UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

AVALIAÇÃO DOS EFEITOS DA SUPERNUTRIÇÃO E DA DESNUTRIÇÃO PROTEICA NO PERÍODO CRÍTICO DO DESENVOLVIMENTO SOBRE A BIOENERGÉTICA MITOCONDRIAL E ESTRESSE OXIDATIVO EM CORAÇÕES DE RATOS JOVENS

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RECIFE, 2016

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

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Nascimento, Luciana Caroline Paulino do
Avaliação dos efeitos da supernutrição e da desnutrição protéica no período crítico do desenvolvimento sobre a bioenergética mitocondrial e estresse oxidativo em corações de ratos jovens / Recife: O Autor, 2016.

87 folhas: il., fig., tab.
Orientadora: Claudia Jacques Lagranha
Coorientadora: Mariana Pinheiro Fernandes
Dissertação (mestrado) – Universidade Federal de Pernambuco. Centro de Biociências. Bioquímica e Fisiologia, 2016.
Inclui referências e anexos

1. Nutrição 2. Coração 3. I. Lagranha, Claudia Jacques (orientadora) II. Fernandes, Mariana Pinheiro (coorientadora) III. Título

572.41 CDD (22.ed.) UFPE/CCB-2017-050

À minha Avó Maria, por todas as palavras encorajadoras e por não deixar morrer em mim a fé em dias melhores.

AGRADECIMENTOS

A Deus pela graça da vida e realização dos meus sonhos;

Aos meus pais, Fátima e Cosme e meus irmãos Luiz e Lucas, por nunca mediram esforços para que eu alcançasse meus objetivos por mais difícil que fosse o caminho;

À Prof.ª Dra Claudia, que pra mim é mais que orientadora científica, mas uma amiga. Seus ensinamentos vão além do conhecimento científico, são lições que me fizeram amadurecer e tornar-me uma pessoa melhor;

À Prof.ª Dra Mariana Fernandes, que sempre esteve à disposição a ajudar em qualquer momento que eu necessitasse;

A Kleyton, pelo companheirismo, paciência, compreensão e incentivos mesmo que na distância;

A Décio Medeiros, que apesar do pouco tempo, sempre se dedicou a me encorajar e não deixar desanimar;

Aos primos Dayane, Diego e Mayara, por todos os momentos de descontração, tornando o caminho menos árduo;

Às "amoras", Talitta e Cristiane pela amizade e pelos momentos que dividimos tanto as preocupações como também as alegrias;

À Cássia Andrade, minha coorientanda, que me mostrou que eu tinha mais a aprender que ensinar;

A todos os integrantes do grupo "Bioenergética Mitocondrial, Metabolismo Celular e Plasticidade Fenotípica" por todo o conhecimento compartilhado e ajuda na realização de todas as etapas deste trabalho;

Aos que fazem parte do Programa de Pós Graduação em Bioquímica e Fisiologia e do Centro Acadêmico de Vitória de Santo Antão, por todo suporte para a realização e conclusão desse trabalho.

Todo aquele que se dedica ao estudo da ciência chega a convencer-se de que nas leis do universo se manifesta um Espírito sumamente superior ao do homem, e perante o qual nós, com os nossos poderes limitados, devemos humilhar-nos.

Albert Einstein

RESUMO

Evidências clínicas e experimentais demonstram que uma nutrição inadequada no início da vida aumenta o risco para o aparecimento de doenças cardiovasculares. Entretanto, o papel da mitocôndria e do desequilíbrio oxidativo, como fator associado, ainda não foi totalmente esclarecido. Dessa forma, o presente estudo teve como objetivo investigar o efeito em curto prazo tanto da supernutrição como da desnutrição proteica durante o período do desenvolvimento, em relação a capacidade respiratória mitocondrial e balanço oxidativo em corações de ratos machos jovens. Para o protocolo de desnutrição e supernutrição utilizamos ratas *Wistar*, seguindo as recomendações do COBEA e aprovação do Comitê de Ética em Estudos com Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco. Para o protocolo de supernutrição, após o nascimento, no primeiro dia de vida, todas as ninhadas foram normalizadas para nove neonatos por mãe, no terceiro dia de vida pelo fato da lactação estar totalmente estabelecida, o tamanho da ninhada foi reduzido para três filhotes por mãe (grupo OF), permitindo que a oferta de leite para os três filhotes seja significativamente maior. Para o grupo controle, o número de filhotes permaneceu com nove neonatos por mãe (grupo NF). No protocolo de desnutrição, os animais foram submetidos à desnutrição no período de gestação e lactação com dieta a base de caseína à 8% (grupo LP) e à 17% para animais controle (grupo NP). Após a lactação, todos os grupos experimentais passaram a receber LABINA. Aos 30 dias de vida, os animais machos foram sacrificados, o sangue foi coletado para análise de glicose em jejum, colesterol e triglicerídeos e o tecido cardíaco para as análises mitocondriais (respiração mitocondrial e produção de espécies reativas) e bioquímicas (nível de peroxidação lipídica, oxidação de proteínas, atividade de enzimas antioxidantes, bem como a avaliação dos níveis de glutationa reduzida). Os valores foram expressos em Média e Erro Padrão da Média (X ± EPM). O teste estatístico utilizado foi o *Student t-test*, mantendo o nível de significância em 5% ($p < 0,05$). Nossos resultados demonstraram que a supernutrição induziu aumento da glicose (21%), colesterol (26%) e triglicerídeos (60%) plasmáticos. Resultado esse também observado nos animais desnutridos onde verificamos um aumento na glicose (49%), colesterol (15%) e triglicerídeos (38%) plasmático. Em relação a função mitocondrial vimos que a supernutrição induziu uma redução de 49% e a desnutrição uma redução de 51% no controle respiratório de mitocôndrias isoladas de coração; já em relação a produção de espécies reativas de oxigênio no supernutrido verificamos uma tendência ao aumento na produção dessas espécies reativas junto com um

aumento de 140% na peroxidação lipídica; ao passo que a desnutrição promoveu um aumento de 124% na produção de espécies reativas de oxigênio e aumento de peroxidação lipídica em 112%. Em ambos os insultos nutricionais verificamos uma redução significativa no sistema antioxidante. Nossos dados sugerem que independente do tipo de insulto nutricional, ou seja, carência de proteína ou excesso de alimento no período do desenvolvimento ocorre uma desregulação metabólica associada a disfunções mitocondriais e estresse oxidativo em coração de ratos jovens.

Palavras chaves: Supernutrição precoce, desnutrição proteica, disfunção mitocondrial, estresse oxidativo, coração.

ABSTRACT

Clinical and experimental evidence show that inadequate nutrition early in life increases the risk of cardiovascular diseases. However, the role of mitochondria and oxidative imbalance, as associated factor, has not yet been completely explained. Thus, the present study aimed to investigate the short-term effect of both overnutrition and protein malnutrition during the developmental period, in relation to mitochondrial respiratory capacity and oxidative status in hearts male young rats. For overfeeding and protein restriction protocols, we used *Wistar* rats following the recommendations of COBEA and approved by the Ethics Committee on Animal studies from the Federal University of Pernambuco-CCB. For the overnutrition protocol, at the first day of life, all litters was normalized to nine puppies per dams, on the third day of life because lactation is fully established, the litter size was reduced to three puppies by mother (OF group), allowing the supply of milk to the three puppies be significantly higher. For the control group, the number of pups remain in nine newborns per dams (NF group). In the malnutrition protocol, the animals were subjected to protein restriction during pregnancy and lactation using diet within casein at 8% (LP group) and 17% for control animals (NP group). After lactation, all groups have received LABINA diet. At 30 days of life, male animals were sacrificed, blood collected for the analysis of fasting glucose, cholesterol and triglycerides; heart tissue were removed for mitochondrial analysis (mitochondrial respiration and production of reactive species) and biochemical (level of lipid peroxidation, protein oxidation, activity of antioxidant enzymes and levels of reduced glutathione). The values were expressed as mean and standard error (X ± SEM). The statistical test used was the *Student t-test*, keeping the level of significance of 5% ($p < 0.05$). Our results showed that overnutrition increases glucose (21%), cholesterol (26%) and triglycerides (60%); similar result was also observed in protein restriction were we observed an increase in glucose (49%), cholesterol (15%) and triglycerides (38%). Related to mitochondrial function we observed that overnutrition decreases 49% of respiratory capacity and malnutrition decreases 51%. Associated with these results we observed in overnourished group a trend to increase ROS production with an increase of 140% in lipid peroxidation; in malnutrition we observer an increase in ROS production of 124% with an increase in lipid peroxidation at 112%. Both nutritional insults induce a significant reduction on antioxidant system. Our data suggest that regardless of the nutritional insult, protein deficiency or over nourishment during the development, potentiates

disruption in the metabolism with dysfunction in mitochondrial bioenergetics and the onset of cardiac oxidative stress.

Key words: Early overnutrition, protein malnutrition, mitochondrial dysfunction, oxidative stress, heart

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LISTA DE ABREVIATURAS

2,4-Dinitrophenylhydrazine = DNPH

2,4-dinitrophenyl-S-glutathione =DNP-SG

2',7'-dichlorodihydrofluorescein diacetate = H_2DCF-DA

Ácido desoxirribonucleico = DNA

Ácido Tiobarbitúrico = TBA

Adenosina difosfato = ADP

Adenosina trifosfato = ATP

Catalase = CAT

Chloro-2, 4-dinitrobenzene = CDNB

Espécies Reativas de Oxigênio = ERO's ou EROS

Espécies Reativas= ER

Ethylenediaminetetraacetic acid =EDTA

Glicose-6-fosfato desidrogenase = G6PDH

Glutationa Peroxidase = GPx

Glutationa redutase = GR

Glutationa reduzida = GSH

Glutatione-S-transferase = GST

Low protein = LP

Malondealdeído = MDA

Normal fed= NF

Normoprotein = NP

Organização Mundial de Saúde = OMS

Ortho-phthaldehyde = OPT

Over fed = OF

Phenylmethanesulfonyl fluoride = PMSF

Poro de Transição de Permeabilidade Mitocondrial = PTPm

Reactive oxygen species = ROS

Respiratory Control Ratio = RCR

Standard Error Mean = SEM

Superóxido dismutase = SOD

Trichloroacetic acid =TCA

World Health Organization = WHO

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1. INTRODUÇÃO

Uma das prioridades atuais da Organização Mundial de Saúde (OMS) refere-se ao combate às doenças crônico-degenerativas. A OMS inclui como estratégias para combater tais doenças, mudanças do estilo de vida, em destaque a alimentação adequada que deve ser priorizada desde o início da vida (WHO, 2016). Essa recomendação se deve ao fato de que um considerável número de estudos epidemiológicos vem mostrando associação entre insultos nutricionais durante o período perinatal e o desenvolvimento de doenças crônicas, tais como obesidade, diabetes e doenças cardiovasculares na vida adulta (SAMUELSON *et al*., 2008; SLATER-JEFFERIES *et al*., 2011).

Entre os insultos nutricionais os modelos de supernutrição ou restrição proteica (i.e desnutrição) no início da vida têm sido amplamente utilizados para tentar entender como esses insultos em períodos críticos do desenvolvimento podem levar a manifestações patológicas anos depois (PATEL; SRINIVASAN, 2010).

Há anos, Barker demonstrou que crianças que nascem com baixo peso apresentam risco maior de morrer por doenças cardíacas isquêmicas do que crianças que haviam nascido com peso normal (BARKER *et al.,* 1989). Estudos posteriores vieram complementar e confirmar que a restrição proteica durante a gestação e lactação leva a alterações no crescimento e desenvolvimento fetal que posteriormente podem ser a causa para o desenvolvimento da hipertensão (LANGLEY-EVANS *et al.*, 1999; DE BRITO ALVES *et al.*, 2013).

Entretanto não apenas a desnutrição pode elevar o risco para o aparecimento de doenças crônicas, mas a maior oferta e consumo de nutrientes levando ao sobrepeso/obesidade nos períodos iniciais da vida, também tem sido usado como um modelo importante para o desenvolvimento de pesquisas que buscam entender a origem dos distúrbios metabólicos e cardíacos na vida adulta (PLAGEMANN; HARDER, 2005; PLAGEMANN *et al*., 2012; HABBOUT *et al.*, 2013; LANGLEY-EVANS, 2014).

Apesar de existir um grande número de trabalhos associando supernutrição, desnutrição e doenças cardíacas, os mecanismos envolvidos na patogênese desses agravos ainda são pouco esclarecidos, porém, é proposto que o estresse oxidativo seja um possível mecanismo que interliga os insultos no período perinatal e o elevado risco de doenças crônicas (LUO *et al*., 2006).

Uma vez que a maioria dos estudos que buscam entender a relação entre desequilíbrios nutricionais no período crítico de desenvolvimento e doenças crônicas, concentram-se em

análises na vida adulta, no presente trabalho objetivamos investigar se os efeitos do excesso de nutrientes e a restrição proteica no período perinatal se manifestam em idade jovem.

O desenvolvimento dessa dissertação permitiu a elaboração de dois artigos científicos. O primeiro intitulado *"Impairment of mitochondrial bioenergetics and oxidative status in postnatal overfeeding rat hearts"*. Este artigo teve como objetivo investigar os efeitos imediatos da supernutrição na função mitocondrial e sistema antioxidante cardíaco. O segundo artigo intitulado "*Early effect of maternal low-protein diet on heart: mitochondrial bioenergetics and oxidative stress status*", teve como objetivo investigar os efeitos imediatos da restrição proteica perinatal na função mitocondrial e sistema antioxidante cardíaco.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Ambiente perinatal e desenvolvimento de doenças

O período de gestação e lactação, também conhecido como período crítico de desenvolvimento, é uma fase de susceptibilidade em que ocorre grande multiplicação e diferenciação celular e maturação de órgãos e sistemas, o que torna o organismo passível de sofrer influências de fatores externos e vir a apresentar modificações bioquímicas e estruturais (MORGANE *et al*., 2002). A epigenética tenta explicar a relação entre o meio ambiente e modificações celulares por meio do estudo de modificações de DNA, por processos de metilação e acetilação ou ainda alteração de proteínas associadas, como as histonas, podendo de maneira permanente alterar os nucleotídeos e predispor o aparecimento de doenças (GLUCKMAN *et al*., 2008; LANGLEY-EVANS, 2009).

Uma diversidade de insultos durante o desenvolvimento pode influenciar na predisposição a doenças, tais como tabagismo, infecção materna, agentes farmacológicos, entre outros (SELCKL; MEANEY, 2004; SALSAMI *et al*., 2010; SCHMIEGELOW *et al*., 2013). Além dessas condições, estudos já mostram que o desbalanço nutricional em períodos críticos do desenvolvimento está associado com a gênese de doenças crônicas, principalmente as doenças cardiovasculares (REMACLE *et al*., 2011; ZOHDI *et al*., 2014).

Segundo Gluckman, o feto se adapta e prediz o ambiente pós-natal no qual ele irá se desenvolver, caracterizando um processo chamado de resposta adaptativa preditiva (GLUCKMAN *et al*., 2009). Estas adaptações, de acordo com a condição ao qual é submetido, podem ser benéficas em curto prazo, garantindo a sobrevivência da espécie, no entanto os custos dessa adaptação, pode ser expressa em longo prazo por alterações fenotípicas aumentando o risco do aparecimento de doenças crônicas como diabetes tipo 2, hipertensão e doença coronariana (HALES; BARKER, 2001; GLUCKMAN *et al*., 2009).

Condições ambientais como a restrição nutricional materna induz mecanismos fetais adaptativos que limitam seu crescimento, a fim de priorizar o desenvolvimento de órgãos e tecidos essenciais, acelerando a maturação destes frente às condições de deficiência nutricional. Sendo assim, há redução no fornecimento de sangue para órgãos como tecido muscular, fígado, pâncreas e rins com o intuito de garantir o aporte sanguíneo para o cérebro, porém levando ao comprometimento antropométrico do feto ao nascimento (LUCAS, 1994; FALL, 2011).

Na restrição de crescimento haveria uma reprogramação das funções metabólicas ajustadas para prever um ambiente pós-natal também de carência nutricional. No entanto, quando o ambiente não coincide com o ambiente fetal, ou seja, ao invés de escassez, há abundância nutricional, aumenta-se então o risco de desenvolvimento de doenças crônicas (GLUCKMAN *et al.,* 2005). Isso se deve a incapacidade do organismo em suportar a alta carga metabólica, o que se torna um fator de risco indutor de processos patológicos na vida adulta (WELL, 2007).

Estudos clínicos e experimentais já demonstraram a correlação direta entre o estado nutricional materno com o comportamento alimentar, adiposidade, homeostase da glicose, além de hipertensão arterial na prole (LANGLEY-EVANS, 2013). Langley-Evans mostrou que a restrição proteica materna levou ao comprometimento do processo de nefrogênese, condição que posteriormente é capaz de causar hipertensão nos animais (LANGLEY-EVANS, 1999).

Na maior parte dos mamíferos, o completo desenvolvimento de vários órgãos não está totalmente realizado ao nascimento e continua durante o imediato período pós-natal, durante a amamentação (KAUNG, 1994; MORRISON *et al.*, 2008). O cérebro, por exemplo, apresenta período crítico de desenvolvimento no início da gestação humana, mas permanece vulnerável por muito mais tempo, uma vez que o extenso crescimento e desenvolvimento das vias neurais estendem-se até a infância (PLAGEMANN *et al*., 2000). Não apenas o período de formação fetal é susceptível a modificações por insultos, mas o período pós-natal precoce também vem apresentando grande associação com o posterior aparecimento de doenças (PLAGEMANN *et al*., 2012).

Embora a relação entre a oferta nutricional perinatal durante o desenvolvimento precoce e doenças na prole tornou-se cada vez mais evidente, os mecanismos biológicos envolvidos mantêm-se em grande parte desconhecidos. Dessa forma é de grande importância estudos sobre os efeitos de insultos nutricionais no início da vida com o objetivo de compreender melhor as relações bioquímicas e fisiológicas que predispõem o desenvolvimento de doenças crônicas na vida adulta, decorrentes do excesso ou da falta de nutrientes.

2.2 Supernutrição no período crítico do desenvolvimento

A supernutrição durante o período pós-natal imediato conduz a alterações em sistemas que controlam a homeostase energética, afeta as concentrações circulantes de hormônios, a

função de vários órgãos e expressão dos genes. Estas alterações podem modificar permanentemente a organização estrutural e o metabolismo podendo induzir alterações posteriores na função e estrutura de órgãos e tecidos, o que aumenta a susceptibilidade a condições patológicas (HABBOUT *et al*., 2013).

Embora a infância não tenha sido alvo de prevenção de sobrepeso/obesidade tanto quanto a idade adulta, várias observações mostraram que o rápido ganho de peso nos primeiros anos de vida pode influenciar na composição corporal na vida adulta, bem como o futuro desenvolvimento de doenças crônicas. Diversos trabalhos têm demonstrado que a supernutrição pós-natal precoce, tem sido associado com a elevação da susceptibilidade ao excesso de peso, obesidade e várias comorbidades relacionadas, tais como as doenças cardiovasculares (STETTLER *et al*., 2005; MORRISSON *et al.*, 2008).

A superalimentação no período pós-natal imediato mostrou-se capaz de causar redução na capacidade de resposta a ação de leptina e insulina, condições que podem contribuir para a ocorrência de hiperfagia, excesso de peso e hiperinsulinemia ao longo da vida (PLAGEMANN *et al*., 1999; PLAGEMANN *et al.*, 2006). Corroborando com esses achados, um estudo recente mostrou que as expressões hepáticas de mediadores da sinalização de insulina estavam reduzidas em animais superalimentados, o que reforça o comprometimento no equilíbrio glicose/insulina (CONCEIÇÃO *et al*., 2013).

As disfunções a nível renal também são relatadas, uma vez que é visto comprometimento na nefrogênese e, em longo prazo, desenvolvimento de proteinúria e glomeruloesclerose em animais precocemente supernutridos (BOUBRED *et al*., 2009). Yim (2012) em seu estudo também mostrou alteração no sistema renina-angiotensina, mostrando que além de danos renais, há uma grande propensão ao aparecimento de doenças cardiovasculares.

As repercussões cardiovasculares da superalimentação durante o período perinatal podem ser demonstradas uma vez que é notada hipertrofia cardíaca com aumento significativo da espessura da parede ventricular, aumento da área dos cardiomiócitos e diminuição da densidade dos vasos coronarianos (MOREIRA *et al*., 2009), além disso, disfunções microvasculares também são relatadas (LEITE *et al*., 2012). Essas mudanças, certamente contribuem para a gênese da hipertensão, já observado em estudos com supernutrição precoce (BOUBRED *et al*., 2009; KAPPELER *et al*., 2009). Estudos histológicos, por sua vez, mostram alta deposição de colágeno nos ventrículos esquerdo e direito em animais adultos supernutridos no período pós-natal (VELKOSKA *et al*., 2008), além de apresentarem alta

expressão de proteínas envolvidas na organização estrutural dos cardiomiócitos (HABBOUT *et al.*, 2013).

Em animais, o modelo de indução de obesidade, caracterizado pela redução do tamanho da prole, têm sido amplamente utilizado. Estudos publicados por Plagemann *et al*. mostraram que este modelo induz aumentos significativos no consumo alimentar devido à maior disponibilidade de leite materno no período pós-natal imediato (DÖRNER; PLAGEMANN, 1994; HEIDEL *et al*., 1999; PLAGEMANN *et al.*, 1999), adicionalmente, a composição do leite é modificada resultando em aumento de calorias e conteúdo lipídico (FIOROTTO *et al*., 1991). Portanto, animais criados em ninhadas reduzidas têm demonstrado ser um modelo experimental útil para estudar as consequências da superalimentação precoce (HABBOUT *et al*., 2013).

2.3 Desnutrição proteica no período crítico do desenvolvimento

Estudos epidemiológicos já mostram que a restrição proteica sofrida no período gestacional pode gerar consequências na vida adulta, tais como obesidade, diabetes, hipertensão e doenças cardiovasculares (RAVELLI, *et al*., 1976; PHILLIPS, 2001; OKEN; GILLMAN, 2003; GONZALEZ ZAPATA *et al*., 2006).

Utilizando animais, Ozanne e Hales evidenciaram que a dieta hipoprotéica durante a gestação e lactação promove alterações fisiológicas e morfológicas que repercutem em danos funcionais podendo refletir por toda a vida (OZANNE; HALES, 2002). Esse déficit nutricional no período perinatal tem ocasionado efeitos deletérios que podem ser observados tanto no crescimento corpóreo, demonstrado pela taxa de crescimento, como também na má formação de órgãos conduzindo ao comprometendo de suas funções (GAMA; CARVALHO; CHAVES, 2007).

No fígado, a restrição proteica na gestação e lactação causou mudanças estruturais e modificações na capacidade metabólica da glicose e gliconeogênese (BURNS *et al.,* 1997; VAN STRATEN *et al*., 2010), assim como alteração na sinalização e metabolismo do colesterol na prole (LIU *et al*., 2008). Alterações também são observadas a nível cerebral onde é encontrada má formação estrutural e neurogênica no cérebro da prole de mães desnutridas (MORGANE *et al*., 2002; AIREY *et al.*, 2015). Em sua revisão, Fanos *et al*. (2010) relata que comprometimentos da função renal devido a restrição proteica perinatal levou a redução do número de néfrons e hipertrofia glomerular com posterior fibrose.

A desnutrição proteica perinatal também afeta negativamente o tecido cardíaco (CHEEMA *et al.,* 2005; CORSTIUS *et al.,* 2005; TOSCANO *et al.*, 2008). Efeitos decorrentes da desnutrição proteica perinatal no coração são evidenciados em estudos epidemiológicos e experimentais (SARAIVA *et al.,* 1992; FIORETTO *et al.,* 2002; CORSTIUS *et al.,* 2005). Avaliando o eletrocardiograma e o ecocardiograma de crianças gravemente desnutridas é observado diminuição do volume cardíaco caracterizado pela redução do diâmetro interno e da espessura da parede do ventrículo esquerdo (SARAIVA *et al.*, 1992). Já em estudos experimentais, a restrição protéico-calórica durante a gestação interfere na proliferação celular do coração (CORSTIUS *et al.,* 2005). Em ratos, cujas mães sofreram restrição proteica durante a gestação, é possível observar aumento da taxa de apoptose de cardiomiócitos (CHEEMA *et al*., 2005; CORSTIUS *et al*., 2005, TOSCANO *et al.*, 2008).

Além disso, também foi demonstrada alterações na dinâmica do Ca^{2+} comprometendo a função de contratilidade do músculo cardíaco (HARVEY *et al.,* 2015) e aumento do conteúdo lipídico (ZHODI *et al*., 2015); alterações metabólicas essas associadas a indução de hipertensão e disfunção vascular (BEAUCHAMP; HARPER, 2015).

Modelos animais têm sido utilizados com a finalidade de investigar os efeitos da desnutrição materna sobre a possibilidade do aparecimento de doença na prole. A indução de desnutrição moderada em ratas durante a gestação e lactação (período crítico do desenvolvimento) tem sido um modelo amplamente utilizado, que consiste em alimentar um grupo de fêmeas com dieta normoproteica (17% de proteína) e outro grupo com uma dieta hipoproteica (8% de proteína) durante o período de gestação e lactação e posteriormente investigar as possíveis repercussões sobre o processo de saúde ou doença da prole (DE MÉLO MONTENEGRO *et al*., 2012; BARROS *et al.,* 2015; FERREIRA *et al*., 2015).

Os efeitos da desnutrição proteica compõem um conjunto de alterações metabólicas que podem predispor as disfunções celulares que desencadeariam as doenças na vida adulta.

2.4 Insultos nutricionais e disfunção mitocondrial: envolvimento do estresse oxidativo

Por desempenharem papel fundamental na homeostase metabólica, principalmente por sua função central na produção de energia na célula, as mitocôndrias são essenciais para a função normal dos órgãos (SHAUGHNESSY *et al*., 2014). Além do seu envolvimento, entre outros processos fundamentais na célula, como sinalização de Ca^{2+} e apoptose, as

mitocôndrias apresentam importante função na homeostase metabólica (SUEN *et al*., 2008; TAIT; GREEN, 2010; CHENG; RISTOW, 2013).

Essa regulação metabólica acontece, uma vez que a mitocôndria é capaz de converter produtos advindos do metabolismo de carboidratos, lipídeos e proteínas em gás carbônico - CO_2 e água com o objetivo de produzir energia. Essa conversão a partir das macromoléculas acontece utilizando-se de enzimas da cadeia de transporte de elétrons, conhecidas por complexos. Os elétrons das coenzimas reduzidas, $NADH^+$ H^+ e $FADH_2$, são transferidos por esses complexos e durante essas reações, prótons são bombeados da matriz mitocondrial para o espaço intermembranas, gerando assim o gradiente próton motriz, também chamado de potencial elétrico de membrana mitocondrial ($\Delta\Psi m$). Uma vez que esses prótons retornam para a matriz, através da enzima ATP sintase, há a força necessária para a geração de ATP (JOHANNSEN; RAVUSSIN, 2009), molécula essa, que é indispensável para a manutenção das funções celulares e orgânicas.

Estima-se que entre 2 e 5% do oxigênio que nossas mitocôndrias utilizam com a finalidade de produzir energia transforme-se em espécies reativas (ER), que devido a sua necessidade de estabilização possuem moderada a alta afinidade a elétrons (TURRENS *et al*., 2003). Dentre essas moléculas, as Espécies Reativas de Oxigênio (ERO's) designam o grupo de uma série de moléculas químicas reativas. Neles estão inclusos os radicais livres como ânion superóxido (O_2^-), radicais hidroxila (OH^-), radicais alcoxila e peroxila, assim como também os não-radicalares como o peróxido de hidrogênio (H_2O_2), hipoclorito, oxigênio singlet e peróxidos lipídicos (SCHMIDT *et al*., 2015). Entre as fontes de produção dessas moléculas, destacam-se as enzimas óxido nítrico sintase (NOS) e xantina oxidase, além das mitocôndrias, consideradas a principal fonte produtora (TURRENS, 2003).

A produção de ERO's mitocondrial é um processo fisiológico e necessário para diversas reações na célula como sinalização e crescimento celular, expressão gênica e apoptose (FUKAI ; OSHIO-FUKAI, 2011). Porém, quando há produção excessiva desses oxidantes ou os antioxidantes apresentam-se em quantidades reduzidas ou ainda suas atividades comprometidas, há a ocorrência do que chamamos de estresse oxidativo. Este desequilíbrio pode levar a alterações da estrutura e função de biomoléculas tais como proteínas, lipídios e ácido desoxirribonucleico (DNA) (NAVARRO-YEPES *et al*., 2014).

Os níveis de ERO's são controlados pelos sistemas antioxidantes, os quais formam um complexo sistema que tem por objetivo proteger as células contra excessiva exposição às ER, através de: diminuição na sua formação; diminuição da sua biodisponibilidade; conversão a biomoléculas menos ativas ou ainda pela substituição de biomoléculas a serem oxidadas

(BLOKHINA *et al*., 2003; HALLIWELL; GUTTERIDGE, 2007). Entre as defesas antioxidantes podem ser destacadas o sistema enzimático; composto por enzimas tais como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa-S-transferase (GST); e o sistema não enzimático, com destaque para a glutationa reduzida (GSH) (BLOKHINA *et al*., 2003; HALLIWELL & GUTTERIDGE, 2007).

As disfunções mitocondriais e o estresse oxidativo vêm sendo apontados como supostos mecanismos moleculares indutores de doenças crônicas que resultam de insultos nutricionais em fases iniciais da vida (LUO *et al*., 2006; REUSENS *et al*., 2011).

Estudos recentes analisando o fígado de ratos superalimentados no período perinatal mostraram uma diminuição nas atividades de várias enzimas antioxidantes, como a catalase, superóxido dismutase e glutationa peroxidase (CONCEIÇÃO *et al.*, 2012). Esses autores também relataram que o marcador de estresse oxidativo, malondealdeído, também estava aumentado tanto no fígado como no plasma (CONCEIÇÃO *et al.*, 2012). Este desequilíbrio entre diminuição de defesas antioxidantes e aumento do dano oxidativo pode predispor os hepatócitos a lesão e pode desencadear o desenvolvimento de resistência à insulina.

Sabe-se que a obesidade também esta associado ao estresse oxidativo plasmático e também cardíaco (LAIGHT *et al*., 1999; VINCENT *et al.*, 1999). O excesso de nutrientes, no período pós-natal precoce, levando a obesidade, aumentou hidroperóxidos e diminuiu as concentrações de antioxidantes não enzimáticos, indicando a ocorrência do estresse oxidativo plasmático, assim como também cardíaco (HABBOUT *et al*., 2012). Além disso, já é relatado que a supernutrição perinatal induz alterações estruturais nas mitocôndrias, com redução do $\Delta\Psi m$ e alta taxa de apoptose nessas células (TURDI *et al*., 2012).

Além desses efeitos da superalimentação, Beauchamp *et al*. (2015) recentemente mostrou que a subnutrição fetal por sua vez, resultou em redução do conteúdo mitocondrial e sua menor capacidade oxidativa tanto no músculo esquelético, como no tecido cardíaco. Evidências experimentais do nosso grupo de pesquisa mostraram que em animais adultos que receberam restrição proteica durante a gestação e a lactação, apresentaram alterações na função mitocondrial com redução do coeficiente respiratório e $\Delta\Psi m$, ao mesmo tempo em que há aumento na produção de ERO's. Além de apresentar diminuição na atividade de enzimas antioxidantes como a SOD e a GPx e aumento de biomarcadores de estresse oxidativo no sistema nervoso (FERREIRA *et al*., 2016)

Em outro estudo do nosso grupo de pesquisa, verificamos que a prole de ratos adultos (100 dias de vida), de mães que sofreram restrição proteica no período da gestação e lactação, apresentaram diminuição da atividade das enzimas antioxidantes SOD, CAT, GST, glutationa

redutase (GR) e glicose-6 fosfato desidrogenase (G6PDH), além de disfunção mitocondrial no tecido cardíaco (NASCIMENTO *et al.*, 2014), caracterizando desequilíbrio oxidativo.

As disfunções na mitocôndria que conduzem ao aumento da produção de espécies reativas é uma importante característica das doenças cardiovasculares (PUDDU *et al*., 2004; DIKALOV; UNGVARI, 2013). É mostrado que desarranjos na função das mitocôndrias associado a menor capacidade antioxidante é capaz de desencadear processos ateroscleróticos (MADAMANCHI; RUNGE, 2007; BONOMINI *et al.*, 2008), disfunção endotelial (SZEWCZYK *et al.*, 2015) e insuficiência cardíaca (MUNZEL *et al.*, 2015).

Componentes mitocondriais são vulneráveis ao ataque de ERO's e o aumento nessa produção pode causar diminuição no $\Delta\Psi m$ e consequentemente a abertura do Poro de Transição de Permeabilidade Mitocondrial (PTPm) aumentando os níveis de Ca^{2+} na mitocôndria. Toda essa sequência de eventos leva a uma resposta que acelera o processo de morte dos cardiomiócitos (MUNTEAN *et al*., 2016) implicando em processos patológicos cardiovasculares (MADAMANCHI; RUNGE, 2007; SZEWCZYK *et al.*, 2015).

Apesar dos relatos da literatura, até o presente momento, não há estudos que buscam entender o efeito em curto prazo tanto da supernutrição como da desnutrição proteica no que se refere à capacidade respiratória mitocondrial, como também a produção de ERO's em associação ao balanço oxidativo cardíaco.

3. OBJETIVOS

3.1. **Geral**

O objetivo do presente projeto é avaliar os efeitos da supernutrição e desnutrição proteica no período crítico do desenvolvimento sobre a bioenergética mitocondrial e indicadores de estresse oxidativo em coração de ratos jovens, aos 30 dias de vida.

3.2. **Específicos**

3.2.1 Avaliar nos animais supernutridos e controles:

- A evolução do peso corporal aos 21 e 30 dias;
- Níveis de glicose de jejum, colesterol e triglicerídeos plasmáticos;
- Nas mitocôndrias cardíacas isoladas:
  O consumo de oxigênio mitocondrial;
  O potencial elétrico de membrana mitocondrial;
  A produção de espécies reativas mitocondriais.
- No tecido cardíaco:
  Níveis de peroxidação lipídica;
  Níveis de oxidação de proteínas;
  Atividade das enzimas antioxidante: superóxido dismutase, catalase e glutationa-S-transferase;
  Níveis do antioxidante não enzimático Glutationa Reduzida.

3.2.2. Avaliar nos animais desnutridos e controles:

- A evolução do peso corporal aos 21 e 30 dias;
- Níveis de glicose de jejum, colesterol e triglicerídeos plasmáticos;
- Nas mitocôndrias cardíacas isoladas:
  O consumo de oxigênio mitocondrial;
  A produção de espécies reativas mitocondriais.
- No tecido cardíaco:
  Níveis de peroxidação lipídica;
  Níveis de oxidação de proteínas;
  Atividade das enzimas antioxidante: superóxido dismutase, catalase e glutationa peroxidase;
  Níveis do antioxidante não enzimático Glutationa Reduzida.

4. ARTIGO 1:

Impairment of mitochondrial bioenergetics and oxidative status in postnatal overfeeding rat hearts

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Running title: Mitochondrial dysfunction in overfeed rats

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Abstract

Clinical and experimental evidences show that early inadequate nutrition improves the risk of heart and vascular diseases. Therefore, this study proposes to investigate the short-term effects of overfeeding at lactation period on the cardiac oxidative capacity of male rats. Reducing the litter size on postnatal day 3 induced postnatal overfeeding. At 21 days of age, the animals were weaned and measured body weight. At 30 days were weighed again and sacrificed for further analysis in cardiac tissue: mitochondrial oxidative capacity, production of ROS, level of oxidative stress biomarkers and enzymatic and non-enzymatic antioxidant capacity. We observed an increase in 52% of the body weight of the animal overfeed to 21 days and 45% at 30 days. Furthermore the biochemical parameters were also found increased: fasting glucose (21%), cholesterol (26%) and triglycerides (60%). The postnatal overnutrition also reduced mitochondrial oxidative capacity (49%) and mitochondrial membrane eletric potential (50%). Concomitantly found increased levels of lipid peroxidation (140%) and reduction of antioxidant enzymes catalase (32%) and glutathione S-transferase (48%) activities and lower levels of reduced glutathione (60%). Our results suggested that in precocious ages has metabolic modification associated with mitochondrial bioenergetics and oxidative status dysfunction.

Keywords: Lactational overfeeding, mitochondrial dysfunction, oxidative status, antioxidant defense, heart.

Introduction

According to a recent publication of the World Health Organization (WHO) in 2012, about 52% of deaths under age 70 were due to chronic diseases and just cardiovascular diseases (CVD) caused 46% of those deaths. Therefore, among global goals to change this situation, WHO proposes a healthy lifestyle which includes physical activity practice and better eating habits [1].

Clinical and experimental evidences show that early inadequate nutrition improves the risk of heart and vascular diseases [2]. Obesity also leads to increased risk of CVD as shown by ZHANG et al. (2012) that found dysfunction in myocardial contractility in overnourished animals at postnatal lactational period [3]. Maternal obesity in female rats resulted in cardiac hypertrophy that was associated with impaired systolic and diastolic function in the young-adult offspring [4,5].

Several research groups have shown that overnutrition induction in early postnatal period, by reduced litter size, causes considerable weight gain until weaning, that may persist into adulthood [6-8]. Similarly, some authors also observed an increase in fasting glucose, plasma insulin and triglycerides [9,10], while others reported increased blood pressure [6,11]. However, the underlying mechanisms related to overweight-induced CVD are not completely understood.

Have been largely proposed that oxidative stress, an imbalance between oxidant agents and antioxidant defense, is a well-established condition in obesity and it is related to onset or aggravation of CVDs [12] such as hypertension, atherosclerosis and heart failure [13]. Moreover, Haboout [14,15] suggested a higher propensity to ischemia/reperfusion injury in

animals overfed during suckling period that showed an increase of circulating markers of oxidative stress in adult ages.

Related to overweight/obesity induced-oxidative stress, most studies using a postnatal overfeeding model have performed at adult or elderly ages. Therefore, this study proposes to investigate the short-term effects of overfeeding at lactation period on the cardiac oxidative capacity of male rats.

Material and Methods

All experiments were performed in accordance with the recommendations of the Brazilian Committee of Animal Experimentation (COBEA) and were previously approved by Ethical Committee for Animal Research at Federal University of Pernambuco (UFPE), Brazil (protocol nº 23076.017808/2014-51).

Drugs and Reagents

All of the drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

After 24 hours of birth, four *Wistar* rats were assigned randomly into two groups: one control group with 9 pups per dam (Normal fed, NF) and three overnourished group with 3 pups per dam (Over fed, OF) as previously described [16-21]. After 21 days of age (weaning)

both groups received commercial laboratory chow (Labina-Purina®) and water *ad libitum* until 30 days of age.

Body weight measurement

Body weights (in grams) were measured in 21st (weaning) and 30rd postnatal day using a digital balance (S-400, with a 1 gram of sensitivity) [22].

Biochemical measurements

After 12 hours of fasting, blood was collected for serum analysis of glucose, cholesterol and triglycerides. The samples were centrifuged at 3500 G for 5 min. All assays were performed according manufactured protocols (Lab Test Kit for glucose, cholesterol and triglycerides).

Mitochondria isolation

Freshly isolated mitochondria were isolated from the homogenized hearts by differential centrifugation as previously described [23,24]. Aliquots of mitochondria were analyzed for the total protein content using the Bradford protocol [25].

Mitochondrial oxygen consumption

The mitochondrial respiration was measured at 28°C in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). The mitochondria were used at 0.5 mg protein/mL buffer. The mitochondrial respiration was measured using Complex I (glutamate 10 mM/malate 2 mM) substrates, and the mitochondrial phosphorylation state was measured by the addition of ADP (0.5 mmol/L) to the breathing medium with pH=7,4 [23,24].

Measurement of mitochondrial membrane electric potential (ΔΨm)

The mitochondrial membrane electric potential was determined by fluorimeter using 2 mM of Safranin-O as a marker with excitation and emission wavelengths of 530 and 590 nm, respectively [26,27].

Reactive oxygen species production

Briefly, the production of ROS was monitored using the fluorescent probe 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Freshly isolated mitochondria (0.1 mg) were incubated in respiratory buffer with 1µM H_2DCF-DA. The DCF fluorescence was monitored at 485 nm and 525 nm via fluorescence spectrophotometry (FLUOStar, Omega, USA) [22,28].

Evaluation of lipids oxidation

A total of 0.5 mg/ml heart homogenate was used to measure the malondialdehyde production (MDA) following the reaction with thiobarbituric acid (TBA) at 100°C according to previously published [24,29].

Evaluation of protein oxidation

The protein oxidation was assessed using the procedures described by Reznick and Packer [30]. With the samples (500mg) on ice, 30% (w/v) TCA was added to the sample and then centrifuged for 14 min at 4000 RPM. The pellet was resuspended in 10 mM 2,4 dinitrophenylhydrazine and immediately incubated in a dark-room for 1h with agitation every 15 min. Samples were washed and centrifuged three times in ethyl/acetate buffer and the final pellet was resuspended in 6 M guanidine hydrochloride, incubated for 30 min at 37 °C and the absorbance read at 370 nm.

Superoxide dismutase assay

The total superoxide dismutase enzyme activity (t-SOD) was determined according to the method of Misra and Fridovichc [31]. The heart homogenates (0.1 mg/ml) were incubated with sodium carbonate buffer and 30 mM epinephrine at 37 ºC and measured at 480 nm.

Catalase assay

A total of 0.3 mg of heart homogenate was used to measure the catalase (CAT) activity according to the method described by Aebi [32]. The absorbance values were obtained 240nm.

Glutathione S-transferase assay

A total of 0.3 mg of heart homogenate was used to measure the glutathione S-transferase (GST) activity according to Habig *et al*. [33] determining the absorbance at 340 nm after the addition of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The GST activity was defined as the amount of protein required to form 1 μmol DNP-SG.

Reduced glutathione (GSH) concentration

GSH was measured by the method described by Hissin and Hilf [34]. The sample (0.3 mg) was diluted 10 fold in a sodium phosphate buffer (pH 8.0), with 5mM EDTA. OPT (ortho-phthaldehyde) and samples were incubated for 15 min at room temperature. The fluorescence was measured at 420 nm with excitation at 350nm.

Statistical Analysis

All of the results were expressed as the mean±SEM. A Student's t-test was used to assess the significant differences between the groups. The data were considered statistically significant for $p \leq 0.05$. All statistical analysis were performed using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

We evaluated the body weight of the animals at 21 and 30 days of age. The OF group showed higher body weight to its controls at 21 days (52%) and at 30 days (45%) [Figure 1].

After the observation that overnutrition increases body weight, we next evaluate some serum parameters. Our data showed that OF group has higher values for fasting glucose (21%), cholesterol (26%) and triglycerides (60%) compared to NF group [Table 1].

Related to mitochondrial function, the basal state of oxygen consumption did not differ between groups [NF= 7.0±0.6 *vs.* OF=5.2±0.6 nmol O_2/mg of protein], although the OF group showed a significant decrease (49.0%) in the phosphorylation capacity compared to NF group [NF=56.6±8.2 *vs.* OF=28.4±3.5 nmol O_2/mg of protein; $p<0.01$; figure 2A]. Associated with the decrease in phosphorylation capacity, the respiratory control rate (RCR) was significantly lower in the OF group compared to NF group [NF=10.0 ± 1.4 *vs.* OF= 3.9 ± 0.7; $p<0.01$; figure 2B]. In addition to the decrease observed in respiration capacity, mitochondrial membrane potential also showed reduced in the OF group compared to NF group [NF=1.0 ± 0.1 *vs*. OF= 0.5 ± 0.1 a.u.; $p<0.05$; figure 2C]. As shown in figure 3, despite of tendency to increase, there was no significant difference in ROS production between groups [NF= 1.6 ± 0.3 *vs.* OF= 2.2 ± 0.5 slope; figure 3A].

Evaluating oxidative stress biomarkers, we observe an increased in lipid oxidation on OF group [140%; figure 3B], but no difference in protein oxidation [NF=100.0 ± 7.2 vs. OF= 92.0 ± 11.0 percentage of control; figure 3C].

Oxidative stress can be established or due to increase in production of pro-oxidant agents (i.e reactive oxygen species) or due to decrease in antioxidant defense. Evaluating

enzymatic antioxidant defense as shown in figure 4, the superoxide dismutase activity was increased in the OF group [163%; figure 4A], catalase activity was decreased [32%; figure 4B] as well as the glutathione-S-transferase activity [48.8%; figure 4C]. Additionally to the decrease in enzymatic defense, the main thiol responsible to the non-enzymatic defense was reduced in the OF group [60%; figure 5], compared to the NF group.

Discussion

The present study was designed to evaluate whether the overnutrition during lactation period affects the cardiac oxidative balance and mitochondrial bioenergetics in young animals. Higher body weight was found in the OF animals compared to NF animals confirming the effectiveness of the overweight/obesity model employed in this study and in accordance to previously articles that also used the same overweight/obesity model [6,35,36].

Alterations in biochemical parameters was also observed with the increase in the serum glucose levels in OF animals, similarly to Rodrigues's findings that analyzed 21-old-day animals [37]. Besides that, the triglycerides and cholesterol levels were also increased in the OF animals, corroborating with the study of Moreira *et al* (2009). The high levels of plasmatic glucose, cholesterol and triglycerides contribute to the impairment of glucose homeostasis and reduced insulin response. OF animals during lactation period show alterations in insulin and fatty acids signaling pathway components, affecting cardiac metabolism [38].

In regard to mitochondrial function, it has been proposed that these organelles are essential in translating the early stress associated with fetal programming into cellular dysfunction observed in later life [39]. Accordingly, our results suggest an impaired mitochondrial respiratory capacity in the OF animals, as already demonstrated by Fleischman

*et al. (*2009) and Bakkman *et al*. (2010) that observed the mitochondrial oxidative capacity in response to an obesity model [40,41]. Moreover, the OF group showed a decreased mitochondrial membrane potential (ΔΨm). Similar findings were reported by Turdi *et al.* (2013), which in addition to reduced mitochondrial membrane potential there was a reduction in the transcriptional factors related to mitochondrial oxidative metabolism in animals that had exposed to overfeeding at postnatal period [42]. As observed, a reduced ΔΨm in OF animals, can induce mitochondrial permeability transition pore (mPTP) opening and thus to facilitate the release of pro-apoptotic proteins, a potentiated process in oxidative stress conditions [43].

The association between overweight/obesity and increased oxidative stress, not only in the bloodstream, as well as in the myocardium, is already consistent in literature [44]. One of the harmful effects of oxidative stress is the lipoperoxidation, which contributes to atherosclerosis development [45] and increases the risk to cardiac dysfunctions [46]. Our results showed increased MDA levels and reduced CAT and GST enzymatic activity, as well as the levels of GSH, a non-enzymatic antioxidant, in the heart of OF animals. Studies with the same obesity experimental model, also found an increase of oxidative stress biomarkers in the heart [15], serum and liver tissue [47]. Related to SOD activity, our result corroborates with those found by Habbout *et al*, in which attribute the elevated SOD activity as an adaptative response to oxidative stress [14,15]

Furthermore, in cardiac histologic analysis of overfed animals there was increase in lipid and fibrous tissue accumulation [14,48] (HABBOUT *et al*., 2012; BERNARDO *et al*., 2016). This effects lead to higher susceptibility to heart failure and ischemia/reperfusion injury [49].

Conclusion

In summary, our data showed impaired mitochondrial function and oxidative stress in the heart of overfed animals during lactation period. This finding emphasizes that the mitochondrial modifications that increase the risk for cardiac dysfunctions in the adulthood, as a postnatal overfeeding effect, may already be initiated still in young ages.

Acknowledgments

The authors are thankful to FACEPE (Foundation for the Support of Science and Research from Pernambuco State—Brazil, APQ-1026-4.09/12) for the financial support to acquire the equipment and reagents used in this work. We are also grateful to CNPQ, CAPES and FACEPE that provided scholarships.

Declaration of interest

The authors report no conflicts of interest.

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Table:

Table 1: **Biochemical parameters of NF (Normal fed) and OF (Over fed) groups**. The assays were performed using colorimetric methods and calculated by Student's *t*-test.

	NF	**OF**
Fasting glucose (mg/dL)	85.2±5.0	102.8±4.2*
Cholesterol (mg/dL)	58.8±4.1	74.4±3.7*
Triglycerides (mg/dL)	50.5±2.8	81.2±6.0***

*p<0.05; ***p<0.001

Figure captions

Figure 1: **Body Weight of Normal fed (NF, whites bars) and Over fed (OF, black bars) groups**. Data at weaning, at 21 days of life, and before the sacrifice, at 30 days of life. Expressed as mean ± SEM. ****p≤ 0.0001.

Figure 2**: Evaluation of mitochondrial respiration capacity in the heart mitochondria of Normal fed (whites bars) and Over fed (blacks bars) groups.** A) Basal respiratory capacity and Phosphorylation capacity ADP-stimulated, B) Respiratory Control Ratio (RCR) and C) Mitochondrial Membrane Potential (ΔΨm) . Expressed as mean ± SEM. **p≤0.01.

Figure 3**: Evaluation of reactive species production and oxidative stress biomarkers in the heart of Normal fed (whites bars) and Over fed (blacks bars) groups.** A) Mitochondrial ROS production, B) Lipid peroxidation, quantified by Malondialdehyde-MDA levels and C) Protein oxidation, quantified by Carbonyl levels. Expressed as mean ± SEM. **p<0.01.

Figure 4: **Enzymatic antioxidant capacity in the heart of Normal fed (whites bars) and Over fed (blacks bars) groups.** A) Quantification of the superoxide dismutase-SOD activity, B) Quantification of the catalase-CAT activity, C) Quantification of Glutathione –S-Transferase-GST activity. Expressed as mean ± SEM. *p≤0.05. **p≤0.01.

Figure 5: **Non-enzymatic GSH antioxidant capacity in the heart of Normal fed (white bars) and Over fed (blacks bars) groups.** Expressed as mean ± SEM. **p<0.01.

Figures:

Figure 1

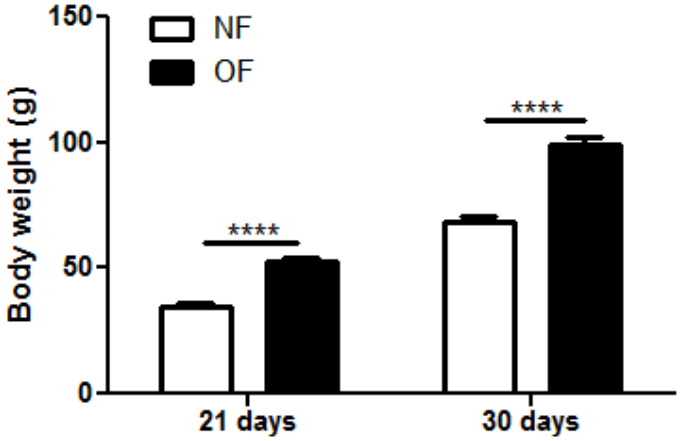


Figure 2

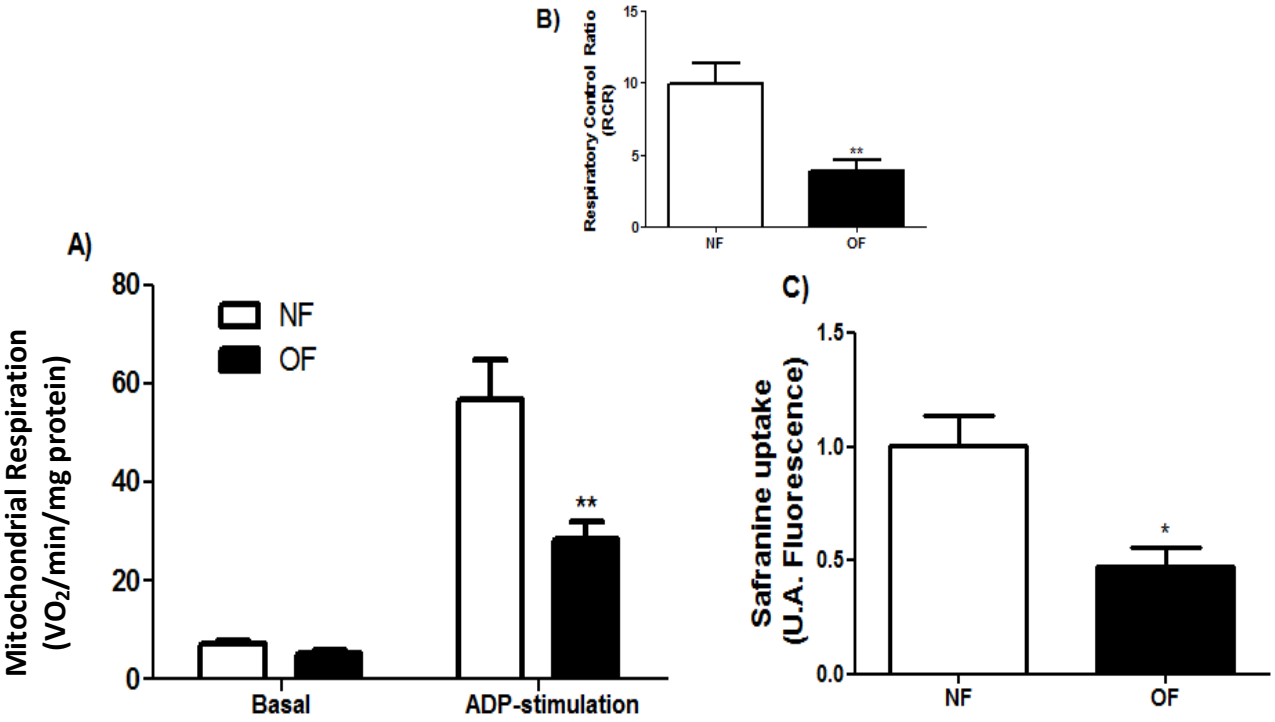


Figure 3

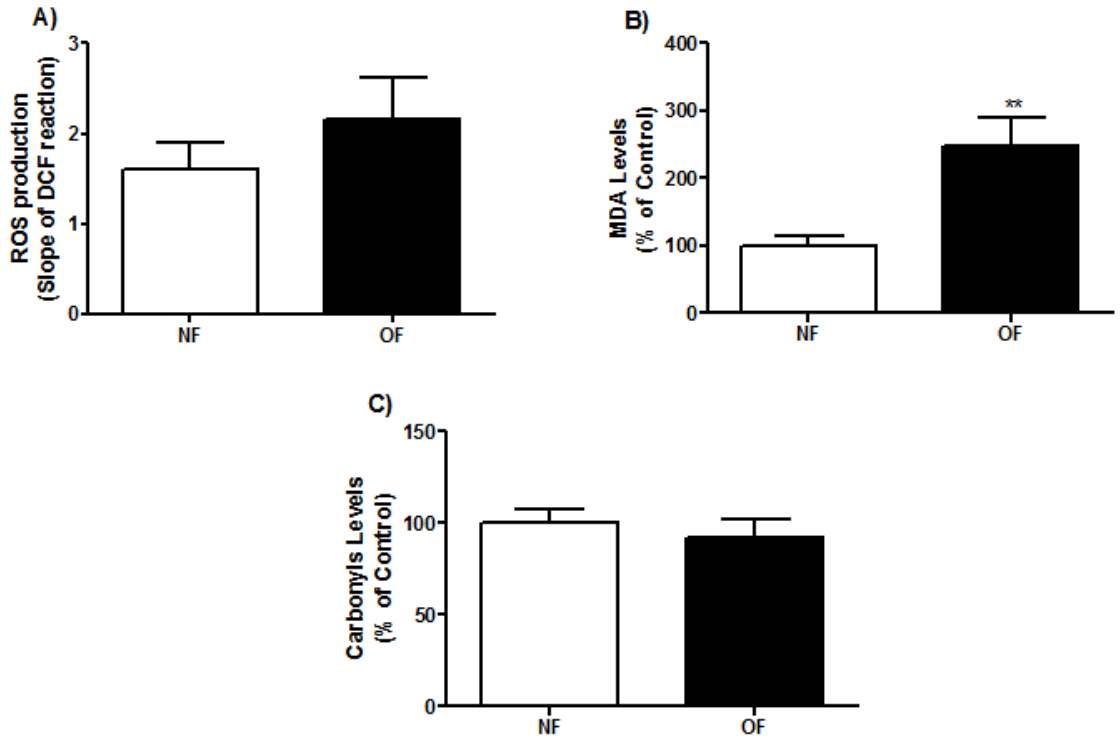


Figure 4

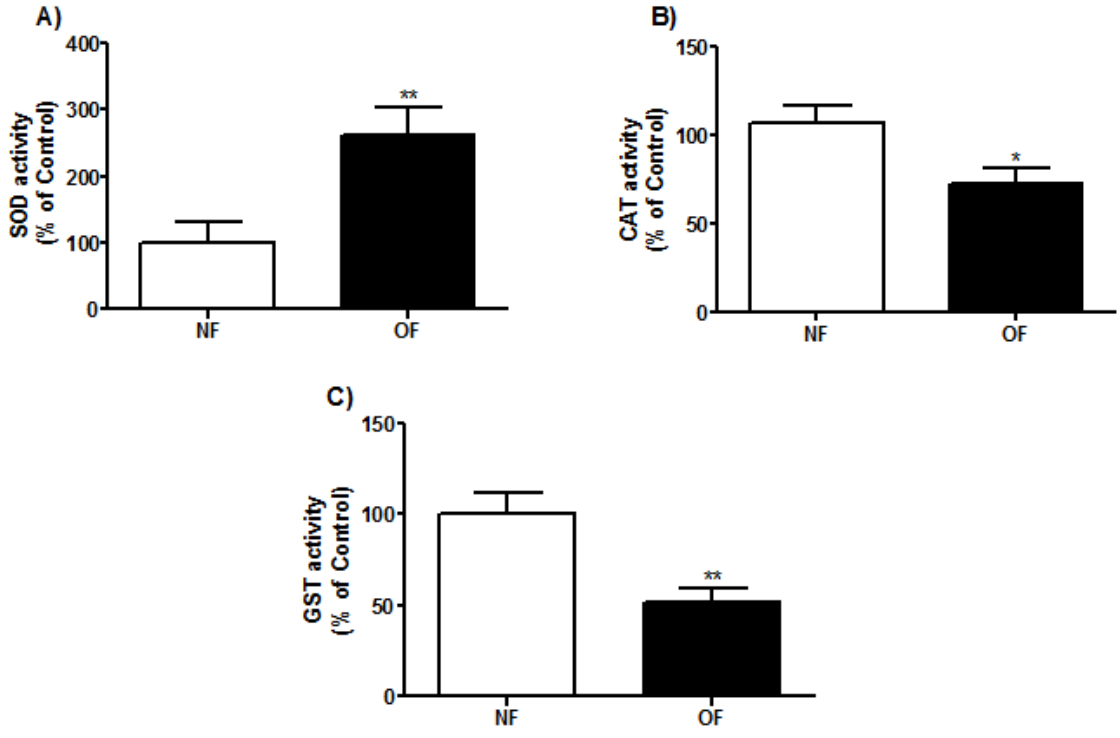
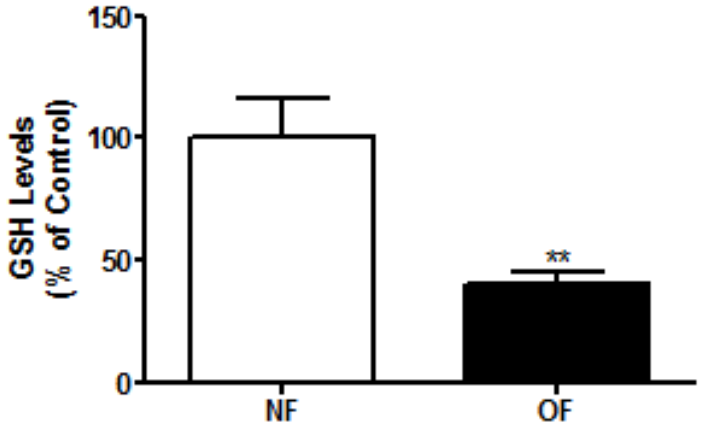


Figure 5



5. ARTIGO 2:

Early effect of maternal low protein diet on heart: mitochondrial bioenergetics and oxidative status

Running title: Maternal low-protein diet on juvenile heart

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Abstract

Protein restriction during critical periods of development is associated with diseases such arterial hypertension in adults. However, the early effects of a low-protein diet (at 30 days of age) remain unknown. The aim of this study was to investigate the effects of a maternal low-protein diet on the mitochondrial function and oxidative stress levels in the heart at 30 days of age. Pregnant Wistar rats received either 17% of casein (normal protein) or 8% of casein (low protein) throughout pregnancy and lactation. Our results show an increase in the fasting glucose (49%), cholesterol (15%) and triglycerides (38%) levels. It was also observed decrease in the respiratory control ratio (51%), an increase in the reactive oxygen species production (124%) and malondialdehyde production (112%) and decrease in the enzymatic antioxidant capacity compared to those of the control. Altogether these findings suggest that the maternal low protein diet induce alteration in metabolism and mitochondrial dysfunction with impair in enzymatic antioxidant system.

Keywords: Maternal protein restriction, mitochondria dysfunction, oxidative status, heart

1. Introduction

World Health Organization (WHO) data show that cardiac disease is the major cause of death in high-, middle-, and low-income countries. To prevent cardiovascular disease, the WHO suggests changes in lifestyle and diet for an optimum benefit beginning early in life [33].

In support of the WHO's call for early intervention, several epidemiologic and experimental studies have demonstrated a close relationship between the diet during perinatal life and the subsequent development of adult cardiovascular diseases [14,15]. Emphasizing the importance of diet in developmental plasticity, many studies have shown that restricted diets at an early age can either promote or retard lipid and/or protein oxidation, depending upon the specific nature of the diet [12]. Underlying this relationship is a process called as phenotypic plasticity, a phenomenon in which different disease phenotypes in later life can be established by maternal influences prenatally and/or environmental stimuli at an early age [4]. One of the hypotheses to explain this relationship is the predictive adaptive response, which postulates that early adversities will provide to the organism under development a predictive adaptation in response to environmental challenges, resulting in anticipated phenotype adaptation for immediate survival [24]. However, according to the hypothesis, the initial adaptation will be only temporary and the inconsistency between the prenatal prediction and postnatal reality will result in detrimental changes to the organism, leading to an increased risk of chronic diseases in adulthood [21,31,32].

Several studies have suggested that cardiovascular disease is induced in part by an increase in mitochondrial dysfunction which leads to an increase in reactive oxygen species production and defect on the capacity to detoxification of these reactive species, resulting in oxidative stress on myocardial/vascular tissues [17,27,28]. During these years our laboratory are devoted to understand the role of mitochondria on maternal undernutrition event and we

have demonstrated in heart and brainstem that maternal low-protein diet during development associated with mismatch diet until 100 days of age induces mitochondrial impair, with an increase in hydrogen peroxide production, coupled with decrease in antioxidant capacity and the incidence of oxidative stress [11,23].

In the present work, we addressed the hypothesis that maternal low-protein diet during development induces mitochondrial dysfunction independent of the long mismatch between diets. To do this, our work was designed to test whether maternal LP diet during gestation and lactation periods affects negatively the mitochondrial bioenergetics and oxidative status in the heart of offsprings closely to the exposure to the maternal protein restriction.

2. Material and Methods

The experimental protocol (protocol nº 23076.016336 / 2012-58) that was used in this study was approved by the Ethical Committee of the Biological Science Center at the Federal University of Pernambuco (UFPE), Brazil. All of the experiments were performed in accordance with the recommendations of the Brazilian Committee of Animal Experimentation (COBEA).

2.1 Drugs and reagents

All of the drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Experimental design and Diet

Pregnant rats were randomly divided into two experimental groups: a normal-protein group fed a 17% casein diet (NP) and a low-protein (LP) diet group fed an 8% casein diet according previous publications from our group [23]. At 22 days of age, the male offspring

were separated into two experimental groups based on maternal diet (NP and LP) and received commercial laboratory chow (Labina-Purina®) and water *ad libitum* until 30 days of age when the hearts were collected.

2.3 Body weight measurement

Body weights (in grams) were measured in 21st (weaning) and 30rd postnatal day using a digital balance (S-400, with a 1 gram of sensitivity) [7].

2.4 Biochemical Measurements

After 12 hours of fasting, blood was collected for serum analysis of glucose, cholesterol and triglycerides. The samples were centrifuged at 3500 rpm for 5 min. All assays were performed according manufactured protocols (Lab Test Kit for glucose, cholesterol and triglycerides).

2.5 Mitochondria isolation

Freshly isolated mitochondria were isolated from the homogenized hearts by differential centrifugation as previously described [19,23]. Aliquots of mitochondria were analyzed for the total protein content using the Bradford protocol [5].

2.6 Mitochondrial oxygen consumption

The mitochondrial respiration was measured at 28°C in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). The mitochondria were used at 0.5 mg protein/mL buffer. The mitochondrial respiration was measured using Complex I (glutamate 10 mM/malate 2 mM) substrates, and themitochondrial phosphorylation state was measured by the addition of ADP (0.5 mmol/L) [19,23].

2.7 Reactive oxygen species production

Briefly, the production of ROS was monitored using the fluorescent probe 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). Freshly isolated mitochondria (100 µg) were incubated in respiratory buffer with 1 µM DCF. The DCF fluorescence was monitored at 485 nm and 525 nm via fluorescence spectrophotometry (FLUOStar, Omega, USA) [13,30].

2.8 Evaluation of lipids oxidation

A total of 0.2 mg/ml heart homogenate was used to measure the malondialdehyde production (MDA) following the reaction with thiobarbituric acid (TBA) at 100°C according to the method of Draper [10,23]. The results are expressed as µmol/mg protein.

2.9 Evaluation of protein oxidation

The measure of protein oxidation was evaluated according to Levine *et al.* (1994). The carbonyl groups react with 2,4-dinitrophenil-hydrazina (DNPH) in a dark room. The absorbance values were obtained 380nm and the results expressed as nmol/mg protein [20].

2.10 Superoxide dismutase assay

The total superoxide dismutase enzyme activity (t-SOD) was determined according to the method of Misra and Fridovich . The heart homogenates (0.1 mg/ml) were incubated with sodium carbonate and 30 mM epinephrine and measured at 480 nm. The data are expressed at U/mg protein [6,23].

2.11 Catalase assay

A total of 0.2 mg/ml heart homogenate was used to measure the catalase (CAT) activity according to the method described by Aebi [1]. The CAT activity was expressed as U/mg protein [6,23].

2.12 Glutathione Peroxidase assay

Glutathione Peroxidase (GPx) activity was performed in accordance to Paglia and Valentine (1967). Briefly, 300 mg of protein was added to a 50mM phosphate buffer, pH7.0 containing 5 mM EDTA, 0.28 mM NADPH, 3.75 mM sodium azide, 5 mM reduced glutathione (GSH) and 16 U glutathione reductase from Sigma (St.Louis,MO). The reaction was started with 2.2 mM H2O2. NADPH oxidation followed at 340 nm absorbance at 20 °C and its coefficient of extinction was used to determine the GPx activity as U/mg protein [26].

2.13 Reduced glutathione (GSH) concentration

GSH was measured by the method described by Hissin and Hilf (1976). The sample was diluted 10 fold in a sodium phosphate buffer (pH 8.0), with 5mM EDTA. OPT (ortho-phthaldehyde) and samples were incubated for 15 min at room temperature. The fluorescence was measured at 420 nm with excitation at 350nm and the results were expressed as units per milligram of protein (U/mg prot) [16].

2.14 Statistical Analyses

All of the results are expressed as the means ± SEM. A Student's t-test was used to assess the significant differences between the groups. The data were considered statistically significant for $p \leq 0.05$. All of the data were plotted, and the statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

When evaluating the body weight of the groups, was observed that the animals in the LP group showed a significant reduction in body weight when compared to their controls, both at 21 days (37%) and 30 days (25%) (Figure 1).

After the evaluation and confirmation of the body weight, we next evaluate some serum parameters that are related to cardiovascular diseases. As showed in table 1, maternal LP increases significantly fasting glucose (49%), total cholesterol (15%) and triglycerides (38%) compared to NP group (Table 1).

The cardiac mitochondrial function differed significantly between the LP and NP groups (figure 2). The offspring of mothers on the LP diet had increased mitochondrial basal respiration (76%; $p<0.05$), no difference observed in the state 3 respiration, however due to the increase in basal respiration (figure 2A), we observe a decrease in respiratory control ratio (51%; $p<0.001$) (figure 2B).

In combination to the negative modulation in respiration, mitochondrial ROS production significantly increased (124%; $p<0.01$) in the LP group compared to that of the NP group (figure 3A); which may be the cause of the increase in lipid (112,5%; $p<0.001$) (figure3B) and protein oxidation (110%; $p<0,01$) (figure 3C). Concordant with the increased ROS and oxidative stress biomarkers in the LP group, we found significant decreases in the activities of antioxidant enzyme in the LP groups, CAT (30% decrease, $p <0.05$) (figure 4B) and GPx (41.35% decrease, $p<0.05$) (figure 4C), but no significant difference was observed on glutathione reduced levels (figure 5).

4. Discussion

The effects of a low-protein diet could induce permanent changes in the heart that could pre-dispose the offspring of protein-deprived mothers to cardiac dysfunction has been

already published [2,29]. The data in literature indicate that a low protein diet pre- and post-natal or only post-natal increases blood pressure [8,9], showing that a low-protein diet modulates cardiac function an adult. Previous data from our laboratory indicate that a low-protein diet in adult rat offspring induce a decrease in the mitochondrial function and an increase in the oxidative stress in the heart [23], although an early effect (i.e., at 30 days of age) of a low-protein diet was not investigated.

Mitochondria for many years were seen only as an energy source. However, current studies indicate that mitochondria can also play a destructive role and initiate or be a final effector of cell death. Recently, Nickel et al. suggested that defects in cardiomyocytes are connected with mitochondrial defects, indicating that mitochondria play a role as definers of life and death [25]. Previous studies have demonstrated that mitochondria are an important source of ROS production in cardiac myocytes and that the accumulation of ROS in the myocardium induces oxidative stress [18,34]. In this study, we observed that a low-protein diet not only increases oxidative stress but also induces mitochondrial dysfunction and increases ROS production.

Enzymes such as superoxide dismutase, catalase, glutathione peroxidase and small molecules (glutathione-GSH) are crucial in detoxifying ROS. In our study, we observed a significant decrease in the activities of catalase and glutathione peroxidase, enzymes that convert H2O2 into H_2O and oxygen [3]. The decrease in these enzymes is crucial since the detoxication of H_2O_2 is a key event in prevention of oxidative stress, because reaction of H2O2 with transition metals (e.g., ferrous iron) may lead to the formation of the $OH^{\cdot}$. Once is not known detoxification system for the $OH^{\cdot}$, the prevention of its formation is a critical antioxidant process. Our data demonstrate that a low-protein diet during development is reflected in the down-regulation of the antioxidant defense capacity, suggesting that mitochondria from hearts from a LP-fed mother produce more ROS and have a lower

enzymatic antioxidant capacity than do mitochondria from an NP-fed mother. Combining our previous data with the present data suggests that the enzymes catalase and glutathione peroxidase appear to be more susceptible to the effects of a low-protein diet, which explains the increase in the oxidative stress biomarkers in both studies. Concomitant with these results, the blood sugar levels, as well as cholesterol and triglyceride levels are increased in LP animals. The increase in these biochemical parameters are strongly associated with increased risk of cardiovascular disease [29].

In summary, our data demonstrate that at 30 days of age, a maternal low-protein diet results in increased in oxidative stress probably due the deleterious effects of protein restriction on the catalase and glutathione peroxidase activities.

Acknowledgments

The acquisition of the reagents that were used in this work was supported financially by the Foundation to Support Science and Research from Pernambuco State—Brazil (FACEPE, APQ-1026-4.09/12). We are also grateful to FACEPE and CAPES, which provided scholarships for LCPN, GRFB, and DFS.

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Legends

Figure 1: Body Weight of juvenile rats that received a normal-protein diet and a low-protein diet during embryonic and early perinatal development. Body weight evaluated after weaning (21 days of life), and at experimental day (30 days of life). The white bars represent the normal-protein diet (NP) group, and the black bars represent the low-protein diet (LP) group (n=7 rats per group). The values are mean ± SEM. **p≤0.01; ****p≤ 0.0001.

***Figure 2*:** Evaluation of mitochondrial respiration capacity in the heart mitochondria of juvenile rats that received a normal-protein diet and a low-protein diet during embryonic and early perinatal development. A) Basal respiratory capacity and State 3 respiration with ADP-stimulation. B) Respiratory Control Ratio. The white bars represent the normal-protein diet (NP) group, and the black bars represent the low-protein diet (LP) group (n= 6 rats per group). The values are mean ± SEM. *p<0.05, ***p < 0.001.

***Figure 3*:** Evaluation of reactive species production and oxidative stress indicators in the heart of juvenile rats that received a normal-protein diet and a low-protein diet during embryonic and early perinatal development. A) Mitochondrial ROS production. B) Malondialdehyde-MDA production. C) Carbonyls production. The white bars represent the normal-protein diet (NP) group, and the black bars represent the low-protein diet (LP) group (n= 6 rats per group). The values are mean ± SEM. *p<0.05. **p<0.01.

Figure 4: Enzymatic antioxidant capacities in the hearts of juvenile rats that received a normal-protein diet and a low-protein diet during embryonic and early perinatal development. A) Quantification of the superoxide dismutase-SOD activity. B) Quantification of the

catalase-CAT activity. C) Glutathione Peroxidase-GPx activity. The white bars represent the normal-protein diet (NP) group, and the black bars represent the low-protein diet (LP) group (n= 6 rats per group). The values are mean ± SEM. *p<0.05.

Figure 5: Non-enzymatic antioxidant level in the heart of juvenile rats that received a normal-protein diet and a low-protein diet during embryonic and early perinatal development. The white bars represent the normal-protein diet (NP) group, and the black bars represent the low-protein diet (LP) group (n= 5 rats per group). The values are mean ± SEM.

Table 1. : **Biochemical parameters of NP (Normal protein) and LP (Low protein) groups**. The assays were performed using colorimetric methods and calculated by Student's *t*-test.

	NP	**LP**
Fasting glucose (mg/dL)	91.3±2.3	135.4±18.5*
Cholesterol (mg/dL)	68.0±2.0	78.4±1.4**
Triglycerides (mg/dL)	41.8±2.9	57.0±2.1**

*p < 0.05; **p < 0.01

Figures:

Figure 1.

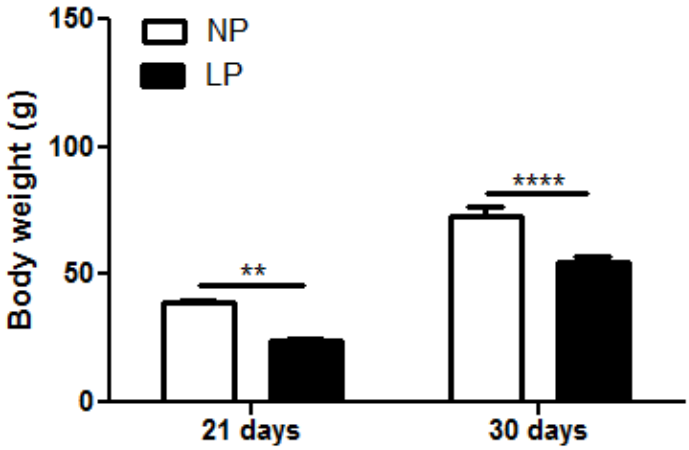


Figure 2

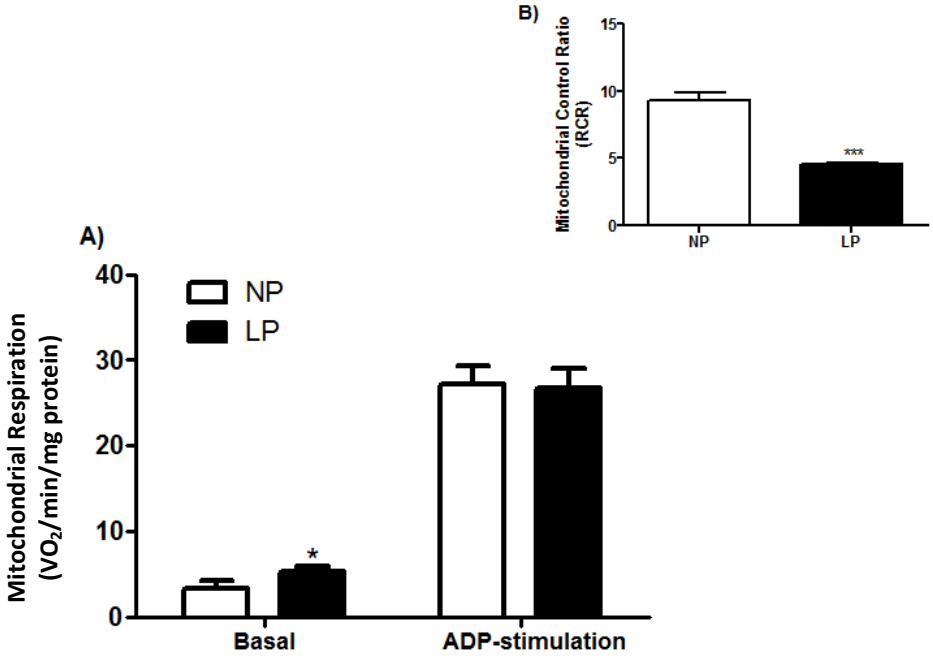


Figure 3

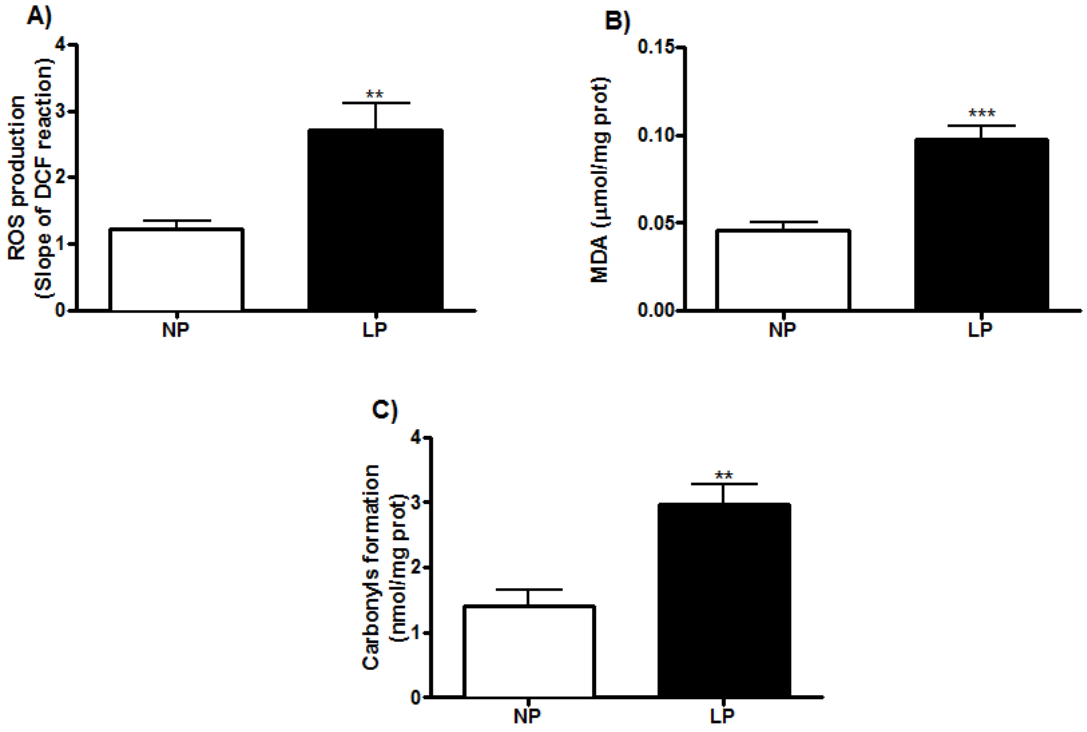


Figure 4

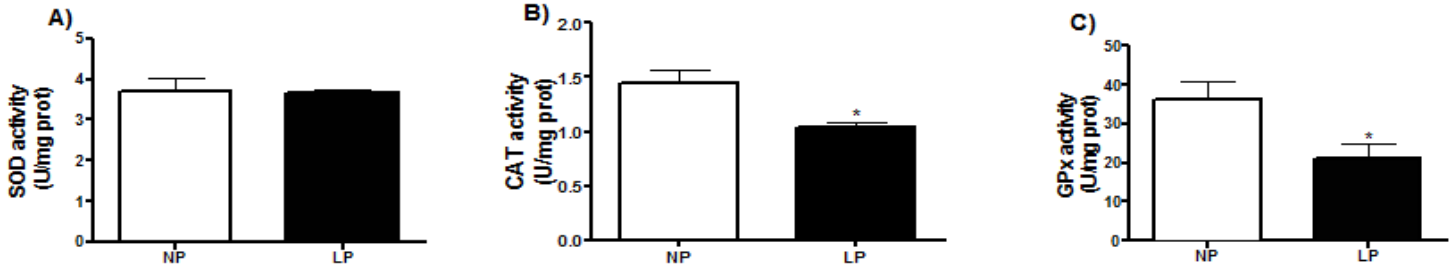
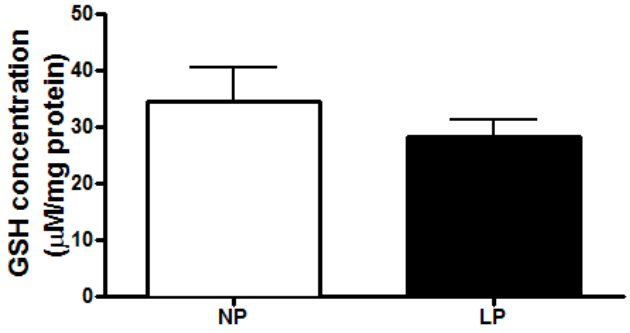


Figure 5



6. CONCLUSÕES

Nossos dados sugerem que independente do tipo de insulto nutricional, ou seja, tanto o excesso de alimento como a carência de proteína no período do desenvolvimento, provoca desregulação metabólica, disfunções mitocondriais e o aparecimento do estresse oxidativo cardíaco em animais ainda jovens. Tais eventos associados podem ser os mecanismos envolvidos na indução de patologias cardíacas observadas na idade adulta.

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ANEXOS

Anexo 1

Dear Dr. Lagranha:

Your manuscript entitled "Impairment of mitochondrial bioenergetics and oxidative status in postnatal overfeeding rat hearts" has been successfully submitted online and is presently being given full consideration for publication in Free Radical Research.

Your manuscript ID is GFRR-OM-2016-0168.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at https://mc.manuscriptcentral.com/gfrr and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to https://mc.manuscriptcentral.com/gfrr.

Thank you for submitting your manuscript to Free Radical Research.

Sincerely,
Free Radical Research Editorial Office

Anexo 2

Dear Ms Luciana Nascimento,

We have received the submission entitled: "Early effect of maternal low protein diet on heart: mitochondrial bioenergetics and oxidative status" for possible publication in Journal of Physiology and Biochemistry, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr Claudia Lagranha who will be able to track the status of the paper through his/her login.

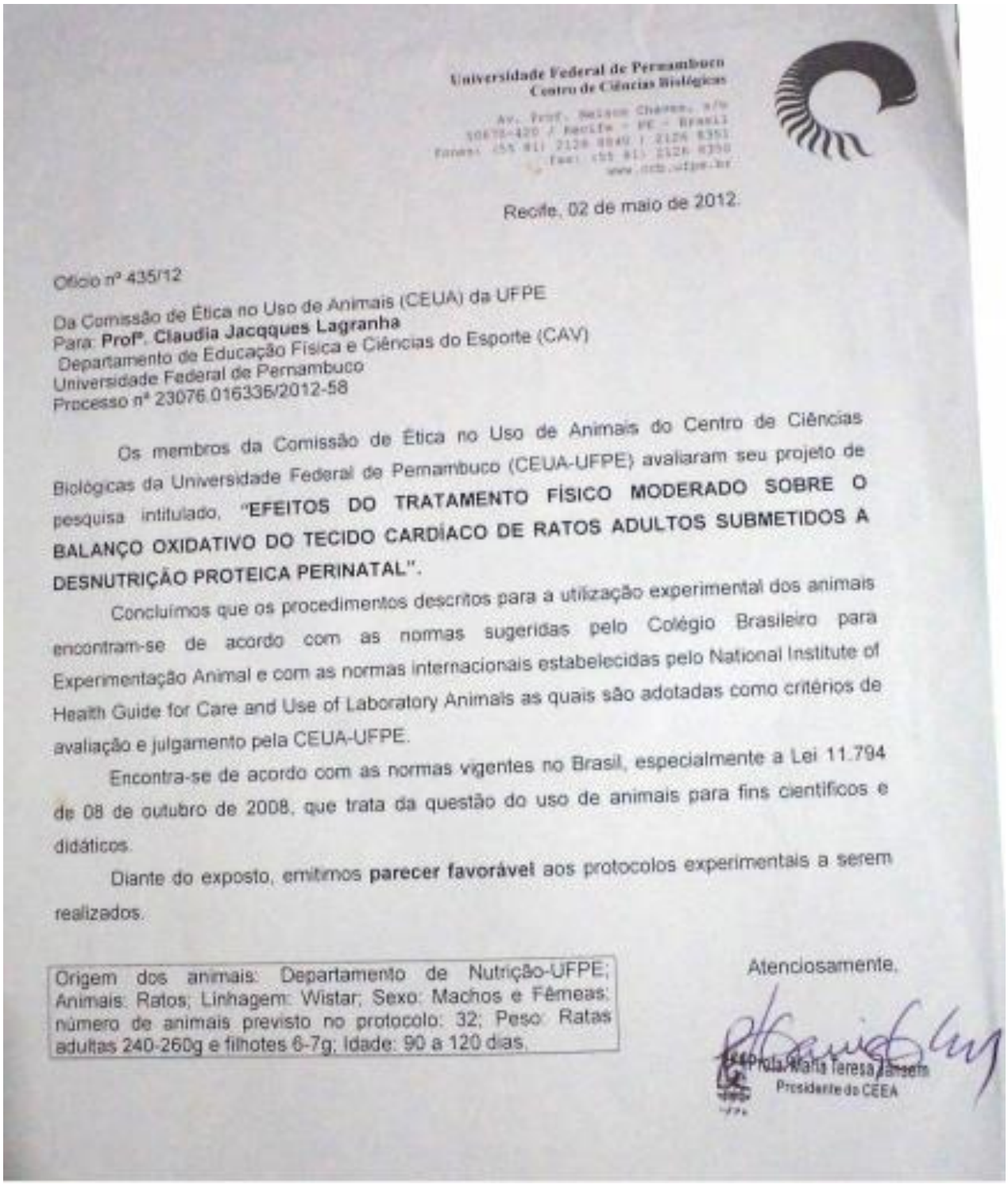
If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Journal of Physiology and Biochemistry

Anexo 3



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Recife, 02 de maio de 2012.

Ofício nº 435/12

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª. Claudia Jacqques Lagranha**
Departamento de Educação Física e Ciências do Esporte (CAV)
Universidade Federal de Pernambuco
Processo nº 23076.016336/2012-58

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"EFEITOS DO TRATAMENTO FÍSICO MODERADO SOBRE O BALANÇO OXIDATIVO DO TECIDO CARDÍACO DE RATOS ADULTOS SUBMETIDOS A DESNUTRIÇÃO PROTEICA PERINATAL"**.

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

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

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

Origem dos animais: Departamento de Nutrição-UFPE; Animais: Ratos; Linhagem: Wistar; Sexo: Machos e Fêmeas; número de animais previsto no protocolo: 32; Peso: Ratas adultas 240-260g e filhotes 6-7g; Idade: 90 a 120 dias.

Atenciosamente,

Profa. Maria Teresa Jansen
Presidente do CEEA

Anexo 4

Universidade Federal de Pernambuco
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Recife, 9 de outubro de 2014.

Ofício nº 56/14

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª. Claudia Jacques Lagranha**
Centro Acadêmico de Vitória - CAV
Universidade Federal de Pernambuco
Processo nº 23076.017808/2014-51

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Estudo da programação fetal de doenças metabólicas da vida adulta nos tecidos vitais: Avaliação dos efeitos da supernutrição"**.

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

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

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

Origem dos animais: Biotério; Animal: ratos; Linhagem: Wistar; Idade: Progenitores adultos e prole após desmame e adulta; Peso: Após desmame:30 – 90g; Sexo: machos e fêmeas; Número total de animais previsto no protocolo: 92.

Atenciosamente,

Profª Marcia Vasconcelos
Vice-Presidente da CEUA/CCB-UFPE
SIAPE 2108035