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MONIQUE ASSIS DE VASCONCELOS BARROS

REPERCUSSÕES DA DESNUTRIÇÃO PROTEICA MATERNA SOBRE OS PARÂMETROS CARDIORRESPIRATÓRIOS EM RATOS: PARTICIPAÇÃO DO SISTEMA RENINA ANGIOTENSINA

RECIFE

2018

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

Área de concentração: Neurociências

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Prof. Dr. João Henrique da Costa Silva

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BANCA EXAMINADORA

_____

Prof. Dr. David Felipe de Santana
Universidade Federal de Pernambuco – UFPE

_____

Profa. Dra. Ana Durce de Oliveira Paixão
Universidade Federal de Pernambuco - UFPE

_____

Prof. Dra. Alice Valença Araújo
Universidade Federal de Pernambuco - UFPE

_____

Prof. Dr. Danilo Augusto Ferreira Fontes
Universidade Federal de Parnambuco – UFPE

_____

Prof. Dr. João Henrique da Costa Silva
Universidade Federal de Pernambuco - UFPE

Com todo meu amor e gratidão, dedico este trabalho aos meus pais:

Célio Meira Neto de Vasconcelos Barros
e
Lenice Regina Assis de Vasconcelos Barros

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“Se chorei ou se sorri, o importante é que emoções eu vivi...”

(CARLOS, R.; CARLOS, E. 1981).

RESUMO

No presente estudo investigamos o envolvimento do sistema renina angiotensina (RAS) sobre o controle cardiorrespiratório na hipertensão arterial induzida pelo déficit de proteínas durante a gestação e lactação. Ratos Wistar machos foram divididos em dois grupos de acordo com as dietas de suas mães durante a gestação e a lactação: grupo controle (grupo NP, 17% caseína) e baixa proteína (grupo LP, 8% caseína). Medida direta da pressão arterial (PA), frequência cardíaca e parâmetros ventilatórios foram registradas em animais acordados com 90 dias de vida durante o período basal e após antagonismo de receptores de angiotensina tipo 1 (AT1) [por losartan potássio, 10 mg/kg, intravenosa (iv) ou infusão intracerebroventricular (icv)] ou inibição da enzima conversora de angiotensina (ECA) por enalapril (5 mg/kg, iv). A variabilidade cardiovascular foi avaliada offline por análise espectral. Além disso, hipóxia citotóxica [injeção em bolus de cianeto de potássio (KCN); 0,04%] foi utilizado para ativar a rede neural cardiorrespiratória. Dosagem sérica de angiotensina II (Ang II) foi realizada por ensaio ELISA. Em nível transcripcional, os componentes de SRA no tronco cerebral foram avaliados por rt-PCR. A prole LP apresentou maior PA média (PAM) e frequência respiratória (FR) do que NP. Na análise espectral, o grupo de LP apresentou aumento das oscilações de baixa frequência (NP: 2,7 ± 0,3 vs. LP: 5 ± 1 mmHg²) da PA sistólica. Contudo, uma relação LF/HF semelhante ao grupo NP. Após o bloqueio dos receptores AT1, os animais LP apresentaram maior delta na PAM (NP: -9,8 ± 2 vs. LP: -23 ± 6 mmHg) e após ativação dos quimiorreceptores, quando comparado com o grupo NP (NP: + 68 ± 8 vs LP: + 98 ± 7 mmHg) e quando comparado com o período basal. A PAM não apresentou alterações significativas em NP e LP após o bloqueio central dos receptores AT1. No entanto, o delta FR foi maior em ambos os grupos quando comparado com o período basal. Não houve alteração nos níveis séricos Ang II entre os grupos NP e LP. Com relação a expressão gênica, não houve alteração na expressão de angiotensinogênio, Ace, At1, At2 e Mas entre os grupos. No entanto, os animais LP demonstraram uma diminuição no mRNA RAc1 no tronco cerebral em comparação com animais NP. O SRA parece participar perifericamente e não centralmente na manutenção da hipertensão arterial induzida por restrição proteica durante a gestação e lactação, independentemente dos níveis de Ang II circulante.

Palavras-chave: Desnutrição proteica. Angiotensina II. Hipertensão arterial.

ABSTRACT

In the present study, we investigate the involvement of renin angiotensin system (RAS) on cardiorespiratory control in the hypertension elicited by maternal low protein diet. Male Wistar rats were divided into two groups according to the diets of their mothers during gestation and lactation: the control (NP Group, 17% casein) and low-protein (LP Group, 8% casein) groups. Direct measurements of arterial pressure (AP), heart rate and ventilatory parameters were recorded from wakeful 90-d-old male offspring at resting and after antagonism of type 1 angiotensin (AT1) receptors [by losartan potassium, 10 mg/kg, intravenous (iv) or intracerebroventricular (icv) infusion] or inhibition of angiotensin converting enzyme (ACE) by enalapril (5 mg/kg, iv). Cardiovascular variability was evaluated off-line by spectral analysis. Besides cytotoxic hypoxia [bolus injection of potassium cyanide (KCN); 0,04%] was used for activating cardiorespiratory neural network. Serum dosage of angiotensin II (Ang II) was performed by ELISA assay. At transcriptional level, RAS components in the brainstem was evaluated by rt-PCR. The LP offspring presented higher mean AP (MAP) and respiratory frequency than NP. In the spectral analysis, the LP group showed higher power at low (NP: 2.7±0.3 vs. LP: 5±1 $mmHg^2$) frequency of systolic AP. However, similar LF/HF ratio. After losartan, the LP animals showed larger delta in the MAP (NP: -9.8±2 vs. LP: -23±6 mmHg), and also bigger delta MAP after KCN when compared with NP group (NP: +68±8 vs. LP: +98±7 mmHg) and when compared with baseline. MAP have no significant change in both NP and LP after central blockade of AT1 receptors. However, the RF delta was bigger in both groups when compared with before drug period. Was not change at levels of serum Ang II between NP and LP groups. Similar gene expression of angiotensinogen, Ace, At1, At2 and Mas receptors was observed in the groups. However, LP animals demonstrated a decrease in RAc1 mRNA in the brainstem compared to NP animals. The RAS seems to participate peripherally and not centrally in the maintenance of arterial hypertension induced by protein restriction during the gestation and lactation, regardless of the levels of circulating Ang II.

Keywords: Protein malnutrition. Angiotensin II. Arterial hypertension.

LISTA DE ABREVIATURAS E SIGLAS

HAS Hipertensão Arterial Sistêmica

COBEA Colégio Brasileiro de Experimentação Animal

CEUA Comitê de Ética em Utilização Animal

AIN Do inglês, *American Institute of Nutrition*

UFPE Universidade Federal de Pernambuco

CAV Centro Acadêmico de Vitória

EPM Erro Padrão da Média

NP Normoproteico

HP Hipoproteico

PA Pressão Arterial

PAP Pressão Arterial Pulsátil

PAM Pressão Arterial Média

FR Frequência Respiratória

FC Frequência Cardíaca

VLF Do inglês, *Very Low Frequency*

LF Do inglês, *Low Frequency*

HF Do inglês, *High Frequency*

PAS Pressão Arterial Sistólica

IP Intervalo de Pulso

UN Unidades Normalizadas

RT-PCR Do inglês, *reverse transcription-polymerase chain reaction*

SRA Sistema Renina Angiotensina

AT1 Receptor de angiotensina tipo 1

AT2 Receptor de angiotensina tipo 2

ECA Enzima conversora de angiotensina

AGTR1A Do inglês, *type 1a angiotensin II receptor*

AGTR2 Do inglês, *type 2 angiotensin II receptor*

AGT Do inglês, *angiotensinogen*

ACE Do inglês, *angiotensin I converting enzyme*

PRKCG Do inglês, *gamma protein kinase C*

RAC1 Do inglês, *ras-related C3 botulinum toxin substrate 1*

PTPN6 Do inglês, *protein tyrosine phosphatase, non-receptor type 6*

MAS1 Do inglês, *proto-oncogene, G protein-coupled receptor*

RPL19 Do inglês, *ribosomal protein L19*

ACTB Do inglês, *actin beta*

MAPK Do inglês, *mitogen-activated protein kinase*

NADPH Do inglês, *nicotinamide adenine dinucleotide phosphatase*

WBC Do inglês, *White blood cells*

RBC Do inglês, *Red blood cells*

RDW CV Do inglês, *Red blood cell distribution width – coefficient of variation*

MCV Do inglês, *Mean corpuscular volume*

MCH Do inglês, *Mean corpuscular hemoglobin*

MCHC Do inglês, *Mean corpuscular hemoglobin concentration*

LYM Do inglês, *Lymphocytes*

LYM ABS Do inglês, *Lymphocytes absolute value*

MPV Do inglês, *Mean platelet volume*

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SUMÁRIO

1 INTRODUÇÃO

A hipertensão arterial é uma condição clínica de natureza multifatorial e caracterizada por elevados níveis de pressão arterial (PA). Associa-se frequentemente a alterações funcionais e/ou estruturais dos órgãos-alvo às alterações metabólicas, com consequente aumento do risco de eventos cardiovasculares (Sociedade Brasileira de Cardiologia, 2006; Malta 2008). Essa patologia afeta mais de 80 milhões de americanos, além de outros milhões no mundo inteiro, permanecendo um grande desafio para a saúde pública mundial (Mozaffarian, Benjamin et al. 2016). A partir de estudos de 154 países que incluíram 8,69 milhões participantes, estima-se que, entre 1990 e 2015 a taxa de pressão arterial sistólica (SBP) de pelo menos 110 para 115 mmHg aumentou de 73.119 para 81.373 por 100.000 pessoas e SBP de 140 mmHg ou superior aumentaram de 17.307 para 20.526 por 100.000 pessoas. Dessa forma, a taxa anual estimada de mortes associadas a SBP de pelo menos 110 a 115 mmHg aumentou de 135,6 para 145,2 por 100.000 pessoas e para a SBP de 140 mmHg ou superior aumentou de 97,9 para 106,3 por 100.000 pessoas. Portanto, nos últimos 25 anos, o número de indivíduos com níveis mundiais de SBP de pelo menos 110 a 115 mmHg e de 140 mmHg ou mais e as mortes associadas estimadas aumentaram substancialmente (Forouzanfar, Liu et al. 2017).

De acordo com as diretrizes de hipertensão da Associação Americana do Coração recentemente publicada, a classificação da PA como normal permanece igual, com PA sistólica <120 mmHg e PA diastólica <80 mmHg). Entretanto, essa diretriz de 2017 substituiu o termo "pré-hipertensão" por "pressão elevada" (PA sistólica de 120 a 129 mmHg e PA diastólica <80 mmHg) e "hipertensão de estágio 1" (PA sistólica de 130 a 139 mm Hg ou PA diastólica de 80 a 89 mmHg). A extremidade superior da pré-hipertensão foi reclassificada como hipertensão do estágio 1 porque os adultos com PA nesta faixa apresentam um aumento de aproximadamente duas vezes no risco de doenças cardiovasculares (DCV) em comparação com adultos com PA normal. Já a hipertensão de estágio 2 ficou definida como uma PA sistólica de pelo menos 140 mmHg ou uma PA diastólica de pelo menos 90 mmHg em vez de uma PA de pelo menos 160/100 mmHg (Carey and Whelton 2018).

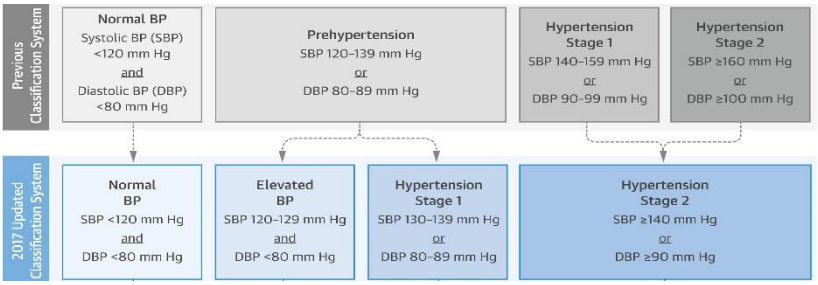

Figura 1: Classificação atualizada da pressão arterial em adultos. Adaptado de P.K. Whelton, R.M. Carey, et al. 2017.

Embora elevados níveis pressóricos seja um cenário comum do mundo industrializado, existe uma crescente consciência da ubiquidade da hipertensão em países de baixa e média renda (Lackland and Weber 2015), chegando a superar a desnutrição infantil como o principal fator de risco para a carga global de doenças, entre os anos de 1990 e 2010 (Lim, Vos et al. 2012). Portanto, estima-se que 29% da população adulta do mundo ou aproximadamente 1,55 bilhões de pessoas terão hipertensão até o ano de 2025 (Kearney et al., 2005). Devido a sua natureza multifatorial, na qual fatores genéticos e ambientais podem predispor o seu desenvolvimento, a causa subjacente da hipertensão arterial torna-se difícil de ser identificada (Hedner, Kjeldsen et al. 2012). No entanto, apesar dos fatores genéticos serem fortemente associados ao desenvolvimento de doenças e determinarem o grau de susceptibilidade individual, recentemente têm sido referenciados que os fatores ambientais são os principais responsáveis pelo aparecimento de doenças, tal como, a hipertensão arterial (Xiao, Zhang et al. 2010).

Nesse sentido, estudos têm reportado que eventos adversos experimentados no útero ou durante o período perinatal (gestação, lactação e primeira infância) podem afetar o desenvolvimento de sistemas fisiológicos e aumentar a predisposição de hipertensão arterial e doenças metabólicas na vida adulta (Barker, Bull et al. 1990; Gluckman and Hanson 2004). O fenômeno biológico subjacente a essa associação é denominado “plasticidade fenotípica”, e refere-se à capacidade de um fenótipo associado a um único genótipo produzir variações no desenvolvimento dos organismos em resposta às circunstâncias ambientais, em termos de comportamento, morfologia e/ou fisiologia (West-Eberhard 1986). Essa plasticidade fenotípica tem sido reconhecida como uma estratégia chave que possibilita o organismo responder às variações ambientais (Brasdshaw 1965). Uma das variações mais bem

documentadas no estudo da plasticidade fenotípica é a nutrição, onde a falta ou o aumento do aporte nutricional durante períodos críticos do desenvolvimento podem resultar em alterações permanentes na estrutura e função de órgãos e predispor o desenvolvimento de doenças não comunicantes (West-Eberhard 1986; Leandro, da Silva Ribeiro et al. 2012; Fidalgo, Falcao-Tebas et al. 2013; de Brito Alves, Nogueira et al. 2014; Barros, De Brito Alves et al. 2015; de Brito Alves, de Oliveira et al. 2016; de Brito Alves and Costa-Silva 2017).

O marco conceitual dessa relação entre insultos ambientais durante o período perinatal e o surgimento de doenças na vida adulta, foi um artigo publicado por Hales e Barker em 1992, onde através de achados epidemiológicos demonstrou uma estreita associação do baixo peso ao nascer com o risco de doenças na vida adulta. A hipótese dos pesquisadores foi chamada de "*Thrifty Phenotype" Hypothesis* e defendia que o neonato com baixo peso representava um "fenótipo de sobrevivência", com um número de características que aumenta sua probabilidade de sobrevivência após uma deficiente experiência nutricional no útero (Hales and Barker 2013). A partir de então, essa hipótese, onde em curto prazo, as adaptações fenotípicas ajudam à sobrevivência, mas em longo-prazo, suscetibilizam o organismo ao aparecimento de distúrbios metabólicos, tem sido amplamente utilizada para investigar o risco de desenvolvimento de doenças.

Uma gama de estudos epidemiológicos e intervenções dietéticas em modelos animais tem fornecido considerável evidência para sugerir que um desbalanço nutricional materno no período crítico do desenvolvimento fetal, pode ter uma persistente e intergeracional efeitos sobre a saúde da prole e sobre o risco para o desenvolvimento de doenças tal como obesidade, diabetes, doenças cardiovasculares e hipertensão arterial (Adair and Cole 2003; Bhargava, Sachdev et al. 2004; Szarc vel Szic, Ndlovu et al. 2010). Sabe-se que esse período crítico se caracteriza por um intenso e complexo processo organizacional e representa uma etapa crucial no desenvolvimento fetal, que não pode ser reajustada posteriormente (Morgane, Austin-LaFrance et al. 1993). Assim, a interação desse período com fatores externos, a exemplo de déficit nutricional, influencia as vias de sinalização desse desenvolvimento e pode culminar com anormalidades no desenvolvimento de órgãos (Hyatt, Budge et al. 2008).

Dessa forma, o surgimento da hipertensão arterial tem sido amplamente demonstrado em estudos com humanos nascidos com baixo peso (Ravelli, Stein et al. 1976; Hales, Barker et al. 1991; Sawaya and Roberts 2003; Sawaya, Martins et al. 2004), bem como em roedores adultos, submetidos à desnutrição proteica (5 a 8% de proteína) durante o período perinatal

(Langley-Evans, Welham et al. 1999; Costa-Silva, Silva et al. 2009; de Brito Alves, Nogueira et al. 2014). No entanto, apesar da evidente relação da desnutrição com o aumento da incidência da hipertensão arterial, os mecanismos envolvidos no desenvolvimento dessa hipertensão ainda permanecem a ser melhor esclarecidos.

Sabe-se que o controle cardiovascular envolve ativação de sistemas de feedback, os quais operam em curto e longo prazo (Shepherd and Mancia 1986; Dampney 1994). Os mecanismos de regulação em curto prazo envolvem os reflexos cardiovasculares, onde a informação detectada através de receptores específicos é processada no sistema nervoso central e retorna para a periferia através das subdivisões do sistema nervoso autônomo eferente: o sistema nervoso simpático e o sistema nervoso parassimpático, para manutenção da homeostase (Machado, Mauad et al. 1997).

Estudos em modelos animais de hipertensão bem como em pacientes hipertensos tem amplamente destacado a hiperativação do sistema nervoso simpático como um dos principais fatores indutores do desenvolvimento da hipertensão (Grassi 1998; Simms, Paton et al. 2009). Essa hiperativação é caracterizada por um aumento na intensidade e na frequência das despolarizações elétricas do nervo simpático e também por um aumento nos níveis plasmáticos de catecolaminas promovendo constrição dos vasos sanguíneos periféricos, aumento na resistência vascular periférica e, consequentemente aumento nos níveis basais da pressão arterial (Malpas 1998; Zoccal, Bonagamba et al. 2009). Assim, uma desinibição ou ativação dos neurônios geradores da atividade simpática localizados na medula ventro lateral rostral (RVLM), pode induzir aumento nos níveis da atividade simpática (Bugenhagen, Cowley et al. 2010; Heusser, Tank et al. 2010).

Nesse sentido, estudo do nosso laboratório previamente demonstrou que a prole adulta de mães expostas a restrição de proteínas durante a gestação e lactação exibem maior tônus simpático cardíaco após antagonismos de receptores b-adrenérgicos, sem alteração no tônus parassimpático após antagonismo de receptores muscarínicos. Associado a isso, esses animais também apresentam um maior tônus simpático após bloqueio ganglionar com hexametônio. Esse imbalanço autonômico com predominância da modulação simpática vasomotora e cardíaca em animais desnutridos durante o período perinatal suporta a hipótese que a hiperatividade do sistema nervoso simpático pode ser associada ao desenvolvimento da hipertensão arterial nesses animais (Barros, De Brito Alves et al. 2015). Interessantemente, essa hiperatividade simpática também foi observada em curto prazo (30 dias de vida) nos

animais submetidos à desnutrição proteica durante os períodos de gestação e lactação. Ou seja, antes do estabelecimento da hipertensão arterial nesses animais (de Brito Alves, Nogueira et al. 2015).

É bem descrito na literatura que disfunções no mecanismo baroreflexo podem levar a uma hiperatividade simpática e consequente desenvolvimento da hipertensão arterial (Souza, Ballejo et al. 2001; Heusser, Tank et al. 2010; Tsyrlin, Galagudza et al. 2013). Para testar essa hipótese nosso grupo de pesquisa analisou o baroreflexo espontâneo, o induzido e a atividade direta do nervo simpático lombar em animais hipertensos adultos que foram desnutridos durante a gestação e lactação. No entanto, não foi evidenciada qualquer alteração desse mecanismo reflexo, indicando que a hipertensão arterial apresentada na prole adulta não está relacionada com disfunção no mecanismo barorreflexo nesses animais (Paulino-Silva and Costa-Silva 2016). Por outro lado, associado a hiperatividade simpática, foi evidenciado que esses animais desnutridos precocemente também exibem em curto prazo importantes modificações no ritmo respiratório, elucidado por um aumento de frequência respiratória e ventilação pulmonar durante o período basal, bem como um aumento da sensibilidade de quimiorreceptores periféricos (de Brito Alves, Nogueira et al. 2014; de Brito Alves, Nogueira et al. 2015). Além disso, nós também observamos que a remoção do corpúsculo carotídeo é capaz de normalizar a frequência respiratória e respostas ao CO_2 em curto prazo, bem como melhora a pressão arterial e hiperatividade simpática em longo prazo, sugerindo que a hipersensibilidade desses receptores pode estar envolvida no desenvolvimento da hipertensão arterial induzida pela desnutrição proteica materna (dados não publicados).

Controle respiratório, hiperatividade simpática e hipertensão arterial

Já tem sido bem descrito que o sistema respiratório pode modular marcadamente a descarga do nervo simpático (Haselton and Guyenet 1989; Malpas 1998; Dick, Hsieh et al. 2004; Zoccal, Simms et al. 2008). Além disso, em estudos com diferentes modelos de hipertensão, já foi demonstrado que alterações na geração ou modulação da função respiratória podem contribuir para o desenvolvimento da hipertensão arterial (Simms, Paton et al. 2009; Simms, Paton et al. 2010; Costa-Silva, Zoccal et al. 2012; Moraes, Bonagamba et al. 2014). Centralmente, essa modulação simpática é realizada por neurônios conhecidos como

"respiratórios" altamente especializados e localizados na região ventral do tronco cerebral (Costa-Silva, Silva et al. 2009; Costa-Silva, Zoccal et al. 2010; Costa-Silva, Zoccal et al. 2012; Moraes, Bonagamba et al. 2014). Esses neurônios são essenciais para geração do ritmo respiratório e suas atividades coordenadas são responsáveis pela manutenção da dinâmica do ciclo respiratório (Bianchi, Denavit-Saubie et al. 1995; Smith, Abdala et al. 2007). Para tal, se faz necessário que essas células neuronais recebam informações precisas e refinadas dos valores arteriais de pressão parcial de oxigênio (PO_2) e de dióxido de carbono (PCO_2) para que ajustes sobre ritmo e amplitude respiratória sejam realizados no intuito de manter a homeostase gasométrica. Os órgãos sensoriais responsáveis por este controle homeostático são os quimiorreceptores periféricos e centrais, estruturas amplamente especializadas, sensível a alterações químicas no sangue (Feldman, Mitchell et al. 2003; Guyenet, Stornetta et al. 2010).

O corpo carotídeo, uma massa tecidual elipsoide localizada na bifurcação das artérias carótidas é o principal quimiorreceptor arterial periférico. Em condições normais essas células monitoram continuamente os níveis de PO_2, PCO_2 e pH plasmático. Em situações onde ocorrem alterações drásticas desses parâmetros como na hipóxia (redução na PO_2), hipercapnia (aumento na PCO_2) e acidose (aumento na concentração de H^+), essas células são estimuladas e promove a liberação de neurotransmissores que determinam a geração dos potenciais de ação nas fibras sensoriais do nervo do seio carotídeo (Fidone, Gonzalez et al. 1988; Gonzalez, Almaraz et al. 1994; Iturriaga and Alcayaga 2004). Esses potenciais de ação ascendem ao tronco cerebral, onde ocorre em diferentes subnúcleos o processamento central das informações provenientes dos quimiorreceptores. A partir destas informações, são gerados os comandos que levarão aos ajustes autonômicos e respiratórios necessários para o retorno aos níveis adequados (Mifflin 1992; Ciriello, Schultz et al. 1994). Ou seja, a ativação desses quimiorreceptores produz uma poderosa ativação da rede neuronal cardiorrespiratória e aumenta a descarga simpática e drive respiratório, os quais são essenciais para estabilidade ventilatória e cardiovascular (Costa-Silva, Zoccal et al. 2010; Costa-Silva, Zoccal et al. 2012; Moraes, Bonagamba et al. 2014).

Nesse sentido, a função dos quimiorreceptores do corpo carotídeo na fisiopatologia das doenças cardiovasculares tem tido bastante destaque (Braga, Soriano et al. 2006; Abdala, McBryde et al. 2012; Costa-Silva, Zoccal et al. 2012). Estudos tem demonstrado que disfunções induzidas pela plasticidade fenotípica no início da vida podem induzir alterações ventilatórias e autonômicas (Nanduri and Prabhakar 2015; Prabhakar, Peng et al. 2015). Em

modelo de desnutrição pós-desmame já foi observado um aumento da resposta cardiovascular à ativação do quimiorreflexo (Penitente, Fernandes et al. 2007) e mais recentemente, em modelo de desnutrição durante a gestação e lactação, animais apresentaram disfunções respiratória associadas com uma hiperatividade simpática e maior sensibilidade dos corpos carotídeos à hipóxia (de Brito Alves, Nogueira et al. 2014; de Brito Alves, Nogueira et al. 2015). Assim, esses dados fortemente sugere a participação do corpo carotídeo nas alterações cardiorrespiratórias observadas nos animais desnutridos precocemente.

Além disso, a presença dos componentes do SRA no corpo carotídeo e sua influência sobre a quimiossensibilidade já tem sido bastante documentada em diferentes modelos experimentais (Heitman and Jennings 1998; Leung, Lam et al. 2000; Lam and Leung 2002; Lam and Leung 2003; Marcus, Li et al. 2010; Peng, Raghuraman et al. 2011; Fung 2014). Os estudos têm preconizado que os receptores de angiotensina tipo 1 (AT1) estão presentes na maioria das células que compõem o corpo carotídeo, os quais permitem que angiotensina II (Ang II) exerça um poderoso efeito na regulação da excitabilidade dos quimiorreceptores (Lam and Leung 2003). De fato, em animais submetidos à hipóxia crônica intermitente, a alta expressão de receptores AT1 no corpo corotídeo induziu uma hipersensibilidade do quimiorreflexo arterial (Leung, Lam et al. 2000; Lam and Leung 2003). Além disso, como resultado da hiper-regulação desse sistema, os níveis de cálcio intracelular mobilizado pela Ang II nas células quimiossensíveis tipo 1 foi aumentado, e em seguida, abolido pelo antagonismo dos receptores AT1. Assim, um sistema local de geração da Ang II está presente no corpo carotídeo de ratos e uma hiper-regulação desse sistema pode atuar aumentando os níveis de atividade simpática (Murali, Zhang et al. 2014), o que demonstra um importante papel do SRA nesse cenário.

Sistema renina angiotensina, hipertensão arterial e desnutrição

O SRA é um dos mais antigos sistemas hormonais e sua descoberta foi um dos achados mais importantes da história da fisiologia (Goldblatt, Lynch et al. 1934). Esse sistema compreende um número de vias enzimáticas e componentes bioativos que estão relacionadas com diversas ações funcionais. O SRA é classicamente definido pela atividade da enzima conversora de angiotensina (ECA) para formar a Ang II e a subsequente ativação do receptor

AT1 para mediar ambos mecanismos centrais e periféricos na regulação da pressão arterial. A Ang II também se liga a outro receptor, o tipo 2 (AT2), descoberto há mais de vinte anos. A ativação desse receptor tem sido reportada como possuindo efeitos opostos ao dos receptores AT1 (de Gasparo, Catt et al. 2000; Johren, Dendorfer et al. 2004; Hernandez Schulman, Zhou et al. 2007; Koid and Campbell 2013). Portanto, a Ang II exerce seus efeitos por se ligar aos seus dois maiores subtipos de receptores, AT1 e AT2, em órgãos ou tecidos alvos. No entanto, novos componentes do SRA e novos mecanismos continuam a ser descobertos com regularidade (Figura 2).

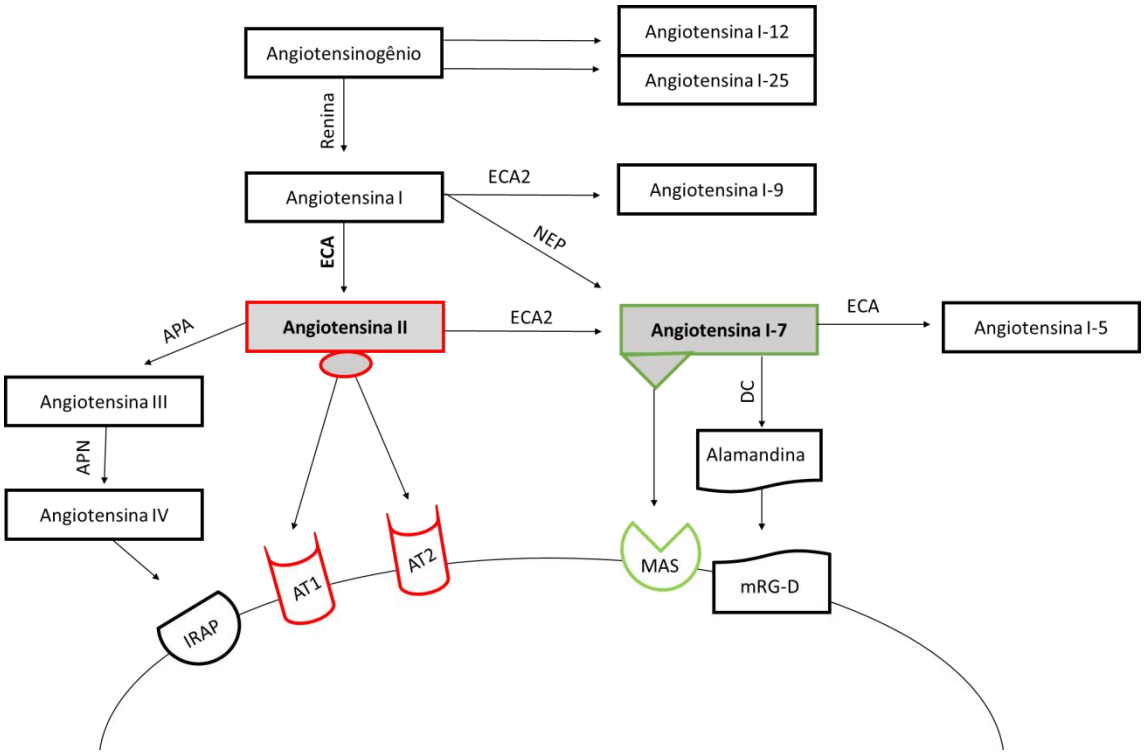

Figura 2: Cascata do sistema renina angiotensina. A renina cliva o angiotensinogênio em angiotensina I, o qual é convertido para o peptídeo biologicamente ativo angiotensina (Ang) II pela enzima conversora de angiotensina (ECA), Ang 1-7 pelo neprilisina e Ang 1-9 pela ECA2. Ang II sofre processamento adicional pela ECA2 para produzir a Ang 1-7 e pela aminopeptidase A (APA) para formar a Ang III. Ang 1-7 é metabolizado pela ECA para formar a Ang 1-5 e a Ang III é ainda hidrolisado pela aminopeptidase N (APN) para produzir a Ang IV. Os novos peptídeos de Ang 1-12 e Ang 1-25 podem ser derivados diretamente do angiotensinogênio. Ang II e Ang III reconhece os receptores AT1 e AT2, enquanto que a Ang 1-9 interage com o receptor AT2. Ang 1-7 e Alamandina reconhecem os receptores Mas e o *receptor* acoplado à proteína G relacionado ao Mas, membro D (mRG-D). ANG IV reconhece a aminopeptidase regulada pela insulina (IRAP). DC, descarboxilase de ácido aspártico. Adaptado de Chappel 2016.

Em nível central, o SRA desempenha um importante papel na regulação da pressão arterial através de sua capacidade de modular a atividade do nervo simpático, especialmente em áreas do cérebro envolvidas na geração e modulação das descargas simpáticas para o sistema cardiovascular (Dampney, Tan et al. 2007). As vias simpatoexcitatórias angiotensinérgicas exercem um papel chave na regulação crônica do tônus simpático e, portanto, um aumento de ativação angiotensinérgica central pode aumentar a ativação da atividade simpática e contribuir para o desenvolvimento da hipertensão arterial (Leenen 2014).

Em estudo com modelo de hipertensão, foi observado que o aumento da atividade do nervo simpático está envolvido com a hiperregulação de receptores AT1 na região do RVLM (Nunes and Braga 2011). Além disso, estudos têm demostrado que sinais da Ang II, incluindo a expressão de receptores AT1 e sua sensibilidade na área do RVLM é hiperregulada em modelos de hipertensão, sugerindo que o aumento de sinalização de Ang II no RVLM é crítico no desenvolvimento e manutenção da hipertensão (Dupont and Brouwers 2010). Também tem sido evidenciado que injeções de antagonista de receptores AT1 dentro dessa região central diminui a pressão arterial em ~14 a 35 mmHg em diferentes modelos animais de hipertensão arterial (Fontes, Baltatu et al. 2000; Ito, Komatsu et al. 2002; Ito, Hiratsuka et al. 2003).

É importante destacar que a Ang II não cruza a barreira hemato encefálica (BHE) e todos os componentes do SRA estão presentes dentro no cérebro, o que permite a sua formação local e utilização como um neurotransmissor central. As suas ações são predominatemente mediadas pela interação com os receptores AT1, os quais estão majoritariamente localizados nos núcleos centrais dentro da BHE, como o núcleo paraventricular do hipotálamo (PVN), núcleo do trato solitário (NTS) e RVLM, o que indica que neurônios nestas regiões podem ser influenciados por Ang II endógena que é formada

dentro do cérebro (Mendelsohn, Quirion et al. 1984; McKinley, McAllen et al. 1996). No entanto, já tem sido demosntrado que os órgãos sensoriais circunventriculares, principalmente o órgão subfornical (OSF) também possui uma alta densidade de receptores AT1 (Mendelsohn, Quirion et al. 1984), sendo então, juntamente com o RVLM, as duas maiores áreas para as ações simpatoexcitatórias da Ang II (Dampney, Tan et al. 2007; Sakai, Agassandian et al. 2007).

Nesse sentido, desde 1961, quando Bickerton and Buckley mostraram pela primeira vez que a Ang II circulante poderia exercer um efeito pressor através de uma ação direta sobre o cérebro, praticamente independente dos seus efeitos sobre os vasos sanguíneos periféricos (Bickerton and Buckley 1961), o consenso é que a Ang II circulante desencadeia alterações no cérebro através de ações sobre os órgãos circunventriculares, os quais possui uma BHE incompleta (Broadwell and Brightman 1976; Shaver, Wall et al. 1992; Daneman 2012). Dessa forma, embora em condições normais a Ang II não cruze a BHE, o OSF possui um importante papel na integração de sinais advindos da Ang II circulante, bem como da Ang II local (Zimmerman, Lazartigues et al. 2004; Sakai, Agassandian et al. 2007). Então, a partir do OSF, informações são enviadas para regiões autonômicas como PVN, RVLM e NTS, uma via chave envolvida na transmissão dos sinais gerados pelo SRA central para a periferia (Ferguson and Bains 1997; Anderson, Smith et al. 2001; Smith and Ferguson 2010). Assim, elevados níveis de Ang II circulante pode resultar em um hiperativação do eixo OSF-PVN-RVLM, o que contribui criticamente para a instalação da hipertensão arterial (Ferguson and Bains 1997; Osborn, Fink et al. 2007).

Sabendo do importante papel da BHE na homeostase central, através da sua atuação como uma barreira física dinâmica na interface sangue-cérebro (Abbott, Ronnback et al. 2006; Abbott, Patabendige et al. 2010), prévios estudos têm demonstrado um rompimento dessa barreira em diferentes modelos experimentais de hipertensão (Mayhan, Faraci et al. 1989; Ueno, Sakamoto et al. 2004; Vital, Terao et al. 2010). Nesse sentido, tem sido proposto uma rota adicional pelo qual a Ang II circulante sinaliza para os centros simpatoexcitatórios durante a hipertensão, através da quebra dessa barreira. Ou seja, essa Ang II pode induzir um aumento na permeabilidade da BHE, facilitando seu acesso direito para áreas centrais críticas envolvidas na regulação da pressão arterial, como o PVN e o RVLM (Biancardi, Son et al. 2014; Biancardi and Stern 2016). Assim, desde as décadas de 70 e 80 já tem sido visto que a Ang II injetada nas artérias vertebrais provoca resposta pressora e ativação simpática (Fukiyama, McCubbin et al. 1971; Collister and Hendel 2005). Bem como, injeções

intracerebroventricular de Ang II também provoca resposta pressora e hipertatividade simpática (Sweet, Columbo et al. 1976; Sumners and Phillips 1983). Portanto, a Ang II seja de origem central ou periférica diretamente afeta os centros vasomotores e geram uma ativação simpática.

Em resposta a uma restrição proteica gestacional, é evidente a grande susceptibilidade do SRA, o qual se encontra alterado em múltiplos órgãos fetais incluindo o cérebro, rim, pulmão e coração. De fato, um acúmulo de evidências indica que o SRA da prole hipertensa submetida a uma restrição proteica está alterado localizadamente ou sistematicamente (Goyal, Galffy et al. 2009; Goyal, Goyal et al. 2010; Moritz, Cuffe et al. 2010; Goyal, Lister et al. 2011). Assim, estudos experimentais têm sugerido que a ativação desse sistema é um importante elemento na instalação da hipertensão programada durante a vida intrauterina (Langley-Evans and Jackson 1995; Sherman and Langley-Evans 2000). Pladys 2004 confirma que o bloqueio periférico de Ang II diminui a pressão arterial de ratos hipertensos desnutridos no início da vida, e, centralmente, observa uma diminuição da pressão arterial após bloqueio dos receptores AT1, bem como, um aumento na expressão desses receptores nas áreas de regulação cardiovascular, demonstrando um maior papel tônico de ambos os SRA periférico e central na manutenção da hipertensão induzida pela desnutrição (Pladys, Lahaie et al. 2004).

Portanto, uma desnutrição proteica induz o aumento da atividade do SRA e contribui para elevação da pressão arterial (Benabe, Wang et al. 1993; Martinez-Maldonado, Benabe et al. 1993; Goyal, Goyal et al. 2010), associada ao aumento da expressão de RNAm de diferentes componentes desse sistema em diversos tecidos (Sangaleti, Crescenzi et al. 2004; Goyal, Galffy et al. 2009). Nesse sentido, foi evidenciado que tratamento com inibidores do SRA, como a losartana (antagonista de recptores AT1) e enalapril (inibidor da enzima conversora de angiotensina - ECA) pode normalizar a pressão arterial e restaurar as alterações cardiovasulares induzidas pela desnutrição intrauterina (Ceravolo, Franco et al. 2007). Em modelo de desnutrição pós-desmame, foi observado um aumento da expressão dos receptores AT1 na aorta, uma redução dos níveis de Ang II circulante, bem como uma menor responsividade a esse peptídeo em ratos desnutridos, apesar desses animais apresentarem uma clara diminuição da pressão arterial após administração endovenosa de losartana e enalapril. Assim, esses dados sugerem que o SRA é um importante fator para manutenção da pressão arterial e que a atividade do sistema nervoso simpático está sobre forte influência da ação da Ang II via receptores AT1 em animais alimentados com déficit de proteínas (Gomide, de Menezes et al. 2013).

Vale destacar que uma importante relação entre estresse oxidativo e hipertensão arterial tem siso recentemente evidenciada em vários modelos animais de hipertensão e que a Ang II tem sido reconhecida como um potente indutor de ambos (Montezano and Touyz 2012). Assim, um aumento intracelular de espécies reativas de oxigênio (EROS) iduzidas pela interação da Ang II sobre seu recpetor AT1 pode atuar aumentando a propagação do pontencial de ação e a atividade neuronal (Zucker and Gao 2005). É bem reconhecido que a produção de EROS derivado da nicotinamida adenina dinucleotídeo fosfatase (NADPH) oxidase desempenha um papel crítico em diferentes modelos de disfunção cardiovascular como hipertensão induza por sal (Su, Huo et al. 2017), insuficiência cardíaca (Gao, Wang et al. 2005), ratos espontaneamente hipertensos propensos a acidente vascular encefálico (SHRPs) (Kishi, Hirooka et al. 2012). Portanto, a modulação da atividade simpática e pressão arterial pelos EROS no tronco encefálico, como a região do RVLM, tem sido evidenciado (Koga, Hirooka et al. 2008; Nishihara, Hirooka et al. 2012; Sousa, Magalhaes et al. 2015). Estudos em modelo de desnutrição proteica durante a gestação e lactação tem demonstrado uma diminuição na atividade de várias enzimas antioxidantes, bem como um aumento de EROS no tronco encefálico da prole (Ferreira, Liu et al. 2016). Esses efeitos foram associados com disfunção oxidativa medular em nível transcripcional e com prejuízo da capacidade antioxidante principalmente na região medular ventral do bulbo (de Brito Alves, de Oliveira et al. 2016). Nesse sentido, o tratamento com fármacos que atuam nos componentes do SRA tem sido bastante utilizado para investigar sua relação com a modulação da atividade simpática. Dentre eles, a losartana potássica, parece ser capaz de diminuir o estresse oxidativo no RVLM, reduzindo assim a simpatoexcitação e hipertensão em modelo de hipertensão renovascular (Nishi, Bergamaschi et al. 2013), bem como preteger o cérebro contra os danos cerebrais relacionados com a hipertensão (He, Zhang et al. 2014).

Portanto, sabendo da importância do SRA sobre a modulação simpática e manutenção da pressão arterial, bem como sua susceptibilidade aos insultos nutricionais maternos, e, considerando que uma desnutrição proteica durante o período perinatal induz importantes alterações cardiorrespiratórias que culminam com a hipertensão arterial, objetivamos investigar a participação do sistema renina angiotensina sobre essas alterações em ratos desnutridos durante a gestação e lactação.

2 HIPÓTESE

- ✓ Desnutrição proteica durante a gestação e lactação induz uma hiperatividade do sistema renina angiotensina, o que contribui para o aumento das atividades simpática-respiratória e consequentemente o desenvolvimento da hipertensão arterial na vida adulta desses animais.

3 OBJETIVOS

O presente estudo teve por objetivo:

3.1 OBJETIVO GERAL:

Investigar a participação do sistema renina angiotensina sobre o controle cardiorrespiratório na hipertensão arterial induzida pela desnutrição proteica durante a gestação e lactação.

3.2 OBJETIVOS ESPECÍFICOS:

Em ratos adultos provenientes de mães submetidas à restrição proteica durante gestação e lactação:

- ✓ Analisar os parâmetros cardiorrespiratórios e variabilidade cardiovascular basal;
- ✓ Avaliar a quimiossensibilidade a hipóxia citotóxica;
- ✓ Averiguar os níveis séricos de angiotensina II;
- ✓ Identificar os efeitos do bloqueio periférico e central dos receptores de angiotensina tipo 1 sobre os parâmetros cardiorrespiratórios e quimiossensibilidade a hipóxia;
- ✓ Identificar os efeitos da inibição periférica da enzima conversora de angiotensina sobre os parâmetros cardiorrespiratórios e quimiossensibilidade a hipóxia;
- ✓ Avaliar a expressão gênica e proteica dos componentes chave da cascata do sistema renina angiotensina no tronco cerebral;

4 MÉTODOS

Todos os protocolos e procedimentos experimentais foram realizados de acordo com Colégio Brasileiro de Experimentação Animal (COBEA) e aprovados pelo Comitê de Ética em Utilização Animal (CEUA) da UFPE (processo nº 23076.0048922/2014-23).

4.1 ANIMAIS

Foram utilizados ratos machos da linhagem Wistar provenientes do biotério do Centro Acadêmico de Vitória da Universidade Federal de Pernambuco. Os animais permaneceram em gaiolas de polipropileno (4 animais/gaiola), com água filtrada e ração *ad libitum.* Eles foram mantidos em ciclo claro escuro de 12h e a temperatura e a umidade foram mantidas dentro dos limites de 22±1°C e 55 a 65 %, respectivamente.

4.2 DIETAS

Foram elaborados dois tipos de dietas a base de caseína: uma dieta normoproteica (17% de proteína) e outra hipoproteica (8% de proteína). Ambas as dietas foram produzidas no Laboratório de Técnica Dietética do Centro Acadêmico de Vitória de acordo com *American Institute of Nutrition* - AIN-93 (Reeves, Nielsen et al. 1993). As dietas são isocalóricas com alteração apenas no conteúdo proteico conforme a tabela 1.

Tabela 1 – Composição nutricional das dietas (g/100g dieta).

Nutriente	**Normoproteica (17% proteína)**	**Hipoproteica (8% proteína)**
Caseína (85%)*	20	9,41
Amido dextrinizado	13,2	13,2
Celulose	5	5
Sacarose	10	10
Amido de milho	39,74	50,34
Óleo de soja	7	7
Colina	0,25	0,25
Metionina	0,3	0,3
Mix vitamínico	1	1
Mix mineral	3,5	3,5

Densidade energética (Kcal/g)	3,89	3,89

**A caseína utilizada na preparação da dieta apresentou 85% de pureza.*

4.3 INDUÇÃO DA DESNUTRIÇÃO PROTEICA DURANTE O PERÍODO PERINATAL (GESTAÇÃO E LACTAÇÃO)

Ratas Wistar virgens com 90 - 120 dias de vida ou com peso acima de 200g foram acasaladas com ratos machos na proporção de 2:1. A observação da presença de espermatozoides no esfregaço vaginal foi utilizada para definir o 1° dia de prenhez.

Posteriormente, as ratas foram colocadas em gaiolas individuais e alocadas randomicamente em dois grupos: grupo normoproteico (NP, recebeu dieta com 17% de proteína) e grupo hipoproteico (HP, recebeu dieta com 8% de proteína) durante a gestação (21 dias) e lactação (21 dias). Ao 2º dia de vida, as proles provenientes destas fêmeas foram reduzidas a oito ratos machos por ninhada. Ao 22º dia de vida, todos os filhotes receberam dieta normoproteica (Presence, Purina Agribands, São Paulo, Brasil). Após o desmame as ratas e os machos utilizados para obtenção da prole foram eutanasiados com overdose de pentobarbital sódico (70 mg/Kg ip). Além disso, a prole de fêmeas também foram eutanasiadas com overdose de pentobarbital sódico (70 mg/kg ip). Nos casos nos quais as ninhadas apresentaram menos de oito ratos machos, filhotes fêmeas foram utilizadas para padronização do tamanho da ninhada. Os estudos funcionais foram realizados aos 90° dia de vida.

4.4 PROCEDIMENTOS PARA AVALIAÇÃO DA VENTILAÇÃO PULMONAR, PRESSÃO ARTERIAL E FREQUÊNCIA CARDÍACA EM ANIMAIS ACORDADOS

As medidas de ventilação foram obtidas por pletismografia de corpo inteiro, em um sistema fechado (Malan 1973). Durante a realização de cada medida de ventilação, o fluxo de ar é interrompido e a câmara do animal permanece totalmente vedada por curtos períodos de tempo (~2 min). As oscilações causadas pela ventilação do animal foram captadas por um dispositivo conectado à câmara que contém o transdutor diferencial de pressão e o amplificador de sinais (ML141 spirometer, PowerLab, ADInstruments). O sinal foi então enviado para o sistema de aquisição e análise dos dados (LabChartTM Pro, PowerLab, ADInstruments). A calibração do volume foi obtida durante cada experimento, injetando-se

um volume conhecido de ar (1 mL) dentro da câmara do animal com o uso de uma seringa graduada. Em seguida foram registradas as medidas de frequência respiratória (FR). Ao 90° dia de vida, os animais foram anestesiados com ketamina (80 mg/kg, i.p.) e xilazina (10 mg/kg, i.p.) para inserção de cateteres de polietileno na artéria e veia femoral, para registro da PA e infusão de drogas, respectivamente. O cateter foi exteriorizado subcutaneamente até a altura do pescoço para facilitar a conexão dele ao transdutor de pressão. Após a cirurgia, os animais receberam uma dose de cetoprofeno (5 mg/kg ip, anti-inflamatório). O registro da PA e da frequência cardíaca (FC) foi realizado 24 horas após o procedimento cirúrgico em animais não anestesiados por meio da conexão da cânula da arterial femoral com o transdutor mecanoelétrico de pressão, cujo sinal foi devidamente amplificado (ML866/P, ADInstruments, Power Lab, Bella Vista, NSW, Australia), digitalizado por meio de uma interface analógico/digital e amostrado a 2000 Hz em um microcomputador equipado com um software apropriado (LabChartTM Pro, ADInstruments, Bella Vista, NSW, Austrália), para posterior análise. A pressão arterial média (PAM) e FC foram derivadas da pressão arterial pulsátil (PAP) por meio deste sistema de aquisição.

4.5 ANÁLISE DA VARIABILIDADE CARDIOVASCULAR

Uma avaliação indireta da modulação autonômica da resistência vascular e da função cardíaca foi realizada através da análise da variabilidade da pressão arterial e da frequência cardíaca no domínio do tempo e da frequência (Barros, De Brito Alves et al. 2015). Para análise no domínio do tempo foi realizada a análise do desvio padrão dessas variáveis. Com relação ao domínio da frequência, oscilações de pressão arterial na faixa de muito baixa frequência (VLF) correspondem a influências hormonais, termorregulação e circadiano, de baixa frequência (LF) são representativos dos efeitos moduladores da atividade simpática, enquanto oscilações na escala de alta frequência (HF) estão associadas a uma modulação respiratória ou parassimpática dos vasos sanguíneos e do coração, respectivamente (Malliani, Pagani et al. 1991; Bernardi, Porta et al. 2001). No presente estudo, as magnitudes dos efeitos moduladores autonômicos e respiratórios no sistema cardiovascular foram avaliadas em ratos NP e HP. Inicialmente, foram efetuados registros basais da PA e FC de ambos os grupos durante 1 hora. Os trechos de registros foram divididos em períodos de segmentos de 350 batimentos e então realizada análise espectral auto regressiva, a fim de determinar os componentes oscilatórios de muito baixa-frequência (VLF, <0.2 Hz), baixa-frequência (LF,

0.20-0.75 Hz) e de alta-frequência (HF, 0.75-3.0 Hz) da pressão arterial sistólica (PAS) e do intervalo de pulso (IP), através de software apropriado (Cardioseries Software v2.4, disponível em https://www.sites.google.com/site/cardioseries/home). Flutuações nas bandas de LF e HF do intervalo de pulso foram expressos em unidades normalizadas (nu). Para avaliação do índice simpato-vagal, a relação LF/HF da variabilidade foi calculada.

Além da análise espectral foi realizada a análise simbólica da variabilidade da FC. Um método não linear baseado na conversão das séries em uma sequência de símbolos. A dinâmica completa da série (o intervalo entre máximo-mínimo) é distribuido por seis caixas, cada uma delas é identificada por um número (símbolo) de 0 a 5. Valores originais dentro de cada caixa são substituídos pelo símbolo para definir o símbolo específico, então, obtem-se uma série simbólica. A série simbólica é convertida para uma série de padrões de três símbolos. Quatro famílias diferentes de padrões podem ser identificadas: 0V (padrões com nenhuma variação, todos os símbolos são iguais), 1V (padrões com uma variação, dois símbolos consecutivos são iguais e o restante é diferente), 2LV (padrões com duas variações, o segundo e o terceiro muda o símbolo com relação ao anterior, as mudanças têm o mesmo sentido) e 2UV (ao contrário dos padrões com duas variações, a segunda e a terceira mudança de símbolo em relação ao anterior, têm sentido oposto). A porcentagem (%) de ocorrência do padrão 0V tem sido considerado como marcador da modulação simpática de FC, enquanto que 2V é considerado um marcador da modulação vagal (Porta, Guzzetti et al. 2001; Porta, Tobaldini et al. 2007).

4.6 ATIVAÇÃO DOS QUIMIORRECEPTORES PERIFÉRICOS (HIPÓXIA CITOTÓXICA)

A hipóxia citotóxica foi induzida através de injeções intravenosa de cianeto de potássio (KCN 0,04 %, 100 ul/rato), com o objetivo de produzir uma forte ativação dos quimiorreceptores periféricos localizados no corpo carotídeo conforme previamente descrito (Franchini and Krieger 1993; Machado and Bonagamba 2005). Todas as respostas cardiorrespiratórias como resposta pressora, bradicárdica e taquipneica foram registradas e a magnitude das alterações na PAM, FC e FR em resposta à ativação do quimioreflexo foi quantificado pelo pico das respostas. Após isso o delta foi calculado pela diferença entre os valores de pico e basal (efeitos sobre PAM, FC e FR). Essa ativação foi realizada antes e após a inibição central e periférica dos receptores de angiotensina tipo 1.

4.7 AVALIAÇÃO DO SISTEMA RENINA ANGIOTENSINA SOBRE A PRESSÃO ARTERIAL

Após o registro basal dos parâmetros cardiorrespiratórios, foi realizado o bloqueio dos receptores tipo 1 de angiotensina (AT1) através da administração de losartana potássica (10 mg/kg, iv) e as repercussões sobre os parâmetros cardiorrespiratórios foram registradas durante os seguintes 30 min. Em outro grupo de animais foi avaliado os efeitos da inibição da enzima conversora de angiotensina (ECA) através da administração de enalapril (5 mg/kg, iv) e as respostas cardiorrespiratórias registradas durante o mesmo período de tempo. Ambas as drogas foram obtidas pela Sigma-Aldrich, St. Louis, USA.

4.8 QUANTIFICAÇÃO DE ANGIONTENSINA II SÉRICA

Aos 85 dias de vida a prole de ambos os grupos foram anestesiadas com ketamina (80 mg/kg) e xilazina (10 mg/kg), e amostras de sangue (aproximadamente 1 ml) foram coletadas através da ruptura do plexo retro-orbital. As amostras do soro foram coletadas para quantificação da Ang II e suas concentrações foram mensuradas utilizando um ensaio de imunoabsorção enzimática (ELISA) de acordo com as instruções do fabricante (Sigma–Aldrich, St. Louis, USA).

Para a estimativa de Ang II em soro de rato, placas de ELISA foram encubadas com 100 ul de anticorpo anti-Ang II, lavado e então encubado com as amostras. Estreptavidina-HRP foi adicionado em seguida o substrato foi adicionado em cada poço de acordo com as instruções do fabricante. No final, foi adicionado solução de parada e posteriormente a absorbância foi mensurada em 450 nm (ELISA kit, Sigma– Aldrich, St. Louis, USA).

4.9 MICROINJEÇÃO INTRACEREBRIVENTRICULAR DE ANTAGOSNISTA DO RECEPTOR AT1

Aos 90 dias de vida, em parte dos animais dos grupos NP (n= 5) e LP (n= 5) os efeitos centrais do bloqueio dos receptores AT1 foram investigados através de microinjeção de losartan no ventrículo lateral. Inicialmente, para implantação de cânula guia para injeção

intracerebroventricular, ratos foram anestesiados com ketamina (80 mg / kg) e xilazina (10 mg / kg). Em seguida, o animal foi colocado em um aparelho estereotáxico e uma cânula guia (tubo de aço inoxidável calibre 26) foi introduzida na posição no ventrículo lateral (1,0 mm de caudal ao bregma, 2,0 mm lateral a linha média e 5,0 mm abaixo da superfície cerebral). Cinco dias após a cirurgia, foram realizados os experimentos. Após o registro inicial da pressão arterial basal, os ratos receberam injeção icv de 50 μg (em 5 μl) de losartan e as respostas cardiorrespiratórias registradas durante 30 min e após a ativação dos quimiorreceptores periféricos. A precisão da canulação icv foi verificada através de autópsia com uma injeção icv de azul de metileno (Chang Gyu Park, Frans H. H. Leenen 2001).

4.10 ENSAIOS MOLECULARES

✓ COLETA DOS TECIDOS

Aos 90 dias de vida, ratos que não passaram por procedimentos cirúrgicos foram eutanasiados por decapitação para coleta do tronco encefálico e fígado para realização dos ensaios moleculares. Os presentes tecidos foram coletados, congelados em nitrogênio líquido e imediatamente armazenados em freezer -80°C até a realização das análises.

✓ EXTRAÇÃO TRÍPLICE: DNA, RNA E PROTEÍNAS PARA ANÁLISES MOLECULARES

Em uma única amostra de tecido foi adicionado Trizol (Tripure Isolamento Reagente, Roche) para extração de RNA e proteína. A solução resultante foi transferida para tubos de rolamento (Bertino, Precellys Lise Kit) e homogeneizadas (Bertino, Precellys 24). Em seguida, foi adicionado ¼ do volume de trizol de clorofórmio e realizado centrifugação durante 15 minutos a 13000 RPM. Após a centrifugação, a fase aquosa contendo o RNA foi recolhida e precipitada em isopropanol (0.5mL para cada 1 mL de trizol) e a fase contendo trizol (DNA e proteínas) foram utilizadas para extração desses componentes.

Após centrifugação do RNA com o isopropanol, o sedimento foi lavado duas vezes com 1 volume de etanol a 75% e 100%. O sedimento de RNA foi ressuspenso em 100 uL de H2O (Versol). A concentração de RNA foi medida num espectrofotômetro Nanodrop2000.

O DNA foi isolado na interfase fenol-clorofórmio. Para isso, foi adicionado 0.3 mL de etanol 100% para cada 1 mL de Trizol usado no início da reação e centrifugado durante 5 minutos a 7000 RPM. O sobrenadante foi coletado em um novo tubo para extração de proteínas.

A extração de proteínas foi realizada no sobrenadante fenol-etanol. Para isso, foi adicionado 1.5mL de isopropanol para cada 1mL de trizol utilizado no início da reação e em seguida centrifugação durante 10 minutos a 13000 RPM. Após, os pelletes foram lavados com guanidina hidrocloride (0.3M) em etanol 95%. Ao final, os pelletes também foram lavados com etanol 100% e as proteínas solubilizadas em SDS 1%. Esse protocolo foi padronizado por José Luiz de Brito Alves no instituto CarMeN – Lyon – França, sob direção do Dr. Luciano Pirola (de Brito Alves, JL e Pirola, L).

- ✓ **TRANSCRIÇÃO REVERSA RT-TAKARA**

A transcrição reversa RT-TAKARA para amostra de RNA foi preparado com 1µg de RNA. A amostra foi então aquecida durante 10 minutos a 65°C e foram adicionados 4µL PrimeScript tampão 5x, 1 ul de mistura de enzimas PrimeScript RT, 1 ul de oligodT (50 microns), 4µl hexâmeros aleatórios (100 uM). RT-ciclo compreende TAKARA 15 minutos a 37°C e 15 segundos a 85°C. A amostra foi então colocada durante 1 minuto em gelo e, em seguida, passou por centrifugação rápida a 4°C. Em seguida, foram adicionados RNase H (1ul) para cada 20 uL de RT puro.

Após, RT puro + RNAH foram colocados para incubar durante 20 minutos a 37°C. RT foi diluído a 1/10 por adição de 179µL de H2O e os tubos armazenados a -20° C. Em seguida, RT 1/10 foi diluído para RT 1/60 (10 ul RT 1/10 + 50 uL H_2O) e a técnica de reação em cadeia da polimerase (PCR em tempo real) foram realizadas para investigação da expressão gênica. A sequência dos primers utilizados para realização dos experimentos de RT-PCR do presente estudo encontram-se descritas na **tabela 2**.

Tabela 2. Sequência de primes usado para realização do qRT-PCR.

Gene	Foward/ Reverse	Tm	Sequence 5'-3'	Amplicon size
Agtr1a	F	60°C	GGCTGGCATTTTGTCTGG	104 bp
	R		TGTTTTTCTGGGTTGAGTTGG	
Agtr2	F	60°C	ACACAAACCGGCAGATAAGC	132 bp
	R		TGCTGGACACCTTTTTAGGG	
Agt	F	60°C	AATTCGGGGATCCTACAACC	145 bp
	R		TTCGAGTTCAAGGAGGATGC	
Ace	F	62°C	CATATGAGTCCGACGACTTGG	178 bp
	R		GTCTGTGCCCACATGTTCC	
Prkcg	F	60°C	CCACGAATTTGTGACCTTCG	100 bp
	R		ACTGCTGTAGCTGTGCAGACG	
Rac1	F	60°C	TCCCAATACTCCCATCATCC	157 bp
	R		GCACTCCAGGTATTTGACAGC	
Ptpn6	F	60°C	CCATCATCCACCTCAAGTACC	137 bp
	R		CTCACGCACAAGAAATGTCC	
Mas1	F	58°C	GTCATGTGTATTGACAGCGG	178 bp
	R		TGTACAGCTTCGAAGAATGG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195 bp
	R		GGACAGAGTCTTGATGATCTC	
Actb	F	60°C	AGCCATGTACGTAGCCATCC	231 bp
	R		TCCCTCTCAGCTGTGCTGGTGAA	

4.10.4 EXTRAÇÃO DE PROTEÍNAS E PROCEDIMENTOS DE WESTERN BLOTTING

Proteínas em sobrenadantes a partir de procedimentos de purificação de RNA foram precipitadas através da adição de isopropanol e centrifugação (15,000 *g*, 15 min at 4 °C). Peletes foram lavados com 0.3 M de hidrocloride guanidina em etanol 95%. Após a centrifugação (3000g, 5min), proteínas foram solubilizadas em dedecil sulfato de sódio (SDS). Concentrações de proteínas foram determinadas usando o método de Bradford. Albumina sérica bovina foi usada como um padrão e densidades óticas foram lidas em 595 nm através de um leitor de microplaca (Multiskan GO, Thermo Fisher Scientific, Waltham, MA).

Amostras de proteínas foram ajustadas para uma concentração final de 1ul/ul. Após adição de tampão Laemmli (150 mM Tris HCl, 36% glicerol, 3% SDS, 12% β-mercaptoetanol, 0.03 % azul bromofenol) e desnaturação, proteínas foram separadas por

padrão SDS-PAGE conforme previamente descrito (Pirola, Bonnafous et al. 2003). Marcadores de tamanho de proteínas (Precision Plus Protein Standards, Biorad) foram depositados em paralelo. Proteínas separadas foram então transferidas para membranas PVDF através de um sistema de transferência (Biorad Transblot Turbo Blotting apparatus). Foram usados os seguintes anticorpos primários: anti p44/42 MAPK (sc-9102, Santa Cruz biotechnologies) e anti phospho p44/42 (sc-9101, Santa Cruz Biotechnologies). O anticorpo secundário anti-coelho foi usado e a revelação foi feita usando o reagente CL Lumina Forte (Merck Millipore, Darmstadt, Alemanha). A quimiluminescência foi adquirida em uma câmera ChemiDocTM XRS + usando o software Image Lab 4.1 (Biorad).

4.11 ANÁLISE ESTATÍSTICA

Os resultados estão expressos como média ± epm (erro padrão da média). Os dados foram testados quanto a sua normalidade e homogeneidade de variância, através dos testes Kolmogorov-Smirnov e Shapiro-Wilk. A comparação entre os grupos normoproteico e hipoproteico foi realizada pelo teste “t” de Student não pareado, e para comparação intergrupo foi utilizado o test “t” de Student pareado. O nível de significância foi considerado quando $p<0,05$.

5 RESULTADOS

Parâmetros cardiorrespiratórios basais

Aos 90 dias de vida, ratos nascidos de mães que receberam dieta hipoproteica exibiram um aumento da pressão arterial média (NP: 102±1 vs. HP: 108±2 mmHg; n=19; p<0.0174), mas sem alterações na frequência cardíaca quando comparado ao grupo normoproteico (NP: 372±4 vs. HP: 384±11 bpm; n=9). A frequência respiratória também foi maior nos animais desnutridos do que no grupo controle (NP 103.5±2 vs. HP 116±4 resp/min; p<0.0122), entretanto, o volume corrente e a ventilação pulmonar foi similar entre os grupos.

Variabilidade cardiovascular

Através da análise espectral não foi observado diferenças nas oscilações de VLF e HF da pressão arterial sistólica, entretanto, as oscilações de LF (NP: 2.7±0.3 vs. HP: 5±1 $mmHg^2$; n=7-16; p=0.0145) foi aumentada nos animais HP durante o período basal. Interessantemente essa alteração foi abolida após o antagonismo dos receptores AT1 e inibição de ACE. Assim, não foi observado diferenças nas oscilações de VLF, LF e HF após losartan e enalapril (Figura 1A). Com relação ao interval de pulso, não foi observado alterações nas oscilações de LF (NP: 20.19±2.21 vs. HP: 24.25±5.59 nu; n=8-16) e HF (NP: 79.81±2.21 vs. HP: 75.75±5.59 nu n=8-16), como mostra a figura 1B, o qual repercutiu em uma razão LF/HF (NP: 0.28±0.03 vs. HP: 0.39±0.11) semelhante entre os grupos, durante o período basal e após as drogas (Figure 1B). Resultados similares também foi observado na variabilidade no

domínio do tempo, onde o desvio padrão da pressão arterial e frequência cardíaca foi similar entre os grupos (Figuras 1C e 1D).

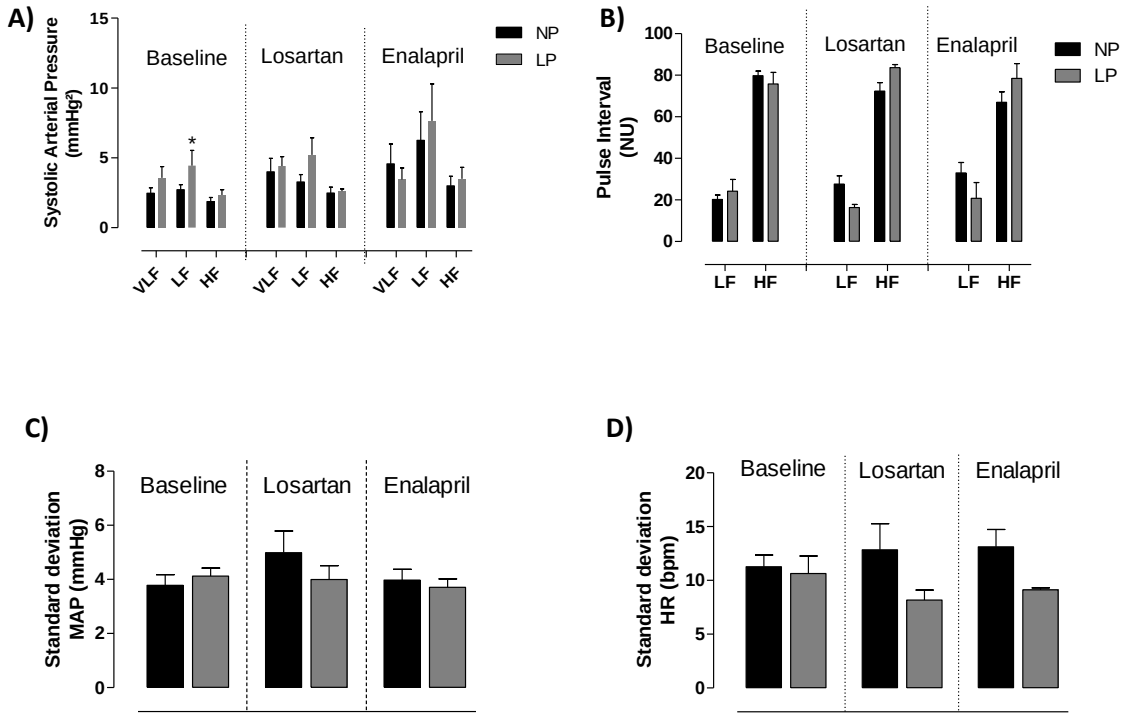

Figura 1. Variabilidade Cardiovascular. Domínio da frequência: Análise spectral da pressão arterial sistólica com as bandas *very low-frequency* (VLF), *low-frequency* (LF) e *high-frequency* (HF) (A) e do intervalo de pulso (B). Domínio do tempo: Desvio padrão da pressão arterial média (PAM, C) e frequência cardíaca (FC, D) dos grupos normoproteicos (NP; barras pretas) e hipoproteicos (HP; barras cinzas). Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP (Teste t de *Student* não pareado).

Avaliação do sistema renina angiotensina sobre a pressão arterial

A figura 2 mostra que os animais desnutridos apresentaram um maior delta de PAM após o bloqueio de receptores AT1 com a losartana (NP: -9.8±2 vs. HP: -23±6 mmHg; n=6-10; p=0.0174), sem alterações na RF e HR comparado com o grupo NP (Figura 2C). Entretanto, após inibição com ECA, nenhuma alteração foi observada na PAM (NP: -14.89±3

vs. HP: 18.6±6 mmHg; n=5-9), FR e FC (Figura 2D), sugerindo um importante envolvimento dos receptores AT1 no aumento da pressão arterial em animais desnutridos.

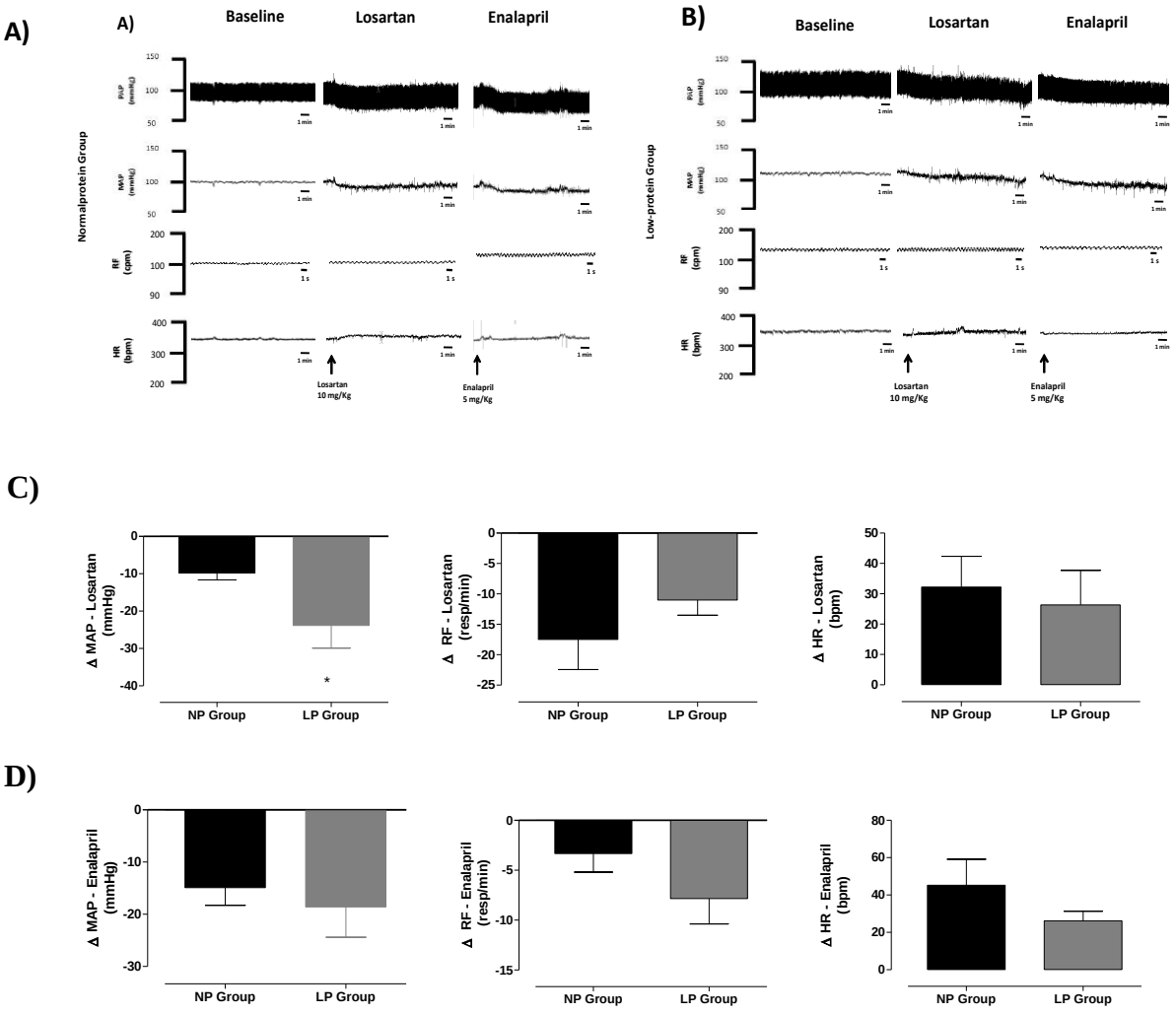

Figura 2. Avaliação do sistema renina angiotensina sobre os parâmetros cardiorrespiratórios. Traçados representativos dos grupos normoproteico (A) e hipoproteico (B) mostrando a pressão arterial pulsátil (PAP), pressão arterial média (PAM), frequência respiratória (FR) e frequência cardíaca (FC) durante o período basal, após bloqueio dos receptores de angiotensina tipo 1 (AT1) com losartana e após inibição da enzima conversora de angiotensina (ECA) através da administração de enalapril. Delta das medias de PAM, FR e FC após bloqueio de AT1(C) e após inibição de ECA (D) dos grupos normoproteicos (NP; barras pretas) e hipoproteicos (HP; barras cinzas). Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP (Teste t de *Student* não pareado).

Respostas cardiorrespiratórias à hipóxia hipóxica

Durante ativação do quimioreflexo através da administração do KCN durante o período basal, não foi observado diferenças nas respostas pressoras, taquipnéicas e bradicárdicas entre os grupos NP e HP. No entanto, após bloqueio de receptores AT1 com a losartana, os animais HP apresentaram maior resposta pressora (delta PAM) quando comparado ao grupo NP (NP: 68±8 vs. HP: 98±7 mmHg; n=5-8) e quando comparado com o período basal. Diferentemente, após inibição de ECA através da administração de enalapril, não foi observada diferença entre os grupos (Figura 3).

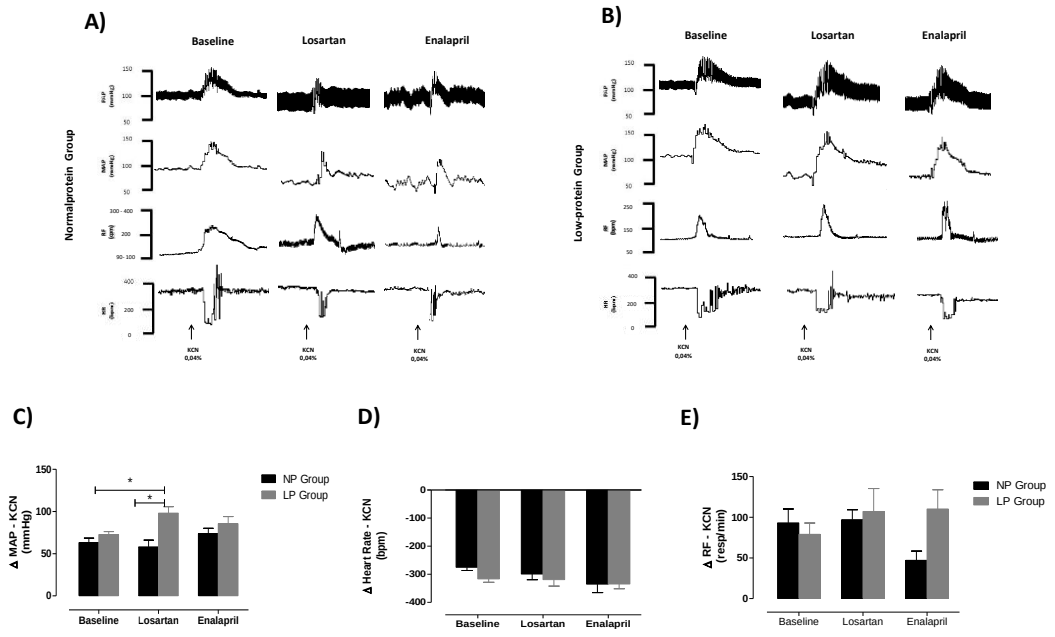

Figura 3**.** Traçados representativos do grupo normoproteico (A) e hipoproteico (B) mostrando a pressão arterial pulsátil (PAP), pressão arterial média (PAM), frequência respiratória (FR) e frequência cardíaca (FC) em resposta à ativação do quimiorreflexo (KCN 0.04%, seta) antes (basal) e após administração de losartana e enalapril. The delta of the MAP (C), HR (D) and RF (E) nesssas consições dos grupos normoproteicos (NP; barras pretas) e hipoproteicos (HP; barras cinzas). Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP (Teste t de *Student* não pareado).

Concentração sérica de angiotensina II

Animais NP e HP apresentaram semelhante concentrações de Ang II (NP: 464.9±63.28 vs. HP: 316.5±39.27 pg/ml; n=19; p=0,0511), sugerindo que uma desnutrição durante o período perinatal não altera os níveis de Ang II circulante.

Microinjeção intracerebroventricular de antagonista de receptors AT1

Em nível central, microinjeção de losartana no ventrículo lateral não alterou a pressão arterial de ambos os grupos. Os animais HP apresentaram os mesmos níveis elevados de pressão arterial quando comparado com os animais NP durante o período basal e após a microinjeção ICV de losartana (NP: 109 ± 6 vs. HP: 129±4 mmHg; n=5). A ativação do quimiorreflexo após o bloqueio dos receptores AT1 não demonstrou diferenças nas respostas pressoras, bradicárdicas e taquipneicas entre os grupos, entretanto, os animais NP e HP apresentaram maio delta de FR quando comparado ao período basal, conforme é mostrado na figura 4.

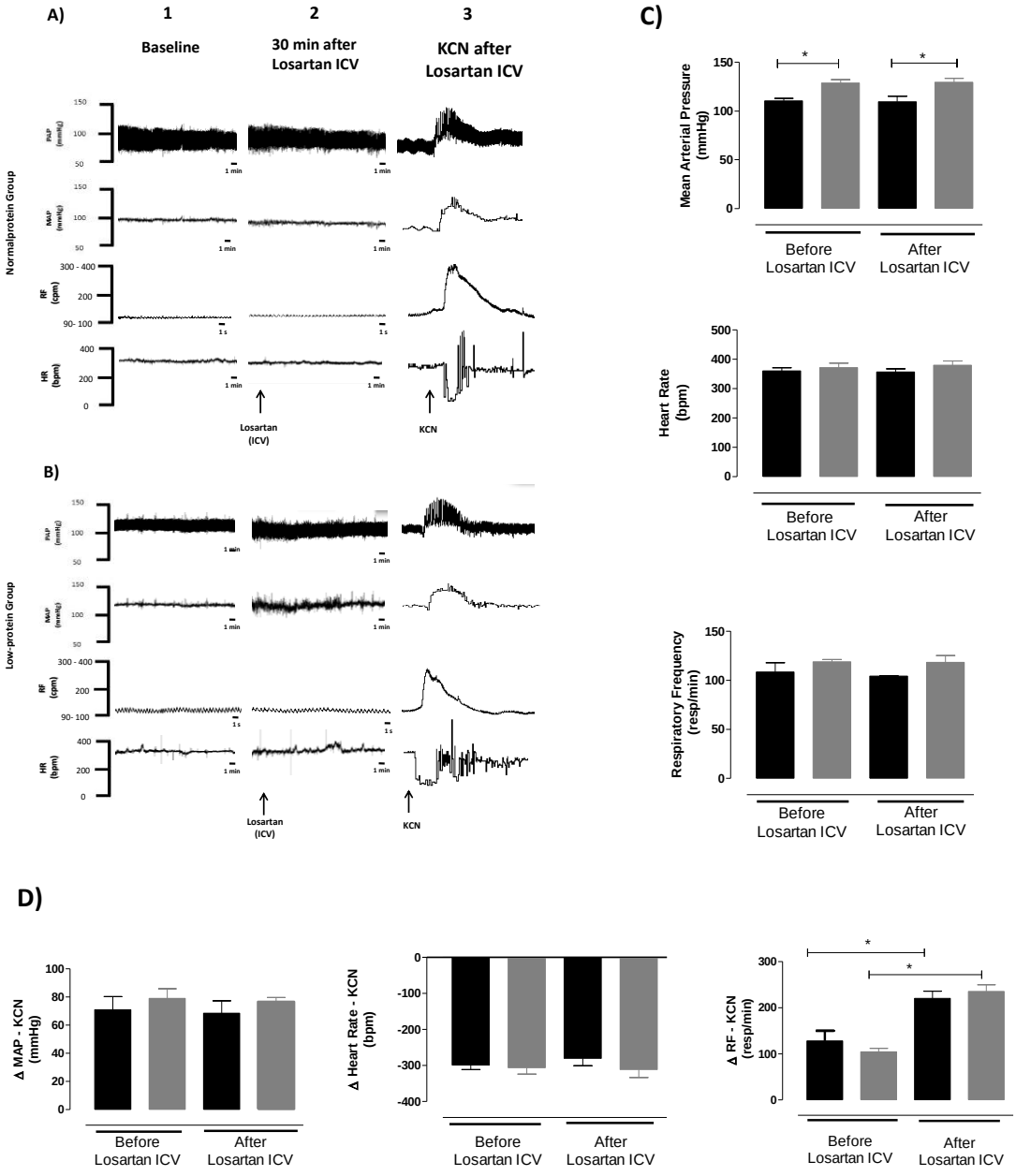

Figure 4. Traçados representativos do grupo normoproteico (A) e hipoproteico (B) mostrando a pressão arterial pulsátil (PAP), pressão arterial média (PAM), frequência respiratória (FR) e frequência cardíaca (FC) durante o período basal, após microinjeção intracerebroventricular de antagonista de receptores AT1, losartan (seta), e durante ativação do quimiorreflexo (KCN 0.04%, seta). Média de PAM, FC e FR. **C.** Médias de PAM, FC e FR antes e após administração ICV de losartana. **D.** Delta de MAP, FC e FR em resposta a ativação do quimioreflexo, antes e após administração ICV de losartana nos grupos normoproteicos (NP; barras pretas) e hipoproteicos (HP; barras cinzas). Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP (Teste t de *Student* não pareado).

Avaliação do RNA_m no fígado e tronco encefálico

No fígado de ratos com 90 dias de vida, a expressão de mRNA de angiotensinogênio foi similar entre os grupos (NP: 1.07 ± 0.09 vs. HP: 0.87 ± 0.07), como mostra a figura 5. Resultados semelhantes foi observado no tronco encefálico desses animais, demonstrando que a desnutrição proteica não altera a expressão gênica de angiotensinogênio em níveis central e periférico. No tronco encefálico, a expressão de RNAm de Ace, Agtr1a e Agtr2 também foram similares entre os grupos NP e HP. Entretanto, quando analisado proteínas das vias de sinalização, a expressão gênica de Rac1 nos animais HP foi menor do que nos animais NP (NP: 1.13± 0.06 vs. HP: 0.88 ± 0.08; n= 5; p= 0,04), enquanto a expressão gênica de prkcg e ptpn6 foram similares entre os grupos. Além disso, não houve alteração na expressão de RNAm de Mas1 no tronco encefálico tanto de animais controles como desnutridos (NP: 1.182 ± 0.05 vs. HP: 1.093 ± 0.13), sugerindo que a via da angiotensina (1-7) pode não estar envolvida nas alterações cardiovasculares presentes em animais hipertensos induzidos pela dieta materna (figura 5).

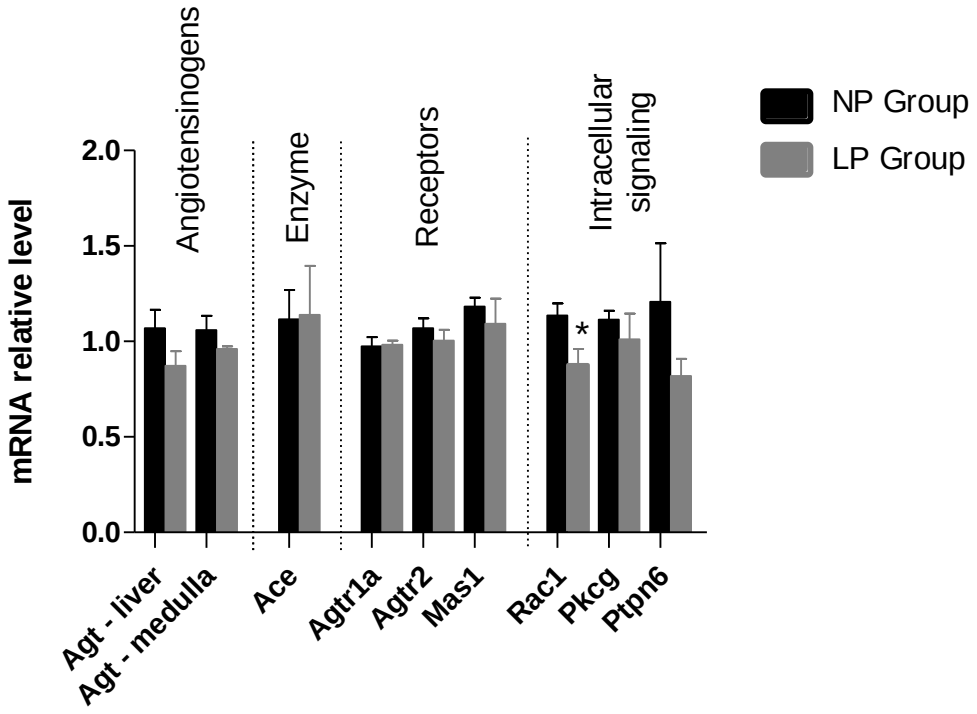

Figure 5. Expressão de RNA_m em animais desnutridos durante gestação e lactação. Avaliação da expressão de RNA_m de angiotensinogênio (Agt) no fígado e no trance encefálico, da *angiotensin I converting enzyme* (Ace), *type 1a angiotensin II receptor* (Agtr1a), *type 2 angiotensin II receptor* (Agtr2), *ras-related C3 botulinum toxin substrate 1* (Rac1), *gamma protein kinase C* (Prkcg), *and protein tyrosine phosphatase, non-receptor type 6 proto-oncogene* (Ptpn6), *G protein-coupled receptor* (Mas1) no tronco encefálico aos 90 dias de vida. Barras pretas representam grupo NP (17% proteína, n=5) e barras cinza grupo HP (8% proteína, n=5). Todos os filhotes foram alimentados com dieta padrão após desmame. Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP através do teste t de *Student* não pareado. (*RPL19 + β-actin Housekeeping gene)*.

Avaliação da expressão proteica no tronco encefálico

Animais desnutridos apresentaram diminuição de MAPK p44 e p42 no tronco encefálico quando comparado ao grupo normoproteico. No entanto, não foi observada diferenças em relação a razão entre MAPK/fosfoMAPK das proteínas p44 e p42, conforme demonstrado na figura 6.

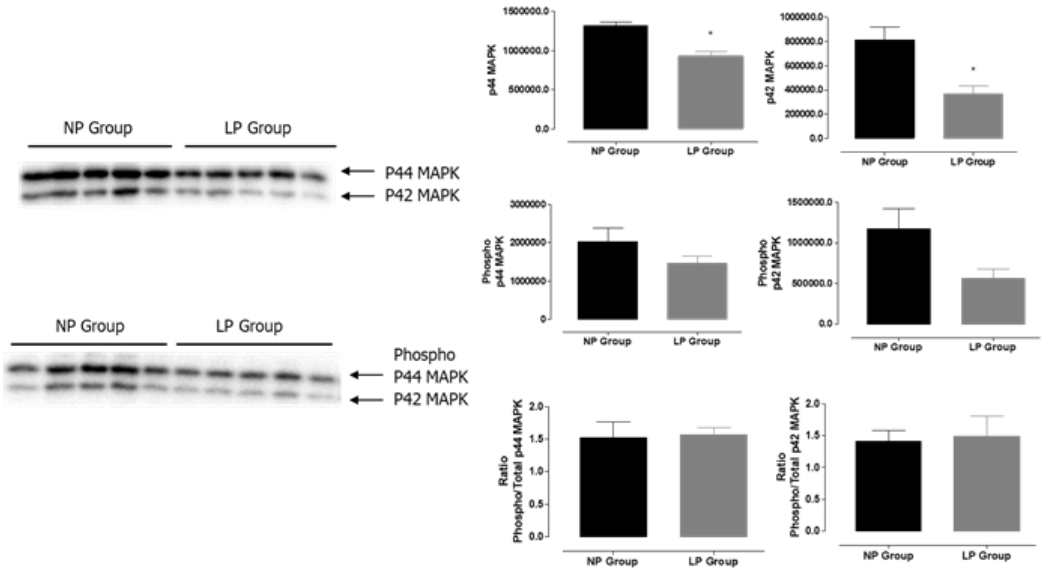

Figura 6. Western blotting para expressão de MAPK e fosfo MAPK no tronco encefálico aos 90 dias de vida. Barras pretas representam grupo NP (17% proteína, n=5) e barras cinza grupo HP (8% proteína, n=5). Todos os filhotes foram alimentados com dieta padrão após desmame. Os valores foram expressos como média ± EPM. (*) P <0.05 comparado com o grupo NP através do teste t de *Student* não pareado.

6 CONCLUSÕES

Nossos dados mostram que os efeitos em longo prazo induzidos pela desnutrição proteica durante os períodos de gestação e lactação incluem maior resposta hipotensora após bloqueio periférico dos receptores angiotensinérgicos tipo 1, bem como em resposta a ativação do quimioreflexo após esse bloqueio, sugerindo um aumento da influência angiotensinérgica na manutenção da hipertensão arterial desses animais, os quais parecem não depender da angiotensina II circulante. Além disso, como não houve alteração pressórica após bloqueio central dos receptores de angiotensina tipo 1 e em resposta a ativação do quimiorreflexo, bem como na expressão gênica dos componentes do sistema renina angiotensina no tronco cerebral desses animais, o sistema renina angiotensina parece ter influência sobre os tecidos periféricos, como sistema vascular, e não centralmente, na manutenção da hipertensão arterial nesses animais.

REFERÊNCIAS

ABBOTT, N. J. et al. Structure and function of the blood-brain barrier. **Neurobiol Dis,** v. 37, n. 1, p. 13-25, 2010.

ABBOTT, N. J. et al. Astrocyte-endothelial interactions at the blood-brain barrier. **Nat Rev Neurosci**, v. 7, n. 1, p**.** 41-53, 2006.

ABDALA, A. P. et al. Hypertension is critically dependent on the carotid body input in the spontaneously hypertensive rat. **J Physiol**, v. 590, n .17, p. 4269-4277, 2012.

ADAIR, L. S.; COLE, T. J. Rapid child growth raises blood pressure in adolescent boys who were thin at birth. **Hypertension,** v. 41, n. 3, p. 451-456, 2003.

ANDERSON, J. W. et al. Subfornical organ neurons projecting to paraventricular nucleus: whole-cell properties. **Brain Res,** v. 921, n**.** 1-2, p. 78-85, 2001.

BARKER, D. J. et al. Fetal and placental size and risk of hypertension in adult life. **BMJ**, v. 301, n. 6746, p. 259-262, 1990.

BARROS, M. A. et al. Maternal low-protein diet induces changes in the cardiovascular autonomic modulation in male rat offspring. **Nutr Metab Cardiovasc Dis**, v. 25, n. 1, p. 123-130, 2015.

BENABE, J. E. et al. Modulation of ANG II receptor and its mRNA in normal rat by low-protein feeding. **Am J Physiol**, v. 265, p. 660-669, 1993.

BERNARDI, L. et al. Modulatory effects of respiration. **Auton Neurosci**, v. 90, n. 1-2, p. 47-56, 2001.

BHARGAVA, S. K. et al. Relation of serial changes in childhood body-mass index to impaired glucose tolerance in young adulthood. **N Engl J Med**, v. 350, n. 9, p. 865-875, 2004.

BIANCARDI, V. C. et al. Circulating angiotensin II gains access to the hypothalamus and brain stem during hypertension via breakdown of the blood-brain barrier. **Hypertension**, v. 63, n. 3, p. 572-579, 2014.

BIANCARDI, V. C.; STERN, J. E. Compromised blood-brain barrier permeability: novel mechanism by which circulating angiotensin II signals to sympathoexcitatory centres during hypertension. **J Physiol**, v. 594, n. 6, p. 1591-1600, 2016.

BIANCHI, A. L. et al. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. **Physiol Ver**, v. 75, n. 1, p. 1-45, 1995.

BRAGA, V. A. et al. Sympathoexcitatory response to peripheral chemoreflex activation is enhanced in juvenile rats exposed to chronic intermittent hypoxia. **Exp Physiol**, v. 91, n. 6, p. 1025-1031, 2006.

BROADWELL, R. D.; BRIGHTMAN, M. W. Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood. **J Comp Neurol**, v. 166, n. 3, p. 257-283, 1976.

BUGENHAGEN, S. M. et al. Identifying physiological origins of baroreflex dysfunction in salt-sensitive hypertension in the Dahl SS rat. **Physiol Genomics**, v. **42**, n. 1, p. 23-41, 2010.

CAREY, R. M.; WHELTON, P. K. Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Synopsis of the 2017 American College of Cardiology/American Heart Association Hypertension Guideline. **Ann Intern Med**, 2018.

CERAVOLO, G. S. et al. Enalapril and losartan restored blood pressure and vascular reactivity in intrauterine undernourished rats. **Life Sciences**, v. 80, n. 8, p. 782-787, 2007.

CIRIELLO, J. et al. Collateral axonal projections from ventrolateral medullary non-catecholaminergic neurons to central nucleus of the amygdala. **Brain Res**, v. 663, n. 2, p. 346-351, 1994.

COLLISTER, J. P.; HENDEL, M. D. Chronic effects of angiotensin II and at1 receptor antagonists in subfornical organ-lesioned rats. **Clin Exp Pharmacol Physiol** v. 32, n. 5-6, p. 462-466, 2005.

COSTA-SILVA, J. H. et al. Chronic undernutrition alters renal active Na+ transport in young rats: potential hidden basis for pathophysiological alterations in adulthood?. **Eur J Nutr**, v. 48, n. 7, p. 437-445, 2009.

COSTA-SILVA, J. H. et al. Glutamatergic antagonism in the NTS decreases post-inspiratory drive and changes phrenic and sympathetic coupling during chemoreflex activation. **J Neurophysiol**, v. 103, n. 4, p. 2095-2106, 2010.

COSTA-SILVA, J. H. et al. Chronic intermittent hypoxia alters glutamatergic control of sympathetic and respiratory activities in the commissural NTS of rats. **Am J Physiol Regul Integr Comp Physiol**, v. 302, n. 6, p. 785-793, 2012.

DAMPNEY, R. A. Functional organization of central pathways regulating the cardiovascular system. **Physiol Rev**, v. 74, n. 2, p. 323-364, 1994.

DAMPNEY, R. A. et al. Cardiovascular effects of angiotensin II in the rostral ventrolateral medulla: the push-pull hypothesis. **Curr Hypertens Rep**, v. 9, n. 3, p. 222-227, 2007.

DANEMAN, R. The blood-brain barrier in health and disease. **Ann Neurol**, v. 72, n. 5, p. 648-672, 2012.

DE BRITO ALVES, J. L.; COSTA-SILVA, J. H. Maternal protein malnutrition induced-hypertension: New evidence about the autonomic and respiratory dysfunctions and epigenetic mechanisms. **Clin Exp Pharmacol Physiol**, 2017.

DE BRITO ALVES, J. L. et al. Maternal protein restriction induced-hypertension is associated to oxidative disruption at transcriptional and functional levels in the medulla oblongata. **Clin Exp Pharmacol Physiol**, v. 43, n. 12, p. 1177-1184, 2016.

DE BRITO ALVES, J. L. et al. Maternal protein restriction increases respiratory and sympathetic activities and sensitizes peripheral chemoreflex in male rat offspring. **J Nutr**, v. 145, n. 5, p. 907-914, 2015.

DE BRITO ALVES, J. L. et al. Maternal protein restriction increases respiratory and sympathetic activities and sensitizes peripheral chemoreflex in male rat offspring. **Journal of Nutrition**, v. 145, n. 5, p. 907-914, 2015.

DE BRITO ALVES, J. L. et al. Short- and long-term effects of a maternal low-protein diet on ventilation, O(2)/CO(2) chemoreception and arterial blood pressure in male rat offspring. **Br J Nutr**, v. 111, n. 4, p. 606-615, 2014.

DE GASPARO, M. et al. International union of pharmacology. XXIII. The angiotensin II receptors. **Pharmacol Rev**, v. 52, n. 3, p. 415-472, 2000.

DICK, T. E. et al. Entrainment pattern betwe en sympathetic and phrenic nerve activities in the Sprague-Dawley rat: hypoxia-evoked sympathetic activity during expiration. **Am J Physiol Regul Integr Comp Physiol**, v. 286, n. 6, p. 1121-1128, 2004.

DUPONT, A. G.; BROUWERS, S. Brain angiotensin peptides regulate sympathetic tone and blood pressure. **J Hypertens**, v. 28, n. 8, p.1599-1610, 2010.

FELDMAN, J. L. et al. Breathing: rhythmicity, plasticity, chemosensitivity. **Annu Rev Neurosci**, v. 26, p. 239-266, 2003.

FERGUSON, A. V.; BAINS, J. S. Actions of angiotensin in the subfornical organ and area postrema: implications for long term control of autonomic output. **Clin Exp Pharmacol Physiol**, v. 24, n. 1, p. 96-101, 1997.

FERREIRA, D. S. et al. Perinatal low-protein diet alters brainstem antioxidant metabolism in adult offspring. **Nutr Neurosci**, v. 19, n. 8, p. 369-375, 2016.

FIDALGO, M. et al. Programmed changes in the adult rat offspring caused by maternal protein restriction during gestation and lactation are attenuated by maternal moderate-low physical training. **Br J Nutr**, v. 109, n. 3, p. 449-456, 2013.

FIDONE, S. J. et al. Mechanisms of chemotransmission in the mammalian carotid body. **Prog Brain Res**, v. 74, p. 169-179, 1998.

FONTES, M. A. et al. Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats. **Physiol Genomics**, v. 2, n. 3, p. 137-142, 2000.

FOROUZANFAR, M. H. et al. Global Burden of Hypertension and Systolic Blood Pressure of at Least 110 to 115 mm Hg, 1990-2015. **JAMA**, v. 317, n. 2, p. 165-182, 2017.

FRANCHINI, K. G.; KRIEGER, E. M. Cardiovascular responses of conscious rats to carotid body chemoreceptor stimulation by intravenous KCN. **J Auton Nerv Syst**, v. 42, n. 1, p. 63-69, 1993.

FUKIYAMA, K. et al. Chronic hypertension elicited by infusion of angiotensin into vertebral arteries of unanaesthetized dogs. **Clinical Science**, v. 40, n. 3, p. 283-291, 1971.

FUNG, M. L. The role of local renin-angiotensin system in arterial chemoreceptors in sleep-breathing disorders. **Front Physiol**, v. 5, p. 336, 2014.

GAO, L. et al. Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and NAD(P)H oxidase in RVLM. **Am J Physiol Heart Circ Physiol**, v. 288, n. 5, p. 2271-2279, 2005.

GLUCKMAN, P. D.; HANSON, M. A. The developmental origins of the metabolic syndrome. **Trends Endocrinol Metab**, v. 15, n. 4, p. 183-187, 2004.

GOLDBLATT, H. et al. Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia. **J Exp Med**, v. 59, n. 3, p. 347-379, 1934.

GOMIDE, J. M. et al. Increased activity of the renin-angiotensin and sympathetic nervous systems is required for regulation of the blood pressure in rats fed a low-protein diet. **Exp Physiol**, v. 98, n. 1, p. 57-66, 2013.

GONZALEZ, C. et al. Carotid body chemoreceptors: from natural stimuli to sensory discharges. **Physiol Rev**, v. 74, n. 4, p. 829-898, 1994.

GOYAL, R. et al. Maternal protein deprivation: changes in systemic renin-angiotensin system of the mouse fetus. **Reprod Sci**, v. 16, n. 9, p. 894-904, 2009.

GOYAL, R. et al. Brain renin-angiotensin system: fetal epigenetic programming by maternal protein restriction during pregnancy. **Reprod Sci**, v. 17, n. 3, p. 227-238, 2010.

GOYAL, R. et al. Antenatal maternal hypoxic stress: adaptations of the placental renin-angiotensin system in the mouse. **Placenta**, v. 32, n. 2, p. 134-139, 2011.

GRASSI, G. Role of the sympathetic nervous system in human hypertension. **J Hypertens**, v. 16, p. 1979-1987, 1998.

GUYENET, P. G. et al. Central respiratory chemoreception. **J Comp Neurol**, v. 518, n. 19, p. 3883-3906, 2010.

HALES, C. N.; BARKER, D. J. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. 1992. **Int J Epidemiol**, v. 42, n. 5, p. 1215-1222, 2013.

HALES, C. N. et al. Fetal and infant growth and impaired glucose tolerance at age 64. **BMJ**, v. 303, n. 6809, p. 1019-1022, 1991.

HASELTON, J. R.; GUYENET, P. G. Central respiratory modulation of medullary sympathoexcitatory neurons in rat. **Am J Physiol**, v. 256, p. 739-750, 1989.

HE, D. H. et al. Long-term prehypertension treatment with losartan effectively prevents brain damage and stroke in stroke-prone spontaneously hypertensive rats. **Int J Mol Med**, v. 33, n. 2, p. 301-309, 2014.

HEDNER, T. et al. State of global health--hypertension burden and control. **Blood Press**, v. 21 Suppl 1, p. 1-2, 2012.

HEITMAN, S. J.; JENNINGS, D. B. Angiotensin II modulates respiratory and acid-base responses to prolonged hypoxia in conscious dogs. **Am J Physiol**, v. 275, p. 390-399, 1998.

HERNANDEZ SCHULMAN, I. et al. Cross-talk between angiotensin II receptor types 1 and 2: potential role in vascular remodeling in humans. **Hypertension**, v. 49, n. 2, p. 270-271, 2007.

HEUSSER, K. et al. Carotid baroreceptor stimulation, sympathetic activity, baroreflex function, and blood pressure in hypertensive patients. **Hypertension**, v. 55, n. 3, p. 619-626, 2010.

HYATT, M. A. et al. Early developmental influences on hepatic organogenesis. **Organogenesis**, v. 4, n. 3, p. 170-175, 2008.

ITO, S. et al. Ventrolateral medulla AT1 receptors support arterial pressure in Dahl salt-sensitive rats. **Hypertension**, v. 41, p. 744-750, 2003.

ITO, S. et al. Ventrolateral medulla AT1 receptors support blood pressure in hypertensive rats. **Hypertension**, v. 40, n. 4, p. 552-559, 2002.

ITURRIAGA, R.; ALCAYAGA, J. Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. **Brain Res Brain Res Rev**, v. 47, n. 1-3, p. 46-53, 2004.

JOHREN, O. et al. Cardiovascular and renal function of angiotensin II type-2 receptors. **Cardiovasc Res**, v. 62, n. 3, p. 460-467, 2004.

KISHI, T. et al. Sympathoinhibition caused by orally administered telmisartan through inhibition of the AT(1) receptor in the rostral ventrolateral medulla of hypertensive rats. **Hypertens Res**, v. 35, n. 9, p. 940-946, 2012.

KOGA, Y. et al. High salt intake enhances blood pressure increase during development of hypertension via oxidative stress in rostral ventrolateral medulla of spontaneously hypertensive rats. **Hypertens Res**, v. 31, n. 11, p. 2075-2083, 2008.

KOID, S. S.; CAMPBELL, D. J. Evolving concepts of the renin-angiotensin system: highlights from the pre-ISH 2012 satellite meeting. **J Renin Angiotensin Aldosterone Syst**, v. 14, n. 1, p. 93-96, 2013.

LACKLAND, D. T.; WEBER, M. A. Global burden of cardiovascular disease and stroke: hypertension at the core. **Can J Cardiol**, v. 31, n. 5, p. 569-571, 2015.

LAM, S. Y.; LEUNG, P. S. A locally generated angiotensin system in rat carotid body. **Regul Pept**, v. 107, n. 1-3, p. 97-103, 2002.

LAM, S. Y.; LEUNG, P. S. Chronic hypoxia activates a local angiotensin-generating system in rat carotid body. **Mol Cell Endocrinol**, v. 203, n. 1-2, p. 147-153, 2003.

LANGLEY-EVANS, S. C.; JACKSON, A. A. Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. **Comp Biochem Physiol A Physiol**, v. 110, n. 3, p. 223-228. 1995.

LANGLEY-EVANS, S. C. et al. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. **Life Sciences**, v. 64, n. 11, p. 965-974, 1999.

LEANDRO, C. G. et al. Moderate physical training attenuates muscle-specific effects on fibre type composition in adult rats submitted to a perinatal maternal low-protein diet. **Eur J Nutr**, v. 51, n. 7, p. 807-815, 2012.

LEENEN, F. H. Actions of circulating angiotensin II and aldosterone in the brain contributing to hypertension. **Am J Hypertens**, v. 27, n. 8, p. 1024-1032, 2014.

LEUNG, P. S. et al. Chronic hypoxia upregulates the expression and function of AT(1) receptor in rat carotid body. **J Endocrinol**, v. 167, n. 3, p. 517-524, 2000.

LIM, S. S. et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. **Lancet**, v. 380, n. 9859, p. 2224-2260, 2012.

MACHADO, B. H. et al. Autonomic processing of the cardiovascular reflexes in the nucleus tractus solitarii. **Braz J Med Biol Res**, v. 30, n. 4, p. 533-543, 1997.

MACHADO, B. H.; BONAGAMBA, L. G. Antagonism of glutamate receptors in the intermediate and caudal NTS of awake rats produced no changes in the hypertensive response to chemoreflex activation. **Auton Neurosci**, v. 117, n. 1, p. 25-32, 2005.

MALAN, A. Ventilation measured by body plethysmography in hibernating mammals and in poikilotherms. **Respir Physiol**, v. 17, n. 1, p. 32-44, 1973.

MALLIANI, A. et al. Cardiovascular neural regulation explored in the frequency domain. **Circulation**, v. 84, n. 2, p. 482-492, 1991.

MALPAS, S. C. The rhythmicity of sympathetic nerve activity. **Prog Neurobiol**, v. 56, n. 1, p. 65-96, 19989.

MARCUS, N. J. et al. Chronic intermittent hypoxia augments chemoreflex control of sympathetic activity: role of the angiotensin II type 1 receptor. **Respir Physiol Neurobiol**, v. 171, n. 1, p. 36-45. 2010.

MARTINEZ-MALDONADO, M. et al. Renal renin, angiotensinogen, and ANG I-converting-enzyme gene expression: influence of dietary protein. **Am J Physiol**, v. 264, p. 981-988, 1993.

MAYHAN, W. G. et al. Role of molecular charge in disruption of the blood-brain barrier during acute hypertension. **Circ Res**, v. 64, n. 4, p. 658-664, 1989.

MCKINLEY, M. J. et al. Physiological actions of angiotensin II mediated by AT1 AND AT2 receptors in the brain. **Clin Exp Pharmacol Physiol**, v. 23 Suppl 3, p. 99-104. 1996.

MENDELSOHN, F. A. et al. Autoradiographic localization of angiotensin II receptors in rat brain. **Proc Natl Acad Sci U S A**, v. 81, n. 5, p. 1575-1579, 1984.

MIFFLIN, S. W. Arterial chemoreceptor input to nucleus tractus solitarius. **Am J Physiol**, v. 263, p. 368-375, 1992.

MONTEZANO, A. C.; TOUYZ, R. M. Molecular mechanisms of hypertension--reactive oxygen species and antioxidants: a basic science update for the clinician. **Can J Cardiol**, v. 28, n. 3, p. 288-295, 2012.

MORAES, D. J. et al. Short-term sustained hypoxia induces changes in the coupling of sympathetic and respiratory activities in rats. **J Physiol**, v. 592, n. 9, p. 2013-2033, 2014.

MORGANE, P. J. et al. Prenatal malnutrition and development of the brain. **Neurosci Biobehav Rev**, v. 17, n. 1, p. 91-128, 1993.

MORITZ, K. M. et al. Review: Sex specific programming: a critical role for the renal renin-angiotensin system. **Placenta**, p. 40-46, 2010.

MOZAFFARIAN, D. et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. **Circulation**, v. 133, n. 4, p. 38-60, 2016.

MURALI, S. et al. Angiotensin II mobilizes intracellular calcium and activates pannexin-1 channels in rat carotid body type II cells via AT1 receptors. **J Physiol, v.** 592, n. 21,p. 4747-4762, 2014.

NANDURI, J.; PRABHAKAR, N. R. Epigenetic Regulation of Carotid Body Oxygen Sensing: Clinical Implications. **Adv Exp Med Biol**, v. 860, p. 1-8, 2015.

NISHI, E. E. et al. Losartan reduces oxidative stress within the rostral ventrolateral medulla of rats with renovascular hypertension. **Am J Hypertens**, v. 26, n. 7, p. 858-865, 2013.

NISHIHARA, M. et al. Oxidative stress in the rostral ventrolateral medulla modulates excitatory and inhibitory inputs in spontaneously hypertensive rats. **J Hypertens**, v. 30, n. 1, p. 97-106, 2012.

NUNES, F. C.; BRAGA, V. A. Chronic angiotensin II infusion modulates angiotensin II type I receptor expression in the subfornical organ and the rostral ventrolateral medulla in hypertensive rats. **J Renin Angiotensin Aldosterone Syst**, v. 12, n. 4, p. 440-445, 2011.

OSBORN, J. W. et al. Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. **Curr Hypertens Rep**, v. 9, n. 3, p. 228-235, 2007.

PAULINO-SILVA, K. M.; COSTA-SILVA, J. H. Hypertension in rat offspring subjected to perinatal protein malnutrition is not related to the baroreflex dysfunction. **Clin Exp Pharmacol Physiol**, v. 43, n. 11, p. 1046-1053, 2016.

PENG, Y. J. et al. Angiotensin II evokes sensory long-term facilitation of the carotid body via NADPH oxidase. **J Appl Physiol (1985)**, v. 111, n. 4, p. 964-970, 2011.

PENITENTE, A. R. et al. Malnutrition enhances cardiovascular responses to chemoreflex activation in awake rats. **Life Sciences**, v. 81, n. 7, p. 609-614, 2007.

PIROLA, L. et al. Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells. **J Biol Chem**, v. 278, n. 18, p. 15641-15651, 2003.

PLADYS, P. et al. Role of brain and peripheral angiotensin II in hypertension and altered arterial baroreflex programmed during fetal life in rat. **Pediatric Research**, v. 55, n. 6, p. 1042-1049, 2004.

PORTA, A. et al. Entropy, entropy rate, and pattern classification as tools to typify complexity in short heart period variability series. **IEEE Trans Biomed Eng**, v. 48, n. 11, p. 1282-1291, 2001.

PORTA, A. et al. Assessment of cardiac autonomic modulation during graded head-up tilt by symbolic analysis of heart rate variability. **Am J Physiol Heart Circ Physiol,** v. 293, n. 1, p. 702-708, 2007.

PRABHAKAR, N. R. et al. Peripheral chemoreception and arterial pressure responses to intermittent hypoxia. **Compr Physiol**, v. 5, n. 2, p. 561-577, 2015.

RAVELLI, G. P. et al. Obesity in young men after famine exposure in utero and early infancy. **N Engl J Med**, v. 295, n. 7, p. 349-353, 1976.

REEVES, P. G. et al. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **J Nutr**, v. 123, n. 11, p. 1939-1951, 1993.

SAKAI, K. et al. Local production of angiotensin II in the subfornical organ causes elevated drinking. **J Clin Invest**, v. 117, n. 4, p. 1088-1095, 2007.

SANGALETI, C. T. et al. Endogenous angiotensin and pressure modulate brain angiotensinogen and AT1A mRNA expression. **Hypertension**, v. 43, n. 2, p. 317-323, 2004.

SAWAYA, A. L. et al. Long-term effects of early malnutrition on body weight regulation. **Nutr Rev**, v. 62, p. 127-133, 2004.

SAWAYA, A. L.; ROBERTS, S. Stunting and future risk of obesity: principal physiological mechanisms. **Cad Saude Publica**, v. 19 Suppl 1, p. 21-28, 2003.

SHAVER, S. W. et al. Regional quantitative permeability of blood-brain barrier lesions in rats with chronic renal hypertension. **Brain Res**, v. 579, n. 1, p. 99-106, 1992.

SHEPHERD, J. T.; MANCIA G. Reflex control of the human cardiovascular system. **Rev Physiol Biochem Pharmacol**, v. 105, p. 1-99, 1986.

SHERMAN, R. C.; LANGLEY-EVANS, S. C. Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. **Clin Sci (Lond)**, v. 98, n. 3, p. 269-275, 2000.

SIMMS, A. E. et al. Is augmented central respiratory-sympathetic coupling involved in the generation of hypertension? **Respir Physiol Neurobiol**, v. 174, n. 1-2, p. 89-97, 2010.

SIMMS, A. E. et al. Amplified respiratory-sympathetic coupling in the spontaneously hypertensive rat: does it contribute to hypertension? **J Physiol**, v. 587, n. 3, p. 597-610, 2009.

SMITH, J. C. et al. Spatial and functional architecture of the mammalian brain stem respiratory network: a hierarchy of three oscillatory mechanisms. **J Neurophysiol**, v. 98, n. 6, p. 3370-3387, 2007.

SMITH, P. M.; FERGUSON, A. V. Circulating signals as critical regulators of autonomic state--central roles for the subfornical organ. **Am J Physiol Regul Integr Comp Physiol**, v. 299, n. 2, p. 405-415, 2010.

SOUSA, L. E. et al. Exercise training restores oxidative stress and nitric oxide synthases in the rostral ventrolateral medulla of renovascular hypertensive rats. **Free Radic Res**, v. 49, n. 11, p. 1335-1343, 2015.

SOUZA, H. C. et al. Cardiac sympathetic overactivity and decreased baroreflex sensitivity in L-NAME hypertensive rats. **Am J Physiol Heart Circ Physiol**, v. 280, n. 2, p. 844-850, 2001.

SU, Q. et al. Renin-angiotensin system acting on reactive oxygen species in paraventricular nucleus induces sympathetic activation via AT1R/PKCgamma/Rac1 pathway in salt-induced hypertension. **Sci Rep**, v. 7, p. 43107, 2017.

SUMNERS, C.; PHILLIPS, M. I. Central injection of angiotensin II alters catecholamine activity in rat brain. **Am J Physiol**, v. 244, n. 2, p. 257-263, 1983.

SWEET, C. S. et al. Central antihypertensive effects of inhibitors of the renin-angiotensin system in rats. **Am J Physiol**, v. 231, n. 6, p. 1794-1799, 1976.

SZARC VEL SZIC, K. et al. Nature or nurture: let food be your epigenetic medicine in chronic inflammatory disorders. **Biochem Pharmacol**, v. 80, n. 12, p. 1816-1832, 2010.

TSYRLIN, V. A. et al. Arterial baroreceptor reflex counteracts long-term blood pressure increase in the rat model of renovascular hypertension. **PLoS One**, v. 8, n. 6, p. 64788, 2013.

UENO, M. et al. Blood-brain barrier disruption in the hypothalamus of young adult spontaneously hypertensive rats. **Histochem Cell Biol**, v. 122, n. 2, p. 131-137, 2004.

VITAL, S. A. et al. Mechanisms underlying the cerebral microvascular responses to angiotensin II-induced hypertension. **Microcirculation**, v. 17, n. 8, p. 641-649, 2010.

WEST-EBERHARD, M. J. Alternative adaptations, speciation, and phylogeny (A Review). **Proc Natl Acad Sci U S A**, v. 83, n. 5, p. 1388-1392, 1986.

XIAO, X. et al. Low birth weight is associated with components of the metabolic syndrome. **Metabolism**, v. 59, n. 9, p. 1282-1286, 2010.

ZIMMERMAN, M. C. et al. Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. **Circ Res**, v. 95, n. 2, p. 210-216, 2004.

ZOCCAL, D. B. et al. Sympathetic-mediated hypertension of awake juvenile rats submitted to chronic intermittent hypoxia is not linked to baroreflex dysfunction. **Exp Physiol**, v. 94, n. 9, p. 972-983, 2009.

ZOCCAL, D. B. et al. Increased sympathetic outflow in juvenile rats submitted to chronic intermittent hypoxia correlates with enhanced expiratory activity. **J Physiol**, v. 586, n. 13, p. 3253-3265, 2008.

ZUCKER, I. H.; GAO, L. The regulation of sympathetic nerve activity by angiotensin II involves reactive oxygen species and MAPK. **Circ Res**, v. 97, n. 8, p. 737-739, 2005.

APÊNDICE A - ARTIGO 1

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FUNCTIONAL AND TRANSCRIPTIONAL EFFECTS OF MATERNAL LOW-PROTEIN DIET ON THE RENIN ANGIOTENSIN SYSTEM AND CARDIORESPIRATORY CONTROL

Monique Assis de Vasconcelos Barros[1]; José Luiz de Brito Alves[2]; Rayssa Gabryella Nery de Barros[1]; José Cândido de Souza Ferraz Junior[1]; Carol Virgínia Gois Leandro[1]; Hubert Vidal[3]; Luciano Pirola[3]; João Henrique Costa-Silva[1*].

[1]Department of Physical Education and Sport Sciences, Academic Center of Vitoria (CAV), Federal University of Pernambuco, 55608-680, Brazil.

[2]Department of Nutrition, Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, 58051900, Brazil.[3]Carmen (Cardiology, Metabolism and Nutrition), Laboratory; INSERM U1060; Lyon-1 University, South Lyon Medical Faculty; 69921 Oullins, France.

***Corresponding author**:

João Henrique Costa-Silva

Núcleo de Educação Física e Ciências do Esporte – CAV - UFPE

Rua Alta do Reservatório, S/N, Bela Vista, Vitória de Santo Antão, PE.

CEP: 55608-680

Phone/fax: 55 81 35233351

Email: joao.hcsilva@ufpe.br

Abstract

Aim**:** To investigate the involvement of renin angiotensin system (RAS) on cardiorespiratory control in the hypertension elicited by maternal low protein diet. Methods and Results: Male Wistar rats were divided into two groups according to the diets of their mothers during gestation and lactation: the control (NP Group, 17% casein) and low-protein (LP Group, 8% casein) groups. Direct measurements of arterial pressure (AP), heart rate and ventilatory parameters were recorded from wakeful 90-d-old male offspring at resting and after antagonism of type 1 angiotensin (AT1) receptors [by losartan potassium, 10 mg/kg, intravenous (iv) or intracerebroventricular (icv) infusion] or inhibition of angiotensin converting enzyme (ACE) by enalapril (5 mg/kg, iv). Cardiovascular variability was evaluated off-line by spectral analysis. Besides cytotoxic hypoxia [bolus injection of potassium cyanide (KCN); 0,04%] was used for activating cardiorespiratory neural network. Serum dosage of angiotensin II (Ang II) was performed by ELISA assay. At transcriptional level, RAS components in the brainstem was evaluated by rt-PCR. The LP offspring presented higher mean AP (MAP) and respiratory frequency than NP. In the spectral analysis, the LP group showed higher power at low (NP: 2.7±0.3 vs. LP: 5±1 $mmHg^2$) frequency of systolic AP. However, similar LF/HF ratio. After losartan, the LP animals showed larger delta in the MAP (NP: -9.8±2 vs. LP: -23±6 mmHg), and also bigger delta MAP after KCN when compared with NP group (NP: +68±8 vs. LP: +98±7 mmHg) and when compared with baseline. MAP have no significant change in both NP and LP after central blockade of AT1 receptors. However, the RF delta was bigger in both groups when compared with before drug period. Was not change at levels of serum Ang II between NP and LP groups. Similar gene expression of angiotensinogen, Ace, At1, At2 and Mas receptors was observed in the groups. However, LP animals demonstrated a decrease in RAc1 mRNA in the brainstem compared to NP animals. Conclusion: The RAS seems to participate peripherally and not centrally in the maintenance of arterial hypertension induced by protein restriction during the gestation and lactation, regardless of the levels of circulating Ang II.

Keywords: Protein undernutrition, developmental plasticity, renin angiotensin system, hypertension.

Introduction

Epidemiological studies and dietetic interventions in animals models have provided considerable evidences suggesting that maternal nutrition deficits occurring during window of human development can influence persistently in the offspring health (Langley-Evans, Welham et al. 1999; Sawaya, Martins et al. 2004; Barker, Osmond et al. 2009; de Brito Alves, Nogueira et al. 2014; Barros, De Brito Alves et al. 2015; de Brito Alves, de Oliveira et al. 2016; de Brito Alves and Costa-Silva 2017). This influence is referring to post-natal phenotype alterations and are associates with increase susceptibility diseases development in adult life (West-Eberhard 1986). Among the pathologies, highlight the arterial hypertension (AH), which remains a big global public health challenge (Mozaffarian, Benjamin et al. 2016; Forouzanfar, Liu et al. 2017).

Many studies with different populations have evidenced a close relationship between perinatal undernutrition and AH development in adult life (McMullen and Langley-Evans 2005; de Brito Alves, Nogueira et al. 2014; Barros, De Brito Alves et al. 2015; Costa-Silva, de Brito-Alves et al. 2015; Costa-Silva, Simoes-Alves et al. 2016). The participation of renal system in this context has been widely consistent among different research groups, demonstrating that protein restriction during development induces a compromise of nephrogenesis, glomerular filtration rate, and dysfunction of the renin angiotensin system (RAS) (Merlet-Benichou, Gilbert et al. 1994; Langley-Evans, Welham et al. 1999; Paixao, Maciel et al. 2001; Costa-Silva, Silva et al. 2009; Nuyt and Alexander 2009; Cornock, Langley-Evans et al. 2010; Vieira-Filho, Cabral et al. 2014). However, a hyperactivity of the sympathetic nervous system has been pointed as a main inductor factor this AH (Bugenhagen, Cowley et al. 2010; Heusser, Tank et al. 2010; Barros, De Brito Alves et al. 2015; Costa-Silva, de Brito-Alves et al. 2015). In post-weaning low-protein diet model, after pharmacological blocks was evidenced an increases the sympathetic and decreased the

parasympathetic tone. This data suggest that this diet may increases the sympathetic efferent activity to the heart and reduce the vagal modulation (Martins, Chianca et al. 2011). In the same way, data from our laboratory corroborates this data showing that protein malnutrition increased sympathetic activity in rats at short and long-term (Barros, De Brito Alves et al. 2015; de Brito Alves, Nogueira et al. 2015).

Within brain, the RAS plays an important role in arterial pressure regulation due to its ability to modulate sympathetic nerve activity (Dampney, Tan et al. 2007) Dupont and Sofie 2010. A lot of evidences has supported the notion that angiotensin II (Ang II) signaling dysregulation is one of the key mechanisms involved in stimulating the sympathetic nervous system (Fink 1997; Leenen 2014). This dysregulation of Ang II can induce production of reactive oxidative stress (ROS) by nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase activation in brain, leading to sympathoexcitation (Dai et al., 2015; Wang, L. H. et al., 2015). It is clear that NADPH oxidase–derived ROS production by Ang II plays a critical role in different models of cardiovascular dysfunction, such as salt-induced hypertension (Su, Huo et al. 2017), heart failure (Gao, Wang et al. 2005), stroke-prone spontaneously hypertensive rats (SHRPs) (Kishi, Hirooka et al. 2012). Thus, the modulation sympathetic activity and blood pressure by ROS in brainstem as rostral ventrolateral medulla (RVLM), key region of cardiovascular control has been well recognized (Koga, Hirooka et al. 2008; Nishihara, Hirooka et al. 2012; Sousa, Magalhaes et al. 2015). Studies from maternal protein undernutrition during gestation and lactation has demonstrated a decrease in the activity of several antioxidant enzymes with increase in the ROS of the offspring's brainstem (Ferreira, Liu et al. 2016). These effects were associated to medullary oxidative dysfunction at the transcriptional level and with impaired antioxidant capacity mainly in the ventral medulla (de Brito Alves, de Oliveira et al. 2016). However, the authors did not evaluate the role played by

RAS in this regions and its impact to the sympathetic tonus and the development of AH in the protein-restricted rats.

On the other hand, recently also have been proposed that generating and modifications in the baseline respiratory rithyms, and changes in the central and pheriferal chemeossensibility can induces a hiperactivity of the sympathetic nervous system and contribute to AH (Zoccal, Simms et al. 2008; Simms, Paton et al. 2009; Costa-Silva, Zoccal et al. 2010; Zoccal and Machado 2011; Costa-Silva, Zoccal et al. 2012). Experimental studies have showed bigger expression of RAS components localized in the in the carotid body which is significantly functional to Ang II effects in the chemorreflex and peripheral chemoreceptors activity regulation (Fung 2014). Studies from our laboratory also have evidenced an increase ventilatory and pressor responses to peripheral chemoreceptor activation, suggesting that an undernutrition might affect the chemoreceptor maturation and predispose the increased arterial blood pressure in the adult life (de Brito Alves, Nogueira et al. 2014; de Brito Alves, Nogueira et al. 2015). Besides that, recently we observed that carotid body removal is able normalize respiratory frequency and response to CO_2 in the short-term as well as improve in arterial pressure and sympathetic hyperactivity over the long-term, suggesting that hyper-sensibility this chemoreceptor can be involved in development protein malnutrition induced-hypertension (data not published).

Therefore, considering the importance of RAS on the cardiorespiratory control, we hypothesized that a maternal protein undernutrition during pregnancy and lactation increases the peripheral and central RASs, which contributes to increase sympathetic-respiratory activities and consequently hypertension development in the offspring.

Methods

Animals and Diets

Rats Wistar were used and all experimental protocols were approved by the Ethical Committee of the Federal University of Pernambuco, Brazil. Two groups were formed based on dietary manipulations: mothers fed a 17% casein diet (normal-protein group, NP) and mothers fed an 8% casein isoenergetic diet (low-protein group, LP) *ad libitum.* During the suckling period, the mothers continued to be provided with the experimental diet of either 8% of casein or 17% of casein. The diets were made according to the American Institute of Nutrition – (AIN-93) (Reeves, Nielsen et al. 1993). At weaning, male offspring received a standard diet (Labina; Purina Agriband) and water *ad libitum* until 90-d-old.

Measurement of arterial pressure and heart rate

Male Wistar rats at 90 days old (d) were anesthetised with ketamine (80 mg/kg) and xylazine (10 mg/kg) to insert femoral artery and vein catheters. After 18-24h, the arterial pressure (AP) and HR were recorded in conscious animals by appropriate system (LabChart 7 Pro; ADInstruments, Bella Vista, NSW, Australia). Each animal was placed in the recording chamber for a period of acclimatisation (approximately 60 min). The pulsatile AP was recorded for 60 min under basal conditions, and the values of the mean arterial pressure (MAP) and HR were calculated by selection of the 10 min of this period.

Spectral and symbolic analyses of cardiovascular variability

The cardiovascular autonomic evaluation was performed using the frequency domain analysis of the heart rate and systolic arterial pressure (SAP) by appropriate software program

(CardioSeries-v.2.4; www.danielpenteado.com). The spectra were integrated in the very-low-frequency (VLF; < 0.2 Hz) oscillations that correspond to influences hormonal, thermoregulation and circadian, low-frequency (LF; 0.2–0.75 Hz) oscillations related to cardiac and vasomotor sympathetic modulation of the heart and blood vessels and a high-frequency (HF; 0.2–0.75 Hz) oscillations corresponding to respiration and associated with cardiac vagal modulation. To assess the sympathovagal index, the LF/HF ratio of the variability was calculated. Moreover, was used the symbolic analysis, a non-linear method based on the conversion of the series into a sequence of symbols (Porta, Tobaldini et al. 2007).

Analysis of respiratory parameters

Measurements of pulmonary ventilation (VE) were obtained using the whole-body plethysmography method as described by Malan (Malan 1973). Before recording the data, the animals were placed into the Plexiglas chamber (5 litres) for a period of acclimatization (approximately 60 min), and the chamber was flushed with humidified air and maintained at 25°C. Afterwards, to record the measurements of VE, air flow was suspended for short periods (3 min), and pressure oscillations caused by the breathing of the animals were captured by an apparatus connected to the chamber, with a pressure differential transducer and a signal amplifier (ML141 spirometer, PowerLab; ADInstruments). Then, the signal was captured into an acquisition system for data analysis (PowerLab; ADInstruments).

Respiratory frequency (RF), tidal volume (VT) and VE were determined in the room air condition (baseline). All data were analysed off-line with LabChart software (LabChart 7 Pro; ADInstruments). A period of 10 s was selected for the determination of mean RF. VT

was calculated from the pressure oscillation caused by the breathing of rats and using the formula described previously by Malan (Malan 1973). VE was obtained by multiplying RF by VT, and it is presented at ambient barometric pressure and at body temperature and pressure saturated.

Evaluation of the renin angiotensin system on arterial pressure

After baseline recordings of cardiorespiratory parameters, it was performed the antagonism angiotensin receptors type 1 (AT1) by losartan potassium (10 mg/kg, iv) and the MAP recorded during the next 30 min. In the other group was evaluated the effects of the angiotensin converting enzyme (ACE) inhibitor by enalapril (5 mg/kg, iv) during the same time. Both drugs were purchased by Sigma– Aldrich, St. Louis, USA.

Cardiorespiratory responses to the cytotoxic hypoxia

The *cytotoxic hypoxia* was induced by intravenous injection of potassium cyanide (KCN, 0.04%, 100 ul/rat) in order to produce a strong activation of the peripheral chemoreceptors located at carotid bodies. All cardiorespiratory responses such pressor, bradycardic, and tachypneic responses were recorded and the magnitude of the changes in MAP, RF and HR in response to chemoreflex activation was quantified at the peak of the responses. The delta was quantified by difference between the peak and baseline values (effects on MAP, HR and RF). This activation was performed before and after central and peripheral inhibition of the AT1 and ACE (de Brito Alves et al 2015).

Measurement of serum Ang II

At 85-d-old, offspring from both NP (n=10) and LP (n=10) groups were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and blood samples (approximately 1 ml) were collected by plexus retro-orbital disruption. Serum samples were collected for the quantification of Ang II and the concentrations was measured by enzyme-linked immunosorbent assay, according to the manufacturer's instructions. For the estimation of angiotensin-II in rat serum, ELISA plates were incubated with 100 µl of anti-Angiotensin-II antibody, washed and then incubated with sample. HRP-streptavidin was added following which substrate was added to each well according to the manufacturer's instructions (rab0010, Sigma Chemicals). After adding stop solution, absorbance was measured at 450 nm (ELISA kit, Sigma– Aldrich, St. Louis, USA).

Intracerebroventricular microinjection of AT1 receptor antagonist

At 90-d-old, part of animals from both NP (n=5) and LP (n=5) groups, the central effects of AT1 receptors blockade was investigate by microinjection of losartan in lateral ventricle. Initially, for guide cannula implantation for intracerebroventricular injection, rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). The rat was placed into a stereotaxic apparatus and a guide cannula (26-gauge stainless-steel tubing) was lowered to a position in the lateral ventricle (1.0 mm caudal to the bregma, 2.0 mm lateral to the midline, and 5.0 mm below the cerebral surface). Five days after surgery, experiments were conducted. After baseline arterial pressure recording rats received by icv injection of 50 µg (in 5 µl) of losartan and the cardiorespiratory responses at resting (during 30 min) and after activation of the peripheral chemoreceptors were recorded. The accuracy of the icv cannulation was

checked at autopsy with an icv injection of methylene blue (Chang Gyu Park, Frans H. H. Leenen 2001).

Medulla-tissue preparation

At 90-d-old, part of animals from both NP (n=5) and LP (n=5) groups were sacrificed by decapitation. Medullas were quickly collected, snap-frozen in liquid nitrogen and stored at – 80°C until RNA extraction. All rats were euthanized between 2:00 and 5:00 pm after a 4-5 hours fasting period. Rat medulla-tissue was collected using the calamus scriptorium (CS) as reference. We used approximately 2 mm rostral and 3 mm caudal from CS, as described in the stereotaxic atlas. For the gene expression analysis, we used the full medullas.

RNA extraction, reverse transcription and quantitative PCR (qPCR)

Total RNA was extracted with Tripure reagent (Roche, Meylan, France) according to the manufacturer's instructions. Briefly, 10µL of Trizol were used per mg of tissue and the resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France). After grinding, ¼ volume of chloroform was added, the preparation vortexed 3 x 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15,000 g at 4°C. The RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba, Val-de-Reuil, France) and centrifugation (15 min at 15,000g at 4°C). RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba), dried, and dissolved in 100 µl of RNase-free water.

Reverse transcription was performed using a RT-TAKARA kit (Primescript TM, Dalian, China) using 1µg of RNA as template and following the manufacturer's instructions. Briefly, samples were heated for 10 min at 65 °C. 4µL PrimeScript Buffer 5x, 1µl oligo-dT (50 µM), 4µl random hexamers and 1µl of PrimeScript RT Enzyme Mix were sequentially added, followed by a 15 min incubation at 37 °C and 15 s at 85 °C. RNA was removed by incubation with 1µL of RNase H for 20 minutes at 37 °C. Reverse transcription reactions were brought to a 200 µl final volume by adding RNase free water, and stored at -20°C. Real-time quantitative PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). Sequences of primers used in this study are reported in table 1.

PCR reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58-62 °C depending on the primer sets, 30 s) and elongation (72 °C, 30 s). mRNA expression levels of type 1a angiotensin II receptor (Agtr1a), type 2 angiotensin II receptor (Agtr2), angiotensinogen (Agt), angiotensin I converting enzyme (Ace), gamma protein kinase C (Prkcg), ras-related C3 botulinum toxin substrate 1 (Rac1) and protein tyrosine phosphatase, non-receptor type 6 (Ptpn6) and proto-oncogene, G protein-coupled receptor (Mas1) were quantified from medulla-derived cDNAs. Beside that, mRNA expression levels of angiotensinogen (Agt) also was quantified from liver-derived cDNA. All results are represented as arbitrary units (A.U.) derived from a standard calibration curve derived from a reference sample. A PCR for each sample was carried out in duplicate for all cDNAs and for the mix of ribosomal protein L19 (RPL19) and actin beta (Actb) as loading control. As a further control, qPCR amplicons were analyzed by electrophoresis on 1% agarose gel (data not shown).

Proteins extractions and Western Blotting procedures

Proteins in supernatants from RNA purification procedures were precipitated by isopropanol addition and centrifugation (15,000 *g*, 15 min at 4 °C). Pellets were washed with 0.3 M guanidine hydrochloride in 95% ethanol. After centrifugation (3000*g*, 5 min), proteins were solubilized in 1% sodium dodecyl sulfate (SDS). Protein concentrations were determined using the Bradford method. Bovine serum albumin was used as a standard and optical densities were read at 595 nm on a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA).

Protein samples were adjusted to a final concentration of 1 µg/µl. After addition of Laemmli sample buffer (150 mM Tris HCl, 36% glycerol, 3% SDS, 12% β- mercaptoethanol, 0.03 % bromophenol blue) and denaturation, proteins were separated by standard SDS-PAGE as previously described (Pirola, Bonnafous et al. 2003). Protein size markers (Precision Plus Protein Standards, Biorad) were deposited in parallel. Separated proteins were then transferred to PVDF membranes by semi-dry blotting using a Biorad Transblot Turbo Blotting apparatus. The following primary antibodies were used: anti p44/42 MAPK (sc-9102, Santa Cruz biotechnologies) and anti phospho p44/42 (sc-9101, Santa Cruz Biotechnologies). Anti-rabbit secondary antibody was used and revelation was made using the ECL reagent Lumina Forte (Merck Millipore, Darmstadt, Germany). Chemiluminescence was acquired on a ChemiDocTM XRS+ camera using the Image Lab 4.1 software (Biorad).

Statistical analysis

The results were expressed as the mean ± the standard error of the mean (SEM) and compared using Student's unpaired or paired t test. These tests were performed after analysis of data distribution (Kolmogorov Smirnov and Shapiro - Wilk tests). The comparisons were

performed using GraphPad Prism software (GraphPad, v.5), and differences were considered significant at $p < 0.05$.

Results

Baseline cardiorespiratory parameters

At 90 d, the male rats born to the low-protein diet dams exhibited an increase in the MAP (NP: 102±1 vs. LP: 108±2 mmHg; n=19; p<0.0174), but similar HR compared with the NP group (NP: 372±4 vs. LP: 384±11 bpm; n=9). The RF also was higher in the LP than NP (NP Group: 103.5±2 vs. LP Group: 116±4 resp/min; p<0.0122), but the VT and VE was similar between the groups.

Cardiovascular variability

The spectral analyses did not reveal differences in the VLF and HF oscillations of the SAP, but the LF oscillations (NP: 2.7±0.3 vs. LP: 5±1 $mmHg^2$; n=7-16; p=0.0145) was increased in the LP animals during baseline period. Interestingly this alteration was abolished after AT1 receptor antagonism and after ACE inhibition. Thus, no difference was observes between groups in VLF, LH and HF oscillations after losartan and enalapril (Figure 1A). Regarding pulse interval not changes was observed in LF (NP: 20.19±2.21 vs. LP: 24.25±5.59 nu; n=8-16) and HF (NP: 79.81±2.21 vs. LP: 75.75±5.59 nu n=8-16) oscillations (Figure 1B), which reverberated in a LF/HF ratio (NP: 0.28±0.03 vs. LP: 0.39±0.11) similar between groups, during beseline and after drugs (Figure 1B). Similar results were also observed in the patterns of sequences 0V (NP: 24.99±3.49 vs. LP: 28.11±6.27 %) and 2V (NP: 28.96±2.60 vs. LP: 28.40±4.72 %; n=8-16) of symbolic analysis, not presenting differences between NP and LP groups at three moments (Figure 1C).

Evaluation of the renin angiotensin system on arterial pressure

The figure 2 shows that the LP animals had bigger delta variation of MAP after AT1 receptor block with losartan (NP: -9.8±2 vs. LP: -23±6 mmHg; n=6-10; p=0.0174), without changes in RF and HR when compared with NP group (Figure 2C). However, not differences it was observed after ACE inhibition with enalapril in MAP (NP: -14.89±3 vs. LP: 18.6±6 mmHg; n=5-9), RF and HR (Figure 2D), suggesting an important involvement of the AT1 receptors in the increased of arterial blood pressure undernutrition animals.

Cardiorespiratory responses to the hypoxic hypoxia

During chemoreflex activation with administration of KCN at baseline period, no change was observed in pressor, tachypneic and bradycardic response between NP and LP groups. However, after block AT1 receptors by losartan, the LP animals presented bigger pressor response (delta MAP) when compared with NP group (NP: 68±8 vs. 98±7 mmHg; n=5-8) and when compared with baseline period. Differently, after inhibition of ACE by enalapril administration, not changes have observed between groups (Figure 3).

Serum concentration of the angiotensin II

NP and LP animals present similar serum concentration of the Ang II (NP: 464.9±63.28 vs. LP: 316.5±39.27 pg/ml; n=19; p=0,0511), suggesting that a perinatal undernutrition not change levels of circulating Ang II.

Intracerebroventricular microinjection of AT1 receptor antagonist

At central level, microinjection of losartan in the lateral ventricle in order to block AT1 receptors did not change the arterial pressure between groups. The LP animals presented the same high arterial pressure when compared with NP group during baseline period and after ICV microinjection of losartan (NP: 109 ± 6 vs. LP: 129±4 mmHg; n=5). The chemoreflex activation after the AT1 receptor block no showed differences in pressor, bradycardic, and tachypneic responses between NP and LP animals, however, the NP and LP animals presented bigger RF delta when compared to baseline period, how show the Figure 4.

Evaluation of mRNA in the liver and medulla tissue

In the liver of 90 d-old rats, de mRNA expression of angiotensinogen was similar between groups (NP: 1.07 ± 0.09 vs. LP: 0.87 ± 0.07), as show the Figure 5. This similar result was observed in medulla tissue these animals, demonstrating that the protein malnutrition no change the gene expression of angiotensinogen at level central and peripheral. In the medulla the mRNA expression of Ace, Agtr1a and Agtr2 also were similar in the NP and LP groups. However, when analyzed pathway signaling proteins, the gene expression of Rac1 in the LP animals was smaller than NP animals (NP: 1.13± 0.06 vs. LP: 0.88 ± 0.08; n= 5; p= 0,04), while the gene expression of Prkcg and ptpn6 were similar between groups (Figure 10). Besides that, the mRNA expression of Mas1 in the medulla also did not change in control and malnutrion animals (NP: 1.182 ± 0.05 vs. LP: 1.093 ± 0.13), suggesting that the angiotensin (1-7) pathway is no involved at cardiovascular alterations presented in hypertensive animals induced by maternal diet (Figure 5).

Evaluation of protein expression in the medulla tissue

Low protein animals presented decrease of p44 and p42 MAPK in medulla tissue when compared with normalprotein group. However, no difference was observed in relation MAPK/phophoMAPK of p44 and p42 proteins.

Discussion

The LP group presented mainly bigger hypotensive response after blockade of AT1 receptors and larger pressor in response to chemoreflex activation also after block AT1 receptor, suggesting an important influence of RAS on cardiovascular system of these animals. It is know of the broad susceptibility of RAS to maternal protein restricted, which present altered in multiple fetal organs including the brain, kidney, lung and heart. In fact, an accumulation of evidence indicates that the RAS of hypertensive offspring subjected to a protein restriction is altered locally or systematically (Goyal, Galffy et al. 2009; Goyal, Goyal et al. 2010; Moritz, Cuffe et al. 2010; Goyal, Lister et al. 2011).

It is known that the cardiovascular homeostasis is achieved by a variety of feedback systems, such as the baroreceptor reflex, chemoreflex, hormonal systems, such as RAS and act controlling the arterial pressure acting on different time scale (Julien, Malpas et al. 2001). The BP regulation by the RAS depends on the synthesis and release of renin and angiotensinogen, which needs to be converted to the active metabolite, Ang II. Thus, it can be expected that the RAS affects BP at lower frequencies than the sympathetic nervous system. Studies by Elghozi et al, who demonstrated in conscious rats that VLF oscillations in BP increased if the RAS was stimulated experimentally and that this increase in VLF could be blocked by AT1 receptor antagonists (Faraci, Baumbach et al. 1989; Ponchon and Elghozi

1996; Ponchon and Elghozi 1997). Therefore, the evaluation of VLF oscillation in systolic BP by spectral analysis strongly indicate a modulation RAS on BP. However, it is important highlight that VLF does not exclusively reflect the RAS, because catecholamines, nitric oxide and myogenic vascular function also affect the BP variability in this range frequency in rats (Stauss 2007). In this sense, not changes has were observed between LP and NP groups in VLF oscillations on systolic BP in the present study, suggesting that this hormonal influence not contribute to increase arterial pressure in the undernutrition offspring.

Data from our laboratory have showed that hypertension offspring submitted to protein restricted diet during pregnancy and lactation present a sympathetic overactivity at 30 and 90 days old (Barros, De Brito Alves et al. 2015; de Brito Alves, Nogueira et al. 2015), and discard that this sympathetic predominance is related to the baroreflex dysfunction (Paulino-Silva and Costa-Silva 2016). Knowing that the sympathoexcitatory effect of Ang II has long been recognized (Fink 1997; Dampney, Tan et al. 2007; Burke, Evans et al. 2008; Nunes and Braga 2011; Leenen 2014), we peripherally blocked the AT1 receptors and inhibited the ECA by losartan and enalapril, respectively, in order to investigate the RAS participation in this context. Although the angiotensinergic influence have not been observed by spectral analysis during baseline, after block AT1 receptor, the BP of LP animals was normalized, resulting at a bigger delta in MAP in the undernutrition offspring. This data tightly indicate the participation of Ang II in the elevated pressor levels these animals. Differently, after block of ACE with enalapril, not changes was observed between groups, indicating that this enzymatic influence not plays a significant role at BP levels in LP animals. These findings are in accordance with previous results reporting that administration of losartan and enalapril either intravenous or by chronic treatment, reduces the MAP of rats undernutrition during pregnancy or post-weaning (Ceravolo, Franco et al. 2007; Gomide, de Menezes et al. 2013).

Besides that, It had already been demonstrate that the respiratory system also can modulate markedly the discharge of the sympathetic nerve (Haselton and Guyenet 1989; Dick, Hsieh et al. 2004; Zoccal, Simms et al. 2008), thus, alterations in the generation and modulation of respiratory function may contribute to the development of hypertension (Simms, Paton et al. 2009; Simms, Paton et al. 2010; Costa-Silva, Zoccal et al. 2012; Moraes, Bonagamba et al. 2014). In this sense, it is important to hightlight that already was demonstrate a abudant presence of Ang II in respiratory areas, as nucleus parabrachialis medialis, Bötzinger complex and Kölliker-Fuse area, suggesting a role for this octapeptide in controlling respiration (Aguirre, Covenas et al. 1989). These regions are broadly influenced by peripheral chemoreceptors, which contain a high density of AT1 receptors located in the glomus cell (Allen 1998). Studies has demonstrated the activation of these receptors by physiological concentrations of Ang II induces predominantly excitatory effect on afferent chemoreceptor activity (Allen 1998) by increased level intracellular calcium (Fung, Lam et al. 2001). Also is evidenced that the chemoreceptor type II cells also express functional AT1 receptors, and stimulation of which causes release of Ca^{2+} from intracellular stores. In addition, AT1 receptors signalling in type II cells activates an inward current carried by pannexin-1 channels which are known to act as conduits for release of ATP, a key CB excitatory neurotransmitter. Thus, the excitatory function of Ang II in the CB seem involve dual actions at both type I and type II cells (Murali, Zhang et al. 2014).

In this sense, our laboratory has evidenced that offspring from protein-restricted dams exhibit enhanced baseline sympathetic and inspiratory motor activities combined with amplified ventilatory and autonomic responses to peripheral chemoreflex activation before the establishment of hypertension, of which the sympathetic hyperactivity and tachypnea remains during stage hypertensive (de Brito Alves, Nogueira et al. 2014; Barros, De Brito Alves et al. 2015; de Brito Alves, Nogueira et al. 2015). Considerating that AT1 receptors are present in

both cells that comprise the carotid body, allowing Ang II exerts a powerful effect in regulating excitability of chemoreceptors (Lam and Leung 2003), we investigated the influence of RAS components on functionally these receptors. It was observed that LP animals present pressor, tachypneic, bradycardic responses similar to NP group during chemoreflex activation at before and after ACE inhibition, suggesting a normal physiologic function of peripheral chemorecetor and absence of angiotensinergic influence in this functionality. However, after AT1 receptors block, the animals undernutrition during pregnancy and lactation presented larger pressor during chemoreflex activation when compared to NP group and to baseline period. It is known that the use of KCN is a recognized tool to evaluted different aspects of the complex pattern of cardiovascular, respiratory, and behavioural responses to chemoreflex activation (Barros, Bonagamba et al. 2002). This activation chemosensitive cells depolarize of chemoreceptor afferents, causing first synapses to occur in the nucleus tractus solitarii (NTS) (Finley and Katz 1992; Mifflin 1992; Vardhan, Kachroo et al. 1993; Ciriello, Schultz et al. 1994) and triggering sympathetic and parasympathetic activity that will result in elevation of blood pressure and bradycardia, respectively. In this sense, the role sympathoexcitatory of Ang II in in the carotid body at response to hypoxia have been previously shown (Li, Xia et al. 2006). However, a study published with chronic kidney disease model was observed that acute administration losartan not altered the pressor response at peripheral chemoreflex activation in animals controls, but it is capable of improving this response in animal with chronic kidney disease, demonstrating a sympathoinhibition by Ang II and suggesting a participation of RAS central in these results (Yao, Hildreth et al. 2015). Thus, it is possible that the increased pressor response observed during chemoreflex activation after AT1 receptor block it is caused by central action of losartan, blocking the action of Ang II central and consequently changing the sympathetic nerve activity response in the LP rats.

First, to verify whether the angiotensinergic predominance observed in the LP animals could be secondary to alteration levels circulating Ang II, we evaluated the serum levels of this peptide. Interestingly, no differences were found between groups, presenting it within the normal range and suggesting that the cardiovascular alterations presented by LP animals seem not to be depend of serum Ang II. Different from our results, at study with post weaning malnutrition model has been found a lower concentration of circulating Ang II associated with a overexpression of AT1 receptors in animals maternal diet induced-hypertension (Gomide, de Menezes et al. 2013). It is known that centrally, the RAS plays an important role in the regulation of arterial pressure through their ability to modulate the activity of the sympathetic nerve, especially in brain areas involved in the generation and modulation of sympathetic discharges to the cardiovascular system (Dampney, Tan et al. 2007), and that that Ang II act how a central neurotransmitter in this pathway. A lot of evidence supports Ang II signalling dysregulation as one of the key mechanisms involved in stimulating the sympathetic nervous system within the brain (Fink 1997; Leenen 2014). Thus, central angiotensinergic activation can increase the sympathetic activity and contribute to AH (Leenen 2014). In this sense, considering that systemic losartan crosses the blood-brain barrier and can acts at AT1 receptors within brain (Li, Bains et al. 1993), it is possible that LP animals would be present central RAS alteration.

In order to investigate the involvement of central Ang II in these alterations, was performed icv administration of losartan, but not change was observed in MAP, RF and HR after central block of AT1 receptor. Besides that, during chemoreflex activation after this angiotensinergic block, both groups presented similar delta pressor and bradycardic. Regarding tachypnea, both groups presented an increase when compared to baseline, however, there was no difference between the groups. Although the literature evidences a series of central angiotensinergic alterations in cardiorespiratory control in models of

malnutrition and hypertension, our data do not suggest the participation of the central RAS in these dysfunctions. However, in model of post-weaning protein restrict it was demonstrate that the increase in the expression of AT1 receptor is associated with sympathetic overactivity, suggesting that RAS and sympathetic nervous system contribute to the high BP observed at malnutrition animals (Gomide, de Menezes et al. 2013). This overactivity of both systems also was observed in later childhood at children with low birthweight (Franco, Casarini et al. 2008). Pladys 2004 also showed that the peripheral blockade of Ang II decreases the blood pressure of malnourished hypertensive rats during gestation. Together, centrally observed a decrease in blood pressure after blocking AT1 receptors, as well as an increase in the expression of these receptors in the areas of cardiovascular regulation, demonstrating a greater tonic role of both peripheral and central RAS in the maintenance of hypertension induced by malnutrition (Pladys, Lahaie et al. 2004).

In this sense, for a better understanding, we investigated the gene expression of the components of the RAS in the brainstem. It is know that AGT is expressed in astrocytes as well as in some neurons, particularly in regions of the brain controlling cardiovascular and metabolic function (Stornetta, Hawelu-Johnson et al. 1988; Yang, Gray et al. 1999; Lavoie, Cassell et al. 2004). There is evidence that AGT expression in brain may be regulated independently of the AGT from liver, besides that, its released from astrocytes into the extracellular space can be cleaved by neuron-derived renin (Schelling, Clauser et al. 1983). Previous studies have demonstrated that an overexpression of the human RAS in glial cells induces hypertension, while a remove this system in the glia avoids the hypertension in mice (Morimoto, Cassell et al. 2001; Sherrod, Davis et al. 2005). In the present study, the mRNA level of ANG was not changed by protein malnutrition at medulla and liver tissues. This similar results was observed in Ace gene expression, a key enzyme of the brain RAS and widely expressed in many regions of the brain including areas that regulate BP (Chai,

Mendelsohn et al. 1987; Mendelsohn, Allen et al. 1990). About receptors, it is know that in the brain the Ang II binds mainly to AT1 and AT2 receptors. In rodents, the AT1 receptor has two subtypes, the AT1A and AT1B. The AT1A accounts for 90% of the total binding and is predominant in some areas of the brain (Balakumar and Jagadeesh 2014). In this sense, we investigate the mRNA level of Agtr1a and Agtr2 genes. Over again was not find differences in gene expression between NP and LP animals, suggesting that low protein diet during gestation and lactation not alter the mRNA levels of RAS components at brainstem in adult life.

It is well recognized that ROS in ventral medulla can modulate sympathetic activity and blood pressure (Chan and Chan 2012; Nishihara, Hirooka et al. 2012; Braga 2013; Sousa, Magalhaes et al. 2015). Recent data from our laboratory have demonstrate that maternal protein restriction induced-hypertension is associated with medullary oxidative dysfunction at transcriptional level and with impaired antioxidant capacity in the ventral medulla (de Brito Alves, de Oliveira et al. 2016). Therefore, ROS in ventral medulla can modulate sympathetic activity and blood pressure. In this sense, worth highlighting that Ang II has been recognized as a potent inducer of ROS (Montezano e Touyz, 2012). It is know that Ang II on AT1 receptor stimulate ROS production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nguyen Dinh Cat, Montezano et al. 2013), which is composed of membrane-bound subunits, gp91phox and p22phox, cytoplasmic subunits, p47phox, p40phox, p67phox, and Rac1 and/or Rac2 (Su, Huo et al. 2017). Several evidence has indicate that the increased Ang II in brain induces the NADPH-dependent ROS production by the PKC which is a critical step in Rac1 activation and subsequent enzyme assembly. Thus, in order to verify if this pathway could be involved in sympathoexcitation presented in LP animals, we evaluated the mRNA of PKC gamma and Rac1. Concerning PKCg, both groups presented similar values of mRNA, however, surprisingly the LP animals presented decrease in gene

expression of Rac1 in brainstem, suggesting that this animals can present a less NADPH oxidase activation.

However, the central actions of Ang II on blood pressure and hypertension are no totally restricted to AT1R, as imagined. Activation of the AT2 receptors has been reported as having opposite effects to that of the AT1 receptors (S. Miura et al, 2013) and appears to have a protective effect in conditions such as hypertension (de Kloet, Steckelings et al. 2017). Overexpression of AT2 receptor solitary-vagal complex prevented renovascular hypertension and reverted the impairment of the baroreflex in rats (Blanch, Freiria-Oliveira et al. 2014). It is know that the in the brain, the main role these receptors is in regulation the sympathetic nervous system, which the AT1R stimulates and AT2 inhibits. In this sense, once activated, the AT2 receptor stimulate the SHP-1, which inhibits the NADPH oxidase promoting oxidative stress defense (Mehta and Griendling 2007). However, in this study the LP and NP animals presented similar mRNA levels of Agtr2 and ptpn6, suggesting that this pathway no influence the sympatho-respiratory alterations observed in malnutrition animals.

Angiotensin-(1–7) is another active hormone within the RAS. Ang-(1–7) is cleaved from angiotensin II by angiotensin-converting enzyme 2 (ACE 2) and binds to the receptor MAS (Santos RAS, et al, 2003). Interestingly, this receptor mediates similar action of AT2 and is considered other protective arm of the RAS. Recently has been questioned the existence of a possible cooperation or interaction between them in brain cardiovascular control centers to lower blood pressure (de Kloet, Steckelings et al. 2017). In this sense, a very recent article demonstrates dimerization between Mas and AT2R, suggesting that both receptors functionally depend on each other (Leonhardt, Villela et al. 2017). Therefore, knowing the importance of this way, we also investigate the mRNA the Mas, however, such as expression of the other receptors of RAS, the low protein diet during perinatal period did not change de gene expression this receptor.

The Ang II produces different actions by acting on its subtypes of receptor, however, the well-known physiological actions of Ang II are mainly mediated through AT1 receptors. This binding induces the signal transduction via different kinases, including the mitogen-activated protein kinases (MAPK) (Higuchi, Ohtsu et al. 2007). Previous studies have demonstrated the critical role of p44/42 MAPK signaling in mediating the central effects of Ang II on blood pressure. In rats with heart failure, the ICV administration of p44/42 MAPK inhibitors, PD98059 and UO126, significantly decreased the mean AP, heart rate, and renal sympathetic nerve activity. Together, losartan pretreatment attenuated the effects of PD98059, suggesting that Ang II may induce the activation of intracellular p44/42 MAPK activity to increase neuronal excitation in the PVN and sympathetic nerve activity (Wei, Yu et al. 2008). In order to investigate the levels this protein in our animals, we performed western blot assay for expression of p44/42 MAPK and phospho p44/42 MAPK in medulla tissue. Interestingly, the LP animals presented a decrease in expression of p44/p42 MAPK associated with similar levels of phospho p44/42 compared with NP animals. This results influenced the ratio phospho/total MAPK which demonstrated similar values between NP and LP groups, suggesting that perinatal protein malnutrition not affect this pathway signaling.

In conclusion, our data suggest that the RAS seems to participate peripherally and not centrally in the maintenance of arterial hypertension induced by protein restrict during the gestation and lactation, regardless of the levels of circulating Ang II.

References

Abbott, N. J., A. A. Patabendige, et al. (2010). "Structure and function of the blood-brain barrier." Neurobiol Dis **37**(1): 13-25.

Abbott, N. J., L. Ronnback, et al. (2006). "Astrocyte-endothelial interactions at the blood-brain barrier." Nat Rev Neurosci **7**(1): 41-53.

Abdala, A. P., F. D. McBryde, et al. (2012). "Hypertension is critically dependent on the carotid body input in the spontaneously hypertensive rat." J Physiol **590**(17): 4269-4277.

Adair, L. S. and T. J. Cole (2003). "Rapid child growth raises blood pressure in adolescent boys who were thin at birth." Hypertension **41**(3): 451-456.

Aguirre, J. A., R. Covenas, et al. (1989). "Immunocytochemical study of angiotensin-II fibres and cell bodies in the brainstem respiratory areas of the cat." Brain Res **489**(2): 311-317.

Allen, A. M. (1998). "Angiotensin AT1 receptor-mediated excitation of rat carotid body chemoreceptor afferent activity." J Physiol **510 (Pt 3)**: 773-781.

Anderson, J. W., P. M. Smith, et al. (2001). "Subfornical organ neurons projecting to paraventricular nucleus: whole-cell properties." Brain Res **921**(1-2): 78-85.

Balakumar, P. and G. Jagadeesh (2014). "Structural determinants for binding, activation, and functional selectivity of the angiotensin AT1 receptor." J Mol Endocrinol **53**(2): R71-92.

Barker, D. J., A. R. Bull, et al. (1990). "Fetal and placental size and risk of hypertension in adult life." BMJ **301**(6746): 259-262.

Barker, D. J. P., C. Osmond, et al. (2009). "Growth and chronic disease: findings in the Helsinki Birth Cohort." Annals of Human Biology **36**(5): 445-458.

Barros, M. A., J. L. De Brito Alves, et al. (2015). "Maternal low-protein diet induces changes in the cardiovascular autonomic modulation in male rat offspring." Nutr Metab Cardiovasc Dis **25**(1): 123-130.

Barros, R. C., L. G. Bonagamba, et al. (2002). "Cardiovascular responses to chemoreflex activation with potassium cyanide or hypoxic hypoxia in awake rats." Auton Neurosci **97**(2): 110-115.

Benabe, J. E., S. Wang, et al. (1993). "Modulation of ANG II receptor and its mRNA in normal rat by low-protein feeding." Am J Physiol **265**(5 Pt 2): F660-669.

Bernardi, L., C. Porta, et al. (2001). "Modulatory effects of respiration." Auton Neurosci **90**(1-2): 47-56.

Bhargava, S. K., H. S. Sachdev, et al. (2004). "Relation of serial changes in childhood body-mass index to impaired glucose tolerance in young adulthood." N Engl J Med **350**(9): 865-875.

Biancardi, V. C., S. J. Son, et al. (2014). "Circulating angiotensin II gains access to the hypothalamus and brain stem during hypertension via breakdown of the blood-brain barrier." Hypertension **63**(3): 572-579.

Biancardi, V. C. and J. E. Stern (2016). "Compromised blood-brain barrier permeability: novel mechanism by which circulating angiotensin II signals to sympathoexcitatory centres during hypertension." J Physiol **594**(6): 1591-1600.

Bianchi, A. L., M. Denavit-Saubie, et al. (1995). "Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters." Physiol Rev **75**(1): 1-45.

Blanch, G. T., A. H. Freiria-Oliveira, et al. (2014). "Increased expression of angiotensin II type 2 receptors in the solitary-vagal complex blunts renovascular hypertension." Hypertension **64**(4): 777-783.

Braga, V. A. (2013). "Reducing oxidative stress in the rostral ventrolateral medulla in renovascular hypertension by peripheral administration of losartan: how and where?" Am J Hypertens **26**(9): 1170.

Braga, V. A., R. N. Soriano, et al. (2006). "Sympathoexcitatory response to peripheral chemoreflex activation is enhanced in juvenile rats exposed to chronic intermittent hypoxia." Exp Physiol **91**(6): 1025-1031.

Broadwell, R. D. and M. W. Brightman (1976). "Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood." J Comp Neurol **166**(3): 257-283.

Bugenhagen, S. M., A. W. Cowley, Jr., et al. (2010). "Identifying physiological origins of baroreflex dysfunction in salt-sensitive hypertension in the Dahl SS rat." Physiol Genomics **42**(1): 23-41.

Burke, S. L., R. G. Evans, et al. (2008). "Levels of renal and extrarenal sympathetic drive in angiotensin II-induced hypertension." Hypertension **51**(4): 878-883.

Carey, R. M. and P. K. Whelton (2018). "Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Synopsis of the 2017 American College of Cardiology/American Heart Association Hypertension Guideline." Ann Intern Med.

Ceravolo, G. S., M. C. Franco, et al. (2007). "Enalapril and losartan restored blood pressure and vascular reactivity in intrauterine undernourished rats." Life Sciences **80**(8): 782-787.

Chai, S. Y., F. A. Mendelsohn, et al. (1987). "Angiotensin converting enzyme in rat brain visualized by quantitative in vitro autoradiography." Neuroscience **20**(2): 615-627.

Chan, S. H. and J. Y. Chan (2012). "Brain stem oxidative stress and its associated signaling in the regulation of sympathetic vasomotor tone." J Appl Physiol (1985) **113**(12): 1921-1928.

Ciriello, J., C. G. Schultz, et al. (1994). "Collateral axonal projections from ventrolateral medullary non-catecholaminergic neurons to central nucleus of the amygdala." Brain Res **663**(2): 346-351.

Collister, J. P. and M. D. Hendel (2005). "Chronic effects of angiotensin II and at1 receptor antagonists in subfornical organ-lesioned rats." Clin Exp Pharmacol Physiol **32**(5-6): 462-466.

Cornock, R., S. C. Langley-Evans, et al. (2010). "The impact of maternal protein restriction during rat pregnancy upon renal expression of angiotensin receptors and vasopressin-related aquaporins." Reprod Biol Endocrinol **8**: 105.

Costa-Silva, J. H., J. L. de Brito-Alves, et al. (2015). "New Insights on the Maternal Diet Induced-Hypertension: Potential Role of the Phenotypic Plasticity and Sympathetic-Respiratory Overactivity." Front Physiol **6**: 345.

Costa-Silva, J. H., P. A. Silva, et al. (2009). "Chronic undernutrition alters renal active Na+ transport in young rats: potential hidden basis for pathophysiological alterations in adulthood?" Eur J Nutr **48**(7): 437-445.

Costa-Silva, J. H., A. C. Simoes-Alves, et al. (2016). "Developmental Origins of Cardiometabolic Diseases: Role of the Maternal Diet." Front Physiol **7**: 504.

Costa-Silva, J. H., D. B. Zoccal, et al. (2010). "Glutamatergic antagonism in the NTS decreases post-inspiratory drive and changes phrenic and sympathetic coupling during chemoreflex activation." J Neurophysiol **103**(4): 2095-2106.

Costa-Silva, J. H., D. B. Zoccal, et al. (2012). "Chronic intermittent hypoxia alters glutamatergic control of sympathetic and respiratory activities in the commissural NTS of rats." Am J Physiol Regul Integr Comp Physiol **302**(6): R785-793.

Dampney, R. A. (1994). "Functional organization of central pathways regulating the cardiovascular system." Physiol Rev **74**(2): 323-364.

Dampney, R. A., P. S. Tan, et al. (2007). "Cardiovascular effects of angiotensin II in the rostral ventrolateral medulla: the push-pull hypothesis." Curr Hypertens Rep **9**(3): 222-227.

Daneman, R. (2012). "The blood-brain barrier in health and disease." Ann Neurol **72**(5): 648-672.

de Brito Alves, J. L. and J. H. Costa-Silva (2017). "Maternal protein malnutrition induced-hypertension: New evidence about the autonomic and respiratory dysfunctions and epigenetic mechanisms." Clin Exp Pharmacol Physiol.

de Brito Alves, J. L., J. M. de Oliveira, et al. (2016). "Maternal protein restriction induced-hypertension is associated to oxidative disruption at transcriptional and functional levels in the medulla oblongata." Clin Exp Pharmacol Physiol **43**(12): 1177-1184.

de Brito Alves, J. L., V. O. Nogueira, et al. (2015). "Maternal protein restriction increases respiratory and sympathetic activities and sensitizes peripheral chemoreflex in male rat offspring." Journal of Nutrition **145**(5): 907-914.

de Brito Alves, J. L., V. O. Nogueira, et al. (2015). "Maternal protein restriction increases respiratory and sympathetic activities and sensitizes peripheral chemoreflex in male rat offspring." J Nutr **145**(5): 907-914.

de Brito Alves, J. L., V. O. Nogueira, et al. (2014). "Short- and long-term effects of a maternal low-protein diet on ventilation, O(2)/CO(2) chemoreception and arterial blood pressure in male rat offspring." Br J Nutr **111**(4): 606-615.

de Gasparo, M., K. J. Catt, et al. (2000). "International union of pharmacology. XXIII. The angiotensin II receptors." Pharmacol Rev **52**(3): 415-472.

de Kloet, A. D., U. M. Steckelings, et al. (2017). "Protective Angiotensin Type 2 Receptors in the Brain and Hypertension." Curr Hypertens Rep **19**(6): 46.

Dick, T. E., Y. H. Hsieh, et al. (2004). "Entrainment pattern between sympathetic and phrenic nerve activities in the Sprague-Dawley rat: hypoxia-evoked sympathetic activity during expiration." Am J Physiol Regul Integr Comp Physiol **286**(6): R1121-1128.

Dupont, A. G. and S. Brouwers (2010). "Brain angiotensin peptides regulate sympathetic tone and blood pressure." J Hypertens **28**(8): 1599-1610.

Faraci, F. M., G. L. Baumbach, et al. (1989). "Myogenic mechanisms in the cerebral circulation." J Hypertens Suppl **7**(4): S61-64; discussion S65.

Feldman, J. L., G. S. Mitchell, et al. (2003). "Breathing: rhythmicity, plasticity, chemosensitivity." Annu Rev Neurosci **26**: 239-266.

Ferguson, A. V. and J. S. Bains (1997). "Actions of angiotensin in the subfornical organ and area postrema: implications for long term control of autonomic output." Clin Exp Pharmacol Physiol **24**(1): 96-101.

Ferreira, D. S., Y. Liu, et al. (2016). "Perinatal low-protein diet alters brainstem antioxidant metabolism in adult offspring." Nutr Neurosci **19**(8): 369-375.

Fidalgo, M., F. Falcao-Tebas, et al. (2013). "Programmed changes in the adult rat offspring caused by maternal protein restriction during gestation and lactation are attenuated by maternal moderate-low physical training." Br J Nutr **109**(3): 449-456.

Fidone, S. J., C. Gonzalez, et al. (1988). "Mechanisms of chemotransmission in the mammalian carotid body." Prog Brain Res **74**: 169-179.

Fink, G. D. (1997). "Long-term sympatho-excitatory effect of angiotensin II: a mechanism of spontaneous and renovascular hypertension." Clin Exp Pharmacol Physiol **24**(1): 91-95.

Finley, J. C. and D. M. Katz (1992). "The central organization of carotid body afferent projections to the brainstem of the rat." Brain Res **572**(1-2): 108-116.

Fontes, M. A., O. Baltatu, et al. (2000). "Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats." Physiol Genomics **2**(3): 137-142.

Forouzanfar, M. H., P. Liu, et al. (2017). "Global Burden of Hypertension and Systolic Blood Pressure of at Least 110 to 115 mm Hg, 1990-2015." JAMA **317**(2): 165-182.

Franchini, K. G. and E. M. Krieger (1993). "Cardiovascular responses of conscious rats to carotid body chemoreceptor stimulation by intravenous KCN." J Auton Nerv Syst **42**(1): 63-69.

Franco, M. C., D. E. Casarini, et al. (2008). "Circulating renin-angiotensin system and catecholamines in childhood: is there a role for birthweight?" Clin Sci (Lond) **114**(5): 375-380.

Fukiyama, K., J. W. McCubbin, et al. (1971). "Chronic hypertension elicited by infusion of angiotensin into vertebral arteries of unanaesthetized dogs." Clinical Science **40**(3): 283-291.

Fung, M. L. (2014). "The role of local renin-angiotensin system in arterial chemoreceptors in sleep-breathing disorders." Front Physiol **5**: 336.

Fung, M. L., S. Y. Lam, et al. (2001). "Functional expression of angiotensin II receptors in type-I cells of the rat carotid body." Pflugers Arch **441**(4): 474-480.

Gao, L., W. Wang, et al. (2005). "Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and NAD(P)H oxidase in RVLM." Am J Physiol Heart Circ Physiol **288**(5): H2271-2279.

Gluckman, P. D. and M. A. Hanson (2004). "The developmental origins of the metabolic syndrome." Trends Endocrinol Metab **15**(4): 183-187.

Goldblatt, H., J. Lynch, et al. (1934). "Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia." J Exp Med **59**(3): 347-379.

Gomide, J. M., R. C. de Menezes, et al. (2013). "Increased activity of the renin-angiotensin and sympathetic nervous systems is required for regulation of the blood pressure in rats fed a low-protein diet." Exp Physiol **98**(1): 57-66.

Gonzalez, C., L. Almaraz, et al. (1994). "Carotid body chemoreceptors: from natural stimuli to sensory discharges." Physiol Rev **74**(4): 829-898.

Goyal, R., A. Galffy, et al. (2009). "Maternal protein deprivation: changes in systemic renin-angiotensin system of the mouse fetus." Reprod Sci **16**(9): 894-904.

Goyal, R., D. Goyal, et al. (2010). "Brain renin-angiotensin system: fetal epigenetic programming by maternal protein restriction during pregnancy." Reprod Sci **17**(3): 227-238.

Goyal, R., R. Lister, et al. (2011). "Antenatal maternal hypoxic stress: adaptations of the placental renin-angiotensin system in the mouse." Placenta **32**(2): 134-139.

Grassi, G. (1998). "Role of the sympathetic nervous system in human hypertension." J Hypertens **16**(12 Pt 2): 1979-1987.

Guyenet, P. G., R. L. Stornetta, et al. (2010). "Central respiratory chemoreception." J Comp Neurol **518**(19): 3883-3906.

Hales, C. N. and D. J. Barker (2013). "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. 1992." Int J Epidemiol **42**(5): 1215-1222.

Hales, C. N., D. J. Barker, et al. (1991). "Fetal and infant growth and impaired glucose tolerance at age 64." BMJ **303**(6809): 1019-1022.

Haselton, J. R. and P. G. Guyenet (1989). "Central respiratory modulation of medullary sympathoexcitatory neurons in rat." Am J Physiol **256**(3 Pt 2): R739-750.

He, D. H., L. M. Zhang, et al. (2014). "Long-term prehypertension treatment with losartan effectively prevents brain damage and stroke in stroke-prone spontaneously hypertensive rats." Int J Mol Med **33**(2): 301-309.

Hedner, T., S. E. Kjeldsen, et al. (2012). "State of global health--hypertension burden and control." Blood Press **21 Suppl 1**: 1-2.

Heitman, S. J. and D. B. Jennings (1998). "Angiotensin II modulates respiratory and acid-base responses to prolonged hypoxia in conscious dogs." Am J Physiol **275**(2 Pt 2): R390-399.

Hernandez Schulman, I., M. S. Zhou, et al. (2007). "Cross-talk between angiotensin II receptor types 1 and 2: potential role in vascular remodeling in humans." Hypertension **49**(2): 270-271.

Heusser, K., J. Tank, et al. (2010). "Carotid baroreceptor stimulation, sympathetic activity, baroreflex function, and blood pressure in hypertensive patients." Hypertension **55**(3): 619-626.

Higuchi, S., H. Ohtsu, et al. (2007). "Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology." Clin Sci (Lond) **112**(8): 417-428.

Hyatt, M. A., H. Budge, et al. (2008). "Early developmental influences on hepatic organogenesis." Organogenesis **4**(3): 170-175.

Ito, S., M. Hiratsuka, et al. (2003). "Ventrolateral medulla AT1 receptors support arterial pressure in Dahl salt-sensitive rats." Hypertension **41**(3 Pt 2): 744-750.

Ito, S., K. Komatsu, et al. (2002). "Ventrolateral medulla AT1 receptors support blood pressure in hypertensive rats." Hypertension **40**(4): 552-559.

Iturriaga, R. and J. Alcayaga (2004). "Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals." Brain Res Brain Res Rev **47**(1-3): 46-53.

Johren, O., A. Dendorfer, et al. (2004). "Cardiovascular and renal function of angiotensin II type-2 receptors." Cardiovasc Res **62**(3): 460-467.

Julien, C., S. C. Malpas, et al. (2001). "Sympathetic modulation of blood pressure variability." J Hypertens **19**(10): 1707-1712.

Kishi, T., Y. Hirooka, et al. (2012). "Sympathoinhibition caused by orally administered telmisartan through inhibition of the AT(1) receptor in the rostral ventrolateral medulla of hypertensive rats." Hypertens Res **35**(9): 940-946.

Koga, Y., Y. Hirooka, et al. (2008). "High salt intake enhances blood pressure increase during development of hypertension via oxidative stress in rostral ventrolateral medulla of spontaneously hypertensive rats." Hypertens Res **31**(11): 2075-2083.

Koid, S. S. and D. J. Campbell (2013). "Evolving concepts of the renin-angiotensin system: highlights from the pre-ISH 2012 satellite meeting." J Renin Angiotensin Aldosterone Syst **14**(1): 93-96.

Lackland, D. T. and M. A. Weber (2015). "Global burden of cardiovascular disease and stroke: hypertension at the core." Can J Cardiol **31**(5): 569-571.

Lam, S. Y. and P. S. Leung (2002). "A locally generated angiotensin system in rat carotid body." Regul Pept **107**(1-3): 97-103.

Lam, S. Y. and P. S. Leung (2003). "Chronic hypoxia activates a local angiotensin-generating system in rat carotid body." Mol Cell Endocrinol **203**(1-2): 147-153.

Langley-Evans, S. C. and A. A. Jackson (1995). "Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets." Comp Biochem Physiol A Physiol **110**(3): 223-228.

Langley-Evans, S. C., S. J. Welham, et al. (1999). "Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat." Life Sciences **64**(11): 965-974.

Lavoie, J. L., M. D. Cassell, et al. (2004). "Adjacent expression of renin and angiotensinogen in the rostral ventrolateral medulla using a dual-reporter transgenic model." Hypertension **43**(5): 1116-1119.

Leandro, C. G., W. da Silva Ribeiro, et al. (2012). "Moderate physical training attenuates muscle-specific effects on fibre type composition in adult rats submitted to a perinatal maternal low-protein diet." Eur J Nutr **51**(7): 807-815.

Leenen, F. H. (2014). "Actions of circulating angiotensin II and aldosterone in the brain contributing to hypertension." Am J Hypertens **27**(8): 1024-1032.

Leonhardt, J., D. C. Villela, et al. (2017). "Evidence for Heterodimerization and Functional Interaction of the Angiotensin Type 2 Receptor and the Receptor MAS." Hypertension **69**(6): 1128-1135.

Leung, P. S., S. Y. Lam, et al. (2000). "Chronic hypoxia upregulates the expression and function of AT(1) receptor in rat carotid body." J Endocrinol **167**(3): 517-524.

Li, Y. L., X. H. Xia, et al. (2006). "Angiotensin II enhances carotid body chemoreflex control of sympathetic outflow in chronic heart failure rabbits." Cardiovasc Res **71**(1): 129-138.

Li, Z., J. S. Bains, et al. (1993). "Functional evidence that the angiotensin antagonist losartan crosses the blood-brain barrier in the rat." Brain Res Bull **30**(1-2): 33-39.

Lim, S. S., T. Vos, et al. (2012). "A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010." Lancet **380**(9859): 2224-2260.

Machado, B. H. and L. G. Bonagamba (2005). "Antagonism of glutamate receptors in the intermediate and caudal NTS of awake rats produced no changes in the hypertensive response to chemoreflex activation." Auton Neurosci **117**(1): 25-32.

Machado, B. H., H. Mauad, et al. (1997). "Autonomic processing of the cardiovascular reflexes in the nucleus tractus solitarii." Braz J Med Biol Res **30**(4): 533-543.

Malan, A. (1973). "Ventilation measured by body plethysmography in hibernating mammals and in poikilotherms." Respir Physiol **17**(1): 32-44.

Malliani, A., M. Pagani, et al. (1991). "Cardiovascular neural regulation explored in the frequency domain." Circulation **84**(2): 482-492.

Malpas, S. C. (1998). "The rhythmicity of sympathetic nerve activity." Prog Neurobiol **56**(1): 65-96.

Marcus, N. J., Y. L. Li, et al. (2010). "Chronic intermittent hypoxia augments chemoreflex control of sympathetic activity: role of the angiotensin II type 1 receptor." Respir Physiol Neurobiol **171**(1): 36-45.

Martinez-Maldonado, M., J. E. Benabe, et al. (1993). "Renal renin, angiotensinogen, and ANG I-converting-enzyme gene expression: influence of dietary protein." Am J Physiol **264**(6 Pt 2): F981-988.

Martins, C. D., D. A. Chianca, Jr., et al. (2011). "Cardiac autonomic balance in rats submitted to protein restriction after weaning." Clin Exp Pharmacol Physiol **38**(2): 89-93.

Mayhan, W. G., F. M. Faraci, et al. (1989). "Role of molecular charge in disruption of the blood-brain barrier during acute hypertension." Circ Res **64**(4): 658-664.

McKinley, M. J., R. M. McAllen, et al. (1996). "Physiological actions of angiotensin II mediated by AT1 AND AT2 receptors in the brain." Clin Exp Pharmacol Physiol **23 Suppl 3**: S99-104.

McMullen, S. and S. C. Langley-Evans (2005). "Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms." Am J Physiol Regul Integr Comp Physiol **288**(1): R85-90.

Mehta, P. K. and K. K. Griendling (2007). "Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system." Am J Physiol Cell Physiol **292**(1): C82-97.

Mendelsohn, F. A., A. M. Allen, et al. (1990). "The brain angiotensin system: insights from mapping its components." Trends Endocrinol Metab **1**(4): 189-198.

Mendelsohn, F. A., R. Quirion, et al. (1984). "Autoradiographic localization of angiotensin II receptors in rat brain." Proc Natl Acad Sci U S A **81**(5): 1575-1579.

Merlet-Benichou, C., T. Gilbert, et al. (1994). "Intrauterine growth retardation leads to a permanent nephron deficit in the rat." Pediatric Nephrology **8**(2): 175-180.

Mifflin, S. W. (1992). "Arterial chemoreceptor input to nucleus tractus solitarius." Am J Physiol **263**(2 Pt 2): R368-375.

Montezano, A. C. and R. M. Touyz (2012). "Molecular mechanisms of hypertension--reactive oxygen species and antioxidants: a basic science update for the clinician." Can J Cardiol **28**(3): 288-295.

Moraes, D. J., L. G. Bonagamba, et al. (2014). "Short-term sustained hypoxia induces changes in the coupling of sympathetic and respiratory activities in rats." J Physiol **592**(9): 2013-2033.

Morgane, P. J., R. Austin-LaFrance, et al. (1993). "Prenatal malnutrition and development of the brain." Neurosci Biobehav Rev **17**(1): 91-128.

Morimoto, S., M. D. Cassell, et al. (2001). "Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter." Circ Res **89**(4): 365-372.

Moritz, K. M., J. S. Cuffe, et al. (2010). "Review: Sex specific programming: a critical role for the renal renin-angiotensin system." Placenta **31 Suppl**: S40-46.

Mozaffarian, D., E. J. Benjamin, et al. (2016). "Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association." Circulation **133**(4): e38-60.

Murali, S., M. Zhang, et al. (2014). "Angiotensin II mobilizes intracellular calcium and activates pannexin-1 channels in rat carotid body type II cells via AT1 receptors." J Physiol **592**(21): 4747-4762.

Nanduri, J. and N. R. Prabhakar (2015). "Epigenetic Regulation of Carotid Body Oxygen Sensing: Clinical Implications." Adv Exp Med Biol **860**: 1-8.

Nguyen Dinh Cat, A., A. C. Montezano, et al. (2013). "Angiotensin II, NADPH oxidase, and redox signaling in the vasculature." Antioxid Redox Signal **19**(10): 1110-1120.

Nishi, E. E., C. T. Bergamaschi, et al. (2013). "Losartan reduces oxidative stress within the rostral ventrolateral medulla of rats with renovascular hypertension." Am J Hypertens **26**(7): 858-865.

Nishihara, M., Y. Hirooka, et al. (2012). "Different role of oxidative stress in paraventricular nucleus and rostral ventrolateral medulla in cardiovascular regulation in awake spontaneously hypertensive rats." J Hypertens **30**(9): 1758-1765.

Nishihara, M., Y. Hirooka, et al. (2012). "Oxidative stress in the rostral ventrolateral medulla modulates excitatory and inhibitory inputs in spontaneously hypertensive rats." J Hypertens **30**(1): 97-106.

Nunes, F. C. and V. A. Braga (2011). "Chronic angiotensin II infusion modulates angiotensin II type I receptor expression in the subfornical organ and the rostral ventrolateral medulla in hypertensive rats." J Renin Angiotensin Aldosterone Syst **12**(4): 440-445.

Nuyt, A. M. and B. T. Alexander (2009). "Developmental programming and hypertension." Curr Opin Nephrol Hypertens **18**(2): 144-152.

Osborn, J. W., G. D. Fink, et al. (2007). "Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension." Curr Hypertens Rep **9**(3): 228-235.

Paixao, A. D., C. R. Maciel, et al. (2001). "Regional Brazilian diet-induced low birth weight is correlated with changes in renal hemodynamics and glomerular morphometry in adult age." Biol Neonate **80**(3): 239-246.

Paulino-Silva, K. M. and J. H. Costa-Silva (2016). "Hypertension in rat offspring subjected to perinatal protein malnutrition is not related to the baroreflex dysfunction." Clin Exp Pharmacol Physiol **43**(11): 1046-1053.

Peng, Y. J., G. Raghuraman, et al. (2011). "Angiotensin II evokes sensory long-term facilitation of the carotid body via NADPH oxidase." J Appl Physiol (1985) **111**(4): 964-970.

Penitente, A. R., L. G. Fernandes, et al. (2007). "Malnutrition enhances cardiovascular responses to chemoreflex activation in awake rats." Life Sciences **81**(7): 609-614.

Pirola, L., S. Bonnafous, et al. (2003). "Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells." J Biol Chem **278**(18): 15641-15651.

Pladys, P., I. Lahaie, et al. (2004). "Role of brain and peripheral angiotensin II in hypertension and altered arterial baroreflex programmed during fetal life in rat." Pediatric Research **55**(6): 1042-1049.

Ponchon, P. and J. L. Elghozi (1996). "Contribution of the renin-angiotensin and kallikrein-kinin systems to short-term variability of blood pressure in two-kidney, one-clip hypertensive rats." Eur J Pharmacol **297**(1-2): 61-70.

Ponchon, P. and J. L. Elghozi (1997). "Contribution of humoral systems to the short-term variability of blood pressure after severe hemorrhage." Am J Physiol **273**(1 Pt 2): R58-69.

Porta, A., S. Guzzetti, et al. (2001). "Entropy, entropy rate, and pattern classification as tools to typify complexity in short heart period variability series." IEEE Trans Biomed Eng **48**(11): 1282-1291.

Porta, A., E. Tobaldini, et al. (2007). "Assessment of cardiac autonomic modulation during graded head-up tilt by symbolic analysis of heart rate variability." Am J Physiol Heart Circ Physiol **293**(1): H702-708.

Prabhakar, N. R., Y. J. Peng, et al. (2015). "Peripheral chemoreception and arterial pressure responses to intermittent hypoxia." Compr Physiol **5**(2): 561-577.

Ravelli, G. P., Z. A. Stein, et al. (1976). "Obesity in young men after famine exposure in utero and early infancy." N Engl J Med **295**(7): 349-353.

Reeves, P. G., F. H. Nielsen, et al. (1993). "AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet." J Nutr **123**(11): 1939-1951.

Sakai, K., K. Agassandian, et al. (2007). "Local production of angiotensin II in the subfornical organ causes elevated drinking." J Clin Invest **117**(4): 1088-1095.

Sangaleti, C. T., A. Crescenzi, et al. (2004). "Endogenous angiotensin and pressure modulate brain angiotensinogen and AT1A mRNA expression." Hypertension **43**(2): 317-323.

Sawaya, A. L., P. A. Martins, et al. (2004). "Long-term effects of early malnutrition on body weight regulation." Nutr Rev **62**(7 Pt 2): S127-133.

Sawaya, A. L. and S. Roberts (2003). "Stunting and future risk of obesity: principal physiological mechanisms." Cad Saude Publica **19 Suppl 1**: S21-28.

Schelling, P., E. Clauser, et al. (1983). "Regulation of angiotensinogen in the central nervous system." Clin Exp Hypertens A **5**(7-8): 1047-1061.

Shaver, S. W., K. M. Wall, et al. (1992). "Regional quantitative permeability of blood-brain barrier lesions in rats with chronic renal hypertension." Brain Res **579**(1): 99-106.

Shepherd, J. T. and G. Mancia (1986). "Reflex control of the human cardiovascular system." Rev Physiol Biochem Pharmacol **105**: 1-99.

Sherman, R. C. and S. C. Langley-Evans (2000). "Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat." Clin Sci (Lond) **98**(3): 269-275.

Sherrod, M., D. R. Davis, et al. (2005). "Glial-specific ablation of angiotensinogen lowers arterial pressure in renin and angiotensinogen transgenic mice." Am J Physiol Regul Integr Comp Physiol **289**(6): R1763-1769.

Simms, A. E., J. F. Paton, et al. (2010). "Is augmented central respiratory-sympathetic coupling involved in the generation of hypertension?" Respir Physiol Neurobiol **174**(1-2): 89-97.

Simms, A. E., J. F. Paton, et al. (2009). "Amplified respiratory-sympathetic coupling in the spontaneously hypertensive rat: does it contribute to hypertension?" J Physiol **587**(3): 597-610.

Simms, A. E., J. F. Paton, et al. (2009). "Amplified respiratory-sympathetic coupling in the spontaneously hypertensive rat: does it contribute to hypertension?" J Physiol **587**(Pt 3): 597-610.

Smith, J. C., A. P. Abdala, et al. (2007). "Spatial and functional architecture of the mammalian brain stem respiratory network: a hierarchy of three oscillatory mechanisms." J Neurophysiol **98**(6): 3370-3387.

Smith, P. M. and A. V. Ferguson (2010). "Circulating signals as critical regulators of autonomic state--central roles for the subfornical organ." Am J Physiol Regul Integr Comp Physiol **299**(2): R405-415.

Sousa, L. E., W. G. Magalhaes, et al. (2015). "Exercise training restores oxidative stress and nitric oxide synthases in the rostral ventrolateral medulla of renovascular hypertensive rats." Free Radic Res **49**(11): 1335-1343.

Souza, H. C., G. Ballejo, et al. (2001). "Cardiac sympathetic overactivity and decreased baroreflex sensitivity in L-NAME hypertensive rats." Am J Physiol Heart Circ Physiol **280**(2): H844-850.

Stauss, H. M. (2007). "Identification of blood pressure control mechanisms by power spectral analysis." Clin Exp Pharmacol Physiol **34**(4): 362-368.

Stornetta, R. L., C. L. Hawelu-Johnson, et al. (1988). "Astrocytes synthesize angiotensinogen in brain." Science **242**(4884): 1444-1446.

Su, Q., C. J. Huo, et al. (2017). "Renin-angiotensin system acting on reactive oxygen species in paraventricular nucleus induces sympathetic activation via AT1R/PKCgamma/Rac1 pathway in salt-induced hypertension." Sci Rep **7**: 43107.

Sumners, C. and M. I. Phillips (1983). "Central injection of angiotensin II alters catecholamine activity in rat brain." Am J Physiol **244**(2): R257-263.

Sweet, C. S., J. M. Columbo, et al. (1976). "Central antihypertensive effects of inhibitors of the renin-angiotensin system in rats." Am J Physiol **231**(6): 1794-1799.

Szarc vel Szic, K., M. N. Ndlovu, et al. (2010). "Nature or nurture: let food be your epigenetic medicine in chronic inflammatory disorders." Biochem Pharmacol **80**(12): 1816-1832.

Tsyrlin, V. A., M. M. Galagudza, et al. (2013). "Arterial baroreceptor reflex counteracts long-term blood pressure increase in the rat model of renovascular hypertension." PLoS One **8**(6): e64788.

Ueno, M., H. Sakamoto, et al. (2004). "Blood-brain barrier disruption in the hypothalamus of young adult spontaneously hypertensive rats." Histochem Cell Biol **122**(2): 131-137.

Vardhan, A., A. Kachroo, et al. (1993). "Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses." Am J Physiol **264**(1 Pt 2): R41-50.

Vieira-Filho, L. D., E. V. Cabral, et al. (2014). "Renal molecular mechanisms underlying altered Na+ handling and genesis of hypertension during adulthood in prenatally undernourished rats." Br J Nutr **111**(11): 1932-1944.

Vital, S. A., S. Terao, et al. (2010). "Mechanisms underlying the cerebral microvascular responses to angiotensin II-induced hypertension." Microcirculation **17**(8): 641-649.

Wei, S. G., Y. Yu, et al. (2008). "Angiotensin II-triggered p44/42 mitogen-activated protein kinase mediates sympathetic excitation in heart failure rats." Hypertension **52**(2): 342-350.

West-Eberhard, M. J. (1986). "Alternative adaptations, speciation, and phylogeny (A Review)." Proc Natl Acad Sci U S A **83**(5): 1388-1392.

Xiao, X., Z. X. Zhang, et al. (2010). "Low birth weight is associated with components of the metabolic syndrome." Metabolism **59**(9): 1282-1286.

Yang, G., T. S. Gray, et al. (1999). "The angiotensinogen gene is expressed in both astrocytes and neurons in murine central nervous system." Brain Res **817**(1-2): 123-131.

Yao, Y., C. M. Hildreth, et al. (2015). "The effect of losartan on differential reflex control of sympathetic nerve activity in chronic kidney disease." J Hypertens **33**(6): 1249-1260.

Zimmerman, M. C., E. Lazartigues, et al. (2004). "Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system." Circ Res **95**(2): 210-216.

Zoccal, D. B., L. G. Bonagamba, et al. (2009). "Sympathetic-mediated hypertension of awake juvenile rats submitted to chronic intermittent hypoxia is not linked to baroreflex dysfunction." Exp Physiol **94**(9): 972-983.

Zoccal, D. B. and B. H. Machado (2011). "Coupling between respiratory and sympathetic activities as a novel mechanism underpinning neurogenic hypertension." Curr Hypertens Rep **13**(3): 229-236.

Zoccal, D. B., A. E. Simms, et al. (2008). "Increased sympathetic outflow in juvenile rats submitted to chronic intermittent hypoxia correlates with enhanced expiratory activity." J Physiol **586**(13): 3253-3265.

Zucker, I. H. and L. Gao (2005). "The regulation of sympathetic nerve activity by angiotensin II involves reactive oxygen species and MAPK." Circ Res **97**(8): 737-739.

Attachments

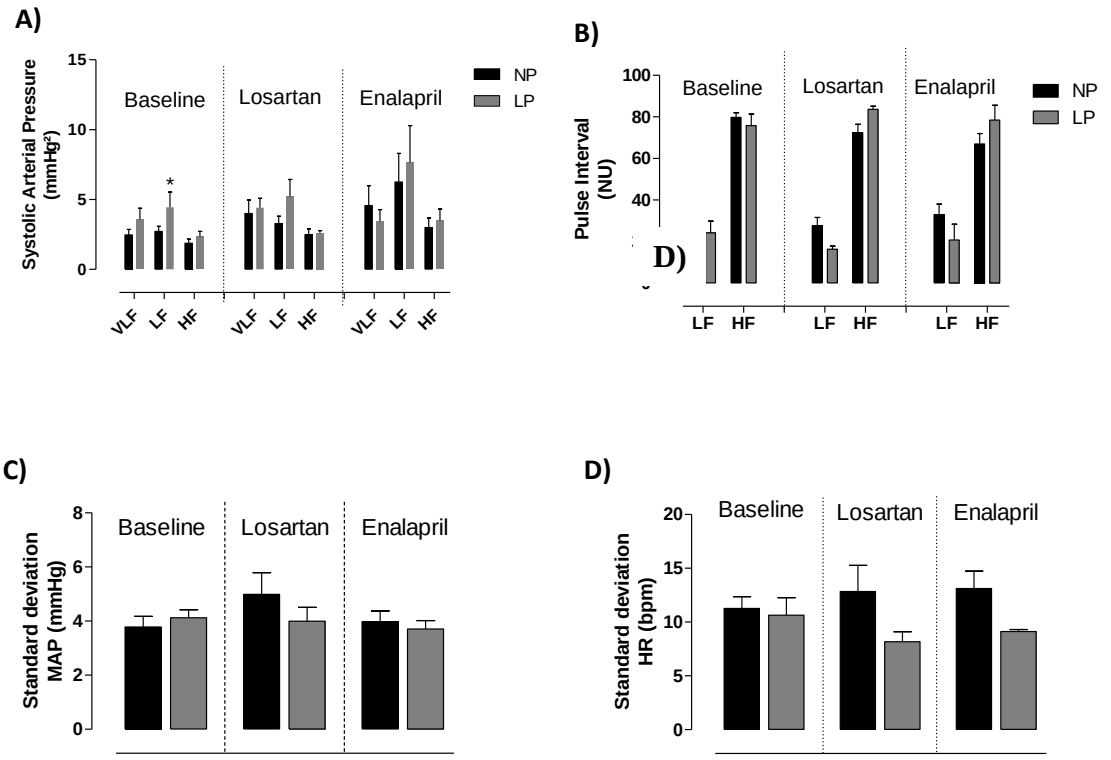

Figure 1. Cardiovascular variability. Frequency domain: Spectral analysis of the systolic arterial pressure with the very low-frequency (VLF), low-frequency (LF) and high-frequency (HF) bands (panel A) and of the pulse interval (panel B). Time domain: Standard deviation of mean arterial blood pressure (MAP, panel C) and heart rate (HR, panel D) of the normoproteic (NP; black bars) and low-protein (LP; grey bars) groups. The values were expressed as the mean ± SEM. (*) P <0.05 compared with the NP group (unpaired Student's t test).

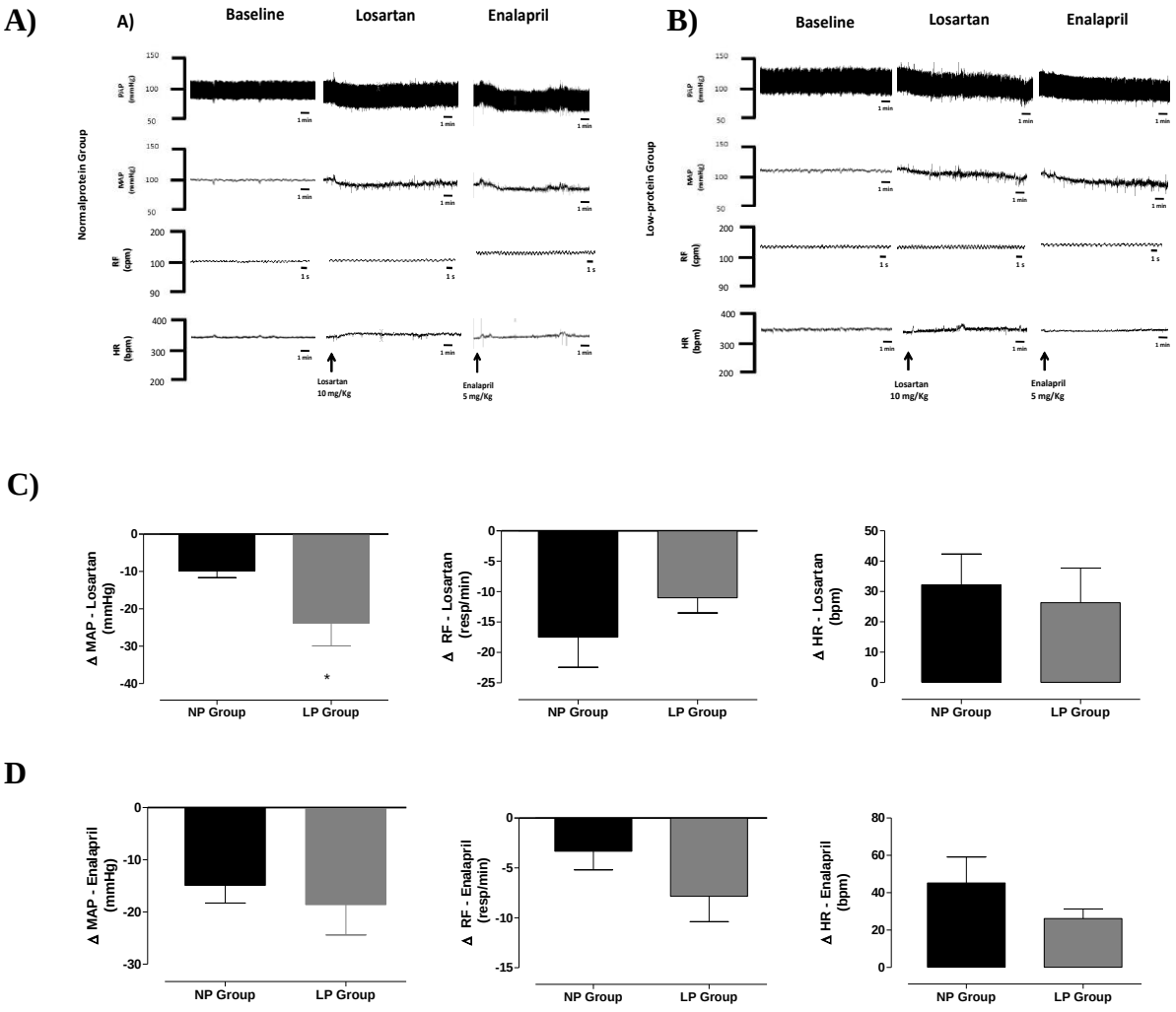

Figure 2. **Evaluation of the renin angiotensin system on cardiorespiratory parameters**. Tracings representative of the normalprotein (panel A) and low-protein group (panel B) showing the pulsative arterial pressure (PAP) mean arterial pressure (MAP), respiratory frequency (RF) and heart rate (HR) during baseline period, after type 1 angiotensin (AT1) receptor block by losartan and after angiotensin converting enzyme (ECA) inhibition by enalapril administration. Means delta of MAP, RF and HR after AT1 block (panel C) and after ECA inhibition (panel D) of the normoproteic (NP, black bars) and low-protein (LP, grey bars) groups. The values are expressed as the mean ± SEM. (*) $P < 0.05$ compared with the NP groups (unpaired Student's t test).

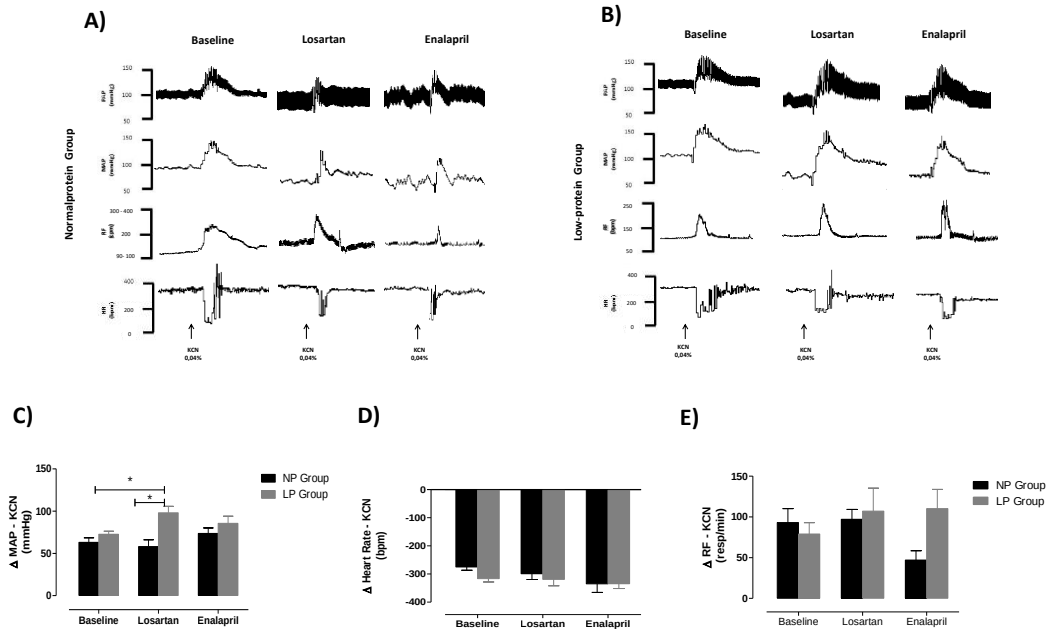

Figure 3. A: Tracings representative of the normalprotein group showing the pulsative arterial pressure (PAP), mean arterial pressure (MAP), respiratory frequency (RF) and heart rate (HR) in response to chemoreflex activation (KCN 0.04%, arrow) before (baseline) and after administration of losartan and enalapril. **B:** Tracings representative of the low-protein group showing the PAP, MAP, RF and HR in response to chemoreflex activation (KCN 0.04%, arrow), before (baseline) and after administration of losartan and enalapril. The delta of the MAP, HR and RF in response to chemoreflex activation, before and after losartan and enalapril administration in the normoproteic (NP, black bars) and low-protein (LP, grey bars) groups. The values are expressed as the mean ± SEM. (*) $P < 0.05$ compared with the NP groups (unpaired Student's t).

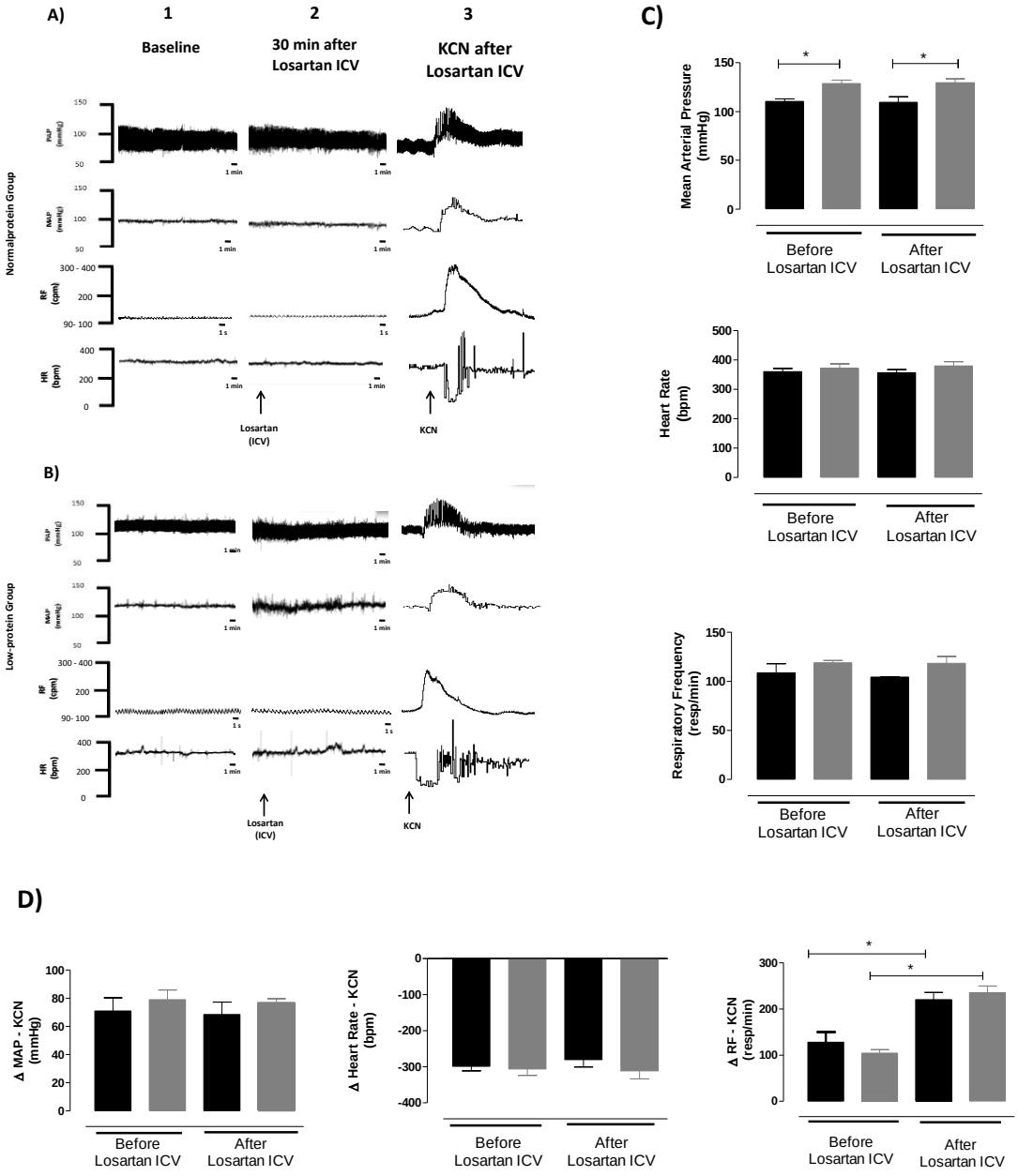

Figure 4. A. Tracings representative of the normalprotein group showing the pulsative arterial pressure (PAP) mean arterial pressure (MAP) and heart rate (HR) during baseline period, after AT1 receptor block with losartan (arrow) microinjection intracerebroventricular (ICV)and during chemoreflex activation (KCN 0.04%, arrow). Mean of MAP, HR and respiratory frequency (RF). **B.** Tracings recordings of the low-protein group showing the PAP, MAP, RF and HR during baseline period, after AT1 receptor block with losartan (arrow) microinjection ICVand during chemoreflex activatio. **C.** Means of the MAP, HR and RF before and after ICV losartan administration. **D.** Delta of the MAP, HR and RF in response to chemoreflex activation, before and after ICV losartan administration in the normalprotein (NP, black bars)

and low-protein (LP, grey bars) groups. The values are expressed as the mean ± SEM. (*) $P < 0.05$ compared with the NP groups (unpaired or paired Student's t test).

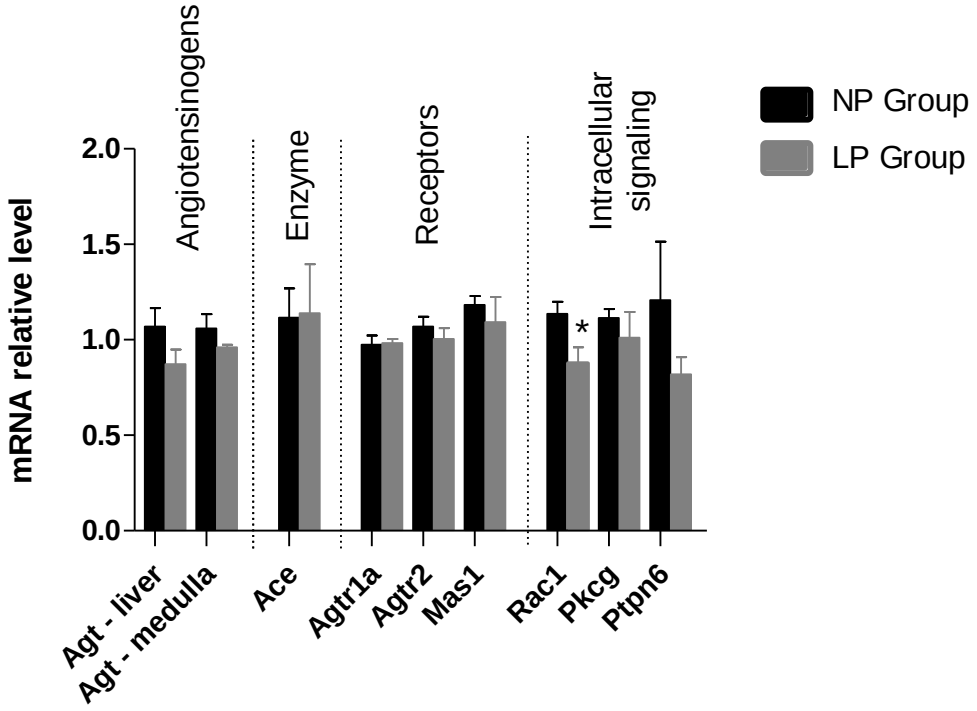

Figure 5. mRNA expression in protein-restricted rats during pregnancy and lactation. Evaluation of mRNA expression of angiotensinogen (Agt) in liver and medulla tissues, of angiotensin I converting enzyme (Ace), type 1a angiotensin II receptor (Agtr1a), type 2 angiotensin II receptor (Agtr2), ras-related C3 botulinum toxin substrate 1 (Rac1), gamma protein kinase C (Prkcg), and protein tyrosine phosphatase, non-receptor type 6 (Ptpn6) and proto-oncogene, G protein-coupled receptor (Mas1) in medulla tissue at 90 days old. Black bar represent NP group (17% protein, n=5) and grey bar LP group (8% protein, n=5). All pups were fed a standard chow diet from weaning. Data are shown in mean±S.E.M. and analyzed by unpaired Student's t test. *$p<0.05$. (*RPL19 + β-actin Housekeeping gene)*.

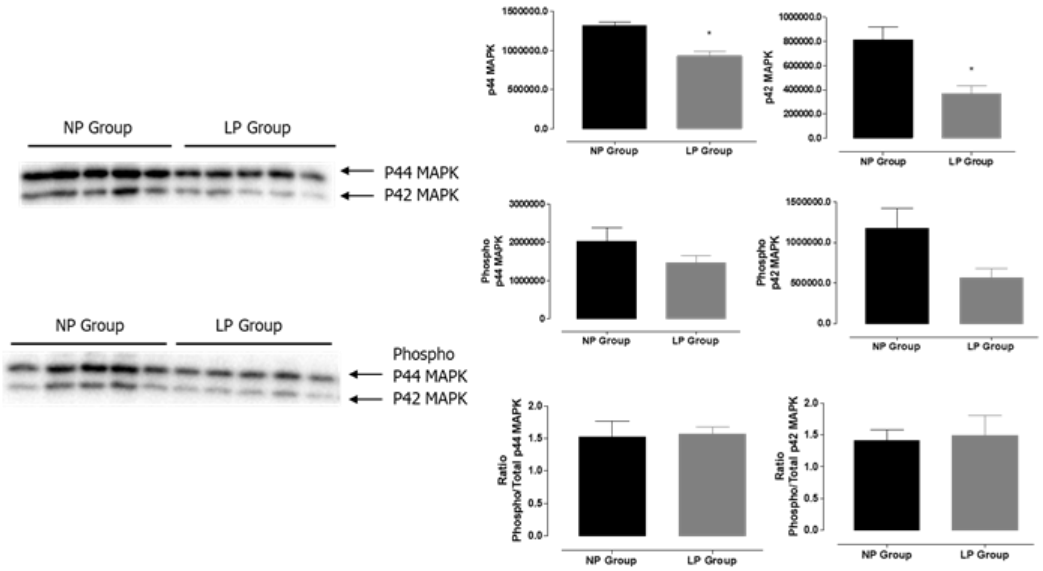

Figure 6. Western blotting assay for expression of MAPK and phospho MAPK in medulla tissue at 90 days old. Black bars represent NP group (17% protein, n=5) and grey bars LP group (8% protein, n=5). All pups were fed a standard chow diet from weaning. Data are shown in mean±S.E.M. and analyzed by unpaired Student's t test. *$p<0.05$.

APÊNDICE B - ARTIGO 2

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Low-protein diet does not alter reproductive, biochemical, and hematological parameters in pregnant Wistar rats

M.A.V. Barros[1], E.B. Andrade[1], R.G.N. Barros[1], I.K.M. Costa[1], I.C.L. Costa[1], G.F.A. Vitorino[1], J.J.C. Andrade[1], K.M. Paulino-Silva[1], V.O. Nogueira[1], J.L. de Brito Alves[2] and J.H. Costa-Silva[1]

[1]Laboratório de Nutrição, Atividade Física e Plasticidade Fenotípica Núcleo de Educação Física e Ciências do Esporte, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE, Brasil
[2]Departamento de Nutrição, Centro de Ciências da Saúde, Universidade Federal de Paraíba, UFPB, João Pessoa, PB, Brasil

Abstract

The aim of this study was to investigate the reproductive, biochemical, and hematological outcomes of pregnant rats exposed to protein restriction. Wistar rat dams were fed a control normal-protein (NP, 17% protein, n=8) or a low-protein (LP, 8% protein, n=14) diet from the 1st to the 20th day of pregnancy. On the 20th day, the clinical signs of toxicity were evaluated. The pregnant rats were then anesthetized and blood samples were collected for biochemical-hematological analyses, and laparotomy was performed to evaluate reproductive parameters. No sign of toxicity, or differences ($P > 0.05$) in body weight gain and biochemical parameters (urea, creatinine, albumin, globulin, and total protein) between NP and LP pregnant dams were observed. Similarly, hematological data, including red blood cell count, white blood cell count, hemoglobin, hematocrit, red blood cell distribution width (coefficient of variation), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % lymphocytes, absolute lymphocyte count, platelet count, and mean platelet volume were similar ($P > 0.05$) at the end of pregnancy. Reproductive parameters (the dam-offspring relationship, ovary mass, placenta mass, number of corpora lutea, implantation index, resorption index, and the pre- and post-implantation loss rates) were also not different ($P > 0.05$) between NP and LP pregnant dams. The present data showed that a protein-restricted diet during pregnancy did not alter reproductive, biochemical, and hematological parameters and seems not to have any toxic effect on pregnant Wistar rats.

Key words: Development; Epigenetics; Fertility; Gestation; Intrauterine growth; Nutrition

Introduction

Gestation and lactation (perinatal period) are characterized by an intense process of hypertrophy, hyperplasia, and cellular differentiation (1). In this period, nutritional supplies are important for adequate intra-uterine growth and development of pups. Epidemiological and experimental reports have demonstrated that nutritional insults, such as the consumption of a low-protein diet during gestation and lactation, produce maternal and offspring metabolic dysfunction (2,3).

Pups from protein-restricted mothers, in the short-term, are able to adapt to a harmful environment to ensure their survival. Though this adaptation is beneficial in the short-term, offspring exposed to maternal malnutrition exhibit several long-term consequences, such as a higher predisposition to the development of non-communicable diseases (4).

In rats, for example, offspring exposed to protein-restriction during pregnancy and lactation exhibit augmentation of arterial blood pressure (5,6), insulin resistance (7), and higher levels of adipose tissue in adult life (8). It is well established that maternal diet induced-hypertension is related to mechanisms that include reduced nephron morphology and function, reduced glomerular filtration rate, dysfunction on the rennin angiotensin-aldosterone system (9), as well as sympathetic-respiratory dysfunctions (10). Besides that, changes in muscle glucose metabolism by expression decrease in protein kinase C (11) and decrease in hexokinase activity (12) are related with insulin resistance and increased susceptibility to diabetes in malnutrition animals.

The phenomenon that links events experienced *in utero* with predisposition to diseases in adulthood is denominated "phenotypic plasticity", and refers to the ability of an organism to react to an internal and external environmental input with a change in the form, state, movement or rate of activity without genetic changes (13,14).

Correspondence: J.H. Costa-Silva: <joao.hcsilva@ufpe.br>

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Low protein diet does not alter reproductive, biochemical, and hematological parameters in pregnant Wistar rats

Barros, M.A.V.[1], Andrade, E.B.[1], Barros, R.G.N.[1], Costa, I.K.M. [1], Costa, I.C.L.[1], Vitorino, G.F.A.[1], Andrade, J.J.C.[1], Paulino-Silva, K.M.[1], Nogueira, V.O.[1], De Brito-Alves[2], J.L. and Costa-Silva, J.H.[1]

[1]Departamento de Educação Física, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE, Brasil

[2]Department of Nutrition, Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, 58051900, Brazil

Correspondence: J.H. Costa-Silva: <joao.hcsilva@ufpe.br>

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Running title: Protein restriction and maternal parameters in rats

Abstract

The aim of this study was to investigate the reproductive, biochemical, and hematological outcomes of pregnant rats exposed to protein restriction. Wistar rat dams were fed a control normal-protein (NP, 17% protein, n=8) or a low-protein (LP, 8% protein, n=14) diet from the 1st to the 20th day of pregnancy. On the 20th day, the clinical signs of toxicity were evaluated. The pregnant rats were then anesthetized and blood samples were collected for biochemical-hematological analyses, and laparotomy was performed to evaluate reproductive parameters. No sign of toxicity, or differences ($P>0.05$) in body weight gain and biochemical parameters (urea, creatinine, albumin, globulin, and total protein) between NP and LP pregnant dams were observed. Similarly, hematological data, including red blood cell count, white blood cell count, hemoglobin, hematocrit, red blood cell distribution width (coefficient of variation), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % lymphocytes, absolute lymphocyte count, platelet count, and mean platelet volume were similar ($P>0.05$) at the end of pregnancy. Reproductive parameters (the dam-offspring relationship, ovary mass, placenta mass, number of corpora lutea, implantation index, resorption index, and the pre- and post-implantation loss rates) were also not different ($P>0.05$) between NP and LP pregnant dams. The present data showed that a protein-restricted diet during pregnancy did not alter reproductive, biochemical and hematological parameters and seems not to have any toxic effect on pregnant Wistar rats.

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Although there are a number of studies showing the relationship between maternal malnutrition and non-communicable disease in adult offspring, none has specifically addressed the effects of a protein-restricted diet on mothers and maternal-fetal coupling. Previous studies have demonstrated that protein-restricted

diets during gestation produce important morphological and functional dysregulation at placental levels (15,16). In addition, protein-restricted pregnant dams exhibit decreased secretion of insulin (17).

It is known that dietary content is often an important environmental determinant of the toxicological activity. Thus, change in maternal and offspring body weight are viewed collectively as indicators of maternal and developmental toxicity, respectively (18). Besides that, clinical observations are an important approach for the identification of maternal toxicity and alterations in general homeostasis (18,19).

Despite these findings, the reproductive, biochemical, and hematological parameters in pregnant rats exposed to protein restriction remain to be clarified. Therefore, the present study aimed to assess the effects of maternal protein restriction on the reproductive, biochemical, and hematological status of pregnant rats.

Material and Methods

Animals

Rats of the Wistar lineage obtained from the Academic Center of Vitoria de Santo Antão (Federal University of Pernambuco, Brazil) and weighing 210–250 g were used and kept under standard environmental conditions (25±2ºC; 12:12 h dark/light cycle). Water and chow diet were available *ad libitum.* The experimental protocol was approved by the Animal Experimentation Ethics Committee of the Centro de Ciências Biológicas, Universidade Federal de Pernambuco (Process No. 23076.016525/2014-92).

Diets

Both the normal protein (17% of protein) and low protein (8% of protein) diets were prepared at the Laboratório de Nutrição Experimental-CAV, Universidade Federal de Pernambuco, according to the American Institute of Nutrition (AIN-97).

The diets were isoenergetic and were offered during pregnancy. Only the amounts of protein and carbohydrate were changed in the diets (Table 1) (20).

Experimental protocol

The rats were first mated (2 females for 1 male). The day on which spermatozoa were identified in a vaginal smear was considered the date of conception (day 1 of pregnancy), and pregnant rats were transferred to individual cages. Two experimental groups were designated according to diet manipulation: mothers fed a 17% protein diet (normal-protein group, NP, n=8) and mothers fed an 8% casein diet (low-protein group, LP, n=14). Water was available *ad libitum* from the 1st to the 20th day of pregnancy (21,22). During pregnancy, body weight and food and water intake were recorded weekly.

On the 20th day of pregnancy, the clinical signs of toxicity (piloerection, diarrhea, salivation, alteration in locomotor activity, changes in behavior or signs of vaginal bleeding) were evaluated. Posteriorly, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and blood samples (about 1–2 mL) were collected by plexus retro-orbital disruption, using capillary tubes for hematological and biochemical studies, with and without anticoagulant, respectively (23). The animals were then laparotomized and their uterine horns removed to determine reproductive parameters (21).

Biochemical and hematological analysis

Hematological analysis was performed using an automatic hematological analyzer (KX-21N, Sysmex, Japan). The parameters included: red blood cell count, white blood cell count, hemoglobin, hematocrit, red blood cell distribution width coefficient of variation, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % lymphocytes, absolute lymphocyte count, platelet count, and mean platelet volume (23). For biochemical analysis, the blood was centrifuged at 1480 *g* for 10 min at room temperature to obtain serum, which was stored at –20°C until determination of the following parameters: total

protein, albumin, globulins, blood urea nitrogen, and creatinine. The dosages were chosen using Cobas Mira (Roche, USA) automation with Boehringer Ingelheim® (USA) biochemical kits.

Reproductive parameters

On the 20th day of pregnancy, the rats were laparotomized and their uterine horns removed. The number of implants, resorptions, and the number of live and dead fetuses was then recorded. The fetuses and placentae were observed for macroscopic abnormality. The ovaries were weighed and the corpora lutea were counted. From these data, the implantation index (total number of implantation sites/total number of corpora lutea ×100), the resorption index (total number of resorption sites/total number of implantation sites ×100), the pre-implantations (number of corpora lutea – number of implantations/number of corpora lutea ×100) and the post-implantation loss rate (number of implantations – number of live fetuses/number of implantations ×100) were calculated.

Statistical analysis

Student's unpaired *t*-test was used to evaluate significant differences between the normal and low protein groups. One-way ANOVA followed by the Newman-Keuls tests were used to evaluate significant differences in hematological parameters. The pre-implantation and post-implantation loss rates and the implantation and resorption indexes were analyzed using Kruskal-Wallis and chi-square tests, respectively. The significance level was set at $P<0.05$.

Results

Low protein diet consumption from the 1st to 20th day of pregnancy did not produce any death or clinical signs of toxicity in the pregnant rats. Maternal food consumption was affected in the 1st week of pregnancy, but no differences were noted in the following weeks. However, malnourished dams had a smaller protein intake than control dams in all weeks analyzed. In relation to water intake, a reduction in the 2nd week of pregnancy (Table 2) was observed in LP animals.

The hematological profiles of NP and LP pregnant rats are presented in Table 3. NP and LP dams exhibited similar ($P>0.05$) hematological parameters in pre- and late pregnancy.

Biochemical analysis revealed that protein-restriction during pregnancy did not alter albumin (NP=1.7±0.1 *vs* LP=1.8±0.1 g/dL; n=7; $P>0.05$), globulins (NP=3.3±0.5 *vs* LP=3.4±0.6 g/dL; n=7; $P>0.05$), and total protein (NP=5.1±0.4 *vs* LP=5.4±0.6 g/dL; n=7, $P>0.05$). Similarly, urea (NP=59±12 *vs* LP=64±9 g/dL; n=7, $P>0.05$) and serum creatinine (NP=0.6±0.1 *vs* LP: 0.8±0.1 g/dL; n=7, $P>0.05$) were similar between the NP and LP groups (Figure 1).

All pregnant females were found to have viable fetuses, observed after a caesarian section. No fetuses with external malformations were observed. In addition, there were no differences between NP and LP dams regarding the number of fetuses of each dam (offspring/dam relationship), the number of corpora lutea, and ovary weights. Likewise, maternal low protein intake did not cause any changes in the implantation and resorption indexes or the pre- and post-implantation loss rates (Table 4).

Discussion

The main finding of this study is that a low protein diet did not produce any death or toxic clinical signs in pregnant rats, nor changed the biochemical, hematological, and reproductive parameters of the animals.

It is known that maternal parameters such as body weight gain, food consumption, and clinical signs of toxicity enable a clear evaluation of the integrity of maternal homeostasis (24). We observed that a protein-restricted pregnant rat had a

significant reduction in protein intake during the entire pregnancy period (about 50%). Interestingly, the low protein intake did not decrease maternal weight gain, suggesting that the homeostatic mechanism is able to provide normal development during pregnancy.

Besides that, in the first week of pregnancy, the LP pregnant rats exhibited higher food consumption compared to pregnant rats fed on a normal diet during the same period. Similarly, in a study with different protein-calorie diets during lactation in rats, was demonstrate an increased diet intake during beginning of lactation in the low protein group (25). It is known that feeding is controlled by a central feeding system that is regulated by a balance between monoamines and neuropeptides. Thus, the animals possibly present a mechanism to compensate for a low protein diet, due to a regulatory system involving gastrointestinal and metabolic aspects mediated by neural structures, which may have stabilized in the second week of pregnancy.

Despite the fact that the protein-restricted dams did not present differences in body weight, offspring from malnourished dams during pregnancy and/or lactation have been shown to have a lower weight and shorter length at birth as well as a higher incidence of mortality in the first days of life (6,26–29). Recent studies have shown that offspring exposed to perinatal protein restriction exhibit a number of dysfunctions in respiratory, cardiovascular, renal, behavioral, and reproductive levels during their lives. As the developing organism is capable of adapting to various environments, these physiological alterations over the course of life appear to be the result of complex gene-environment interactions, resulting from epigenetic changes during their critical developmental time window (30,31).

In the same manner, studies have also shown that maternal-fetal coupling suffers injury under maternal malnutrition, with the placenta being the focus of these studies. For example, a low protein diet during pregnancy induced placental oxidative stress (32) as well as mitochondrial alterations and degenerative processes, suggesting a premature aging of the placenta (33). Although previous studies found no change in the weight of the placenta (34), function of the placenta appears to be compromised by low protein diet intake.

To understand this, we investigated if this diet could change the biochemical and hematological parameters as well as compromise the reproductive capacity of the mother. Our data provided new insights into the effects of protein-restriction during pregnancy, demonstrating that after exposure to a protein-restricted diet, NP and LP pregnant rats showed similar hematological profiles. Thus, all parameters remained within the reference range for the species. Prestes-Carneiro et al. (35) have reported that exposure to a low protein diet from 12 days of lactation can induce alterations in red blood cell count in the offspring, which is never restored completely even after a normal protein diet is supplied. Our study shows clearly that protein restriction during pregnancy does not modify maternal hemostasis.

On the other hand, our data also showed that a low protein diet during pregnancy did not change maternal serum levels of the albumin, total protein, globulin, urea, and creatinine. We hypothesized that physiological synthesis of the protein and its metabolism during pregnancy is not affected by lower protein and amino acid intakes.

Regarding reproductive parameters, we also found similar ovarian mass and the number of corpora lutea in both the protein restriction group and control group. These findings indicated normal development of corpora lutea and suggested that the production of progesterone is not influenced by low protein diet (36).

Implantation index and pre-implantation loss rate evaluate blastocyst implantation in the uterus. These parameters were similar in both control and malnourished groups, suggesting normal reproductive capacity (36). The resorption index and post-implantation loss rate establish correlations between the number of implanted blastocysts and those that do not develop (24). When the implanted blastocysts do not develop, they are known as "resorptions", which indicate failure in the development of the embryo. In this study, there was no statistical difference between control and malnourished groups for the resorption index and post-implantation loss rate, indicating normal development of the implanted blastocysts.

Although our results showed no damage in low protein dams, it is known that a restriction of protein during pregnancy may induce changes in other pathways, such as glucoses metabolism and insulin secretion (37,38). A decrease in $(Ca^{2+})i$, as well as changes in gene expression in pancreatic islets (39) can explain the decreased

insulin secretion in malnourished animals. A study also demonstrated that physical training before and during pregnancy attenuated the effects of a low-protein diet on the secretion of insulin (17).

In conclusion, the present study showed that a low protein diet during pregnancy did not change the hematological, biochemical, and reproductive parameters, and seems not to have any toxic effect on pregnant Wistar rats. These data strengthen the plasticity phenotype theory, in which the adaptive mechanisms elicited by the maternal organism are responsible for providing normal nutrient contents to the fetus during exposure to maternal protein restriction. However, other parameters indicate alterations in maternal-fetal coupling induced by protein restriction in the literature and therefore, the long-term effects of this maternal physiological adaptation on the offspring needs to be further studied.

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References

1. Luther JS, Redmer DA, Reynolds LP, Wallace JM. Nutritional paradigms of ovine fetal growth restriction: implications for human pregnancy. *Hum Fertil* 2005; 8: 179–187.

2. Barker DJP, Osmond C, Kajantie E, Eriksson JG. Growth and chronic disease: findings in the Helsinki Birth Cohort. *Ann Hum Biol* 2009; 36: 445–458.

3. Ozanne SE, Hales CN. Lifespan: catch-up growth and obesity in male mice. *Nature* 2004; 427: 411–412.

4. Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo. *Reprod Fertil Dev* 2015; 27: 684–692.

5. Barros MA, De Brito Alves JL, Nogueira VO, Wanderley AG, Costa-Silva JH. Maternal low-protein diet induces changes in the cardiovascular autonomic modulation in male rat offspring. *Nutr Metab Cardiovasc Dis* 2015; 25: 123–130.

6. de Brito Alves JL, Nogueira VO, de Oliveira GB, da Silva GS, Wanderley AG, Leandro CG, et al. Short- and long-term effects of a maternal low-protein diet on ventilation, O(2)/CO(2) chemoreception and arterial blood pressure in male rat offspring. *Br J Nutr* 2014; 111: 606–615.

7. Dunlop K, Cedrone M, Staples JF, Regnault TRH. Altered fetal skeletal muscle nutrient metabolism following an adverse in utero environment and the modulation of later life insulin sensitivity. *Nutrients* 2015; 7: 1202–1216.

8. Hult M, Tornhammar P, Ueda P, Chima C, Bonamy AKE, Ozumba B, et al. Hypertension, diabetes and overweight: looming legacies of the Biafran famine. PLoS One 2010; 5: e13582.

9. Nuyt AM. Mechanisms underlying developmental programming of elevated blood pressure and vascular dysfunction: evidence from human studies and experimental animal models. *Clin Sci* 2008; 114: 1–17.

10. de Brito Alves JL, Nogueira VO, Cavalcanti Neto MP, Leopoldino AM, Curti C, Colombari DS, et al. Maternal protein restriction increases respiratory and sympathetic activities and sensitizes peripheral chemoreflex in male rat offspring. *J Nutr* 2015; 145: 907–914.

11. Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, and Ozanne SE, Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *Am J Physiol Regul Integr Comp Physiol* 2005; 288: R368–R373.

12. da Silva Aragao R, Guzman-Quevedo O, Perez-Garcia G, Toscano AE, Gois Leandro C, Manhaes-de-Castro R, et al. Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre-type composition. *Acta* 2014; 210: 70–83.

13. Barker DJP, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Trajectories of growth among children who have coronary events as adults. *N Engl J Med* 2005; 353: 1802–1809.

14. West-Eberhard MJ. Alternative adaptations, speciation, and phylogeny (A Review). *Proc Natl Acad Sci U S A* 1986; 83: 1388–1392.

15. Saif Z, Hodyl NA, Hobbs E, Tuck AR, Butler MS, Osei-Kumah A, et al. The human placenta expresses multiple glucocorticoid receptor isoforms that are altered by fetal sex, growth restriction and maternal asthma. *Placenta* 2014; 35: 260–268.

16. He ZX, Sun ZH, Beauchemin KA, Yang WZ, Tang SX, Zhou CS, et al. Effect of protein or energy restriction during late gestation on hormonal and metabolic status in pregnant goats and postnatal male offspring. *Animal* 2015; 9: 1843–1851.

17. Leandro CG, Fidalgo M, Bento-Santos A, Falcao-Tebas F, Vasconcelos D, Manhaes-de-Castro R, et al. Maternal moderate physical training during pregnancy attenuates the effects of a low-protein diet on the impaired secretion of insulin in rats: potential role for compensation of insulin resistance and preventing gestational diabetes mellitus. *J Biomed Biotechnol* 2012; 2012: 805418.

18. EPA. Guidelines for developmental toxicity risk assessment. 1991; 56: 63798–63826.

19. Kimmel CA, Price CJ. Developmental toxicity studies. In: Arnold D L, Grice HC, Krewski DR (Editors). Handbook of *in vivo* toxicity testing. San Diego: Academic Press*;* 1990. p 271–301.

20. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet*. J Nutrition* 1997; 127: S838–S841.

21. Costa-Silva JH, Lyra MM, Lima CR, Arruda VM, Araujo AV, Ribeiro e Ribeiro A, et al. A toxicological evaluation of the effect of *Carapa guianensis* Aublet on pregnancy in Wistar rats*. J Ethnopharmacol* 2007; 112: 122–126.

22. de Almeida ER, Melo AM, Xavier H, Toxicological evaluation of the hydro-alcohol extract of the dry leaves of *Peumus boldus* and boldine in rats*. Phytother Res* 2000; 14: 99–102.

23. Costa-Silva JH, Lima CR, Silva EJ, Araujo AV, Fraga MC, Ribeiro ERA, et al. Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliaceae) seed oil*. J Ethnopharmacol* 2008; 116: 495–500.

24. Almeida FCG, Lemonica IP. The toxic effects of *Coleus barbatus* B. on the different periods of pregnancy in rats*. J Ethnopharmacol* 2000; 73: 53–60.

25. Cambraia RP, Vannucchi H, De-Oliveira LM. Food intake and weight of lactating rats maintained on different protein-calorie diets, and pup growth*. Braz J Med Biol Res* 1997; 30: 985–988.

26. Otani L, Shirasaka N, Yoshizumi H, Murakami T. The effects of maternal mild protein restriction on stroke incidence and blood pressure in stroke-prone spontaneously hypertensive rats (SHRSP)*. Biosci Biotechnol Biochem* 2004; 68: 488–494.

27. Gangula PR, Reed L, Yallampalli C. Antihypertensive effects of flutamide in rats that are exposed to a low-protein diet in utero*. Am J Obstet Gynecol* 2005; 192: 952–960.

28. Vieira-Filho LD, Lara LS, Silva PA, Luzardo R, Einicker-Lamas M, Cardoso HD, et al. Placental oxidative stress in malnourished rats and changes in kidney

proximal tubule sodium ATPases in offspring. *Clin Exp Pharmacol Physiol* 2009; 36: 1157–1163.

29. Guzman C, Cabrera R, Cardenas M, Larrea F, Nathanielsz PW, Zambrano E. Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. *J Physiol* 2006; 572 (Part 1): 97–108.

30. Gallo LA, Tran M, Moritz KM, Wlodek ME, Developmental programming: variations in early growth and adult disease. *Clin Exp Pharmacol Physiol* 2013; 40: 795–802.

31. Van Soom A, Fazeli A. Epigenetics and periconception environment: an introduction. *Reprod Fertil Dev* 2015; 27 iii–v.

32. Vega CC, Reyes-Castro LA, Rodriguez-Gonzalez GL, Bautista CJ, Vazquez-Martinez M, Larrea F, et al. Resveratrol partially prevents oxidative stress and metabolic dysfunction in pregnant rats fed a low protein diet and their offspring. *J Physiol* 2016; 594: 1483–1499.

33. Rebelato HJ, Esquisatto MA, Moraes C, Amaral ME, Catisti R. Gestational protein restriction induces alterations in placental morphology and mitochondrial function in rats during late pregnancy. *J Mol Histol* 2013; 44: 629–637.

34. Otani L, Shirasaka N, Yoshizumi H, Murakami T. The effects of maternal mild protein restriction on stroke incidence and blood pressure in stroke-prone spontaneously hypertensive rats (SHRSP). *Biosci Biotechnol Biochem* 2004; 68: 488–494.

35. Prestes-Carneiro LE, Laraya RD, Silva PRC, Moliterno RA, Felipe I, Mathias PC. Long-term effect of early protein malnutrition on growth curve, hematological parameters and macrophage function of rats. *J Nutr Sci Vitaminol* 2006; 52: 414–420.

36. Chang CV, Felicio AC, Reis JED, Guerra MD, Peters VM. Fetal toxicity of *Solanum lycocarpum* (Solanaceae) in rats. *J Ethnopharmacol* 2002; 81: 265–269.

37. Milanski M, Arantes VC, Ferreira F, de Barros Reis MA, Carneiro EM, Boschero AC, et al. Low-protein diets reduce PKAalpha expression in islets from pregnant rats. *J Nutr* 2005; 135: 1873–1878.

38. Souza Dde F, Ignacio-Souza LM, Reis SR, Reis MA, Stoppiglia LF, Carneiro EM, et al. A low-protein diet during pregnancy alters glucose metabolism and insulin secretion. *Cell Biochem Funct* 2012; 30: 114–121.

39. de Siqueira KC, de Lima FM, Lima FS, Taki MS, da Cunha CF, de Lima Reis SR, et al. miR-124a expression contributes to the monophasic pattern of insulin secretion in islets from pregnant rats submitted to a low-protein diet. *Eur J Nutr* 2017; DOI: 10.1007/s00394-017-1425-z

Tables and figure

Table 1. Nutritional composition of the experimental diets.

Nutrient	Normal protein (17% protein)	Low protein (8% protein)
Casein (85% purity)	20	9.41
Dextrin cornstarch	13	13.2
Cellulose	5	5
Sucrose	10	10
Cornstarch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0,25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

Data are reported as g/100 g diet.

Table 2. Consumption of female rats submitted to normal (NP group, 17% protein) or low-protein diet (LP group, 8% protein) during pregnancy.

Period of	Food Intake (g)		Protein Intake (g)		Water Intake (mL)	
pregnancy	NP	LP	NP	LP	NP	LP
Week 1	111 ± 14	146 ± 9*	18 ± 1	12 ± 1*	166 ± 14	149 ± 8
Week 2	123 ± 7	130 ± 4	21 ± 1	10 ± 1*	177 ± 7	151 ± 7*
Week 3	93 ± 9	89 ± 4	15 ± 1	6 ± 1*	109 ± 9	115 ± 6

Data are reported as means±SE. *P<0.05 compared with NP group (unpaired Student's *t*-test).

Table 3. Hematological parameters of female rats subjected to normal (NP group, 17% protein) or low-protein diet (LP group, 8% protein) during pregnancy.

Item (unit)	Baseline (before mating)		Pregnancy	
	NP	LP	NP	LP
WBC (×10³/μL)	14.3 ± 1.8	16.7 ± 1.4	9.2 ± 2.1	11.4 ± 1.9
RBC (×10⁶/μL)	7.6 ± 0.3	7.6 ± 0.1	7.6 ± 0.2	7.6 ± 0.3
Hemoglobin (g/dL)	13.9 ± 0.2	13.9 ± 0.2	14.4 ± 0.6	14.3 ± 0.5
Hematocrit (%)	46.3 ± 0.6	45.9 ± 0.6	43.2 ± 1.1	43.6 ± 0.9
RDW CV (%)	11.9 ± 0.2	13.1 ± 0.4*	14.6 ± 0.8	13.9 ± 0.9
MCV (fL)	60.8 ± 0.5	60.1 ± 0.6	56.7 ± 0.7	57.5 ± 0.9
MCH (pg)	18.3 ± 0.2	18.2 ± 0.3	18.9 ± 0.2	18.9 ± 0.2
MCHC (g/dL)	30.1 ± 0.1	30.2 ± 0.2	33.4 ± 0.6	32.8 ± 0.6
LYM (%)	70.9 ± 1.1	75.1 ± 1.8	71.8 ± 2.6	73.1 ± 2.9
LYM ABS (×10³/μL)	10.1 ± 1.2	12.5 ± 1.1	11.6 ± 0.7	10.9 ± 1.3
Platelets (×10³/μL)	513.1 ± 43.6	655.2 ± 48.8*	553.5 ± 48.3	640.9 ± 55.5
MPV (fL)	7.1 ± 0.1	6.8 ± 0.1*	6.9 ± 0.2	6.8 ± 0.2

Data are reported as means±SE. WBC: white blood cells; RBC: red blood cells; RDW CV: red blood cell distribution width coefficient of variation; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; LYM: lymphocytes; LYM ABS: lymphocytes absolute value; MPV: mean platelet volume. *P<0.05 compared with NP group (unpaired Student's *t*-test).

Table 4. Reproductive parameters of female rats subjected to normal (NP group, 17% protein) or low-protein diet (LP group, 8% protein) during pregnancy.

Reproductive parameters	NP group	LP group
Pregnant rats (n)	8	14
Mass gain in the pregnancy period (g)	75 ± 11	70 ± 5
Mass gain in the organogenic period (g)	35 ± 4	24 ± 3
Offspring/dam relationship	12 ± 1	11 ± 1
Ovary mass (mg/100g)	37.9 ± 3.6	37.2 ± 4.5
Fetus mass (g)	2.5 ± 0.1	2.6 ± 0.2
Placentae mass (g)	37 ± 4	38 ± 3
Number of corpora lutea	13 ± 1	12 ± 1
Implantation index (%)	94 ± 2	93 ± 2
Resorption index (%)	2 ± 1	4 ± 2
Pre-implantation loss (%)	9	6
Post-implantation loss (%)	0	7

Data are reported as means±SE or median. There were no significant differences between groups.

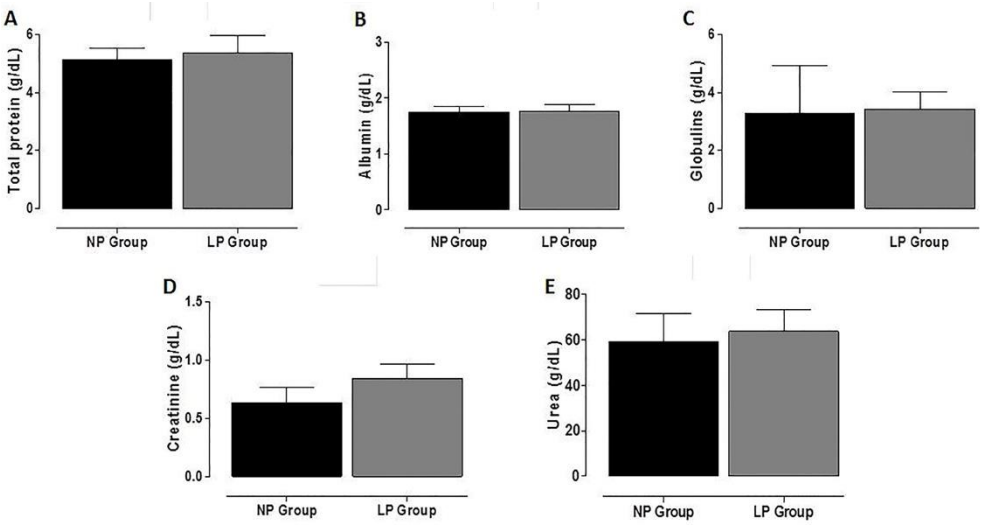

Figure 1. Serum biochemical parameters. Total protein (*panel A*), albumin (*panel B*), globulins (*panel C*), creatinine (*panel D*), and urea (*panel E*) of female rats subjected to normal (NP group, 17% protein) or low-protein diet (LP group, 8% protein) during pregnancy. Data are reported as means±SE and the comparison between groups was done by unpaired Student's *t*-test (P>0.05).