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DISSERTAÇÃO DE MESTRADO

EFEITOS DA SUPERNUTRIÇÃO NA FUNÇÃO MITOCONDRIAL CARDÍACA: ESTUDO COM ISQUEMIA-REPERFUSÃO

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sempre me incentivou a buscar meus sonhos, colocando a
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”Se enxerguei mais longe, foi porque estava sobre os ombros de gigantes.”

(Isaac Newton)

RESUMO

Evidências clínicas e experimentais demonstram que uma supernutrição no início da vida aumenta o risco para o desenvolvimento da obesidade e de doenças correlatas, como doenças cardiovasculares. Apesar disso, até o presente momento poucos são os estudos com o objetivo de avaliar a função mitocondrial e o balanço oxidativo no coração de ratos que foram supernutridos na lactação e submetidos ao insulto isquêmico. O presente estudo teve como objetivo investigar o efeito da supernutrição durante a lactação, avaliando a capacidade respiratória mitocondrial, produção de ERs, potencial elétrico de membrana mitocondrial, balanço oxidativo e expressão de UCP2 no coração de ratos machos submetidos ao insulto de isquemia e reperfusão. Utilizamos ratas Wistar, seguindo as recomendações do COBEA e aprovação do Comitê de Ética em Estudos com Animais do Centro de Biociências da Universidade Federal de Pernambuco (Processo nº 23076.017808/2014-51). Para a indução da 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, permitindo uma oferta significativamente maior de leite para os três filhotes. Para o grupo controle, o número de filhotes permaneceu em nove neonatos por mãe. Aos 60 dias de vida, os animais machos foram sacrificados e o tecido cardíaco foi retirado e submetido ao processo isquemia e reperfusão. Então foram realizadas as análises mitocondriais (respiração mitocondrial, potencial elétrico de membrana mitocondrial, produção de espécies reativas de oxigênio), bioquímicas (nível de peroxidação lipídica, oxidação de proteínas, atividade de enzimas antioxidantes, e conteúdo total de tióis) e moleculares (quantificação da expressão gênica da UCP2 e do gene normalizador, B2M). Nossos resultados demonstraram que a supernutrição induziu uma redução de 43% da capacidade respiratória, com aumento na produção de espécies reativas de 144% e redução no potencial elétrico de 52%. Associado a isso, verificamos um aumento da oxidação de proteínas (32%) e diminuição na expressão de UCP2 (40%). Em relação ao sistema antioxidante verificamos uma redução significativa na atividade da enzima SOD (42,7%) e na quantidade totais de tióis (15%). No que se refere a injúria da isquemia-reperfusão, verificamos uma redução na capacidade respiratória tanto no controle (81%) como no obeso (62,5%), associado com uma tendência ao aumento da oxidação de proteínas. A I/R induz redução na expressão da UCP2 em ambos os grupos. Em relação à atividade antioxidante observamos redução apenas na quantidade total de tiois (22,3%). Nossos dados sugerem que o excesso de alimento no período crítico do desenvolvimento, promove uma desregulação nas funções mitocondriais e no balanço

oxidativo cardíaco, e as injurias induzidas pelo insulto de isquemia-reperfusão são potencializados pela supernutrição precoce.

Palavras-chave: Estresse oxidativo. Isquemia miocárdica. Mitocôndria. Supernutrição.

ABSTRACT

Clinical and experimental evidences have shown that early overnutrition increases the risk for the development of obesity and related diseases such as cardiovascular disease. However, to date, few studies have been conducted to evaluate the mitochondrial function and oxidative balance in rat heart heart of rats that were overnourished during lactation, and submitted to ischemic insult. The present study aimed to investigate the effect of overnutrition during the lactation period, evaluating mitochondrial respiratory capacity, ROS production, membrane mitochondrial potential, oxidative balance and UCP2 expression in the heart of male rats after I/R. We used Wistar rats, following the recommendations of Brazilian Animal Care Committee and approval of the Ethics Committee in Animal Studies of the Center of Biological Sciences of the Federal University of Pernambuco (Process nº 23076.017808 / 2014-51). For the induction of overnutrition, after birth, on the first day of life, all litters were normalized to nine puppies per dams, on the third day of life because the lactation was fully established, the size of the litter was reduced to three pups per, allowing a significant increase in milk supply to the three puppies. For the control group, the number of pups remained in nine neonates per dams. At 60 days of age, male animals were sacrificed and cardiac tissue removed and submitted to ischemia and reperfusion. Mitochondrial (mitochondrial respiration, membrane mitochondrial potential and production of reactive oxygen species), biochemical analyzes (quantification of lipid peroxidation, protein oxidation, antioxidant enzyme activity, and total thiol) and molecular analysis (quantification of gene expression of UCP2 and the normalizing gene, B2M). Our results showed that excess of food during lactation induce body weight increase in adulthood (18% higher), in addition to mitochondria dysfunction; overnutrition induces a reduction of 43% in respiratory capacity, an increase in ROS production of 144%, and a decrease in membrane mitochondrial potential (52%). Associated, we observed an increased oxidation of proteins (32%), and decrease in UCP2 expression (40%) in the overnourished group. In antioxidant defense, we observed a significant reduction in the activity of the SOD enzyme (42.7%) and total thiols (15%). Regarding the I/R injury, we observe a reduction in respiratory capacity in both control (81%) and overnourished groups (62.5%), associated with a tendency to increase protein oxidation. I/R injury induces reduction in UCP2 expression in both groups. Regarding antioxidant defense, we observed reduction in total thiols (22.3%). Our data suggest that excess of food during developmental period promotes dysregulation of mitochondrial functions and cardiac oxidative status and the injuries induced by I/R are potentiates by overnutrition during development.

Keyboards: Mitochondria. Myocardial ischemia. Overnutrition. Oxidative stress.

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ADP – Adenosina difosfato

ATP – Adenosina trifosfato

BSA – Albumina de soro bovino

CAT - Catalase

CDNB – 1-Chlro-2,4-dinitrobenzene

COBEA – Colégio Brasileiro de Experimentação Animal

DCV – Doenças cardiovasculares

DNPH – 2,4 - Dinitrophenylhydrazine

DOHaD - Developmental Origins of Health and Disease

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol-bis (2-aminoethylether)

g - Gravidade

GSH – Glutatione reduced

GSSG – Oxidized glutathione

GST – Glutatione-S-Transferase

H2O2 - Hydrogen peroxide

H2DCF – dichlorofluorescindiacetate

IBGE – Instituto Brasileiro de Geografia e Estatística

IM – Infarto do miocárdio

IMC – Índice de Massa Corpórea

MDA - Malondialdehyde

NHLBI - National Heart, Lung, andBloodInstitute

OMS – Organização mundial de saúde

OPT- Phthaldialdehyde

PA – Pressão arterial

POF – Pesquisa de orçamentos familiares

PTP – Poro de transição de permeabilidade

SOD – Superoxide dismutase

TBA – 2-Thiobarbituric acid

TCA - Trichloroacetic Acid

TTC - Triphenyltetrazolium chloride

WHO – World Health Organization

SUMÁRIO

1. INTRODUÇÃO

O sobrepeso e a obesidade podem ser definidos como o acúmulo excessivo de gordura no tecido adiposo, de forma que possa comprometer a saúde do indivíduo (WHO, 2016). Como indicador de obesidade, são utilizados valores iguais ou acima de 30 do índice de Massa Corporal (IMC) (WHO, 1995). O desenvolvimento da obesidade é complexo e multifatorial, podendo ser a partir de fatores genéticos, endócrinos, metabólicos e ambientais (FISBERG, 1995). Estudos mostram que dentre os fatores ambientais os principais fatores são: o aumento do sedentarismo e a má alimentação, com excesso de calorias (SILVEIRA *et al.*, 2006; SABIN, WERTHER e KIESS, 2011; WHO, 2016).

Dados da OMS, mostram que há um aumento significativo da prevalência da obesidade em diversas populações do mundo. Pela primeira vez no ano de 2004, o número de pessoas com excesso de peso ultrapassou o número de pessoas desnutridas (OMS, 2004), expondo o momento de transição epidemiológica mundial (BATISTA-FILHO e RISSIN, 2003). Em 2014, mais de 1,9 bilhões de adultos com mais de 18 anos estavam acima do peso e destes, mais de 600 milhões eram obesos (WHO, 2014b). Anteriormente considerado um problema apenas em países desenvolvidos, o excesso de peso e a obesidade estão em ascensão em países subdesenvolvidos, especialmente em ambientes urbanos (OMS, 2004). No Brasil, estimativas de indicadores antropométricos calculados a partir de inquéritos nacionais, mostram que cerca de metade da população analisada apresentou excesso de peso e aproximadamente 15% obesidade (IBGE, 2010).

O excesso de peso durante a infância tem aumentado em ritmo alarmante, tendo alcançado em 2014 mais de 41 milhões de crianças com menos de cinco anos no mundo, e podendo chegar a 75 milhões em 2025 (WHO, 2016). Os dados nacionais relacionados à obesidade infantil ainda são limitados, porém segundo os últimos dados da Pesquisa de Orçamentos Familiares, do Instituto Brasileiro de Geografia e Estatística (IBGE), cerca de 15% de crianças apresentam obesidade e 30% excesso de peso (IBGE, 2010). Assim como a obesidade adulta, a obesidade pediátrica é oriunda de diversos fatores, tendo a influência familiar um papel significativo sobre o estilo de vida e o padrão alimentar (WHO, 2000; AGOSTONI *et al.*, 2011).

O sobrepeso e a obesidade infantil preocupam devido o risco aumentado que esses indivíduos têm de tornarem-se adultos obesos (HANKS *et al.*, 2015). Freedman e cols, correlacionaram o IMC da infância com o da vida adulta, demonstrando que 77% das crianças com excesso de peso tornaram-se obesas na vida adulta (FREEDMAN *et al.*, 2008).

Consequentemente aumentando o risco de desenvolver complicações neuro-metabólicas e endócrinas que podem estar associadas a gênese de doenças cardiovasculares com o aumento da pressão arterial e disfunção autonômica na idade adulta (SKINNER *et al.*, 2015).

Estudos sugerem que o excesso no consumo alimentar caracterizando um estado de supernutrição, aumenta significativamente o risco do desenvolvimento e progressão de patologias correlacionadas com a obesidade, tais como hipertensão, infarto do miocárdio ou doenças coronarianas na vida adulta. (PLAGEMANN, HARDER, RAKE, MELCHIOR, *et al.*, 1999; DAVIDOWA e PLAGEMANN, 2000b; STETTLER *et al.*, 2005; VELKOSKA, COLE e MORRIS, 2005; JI *et al.*, 2014; BEI *et al.*, 2015) Apesar de alguns estudos já mostrarem o efeito deletério da supernutrição e ou obesidade na função mitocondrial, nenhum estudo foi publicado até o presente momento associando supernutrição no período do desenvolvimento (i.e durante a lactação), com a função mitocondrial e o estresse oxidativo no ventrículo com ou sem o insulto isquêmico. Dessa forma, o intuito do presente estudo é verificar os efeitos da supernutrição infantil em corações de ratos que sofreram o insulto de isquemia e reperfusão. Como hipótese trabalharemos a seguinte questão:

- A supernutrição no período crítico do desenvolvimento potencializa disfunção de mitocôndrias cardíacas de ratos jovens após o insulto de isquemia e reperfusão.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. PERÍODO CRÍTICO DO DESENVOLVIMENTO E SURGIMENTO DE DOENÇAS

Durante seu desenvolvimento, o organismo pode sofrer influência de fatores externos e apresentar modificações em nível bioquímico e estrutural, podendo influenciar na maturação e função de órgãos e sistemas (PASSOS e RAMOS, 2000; MORGANE, MOKLER e GALLER, 2002). Essa fase onde ocorre rápida proliferação e diferenciação celular é conhecida como período crítico do desenvolvimento (DOBBING, 1964; MORGANE, MOKLER e GALLER, 2002). A depender da espécie, tais períodos de maior susceptibilidade a alterações ambientais podem ocorrer durante a gestação, lactação ou na primeira infância (DOBBING e SANDS, 1985).

O termo "plasticidade fenotípica", denomina a capacidade que o organismo tem de modificar seu fenótipo a partir de um estímulo ou insulto, quando aplicado em períodos críticos do desenvolvimento, podendo posteriormente levar a modificações na estrutura e função dos tecidos (PIGLIUCCI, 1998; LUCAS, FEWTRELL e COLE, 1999; GLUCKMAN e HANSON, 2007; HANSON e GLUCKMAN, 2011). No livro *On the origin of species* (1859), Charles Darwin apresenta evidências a cerca da evolução das espécies através da seleção natural e da adaptação gradual ao meio ambiente em que os organismos estão expostos (WEST-EBERHARD, 2005). A epigenética procura explicar a relação entre o ambiente e modificações celulares por meio do estudo sobre modificações de DNA, de processos de metilação e acetilação ou ainda alterações de proteínas associadas, como as histonas, podendo alterar os nucleotídeos e predispor o aparecimento de doenças (GLUCKMAN e HANSON, 2008; LANGLEY-EVANS, 2009).

A hipótese do *DOHaD* (Origem Desenvolvimentista da Saúde e da Doença) defende que se durante os períodos iniciais da vida o organismo for exposto a fatores que o acometam direta ou indiretamente, o seu desenvolvimento pode ser alterado tendo consequências funcionais quando adulto (VICKERS, 2011). Diversos mecanismos foram propostos na tentativa de elucidar esta relação entre a capacidade adaptativa aos insultos na fase perinatal e doenças na vida adulta. Dentre eles, sugere-se que o indivíduo desenvolva uma resposta adaptativa preditiva, onde determinado estímulo promoveria alterações metabólicas para prepará-lo para o ambiente que seria exposto na vida adulta. Porém, quando há incompatibilidade entre o ambiente previsto e o real, o organismo se predispõe ao surgimento

e/ou progressão de doenças crônico-degenerativas (GLUCKMAN e HANSON, 2004; GODFREY *et al.*, 2007; HANSON e GLUCKMAN, 2008; KNIGHT *et al.*, 2009; GODFREY, GLUCKMAN e HANSON, 2010).

Insultos durante o período crítico de desenvolvimento como tabagismo (SALMASI *et al.*, 2010), agentes farmacológicos (SECKL e MEANEY, 2004) infecções maternas (SCHMIEGELOW *et al.*, 2013) podem aumentar a predisposição a doenças crônicas. Atualmente um grande número de estudos tem mostrado que o desbalanço nutricional positivo (maior oferta de nutrientes) (REMACLE *et al.*, 2011) ou negativo (redução oferta de nutrientes específicos) (ZOHDI *et al.*, 2014) pode influenciar significativamente no aparecimento e progressão de doenças cardiovasculares. Um grande número de estudos vem mostrando que não apenas o período de formação fetal é susceptível a modificações por insultos, mas o período pós-natal precoce também apresenta associação com o aparecimento de doenças na vida adulta (PLAGEMANN, HARDER, RAKE, MELCHIOR, *et al.*, 1999; DAVIDOWA e PLAGEMANN, 2000a; 2001; LI, PLAGEMANN e DAVIDOWA, 2002; PLAGEMANN *et al.*, 2009; PLAGEMANN *et al.*, 2010).

2.2 SUPERNUTRIÇÃO

Dados recentes da Organização Mundial da Saúde mostram que mais de 1.9 bilhões de pessoas estão com sobrepeso e desses mais de 600 milhões estão obesas (WHO, 2014a). Dados adicionais da OMS reporta que pelo menos 2.8 milhões de pessoas morrem a cada ano devido a doenças associadas ao sobrepeso e obesidade (WHO, 2014a). Antigamente a obesidade era considerada apenas uma problemática de países desenvolvidos, atualmente tanto o sobrepeso como a obesidade aumentaram significativamente em países subdesenvolvidos e em desenvolvimento (WHO, 2014b). O maior problema associado a obesidade está relacionado ao elevado risco de morbidade e mortalidade, e redução da expectativa de vida devido a inúmeras co-morbidades associadas, tais como as doenças cardiovasculares, diabetes, hipertensão, certos tipos de câncer, entre outras (WHO, 2014b).

Estudos mostram que o crescente problema do excesso de peso pode estar associado a fatores epigéneticos como uma nutrição inadequada pós-natal (DIETZ, 1994). Stettler demonstrou em seus estudos epidemiológicos que o rápido ganho de peso no início da vida pode estar associado ao aumento do risco de sobrepeso e obesidade na vida adulta, independente do peso ao nascer e do peso com 1 ano de idade (STETTLER *et al.*, 2002).

Modelos utilizando ninhadas reduzidas de ratos têm sido bastante utilizados para estudar as consequências de uma superalimentação pós-natal precoce, pois estas desenvolvem sobrepeso e obesidade de forma rápida, por promover uma maior oferta de alimentos para os filhotes (DORNER e PLAGEMANN, 1994; PLAGEMANN, HARDER, RAKE, VOITS, *et al.*, 1999; PLAGEMANN, HARDER, RAKE, WAAS, *et al.*, 1999). Estudos realizados na década de 90 mostraram que ratos de ninhadas reduzidas apresentam malformações nos núcleos hipotalâmicos, sendo essas más formações persistentes até a vida adulta, sugerindo que a supernutrição neonatal altera o desenvolvimento de populações neuronais responsáveis pela regulação da ingestão de alimentos, apresentando hiperfagia e com a indução de sobrepeso e obesidade (PLAGEMANN *et al.*, 1992; PLAGEMANN, HARDER, RAKE, WAAS, *et al.*, 1999)

Esta supernutrição e consequente obesidade aumenta o risco para o aparecimento de comorbidades associadas incluindo síndrome metabólica e doenças cardiovasculares em adultos (PLAGEMANN, HARDER, RAKE, MELCHIOR, *et al.*, 1999; DAVIDOWA e PLAGEMANN, 2000b; STETTLER *et al.*, 2005; VELKOSKA, COLE e MORRIS, 2005; JI *et al.*, 2014; BEI *et al.*, 2015). Corroborando com essas observações, estudos adicionais mostraram que a supernutrição precoce aumentou a glicemia, induziu resistência a insulina e aumentou triglicerídeos (KUDO *et al.*, 1995; BOULLU-CIOCCA *et al.*, 2005; BOULLU-CIOCCA *et al.*, 2008; COSTA *et al.*, 2016).

2.2.1 Supernutrição e Doença Cardiovascular

A associação entre o aumento de peso corporal e pressão arterial (PA), em adultos já está bem estabelecida na literatura. (SAMUELSSON *et al.* , 2008; LIANG *et al.,* 2009; GUBERMAN *et al.,* 2013). Atualmente estudos vem mostrando que em jovens obesos essa relação também esta aumentando, elevando assim o risco do aumento da pressão arterial nesse grupo populacional. É cada vez mais conhecido que além do aumento do risco de hipertensão, a obesidade infanto-juvenil pode aumentar o risco de doenças cardiovasculares (DCV) em geral (KENCHAIAH *et al.,* 2002).

Dados recentes de mostram que crianças obesas tem níveis elevados de lipídios, os valores de glicose em jejum aumentados, maior resistência à insulina, e valores elevados de insulina em jejum do que crianças e adolescentes com peso normal. As crianças obesas também têm maior espessura media camada íntima da carótida, uma medida associada com a doença vascular, quando comparadas a crianças com peso normal (KUMAR, 2017). Outros autoresmostram que, a supernutrição no início da vida pode estar associada com o aumento da

pressão arterial (SAMUELSSON *et al.* , 2008; LIANG *et al.*, 2009; GUBERMAN *et al.*, 2013), colesterol elevado (ELAHI *et al.*, 2009), disfunção endotelial (GHOSH *et al.*, 2001; MOREIRA *et al.*, 2009 ; FAN *et al.*, 2013) e hipertrofia cardíaca (FERNANDEZ-TWINN *et al.*, 2012; MOREIRA *et al.*, 2009) em adultos jovens. Sendo a obesidade um fator de risco independente para doenças cardiovasculares (HUBERT *et al.*, 1983; ARTHAM *et al.*, 2009).

Bernardo et al. (2016) e Vieira et al. (2015) estudando os efeitos da supernutrição pós-natal em coração de camundongos com 21 e 120 dias de idade, demonstraram que precocemente (21 dias) o aumento de peso induzido pela supernutrição pós-natal aumentou o estresse oxidativo, a razão BAX/BCL2 e induzia fibrose cardíaca, além de prejuízo na sinalização insulínica cardíaca, regulação de GLUT-1, em adição a uma lesão mitocondrial sem prejuízo nos complexos mitocondriais. Os autores concluíram que em curto prazo a supernutrição durante a lactação induz distúrbios metabólicos e altera a preferência do substrato cardíaco em camundongos jovens (VIEIRAA.K.G. *et al.*, 2015; BERNARDO *et al.*, 2016).

A longo prazo (120 dias), os autores mostraram que a supernutrição durante a lactação modula varias proteínas e genes da sinalização de insulina e da via da AMPK. Em adição, o estudo também avaliou o efeito da injuria de isquemia e reperfusão em camundongos supernutridos, onde autores mostraram que essa injuria em camundongos obesos de 120 de idade diminui a p-AMPK, FABP e CPT1 e UCP3, sugerindo que a supernutrição na infância induz efeito prejudicial em longo prazo no metabolismo cardíaco (VIEIRAA.K.G. *et al.*, 2015; BERNARDO *et al.*, 2016). Turdi *et al.* (2013) relata a redução do potencial de membrana mitocondrial e uma redução nos fatores transcripicionais relacionados com metabolismo oxidativo mitocondrial em animais que foram expostos a supernutrição no período pós-natal (TURDI *et al.*, 2013)

A patogênese da falha cardíaca provocada pelo o infarto do miocárdio é caracterizada pela perda de cardiomiócitos funcionais em decorrência da injúria isquêmica (LOVELL e MATHUR, 2004). A injuria de isquemia-reperfusão (IR) ocorre quando o suprimento de sangue para um tecido é bloqueado por alguns minutos a horas (isquemia) e posteriormente o suprimento de sangue é restaurado (reperfusão). As células isquêmicas morrem se o fluxo sanguíneo não for restaurado, mas é na reperfusão em si que ocorre a maior parte do dano de IR. (ZWEIER, FLAHERTY e WEISFELDT, 1987; CHOUCHANI *et al.*, 2016). Os mecanismos celulares envolvidos na patogênese da cardiopatia isquêmica são complexos e envolvem a interação de inúmeros processo e tipos celulares, incluindo a função das mitocôndrias e a capacidade contrátil dos cardiomiócitos.

2.3 MITOCÔNDRIA

As mitocôndrias são organelas que possuem duas membranas em sua estrutura, onde as mesmas possuem permeabilidades distintas. A membrana externa é permeável a moléculas pequenas, enquanto a interna é extremamente impermeável a maioria das moléculas (KALUDERCIC e GIORGIO, 2016). A função central das mitocôndrias é a produção de energia através da oxidação de nutrientes, um processo conhecido como fosforilação oxidativa. Piruvato e ácidos graxos são convertidos em acetil-CoA na matriz mitocondrial. Os grupos acetil alimentam o ciclo de Krebs e o processo culmina com transferência de elétrons para a cadeia respiratória ou cadeia transportadora de elétrons. Este processo é acoplado à produção de ATP com o retorno dos prótons à matriz mitocondrial pelo complexo ATP-sintase através da membrana mitocondrial interna (PEEK *et al.*, 2013)

Durante o transporte de elétrons mitocondrial espécies reativas de oxigênio (EROs) são formados, sendo assim a mitocôndria uma importante fonte (PEEK *et al.*, 2013). EROs são moléculas de oxigênio quimicamente reativas tais como o ânion superóxido ($O^2 \cdot -$), radical hidroxila ($OH^{\cdot}$) e peróxido de hidrogênio (H^2O^2). Aproximadamente de 1 a 3% d0 oxigênio molecular são convertidos em compostos reativos, principalmente, nos complexos mitocondriais I e III através da fosforilação oxidativa (MURPHY, 2009). O excesso de EROs contribui para o desenvolvimento de danos que podem levar a disfunção mitocondrial e morte celular (MURPHY, 2009). A quantidade de EROs depende das condições fisiopatológicas variáveis de cada célula e é determinada pelo equilíbrio de muitos agentes pró-oxidantes endógenos e exógenos (PATEL, 2016).

Para evitar danos oxidativos, o equilíbrio redox celular é mantido por antioxidantes enzimáticos e não enzimáticos notavelmente eficientes, tais como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidases (GPx), glutationa S-transferase (GST), vitaminas A, C e E, Ubiquinona (Q10), entre outras moléculas (ANDREYEV, KUSHNAREVA e STARKOV, 2005; VALKO *et al.*, 2007; KLAUNIG *et al.*, 2011; BHAT *et al.*, 2015). O desequilíbrio entre os mecanismos de defesa e a produção de EROs leva ao estresse oxidativo e condições patológicas subsequentes (HEITZER *et al.*, 2001). Em geral, os miócitos consomem um alto nível de oxigênio devido ao considerável número de mitocôndrias presentes, em comparação com outros tipos celulares (ZOROV, JUHASZOVA e SOLLOTT, 2014). Por esta razão, os cardiomiócitos também produzem uma grande quantidade de EROs, podendo gerar o estresse oxidativo em células vizinhas (HANDY e LOSCALZO, 2012).

Por conta da sua dependência do metabolismo e da bioenergética mitocondrial, o músculo cardíaco é especialmente vulnerável a distúrbios mitocondriais (JENNINGS e GANOTE, 1976; HALESTRAP *et al.*, 1998). A insuficiência da oxigenação celular pode levar a inibição do fluxo de elétrons pela cadeia respiratória, comprometendo assim a conservação de energia e o metabolismo oxidativo. Além disso, a homeostase do cálcio (Ca^{++}) também é prejudicada. Isso tipicamente ocorre durante a lesão de isquemia e reperfusão (I/R). Durante a reperfusão, há um acúmulo de Ca^{++} dentro da matriz mitocondrial devido ao aumento de Ca^{++}citosólico, podendo levar a uma sobrecarga que pode resultar em abertura do poro de transição de permeabilidade (PTP), despolarização mitocondrial e morte celular (WEISS *et al.*, 2003; BERNARDI *et al.*, 2006).

Os poros de transição de permeabilidade mitocondrial (PTP), são canais não seletivos localizados na membrana interna mitocondrial. A superprodução de EROs e recaptação exacerbada de cálcio podem dissipar o gradiente eletroquímico de prótons e abrir o PTP, levando ao desacoplamento do processo de fosforilação oxidativa e a uma produção ainda maior de EROs (HAUSENLOY e YELLON, 2003; BAINES, 2009). A abertura do PTP, com a dissipação do potencial elétrico de membrana mitocondrial, levará a liberação de proteínas pró-apoptóticas, tais como as pro-caspases e as proteínas indutoras de apoptose, induzindo a morte celularpor apoptose ou necrose (BAINES, 2009; ZHU *et al.*, 2011). Vários estudos tem mostrado que a inibição do PTP pode atenuar a perda celular em patologias cardíacas como a isquemia e reperfusão cardíaca (ZHU *et al.*, 2011), falência cardíaca (DEDKOVA, SEIDLMAYER e BLATTER, 2013) e cardiomiopatia diabética (OLIVEIRA *et al.*, 2003). A inibição da abertura do PTP, durante os primeiros minutos de reperfusão, protege os miócitos do estresse oxidativo e limita o tamanho do infarto (HAUSENLOY, DUCHEN e YELLON, 2003). Entretanto, Saotome et al. revela que a abertura transiente do PTP induzida pelas EROs, pode proteger o miocárdio da injúria de I/R (SAOTOME *et al.*, 2009) revelando uma função específica do PTP dependente do grau e do tempo de abertura do PTP.

Proteínas desacopladoras (UCPs) são transportadores localizados na membrana interna da mitocôndria que promovem o transporte de prótons do espaço inter-membrana para a matrix mitocondrial dissipando o gradiente de prótons. Em adição ao sistema antioxidante, têm emergido relatos mostrando que as UCPs atuam também como um modulador essencial da função mitocondrial (AZZU e BRAND, 2010; MAILLOUX e HARPER, 2011; SLUSE, 2012). Esta regulação da função mitocondrial mediada por UCPs participa de um importante papel fisiológico para prevenir a sobrecarga de elétrons dentro da cadeia transportadora de

elétrons, ajustando o metabolismo energético e prevenindo a produção excessiva de EROs mitocondrial e diminuindo o gradiente eletroquímico de prótons através da membrana mitocondrial interna e regulando a produção de EROs mitocondrial associado a varias desordens, incluindo obesidade e doenças cardiovasculares (AZZU e BRAND, 2010; MAILLOUX e HARPER, 2011; SLUSE, 2012).

2.4 MITOCÔNDRIAS, INJÚRIA DE ISQUEMIA E REPERFUSÃO E SUPERNUTRIÇÃO

Como em muitos outros tipos celulares, nos cardiomiócitos, as mitocôndrias desempenham um papel fundamental, pois através da metabolização de glicose ou ácidos graxos são o principal sítio produtor de ATP importante na contração cardíaca, além de ser o principal sítio produtor de espécies reativas de oxigênio (HALESTRAP, CLARKE e JAVADOV, 2004). Diversos estudos (SCHULZ *et al.*, 2007; MURPHY e STEENBERGEN, 2008) vêm demonstrando que as mitocôndrias têm participação importante na susceptibilidade cardíaca devido ao desequilíbrio entre produção e remoção de espécies reativas de oxigênio, como também na coparticipação do processo de progressão da injúria da isquemia e reperfusão e na falência cardíaca.

As mitocôndrias em cardiomiócitos sadios apresentam um ajuste altamente regulado em relação a oxidação de substratos como glicose e ácidos graxos, entretanto em doenças cardíacas, tais como injúria isquêmica, a relação de oxidação de glicose e ácidos graxos estão alterada (LIU *et al.*, 2002; BUCHANAN *et al.*, 2005; LOPASCHUK *et al.*, 2010). Essa alteração inclui um aumento relativo na proporção de oxidação de ácidos graxos pelas mitocôndrias em comparação à quantidade de carboidratos oxidados (LIU *et al.*, 2002; BUCHANAN *et al.*, 2005; LOPASCHUK *et al.*, 2010). Estudos prévios já demonstraram que aumento na quantidade de oxidação de ácidos graxos induz redução na eficiência cardíaca e contribui com efeitos deletérios observados em doenças cardíacas como hipertensão pulmonar, falência cardíaca e isquemia coronariana (LIONETTI *et al.*, 2005; ONAY-BESIKCI *et al.*, 2007; FANG *et al.*, 2012; PIAO *et al.*, 2013)

As células miocárdicas que sofreram isquemia morrem se o fluxo sangüíneo não for restaurado, mas é na reperfusão em si que ocorre o maior dano induzido pela injúria de isquêmica. O primeiro evento prejudicial após reperfusão é uma produção exacerbada de EROs mitocôndriais que inicia a patologia que se desenvolve ao longo dos minutos, dias e semanas após a reperfusão (ZWEIER, FLAHERTY e WEISFELDT, 1987; CHOUCHANI *et al.*, 2016). Esta explosão inicial na produção de EROs após a reperfusão causa diretamente

danos às mitocôndrias, e em conjunto com a desregulação dos níveis de cálcio, podem levar à indução da transição de permeabilidade mitocondrial e a morte celular após reperfusão (MURPHY e STEENBERGEN, 2011; CHOUCHANI *et al.*, 2016).

Além disso, devido ao insulto isquêmico, o metabolismo oxidativo mitocondrial diminui; já durante o processo de reperfusão, o coração apresenta um aumento nas taxas de oxidação de ácidos graxos, em parte, devido ao aumento dos níveis circulantes de ácidos graxos (FOLMES *et al.*, 2009). Em adição, o controle subcelular de oxidação de ácidos graxos em corações isquêmicos ficam descompensados, isso inclui diminuição de malonil-CoA que normalmente é um potente inibidor da captação mitocondrial de ácidos graxos (KUDO *et al.*, 1995). Os elevados níveis circulantes de ácidos graxos somado a redução dos níveis de malonil-CoA, resulta em um aumento da oxidação de ácidos graxos no período de reperfusão induzindo uma marcante redução na oxidação de glicose (LIU *et al.*, 2002). Esses efeitos somados resultam em um aumento no desacoplamento do processo da glicólise com um subsequente aumento na produção de lactato e na concentração de prótons que resulta em uma diminuição na eficiência cardíaca e prejuízo na função dos cardiomiócitos (LIU *et al.*, 2002; ONAY-BESIKCI, 2007).

3. OBJETIVOS

3.1 GERAL

Investigar se a supernutrição pós-natal aumenta a susceptibilidade à lesão cardíaca após insulto de isquemia e reperfusão em ratos aos 60 dias de vida.

3.2 ESPECÍFICOS

Avaliar **nas mitocôndrias** do tecido cardíaco de ratos controle e supernutridos com 60dias de vida **submetidas e não submetidas** ao insulto de isquemia e reperfusão:

- O consumo de oxigênio mitocondrial;
- A produção de espécies reativas de oxigênio das mitocôndrias isoladas;

Avaliar **no tecido cardíaco** de ratos controle e supernutridos com 60 dias de vida **submetidas e não submetidas** ao insulto de isquemia e reperfusão:

- A expressão gênica da proteína desacopladora mitocondrial-2;
- Biomarcadores de estresse oxidativo;
- Atividade das enzimas antioxidantes (SOD, CAT e GST);
- Quantidade total de tióis.

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5. ARTIGO

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Mitochondrial bioenergetics and oxidative damage in hearts of post-natal overnourished rat: With and without ischemia-reperfusion insult

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Running title: Overnutrition induces cardiac mitochondrial dysfunction in adulthood

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Abstract

Clinical and experimental evidences have shown that early overnutrition increases the risk for the development of obesity and related diseases such as cardiovascular disease. Despite studies showing the deleterious effects associated with obesity and cardiovascular disease, there are few studies to evaluate the mitochondrial function and oxidative balance in rat heart at 60 days of age, that were overnourished during lactation, and submitted to ischemia-reperfusion (I/R) injury. The present study aimed to investigate the effect of overnutrition during the lactation period, evaluating mitochondrial respiratory capacity, ROS production, membrane mitochondrial membrane electric potential, oxidative balance and UCP2 expression in the heart of male rats after I/R. We used Wistar rats, following the recommendations of Brazilian Animal Care Committee and approval of the Ethics Committee in Animal Studies of the Center of Biological Sciences of the Federal University of Pernambuco (Process nº 23076.017808 / 2014-51). For the induction of overnutrition, after birth, on the first day of life, all litters were normalized to nine puppies per dams, on the third day of life because the lactation was fully established, the size of the litter was reduced to three pups per, allowing a significant increase in milk supply to the three puppies. For the control group, the number of pups remained in nine neonates per dams. At 60 days of age, male animals were sacrificed and cardiac tissue removed for evaluate mitochondrial bioenergetics (mitochondrial respiration, mitochondrial membrane electric potential ($\Delta\Psi m$) and production of reactive oxygen species), biochemical analyzes (quantification of lipid peroxidation, protein oxidation, antioxidant enzyme activity, and total thiol) and molecular analysis (quantification of gene expression of UCP2 and the normalizing gene, B2M). Our results showed that excess of food during lactation induce body weight increase in adulthood (18% higher), in addition to mitochondria dysfunction; overnutrition induces a reduction of 43% in respiratory capacity, an increase in ROS production of 144%, and a decrease in $\Delta\Psi m$ (52%). We observed an increased oxidation of proteins (32%), and decrease in UCP2 expression (40%) in the overnourished group. The evaluation of antioxidant defense showed a significant reduction in the activity of the SOD enzyme (42.7%) and total thiols (15%). Regarding the I/R injury, we observed a reduction in respiratory capacity in both control (81%) and overnourished groups (62.5%), associated with a tendency to increase protein oxidation. I/R injury induces reduction in UCP2 expression in both groups. Regarding antioxidant defense, we observed reduction in total thiols (22.3%). Our data suggest that excess of food during developmental period promotes dysregulation of mitochondrial functions and cardiac oxidative status and the injuries induced by I/R are potentiates by overnutrition during development.

Keywords: Early overnutrition, mitochondrial dysfunction, ischemia-reperfusion injury.

INTRODUCTION

According to a World Health Organization publication more than 1.9 billion of people are overweight, of these over 600 million were obese [1]. Additional data from WHO reports that at least 2.8 million people dying each year as a result of being overweight or obese [1,2]. Previously, obesity was considered a problem only in high-income countries, now overweight and obesity dramatically increase in low- and middle-income countries, particularly in urban settings [3]. The main problem associated with obesity is related to the increased risk of morbidity and mortality as well as reduced life expectancy due to the numerous comorbidities associated, such as cardiovascular diseases (CVD), type 2 diabetes, hypertension, certain cancers, and sleep apnea/sleep-disordered breathing.

With the intention of understand the mechanism involved in obesity and cardiovascular diseases, several studies have pointed that mitochondrial dysfunction play a key role in the genesis of the pathology. Pipatpiboon et al. (2015) studying the effect of obesity induced by high-fat diet on cardiac mitochondria showed that this type of diet induces an increase the mitochondrial ROS production[4]. In the same year Kang et al (2015) also studying the effect of high-fat diet on cardiac mitochondria showed that the body weight of the group that received high-fat diet was significantly higher than control group and in the myocardium of the obese group, the expression of complex I subunit and PGC1α were decreased in association to a decrease in the mitochondrial DNA number; the authors associates these results to the oxidative stress damage accompanied with reduced ATP levels[5]. Yuan et al (2015) also studying the effect of high fat diet-induced obesity demonstrated that high fat diet reduces myocardial aconitase activity, down regulates levels of mitochondrial protein PGC-1α and SOD1, in addition to decrease the gene and protein expression of UCP2[6].

Riojas-Hernandez et al. (2015) using a different approach to study obesity effect in heart, demonstrated in Zucker obese rats, that systolic Ca^{2+} transient was slower, while mitochondrial H^2O^2 production increased and mitochondrial permeability transition pore opening was more sensitive to Ca^{2+} than in lean rats. In addition, the authors showed in obese rats, decrease in oxidative phosphorylation capacity with decrease in ATP content, combined with increased caspase 9 activity. The authors suggested that obesity induce mitochondrial dysfunction and apoptosis, leading to oxidative stress and diastolic dysregulation [7].

Early postnatal life is considered to be a critical period of development in which the individual remains particularly sensitive to environmental and nutritional influences [8,9]. In addition to the deleterious effects of nutritional imbalance on growth rate and morphogenesis during this time, overnutrition during early postnatal life can also place the individual at risk for developing obesity and metabolic syndrome in adulthood [9,10]. 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 [11-13]. Additional studies, observed an increase in fasting glucose, plasma insulin and triglycerides [14-16], while others reported increased blood pressure [17,18]. Studying the effect of overnutrition during lactation, Zhang et al. demonstrated myocardial contractility dysfunction in overnourished animals [19]. However, the association of postnatal overnutrition and cardiac dysfunction are not completely understood.

Bernardo et al. (2016) and Vieira et al. (2015) studying the effect of post-natal overnutrition in mice heart with 21 and 120 days of age, demonstrate that in short-term obesity induced by overnutrition increased oxidative stress, BAX/BCL2 ratio and cardiac fibrosis, impairment in heart insulin signaling, up-regulates GLUT-1, in addition to mild mitochondrial damage without alterations in OXPHOS complexes. The authors conclude that in short-term, overnutrition during early life induces metabolic disturbances and switch cardiac fuel preference in juvenile mice. In long-term, the authors showed that overnutrition during early life modulates several protein and gene expression, in addition the study also evaluates the effect of ischemia and reperfusion injury in overnourished mice; the authors claimed that the ischemic injury in obese mice at 120 days of age decreases p-AMPK, FABP and CPT1 and UCP3, suggesting that overnutrition in childhood induces a long-term detrimental effect on cardiac metabolism [20,21].

Besides several researchers have demonstrated the detrimental effect of obesity induced of by genetic modification or diet, until now, there is a lack in the literature studying the effect of overnutrition during lactation in mitochondrial bioenergetics, UCP2 expression and oxidative status in rat heart at 60 days of age. Thus, in the present study, we aimed test the hypothesis that overnutrition in childhood induce mitochondrial dysfunction that may increase the ventricular damage after ischemia-reperfusion insult.

MATERIALS AND METHODS

Ethical Standards

Experiments were carried out in accordance with the recommendations of the Brazilian Committee of Animal Care (COBEA) and endorsed by the Ethical Committee of the Biological Science Center of the Federal University of Pernambuco, Brazil, under the process number 23076.017808/2014-51.

Animals and experimental groups

Wistar rats obtained from our Academic Center (Federal University of Pernambuco), were maintained at a room temperature of 23 ± 1 °C, on a light–dark cycle of 12:12 h, with free access to water and food (diet Labina - Purina S / A) *ad libitum*. The females selected (n = 8) between 220-250 g underwent a 15-day adaptation period to synchronize the circadian cycle. After adaptation, the rats in the estrus period were mated in the proportion 1 female to 2 males. With pregnancy detection the pregnant rats were transferred to individual cages and divided into two experimental groups: Control group (N, 9 pups per mother) and the Overnourished group (O, 3 pups per mother) according to the studies from Plagemann's group [22-27]. After 21 days of age (weaning) both groups received commercial laboratory chow (Labina-Purina®) and water *ad libitum*. At 60 days of life, the rats were euthanized by decapitation.

Body weight measurement

Body weights (in grams) were measured at 60rd postnatal day using a digital balance (S-400, with a 1 gram of sensitivity) [28].

Blood collection and biochemical analysis

Blood was collected for serum analysis of glucose, cholesterol and triglycerides. The samples were centrifuged at 900 g for 5 min. All assays were performed according manufactured protocols (Lab Test Kit for glucose, cholesterol and triglycerides) using an automatic chemistry analyzer (Pioway Medical Lab Equipment, Nanjing, Jiangsu, China).

Mitochondrial Isolation

After the hearts were removed, left ventricle were immediately minced and homogenized, in a mitochondrial isolation buffer containing 225 mM mannitol, 75 mM sucrose, 4 mM HEPES, 2 mM Taurine and 0.5 mM EGTA, pH 7.4. Subsequently, the samples were centrifuged at 4°C for 5 min at 1180 g, the supernatants were collected and centrifuged once more at 4°C for 10 min at 12470 g. After the last centrifugation, the pellets

were re-suspended in the buffer consisting of 250 mM sucrose, 5 mM HEPES (w/v), pH 7.4. Aliquots of mitochondria were analyzed for the total protein content using the Bradford protocol [29,30].

Simulated ischemia model

The cardiac mitochondria were subjected to simulated ischemia by pelleting the mitochondria as described above. Briefly, freshly isolated mitochondria from control or overnourished rat heart were divided into normoxia and IR. Simulated ischemia was carried out in respiration buffer with complex I substrate for 5 min stabilization and then covered with mineral oil and incubated for 30 min. After ischemia, mineral oil was removed and mitochondria were quickly centrifuged and supernatant replaced by fresh isolation buffer for followed assays (i.e respiration, ROS production and $\Delta\Psi m$).

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 mitochondrial respiration buffer (RB) containing 120 mM KCl, 4 mM HEPES, 5 mM K2HPO4, 0.2% BSA. 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) [29,30].

Reactive species production

The production of ROS was monitored using the fluorescent probe 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA). Freshly isolated mitochondria (100 µg) were incubated in respiratory buffer with 1 µM H2DCF-DA. The DCF fluorescence was monitored at 485 nm and 525 nm via fluorescence spectrophotometry (FLUOStar, Omega, USA)[28,31].

Mitochondrial membrane electric potential ($\Delta\Psi m$)

The mitochondrial electrical membrane potential was performed as previously described [32,33]. This assay relies on the electric affinity of safranin-O to anions, where the fluorescence decay is related to electrophoretic transport at ion into the mitochondria. The assay consisted of the addition of 5 mM dye to 0.1 mg mitochondria incubated in RB with

complex I substrates, with the safranin fluorescence monitored at 485 nm excitation and 590 nm emission. The analysis was performed at 28 °C in a FLUOstar OMEGA (BMG Labtech, USA) with gentle agitation [34].

Global ischemia-reperfusion (I/R) injury

The heart was quickly excised and perfused retrograde via the aorta with Krebs-Henseleit (K-H) buffer (120 nM NaCl, 4.6 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) oxygenated. Hearts were submitted to global ischemia for 30 minutes and 15 minutes of reperfusion at 38 °C [29].

Tissue homogenate preparation

After I/R, heart for biochemical analysis were quickly collected and stored at −20 °C. Left ventricles were placed in small glass homogenizers with extraction buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing protease and phosphatase inhibitors. The ventricle was homogenized on ice and centrifuged at 1180 g for 10 min at 4 °C. The supernatant was collected and stored at −20 °C for the following assay [29,30].

Protein determination

Aliquots of tissue homogenate were used for measuring the total protein content as described by Bradford [35] using known concentrations of BSA for standard curve.

Evaluation of malondialdehyde production

Lipid peroxidation is a process which reactive oxygen species attack lipids, especially polyunsaturated fatty acids. Among the many different aldehydes formed as secondary products during lipid peroxidation, malondialdehyde (MDA) appears to be the most mutagenic product of lipid peroxidation [26]. For the evaluation of lipid peroxidation, a total of 0.2 mg/ml homogenate was used to measure malondialdehyde production (MDA) following reaction with thiobarbituric acid (TBA) at 100 °C according previously published [30,36,37]. The results are expressed as µmol/mg protein.

Evaluation of protein oxidation

Reactive oxygen species can induce the oxidation of amino acid residues on proteins, thus yielding protein carbonyls. The protein carbonyl content is the most widely used marker of oxidative modification of proteins [38]. The protein oxidation was assessed using the

procedures highlighted by Reznick and Packer [39]. 2,2,2-Trichloroacetic acid (TCA) it 30% (w/v) was added to the sample on ice, and then this mixture was centrifuged for 14 min at 1180 g. The pellet was suspended in 10 mM 2,4-dinitrophenylhydrazine (DNPH) and immediately incubated in a dark room for 1 h with shaking every 15 min. Thereafter, the samples were centrifuged and washed thrice with ethyl/acetate buffer; then, the final pellet suspended in 6 M guanidine hydrochloride was incubated for 5 min in a water bath at 37 °C and the absorbance was measured at 370 nm [40].

Superoxide dismutase activity

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide anion into hydrogen peroxide (H_2O_2) [41]. The determination of total SOD enzyme activity (t-SOD) was performed according to the method of Misra and Fridovich[42]. Samples (0.3 mg/ml homogenate) were incubated in sodium carbonate buffer (0.05%, pH 10.2, 0.1 mM EDTA; ethylenediaminetetraacetic acid) in a water bath at 37 °C. The reaction was started by the addition of 30 mM epinephrine dissolved in 0.05% acetic acid. Absorbance at 480 nm was determined after 4 min. One unit of t-SOD was defined as the amount of enzyme causing a 50% inhibition of epinephrine oxidation. Tissue t-SOD activity was expressed as units per milligram protein (U/mg protein) [30,40].

Catalase activity

Catalase (CAT) catalyzes the decomposition of hydrogen peroxide into water and oxygen [41]. Catalase activity was measured according to the method described by Aebi [43]. Briefly, 0.3 M H_2O_2 was added to a mixture containing 0.3 mg/ml of homogenate and 50 mM phosphate buffer (pH 7.0). A decrease in the H2O2 levels was noted, following which the absorbance was measured at 240 nm for 3 min; the CAT activity was expressed as U/mg protein [30,40,43].

Glutathione S-transferase activity

Glutathione S-transferase (GST) is an antioxidant enzyme involved in the detoxification of a wide range of toxic agents including peroxide and alkylating agents present in the tissues. The activity of GST was measured by the method described by Habig et al. [44]. The principle of the assay is based on the determination through absorbance spectroscopy of the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced

glutathione (GSH). Absorbance was measured at 340 nm. One unit of enzyme conjugates 10.0 nmol of CDNB per minute with reduced glutathione [30,40].

Total and protein-bound sulfhydryl group content

Total and protein-bound sulfhydryl group content were determined as described by Aksenov and Markesbery [45]. The reduction of 5,5-dithiobis(2-nitrobenzoic acid) by thiol groups was measured in homogenates of 0.5 mg/mL heart, resulting in the generation of a yellow-stained compound, TNB, whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins [16].

RNA extraction

Total RNA was obtained from left ventricle by the guanidine isothiocyanate extraction method [46], using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, homogenized samples were incubated 5 minutes at RT in order to allow the complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL) was added and the tubes centrifuged at 12.000 x g for 15 minutes at 4°C. The aqueous phase was transferred and the RNA precipitate was obtained by centrifugation with 0.5 mL of cold isopropyl alcohol [47]. The RNA pellet was washed with 75% ethanol and centrifuged at 7.500 x g for 5 minutes at 4°C. At the end, the RNA pellet was air-dried, dissolved in RNase-free water and stored at -20°C. RNA was quantified by measuring absorbance at 260 nm and it is purity assessed by the 260/280 nm ratio [48,49].

Reverse transcription-polymerase chain reaction (RT-PCR)

PCR reactions were carried out for all genes of interest in each sample using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA). Each reaction was performed in a final volume of 25 μL containing: 200ng of RNA, 12.5 μL 2x Reaction mix, 10 μM of each primers, 2 μL SuperScript® III RT/Platinum® Taq mix and up to 25 μL of DEPC ultrapure water. cDNA synthesis were performed starting with a 60°C for 30 min, Denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 57°C for 30 sec and 68°C for 1 min, and as final extension 68°C for 5 min. Each group were run in quadruplicate and average values were calculated using β2-microglobulin (β2M) gene as internal control [50-52]. For the analysis of the PCR amplification products, aliquots of amplification reaction were added in pre-cast Ex-Gel® with SYBR® Safe system and following manufacture protocol.

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

Statistical analysis

Results were expressed as the mean ± the standard error of the mean (SEM). For the analyses of blood analysis and body weight were used Student's unpaired t-test, for all others analyses were used two-way ANOVA with multiple comparisons. The tests of significance were recommended based on the results of the tests of normality (Kolmogorov–Smirnov test). Comparisons were performed using GraphPad Prism software (GraphPad Software Inc., v.5, La Jolla, CA, USA), and differences were considered to be significant at p b 0.05.

RESULTS

Body weight and serum analysis

We found a significant effect of the nutritional state since 21 days of age on the body weight (data not show). Overnutrition induced a significant difference in postnatal body weight and this difference was maintained until 39 days after weaning (Animals with 60 days of age, Control: 232.9 ± 3.2; Overnourished: 274.3 ± 5.8 grams, $p<0.001$). In addition to the increase in body weight, overnutrition induces a significant increase in glycaemia (Control: 93.2 ± 4.9; Overnourished: 139.9 ± 15.1 grams, $p<0.05$) and triglycerides (Control: 147.0 ± 3.4; Overnourished: 168.7 ± 3.1 mg/dl, $p<0.001$) (table 1).

Mitochondrial respiratory capacity

Mitochondrial respiration can be measured when isolated mitochondria in the presence of substrates (state 2 or St2) and ADP (state 3 or St3) start the process of the phosphorylation (i.e ATP formation). After the phosphorylation of ADP, mitochondria respire slowly, which is kneed as resting respiration state (state 4 or St4) and when an uncoupler was added in this preparation, the maximum stimulated mitochondrial respiration (Vmax) can be measured. The respiratory control ratio (RCR) is the main indicator of the oxidative phosphorylation efficiency of the mitochondria, in another words, an index of how well these mitochondria coupling the substrate oxidation to ATP synthesis. In our study we observed that in overnourished group St2 was already higher than control group (Control: 6.75 ± 1.1;

Overnourished: 9.8 ± 1.2 nmols O2/mim/mg prot, $p<0.05$), associated to these results we observed a lower St3 in overnourished (Control: 55.3 ± 7.3; Overnourished: 34.6 ± 5.7 nmols O2/mim/mg prot, $p<0.05$). Associated with detrimental alterations in respiratory state, RCR was lower in overnourished group (Control: 7.7 ± 0.51; Overnourished: 4.4 ± 0.45, $p<0.001$). As expected, simulated I/R induce a marked decrease in all respiration state either in control and overnourished groups (figure 1).

Reactive species production and mitochondrial transmembrane potential

According to previous studies ROS production and mitochondrial ΔΨm, are tightly related and with mitochondrial respiration controls of the mitochondrion integrity[53,54]. Assessing these additional indicator of mitochondrial bioenergetics, we observed that the overnourished group produced 196.3% more ROS than control group (Control: 2.8 ± 0.46; Overnourished: 8.3 ± 0.5 a.u. of fluorescence, $p<0.001$, Fig. 2A), while the ΔΨm decreased 53.3% in overnourished group (Control: 286.7 ± 45.8; Overnourished: 133.8 ± 41.3 a.u. of fluorescence, Fig. 2B). In addition our results demonstrate that I/R injury increase ROS production (Control: 2.8 ± 0.46; Control+I/R: 5.8 ± 0.5 a.u. of fluorescence, $p<0.05$, Fig. 2A), while the ΔΨm decreased 64.4% in control group (Control: 278.9 ± 38.1; Control+I/R: 99.3 ± 18.9 a.u. of fluorescence, $p<0.05$, Fig. 2B)

Uncoupling protein 2 expression

Uncoupling proteins (UCPs) are transporters located in inner mitochondrial membrane that promote the proton transport from the inter-membrane space to the mitochondrial matrix dissipating proton gradient (Δ pH) [55]. Our data demonstrated that overnutrition during lactation decreases the mRNA levels of UCP2 (Control: 1.18 ± 0.15; Overnourished: 0.46 ± 0.11 UCP2/β2M ratio, $p<0.05$ Fig. 3), and also after ischemia (Control + IR: 1.0 ± 0.11; Overnourished + IR: 0.30 ± 0.08 UCP2/β2M ratio, $p<0.05$ Fig. 3). When the comparison is between control and overnourished + IR, the decrease in the UCP2 mRNA is around 75% ($p<0.01$, figure 3).

Oxidative stress biomarkers and antioxidant system

The excess of reactive species causes the oxidation of cellular phospholipids, proteins, and DNA; in some pathological conditions increased levels of reactive species production exceed the anti-oxidant defense system leading to oxidative stress [41,56]. Taken these in consideration, we next investigate whether overnutrition during lactation could induce

oxidative stress due also by decrease in antioxidant system. As can be observed in Figures 4-6, overnourished group showed an increase in protein oxidation (Control: 5.4 ± 0.5; Overnourished: 7.1 ± 0.4 µmols /mg protein, $p<0.05$, Fig. 4B), decrease in superoxide dismutase activity (Control: 11.7 ± 0.9; Overnourished: 6.1 ± 0.4 U/mg protein, $p<0.05$, Fig. 5A) and decrease in total intracellular thiols (Control: 100 ± 2.1; Overnourished: 85.3 ± 4.0 % of control, $p<0.05$, Fig. 6); whether the comparisons are between control and overnourished + I/R, the oxidative stress are more pronounced (MDA=Control: 83.5 ± 7.3; Overnourished: 122.5 ± 8.0 µmols /mg protein, $p<0.01$, Fig. 4A; Carbonyls= Control: 5.4 ± 0.5; Overnourished: 8.1 ± 0.5 µmols/mg protein, $p<0.001$, Fig. 4B; Total Thiols= Control: 100 ± 2.1; Overnourished: 85.4 ± 4.2 % of control, $p<0.001$, Fig.6).

DISCUSSION

To the best of our knowledge, this study was the first to investigate the effect of overnutrition during lactation and ischemia-reperfusion on mitochondrial bioenergetics, UCP 2 expression and oxidative status in rat heart at 60 days of age. Our data demonstrate that excess of food during development, alters cardiac bioenergetics and may contribute to the increase in the risk for cardiac injury in adulthood.

Overnutrition increases body weight since lactation and maintain until adulthood (60 days). These data corroborates with our previous study [16] and studies from other research groups [11,22,57,58]. In addition to the increase in body weight, overnutrition induces a significant alteration in glycaemia and triglycerides, which are comorbidities that can increase the risk for cardiovascular diseases[59]. As recently reported overnutrition during lactation, increase levels of plasmatic glucose, cholesterol and triglycerides that contribute to the impairment of glucose homeostasis and reduced insulin response, in addition to defect in insulin and fatty acids signaling pathway components, affecting cardiac metabolism [60].

Several studies have reported that metabolic alteration, in special metabolic syndrome, shows as a common characteristic an increase in oxidative stress in the heart, as a result of an overproduction of ROS and decrease in antioxidant defense [61-63]. With these in mind, we next evaluate mitochondrial respiration and membrane potential, since this two analyses tells us how couple are mitochondrial and indirectly suggest whether these mitochondrion are producing increased levels of ROS. Our results suggest impairment in mitochondrial respiratory capacity in the overnourished animals, corroborating with previous data from Fleischman et al. (2009) and Bakkman et al. (2010) where they demonstrates that

mitochondrial oxidative capacity was deteriorated in response to an obese model [64,65]. Additionally, overnourished group shows decrease in mitochondrial membrane electric potential (ΔΨm). Similar findings were reported by Turdi et al. (2013), which in addition to reduced ΔΨm there was a reduction in the transcriptional factors related to mitochondrial oxidative metabolism in animals that had exposed to overfeeding at postnatal period [66]. As observed, a reduced ΔΨm in overnourished group, 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 [67]. In addition to the ΔΨm increases in release of pro-apoptotic proteins, Starkov and Fiskum (2003), suggest that the decrease in mitochondrial membrane electric potential, can induce a deceleration in the electron flow through mitochondrial complexes which increases ROS generation[54].

According to Halliwell, oxidative stress is a condition where there are elevated levels of reactive oxygen species (ROS) and/or decrease in antioxidant system, results an increased pro-oxidant environment[41]. In our study we demonstrate that overnutrition and I/R injury increases ROS production, with these we further evaluate the biomarkers for oxidative stress and the antioxidant molecules. As showed in Figures 4-6 we observe that overnutrition and/or I/R injury significantly increases protein oxidation, decreases SOD activity and total intracellular thiols, all together suggests that the oxidative stress in early overnutrition is induced by the increase of pro-oxidant agents combined with a decrease in antioxidant molecules and I/R injury also negative modulates all these molecules.

In addition to antioxidant system as ROS scavengers, uncoupling proteins (UCPs) has emerged as an essential modulator of mitochondrial function. UCPs located in the inner membrane promote proton transport from the inter-membrane space to the mitochondrial matrix dissipating the proton gradient[68-70]. This UCP-regulated mild uncoupling plays an important physiological role to avoid oversupply of electrons into the electron transport chain adjusting energy metabolism and preventing excessive mitochondrial ROS generation and decrease the proton electrochemical gradient across the inner mitochondrial membrane regulating mitochondrial ROS production associated with various disorders, including obesity and cardiovascular diseases[68-70]. As showed in our results, early overnutrition deregulates UCP levels that may contribute to the increase in ROS overproduction and induction of oxidative stress, as a detrimental vicious cycle.

Taken together our data with the literature, we can suggest that overnutrition during lactation deregulates mitochondrial bioenergetics, potentiates oxidative stress condition and increasing the cardiac damage with or without an ischemic insult.

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TABLE

	Control	Overnourished
Body weigh	232.9 ± 3.2 g	274.3 ± 5.8 g***
Glycemia	93.2 ± 4.9 mg/dL	139.9 ± 15.1 mg/dL *
Triglyceride	147.0 ± 3.4 mg/dL	168.7 ± 3.2 mg/dL ***
Cholesterol total	83.2 ± 2.5 mg/dL	84.6 ± 2.7 mg/dL

TABLE AND FIGURE CAPTIONS

Table: Body weight and serum determinations in control rats (offsprings from dams that maintain 9 puppies until weaning) and overnourished rats, (offsprings from dams that had only 3 puppies until weaning). Values are expressed as mean ± SEM of eight rats per group. * $p < 0.05$, **$p < 0.01$; *** $p < 0.001$. g: grams; mg/dL: miligrams per deciliters.

Figure 1: Mitochondrial respiratory capacity in control and overnourished rat hearts in normoxia and after simulated ischemia-reperfusion. A) Basal respiratory capacity (State 2 respiration), B) Phosphorylation capacity (State 3 respiration), C) Resting state (State 4 repiration), D) Maximum respiratory capacity (Vmax), E) Respiratory control ratio (RCR). Control: white bar, Overnourished: black bar. Values are expressed as mean ± SEM of eight rats per group. * $p < 0.05$, *** $p < 0.001$.

Figure 2: Mitochondrial reactive species production (A) and mitochondrial membrane potential (B) in control and overnourished rat hearts in normoxia and after simulated

ischemia-reperfusion. Control: white bar, Overnourished: black bar. Values are expressed as mean ± SEM of eight rats per group. *p<0.05.

Figure 3: Uncoupling protein 2 (UCP2) gene expression in control and overnourished rat hearts in normoxia and after ischemia-reperfusion. Data are in relative expression ratio of UCP2/□2M mRNA in mean ± SEM of two separate experiments with three rats per group. * p <0.05; ** p <0.01.

Figure 4: Oxidative status in control and overnourished rat hearts in normoxia and after ischemia-reperfusion. A) Lipoperoxidation and B) Protein oxidation. Values are expressed as mean ± SEM of eight rats per group. Control: white bar, Overnourished: black bar. * p<0.05, ** p <0.01.

Figure 5: Enzymatic antioxidant system in control and overnourished rat hearts in normoxia and after ischemia-reperfusion. A) Superoxide Dismutase activity, B) Catalase activity, C) Glutathione s-transferase activity. Values are expressed as mean ± SEM of eight rats per group. Control: white bar, Overnourished: black bar. *p <0.05.

Figure 6: Total and protein-bound thiol group in control and overnourished rat hearts in normoxia and after ischemia-reperfusion. Values are expressed as mean ± SEM of eight rats per group. * p <0.05, ** p <0.01.

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FIGURES:

Figure 1:

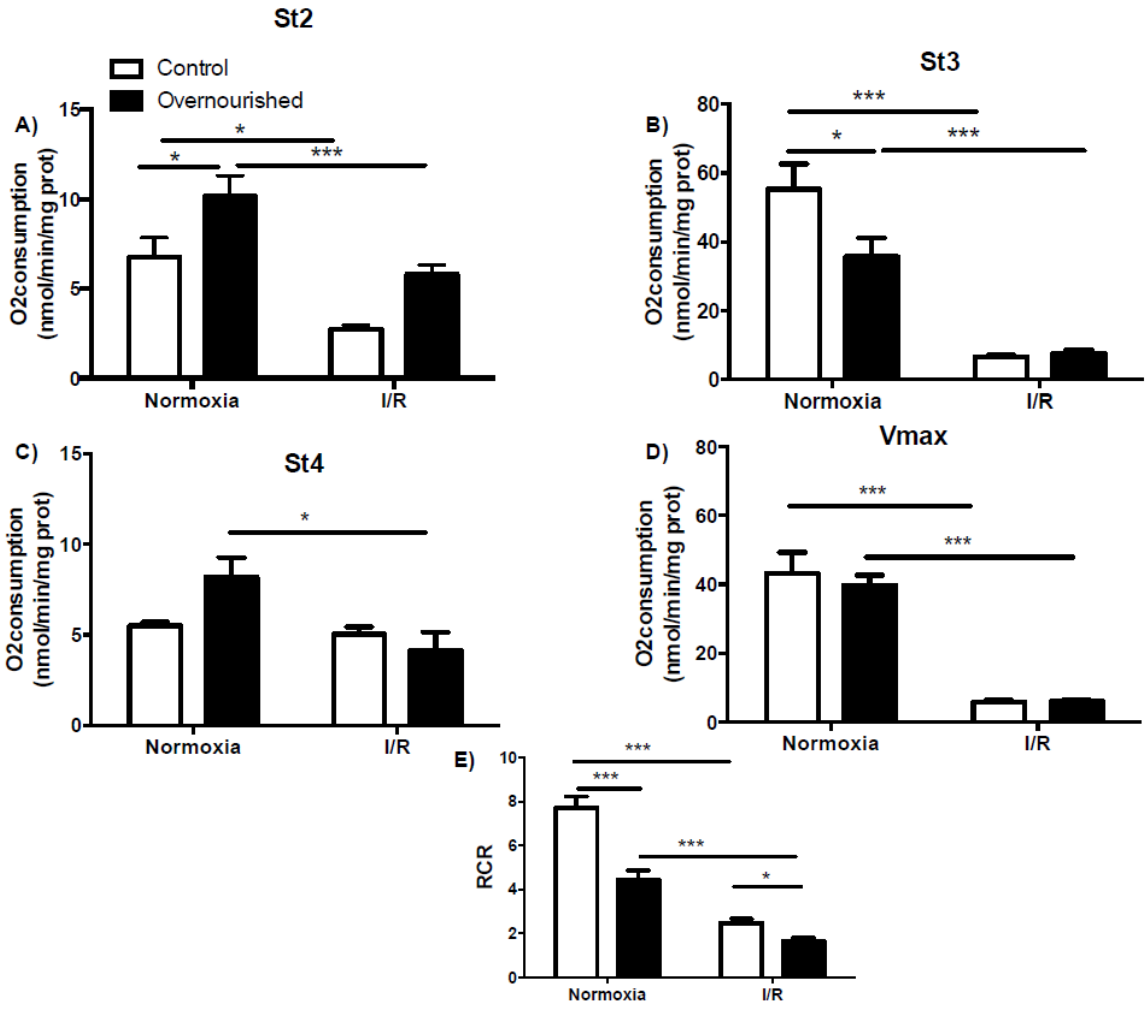
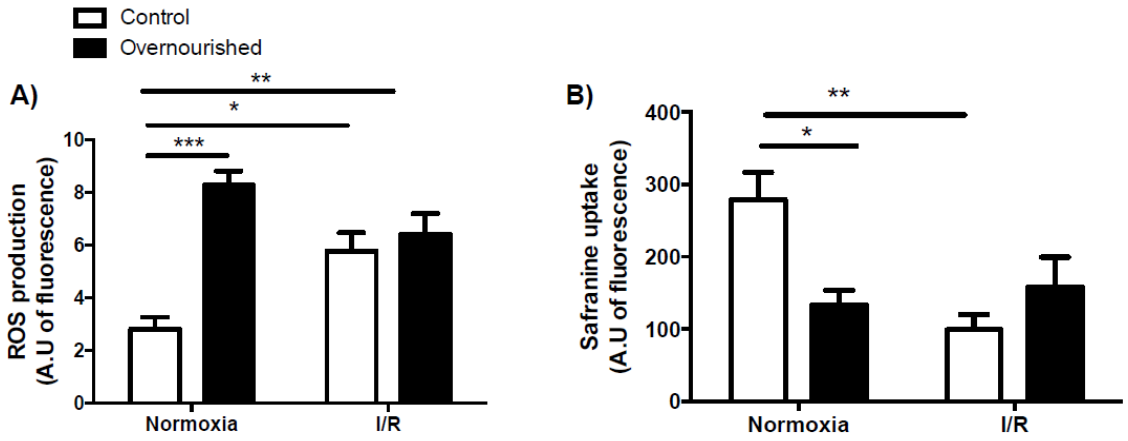

St2
Control
Overnourished
A)
O2consumption (nmol/min/mg prot)
Normoxia
I/R
St3
B)
St4
C)
Vmax
D)
E)
RCR

Figure 2:



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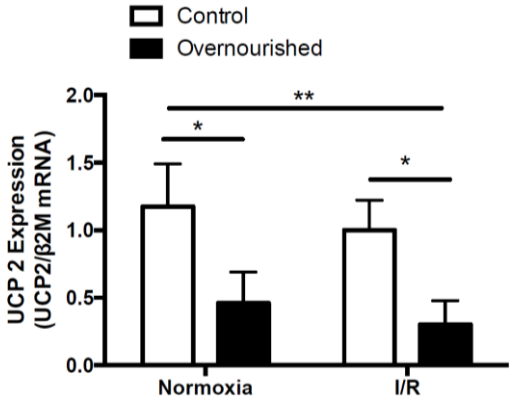

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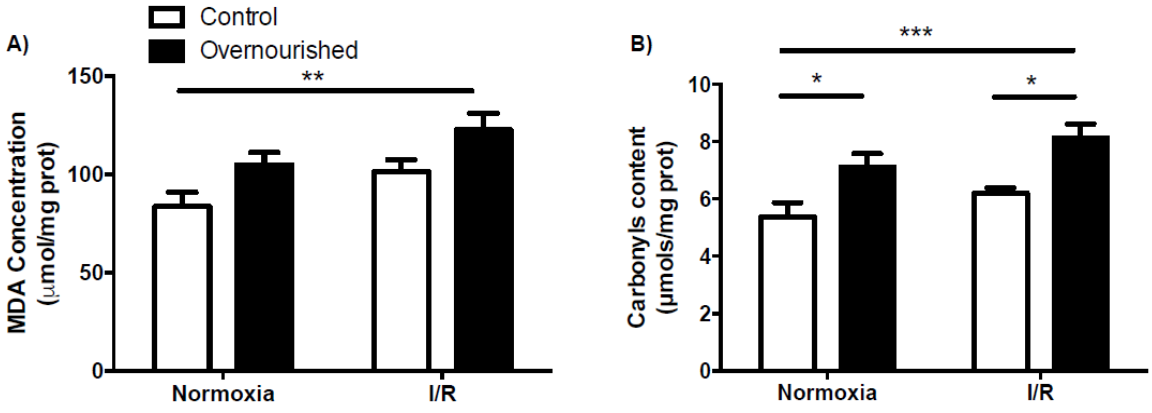

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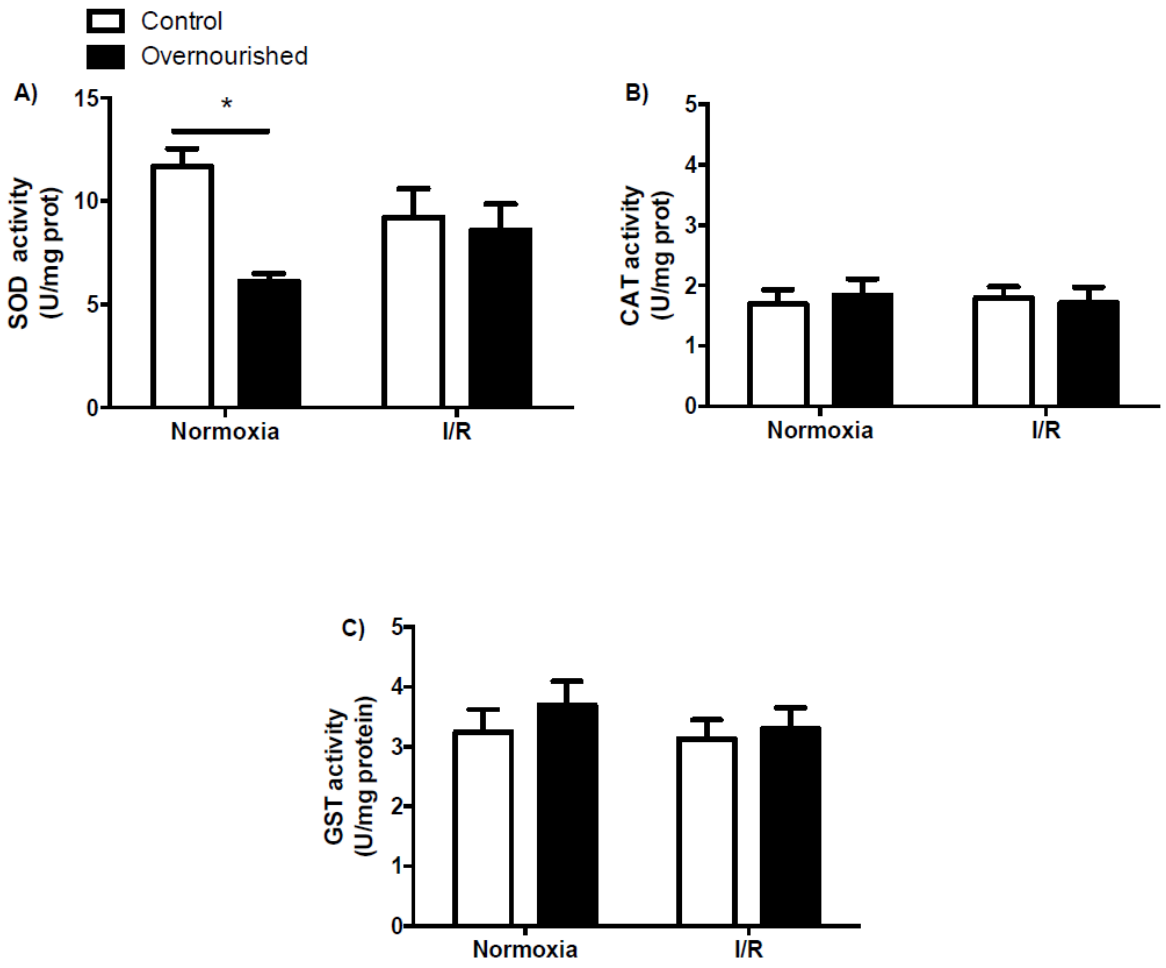

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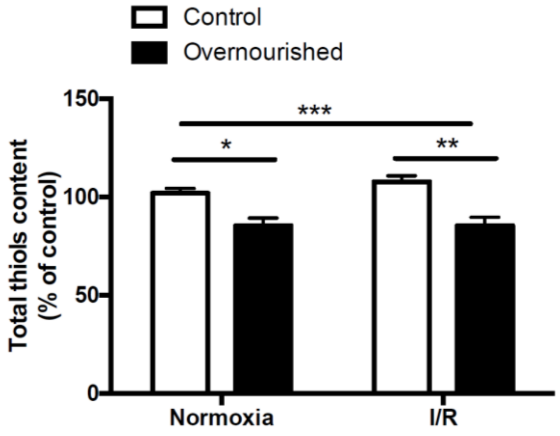

ANEXO I – Comitê de Ética

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

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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-Presidenta do CEUA/CCB-UFPE
SIAPE 2199635

CCB: Integrar para desenvolver

ANEXO II – Publicações no Período do Mestrado

1. Da Silva AI, Braz GRF, Nascimento L, Freitas CM, Pedroza A, Ferreira DS, Manhães-de-Castro R, Lagranha C. **Fluoxetine induces lean phenotype in rat by increasing the brown/white adipose tissue ratio and UCP1 expression.** Journal of Bioenergetics and Biomembranes 2015 Aug;47(4):309-18.

2. Braz GR, Pedroza AA, Nogueira VO, de Vasconcelos Barros MA, de Moura Freitas C, de Brito Alves JL, da Silva AI, Costa-Silva JH, Lagranha CJ. **Serotonin modulation in neonatal age does not impair cardiovascular physiology in adult female rats: Hemodynamics and oxidative stress analysis.** Life Sci. 2016 Jan; 145:42-50.

3. Braz GR, Freitas CM, Nascimento L, Pedroza AA, da Silva AI, Lagranha C. **Neonatal SSRI exposure improves mitochondrial function and antioxidant defense in rat heart.** ApplPhysiol, NutrMetab, 2016 Apr; 41(4):362-6.

4. Costa LR, Macêdo PC, Vasconcelos de Melo JS, Freitas CM, Alves AS, Barbosa HM, Lira E, Fernandes MP, Batista-de-Oliveira-Hornsby M, Lagranha C. **Safflower (Catharmustinctorius) oil supplementation in overnourished rats during early neonatal development: effects on heart and liver function in the adult.** Appl Physiol, Nutr Metab.2016 Dec;41(12):1271-1277.