiamina, 40; riboflavina, 30; ácido pantotênico 140; piridoxina, 20; inositol, 300; cianocobalamina, 0,1; menadiona, 80; ácido nicotínico, 200; colina, 2720; ácido fólico, 10; ácido p-aminobenzoico, 100 e biotina, 0,6.
**O Mix Mineral é composto pelos seguintes reagentes (em mg/Kg de dieta): $CaHPO_4$, 17.200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_2, 120; $FeSO_4.7H_2O$, 200. Fonte: REEVES, 1993.
Fonte: BARROS JÚNIOR, I. B. de, 2019.

Tabela 2 - Composição da dieta High-fat (g/100 g de dieta)

Ingredientes	Dieta com teor normal de lipídeos (18 %)	Dieta com alto teor de lipídeos (32 %)
Amido de milho (g)	52,9	11,8
Farinha de trigo (g)	-	12
Amido de biscoito (g)	-	7,2
Farinha de soja (g)	-	8,5
Goma (g)	-	0,1
Banha (g)	-	5,5
Margarina (65% de gord.) (g)	-	3,5
Creme de leite (20% de gord.) (g)	-	3
Caseína (>85%) (g)	20	20

Continua.
Cont. Tabela 2

Sacarose (g)	10	20
Óleo de soja (g)	7	4
Fibras (celulose) (g)	5	0,3
Mix mineral* (g)	3,5	2,5
DL-metionina (g)	0,3	0,3
Bitartarato de colina (g)	0,25	0,25
Cloreto de sódio (g)	-	0,36
Energia total (kcal/g)	3,6	4,2

*O Mix Mineral é composto pelos seguintes reagentes (em mg/Kg de dieta): $CaHPO_4$, 17.200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_2, 120; $FeSO_4.7H_2O$, 200.
Fonte: REEVES, 1993.
Dieta ocidentalizada adaptada do Orçamento Familiar de Pesquisa (SFB) 2002/2003; Dieta AIN-93G adaptada de Reeves, 1997. Os cálculos foram baseados nas informações de composição nutricional enviadas pelo fornecedor do produto e pela Tabela Brasileira de Composição de Alimentos (TFC) (FERRO CAVALCANTE, 2013).
Fonte: BARROS JÚNIOR, I. B. de, 2019.

3.2 Coleta do tecido hepático para análises bioquímicas

Todos os animais foram eutanasiados através do uso da guilhotina, aos 90 dias de vida, e em seguida foi realizada dissecação para a remoção do tecido hepático.

3.3 Preparo do homogeneizado do tecido hepático para utilização nas técnicas bioquímicas

O tecido hepático foi homogeneizado em tampão de extração (Tris base 50 mM e EDTA 1 mM, pH 7,4; com adição de ortovanadato de sódio 1 mM e PMSF 2 mM). Após a homogeneização, as amostras foram centrifugadas (Sigma 1-14K) a 1180 x *g*, a 4° C, por 10 minutos e os sobrenadantes submetidos à quantificação proteica.

3.4 Isolamento das mitocôndrias hepáticas

As mitocôndrias foram isoladas a partir do fígado de ratos utilizando a técnica de centrifugação diferencial, segundo Schneider e Hogeboom (1951). O fígado foi retirado após a morte do animal, lavado em solução de sacarose 250 mM contendo tampão 10 mM de HEPES (pH 7,2) e 0,5 mM de EGTA, e cortado com tesoura e homogeneizado em homogeneizador *Potter-Elvehjem*. O material foi centrifugado a 461 x *g* por 10 minutos. O sobrenadante resultante foi centrifugado durante 10 minutos a 4722 x *g* sendo a fase lipídica

superior retirada com pipeta de *Pasteur*. O sobrenadante foi descartado e o precipitado ressuspenso em 250 mM de sacarose, 5 mM de HEPES (pH 7,2) e 0,3 mM de EGTA, e novamente centrifugado como na condição anterior. A fração mitocondrial foi ressuspensa na mesma solução, porém isenta de EGTA, numa concentração de aproximadamente 80 mg de proteína por mililitro de suspensão mitocondrial.

3.5 Dosagem de proteína

A concentração de proteína foi determinada pelo método de *Bradford* (Bradford, 1976). O princípio do método baseia-se na determinação da concentração de ligações peptídicas através da medida da absorbância do complexo proteína-corante. Este complexo absorve em comprimento de onda de 595 nm. A absorbância é considerada diretamente proporcional à concentração de proteína na solução analisada, onde uma solução de *Bovine Serum Albumin* (*BSA)* (2mg/mL) foi utilizada como padrão.

3.6 Condições experimentais

Os experimentos com mitocôndrias isoladas foram realizados a 28 °C em meio de respiração, o qual continha 125 mM sacarose, 10 mM HEPES (pH 7,2), 65 mM KCl, 2 mM K_2HPO_4 e 1 mM $MgCl_2$. Como substrato respiratório foi utilizado 5mM de succinato + 2 μM rotenona. Os experimentos foram feitos na presença e/ou ausência de 0,5 mM de EGTA.

3.7 Avaliação do inchamento mitocondrial (TPM)

As suspensões mitocondriais são turvas e espalham a luz incidente. A luz espalhada é uma função da diferença entre o índice de refração da matriz e do meio, e, qualquer processo que diminua esta diferença irá diminuir a luz espalhada e aumentar a transmitância (Nicholls e Brand, 1980). Assim, um aumento no volume da matriz mitocondrial, associado com a entrada de solutos permeáveis, resulta numa aproximação entre o índice de refração da matriz e do meio de reação com a consequente diminuição da luz espalhada. Esta propriedade das mitocôndrias fornece um método qualitativo simples para se estudar o fluxo de solutos através da membrana mitocondrial interna. As mitocôndrias são ideais à aplicação desta técnica porque sua matriz pode sofrer grandes variações de volume, já que a membrana interna sofre desdobramento de suas pregas. O acompanhamento espectrofotométrico da redução da absorbância a 520 nm (Macedo *et al.*, 1988) foi feito em um espectrofotômetro utilizando mitocôndrias isoladas (0,5 mg de proteína/mL). Essa técnica também pode ser utilizada para

avaliar o fenômeno de *TPM*, o qual resulta em inchamento da organela. A confirmação do aumento do volume mitocondrial em decorrência da *TPM* foi feita através do uso de inibidores do poro de transição de permeabilidade (como ciclosporina A e EGTA, quelante de cálcio).

3.8 Medida do consumo de oxigênio mitocondrial

O consumo de oxigênio mitocondrial foi medido polarograficamente utilizando-se um eletrodo do tipo *OXIGY* conectado a um oxígrafo (Hansatech Instrument), em uma câmara de vidro fechada (1 mL) e termostatizada (28º C), equipada com agitador magnético (ROBINSON; COOPER, 1970). Esse tipo de eletrodo compreende um cátodo de platina e um ânodo de prata, imersos numa solução eletrolítica (geralmente KCl). A superfície do cátodo é revestida por uma fina membrana de teflon ou polietileno, que são permeáveis ao oxigênio. Quando uma pequena voltagem é aplicada entre os eletrodos, a platina torna-se negativa em relação à prata, tornando-se polarizada. O oxigênio é então reduzido a peróxido de hidrogênio na superfície da platina, funcionando como aceptor de elétrons, segundo as reações:

$$O_2 + 2\,H_2O + 2e^- \rightarrow H_2O_2 + 2\,OH^-$$

$$H_2O_2 + 2e^- \rightarrow 2\,OH^-$$

Na superfície do ânodo a prata é oxidada, gerando cloreto de prata, segundo as reações:

$$4\,Ag \rightarrow Ag^+ + 2e^-$$

$$4\,Ag^+ + 4\,Cl^- \rightarrow 4\,AgCl$$

A corrente gerada pela diferença dos eletrodos é relacionada estequiometricamente à concentração de O_2 na superfície do cátodo. Os impulsos elétricos são transmitidos ao oxígrafo, onde foi feita a leitura.

3.9 Avaliação dos níveis de peroxidação lipídica pela metodologia da substância Reativa ao Ácido Tiobarbitúrico

Para a dosagem de *TBARS* foi utilizada a técnica colorimétrica de Buege e Aust (Buege e Aust, 1978), sendo uma técnica muito utilizada para avaliar a lipoperoxidação (LPO), pois o ácido tiobarbitúrico reage com os produtos da LPO, entre eles o malondialdeído e outros aldeídos. Adicionou-se a uma alíquota (300 μg proteína) do homogeneizado ao ácido tricloroacético (TCA) a 30%, o material foi centrifugado a 1180 x *g* por 10 minutos, o sobrenadante retirado e adicionado ao ácido tiobarbitúrico a 0,73% que reage com os produtos da lipoperoxidação formando um composto de coloração rosada. A mistura foi incubada por

15 minutos a 100ºC e em seguida resfriada, utilizada para a leitura da absorbância a 535 nm, utilizando cubetas de quartzo. Os resultados foram expressos em µM/mg de proteína.

3.10 Medida dos níveis de oxidação de proteínas (Carbonilas)

A avaliação da oxidação de proteínas foi feita através da reação com o composto 2,4-dinitrofenilhidrazina (DNPH) descrito por Zanatta *et al.* (2013). As proteínas (300 µg) foram precipitadas em TCA a 30 % seguido de centrifugação a 1180 g por 15 minutos, o *pellet* foi ressuspenso e adicionado DNPH 10 mM dissolvido em HCℓ 2,5 M e incubado em local livre de luz a temperatura ambiente durante uma hora. Posteriormente, as proteínas precipitadas em adição ao TCA 20% foram centrifugadas e passaram por uma série de lavagens com tampão contando acetato de etila e etanol, até o precipitado ser ressuspenso em cloridrato de guanidina e levado ao espectrofotômetro para leitura em um comprimento de onda de 370 nm. Os resultados foram expressos em µM/mg de proteína.

3.11 Avaliação do conteúdo de sulfidrilas-SH

O conteúdo de sulfidrila foi determinado a partir da reação com o composto DTNB (ácido 5,5'-ditiobis- (2-nitrobenzóico), ou reagente de Ellman). A alíquota do homogenato (450 µg) foi incubada no escuro após a adição de DTNB 10mM e o volume final foi de 1 mL completado com tampão de extração pH 7,4 e realizada a leitura com absorbância a 412 nm em espectrofotômetro *UV/VIS SPECTROPHOTOMETER* Modelo IL-592 IONLAB (ELLMAN, 1959). Os resultados foram expressos em µM/mg de proteína.

3.12 Avaliação da atividade enzimática da Superóxido dismutase (SOD)

A determinação da atividade de SOD foi feita de acordo com Misra e Fridovich (MISRA; FRIDOVICH, 1972). O homogenato de fígado (100 µg de proteína) foi incubado no tampão carbonato de sódio (0,05 %, pH 10,2, 0,1 mM de EDTA) em banho-maria a 37ºC, antes da avaliação da atividade enzimática. A reação teve início pela adição de 20 µM de epinefrina (150 mM), em ácido acético (0,05 %). A absorbância foi lida a 480nm por 3 min. Os resultados foram expressos em U/mg de proteína. Uma unidade de SOD foi definida como a quantidade de proteína requerida para inibir a auto-oxidação de 1µmol de epinefrina por minuto.

3.13 Avaliação da atividade enzimática da Catalase (CAT)

A atividade da catalase foi monitorada de acordo com Aebi (1984). Resumidamente, 0,3M de H_2O_2 foi adicionado a amostra (80µg de proteína), seguido de adição do tampão fosfato 50 mM, pH 7,0 a 20° C, a absorção de decaimento foi monitorizada por 100 segundos a 240 nm. Os resultados foram expressos em U/mg de proteína. Uma unidade de catalase foi definida como a quantidade necessária para converter 1 µmol de H_2O_2 por minuto a H_2O.

3.14 Avaliação da atividade enzimática da Glutationa peroxidase (GPx)

A atividade da enzima GPx foi monitorada pela diminuição de absorbância do NADPH a 340 nm em espectrofotômetro (UV/VIS SPECTROPHOTOMETER (Modelo IL-592) IONLAB), durante 4 minutos, a 20ºC, em um meio contendo: 100µg de proteínas, tampão fosfato 0,05M (pH 7,0) e EDTA 0,005 M, NADPH 0,0084 M, Azida sódica 1,125 M, GSH 0,15 M, Glutationa redutase (GR) 3 mM e peróxido de hidrogênio 0,0022 M (PAGLIA; VALENTINE, 1967). A atividade da GPx foi expressa em U/mg de proteína. Uma unidade de GPx foi definida como a quantidade de proteína necessária para oxidar 1 µmol de NADPH por minuto, baseado na sua absorbância molecular.

3.15 Avaliação da atividade enzimática da Glutationa-S-Transferase (GST)

A atividade da glutationa-S-transferase é diretamente proporcional a taxa de formação do composto DNP-SG (dinitro fenil S-glutationa), podendo desta forma ser medida através do monitoramento da taxa de formação do composto. Em uma cubeta de quartzo de 1 mL, adicionou-se a amostra (400µg de proteína) ao tampão fosfato (0.1M) e EDTA (1mM), amostra, GSH (1mM) e CDNB (1mM). A absorbância (340 mm) foi registrada por um período de aproximadamente 3 min com controle da temperatura (30º C). Os resultados foram expressos em U/mg proteína. Uma unidade de atividade enzimática da GST foi definida como a quantidade necessária para catalisar a formação de 1µmol do composto DNP-SG por minuto (HABIG *et al.*, 1974).

3.16 Níveis de glutationa reduzida (GSH)

Os níveis de glutationa reduzida foram avaliavas através da quantificação dos níveis de GSH segundo o método de Hissin e Hilf. Em tampão 0,1M fosfato contendo 5 mM de EDTA (pH 8,0) foi adicionado 100 µg do homogenato do fígado e o fluorescente

ortoftaldeido (1μM), e então incubado a temperatura ambiente, por 15 minutos e lido em espectrofluorímetro utilizando os comprimentos de onda de 350 nm de excitação e 420 nm de emissão (Hissin e Hilf, 1976).

3.17 Análise estatística

Para a análise estatística, foi utilizada *GraphPad Prism*® 6.0 para Windows, e os resultados foram expressos como média ± epm (erro padrão da média). Para comparação entre os grupos foi utilizado o teste ANOVA™ *Two-way*. Quando necessário, foi utilizado o pós-teste *Bonferroni*. O nível de significância foi considerado quando $p<0,05$.

4 RESULTADOS

4.1 Artigo original a ser submetido à revista Cells (FI: 4,82), qualis A1 na área de Nutrição.

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Article

Saturated fatty acid enriched diet impaired mitochondrial bioenergetics in liver from undernourished rats during critical periods of development

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Abstract: The nutritional transition that the western population has undergone is increasingly associated with chronic metabolic diseases. In this work, we evaluated a diet rich in saturated fatty acids (hyperlipidic, HL) after weaning of the offspring rats submitted to maternal protein restriction on the hepatic mitochondrial bioenergetics. Wistar rats were mated and during gestation and lactation, mothers received control diets (NP, normal protein content 17%) or low protein (LP, 8% protein). After weaning, rats received either NL (normolipidic) or HL (+59% SFA) diets up to 90 days of life. It was verified that all respiratory states of hepatic mitochondria showed a reduction in the LP group submitted to the post-weaning HL diet. This group also presented greater mitochondrial swelling compared to controls, potentiated after Ca^{2+} addition and prevented in the presence of EGTA (calcium chelator) and cyclosporin A (mitochondrial permeability transition pore inhibitor). There was also an increase in liver protein oxidation and lipid peroxidation and reduction in catalase and glutathione peroxidase activities in the LP group fed HL diet after weaning. Our data suggest that adult rats subjected to maternal protein restriction were more susceptible to hepatic mitochondrial damage caused by a diet rich in saturated fatty acids post-weaning.

Keywords: Hyperlipidic diet; oxidative metabolism; protein restriction

1. Introduction

According to the World Health Organization, thousands of people, mostly children, die annually or present a delay in physiological development due to nutritional deficiencies and changes in the perinatal environment (during gestation and lactation). Studies have shown that protein reduction during gestation associated with a postnatal environment with increased availability of nutrients seems to induce the early onset of hypertension, type 2 diabetes, dyslipidemia and arterial hypertension [1-4]. The underlying mechanisms are based on "phenotypic plasticity" [5,6]. This phenotypic plasticity refers to the ability of an organism to react to changes in the environment with changes in shape, state, movement, or activity rate, without genetic alterations [7].

In the last decades, a new nutritional paradigm has been configured, in which undernutrition has been replaced by overnutrition, characterizing a nutritional transition [8]. This favorable situation for the early onset of chronic-metabolic diseases is strongly associated with the consumption of hypercaloric/hyperlipidic diets [9,10], mainly, westernized diets enriched in saturated fatty acids (SFA). In many western countries, the nutritional transition and the inversion of dietary patterns are noteworthy, where there is a high prevalence of excessive food consumption with higher palatability, such as those rich in fats and sugars [11] leading to greater prevalence of overweight and obesity.

The liver during fetal development is very vulnerable to maternal changes, besides being an essential organ in the metabolism of carbohydrates, lipids and proteins [12]. The literature has reported various effects of protein restriction during critical periods of development on the liver; these include morphological and metabolic alterations, such as content of triglycerides and changes in activity of fatty acid metabolism enzymes, production and use of energy. Moreover, hepatic dysfunction-related studies have shown that liver injury can be assessed by oxidative stress [13-15]. It is believed that oxidative stress is a major cause of developing metabolic disorders as hepatic steatosis, non-alcoholic fatty liver disease and type 2 diabetes, associated to mitochondrial dysfunction [16-18]. Another study showed that exposure to a diet with 45% lipid content during gestation, lactation and postnatal period in the offspring caused changes in the redox state and disturbances in the gene expression related to lipid metabolism in the liver of adult rats [19].

Mitochondria are the main organelles of cellular energy metabolism, responsible for the vast majority of adenosine-5-triphosphate (ATP) synthesis via oxidative phosphorylation. In recent decades, mitochondria have emerged as organelles equipped with sophisticated machinery to mediate the Ca^{2+} flow through the internal mitochondrial membrane and are involved in signaling pathways, injury and cell death (i.e. apoptosis) [20]. Excess in ROS production associated with a decrease on its elimination may generate a situation known as oxidative stress [21], often related to mitochondrial dysfunction.

It is well documented that exposure to dietary factors may cause an increase in oxidative stress, making the body vulnerable to reactive oxygen species, mitochondrial dysfunction, reduction of antioxidant genes expression and related to mitochondrial biogenesis [22]. In vitro assays showed that saturated fatty acids may induce oxygen species production related to long chain fatty acid metabolism [23,24]. Overall, accumulation of palmitic acid in tissues has been described as a risk factor in developing metabolic and mitochondrial dysfunctions [25]. However, the physiological effects of saturated fatty acid enriched diet consumption in malnourished individuals and its repercussion on bioenergetics and mitochondrial function have not been assessed.

Studies show that alterations in mitochondrial biogenesis co-activators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) contribute to the arising of metabolic diseases including diabetes, obesity, cardiomyopathy, Alzheimer's disease and Parkinson's disease [26-28]. In addition, some studies have emphasized the role played by the mitochondrial calcium in order to maintain cellular homeostasis in a condition of nutritional transition [29]. In this context, the proteins VDAC, GRP75, MFN2 and CypD have emerged as essential to the control of the calcium flow into the mitochondria and disruption in their transcriptional pattern is involved in calcium accumulation in the mitochondria and cellular damage in metabolic diseases [30-33].

Although the literature shows an association between protein restriction and lipid overnutrition, so far, few studies have shown the effects of a nutritional transition from protein restriction to saturated fatty acid enriched diet, focusing on bioenergetics and mitochondrial function. The main aim of this study was to evaluate the effects of a diet rich in saturated fatty acids on the liver mitochondrial bioenergetics from protein-undernourished rats.

2. Materials and Methods

2.1 Animals

Male and female Wistar rats were provided by Animal Care and Facilities from the Academic Center of Vitória de Santo Antão, UFPE, Brazil. The animals were maintained at a room with controlled temperature (22 ± 1ºC), light-dark cycle (dark: 18:00-06:00 hours). All experimental protocols were approved by the Ethical Committee of the UFPE, Brazil (23076.016634/2017-52). The body weight of animals was recorded at 90 days on an AS-1000 balance (Marte, São Paulo, Brazil), having an error range of 0.01 g. The food consumption were measured at 90 days, calculated from the difference between the offered diet and what remained after 24 hours in g/day.

2.2 Diets

Three different diets were used in the study, control (normal contents of protein and lipid, NP and NL), low-protein (LP) and SFA enriched diets (hyperlipidic - HL). Control and low-protein diets were prepared according to the American Institute of Nutrition [34]. The hyperlipidic diet was prepared as described by Ferro Cavalcante, *et al.* [35] with modifications. Regarding the composition of the macronutrients, the diets presented, in g/100g: control (NP and NL) (17% protein, 6% lipid and 67% carbohydrate; energy density 3.7 Kcal /100g), low-protein (LP) (8% protein, 6% lipid and 76% carbohydrate; energy density 3.7 Kcal /100g) and hyperlipidic (HL) (22% protein, 15 % lipid and 55 % carbohydrate; energy density 4.5 Kcal /g).

2.3 Nutritional protocols

The rats were placed for mating (one male: two female) and confirmation of pregnancy was assessed by the microscopic presence of spermatozoa in the vaginal smear, which was considered the first day of gestation. After, mothers were randomly transferred to individual cages and fed a diet with 17% protein (Control diet, n=5) or with 8% protein (low-protein – LP, n=5) ad libitum during pregnancy and lactation. After birth (24h), the offspring were grouped into litters with 8 pups. After weaning (postnatal day 22), one or two male offspring from each litter were randomly housed in collective cages (up to four animals per cage). The puppies of each offspring (control and LP groups) were fed with control or with hyperlipidic diet (SFA enriched) until 90d-old, where they were euthanized for liver collection.

2.4 Collection of hepatic tissue, homogenization and protein dosage

The animals were euthanized by decapitation (at 90 days of life) and hepatic tissue was dissected and stored at -80° C until use. Hepatic tissue was homogenized in an extraction buffer (50 mM Tris base and 1 mM EDTA, pH 7.4, with addition of 1 mM sodium orthovanadate and 2 mM PMSF). After homogenization, the samples were centrifuged at 1180g at 4° C for 10 minutes. The total protein analysis was performed by the Bradford method, with BSA solution (2mg/mL) used as standard [36].

2.5 Mitochondria isolation

Liver mitochondria were prepared by homogenization followed by differential centrifugation [37]. After decaptation, the rat's liver was removed immediately and homogenized in a mixture containing 125 mM sucrose, 10 mM HEPES (pH 7.2), 65 mM potassium chloride, 2 mM potassium phosphate and 1 mM magnesium chloride. The homogenate was centrifuged at 461 g for 10 min at 4°C, the resulting supernatant was carefully removed and centrifuged at 4722 g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 250 mM sucrose, 5 mM HEPES (pH

7.2) and 0.3 mM EGTA, and centrifuged as in previous condition. The pellet containing isolated mitochondria was resuspended in a buffer containing 250 mM sucrose and 5 mM HEPES (pH 7.2). Mitochondrial protein concentration was determined spectrophotometrically according to Bradford[21].

2.6 Mitochondrial oxygen consumption

Measurement of mitochondrial respiration was performed at 28°C in a closed thermostatic glass chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) as described previously by Robinson and Cooper [38]. Mitochondria were suspended at a concentration of 0.5 mg of protein/mL in respiration buffer containing contained 125 mM sucrose, 10 mM HEPES (pH 7.2), 65 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 2 μM rotenone, 5 mM succinate and 0.5 mM EGTA. Mitochondrial respiration was measured with Complex II substrate. The following were added to the cell respiration experiments: ADP (200 μM), oligomycin (1 μg/mL) and CCCP (1 μM) [38].

2.7 Mitochondrial permeability transition pore (MPTP) opening

MPTP was determined as described previously [39]. Opening of the pore induces mitochondrial swelling, which is measured spectrophotometrically as a reduction in absorbance at 520 nm. Isolated mitochondria (0.5 mg/mL of protein) were added into swelling buffer that contained 125 mM sucrose, 10 mM HEPES (pH 7.2), 65 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$ and 5 mM succinate. The confirmation of mitochondrial volume increase as a consequence of MPTP was performed through the use of 0.1 μM cyclosporin A (CsA), classical inhibitor of the mitochondrial permeability transition pore, and 0.5 mM EGTA, a calcium chelator [39].

2.8 Oxidative stress evaluation in liver

2.8.1 Evaluation of substances reactive to thiobarbituric acid (TBARS) levels

For the dosage of TBARS, the colorimetric technique of Buege and Aust [40] was used. An aliquot (0.3 mg/mL) of the liver homogenate was added to 30% trichloroacetic acid (TCA) and Tris-HCl (10 mmol/L) followed by thorough mixing and centrifugation at 1180 g for 10 min. The supernatant was transferred to another tube and 0.73% TBA (v/v) was added before mixing and boiling for 15 min. The pink pigment yield was then measured at 535 nm in a spectrophotometer (LIBRA S12 UV/VISIBLE, USA) at RT and expressed as mmol/mg of protein.

2.8.2 Evaluation of protein oxidation

The protein oxidation was assessed using the procedures highlighted by Zanatta, *et al.* [41]. With the samples on ice, 30% (w/v) TCA was added to the sample (0.3 mg/mL) and then centrifuged for 15 min at 1180 g. The pellet was re-suspended in 10 mM 2,4-dinitrophenylhydrazine (DNPH) and immediately incubated in a darkroom 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 5 min at 37 °C and the absorbance read in a spectrophotometer (LIBRA S12 UV/VISIBLE, USA) at 370 nm. The results were expressed as mmol/mg protein.

2.8.3 Superoxide dismutase (SOD) assay

173 SOD determination was performed in accordance with the protocol developed by Misra and
174 Fridovich [42]. In brief, 0.1 mg of protein was added to a 0.05 M Carbonate buffer with 0.1 mM
175 EDTA, pH 10.2. The reaction was started with 150 mM epinephrine and the SOD activity at 37 °C
176 was measured by the kinetics of inhibition of 1 epinephrine auto-oxidation at 480 nm. One unit of
177 SOD was defined as the amount of protein required to inhibit the auto-oxidation of 1 μmol de
178 epinephrine per minute. The results were expressed in U/mg protein.

179 2.8.4 Catalase (CAT) assay

180 CAT activity was monitored according to Aebi [43]. Briefly, 0.3M hydrogen peroxide (H_2O_2)
181 was added to the sample (0.08 mg of protein) followed by addition of the 50 mM phosphate buffer,
182 pH 7.0 at 20 °C. The decay curve absorption was monitored for 100 seconds at 240 nm. One unit of
183 CAT was defined as the amount of protein required to convert 1 μmol of H_2O_2 per minute to H_2O.
184 The results were expressed in U/mg protein.

185 2.8.5 Glutathione peroxidase (GPX) assay

186 GPx activity was performed in accordance with Paglia and Valentine [44]. Briefly, 0.1 mg of
187 protein was added to a 50 mM phosphate buffer, pH 7.0, containing 5 mM EDTA; 0.28 mM NADPH;
188 3.75 mM sodium azide; 5 mM glutathione reduced (GSH) and glutathione reductase, acquired from
189 Sigma (St. Louis, MO). The reaction was started with 2.2 mM H_2O_2. NADPH oxidation followed at
190 340 nm absorbance at 20 °C and its coefficient of extinction was used to determine the GPx activity as
191 U/mg protein. One unit of GPx was defined as the amount of protein required to oxidize1 μmol of
192 NADPH per minute, based on its molecular absorbance.

193 2.8.6 Glutathione S-Transferase (GST) assay

194 The liver homogenate was used to measure GST activity according to the method of Habig *et al.*
195 [45] by determination of absorbance at 340 nm after addition of 1 mmol/L of
196 1-chloro-2.4-dinitrobenzene (CDNB). GST activity was calculated using a
197 2.4-dinitrophenyl-S-glutathione (DNP-SG) substrate at 30 °C. GST activity was expressed as U/mg of
198 protein. Based on its molecular absorbance, 1 enzymatic unit was defined as the amount of protein
199 required to produce 1 μmol/L DNP-SG per minute.

200 2.8.7 Glutathione reduced (GSH) levels

201 To determine GSH levels, the liver homogenate (0.1 mg protein) and the o-Phthaldialdehyde
202 (OPT) fluorescent (1 mg/mL) were added in 0.1M phosphate buffer containing 5 mM EDTA (pH 8.0)
203 and incubated at room temperature for 15 minutes. Fluorescence intensity was measured at 350 nm
204 excitation and 420 nm emission wavelengths and compared with a known standard GSH curve
205 according to the method of Hissin and Hilf [46]. The results were expressed as μmol/mg of protein.

206 2.8.8 Evaluation of total thiols (SH) groups

207 The sulfhydryl content was determined by reaction with DTNB (5,5'-dithiobis (2-nitrobenzoic
208 acid) as described by Ellman [47]. Liver homogenate was incubated in the dark after addition of 10
209 mM DTNB and the final volume was completed to 1 mL with an extraction buffer (pH 7.4). The
210 absorbance reading was then made in a spectrophotometer at 412 nm (LIBRA S12 UV/VISIBLE,
211 USA). The results were expressed as μmol/mg of protein.

212 2.8.9 RNA extraction, reverse transcription and quantitative PCR (qPCR)

213 Total RNA was extracted with Tripure reagent (Roche, Meylan, France) according to the
214 manufacturer's instructions. Briefly, 10 μL of Trizol were used per mg of liver tissue and the

resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France). After grinding, ¼ volume of chloroform was added, the preparation vortexed 3 x 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15,000 g at 4°C. The RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba reagents, Val-de-Reuil, France) and centrifugation (15 min at 15,000 g at 4°C). RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba reagents, Val-de-Reuil, France), dried, and dissolved in 100 µl of RNase-free water. RNA concentration and purity (260/280 nm absorbance ratio) was determined on a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Waltham, MA, USA).

Reverse transcription was performed using a RT-TAKARA kit (Primescript TM, Dalian, China) to generate cDNA for Real Time PCR. The quantitative amplification (qPCR) was measured by Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). All results are represented as arbitrary units (A.U.) derived from a standard calibration curve derived from a reference sample. A PCR for each sample was carried out in duplicate for all genes and TBP was used as a housekeeping gene. As a further control, qPCR amplicons were analyzed by electrophoresis on 1% agarose gel (data not shown). Sequences of primers used in this study are reported in Table 1.

Table 1 - Sequences of primers used for the real-time RT-PCR analysis

Gene Sequence	F/R	5' - 3'	Tm (°C)	Amplicon Size	Ref NCBI
Tbp	F	TGGTGTGCACAGGAGCCAAG	62	139pb	NM_001004198
	R	TTCACATCACAGCTCCCCAC			
Mfn2	F	TTGGATGGACTATGCTAGTG	60	230pb	NM_130894
	R	TCCTCCGACCACGAGAATG			
Hspa9 (Grp75)	F	TGATGCCAATGGGATTGTGC	60	175pb	NM_001100658
	R	CTGCTTCAACACGTTCCTTC			
Ppif (CypD)	F	GGCTACAAAGGCTCCACCTTC	62	112pb	NM_172243
	R	GAAAGCGGCTTCCGTAGATG			
Vdac1	F	AACAGTAACACTCGCTTTGG	60	167pb	NM_031353
	R	TTGACGTTCTTGCCATCCAG			
Tfam	F	GCTTGGAAAACCAAAAAGAC	60	201pb	NM_031326
	R	CCCAAGACTTCATTTCATT			
Pgc-1α	F	TCCTCTGACCCCAGAGTCAC	60	143pb	NM_031347
	R	CTTGGTTGGCTTTATGAGGAGG			

Table 1. mRNA expression levels were quantified from liver-derived cDNAs for the following genes, TATA box binding protein (Tbp; housekeeping), mitofusin 2 (Mfn2); Heat shock protein 9 (Hspa9); Peptidylprolyl isomerase F (Ppif); voltage-dependent anion channel 1 (Vdac); Transcription factor A, mitochondrial (Tfam); Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Pgc-1α).

2.9 Statistical analysis

For statistical analysis, GraphPad Prism® 6.0 for Windows was used, and the results were expressed as mean ± SEM (standard error of the mean). Kolmogorov-Smirnov and Shapiro-Wilk normality tests were applied to all groups. Two-way ANOVA™ test was used to compare all the groups followed by Bonferroni post-test. The level of significance was considered $p < 0.05$.

3. Results

3.1 Body weight and food consumption

The body weight at 90 days was reduced in LP-NL and LP-HL when compared to NP-NL [NP-NL 371.2±7.2 *vs.* LP-NL 317.4±5.1 g (-14.5%, $p<0.0001$), and *vs.* LP-HL 328.9±11.9 g (-11.4%, $p<0.01$)]. In daily food consumption it was observed a decrease in LP-NL when compared to NP-NL [LP-NL 15.5±0.5 *vs.* NP-NL 20.0±1.4 g (-22.5%, $p<0.001$)]; while, NP-HL showed an increased food consumption when compared to LP-HL [NP-HL 24.3±0.2 *vs.* LP-HL 18.4±0.5 g (+24.3%, $p<0.001$)].

3.2 Mitochondrial respiration

The mitochondrial respiration during basal state was reduced in LP-HL when compared to LP-NL (-43.7%, $p<0.05$) and NP-HL groups (-48.7%, $p<0.01$) (Figure **1a**); with ADP stimulated (state 3), HL diet was able to increase ADP phosphorylation (NP-NL vs. NP-HL, +43.01%, $p<0.01$). However, the association of diets during different times led to a decrease in this parameter (LP-HL vs. NP-HL, -37.3%, $p<0.01$) (Figure **1b**). In the resting condition (state 4), there was an increase in LP-NL (+63.1%, $p<0.01$) and NP-HL (+75.1%, $p<0.001$) compared to respective control group (NP-NL); and a decrease in LP-HL when compared with LP-NL (-32.1%, $p<0.05$) and NP-HL groups (-36.8%, $p<0.01$) (Figure **1c**). Similarly, on the uncoupled state the LP-NL (+128.9%, $p<0.001$) and NP-HL (+59.1%, $p<0.05$) groups were higher than NP-NL; and there was a decrease in LP-HL when compared with the NP-HL (-43.0%, $p<0.01$) and LP-NL (-60.4%, $p<0.001$) groups (Figure **1d**). Respiratory control was significantly higher in the LP-NL compared to NP-NL (+36.8%, $p<0.05$) and lower between the LP-HL group compared with the LP-NL group (-25.7%, $p<0.01$) (Figure **1e**).

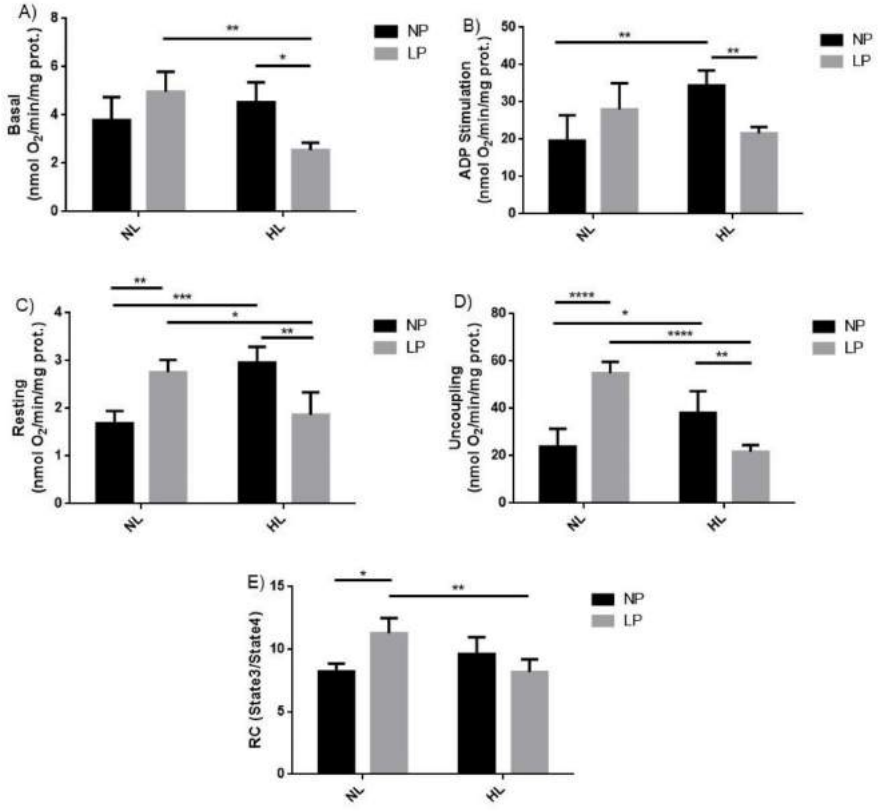

Figure 1. Evaluation of mitochondrial respiration in adult rats subjected to low protein diet during gestation and lactation and post-weaning hyperlipidic diet. Mitochondrial respiration on the basal state (**a**), with ADP (**b**) stimulus, resting state (**c**), uncoupling state (**d**) and respiratory control (**e**), in liver homogenate of male 90 day old rats born to mothers fed on normoprotein (NP) or low protein (LP) diets, during gestation and lactation, and normolipidic (NL) or hyperlipidic diets (HL) after weaning up to adulthood. n=6-9 animals per group. All values were expressed as mean ± SEM. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001 performed with two-way ANOVA followed by the Bonferroni multiple comparison test.

3.3 Mitochondrial swelling

The mitochondrial swelling data revealed that in the presence of calcium ion (10 μM), the area under curve of the LP-HL was higher than the NP-HL (+49.2%, p<0.05) and LP-NL groups (+67.1%, p<0.01) (**2a**). These findings indicate a higher swelling of the organelle in animals submitted to a low protein diet during gestation and lactation and a post-weaning HL diet (**2b**). After addition of higher calcium concentrations (20 μM), the effect of swelling in the LP-HL group was potentiated (272.1%, p<0.01). It was observed that in the presence of CsA and EGTA the mitochondrial swelling was prevented [LP-HL=2.525±0.25 *vs.* LP-HL+CsA=1.112±0.26 (-56%, p<0.0001) and LP-HL=2.525±0.25 *vs.* LP-HL+EGTA=1.212±0.12 (-52%, p<0.001)].

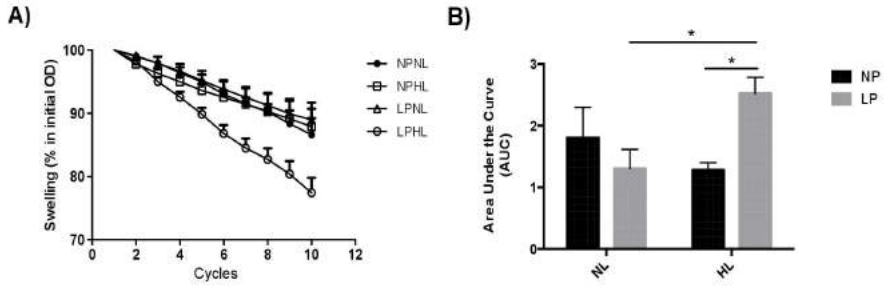

Figure 2. Evaluation of mitochondrial swelling in adult rats subjected to the low protein diet during gestation and lactation and post-weaning hyperlipidic diet. (**a**) Mitochondrial swelling (% of initial OD) and (**b**) Area under the curve in mitochondria isolated from liver of 90-day male rats born to mothers fed a normoprotein (NP) or low protein (LP), during gestation and lactation, and normolipidic (NL) or hyperlipidic diets (HL) after weaning up to adulthood. n =6-9 animals per group. All values were expressed as mean ± SEM. *p<0.05 performed with two-way ANOVA followed by the Bonferroni multiple comparison test.

3.4 Oxidative stress biomarkers

The biomarkers of oxidative stress showed that low protein diet during gestation and lactation followed by a hyperlipidic diet after weaning increased the levels of malondialdehyde (MDA) in LP-NL vs. LP-HL groups (+42.5%, p<0.01) as well as in NP-HL vs. LP-HL groups (+54.3%, p<0.001) (**3a**). Carbonyl levels increased in LP-NL vs. LP-HL (+72.2%, p<0.01), as also in NP-HL vs. LP-HL groups (+60.07%, p<0.05) (**3b**).

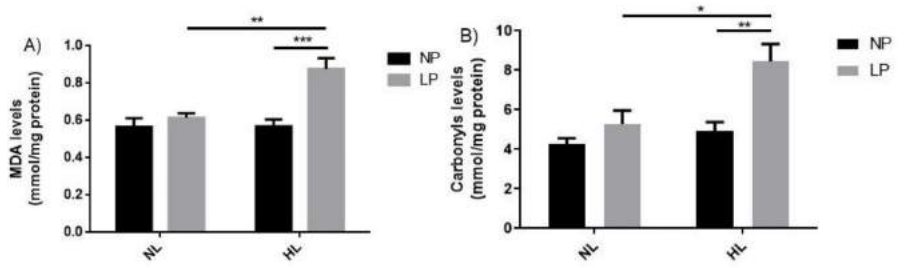

Figure 3 - Evaluation of levels of oxidative stress biomarkers in adult rats subjected to hypoproteic diets during gestation and lactation and post-weaning hyperlipidic diets. (**a**) Levels of malondialdehyde and (**b**) Levels of carbonyls in liver homogenate of 90-days male rats born to mothers fed a normoprotein (NP) or low protein (LP), during gestation and lactation, and normolipidic (NL) or hyperlipidic diets (HL) after weaning up to adulthood. n =5-7 animals per group. All values were expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; performed with two-way ANOVA followed by the Bonferroni multiple comparison test.

3.5 Enzymatic and non-enzymatic antioxidant responses

The evaluation of the enzymatic antioxidant response revealed that the nutritional manipulations did not change significantly the SOD activity (**4a**). However, it was observed that CAT (-48.1%, p<0.05) and GPx (-21.0%, p<0.05) were found reduced in LP-NL vs. LP-HL groups (**4b** and **4c**, respectively). The GST activity showed increase when compared NP-NL with LP-NL (+38.1%, p<0.05) and NP-NL with NP-HL (+44.5%, p<0.01) groups (**4d**). HL diet led a decrease of GSH levels in NP-HL (-29.7%, p<0.05) related to control group (NP-NL) (**4e**). There was no significant change in the total thiol levels (**4f**).

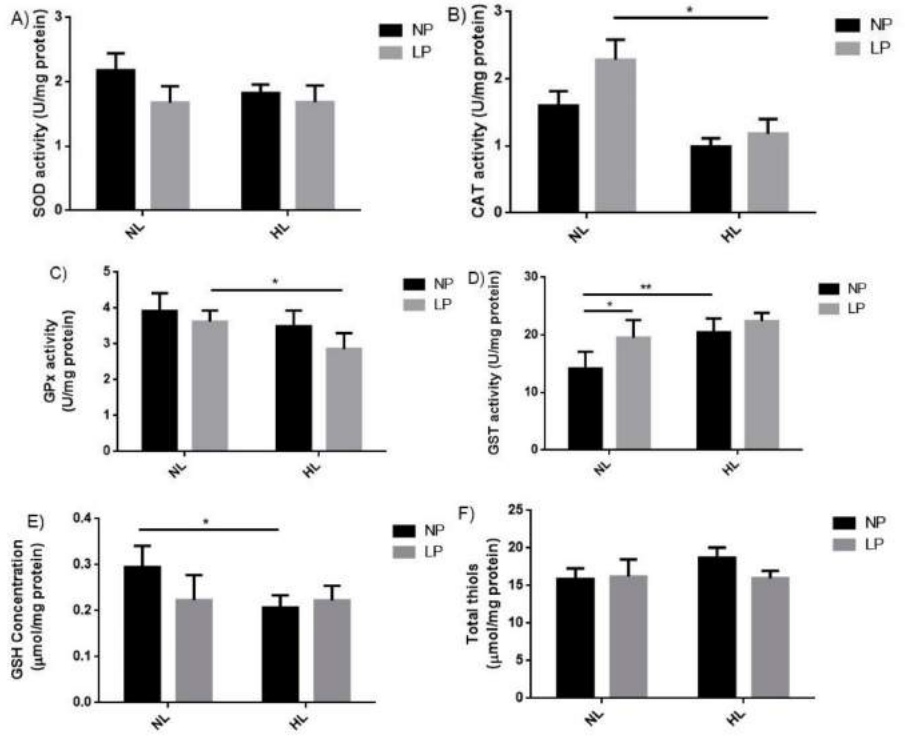

Figure 4 - Evaluation of enzymatic and non-enzymatic antioxidant systems in adult rats subjected to the low protein diet during gestation and lactation and post-weaning hyperlipidic diet. (**a**) Superoxide dismutase activity (SOD), (**b**) Catalase activity (CAT), (**c**) Glutathione peroxidase activity (GPx), (**d**) Glutathione-S-transferase (GST) activity, (**e**) Reduced glutathione levels (GSH) and (**f**) Total thiols in liver homogenate of 90-days male rats born to mothers fed a normoprotein (NP) or low protein (LP), during gestation and lactation, and normolipidic (NL) or hyperlipidic diets (HL) after weaning up to adulthood. n = 5-7 animals per group. All values were expressed as mean ± SEM. *p<0.05; **p<0.01, performed with two-way ANOVA followed by the Bonferroni multiple comparison test.

3.6 Gene expression by RT-PCR

PGC-1α expression was higher in LP-HL when compared to LP-NL (+178.1%, p<0.0001) and LPHL (+95.9%, p<0.001) groups (**5a**). Conversely, Tfam showed a decrease between LP-HL vs. LP-NL (-22.5%, p<0.001) as also between the LP-HL vs. LP-NL (-18.6%, p<0.01) groups (**5b**). Gene expression of MFN2, CypD and GRP75 did not show differences among groups (**5c**, **5d** and **5e**, respectively). In the VDAC analysis, there were increases comparing the NP-HL vs. NP-NL groups (+16%, p<0.05), LP-NL vs. LP-HL (+18.0%, p<0.01), as well between LP-HL vs. NP-HL (+15.1%, p<0.05) (**5f**).

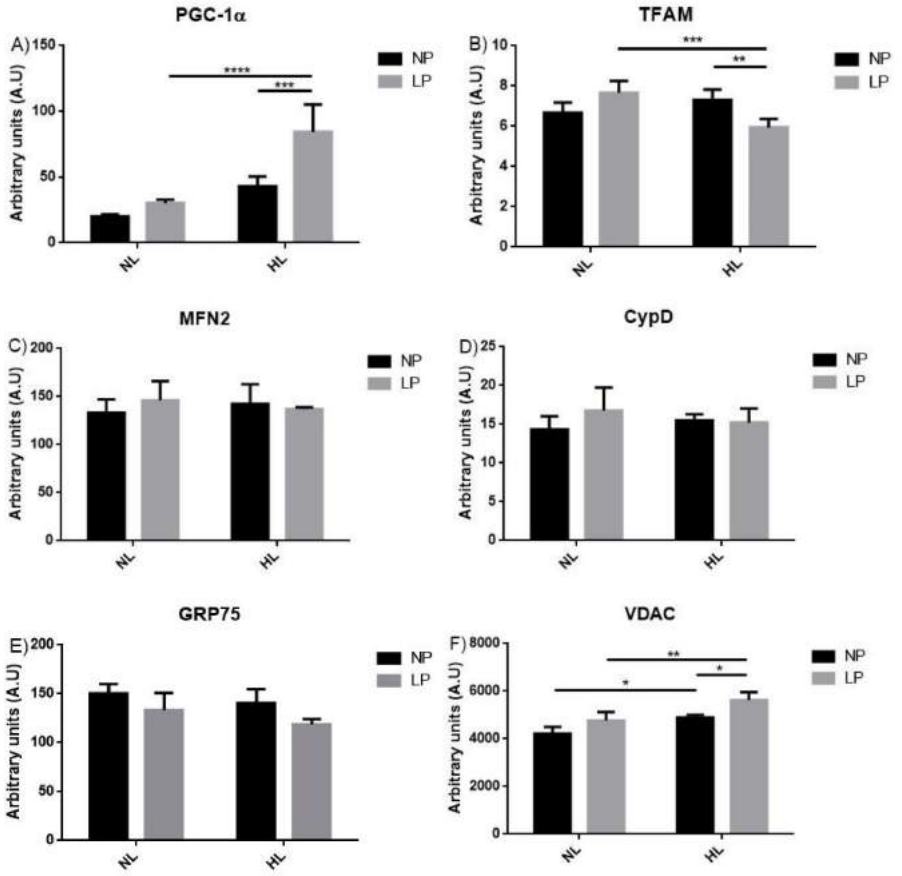

Figure 5 - Evaluation of liver gene expression of adult rats subjected to the low protein diets during gestation and lactation and post-weaning hyperlipidic diet. Genic expression of (**a**) Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α), (**b**) Transcription factor A, mitochondrial (Tfam), (**c**) Mitofusin 2 (MFN2), (**d**) Cyclophilin D (CypD), (**e**) Glucose regulated protein 75 (GRP75) and (**f**) Voltage-dependent anion channel 1 (VDAC) in liver of 90-day male rats born to mothers fed a normoprotein (NP) or low protein (LP), during gestation and lactation, and normolipidic (NL) or hyperlipidic diets (HL) after weaning up to adulthood. All values were expressed as mean ± SEM. *p<0.05; **p <0,01; ***p<0.001; ****p< 0.0001, performed with two-way ANOVA followed by the Bonferroni multiple comparison test.

4. Discussion

In the last decades, a new nutritional paradigm has been identified, in which undernutrition has been replaced by overnutrition, characterizing a nutritional transition. This has been associated to the consumption of westernized diets enriched in saturated fatty acids (SFA), leading to increased risk factor for chronic-metabolic diseases in adulthood [8]. In the present study, we evaluated the impact of a saturated fatty acid enriched diet in the post-weaning offspring subjected to maternal protein restriction on the liver mitochondrial bioenergetics.

354 In our study, it was observed that offspring from groups feeding normal protein diets showed
355 high food intake consumption, with direct association to body weight gain. The body weight from
356 low protein groups that received or not a diet rich in saturated fatty acids were reduced in relation to
357 the control group (NPNL). These data confirm the impact protein malnutrition in early life on the
358 growth [48,49] and reveal that a post-weaning consumption of saturated fatty acids enriched-diet
359 was not able to induce overweight in juvenile rats. It is well known that hyperlipidic diets may
360 impact the growth and fat deposition in adulthood. However, at 90 days of life no sign of
361 overweight was noted. Thus, all results related to hepatic mitochondrial bioenergetics showed in the
362 present study were observed before the progressive dysfunction in body weight.

363 The significant increase on the respiratory control ratio (RCR) of rats' liver mitochondria
364 submitted to low protein diet during gestation and lactation showed higher electron transport chain
365 activity, as a form of metabolic adaptation to the nutritional insult suffered at early life of these
366 animals. This result corroborates with previous study conducted on the offspring from rats
367 subjected to protein restriction during gestation that showed increase in oxygen uptake on isolated
368 rat liver mitochondria on 45, 60 and 120 days of life [50]. However, when the animals were fed with
369 diet rich in SFA, we observed a slower mitochondrial respiration rate and RCR suggesting
370 mitochondrial dysfunction (Figure 1).

371 The literature has shown that inner mitochondrial permeability induced by the calcium ion can
372 be associated with a nonspecific increase in membrane permeability, increasing that increases
373 respiratory rates and decreasing the coupling between oxygen consumption and oxidative
374 phosphorylation [51,52]. Mitochondrial swelling may be due to mitochondrial cristae unfolding, as
375 result of physiological response, or occurs by opening the mitochondrial permeability transition
376 pore (MPTP), a high-conductance channel that can induce loss of mitochondrial membrane
377 potential, impairment of cellular calcium homeostasis, oxidative stress, and a decrease in ATP
378 production upon pathological activation [53]. The mitochondrial swelling experiments showed that
379 animals that received a low protein diet during gestation and lactation were more resistant to the
380 swelling induced by calcium ion; but when the animals were submitted to low protein diet and a
381 SFA enriched diet post-weaning up to adulthood, a higher swelling of the organelle (Figure 2) was
382 observed. This effect was potentiated after the addition of higher calcium concentrations. Generally,
383 mitochondrial impairment is triggered by calcium deregulation that can lead to MPTP opening and
384 cell death [53,54]. After addition of cyclosporin A (mitochondrial permeability transition pore
385 inhibitor) and EGTA (calcium chelator) the mitochondrial swelling was totally prevented, showing
386 that the increased mitochondrial volume observed in the LP-HL was due to the MPTP opening. The
387 MPTP has been considered a key contributor to cell death, inducing neurodegenerative diseases, as
388 Alzheimer's, Parkinson and Huntington's diseases **[55]**, beside cardiomyopathies **[56]**, some types of
389 cancer **[57]**, diabetes [58] and nonalcoholic steatohepatitis **[59]**.

390 Adequate perinatal nutrition is important because macro- and/or micronutrient deficiency
391 during critical periods of development increases oxidative stress by the generation of reactive
392 oxygen species (ROS) and decreases anti-oxidant activity associated with offspring insulin resistance
393 [60,61]. Our results showed that protein restriction during gestation and lactation did not alter the
394 oxidative stress biomarkers levels in liver of adult rats. However, when these animals were
395 submitted to diet rich in SFA from the post-weaning period to adulthood, there was an increase in
396 the lipid peroxidation and protein oxidation levels (Figure 3). A high fat diet (HFD) is shown to
397 increase free radical production that may generally lead to systemic production of oxidative stress
398 [62] and generate hepatic steatosis in rodents and humans by increased hepatic lipid uptake [63,64].

399 Under normal developmental conditions, ROS production is balanced by the removal of free
400 radicals by anti-oxidant mechanisms [65]. Oxidative stress occurs when ROS generation exceeds the
401 scavenging capacity of cellular anti-oxidant mechanisms as a result of excessive ROS production
402 and/or inadequate anti-oxidant intake or synthesis [60]. The literature relates that inadequate

403 maternal nutrition and other challenges during development increase maternal and fetal oxidative
404 stress and are responsible for onset and progression of metabolic diseases [66-68].

405 The evaluation of antioxidant systems showed decrease in CAT and GPx activities in the
406 animals that received HL post-weaning (Figures **4b** and **4c**). Lower CAT activity increases hydrogen
407 peroxide levels and can cause cellular damage [69]. Our results corroborate Tanrikulu Küçük, *et al.*
408 [70] who also observed a decrease on CAT activity and GPx expression in rat's liver fed with HFD
409 during 16 weeks. Another study demonstrated that catalase deficiency did not cause noticeable
410 changes in mice phenotype up to 10 weeks of age, but accelerated HFD-induced systemic and liver
411 insulin resistance, liver inflammation along with increased oxidative stress as early as after 2 weeks
412 of HFD feeding [71]. Data from literature also show that GPx depletion induces lipid peroxidation in
413 various tissues, including the liver [72], and is associated to hepatocellular carcinoma [73]. The
414 glutathione-S-transferase (GST), which is an important antioxidant enzyme, acts on the xenobiotics
415 metabolism, participates in redox signaling because it regulates a lipid peroxidation product,
416 4-hydroxy-trans 2-nonenal (HNE), besides involved on the modulation of gene expression, cell
417 proliferation and apoptosis [74]. Our data showed that independent of nutritional insult, protein
418 restriction or HL diet, the GST activities were higher than in the control groups (Figure 4D). These
419 results may be related to increase liver activity as a form of metabolic compensation.

420 The non-enzymatic antioxidant system was evaluated by GSH dosage, a non-protein thiol with
421 a wide range of antioxidant properties, it can eliminate O2- and OH- radical non-enzymatically,
422 regenerate other antioxidants to their active form, and can be conjugated and excreted with toxins
423 through the reaction catalyzed by glutathione S-Transferases (GST) [66]. GSH levels were decreased
424 in animals that received HL diet post weaning (Figure 4), which suggests reduction in the
425 non-enzymatic antioxidant system. Intracellular GSH homeostasis is affected by increased ROS
426 production, being associated with alcoholic and non-alcoholic liver diseases [75]. Increased lipid and
427 protein oxidations observed in present study may be considered good biomarkers of cellular
428 homeostasis and suggest that the oxidative balance might be impaired in animals subjected to
429 perinatal low protein and post-weaning HL diets. These data reinforce the hypothesis that subjects
430 exposed to nutritional transition are unable to restore oxidative homeostasis. At least in part,
431 changes in oxidative gene transcription and expression should be involved in these functional
432 effects. However, this hypothesis was not tested in the present study and may be evaluated in future
433 studies.

434 Nutritional deficiency may affect several essential metabolic processes and can interfere with
435 genome stability. There is evidence suggesting that a reduction in the basal metabolic rate, which
436 could be caused by the decreased rate of cellular oxygen consumption, mitigating DNA damage
437 arising from oxidative stress [76]. Members of the peroxisome proliferator-activated receptor gamma
438 coactivator 1 (PGC-1) family of coactivators have been revealed as key players in the regulation of
439 the energy metabolism. PGC-1 coactivators coordinate the activity of transcription factors to
440 modulate energy metabolism and other cellular processes in response to a variety of environmental
441 and physiological signals [77]. Mitochondrial biogenesis is regulated to adapt the mitochondrial
442 population to cell energy demands. The mitochondrial transcription factor A (Tfam) performs
443 several functions for mtDNA and interactions between Tfam and mtDNA participate to regulation
444 of mitochondrial biogenesis [78]. Our results showed higher PGC-1α expression and Tfam decrease
445 in the animal's liver submitted to protein restriction during critical periods of development and then
446 received a SFA enriched diet from the post weaning to adulthood (Figures **5a** and **5b**). Initially
447 described as key regulators of the process of mitochondrial biogenesis, recent studies using
448 genetically engineered animal models have uncovered new functions for PGC-1 coactivators beyond
449 the regulation of gene networks strictly related to mitochondrial oxidative metabolism. Lipid
450 synthesis, lipoprotein secretion, muscle fiber type specification, angiogenesis, brite/breige adipocyte
451 differentiation, hematopoiesis and the immune response are among the new cellular and
452 physiological processes that have been described to be regulated by members of the PGC-1 family

453 [77]. Our Tfam results corroborate with Sheldon, *et al.* [79] that showed lower Tfam expression in the
454 liver of rats fed with HFD (45% fat, 35% carbohydrate and 20% protein) post-weaning. However, in
455 the present study, a mild hyperlipidic diet with 33% of fat, but enriched in saturated fatty acids, was
456 able to induce modification of genes related to mitochondrial biogenesis, such Tfam and PGC-1.
457 These results support the notion that saturated fatty acids have an impact on mitochondria,
458 especially those subjected to protein restriction during fetal development.

459 Mitochondrial calcium handling is essential to maintain cellular homeostasis under condition of
460 nutritional transition. In this context, proteins VDAC, GRP75, MFN2 and CypD have emerged as
461 essential to the control of the calcium flow into the mitochondria. Disruption in their transcriptional
462 pattern have been involved in calcium accumulation in the mitochondria and cellular damage in
463 metabolic diseases.

464 The voltage-dependent anion channel 1 (VDAC1), located in the outer mitochondrial
465 membrane (OMM), serves as a mitochondrial gatekeeper, controlling the metabolic and energy cross
466 talk between mitochondria and the rest of the cell. VDAC1 is highly Ca^{2+}-permeable, transporting
467 Ca^{2+} to the inter-membranes space and thus modulating Ca^{2+} access to Ca^{2+} transporters in the inner
468 mitochondrial membrane [31]. VDAC plays key role in the interactions between mitochondria with
469 endoplasmic reticulum (ER). This interaction is very important to mitochondrial calcium handling in
470 order to maintain cellular homeostasis, especially under conditions of nutritional transition. Other
471 proteins are implicated in this process, such as GRP75, MFN2 and CypD. The GRP75 is part of the
472 complex involved in ER and mitochondria Ca^{2+} flow and is composed of the
473 inositol-1,4,5-triphoaphate receptor (IP3R) in the ER membrane and of the VDAC at OMM, and
474 coupled by the GRP75[80]. It is known that reduction in GRP75 expression affects interaction
475 between organelles and impairs mitochondrial calcium handling. Our results showed increased
476 VDAC transcription in the liver of animals that received HL diet enriched with SFA. This effect was
477 potentiated when these animals had been previously subjected to protein restriction diet during
478 critical periods of development (Figure 5f). However, no changes were noted in the transcriptional
479 levels of GRP75, showing that the transcriptional level of VDAC and GRP75 are affected differently
480 by the nutritional transition evaluated in the present study. Studies of Le, *et al.* [81] showed greater
481 VDAC1 expression in mice liver fed with HFD by 16 weeks. The authors suggest that VDAC1
482 inhibition may be an underlying mechanism of drugs, such as sennoside A, a commonly used
483 clinical laxative stimulant, for protecting mitochondria in HFD-induced hepatic steatosis in mice.
484 VADC may be a promising target for treating fatty liver disease [81].

485 Mfn2[82] and CypD[83] have also been cited as involved in the interactions between ER and
486 mitochondria. Loss of these proteins affects the interactions and induces ER stress. However, the
487 interactions can increase in acute responses to ER stress, leading to increased mitochondrial
488 respiration and Ca2+ accumulation with swelling and dysfunction[84]. One of our hypotheses tested
489 after the mitochondrial function essays was in what way the nutrition transition from perinatal
490 protein restriction to a post-weaning SFA enriched diet would affect mitochondrial respiration and
491 swelling, at least in part, by altering the transcriptional levels of these proteins. The data obtained in
492 this paper, however, indicated that the harmful effects observed in mitochondrial bioenergetic
493 functions seems not to be related to dysfunction at transcriptional levels of Mfn2 and CypD. In order
494 to evaluate detail in this process, additional and specific experiment will be needed.

495 **5. Conclusions**

496 This study showed that SFA-enriched HFD induces mitochondrial dysfunction in liver of
497 young rats and that this effect was potentiated when the animals were submitted to low protein diet
498 during critical periods of development. Our results suggest that this mitochondrial dysfunction is
499 associated with oxidative stress, greater expression of VDAC that potentiates the opening of the
500 mitochondrial permeability transition pore and lower mitochondrial biogenesis.

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5 CONSIDERAÇÕES FINAIS

Recentes pesquisas sugerem que disfunção mitocondrial desempenha um importante papel na patogênese e desenvolvimento de doenças hepáticas gordurosas. Este estudo mostrou que dieta hiperlipídica rica em ácidos graxos saturados induz disfunção mitocondrial em fígado de ratos jovens e que este efeito foi potencializado quando os animais foram submetidos previamente a uma dieta com baixo teor de proteínas durante períodos críticos do desenvolvimento (gestação e lactação). Nossos resultados sugerem que esta disfunção mitocondrial está associada com o estresse oxidativo, maior expressão de VDAC que potencializa a abertura do poro de transição de permeabilidade mitocondrial e menor biogênese mitocondrial.

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APÊNDICE A - ARTIGO DE REVISÃO A SER ENVIADO PARA PUBLICACÃO

The impact of High Fat and Low Protein diets on expression regulation of superoxide dismutase, catalase and glutathione peroxidase through microRNAs in the liver

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Running Title: The impact of High Fat and Low Protein diets on expression of antioxidant enzymes through microRNAs in the liver

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Introduction

Although it is necessary for the production of energy in the form of ATP, the oxygen gas (O_2) ends up forming free radicals, called reactive oxygen species-ROS (Halliwell e Gutteridge, 2007). The ROS imbalance may alter mitochondrial bioenergetics, which can cause problems to the body, such as damage to the mitochondrial genome and reduction of ATP production (Shaughnessy *et al.*, 2014).

There are definitions and studies on oxygen toxicity, being able to act in the aging process (Harman, 1956). Damage to cellular structures is related to redox control, and knowledge about oxidative stress has been evolving considerably over the years, both in the higher production of ROS and in the lower antioxidant defense (Allen e Tresini, 2000; Valko *et al.*, 2007). The organism has its own defense to these ROS, being called the antioxidant defense, divided into enzymatic and non-enzymatic (Bianchi e Antunes, 1999). In relation to enzymatic defense, three enzymes are important: superoxide dismutase, catalase and glutathione peroxidase. The latter enzyme uses reduced glutathione to form the oxidized glutathione. Multiple factors influence oxidative stress, such as nutritional imbalance as

obesity and undernutrition; and external factors such as cigarette use and pollutants (Machlin e Bendich, 1987; De Mello *et al.*, 2017).

Nutritional diets as Low-protein and High Fat are widely used inducing metabolic problems, such as chronic diseases, hypertension, obesity, cardiovascular disease, diabetes, as well as impacting some biological processes. In this way, several experimental studies showed the effects of nutritional insult on the antioxidant defense system, including the proteins expression in the diverse tissues (Theys *et al.*, 2009; Boden *et al.*, 2012; Rindler *et al.*, 2013; Tarry-Adkins *et al.*, 2013; Yuzefovych *et al.*, 2013; Liu *et al.*, 2015; Brito Alves *et al.*, 2016; Zhou *et al.*, 2018).

The epigenetic mechanisms are involved in gene expression by regulating the information contained in the genome. It has been demonstrated an association between microRNA regulating protein synthesis (Jablonka e Lamb, 2002; Ambros, 2004; Bartel, 2004; Haig, 2004; Godfrey *et al.*, 2007), in various organs (Dumortier *et al.*, 2013), and can be called "epi-microRNAs" (Valeri *et al.*, 2009). The works have shown that antioxidant enzymes are regulated by microRNAs showing their importance in the epigenetic (Haque *et al.*, 2012; Howell *et al.*, 2013; Matoušková *et al.*, 2018).

Studies have shown the effects of the impact of a nutritional insult on the antioxidant defense systems in the most diverse tissues (Theys *et al.*, 2009; Boden *et al.*, 2012; Rindler *et al.*, 2013; Tarry-Adkins *et al.*, 2013; Yuzefovych *et al.*, 2013; Liu *et al.*, 2015; Brito Alves *et al.*, 2016; Zhou *et al.*, 2018), and the influence of miRNAs with the synthesis of antioxidant enzymes (Carrer *et al.*, 2012; Wang *et al.*, 2014; Yang *et al.*, 2015; Lim *et al.*, 2016; Wan *et al.*, 2016; Akbari *et al.*, 2017; Firmin *et al.*, 2018; Qin *et al.*, 2018; Yang *et al.*, 2018). Hence, the goal of this review is to focus on the impact of High Fat and Low Protein diets on antioxidant proteins expression in different tissues; and how microRNAs may modulate the expression of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in the liver.

Antioxidant systems and oxidative stress

Aerobic beings need O_2 to perform efficient energy production. However, the need for O_2 eventually masks the fact that it is a toxic molecule, therefore aerobic beings only survive because of their antioxidant defenses. In mammals, O_2 is essential. In tissues, O_2 is essential for energy production, however, this molecule can form free radicals in mitochondria. Free radicals can be defined as “molecules, or fragments of molecules, that contain one or more unpaired electrons in their orbitals” (Halliwell e Gutteridge, 2007).

Mitochondria are the cellular organelles responsible for the production of energy (ATP), through oxidative phosphorylation coupled to electron transport chain, both occur in the internal mitochondrial membrane (Porter e Berdanier, 2002). This sequence provides some free radicals. Approximately 2 to 5% of the oxygen that our mitochondria use turns into free radicals, because they need a stabilization for their high affinity to electrons (Turrens, 2003). Also, external sources of free radicals include the use of cigarettes, pollutants in the atmosphere, organic solvents, and pesticides, as well as radiation (Machlin e Bendich, 1987).

Initially, the causes of the negative properties of oxygen became obscure until the publication of the free radical theory of Gerschman *et al.* in 1954, dealing with the toxicity of oxygen is due to its reduced form (Gerschman *et al.*, 1954). Subsequently, Harman in 1956 proposed that free radicals in biology would play a role in the aging process (Harman, 1956).

After that, in 1969, McCord and Fridovich discovered the enzyme superoxide dismutase (SOD), providing evidence on the importance of free radicals in organisms (Mccord e Fridovich, 1969). Since then, various evidences of how living beings have adapted to free radicals developing various defense mechanisms against them have been discovered and analyzed. Oxidative stress is noted as an important in aging and in several biochemical processes. The redox homeostasis, like pH control, is central to life. In bioenergetics and vital functions, oxidation-reduction reactions, known as redox state, are practically involved in all fundamental processes (Sies *et al.*, 2017). Sies e Cadenas (1985) proposed that oxidative stress corresponds to the perturbation of the balance between pro-oxidants and antioxidants, to the detriment of the former, resulting in the oxidation of biomolecules. More recent Halliwell e Gutteridge (2007) proposed that oxidative stress is the biomolecular damage caused by an attack of reactive species on the constituents of living organisms.

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al.*, 2007). Mitochondrial proteins and mitochondrial DNA (mtDNA) are vulnerable to ROS damage by altering the integrity of mtDNA, inhibiting complexes in the electron transport chain, affecting Ca^{2+} transport and activating proaptotic signaling because they are produced during production normal energy by oxidative phosphorylation and generation of ATP by the electron transport chain (Shaughnessy *et al.*, 2014).

The excessive production of free radicals is harmful and may cause damage to some molecules, for example mitochondrial constituents or components of the cell structure, such as membrane lipids and DNA, such as in a chain reaction (Halliwell, 1994). The antioxidant defense can be divided into non-enzymatic, which involves substances such as vitamin E, vitamin C, β-carotene, glutathione (which has important role associated with an antioxidant enzyme), among others (Bianchi e Antunes, 1999); and enzymatic, which will be more focused here. The superoxide dismutase (SOD), an enzyme found in mitochondria and cytosol, converts the superoxide ion to hydrogen peroxide in a reaction involving hydrogen ions and also releases oxygen (Halliwell, 1994; Babior, 1997). Catalase (CAT), in turn, uses two molecules of hydrogen peroxide to form two molecules of water, and one of oxygen gas (Jacob, 1995). Glutathione peroxidase (GPx) uses two molecules of reduced glutathione (GSH) and hydrogen peroxide, forming two molecules of water and one of oxidized glutathione (GSSG) (Huber *et al.*, 2008; Raza, 2011):

Table 1 – Enzymatic antioxidant system reactions

ENZYMES	***REACTION***
SOD	$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$
CAT	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
GPX	$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$

Adapted from Jacob (1995).

The non-enzymatic system derives from the compound of food origin, such as vitamin E, vitamin C, carotenoids, among others. Increased ROS production is one of the responses to excess nutrients that occurs in obesity. This excess of nutrients may be accompanied by a vitamin-poor diet, as has been the trend in the diet West, and may be

aggravated by physical inactivity. Oxidative stress can be caused by a condition such as obesity. As well as, a diet of lower nutritional quality increases the ROS production and can lead to mitochondrial dysfunction (De Mello *et al.*, 2017).

Some factors may be modulatory to oxidative stress, such as diet, lifestyle, physical activity, smoking, use of alcoholic beverages, among others (Barbosa *et al.*, 2010); besides diseases that may be related to the generation of free radicals, including atherosclerosis, diabetes, heart disease, cancer and diseases of the immune system, besides aging (Bianchi e Antunes, 1999). Understanding how antioxidant systems acts in situations, such as deprivation or nutritional excess, specially the gene regulation of proteins involved in this process can show ways of adapting to challenges.

Antioxidant proteins regulated by epigenetic mechanisms

Various epigenetic mechanisms modulate proteins that participate in the antioxidant processes. Epigenetic regulation plays an important role on the functional transduction of the information contained in the genome. Some processes involved this regulation are called post-translational, for instance the modifications of histones, impacting gene expression by altering the structure of the chromatin. The most known modifications are acetylation, in which the addition of an acetyl group promoted by acetyl coenzyme A, involved in the regulation of cellular processes, DNA replication and apoptosis occurs; and methylation, defined as the transfer of a methyl group by histone methyltransferases, where some genes can be evidenced or silenced (Jablonka e Lamb, 2002; Haig, 2004; Godfrey *et al.*, 2007). There are also post-transcriptional changes, which can occur on the three main RNA types, resulting in a process for the correct translation of the genome that will serve as the basis for protein synthesis, containing both exons and introns, responsible for the formation of a new RNA sequence. One type of RNA studied is microRNAs, which are recognized as fundamental in the regulation of gene expression, where a single micro-RNA acts on several mRNAs, influencing multiple signaling pathways and with enormous regulatory potential (Muramatsu *et al.*, 1988; Kiss, 2001; Ricarte Filho e Kimura, 2006). The study from García-Giménez *et al.* (2017) shows that GSH metabolism can control the epigenetic mechanism at different levels, including enzymatic activity of DNA methylation and modifications in the expression of microRNAs showing how oxidative stress and epigenetics are associated.

Some studies show an association between microRNA and epigenetics. MicroRNAs (miRNAs) constitute a newly discovered class of non-coding RNAs that play key roles in the regulation of gene expression. By acting at the post-transcriptional level, these molecules can adjust the expression of up to 30% of all genes encoding mammalian proteins. The miRNAs are short single stranded RNA molecules with approximately 22 nucleotides in length. The maintenance of metabolic homeostasis depends of gene expression regulation, and miRNAs play an essential role in this expression in tissues. For instance, in the liver, the miR-122 is important for lipid metabolism (Lynn, 2009).

As the proteins, the miRNAs are also targets of epigenetic regulation and they can regulate various components of the epigenetic machinery. In cancer, for example, many miRNAs have diminished expression, impacting the epigenetic regulation of genes involved in cell proliferation. Also, in the case of colorectal cancer, the miR-342 was the methylated miRNA methylated (Silahtaroglu e Stenvang, 2010). The miRNA has a role in the regulation of epigenetic processes, controlling the structure of chromatin by the post-transcriptional regulation of chromatin-modifying enzymes. Thus, it being able to be directly or indirectly

regulating the levels of gene expression of the epigenetic processes, it being called "epi-miRNAs" (Valeri *et al.*, 2009).

The regulation of the gene expression in the antioxidant enzymes is affected via post-transcriptional miRNA modulation. A study showed that one of the microRNAs of the miR-30 family, the miR-30b was inhibited, while inhibiting the expression of catalase in the human retinal pigment epithelial cell line by administration of exogenous H_2O_2 showing a cytoprotective mechanism against oxidative stress (Haque *et al.*, 2012) Another work that used the same culture of epithelial cells, however with treatment of curcumin, compound of exogenous catalase and has antioxidant effects, resulted in altering of the expression of miRNAs modulated by the administration of H_2O_2, regulating the antioxidant defense (Howell *et al.*, 2013). Glutathione peroxidase also appears to have a regulation of several miRNAs, in particular miR-185-5p, where this miRNA has been altered by selenodeficiency, and may influence the feedback control of selenoprotein production, of which GPx is included (Matoušková *et al.*, 2018). These data reveal that, these enzymes may have their expression regulated by the miRNAs.

miRNAs and gene regulation of antioxidant enzymes in liver

miRNAs have been shown to be involved in a wide range of biological processes, such as cell cycle control, apoptosis and various physiological and developmental processes, in addition to hematopoiesis and standardization of the central nervous system (Ambros, 2004; Bartel, 2004; He e Hannon, 2004). microRNA can participate in the synthesis of 30% of all human genes, because a simple miRNA regulates hundreds of different mRNAs. The miRNAs have been related to one or more tissues, participating in the metabolic processes and regulators of energy metabolism (Dumortier *et al.*, 2013). Multiple studies have showed that a large number of miRNAs participate in signaling cascades and can be modulated or repressed (Carrer *et al.*, 2012; Wang *et al.*, 2014; Yang *et al.*, 2015; Lim *et al.*, 2016; Wan *et al.*, 2016; Akbari *et al.*, 2017; Firmin *et al.*, 2018; Qin *et al.*, 2018; Yang *et al.*, 2018).

In liver, specific studies have published analyzes related to the miRNAs involved in the antioxidant enzymatic synthesis. Mitochondrial dysfunction is associated with metabolic syndrome and obesity, and the proliferator-activated receptor γ coactivator 1β (PGC-1β) gene encodes two miRNAs, miRNA-378 and miRNA-378*, participating in systemic control and energetic homeostasis under stress conditions, demonstrating as possible targets for pharmacological intervention in obesity (Carrer *et al.*, 2012). The work of Firmin *et al.* (2018) examined the impact of glucose as a pro-oxidant molecule (Maillard reaction products), such as dietary advanced glycation end products, on pigs' liver; analyzing the expression of miRNA-21 and miRNA-155. The results demonstrated that there was a greater expression of SOD and CAT, but not of GPx, demonstrating that the antioxidant mechanism was not enough to detoxify ROS, probably due to the age of the animals were 54 days of life in association with the diet with thid diet.

Compounds with protective antioxidant or pro-oxidant effects are studied as well. The use of xylobiosis, which can regulate diabetes-related metabolism, was analyzed in rats subjected to energy restriction evaluating the expression levels of miR-122a and miR-33a. On the group which was exposed to energy restriction, the levels of SOD expression increased, and this increase was reverted on the group that received disaccharide, similar effects were observed with CAT and GPx (Lim *et al.*, 2016). The diterpene paclitaxel, originating from an Asian tree, was studied by Yang *et al.* (2018) for being known in the fight against hepatic septic damage. Analysis of rat liver miRNA-27a relative to the synthesis of SOD and GPx showed that this compound upregulated the expression of this miRNA, accompanied by an increase in the levels of these enzymes. The hepatotoxicity of perfluorooctane sulfonate (PFOS) is related to the fact that its concentration in this organ is related to centrilobular

hypertrophy, hepatic steatosis and hepatomegaly, being able to promote the expression of miRNA-155. However, in the animals subjected to pre-treatment with exogenous CAT, its expression was similar to normal values, serving as a biomarker for detection of PFOS, and specific inhibition of miRNA can prevent oxidative damage in the liver (Wan *et al.*, 2016). Similar effect was observed with miRNA-21, relative to betulinic acid, a natural compound related to pro-apoptotic activity and hepatocellular carcinoma. The treatment down-regulated SOD expression, and increased ROS production leading to apoptotic cell death (Yang *et al.*, 2015). Also, in response to hepatocellular carcinoma, miRNA-503 was overexpressed in reversal a treatment with Adriamycin®, significantly decreasing SOD activity. This miRNA was known to be a target against the drug fighting this carcinoma, preventing cell progression and cellular apoptosis (Wang *et al.*, 2014). In addition, the crocin carotenoid, obtained from saffron, has antioxidant and antiapoptotic role. A treatment that leads to increase in GPx, CAT and SOD activities, followed by down-regulating miRNA-122 and miRNA-34a, being effective in combating oxidative damage and ischemia-reperfusion injury (Akbari *et al.*, 2017). The miRNA-34a was also studied by Qin *et al.* (2018), related to ischemia-reperfusion injury, and the application of an inhibitor of this miRNA was able to recover the levels of SOD, reverting the damage not only in the liver, but in other organs.

Regulation of antioxidant proteins by low-protein diets

Low-protein diets are widely used on the diverse types of animal models because protein is essential in tissue growth and development, and its deficiency is associated with several metabolic problems, such as reduced synthesis of hormones and enzymes, impaired immune function, loss of muscle and decreased growth (Arnold *et al.*, 2002; Campbell *et al.*, 2002; Li *et al.*, 2007; Wu, 2016).

Previous studies have been demonstrating the physiology and molecular effects of the low-protein diet in different tissues, such as testicles (Rodríguez-González *et al.*, 2014), in the blood (Takemori *et al.*, 2013), kidneys (Bem *et al.*, 2014; Liu *et al.*, 2018), in the heart (Nascimento *et al.*, 2014; Braz *et al.*, 2017; Carthagenes *et al.*, 2017), in the cerebellum (Augusto *et al.*, 2017) and in the brainstem (Ferreira, D. J. S. *et al.*, 2016; Ferreira, D. S. *et al.*, 2016; De Sousa *et al.*, 2017), beyond the liver (Maddineni *et al.*, 2013; Lívero *et al.*, 2014; Mitchell *et al.*, 2015; Vega *et al.*, 2016). Most of them showed the malefic effects of this nutritional insult, and the others showed some protective effect for some specific substance in an attempt to reverse oxidative damage.

Effects on protein expression also were observed by De Brito Alves *et al.* (2016) studying a model of protein restriction during the gestation and lactation periods in the ventral medulla of rats. The data showed that the expression of SOD and GPx was lower, revealing that the enzymatic activity was affected. In cardiac tissue, an up-regulation of SOD and CAT was observed, configuring a compensatory response to oxidative stress (Tarry-Adkins *et al.*, 2013). In another paper, analyzing the pancreatic islets, a greater transcription of SOD, CAT and GPx was observed, demonstrating a mitochondrial dysfunction (Theys *et al.*, 2009). Supplementation of the antioxidant enzyme coenzyme Q10 (CoQ_{10}) altered the enzymatic antioxidant capacity in the liver, even with the decrease of GPx, showing a potential preventive effect of CoQ_{10} against oxidative stress and hepatic fibrosis (Tarry-Adkins, Fernandez-Twinn, Hargreaves*, et al.*, 2015). In skeletal muscle, the low-protein diet had its adverse effect reverted in animals that obtained accelerated growth, even with a poor maternal diet. The data showed that these animals had a greater SOD and CAT expression than the animals that did not grow satisfactorily, configuring a satisfactory response to the higher oxidative stress as a consequence of the nutritional insult (Tarry-Adkins *et al.*, 2016). As observed, the adverse effects of the low-protein diet can negatively influence the antioxidant enzymatic activity.

Also the kidneys are inversely affected by low-protein diets, as shown in the paper of Tarry-Adkins *et al.* (2007). The diet was able to protect the organ, even with a protein restriction in lactation, being a result of the decrease of the renal damage accompanied by an up-regulation of the SOD and GPx, improving until the longevity of the animals.

Interestingly, the low-protein diet may also influence miRNAs, as demonstrated in the Tarry-Adkins, Fernandez-Twinn, Madsen*, et al.* (2015) paper's, analyzing whether a supplementation with CoQ_{10} has an antioxidant role on the epididymal adipose tissue in animals that had a rapid growth, even with nutritional insult. The data showed that none of the analyzed miRNAs (25, 301a, 19a, 130a, 130b, 126 and 143) had their expression modified even with the diet, not directly interfering with SOD and CAT activity. The authors speculated that the miRNAs chosen are not related to the expression of the antioxidant proteins, or that other post-transcriptional mechanisms are involved.

Regulation of antioxidant proteins by High Fat Diets

As well as low-protein diets, the High Fat diets are widely studied in experimental models, mainly because of the energetic excess offered, associated with insulin resistance and obesity. The use of high fat diet (HFD) is followed by disorders in adult life, mainly due to changes in dietary style in the life of the world population (Sullivan *et al.*, 2014; Cordner e Tamashiro, 2015).

Several papers showed the effects of a HFD on different tissues, analyzing the potential positive and negative effects with physiology and molecular changes, such as in the brain (Che *et al.*, 2018), in the hippocampus (Alzoubi *et al.*, 2018), in the spleen (Cui *et al.*, 2012), in white adipose tissue (Amengual-Cladera *et al.*, 2012), and especially in the liver (Raffaella *et al.*, 2008; Nadal-Casellas *et al.*, 2010; De Assis *et al.*, 2012; Mahmoud *et al.*, 2012; Satapati *et al.*, 2012; Valenzuela *et al.*, 2012; Macedo *et al.*, 2013; Zhang *et al.*, 2013; Enos *et al.*, 2014; Franko *et al.*, 2014; Crescenzo *et al.*, 2015; Hou *et al.*, 2016; Zhong e Liu, 2018), each one with its specific experimental design and different analyzes in relation to the antioxidant system.

In this way, the relationship between HFD and protein expression were studied as well. The cardiac tissue was also analyzed by Rindler *et al.* (2013). The authors showed that HFD can influence the expression of CAT, resulting in an up-regulation of this enzyme, as a way of trying to reverse the increased production of ROS. An increase in fat metabolism was observed showing a possible protective metabolic effect against oxidative damage.

It has been demonstrated that SOD can also be overexpressed, elevating mitochondrial antioxidant enzymatic activity in skeletal muscle, protecting against diet-induced insulin resistance (Boden *et al.*, 2012). In the liver, the epigenetic effects modulating oxidative stress function were also observed in a paper which examined whether the amino acid serine could prevent hypermethylation of promoters of genes related to glutathione synthesis (Zhou *et al.*, 2018). In the Yuzefovych *et al.* (2013) work, ROS damage to mtDNA was sufficient to decrease SOD expression in both skeletal muscle and liver resulting from mitochondrial dysfunction generated by increased oxidative stress. Also in skeletal muscle, the adiponectin improved the GPx and SOD gene expression, revealing a potential antioxidant role of adiponectin. (Liu *et al.*, 2015).

A study analyzed the relationship between miRNA-34a and apoptosis in the liver fatty liver disease (NAFLD) High Fat diet-induced. It was demonstrated that hepatocytes accumulate fat and cause hepatic inflammation and may be irreversible. Also, it was verified whether carnosic acid, obtained from rosemary, induces apoptosis in cancer cells affecting the expression of genes that regulate apoptosis, presenting protective role against tissue damage. Thus, the results showed that the treatment with this substance is associated with the down-regulation of miRNA-34a and SOD levels increased, constituting a treatment against the

progression of NAFLD (Shan *et al.*, 2015). All these data show how much HFD can influence the enzymatic system of antioxidant defense through gene expression regulation.

Conclusion

Antioxidant enzymatic defense is one of the ways to avoid oxidative stress, however nutritional insults and certain compounds in the critical period of development may influence the expression of these proteins, both by epigenetic processes and miRNAs modulation, which play an important role in the synthesis of proteins. The studies that relate miRNAs to antioxidant enzymes are quite recent, revealing that several miRNAs are related to antioxidant enzyme defense in organs such as the liver, and that nutritional insults, at various stages of development, influence the antioxidant enzyme system; showing how epigenetics factors plays an important role in the defense against ROS

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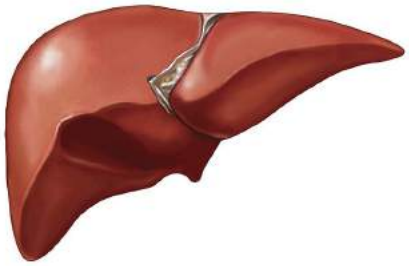
Figures

HighFat Diets

Tissue	Expression	References
Heart	↑CAT	Rindler *et al.*, 2013
Skeletal muscle	↑SOD	Boden *et al.*, 2012
Sk. muscle and liver	↓SOD caused by ROS damage to mitochondrial DNA	Yuzefovich *et al.*, 2013
Skeletal muscle	Adiponectin improved the GPx and SOD gene expression	Liu et al., *2015*
Liver	Serine prevent hypermethylation of genes related to glutathione synthesis	Zhou et al., *2018*

Low-protein Diets

Tissue	Expression	References
Ventral medula	↓SOD and GPX expression	de Brito Alves *et al.*, 2016
Heart	↑CAT and SOD	Tarry-Adkins *et al.*, 2013
Pancreas	↑transcription of SOD, CAT and GPx	Theys *et al.*, 2009
Liver	↓GPx, the suplementation with CoQ10 prevented the oxidative stress	Tarry-Adkins *et al.*, 2015
Skeletal muscle	↑SOD and CAT expression, consequence of the diet	Tarry-Adkins *et al.*, 2016
Kidneys	↑SOD and GPx expression, acompanied with a decrease of the renal damage	Tarry-Adkins *et al.*, 2007



miRNAs	Compound	Expression	References
122a; 33a	Xylobiosis	↑SOD, CAT and GPx	Lim *et al.*, 2016
27a	Paclitaxel	↑SOD and GPX	Yang *et al.*, 2018
122; 34a	Crocin	↑SOD, CAT and GPx	Akbari *et al.*, 2017
34a	Carnosic acid	↑SOD	Shan *et al.*, 2015
34a	Inhibitor of this miRNA	↑SOD	Qin *et al.*, 2018
155	Perfluorooctane sulfonate	↓SOD	Wan *et al.*, 2016
21	Betulinic acid	↓SOD	Yang *et al.*, 2015
503	Adriamycin®	↓SOD, CAT and GPx	Wang *et al.*, 2014

Figure 1. The summary of the action of High Fat and Low-Protein diets in various organs and tissues, affecting positively or negatively the action of antioxidant enzymes; and the microRNAs that were analyzed in the liver, influencing the expression of these enzymes

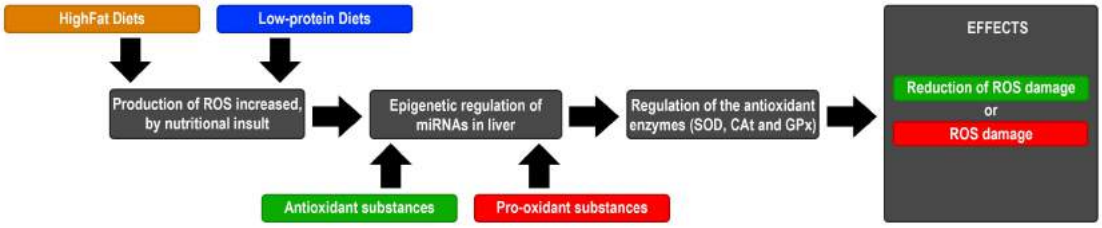


Figure 2. The brief summary of the action of diets on ROS production, and how the epigenetic regulation of microRNAs may influence the action of antioxidant enzymes

ANEXO A – COMITÊ DE ÉTICA

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Recife, 01 de setembro de 2017.

Ofício nº 87/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Mariana Pinheiro Fernandes**
Núcleo de Educação Física e Ciências do Esporte
Centro Acadêmico de Vitória
Universidade Federal de Pernambuco
Processo nº **23076.016634/2017-52**

Certificamos que a proposta intitulada **"Avaliação dos efeitos de dieta hiperlipídica pós-desmame sobre parâmetros cardiometabólicos de ratos submetidos à dieta hipoproteica perinatal"**, registrada com o nº **23076.016634/2017-52**, sob a responsabilidade de Prof.ª **Mariana Pinheiro Fernandes** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 30/08/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	01/09/2017 a 01/03/2021
Espécie/linhagem/raça	Ratos heterogênicos Wistar
Nº de animais	84
Peso/Idade	220-260g / 80-90 dias
Sexo	Machos (68) e fêmeas (16)
Origem	Biotério do CAV-UFPE

Atenciosamente,