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AUCELIA CRISTINA SOARES DE BELCHIOR

EFEITOS DA DESNUTRIÇÃO INTRA-ÚTERO E APÓS O DESMAME SOBRE A MECÂNICA MIOCÁRDICA DE RATOS

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.
Orientador: Prof°. Dr°. Carlos Peres da Costa
Co-orientadores: Profº. Drº. Dalton Valentim Vassallo e
Profª. Drª. Alessandra Simão Padilha.

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RESUMO

A desnutrição durante períodos críticos no início da vida pode aumentar o risco subsequente de hipertensão e doenças metabólicas na vida adulta, mas os mecanismos subjacentes ainda não estão claros. Tivemos como objetivo avaliar os efeitos da desnutrição protéica pós-desmame (DPD) sobre a pressão arterial e contratilidade dos músculos papilares de ratos controle e DPD (descendentes que receberam uma dieta com baixo teor de proteína por três meses). Com a idade de 3 meses, os animais foram anestesiados e sacrificados, e os parâmetros relacionados com a contractilidade do músculo papilar isolado foram registrados. A pressão arterial sistólica e diastólica foram aumentados nos ratos DPD quando comparado com. DPD diminuição de força e tempo de derivados em músculos papilares isolados. Potenciação pós-pausa, a expressão da proteína SERCA e da fosfolambam e suas subunidades fosforilados foram reduzidas no grupo DPD, sugerindo comprometimento da absorção de Ca2+ pelo retículo sarcoplasmático. Além disso, as respostas inotrópicas induzidas por um aumento da concentração extracelular de cálcio foi reduzida no grupo de DPD. Avaliação in vitro do envolvimento dos canais de Ca2 + do tipo L, utilizando verapamil revelou uma diminuição semelhante da força em ambos os grupos. Estes resultados sugerem que a redução da resposta inotrópica ao Ca2 + não está relacionada com alterações no Ca2 + do sarcolema trans-fluxo, mas para uma possível alteração de proteínas contrácteis. Além disso, a expressão da proteína de isoforma α2 de Na +, K +, ATPase foi reduzida e a produção de anion superóxido foi aumentada no grupo de DPD. Estes resultados sugerem que DPD induz um aumento da pressão sanguínea e uma redução da força do músculo papilar isolado, que está relacionado com mudanças na absorção de Ca2 + pelo retículo sarcoplasmático. Produção de ânion superóxido parecem estar envolvidos nesta resposta.
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Palavras-chaves: desnutrição, hipertensão arterial, contratilidade cardíaca, manuseio de cálcio, proteínas contráteis.

ABSTRATC

Malnutrition during critical periods in early life may increase the subsequent risk of hypertension and metabolic diseases in adulthood, but the underlying mechanisms are still unclear. We aimed to evaluate the effects of post-weaning protein malnutrition (PWM) on blood pressure and papillary muscle contractility from control and PWM rats (offspring that received a diet with low protein content for three months). At the 3 months age, the animals were anesthetized and euthanized, and parameters related to isolated papillary muscle contractility were recorded. Systolic and diastolic blood pressure were increased in the PWM rats when compared to. PWM decreased force and time derivatives in isolated papillary muscles. Post-rest potentiation, protein expression of SERCA and phospholamban and its phosphorylated subunits were reduced in PWM group, suggesting impairment in uptake of Ca2+ by the sarcoplasmic reticulum. In addition, the inotropic responses induced by an increase in the extracellular calcium concentration was reduced in the PWM group. In vitro assessment of the involvement of L-type Ca2+ channels using verapamil revealed a similar decrease of force in both groups. These findings suggest that the reduction of the inotropic response to Ca2+ is not related to changes in the trans-sarcolemmal Ca2+ flux but to a possible alteration of contractile proteins. Moreover, the protein expression of isoform α2 of Na+,K+, ATPase was reduced and superoxide anion production was increased in PWM group. These results suggest that PWM induces an increase of blood pressure and a reduction of the force in isolated papillary muscle, which is related to changes in the uptake of Ca2+ by the sarcoplasmic reticulum. Superoxide anion production seem to be involved in this response.

Keywords: malnutrition, hypertension, cardiac contractility, calcium handling, and contractile proteins.

SUMÁRIO

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1 INTRODUÇÃO

1. 1 DESNUTRIÇÃO

A alimentação e a nutrição constituem requisitos básicos para a promoção e a proteção da saúde, possibilitando a capacidade plena do potencial de crescimento e desenvolvimento humano, com qualidade de vida e cidadania (BRASIL, 2011).

A nutrição pode ser definida como um grupo de processos pelo qual um indivíduo obtém, ingere, absorve e metaboliza as substâncias essenciais para a vida sob as mais variadas condições ambientais (SERAPÍÃO, 1986).

A nutrição adequada, em qualidade e quantidade, é fundamental para o crescimento e desenvolvimento dos seres vivos inclusive do homem. Por outro lado, deficiências nutricionais, decorrentes do menor aporte ou ausência de macro e micronutrientes na alimentação, levam a estados de má nutrição e desnutrição cujos graus dependem do tipo da dieta, do período da vida em que foi consumida e da extensão temporal do consumo, o que pode causar efeitos lesivos, reversíveis ou não (MORGANE *et al*.,1992). Segundo Andrade (1988), a desnutrição não se constitui em um fato isolado, mas no efeito da ação recíproca de fatores socioeconômicos, políticos, culturais e ambientais os quais atingem com maior intensidade as crianças que vivem em situações de extrema pobreza.

O estado nutricional dos indivíduos é caracterizado por grande dinamismo e decorre essencialmente do equilíbrio entre três fatores: composição da alimentação (tipo e quantidade dos alimentos ingeridos), necessidades do organismo em energia e nutrientes, e eficiência do aproveitamento biológico dos alimentos (ou da nutrição propriamente dita). Combinações ótimas desses três fatores, comportando razoáveis margens de variação para cada fator, propiciam ao indivíduo um estado nutricional ótimo, compatível com o pleno exercício de todas as suas funções vitais. A junção não equilibrada da ingestão alimentar, deficiências nutricionais e baixo aproveitamento biológico dos alimentos, podem produzir a má-nutrição ou a desnutrição (BRASIL, 2010).

Os termos má-nutrição e desnutrição são frequentemente usados imprecisamente e de maneira dúbia (SHETTY, 2006). O termo “má-nutrição” se refere a todos os desvios do *status* nutricional adequado e ótimo (SHETTY, 2006), e assim implica que um ou mais nutrientes essenciais estão faltando ou que podem estar presentes, mas, em proporções erradas (MORGANE *et al*., 2002). Desta forma, tanto a desnutrição quanto a obesidade

são exemplos de má-nutrição. O termo “desnutrição” é usado geralmente para *status* nutricional pobre (SHETTY, 2006), e indica que embora os nutrientes necessários para a espécie estejam disponíveis na dieta durante todo o tempo, estão em quantidades insuficientes (MORGANE *et al*., 2002). Suas principais manifestações são: o baixo peso, a baixa estatura e, em casos mais graves, caracterizam condições conhecidas como *Kwashiokor* e marasmo.

Kwashiokor é o nome dado para a severa desnutrição protéica (CASTIGLIA, 1996). Suas principais características são retardo no crescimento, perda de gordura subcutânea e muscular menos intensa que no marasmo, edema depressível que se localiza, principalmente, nas pernas, das crianças que caminham, mas que pode atingir todo o corpo, hepatomegalia acentuada devido a esteatose hepática, e alterações mentais e de humor. Podem ocorrer lesões de cabelos (textura, cor, sem brilho, queda) generalizadas ou localizadas (sinal da bandeira), e também lesões de pele, tais como: despigmentação, dermatose de áreas de fricção, descamação, etc. (MONTE, 2000).

Já o marasmo é o resultado da de privação calórica (CASTIGLIA, 1996), e decorre do aporte alimentar insuficiente para suprir as necessidades energéticas e proteicas do indivíduo, processo que se acompanha de emagrecimento progressivo, com diminuição do peso corporal e das reservas de gordura e de massa muscular (CUNHA *et al.,* 1998).

A desnutrição calórico-protéica se caracteriza pela existência de um desequilíbrio celular entre o fornecimento de nutrientes e energia por um lado, e por outro, a demanda corporal para assegurar o crescimento, manutenção e funções específicas do organismo (SERAPIÃO, 1986).

A desnutrição no período intrauterino advém do aporte materno inadequado de nutrientes ou de fatores intrínsecos como deficiência no transporte destes nutrientes através da placenta, o que produz alterações observadas ao nascimento, bem como no desenvolvimento pós-natal.

Segundo a Organização das Nações Unidas, a desnutrição engloba um grupo de condições patológicas que resulta da falta concomitante de calorias e proteínas. A desnutrição calórico-protéica pode alterar a função de uma série de órgãos e tecidos, tendo em vista que as proteínas são componentes importantes para a estrutura e função específicas das células do organismo (JEOR et al., 2001).

Dados da Organização Mundial de Saúde (OMS) revelam que, no mundo, aproximadamente 33% (182 milhões) de crianças com menos de cinco anos de idade estavam desnutridas no ano 2000. No Brasil, esse índice foi estimado em 10,5%. Já entre

aquelas vivendo em áreas rurais no Nordeste, a taxa foi de 39,8%. A desnutrição calórico-protéica continua a ser a doença que mais causa mortes em crianças.

Estudos realizados em humanos demonstraram a existência de uma relação entre a desnutrição fetal e a incidência de doenças crônicas na vida adulta (SCRIMSHAW, 1997; SLOAN *et al.,* 2001), pontuando uma associação entre retardo do crescimento fetal, consequência da desnutrição intra-útero, com o desenvolvimento da hipertensão arterial sistêmica (HAS) (ERIKSSON *et al.,* 2000) e doenças coronarianas na idade adulta (LEISTIKOW, 1998).

A desnutrição, especialmente a calórico-proteica, tem sido um problema para grande parte da humanidade e, frequentemente, aparece como causa de várias alterações no desenvolvimento humano (SILVA; ALMEIDA, 2006). Para Lucas (1997), a desnutrição quando ocorre nos períodos críticos ou sensíveis do desenvolvimento, pode programar por toda a vida a estrutura e função do organismo.

Para alguns autores, o aumento de pressão arterial na prole resultante de uma restrição proteica materna poderia aumentar a pós-carga cardíaca, resultando em hipertrofia e disfunção cardíaca (TAPPIA; GABRIEL, 2006). Assim, além de prejuízos na função cardíaca resultantes de aumento da pós-carga pressórica, a restrição proteica materna pode acarretar, na prole, alterações cardíacas advindas da programação fetal (BARKER, 2007).

Estudos realizados para avaliar os efeitos da desnutrição intrauterina sobre os níveis de pressão arterial (PA), têm demostrado um aumento da mesma. Langley-Evans et al., (1998), ao estudar ratos submetidos à dieta hipoprotéica, durante o desenvolvimento intrauterino, demonstraram que os animais apresentavam baixo peso corporal ao nascimento e pressão arterial sistólica (PAS) elevada. Ratos filhos de mães normotensas, expostas a uma dieta de baixo teor protéico durante a gestação, apresentaram diminuição do peso corporal ao nascer, e elevação da PA, detectada na quarta semana de vida, o que permanecia até a vida adulta (LANGLEY-EVANS; JACKSON, 1995; WOODALL *et al*., 1996; PÉREZ *et al*., 2002).

No adulto, a desnutrição crônica é um risco para a saúde e prejudica várias funções, incluindo a redução da habilidade para o trabalho e disponibilidade para atividades físicas, acarretando prejuízo na função imune e aumento na predisposição para infecções (SHETTY, 2006). E possui como causa principal, as condições socioeconômicas, embora, possa também surgir pela falta de nutrição adequada, em termos qualitativos, ou associada a doenças como fibrose cística, doença renal crônica,

AIDS e alcoolismo entre outras (OLIVARES *et al.*, 2005), ou ainda devido a uma redução voluntária da dieta por várias razões, inclusive psicológicas, como por exemplo, a anorexia nervosa (SHETTY, 2006).

Utilizando um modelo de desnutrição qualitativa, com baixo teor de proteínas, em ratos, Belchior et al. (2012) demonstraram que a desnutrição após o desmame, ou seja, a desnutrição crônica, promove aumento da PA associado a disfunção endotelial em artérias de condutância e aumento de reatividade vascular, em artérias de resistência.

1.2 DESNUTRIÇÃO E O SISTEMACARDIOVASCULAR

Como mencionado anteriormente, a desnutrição aumenta o risco de doenças cardiovasculares, e estas constituem um grave problema de saúde pública, que atualmente vêm se agravando.

Diversos estudos epidemiológicos têm demonstrado que a restrição proteica materna predispõe a prole ao aumento na incidência de doenças cardiovasculares na vida adulta (BARKER ; OSMOND, 1988; MARTYN; BARKER, 1994; LAMIREAU et al., 2002; HOVI et al., 2010). A hipótese é que a privação de nutrientes durante períodos distintos de desenvolvimento dos órgãos pré-natal pode programar a prole para doenças cardiovasculares na vida adulta (RICH-EDWARDS et al., 1976; LAMIREAU et al., 2002; ALEXANDER, 2006; BARKER, 2007). Em um dos modelos de desnutrição experimental mais frequentemente utilizados, ratas gestantes recebem uma dieta com baixa proteína durante a gestação. O fenótipo da prole é o baixo peso ao nascer e elevação da pressão arterial que ocorre geralmente após 4 semanas de vida e aumenta progressivamente com a idade (LANGLEY-EVANS et al., 1996; MANNING ; VEHASKARI, 2001).

Estudos anteriores têm demonstram que a restrição nutricional materna causa baixo peso ao nascer (BARKER; CLARK, 1997; FALKNER, 2002; LACKLAND *et al*., 2003; HOLEMANS *et al*., 2003), com desenvolvimento inadequado de órgãos como o pâncreas (GAROFANO *et al*., 1998) e o rim (LUCAS *et al*., 1997; VIEIRA-FILHO, 2011), ao contrário do cérebro e pulmões que são relativamente protegidos (DESAI; HALES, 1997). Essas alterações na organogênese predispõem a prole, na fase adulta, ao desenvolvimento de hipertensão (FALKNER, 2002) e de doenças cardiovasculares (BARKER *et al*., 1993).

A “ThriftyPhenotypeHypothesis”, proposta por Hales e Barker em 1992, postula que o diabetes tipo 2 e outras anormalidades metabólicas, como a síndrome X, possuem uma

forte influência ambiental. A programação da organogênese é influenciada pelo ambiente nutricional fetal e pós-natal e pode determinar como já foi relatada, a susceptibilidade do indivíduo ao desenvolvimento de alterações metabólicas e doenças cardiovasculares na vida adulta.

Segundo Jiménez-Chillarón *et al.* (2012), a nutrição adequada é particularmente essencial em períodos críticos, no início da vida, tanto pré como pós-natal. Dietas desequilibradas são as principais determinantes de doenças crônicas, incluindo doenças cardiovasculares, obesidade, diabetes e câncer. Os mesmos defendem a hipótese de que, mecanismos epigenéticos, podem ser responsáveis por vincular esses desequilíbrios nutricionais com os riscos de desenvolver essas dessas doenças.

O epigenoma é o substrato, onde o ambiente pode induzir em longo prazo, modificações fisiológicas permanentes. Assim, o epigenoma fetal / neonatal é modificado como uma reação ao ambiente materno, para preparar a prole para futuras pistas ambientais após o nascimento e, portanto, aumentar a sua aptidão evolutiva. Mas, ainda não está claro se desnutrição materna leva a modificações epigenéticas. Em estudos com animais, a desnutrição materna levou a hipometilação de vários genes (LILLYCROP *et al*., 2005)

Vários trabalhos têm demonstrado que, tanto a desnutrição intra-útero quanto a pós-desmame, predispõe ao desenvolvimento da hipertensão arterial em ratos adultos (LANGLEY-EVANS *et al*., 1994;. BELCHIOR *et al*., 2012).

Hu *et al*., (2000), estudando ratos submetidos à privação proteica, no período intrauterino, nos quais o comportamento hemodinâmico foi avaliado nos diferentes estágios de desenvolvimento, de adulto jovem à idade senil, demonstraram retardo no crescimento associado com aumento da pressão arterial diastólica, além de predisposição ao aparecimento de arritmias cardíacas, mais evidentes nos ratos mais velhos.

Investigação realizadas por Saraiva *et al.,* (1992) em crianças procedentes de favelas recifenses ou da Zona da Mata de Pernambuco com desnutrição calórica proteica, demonstraram que dos estudos eletrocardiográficos realizados, 95% dos enfermos apresentavam alteração na repolarização ventricular e baixa voltagem do QRS no plano frontal e o estudo ecocardiográfico apresentou diminuição da massa estimada para o ventrículo esquerdo entre outros achados. Cunha *et al.,* (1998),estudaram os efeitos da desnutrição calórico-protéica na morfologia do miocárdio e observaram existir menor espessura dos cardiomiócitos nos indivíduos desnutridos.

A desnutrição calórico-protéica também pode causar danos ao coração. Em animais de experimentação (Cicogna *et al*., 1999). Pissaia *et al*., (1990) ao estudar os

efeitos experimentais deste tipo de desnutrição sobre o coração de ratos, concluíram que esta induz alterações morfológicas e eletrofisiológicas, havendo também aumento dos níveis circulantes de catecolaminas que têm importantes efeitos inotrópicos e metabólicos sobre o coração.

O miocárdio ventricular tem sido um dos principais alvos das alterações provenientes da restrição alimentar. Em humanos, a alimentação pobre em teores proteicos e calóricos pode acarretar em perda de peso (JEOR *et al*., 2001), bradicardia, redução da contratilidade miocárdica, insuficiência cardíaca (OLUBODUN, 1992), e em animais, redução da distensibilidade ventricular e da função cardíaca (CICOGNA *et al*., 1999 e 2000; OKOSHI *et al*., 2002).

Alguns autores têm demonstrado que, a restrição alimentar, promove redução da distensibilidade ventricular e da função cardíaca (CICOGNA *et al*., 1999 e 2001), diminuição da complacência do ventrículo esquerdo, redução da contratilidade do miocárdio e remodelamento ventricular excêntrico (ABEL *et al*., 1979; OKOSHI et *al*., 2002). Essas alterações provavelmente conduzem a um prejuízo da função sistólica do ventrículo esquerdo (OKOSHI *et al*., 2002), disfunção miocárdica e alterações na função ventricular (KYGER *et al*., 1978; MCKNIGHT *et al*., 1999).

Estudos realizados em nosso laboratório com ratos desnutridos mostraram alterações histológicas nos ventrículos, caracterizadas por células em apoptose, necrose, e sinais de processos inflamatórios, esses achados histológicos também são observados na insuficiência cardíaca congestiva (SANT'HELENA, 2009).

Embora as modificações miocárdicas tenham valor expressivo na função cardíaca, outras alterações tais como, o aumento da concentração de catecolaminas no plasma e na fibra miocárdica, encontradas em muitos estudos com protocolo de desnutrição, sugerem que, o estresse nutricional e a exposição continuada do coração aos níveis elevados de catecolaminas, causam danos à contratilidade miocárdica e às membranas celulares (ROSSI *et al*., 1980; PISSAIA *et al*., 1990; DAVIS; JOHNSTON, 1990).

O sistema cardiovascular em situações de estresse, respondem com um aumento da PA e da frequência cardíaca, pela estimulação do eixo-hipotálamo-hipofise-adrenal, e liberação hormonal de catecolaminas (FAZAA *et al.,* 1999). No entanto, os resultados das pesquisas realizadas sobre os efeitos da desnutrição sobre a biossíntese e liberação de catecolaminas são conflitantes. Alguns autores evidenciaram um aumento dos níveis de catecolaminas circulantes no período de má nutrição (NUTTER *et al*., 1979; PISSAIA *et al*., 1980; KIM *et al.,* 1994). Embora outros, em contraste, observaram uma diminuição na

síntese de noradrenalina em ratos submetidos à má nutrição no período pré e pós-natal (MARICHICH *et al*., 1979)

Como mencionado anteriormente, tanto a desnutrição intra-útero quando a desnutrição pós desmame, acarretam alterações do sistema cardiovascular, como por exemplo, redução da distensibilidade ventricular, perda da função cardíaca e diminuição da complacência do ventrículo esquerdo (CICOGNA *et al*., 1999 e 2001). No entanto, poucos estudos são direcionados as investigações dos efeitos desses tipos de desnutrição sobre a maquinaria contrátil do coração. Sendo assim, como objetivamos estudar tais efeitos, faz-se necessário uma breve introdução dos mecanismos envolvidos no acoplamento excitação-contração do coração.

1.3 MECANISMO DE ACOPLAMENTO EXCITAÇÃO-CONTRAÇÃO

O mecanismo de acoplamento excitação-contração (AEC) consiste em um conjunto de mecanismos, desencadeados pela estimulação elétrica, que culmina na contração cardíaca (proporcionando a ejeção do sangue). O íon cálcio é essencial para a atividade elétrica cardíaca e é um ativador direto dos miofilamentos (BERS, 2002).

Durante o potencial de ação cardíaco o cálcio entra na célula através dos canais de cálcio ativados por despolarização e gera a corrente de cálcio, chamada ICa, que geralmente se refere à entrada através dos canais de cálcio do tipo L. A entrada de cálcio pelo sarcolema desencadeia a liberação de cálcio-cálcio induzida do retículo sarcoplasmático (RS), pelos canais de rianodina. A combinação entre o influxo e a liberação de cálcio aumenta a concentração de cálcio intracelular ([Ca]i), possibilitando a ligação do cálcio com a troponina C (TnC), e desta forma, desencadeia o processo contrátil.

O relaxamento do cardiomiócito ocorre com o desprendimento do cálcio da TnC e a posterior redução da [Ca]i. Esta redução é possibilitada por ação de 4 mecanismos, envolvendo: a bomba de cálcio do RS (SERCA ou RS Ca++-ATPase), a qual recapta ativamente este íon para seu interior; a Ca^{2+}-ATPase do sarcolema; o trocador Na^{+} Ca^{2+} do sarcolema; e o uniporte de cálcio na mitocôndria. O fosfolambam (PLB) é um inibidor endógeno da SERCA que quando fosforilado, devido ao aumento do AMPc (ativando PKA) ou cálcio calmodulina quinase (CaCKII), deixa de inibir a bomba e permite o influxo ativo de cálcio para dentro do RS. Um dos locais de fosforilação do PLB é a serina 16 (SCHWINGER *et al.*, 1999; BERS, 2002).

A *figura 1* ilustra estes mecanismos envolvidos na cinética do cálcio durante o processo de contração e relaxamento do miócito.

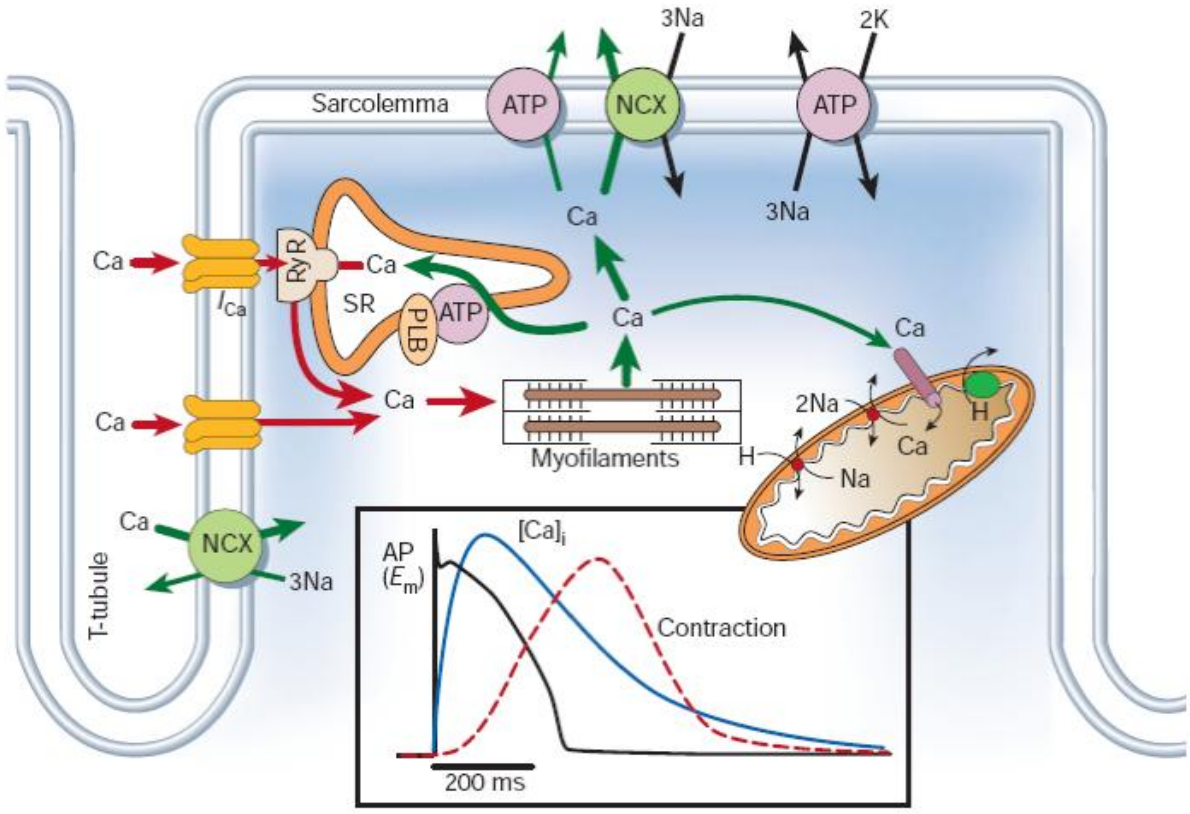

Figura 1: Transporte de cálcio nos miócitos ventriculares. Destaque para o curso temporal do potencial de ação, o transiente de cálcio e a contração, mensurados em miócitos ventriculares de coelhos a 37°C. NCX: trocador Na^+ Ca^{2+} do sarcolema; PLB: fosfolambam; SR: retículo sarcoplasmático.
Fonte: Bers, 2002.

O trocador Na^+/Ca^{++} do sarcolema é reversível e, desta forma, além de participar do processo de contração, também auxilia no processo de relaxamento. Este trocador possui uma estequiometria de 3 Na^+ para cada 1 Ca^{2+}, e gera a corrente $I_{Na/Ca,}$ a qual pode resultar da extrusão ou do influxo de cálcio. Na repolarização ou no repouso celular o trocador trabalha em seu modo normal, ou seja, favorecendo a extrusão de cálcio e o influxo de sódio. Durante a despolarização, o potencial de membrana torna-se positivo e atinge valores maiores que o do potencial de equilíbrio do trocador. Esta alteração elétrica causa inversão do NCX, portanto, o mesmo trabalha retirando sódio e favorecendo o influxo de cálcio no miócito.

Outra forma de controlar a atividade do NCX, além das mudanças do potencial de membrana, consiste nas alterações na disponibilidade destes íons para o transporte. A

elevação do Na^+_i dificulta, rápida e intensamente, a troca Na^+/Ca^{2+} no estado de repouso, basicamente porque o aumento do Na^+_i diminui o gradiente difusional do Na^+ através da membrana. Além disso, o trocador possui 2 sítios intracelulares onde se ligam Na^+ e Ca^{2+}. A ligação do Na^+ provoca redução da atividade da troca, enquanto a ligação com o Ca^{2+} a estimula. Desta forma, condições que promovem elevação dos níveis de Na^+_i, tais como os digitálicos, dificultam a extrusão do Ca^{2+} via troca Na/Ca, aumentando a força de contração (BLAUSTEIN *et al.*, 1991).

A região de junção entre o RS e as membranas dos túbulos transversos ou sarcolema é, atualmente, alvo de muitos estudos envolvendo a modulação da atividade contrátil. Estudos recentes demonstraram que a este nível são expressas moléculas de NCX e da Na/K-ATPase (NKA). Devido a esta proximidade, efeitos inibidores sobre a NKA, como os da ouabaína em pequenas concentrações, promovem aumento local do Na^+_i sem, entretanto, afetar a concentração global de Na^+ intracelular. Este aumento local do Na^+ inibe parcialmente o NCX aumentando a concentração local do Ca^{2+}. Este, por sua vez, é captado pelo RS e, frente a uma ativação de um músculo, é liberado em maior quantidade aumentando a força de contração (BLAUSTEIN; LEDERER, 1999).

Há três formas de alterar a força de contração cardíaca: alterando a amplitude ou a duração do transiente de cálcio; modificando a sensibilidade dos miofilamentos ao cálcio; ou através da mudança na força máxima ativada por cálcio que pode ser alcançada pelos miofilamentos, o que corresponde à variação no número de pontes cruzadas (BERS, 2002, VASSALLO *et al*., 2008).

Uma das intervenções mais comuns que afeta, tanto a disponibilidade do cálcio quanto a responsividade miofibrilar ao cálcio, é a estimulação β-adrénergica. Fisiologicamente a estimulação simpática dos β-receptores resulta em aumento da força contrátil desenvolvida (efeito inotrópico positivo) e aceleração do tempo de relaxamento (efeito lusitrópico positivo). Os agonistas β-adrenérgicos, aumentam a produção intracelular do segundo mensageiro, AMPc, que ativa a proteína cinase A (PKA), causando fosforilaçao da troponina I. Este mecanismo envolve interações alostéricas entre as proteínas do filamento fino, culminando na redução da afinidade da TnC pelo Ca^{2+}. A *figura 2* ilustra a ativação do receptor β-adrenérgico no músculo cardíaco e os mecanismos intracelulares desencadeados por esta ativação.

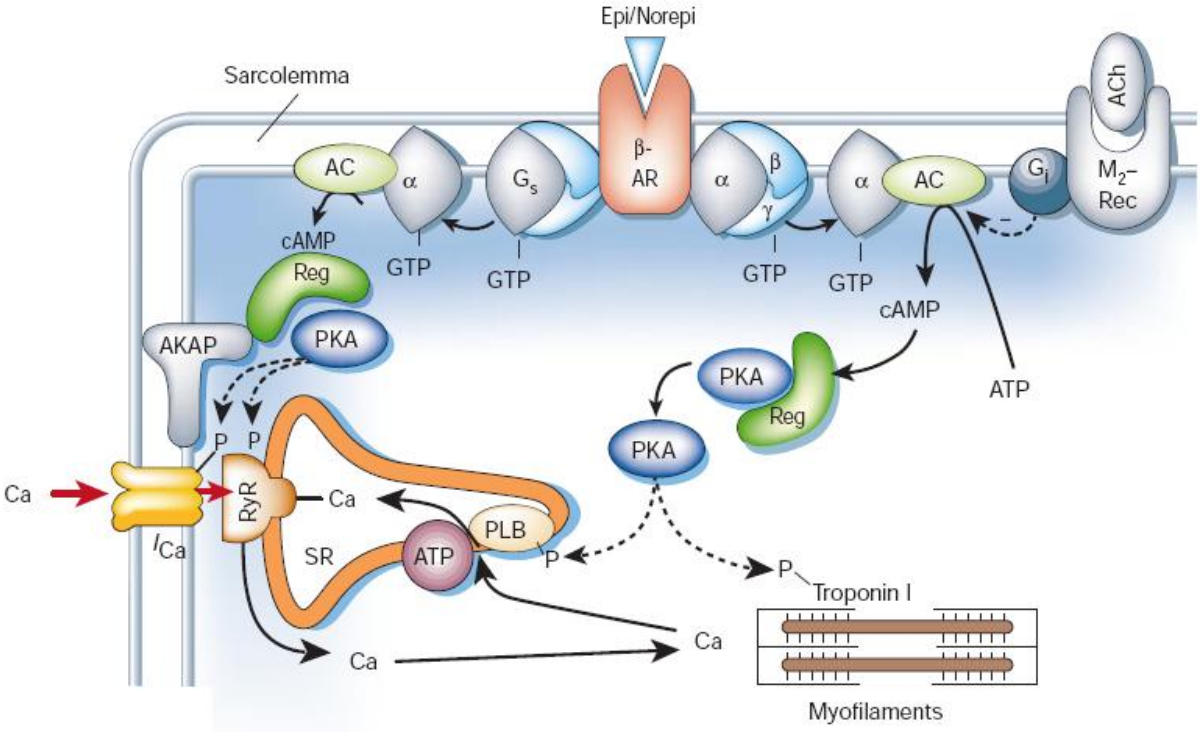

Figura 2: Ativação do receptor adrenérgico e fosforilação dos alvos relevantes para o acoplamento excitação-contração. AC: adenilatociclase; Ach: acetilcolina; AKAP: proteína ancoradora de cinase A; β AR: β –receptores; M_2-Rec: receptor muscarínico M_2; PLB: fosfolambam; SR: retículo sarcoplasmático.

Fonte: Bers, 2002.

Para a realização deste estudo, a desnutrição foi provocada por um tipo de dieta proposta por Teodósio *et al.* (1990), que baseado em uma enquete alimentar, desenvolveram um modelo experimental, que reproduz no rato, quadro muito semelhante ao da desnutrição calórico-protéico que já foi prevalente no Nordeste brasileiro.

Levando-se em consideração que, a dieta básica regional (DBR), já representou o consumo básico diário de uma significativa parcela da população da zona da Mata de Pernambuco. E que este modelo de desnutrição qualitativa ainda expressa o consumo de algumas populações em determinadas situações, a importância deste estudo, realizado em ratos desnutridos, foi demonstrar possíveis relações entre essa dieta e alterações na maquinaria contrátil do coração. Assim, avaliamos no presente trabalho as repercussões da DBR em ratos submetidos a mesma, na vida intrauterina e após o desmame, sobre alguns parâmetros hemodinâmicos e a contratilidade miocárdica que foi estudada no músculo papilar isolado. Os resultados foram comparados com ratos controle submetidos a dieta padrão Labina®.

2 OBJETIVOS

2.1 – GERAIS

Avaliar em ratos adultos, os efeitos produzidos pela desnutrição intra-útero e pós desmame sobre a hemodinâmica e contratilidade miocárdica.

2.2 – ESPECÍFICOS

- Avaliar os efeitos da desnutrição intra-útero e após o desmame sobre o ganho ponderal;
- Comparar os parâmetros hemodinâmicos, tais como, pressão arterial, pressão intraventricular, derivadas de pressão positiva e negativa e pressão diastólica final do ventrículo esquerdo *in vivo* de ratos desnutridos com ratos normonutridos;
- Avaliar os efeitos desnutrição sobre a força isométrica de papilares isolados ;
- Averiguar, *in vitro*, os efeitos da exposição da desnutrição na contratilidade miocárdica, avaliando: o inotropismo cardíaco, os parâmetros temporais da contração, a atividade do retículo sarcoplasmático, a permeabilidade da membrana sarcoplasmática ao cálcio, a resposta das proteínas contráteis ao cálcio e a participação da ativação β-adrenérgica nos músculos papilares do ventrículo esquerdo;
- Investigar se a desnutrição altera a expressão de proteínas envolvidas na regulação do ciclo de cálcio no miócito cardíaco.

3 CONCLUSÕES

Neste estudo, foram investigados os efeitos da desnutrição sobre a mecânica cardíaca em dois períodos importantes do desenvolvimento - na vida intrauterina e após o desmame - a fim de elucidar alguns mecanismos que envolvem questões relevantes em saúde pública, desnutrição e os mecanismos envolvidos no *défict* de força do miocárdio ventricular promovido pela desnutrição.

Principais achados da desnutrição intra-útero:

- Redução do peso corporal e elevação da PAS

- Não houve alteração de força desenvolvida pelos músculos papilares isolados

- Redução das dF/dt_{max} e dF/dt_{min} e do tempo de ativação e de relaxamento em músculos papilares isolados

- Redução da potenciação pós-pausa, sugerindo redução da função do retículo sarcoplasmático.

- Redução da expressão proteica da SERCA-2a, do fosfolambam e do fosfolambam fosforilado na serina-16

- Redução da força em maior magnitude no grupo desnutrido, após incubação com verapamil, um bloqueador dos canais para Ca^{2+} do tipo L, sugerindo maior influxo de cálcio ou maior atividade dos canais do tipo L.

Sugerimos que ratos jovens nascidos de ratas submetidas a desnutrição intra-útero apresentam aumento de pressão arterial e mudanças na maquinaria contrátil e expressão alterada de proteínas que regulam a contratilidade cardíaca. Essas alterações propiciam ao desenvolimento de doenças cardiovasculares.

Principais achados da desnutrição crônica ou pós-desmame:

- Redução do peso corporal e aumento da pressão arterial e da frequência cardíaca;
- Diminuição da força desenvolvida e redução das dF/dt positiva (+) e negativa (-) em músculos papilares isolados;
- Redução da potenciação pós-pausa, sugerindo redução da função do retículo sarcoplasmático.
- Redução da expressão proteica da SERCA-2a, do fosfolambam e do fosfolambam fosforilado na serina-16; e aumento da expressão da isoforma α1 da NKA.
- Redução da resposta inotrópica ao cálcio.
- Não houve diferenças nas contrações PRC, na presença e na ausência de Verapamil, um bloqueador dos canais para Ca^{2+} do tipo L, sugerindo que não há prejuízo no influxo de cálcio ou na atividade dos canais do tipo L.
- Aumento da produção de espécies reativas de oxigênio.

Em conjunto, esses resultados sugerem que a desnutrição crônica induz aumento de pressão arterial e redução da força desenvolvida por músculos papilares isolados, o qual parece estar associado a mudanças na recaptação de cálcio pelo retículo sarcoplasmático. O aumento da produção de anion superóxido parece estar envolvido neste efeito.

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ANEXO I

Artigo 1 – Submetido - Maternal Protein Restriction Compromises Cardiac Function in Adult Offspring

Maternal Protein Restriction Compromises Cardiac Function in Adult Offspring

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Running Head: MATERNAL PROTEIN RESTRICTION COMPROMISES CARDIAC FUNCTION

Abstract

Maternal protein restriction (MPR) during pregnancy is associated with the occurrence of arterial hypertension in the offspring in adulthood. In turn, arterial hypertension might cause cardiac dysfunction. Therefore, we sought to investigate the cardiac function of young rats born ("MPR" group) or not ("control" group) from mothers subjected to protein restriction during pregnancy. The following parameters were assessed: systolic arterial pressure (SAP) in the waking state; arterial and intraventricular pressures, as well as the positive and negative derivatives of intraventricular pressure (dP/dt_{max} and dP/dt_{min}, respectively) under anesthesia; and cardiac contractility in isolated papillary muscles. The body weight was lower and SAP higher in the MPR group compared to the control; dP/dt_{max} and dP/dt_{min} exhibited increases in the MPR group. The isometric force of the isolated papillary muscles did not exhibit changes; however, the time to peak and the relaxation time were higher, and the time derivatives of force were lower in the MPR group. The isolated papillary muscles of the animals in the MPR group exhibited decreased post-rest potentiation, which suggests reduced activity of the sarcoplasmic reticulum. Corroborating those findings, the protein expression levels of SERCA-2a, phospholamban, and serine^{16} phosphorylated phospholamban were decreased in the MPR group. Based on the assessment of the participation of L-type Ca^{2+} channels using blocker verapamil, greater force reduction was found in the papillary muscles of the animals from the MPR group, which suggests increased calcium influx or greater activity of the L-type channels. We suggest that young animals born from rats subjected to protein restriction during pregnancy exhibit elevated blood pressure, changes in the contractile machinery, and altered expression of the proteins that regulate cardiac contractility. Those changes are favorable for the development of cardiovascular diseases.

Keywords: Maternal protein restriction, cardiac contractility, calcium homeostasis

Introduction

Several epidemiological studies showed that maternal protein restriction predisposes the offspring to an increased incidence of cardiovascular diseases in adulthood [1-4]. It is hypothesized that nutrient deprivation in certain periods of the prenatal development of organs might program the offspring to develop cardiovascular diseases in adulthood [3,5-7]. In one of the most widely used models of experimental malnutrition, pregnant rats are fed a low-protein diet throughout pregnancy. The resulting offspring phenotype is characterized by low birth weight and elevated blood pressure, which usually appears after four weeks of age and increases progressively with further age [8,9].

The elevation in the blood pressure due to maternal protein restriction might increase the afterload, resulting in cardiac hypertrophy and dysfunction [10]. In addition to the impairment of heart function caused by the elevated afterload, maternal protein restriction might be associated with heart problems derived from fetal programming [7]. Indeed, one study showed that maternal protein restriction induced considerable depression of the heart contractile function in the offspring, most likely due to loss of cardiomyocytes caused by increased apoptosis [11]. According to other authors, maternal protein restriction might induce alterations in the functional components of the neonatal heart, including genomic changes in the proteins involved in the homeostasis of the myoplasmic calcium, which might contribute to the depression of the heart contractile function [12,13].

The few studies that have investigated the effects of maternal protein restriction on the heart function were conducted with neonates. According to Barker's [7] theory, maternal protein restriction might predispose the offspring to developing cardiovascular diseases in adulthood. Thus, the aim of this study was to assess cardiac contractility and the proteins involved in cardiac contractility and in calcium homeostasis in the young offspring of rats subjected to protein restriction. For that purpose, the diet suggested by Teodósio et al. [14] was used, which is based on a nutrition survey that reproduces in rats a situation similar to the protein malnutrition that still exists in some areas of Northeastern Brazil.

Materials and Methods

Diets

Two types of diets were used for this study. The diet used to induce malnutrition was the regional basic diet (RBD), as described previously [14-16]. The RBD consists of beans (*Phaseolus vulgaris)*, sweet *potatoes (Ipomea batatas)*, jerked beef, and manioc flour *(Manihot esculenta)*. These components were macerated, molded into "pellets", and heated to 50°C. The RBD pellets provide a total of (g/g%) proteins 9, carbohydrates 78, lipids 1.1, fiber 7, minerals 4, sodium chloride 0.17 and kilocalories 356. No vitamin supplement was added. Moreover, part of the diet was supplemented with 0.2% (g/g) sodium chloride. The control diet was a commercial diet (Labina®). The standard diet contains the following content (g/g%): protein 23, carbohydrates 41, lipids 2.5, fibers 9, minerals 8, sodium chloride 0.37 and kilocalories 278.

Animals and experimental groups

This study used *Wistar* rats bred at the vivarium of the Department of Physiology and Pharmacology, Center of Biological Sciences, Federal University of Pernambuco. The animals were kept in cages under controlled temperature and a 12-hour light-dark cycle, with free access to water and food.

Use and care of the animals complied with the ethical principles for animal research formulated by the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal/COBEA-1991). All the experimental protocols were approved by the animal experimentation ethics commission, Center of Biological Sciences, Federal University of Pernambuco (no. 23076.008507/2010-16).

The animals were allocated to two experimental groups: the control group (Ct), which was fed a standard diet (Labina®), and the group subjected to maternal protein restriction (MPR) as follows:

- Ct Group – The mothers were fed the standard diet during the pre-mating and mating periods, pregnancy, and lactation. After weaning (day 21), the pups were fed the same standard diet and subjected to the experimental protocol at age three months old.
- MPR Group – The mothers were fed the standard diet in the pre-mating period and RBD during the mating period and pregnancy. After weaning, the offspring was fed the standard diet until age three months old, when it was subjected to the experimental protocol.

Assessment of weight

The animals were weighed at birth, immediately after weaning, and at age 90 days old (onset of the experimental protocol). Following dissection of the left papillary muscle, the right and left ventricles were separated and weighed.

Hemodynamic measurements

At age three months old, the rats were anesthetized with urethane (1.2 g/kg IP), the left carotid artery was dissected, and a polyethylene catheter (PE 50, Clay-Adams) filled with heparinized saline (50 IU/ml) was inserted to measure the systolic (SAP) and diastolic (DAP) arterial pressure. Then, the catheter was introduced into the left ventricle to measure the left ventricular systolic pressure (LVSP) and the positive and negative derivatives of intraventricular pressure (dP/dt_{max} and dP/dt_{min}, respectively). Next, the catheter was placed in the carotid artery again, and the blood pressure was measured again to establish whether aortic valve damage had occurred. The rats that exhibited reduced DAP were discarded. The records were made over 30 minutes using a pressure transducer (model TSD 104A) connected to a preamplifier and a data acquisition system (model MP30, BIOPAC System, Inc. Santa Barbara, CA).

SAP was measured indirectly in both groups at the end of treatment using the tail-cuff plethysmography method (IITC Life Science non-invasive blood pressure, version 1.35). In a conscious state, the rats from both groups were kept five to 10 minutes in a warm chamber and in a silent environment to record the cuff inflation-deflation cycles. Next, SAP was measured, and the average values of three measurements were recorded and analyzed, as recommended by Grizzo et al. [17].

Isolated papillary muscles

After the hemodynamic parameters were measured, the hearts of the animals in both groups were quickly removed and perfused through the aortic stump before dissection of the papillary muscles on the anterior and posterior walls of the left ventricle. To measure the isometric tension, the papillary muscles were mounted and kept in a 20-mL organ bath containing Krebs-Henseleit solution (in mM: 118 NaCl, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 23 $NaHCO_3$, and 11 glucose) at 26 ± 0.5ºC to prevent hypoxia [18] and at pH 7.4, being continuously aerated with 95% O_2 and 5% CO_2. The specimens were connected to an isometric transducer (TSD125-Biopac Systems, Inc., CA). The muscle was stimulated by means of isolated rectangular pulses (10-15 V, 12 ms long) applied through a pair of platinum electrodes placed along the full muscle length. The standard stimulation rate was 0.5 Hz. The records began 45-60 minutes later to allow the specimens to adapt to the new environmental conditions. The strength developed during contractions was measured as g/mg (strength developed in g divided by the muscle weight in mg). Correction for the papillary weight was performed to normalize the data from the various specimens. Following a period of stabilization, the experimental protocols were begun as follows:

1. The effects of MPR on the development of isometric force were compared to the values exhibited by the control animals. Force time parameters (time to peak – TP; relaxation time – TR) were also evaluated. TP represents the time elapsed from the onset of contraction to

the maximum peak force, and RT represents the time elapsed from the maximum peak to the period of isometric relaxation.

2. Post-rest potentiation (PRP) was used to obtain information on the sarcoplasmic reticulum (SR) function. The contractions of the cardiac muscle that occur after short rest intervals are potentiated; such contractions depend on the duration of the rest intervals and the amount of calcium stored in intracellular sites. Various rest interval lengths were tested (15, 30, and 60 s). The results are expressed as relative potentiation (amplitude of post-rest contractions divided by contraction in basal conditions, i.e., steady state) to normalize the data from the various specimens [19].
3. Under basal conditions (steady state), force was assessed under various isoproterenol concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M) in the specimens from both groups.
4. Post-rest contractions (PRC) were obtained after 10 min without stimulation. The muscles were kept in calcium-free solution with (5 mM) caffeine. To induce PRC, the calcium-free solution was replaced by modified Krebs solution (with 1.25 mM calcium) a few seconds before the onset of electrical stimulation. PRC was induced before and after blockade of the calcium transarcolemmal influx. Blockade was performed by adding verapamil (10 μM) to the bath over 20 minutes before induction of the subsequent PRC [20].
5. Tetanic tension is a protocol used to investigate the contractile response of intact myocardium with non-functional SR [21]. Tetanic tension was induced through high-frequency stimulation (10 Hz over 15 s) following pre-incubation with 5 mM caffeine over 30 minutes [21]. Tetanic tension was assessed before and after incubation with verapamil (10 μM), which was added to the bath 20 minutes before the subsequent tetanus [21].

Western blot analysis

The hearts of the Ct and MPR rats were homogenized, and protein (80 µg) was separated using 10% SDS-PAGE gel to quantify SR Ca^{++}-ATPase (SERCA-2a) and sodium-calcium exchanger (NCX). The low-molecular-weight proteins phospholamban (PLB) and serine 16 phosphorylated PLB (PLBp) (phospho-ser^{16}-PLB) were separated using 15% SDS-PAGE gel.

The proteins were transferred to nitrocellulose membranes (Amersham, UK), which were incubated with mouse monoclonal antibodies to SERCA-2a (1:1,000, Thermo Scientific, Rockford, USA), NCX (1:1,000, Thermo Scientific, Rockford, USA), PLB (1:1,000, Thermo Scientific, Rockford, USA) and PLBp (1:5,000, Thermo Scientific, Rockford, USA). After rinsing, the membranes were incubated with anti-mouse IgG antibody (1:5,000, Sigma Chemical, Co., St Louis, MO, USA).

After thorough rinsing, the immune complexes were detected using a chemiluminescence system (ECL Plus Amersham International, Little Chalfont, UK) and film (Hyperfilm ECL International). The immunoblot signals were quantified using the software National Institutes of Health Image V1.56. The same membrane was used to measure the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using rat monoclonal antibody to GAPDH (1:5,000, Abcam Cambridge MA, USA). After rinsing, the membrane was incubated with anti-mouse antibody (1:5,000, Ensaio designers, Hines Drive, Ann Arbor, MI). All the reagents used in western blot transfer were purchased from Sigma Chemical Co., USA.

Drugs used

The following drugs were used: heparin (Roche Q.F.S.A., Brazil), anhydrous caffeine (B. Herzog, Brazil), urethane, bovine serum albumin, and verapamil (Sigma Chemical Co., USA). The other reagents used were analytical grade and purchased from Sigma and Merck (Germany) or Reagen (Brazil).

Data analysis and statistics

All the results are expressed as the mean ± standard error of the mean (SEM) and were analyzed using the unpaired Student's t-test and one-way or two-way ANOVA. When the results on ANOVA were statistically significant, the Bonferroni *post hoc* or Tukey's test was used to compare individual means. The significance value was set to p-value < 0.05. The data were analyzed and the graphics plotted using the software GraphPad Prism (Version 5.0, GraphPad Software, USA).

Results

Assessment of weight gain

Maternal protein restriction caused a delay in the weight gain of the offspring (Table 1). The birth weight was lower in the MPR group than in Ct (MPR: 3.51 ± 0.07 g vs. Ct: 7.36 ± 0.09 g, p< 0.05). Upon weaning, on day 21, the body weight was still lower in the MPR group than in Ct (MPR: 42.4 ± 2.3 g vs. Ct: 64.46 ± 1.3 g, p < 0.05). The reduction in weight exhibited by the MPR group remained until day 120 (MPR: 267.25 ± 2.7 g vs. Ct: 357.85 ± 7.7 g, p < 0.05).

Hemodynamic parameters

SAP, as indirectly measured with the animals awake, was higher in the MPR group compared to Ct (Table 2). However, when SAP was measured with the animals anesthetized, no difference was found (Table 2). The remainder of the pressure parameters (DAP and LVSP) did not differ between the groups; however, the positive and negative derivatives of the left intraventricular pressure were higher in the MPR group than in Ct.

Effects of maternal protein restriction on the contractility of the papillary muscles

Although maternal protein restriction caused elevation of the systolic arterial pressure in the offspring, no changes were found in the force developed by the isolated papillary muscles (Figure 1 A). However, TP and 90%RT were higher in the papillary muscles of MPR rats compared to Ct (Figure 1 B and C). In contrast to the in vivo data, the positive and negative force derivatives (dF/dt+ and dF/dt-, respectively) were lower in the MPR group compared to Ct (Figure 1 D and E).

To establish whether the SR function of the offspring was altered by maternal protein restriction, PRPs were recorded and assessed. As Figure 2 A shows, the force decreased after 30- and 60-s rest intervals in the MPR group.

We further investigated whether maternal protein restriction altered the inotropic response of the offspring through the isoproterenol concentration-response curve (Figure 3). The results showed that the inotropic intervention of isoproterenol was similar in both groups.

The results described up to this point show that although maternal protein restriction did not alter the force developed by the isolated papillary muscles of the offspring, it promoted increased TP and 90%RT and reduced positive and negative derivatives of force. Those changes might be related to impairment of the SR, as suggested by the data obtained through PRP. However, changes in the transarcolemmal calcium influx and in the proteins involved in the cardiac contractility and calcium homeostasis might also account for the alterations found in PT, 90%RT and the positive and negative derivatives of force.

Therefore, to investigate indirectly whether intrauterine malnutrition alters the transarcolemmal calcium influx, PRCs, in the presence and absence of verapamil (1 μM), an L-type calcium channel blocker, were assessed. Figure 4 A shows that PRC was higher in the MPR group compared to Ct. After treatment with verapamil, PRC decreased in both groups (Figure 4 B), and the effect was greater in the MPR group (Figure 4 C).

Tetanic contraction also depends on the transarcolemmal calcium influx as well as on the sensitivity of the contractile proteins to calcium. Figure 5 A and B shows that the tetanus plateau and peak were similar in both groups. However, following incubation with verapamil, both the tetanus plateau (Figure 5 C) and peak (Figure 5 D) exhibited greater reduction in the MPR group.

The amounts of some of the main proteins involved in the regulation of calcium homeostasis, and thus in cardiomyocyte contractility, were assessed through western blot, including the protein expression of SERCA-2a, NCX, PLB, and PLBp. Corroborating the functional data obtained through PRP, MPR promoted reduction of SERCA-2a expression and reduction of PLB and PLBp in the offspring, although the PLB/PLBp ratio remained unchanged (Figure 6 A, B, C, D). In addition, the protein expression of NCX (Figure 6 E) was increased in the MPR group compared to Ct.

Discussion

The results of this study suggest that MPR promotes reduction of the SR function in the isolated papillary muscle of the adult offspring, in association with a reduction of SERCA-2a protein expression and increased participation of the L-type calcium channels, with increased protein expression of NCX. These data, together with the elevation in the blood pressure exhibited by those animals, suggest that MPR predisposes the offspring to cardiovascular diseases in adulthood. These data are relevant because cardiovascular diseases are among the main causes of morbidity and mortality worldwide and the incidence of MPR is still significant in several countries, the developing ones in particular.

This study used the model of experimental qualitative malnutrition induced by the regional basic diet (RBD) described by Teodósio et al. [14]. RBD is based on a nutrition survey that reproduces in rats a situation similar to the protein malnutrition that still exists in certain areas of Northeastern Brazil. Low birth weight is a significant feature exhibited by the offspring of mothers subjected to protein restriction. In this study, the body weight in adulthood for the offspring of rats

subjected to protein restriction was lower compared to the control group, despite the offspring having been fed a normal diet after birth.

Many authors suggest that low birth weight due to inadequate nutrient supply during fetal development might program the offspring to develop a predisposition for cardiovascular diseases, such as systemic arterial hypertension, in adulthood [5-7,22,23]. Our data confirm those reports, as we found elevation of SAP in the adult offspring of rats subjected to protein restriction. In the case of the offspring of mothers with low-protein energy-restricted diets, hypertension seems to be caused by several mechanisms, including increased sympathetic activity [24,25], increased oxidative stress and activation of the renin-angiotensin system [26,27], and impaired secretion of sodium and retention of water and sodium by the kidneys, with reduction in the number of nephrons [28,29], among other factors. The fact that we did not find a significant difference in the blood pressure between the MPR and Ct rats under anesthesia might be accounted for by the depression of the central nervous system induced by urethane [30]. However, hemodynamic analysis provided some relevant data, to wit, the increase of the positive and negative time derivatives of intraventricular pressure in the MPR group, which suggests cardiac inotropism. Several factors might increase the cardiac contractility, including an increase of the sympathetic activity and changes in the contractile machinery and/or in the proteins that regulate the cardiac contractility. Previous studies demonstrated that sympathetic activity is increased in the offspring of rats subjected to protein restriction during pregnancy [24,25], which might account for the increased positive and negative derivatives of intraventricular pressure found in the MPR rats. Based on those findings, we sought to investigate possible changes in the contractile machinery and/or in the proteins that regulate cardiac contractility in the MPR group.

Although maternal protein restriction did not alter the isometric force of the isolated papillary muscles of the adult offspring, it promoted reduction of the positive and negative derivatives of force as well as increases of PT and 90%RT. The increases in PT and 90%RT and the reduction of the derivatives of force might be due to alterations in the transarcolemmal calcium

influx and calcium uptake by the SR. Indeed, upon analyzing the SR function via PRP, we found a reduction of that parameter in the papillary muscle of the MPR rats, which suggests reduced participation of SR in the myoplasmic calcium uptake. Corroborating the functional data, the protein expression levels of SERCA-2a, PLB and PLBp were reduced in the MPR group. Additionally, Tappia and colleagues [13] found reduction of the protein expression of SERCA-2a in the neonate offspring of rats subjected to protein restriction during pregnancy, suggesting that calcium uptake by the SR could be impaired. Therefore, we suggest that the alteration in SERCA-2a expression found by Tappia et al. [13] in neonates might persist into adulthood, as observed in this study.

Another relevant point of our study was the investigation of possible alterations in the inotropic response of the papillary muscles of the young MPR rats to isoproterenol. The results showed that the inotropic intervention of isoproterenol did not cause any change in the force increase between the MPR and control groups. Those findings disagree with the results of Fernandez-Twinn and colleagues [31], who found reduction of the beta-adrenergic responsiveness and attenuation of the adrenergic signaling pathway in young animals (three months old) born from rats fed a low-protein diet during pregnancy and lactation. However, Fernandez-Twinn and colleagues [31] assessed the pressure response to isoproterenol in vivo, while we assessed the inotropic response of papillary muscles in vitro.

In addition to changes in the calcium uptake by the SR, modifications in the calcium influx through L-type channels might also increase TP and reduce the positive derivative of force, as found in the isolated papillary muscle of the MPR rats. The results relative to PRCs and tetanic contraction, both in the presence and absence of verapamil, suggest that the calcium influx through the L-type channels was greater in the heart of the MPR rats or that those channels exhibited greater functionality. However, in the study conducted by Tappia and colleagues [13], intrauterine malnutrition promoted reduction of L-type channel expression. According to those authors, reduction of L-type channels and SERCA-2a expression might impair the heart contractile function

in neonate rats. As we assessed the cardiac function of adult rats, one might speculate that the increase in calcium influx or in the activity of the L-type channels might result from a compensatory mechanism seeking to preserve the heart contractile strength. Further evidence for such a compensatory mechanism is provided by the increase in the protein expression of NCX found in the hearts of the MPR rats in this study. NCX might represent a significant cell defense mechanism to prevent calcium overload when the SERCA-2a function is decreased. Such "compensatory actions" might play a relevant role when the blood pressure is elevated (as in the case of the MPR animals) to preserve the cardiac output. However, in the long run, such actions might result in considerable structural alterations, with consequent cardiac dysfunction. Therefore, this point gives further support to the idea that MPR might be a risk factor for cardiovascular diseases in the offspring in adulthood.

To conclude, we suggest that young animals born from rats fed a low-protein diet during pregnancy exhibit elevated blood pressure, changes in the contractile machinery, and alterations in the expression of the proteins that regulate cardiac contractility. Those changes are favorable for the development of cardiovascular diseases and thus corroborate the fetal programming theory.

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Figure Legends

Figure 1. (A) Isometric force; (B, C) positive (dF/dt +) and negative (dF/dt -) derivatives of force; (D, E) time to peak and relaxation time of isolated papillary muscles from control rats (Ct, n = 10) and animals born from rats subjected to protein restriction (MPR, n = 8). The results are expressed as the mean ± SEM. Unpaired *Student's t*-test (*p < 0.05 Ct vs. MPR).

Figure 2. Relative potentiation after 15-, 30- and 60-s rest intervals of the isometric force of isolated papillary muscles from control rats (Ct, n = 10) and animals born from rats subjected to protein restriction (MPR, n = 8). The results are expressed as the mean ± SEM. Two-way ANOVA (*p < 0.05 Ct vs. MPR).

Figure 3. Inotropic effect induced by increasing isoproterenol concentrations on isolated papillary muscles from control rats (Ct, n = 14) and animals born from rats subjected to protein restriction (MPR, n = 8). The results are expressed as the mean ± SEM. Unpaired *Student's t*-test (*p < 0.05 Ct vs. MPR).

Figure 4. (A) Post-rest contraction (PRC) obtained after 10 min without stimulation and in calcium-free solution containing 5 mM caffeine in the absence or presence of 1 µM verapamil (B) on papillary muscles from control (Ct, n = 10) and maternal protein malnutrition (MPR, n = 8) rats. (B) PRC delta from % reduced force before and after verapamil incubation. The results represent the mean ± SEM. *Student's t*-test. *p < 0.05 vs. MPR.

Figure 5. Tetanic peak force (A) and tetanic plateau force (B) before and after verapamil incubation on papillary muscles from control (Ct, n = 10) and maternal protein malnutrition (MPR, n = 8) rats. Delta from % reduced tetanic peak (C) and plateau force (D) before and after verapamil incubation. The results represent the mean ± SEM. *Student's t*-test. *p < 0.05 vs. MPR.

Figure 6. Densitometric analysis of the western blot for (A) SERCA-2a, (B) **phospholamban (PLB)**, (C) **phospho-Ser^{16}-PLB (PLBp)**, (D) **ratio phospho-Ser^{16}-PLB/PLB,** and (E) NCX from control (Ct, n = 10) and maternal protein malnutrition (MPR, n = 8) rats. The results represent the mean ± SEM. *Student's t*-test. *p < 0.05 vs. MPR.

Table 1. Weight data corresponding to the control animals (Ct) and the young offspring of rats subjected to protein restriction (MPR).

Weight	**Ct (N = 24)**	**MPR (N = 24)**
Birth (g)	7.36 ± 0.09	3.51 ± 0.07*
21 days (g)	64.46 ± 1.3	42.4 ± 2.3*
90 days (g)	357.85 ± 7.7	267.25 ± 2.7*
Heart (g)	1.03 ± 0.025	0.87 ± 0.027
Heart-to-body weight ratio (mg/g)	3.0 ± 0.05	4.0 ± 0.04
Right ventricle (g)	0.23 ± 0.009	0.19 ± 0.01
Right ventricle-to-body weight ratio (mg/g)	1.0 ± 0.05	1.0 ± 0.04
Right ventricle-to-tibia ratio (mg/mm)	6.0 ± 0.05	6.0 ± 0.04
Left ventricle (mg)	0.587 ± 0.01	0.466 ± 0.02
Left ventricle-to-body weight ratio (mg/g)	2.0 ± 0.05	2.0 ± 0.04
Left ventricle-to-tibia ratio (mg/mm)	230 ± 0.09	150 ± 0.01*
Liver (g)	13.95 ± 0.57	10.35 ± 063
Liver-to-body weight ratio (mg/g)	35.0 ± 0.01	37.0 ± 0.04

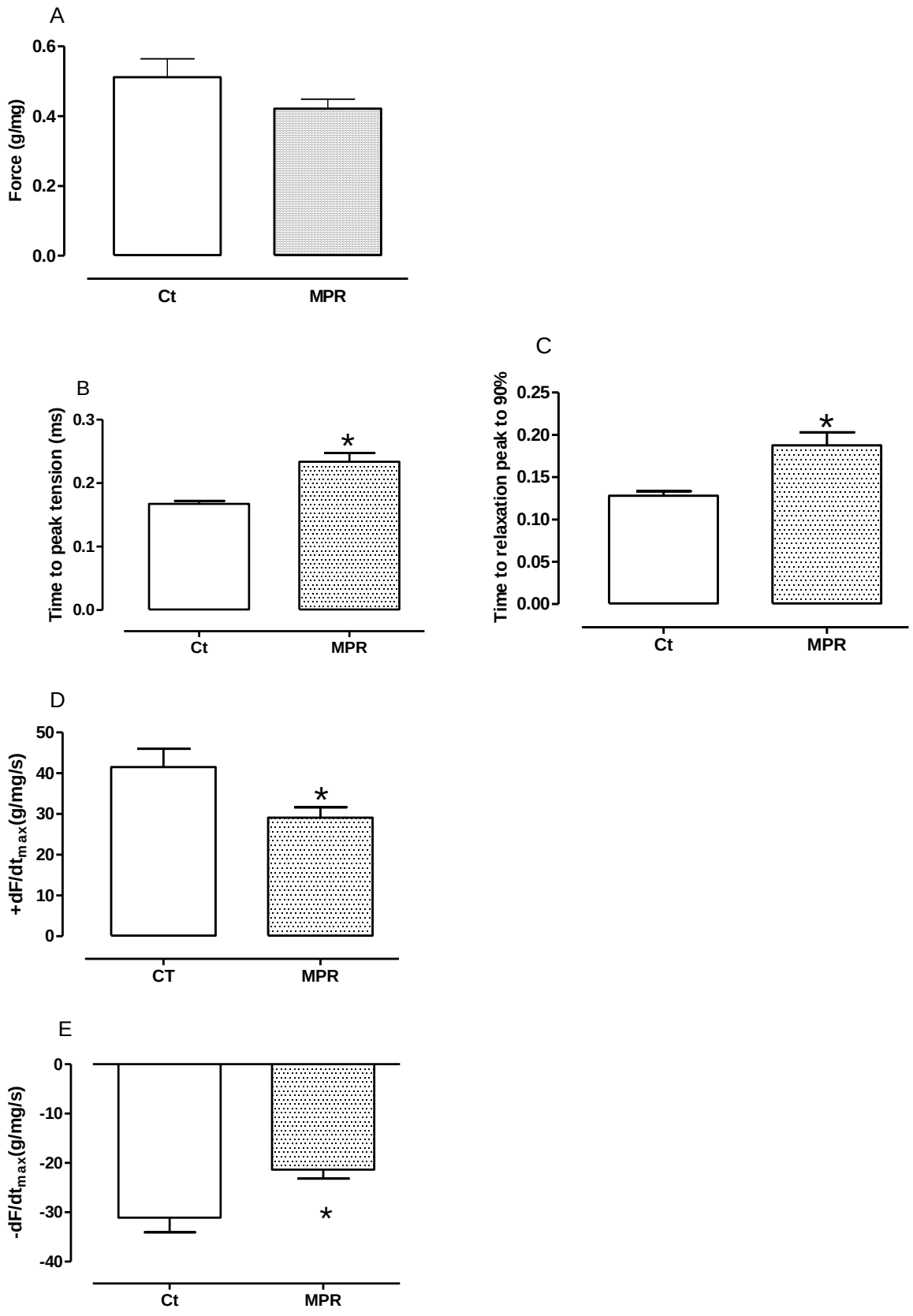
Values expressed as the mean ± SEM. Data analyzed by means of unpaired *Student's t*-test (MPR vs. Ct, *$p < 0.05$).

Table 2. Hemodynamic parameters corresponding to the control animals (Ct) and the young offspring of rats subjected to protein restriction (MPR).

Pressure indirect measurement	Ct (N = 12)	MPR (N = 12)
SAP (mmHg)	126 ± 2	147 ± 2.42*
Pressure direct measurement	Ct	MPR
SAP (mmHg)	103 ± 3.7	102 ± 7
DAP (mmHg)	54 ± 4	54 ± 7
MAP (mmHg)	72 ± 4	69 ± 8
LVSP (mmHg)	102 ± 6.71	103 ± 5.43
LVeDP (mmHg)	2.46 ± 0.54	2.52 ± 04
Dp/dt+LV (mmHg/s)	3685 ± 282	5678 ± 321*
Dp/dt-LV (mmHg/s)	- 4782 ± 183	-6824 ± 320*
HR (bpm)	302 ± 9	300 ± 12

Indirect measurement of the systolic arterial pressure (SAP) and direct measurement of SAP, diastolic arterial pressure (DA), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVeDP), left ventricular time positive (dP/dt + LV) and negative (dP/dt - LV) derivatives, and heart rate (HR). Values expressed as the mean ± SEM. Data analyzed by means of unpaired *Student's t*-test (MPR vs. Ct, *$p < 0.05$).

Figure 1.


A
Force (g/mg)
0.6
0.4
0.2
0.0
Ct
MPR
B
Time to peak tension (ms)
0.3
0.2
0.1
0.0
*
Ct
MPR
C
Time to relaxation peak to 90%
0.25
0.20
0.15
0.10
0.05
0.00
*
Ct
MPR
D
+dF/dtmax(g/mg/s)
50
40
30
20
10
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CT
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Ct
MPR

Figure 2.

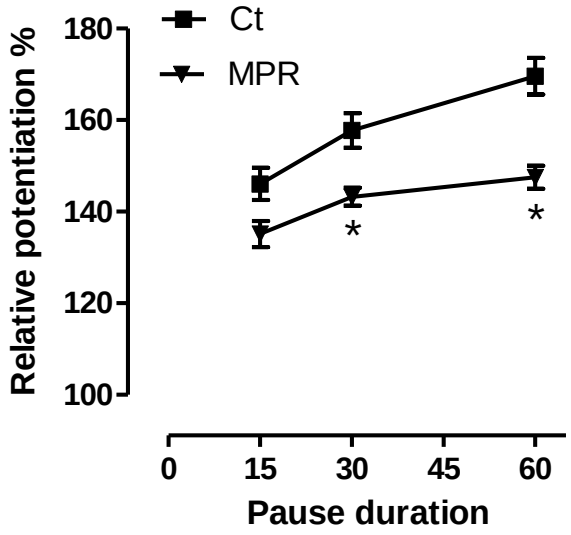


Figure 3.

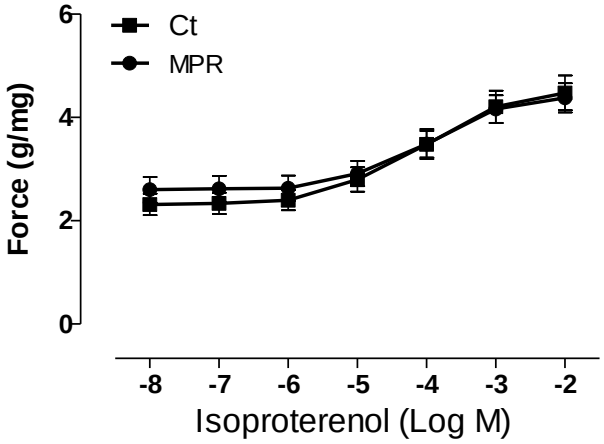


Figure 4.

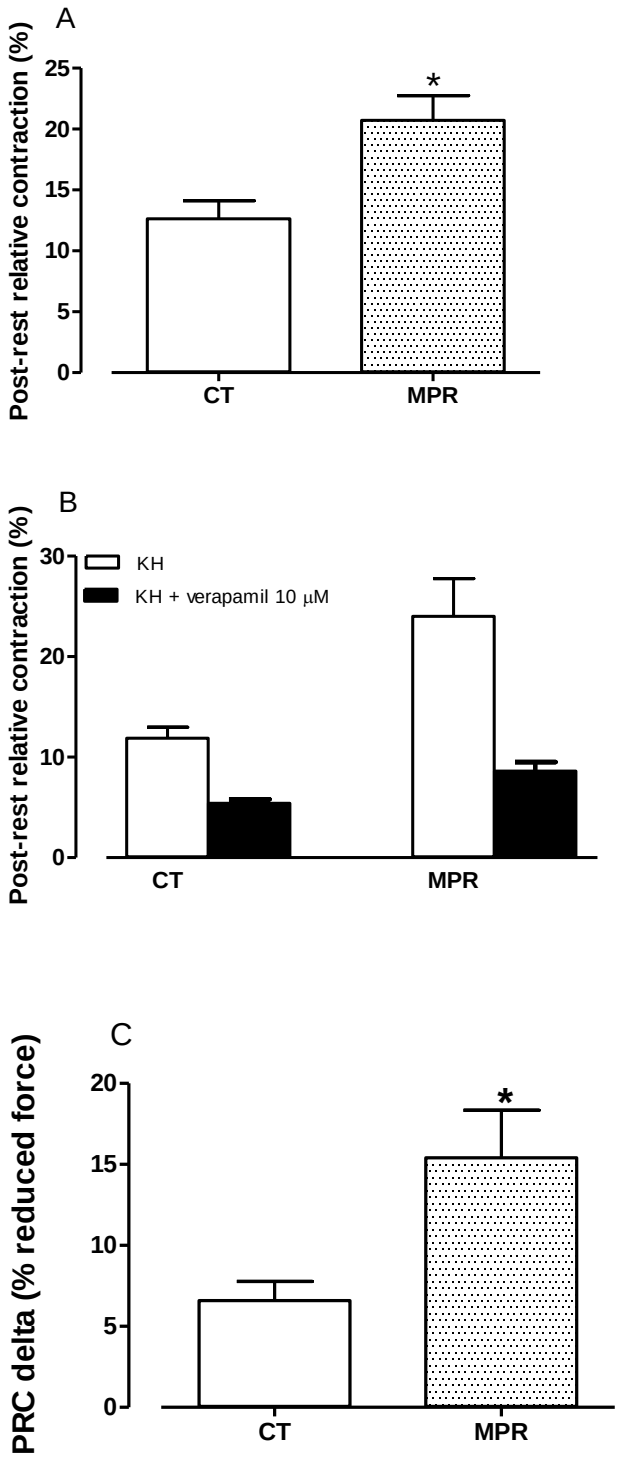

A
Post-rest relative contraction (%)
25
20
15
10
5
0
*
CT
MPR
B
Post-rest relative contraction (%)
30
20
10
0
KH
KH + verapamil 10 μM
CT
MPR
C
PRC delta (% reduced force)
20
15
10
5
0
*
CT
MPR

Figure 5.

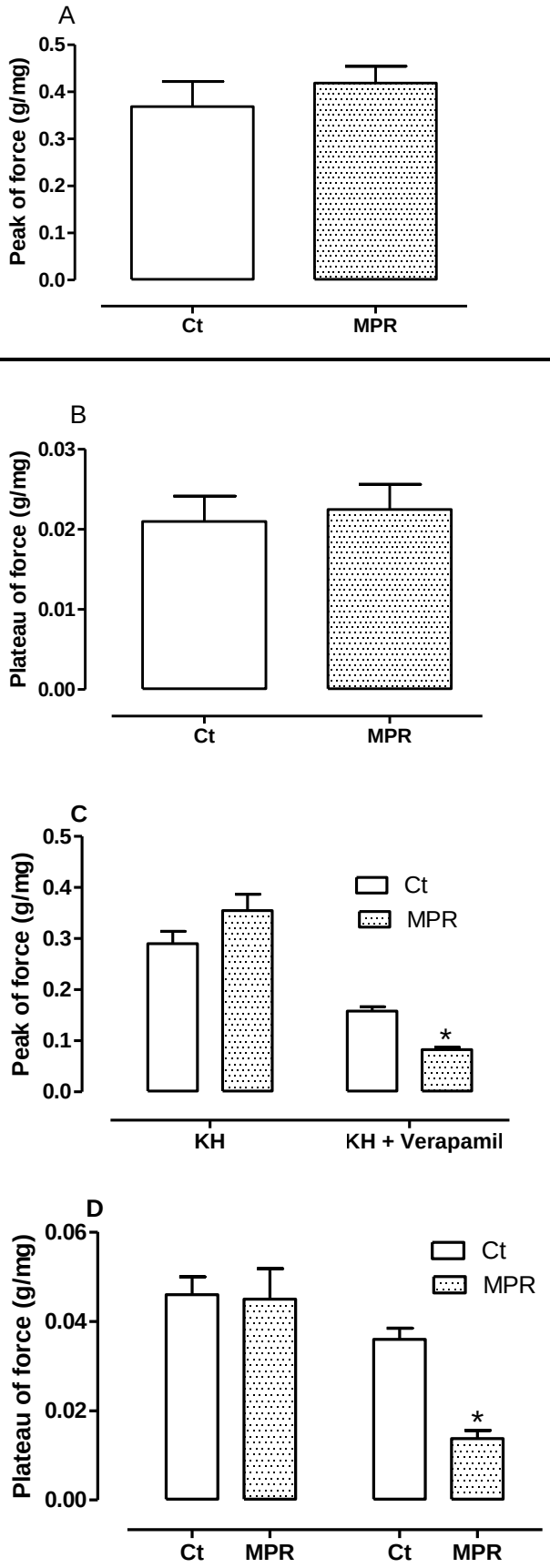
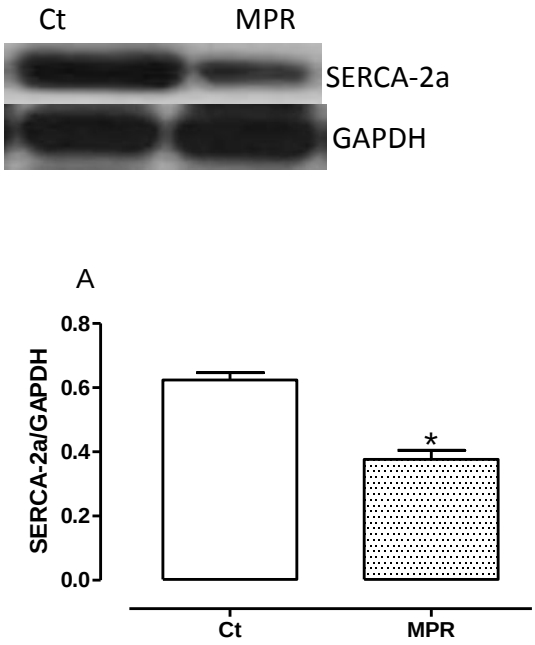
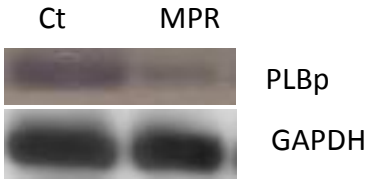
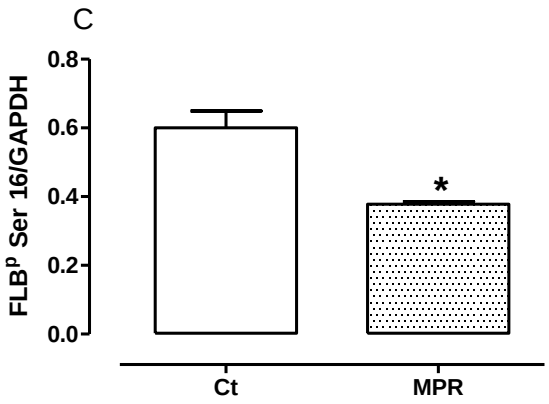
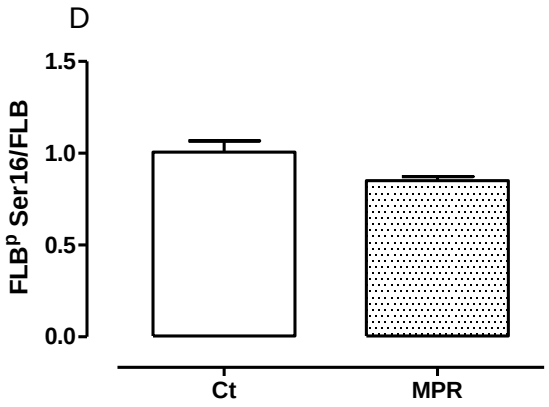

A
Peak of force (g/mg)
0.5
0.4
0.3
0.2
0.1
0.0
Ct
MPR
B
Plateau of force (g/mg)
0.03
0.02
0.01
0.00
Ct
MPR
C
Peak of force (g/mg)
0.5
0.4
0.3
0.2
0.1
0.0
Ct
MPR
*
KH
KH + Verapamil
D
Plateau of force (g/mg)
0.06
0.04
0.02
0.00
Ct
MPR
*
Ct
MPR
Ct
MPR

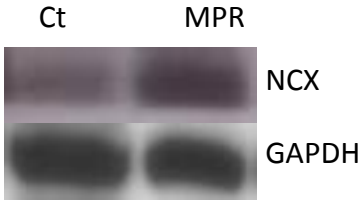
Figure 6.

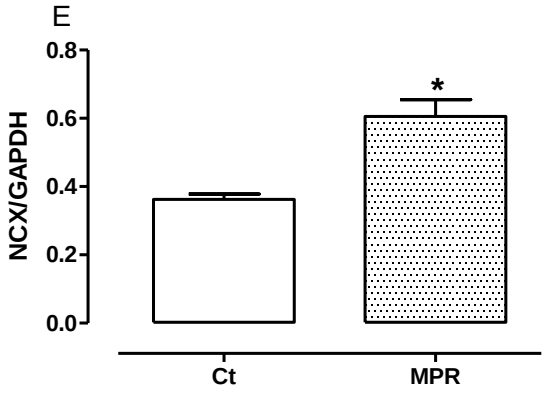

Ct
MPR
SERCA-2a
GAPDH
A
SERCA-2a/GAPDH
0.8
0.6
0.4
0.2
0.0
*
Ct
MPR
Ct
MPR
PLB
GAPDH
B
PLB/GAPDH
0.8
0.6
0.4
0.2
0.0
*
Ct
MPR


Ct
MPR
PLBp
GAPDH


C
FLB^{P} Ser 16/GAPDH
0.8
0.6
0.4
0.2
0.0
*
Ct
MPR


D
FLB^{P} Ser16/FLB
1.5
1.0
0.5
0.0
Ct
MPR


Ct
MPR
NCX
GAPDH


E
NCX/GAPDH
0.8
0.6
0.4
0.2
0.0
*
Ct
MPR

ANEXO II

Artigo 2 - Submetido - Post-weaning protein malnutrition increases blood pressure and reduce reduces papillary muscle contractility in rats

Post-weaning protein malnutrition increases blood pressure and reduce reduces papillary muscle contractility in rats

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Running Head: Malnutrition, hypertension and papillary muscle contractility

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Abstract

Malnutrition during critical periods in early life may increase the subsequent risk of hypertension and metabolic diseases in adulthood, but the underlying mechanisms are still unclear. We aimed to evaluate the effects of post-weaning protein malnutrition (PWM) on blood pressure and papillary muscle contractility from control and PWM rats (offspring that received a diet with low protein content for three months). At the 3 months age, the animals were anesthetized and euthanized, and parameters related to isolated papillary muscle contractility were recorded. Systolic and diastolic blood pressure were increased in the PWM rats when compared to. PWM decreased force and time derivatives in isolated papillary muscles. Post-rest potentiation, protein expression of SERCA and phospholamban and its phosphorylated subunits were reduced in PWM group, suggesting impairment in uptake of Ca^{2+} by the sarcoplasmic reticulum. In addition, the inotropic responses induced by an increase in the extracellular calcium concentration was reduced in the PWM group. *In vitro* assessment of the involvement of L-type Ca^{2+} channels using verapamil revealed a similar decrease of force in both groups. These findings suggest that the reduction of the inotropic response to Ca^{2+} is not related to changes in the trans-sarcolemmal Ca^{2+} flux but to a possible alteration of contractile proteins. Moreover, the protein expression of isoform α2 of Na^{+},K^{+}, ATPase was reduced and superoxide anion production was increased in PWM group. These results suggest that PWM induces an increase of blood pressure and a reduction of the force in isolated papillary muscle, which is related to changes in the uptake of Ca^{2+} by the sarcoplasmic reticulum. Superoxide anion production seem to be involved in this response.

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Keywords: post-weaning protein malnutrition, hypertension, cardiac contractility, papillary muscles, calcium handling, and contractile proteins.

Introduction

Nutritional deficiencies due to a decrease in or absence of the consumptions of micro or macronutrients in food causes malnutrition. The effects f these malnutrition for organisms include reduction of the growth and development of organisms and depend of the type of diet, age and the length of decrease consumption.

Malnutrition is a public health problem and can affect children more strongly (1). It is well established that intrauterine malnutrition or its occurrence during childhood might be a risk factor for the development of hypertension and metabolic imbalance in adult life (2-5). These observations are in accordance with the "nutritional programming" hypothesis for cardiovascular diseases (6-9).

Some mechanisms there are proposed to explain these hypertension are endothelial dysfunction (5,8, 10), hyperactivity of sympathetic (11-14), levels of circulating catecholamines increase (12) and cardiac electric remodeling (15). In addition, previous reports demonstrated that the malnutrition decreased myocardial fibers diameters and decrease, enhance or did not alter the myocardial contractility of the hearts in rats (16-18). Thus, the effects of malnutrition on myocardial contractility were controversy. A previous report has demonstrated that adult with acute malnutrition had smaller cardiac output when compared with controls, yet markedly elevated peripheral resistance (19). In fact, the increase in peripheral resistance would induce a higher cardiac afterload , and if it persists can promote a reduction in cardiac output and cardiac dysfunction.

Although there is evidence that malnutrition affects the cardiovascular system, there are few studies addressing the impact of chronic malnutrition on the cardiac function. Therefore, we proposed to study the effects of post-weaning protein malnutrition on the systolic and diastolic function of the left ventricle (LV) and cardiac function of rats with after 3 months. For this, we used a diet prepared according to data from food consumption

surveys in different geographic zones of Northeast Brazil (the Basic Regional Diet/BRD) (20). This diet mimics other deficient diets that are consumed in many parts of the world. Both essential and non-essential amino acids are extremely limited in this diet, in addition to calories, fat, vitamins and minerals.

Materials and Methods

Animals and experimental groups

The studies were performed on male Wistar rats. All experiments were conducted in compliance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Experimentation of the Biological Sciences Center of the Federal University of Pernambuco (Process Number N^{o}23076.008507/2010-16). All rats had free access to water and were fed rat chow *ad libitum*.

Animals were divided into two experimental groups: control (Ct) and post-weaning protein malnutrition over 3 months. In the control group, the mothers were fed a chow diet during the pre-mating, mating, pregnancy and lactation. After weaning (21 days), the offspring had the same diet as their mothers (Labina®). In the post-weaning protein malnutrition group, mothers were fed a Labina® diet during the pre-mating, mating, pregnancy and lactation phases. The offspring received the RBD ration post weaning for 3 months.

Diets

Two types of diets were used for this study. The diet used to induce malnutrition was the RBD as described previously (5, 20-23). The RBD consisting of beans (*Phaseolus vulgaris)*, sweet *potatoes (Ipomea batatas)*, jerked beef and manioc flour *(Manihot*

esculenta). These components were macerated, molded into “pellets” and heated to 50^{o}C. The RBD pellets providing a total of (g/g%): proteins 9, carbohydrates 78, lipids 1.1, fiber 7, minerals 4, sodium chloride 0.17 and Kcalorie 356. No vitamin supplement was added. Moreover, part of the diet was supplemented with 0.2% (g/g) sodium chloride. The control diet was a commercial diet (Labina®). The standard diet contains the following content (g/g%): protein 23, carbohydrates 41, lipids 2.5, fibers 9, minerals 8, sodium chloride 0.37 and Kcalorie 278.

Measurement of arterial pressure

The systolic arterial pressure (SAP) of the animals in both groups was measured indirectly every week using noninvasive tail-cuff plethysmography method (IITC Life Science noninvasive blood pressure, version 1.35). In a conscious state, the rats from both groups were placed in a heated chamber for 5-10 minutes in a silent environment to perform measurements by means of cuff inflation-deflation cycles. Then, the SAP was measured, and the average of three measurements was recorded and analyzed, as reported (5).

The arterial blood pressures were also measurement directly. For this, at 3 months age, rats were anesthetized with urethane (1.2 g/kg, i.p.). A polyethylene catheter (PE50) filled with heparinized saline (50 U/mL) was introduced into the carotid artery to measure systolic (SBP) and diastolic (DBP) arterial pressures. Recordings were performed using a pressure transducer (TSD 104A- Biopac) and data were collected with computer software (MP 30 Biopac Systems, Inc; CA). Heart rate (HR) was determined from the inter-beat intervals.

Isolated papillary muscles

At the end of treatment, the animals were anesthetized with urethane (1.2 g/kg). Their hearts were removed quickly and perfused through the aortic stump before the papillary muscles from the left ventricular anterior and posterior walls were dissected. To record isometric tension, the papillary muscles were mounted and kept in a 20-mL organ bath with Krebs-Henseleit solution (118 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM MgSO**4**, 23 mM $NaHCO_3$ and 11 mM glucose, pH 7.4) at 26 ± 0.5 ºC to avoid the occurrence of hypoxia (24); the solution was continuously aerated with 95% O_2 and 5% CO_2. The preparations were connected to an isometric transducer (TSD125-Biopac Systems, Inc; CA). The muscle was stimulated by means of isolated rectangular pulses (10 to 15 V, duration 12 ms) applied through a pair of platinum electrodes placed along the full extension of the muscle. The standard stimulation rate was 0.5 Hz. The records were initiated 45 to 60 minutes later to allow the preparations to adapt to the new environmental conditions. The force that developed during contraction was measured in g/mg (developed force in g divided by the muscle weight in mg). The values were adjusted to the papillary muscle weight to normalize the data corresponding to the various preparations. Following the period of stabilization, the experimental protocols described below were initiated:

6. The effects of treatment with post-weaning malnutrition on isometric force development were studied.

7. Post-rest potentiation (PRP) was used to gather information on sarcoplamic reticulum (RS) function. In the cardiac muscle, the contractions that occur after short pauses are potentiated; post-rest contractions depend on the duration of pauses and on the amount of calcium stored at intracellular sites. Various rest periods were applied (15, 30 and 60 s). The results are presented as the relative potentiation (amplitude of post-rest contractions divided by contraction under basal conditions – steady state) to normalize the data corresponding to the various preparations (24).

8. Force was assessed at various concentrations of calcium (0.5, 1.0, 1.5 and 2.0 mM) and isoproterenol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M) in preparations from rats from both the CT and PWM experimental groups.

9. Post-rest contractions (PRCs) were determined after a period of 10 minutes without stimulation. The muscles were kept in a calcium-free solution with caffeine (5 mM). To achieve PRCs, the calcium-free solution was replaced with a modified Krebs solution (containing 1.25 mM calcium) a few seconds before the onset of electrical stimulation. PRCs were measured before and after trans-sarcolemmal calcium influx blockade. To induce this blockade, verapamil (10 µM) was added to the bath for 20 minutes before the following PRCs (25).

10. The tetanic tension protocol was applied for investigation of the contractile response of an intact myocardium with a non-functional SR (26). Tetanic tension was developed by means of high-frequency stimulation (10 Hz over 15 s) following pre-incubation with 5 mM caffeine over 30 minutes (26). The tetanic tension was assessed before and after incubation with verapamil (10 µM), which was added to the bath 20 minutes before the subsequent tetanus (26).

Western blot analysis

The hearts of animals from the CT and PWM groups were homogenized, and proteins (80 µg) were separated from the tissues via 10% SDS-PAGE for quantification of RS Ca^{++}-ATPase (SERCA-2a), sodium-calcium exchanger (NCX) levels and α-2 isoform of Na^{+},K^{+}-ATPase. The low-molecular weight protein phospholamban (PLB) and fosfolambam fosforized (PLBp) on serina-16 (fosfo-Ser^{16}-PLB) were separated via 15% SDS-PAGE.

The proteins were subsequently transferred to nitrocellulose membranes (Amersham, UK), which were incubated with mouse monoclonal antibodies against

SERCA-2a (1:1000, Thermo Scientific, Rockford, USA), NCX (1:1,000, Thermo Scientific, Rockford, USA), PLB (1:1000, Thermo Scientific, Rockford, USA), fosfo-Ser^{16}-PLB 1:5000, Thermo Scientific, Rockford, USA) and α-2 isoform of Na^+K^+ATPase 1:1000, Upstate Biotechnology, Lake Placid, NY). Following rinsing, the membranes were incubated with an anti-mouse IgG antibody (1:5000, Sigma Chemical, Co., St Louis, USA).

After thorough rinsing, the detection of immune complexes was performed using a chemiluminescence system (ECL Plus Amersham International, Little Chalfont, United Kingdom) and film (Hyperfilm ECL International). The immunoblot signals were quantified using National Institutes of Health Image V1.56 software. The same membrane was subjected to assessment of the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using a mouse monoclonal antibody against GAPDH (1:5000, Abcam Cambridge MA, USA). Following rinsing, the membrane was incubated with an anti-mouse antibody (1:5000, Assay Designers, Hines Drive, Ann Arbor, MI). All of the reagents employed for transfer to the Western blots were purchased from Sigma Chemical Co., USA.

In situ detection of heart $O_2^{\cdot -}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate $O_2^{\cdot -}$ production in situ, as previously described (27). Hydroethidine freely permeates cells and is oxidized in the presence of $O_2^{\cdot -}$ to ethidium bromide, which is trapped by intercalation into DNA. Ethidium bromide is excited at 546 nm and emits light at 610 nm. Frozen tissue were cut into 10-µm-thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, and 11 glucose, pH=7.4). Fresh buffer containing DHE (2 µM) was applied topically to each slide tissue section. Then, the slide sections were covered with a cover slip; incubated for 30 min in a light-protected

humidified chamber at 37° C; and viewed with an inverted fluorescence microscope Leica DM 2500 with a x40 objective and Leica DFC 310 FX camera, using the same imaging settings in control and PWM rats. Fluorescence was detected with a 585-nm long-pass filter. For quantification, eight frozen slide tissue per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated using ImageJ software.

Drugs

The following drugs were used: heparin (Roche Q.F.S.A., Brazil), anhydrous caffeine (B. Herzog, Brazil), urethane, bovine serum albumin, lead acetate and verapamil (Sigma Chemical Co., USA). All other chemicals were of analytical reagent grade and were obtained from Sigma and Merck (Germany) or Reagen (Brazil).

Data analysis and statistics

All results were expressed as the mean ± standard error of the mean (SEM) and were analyzed by means of unpaired Student's t-test or one- or two-way ANOVA. Whenever the results of ANOVA were statistically significant, the Bonferroni or Tukey's *post hoc* test was used to compare individual means. The significance level was set at $p < 0.05$. The data were analyzed and figures were plotted using GraphPad Prism software (Version 5.01, GraphPad Software, USA).

RESULTS

No difference in body weight between the groups was observed either at birth or 21 days after birth (Table 1). However, 90 days after birth, the body weight was lower in the post-weaning protein malnutrition rats compared to the controls (Table 1). These results

demonstrated that the post-weaning protein malnutrition was able to induce a loss in weight gain, as previously described (5).

In order to verify if the loss in weight gain induced by hypoproteic diet produces arterial pressures alterations, blood pressure was measured. At the end of treatment of malnutrition (90 days), significant increases in systolic blood pressure was observed in the post-weaning protein malnutrition group compared to the controls (Table 2). Confirming this result, the systolic and diastolic blood pressures, and heart rate, were also increased in anesthetized rats from PWM group (Table 2). Therefore, this results confirm those previously reported (5), and indicate that the post-weaning protein malnutrition induces a weight defect and hypertension.

Effects of post-weaning protein malnutrition on papillary muscle contraction

Isometric force (Figure 1 A) and the positive and negative force derivatives (dF/dt+ and dF/dt-, respectively; Figures 1B and 1C) were reduced in muscle from malnutrition rats when compared to controls. Nevertheless, the time to peak (TP) and the 90% relaxation (TR90%) time were similar in the two groups (Figure 1D and 1E).

To establish whether SR function was altered following chronic malnutrition, the PRP was recorded and assessed. As shown in Figure 2, post-weaning protein malnutrition decrease the development force after PRP.

Thus, the next step was to investigate whether chronic malnutrition also alters the cellular mechanisms involved in the cardiac inotropic response by means of isoproterenol and calcium concentration-response curves. Inotropic intervention induced by isoproterenol and an increase in the extracellular calcium concentration were associated with lower force development in the isolated papillary muscles of the animals from the PWM group compared with CT (Figure 3A and 3B).

These results, together with those previously reported (26), led to the following question: do the cardiomyocytes of rats exposed to chronic malnutrition exhibit less permeability to calcium, or do the contractile proteins display a lower affinity for calcium ions?

To establish whether the smaller increase in the force developed by the animals in the PWM group in response to calcium was due to a lower trans-sarcolemmal calcium influx, PRCs were assessed in the presence and absence of verapamil (1 µM), which is an L-type calcium channel blocker. Figure 4A shows that the PRCs were similar in the two groups before and after verapamil (Figure 4B).

Tetanic contractions also depend on the trans-sarcolemmal calcium influx and the sensitivity of the contractile proteins to calcium. Figures 5A and 5B show that both plateau and peak of tetanic contractions were lower in the PWM group compared with CT. When the preparations were exposure to verapamil, the tetanic contractions were reduced similarly in both groups. This finding corroborate the responses that were obtained in PRC protocols, suggesting that the cardiomyocytes that were isolated from animals that were submitted of post-weaning malnutrition no have increased calcium influx but might possible alteration of contractile proteins.

Expression of proteins involved in Ca^{2+} handling

The levels of some proteins with relevant roles in the regulation of cardiomyocyte contractility were assessed by means of Western blotting, including the protein expression of SERCA-2a, NCX, PLB, fosfo-Ser^{16}-PLB and α2 isoform of Na^+,K^+,ATPase. Post-weaning protein malnutrition reduced protein expression of SERCA-2a (Figures 6A) and PLB or its phosphorylated subunits (Figures 6B, C and D). Moreover, also reduced protein expression of α2 isoform of Na^+,K^+,ATPase although did not modified the protein expression of NCX (Figures 6 E, F).

In situ detection of heart $O_2^{\cdot -}$ production

In addition to changes in cardiac force, we evaluated whether the superoxide anion production would be altered after post-weaning protein malnutrition. Figure 7 shows that there was a significant increase in the local superoxide anion production in hearts of PWM rats.

DISCUSSION

This study demonstrated that post-weaning protein malnutrition increased blood pressure, which was accompanied by impairs the heart contractile machinery. Such a deleterious effect on the heart appears to be associated with a reduction in the uptake of Ca^{2+} by the sarcoplasmic reticulum and reduction in the sensitivity of contractile proteins to calcium. Moreover, the increase of superoxide anion in hearts from post-weaning malnutrition rats also contributes to deleterious effects. Our data corroborated the hypothesis that chronic malnutrition is a risk factor for cardiovascular diseases.

In this study, we investigated the effects of post-weaning protein malnutrition on blood pressure and cardiac function to elucidate some mechanisms that are correlated with two relevant issues in public health: malnutrition and cardiovascular diseases. Post-weaning protein malnutrition was induced by a diet that reproduces a regional basic diet (RBD) (5, 20-23) widely consumed by inhabitants living in an area of sugar cane plantations along the coast of the State of Pernambuco, Brazil. This diet has been associated both with lower weight gain and the malfunction of various organs, probably because of its low protein content and vitamin and mineral deficiencies (5, 20-23). It is important to mention that this diet is deficient in a qualitative rather than a quantitative nature, the latter of which is used by most researchers and represents the real consumption of this population. Depending on the region and season, populations in

northeast Brazil and in other regions of the world might consume a similar diet. Therefore, the importance of knowing its effects on the organism might help to introduce future corrective interventions to minimize the risk of diseases associated with malnutrition.

Animals submitted to post-weaning protein malnutrition showed a marked reduction in weight gain. These results highlight the importance of nutritional status in the growth phase of the animal. They show a greater impact of the post-weaning protein malnutrition period including the stage of growth and development into adulthood. Similar results were previously observed (5, 13, 20-23) with use of the same diet.

An alteration in body weight is an indicator of the nutritional status of the individual and might be associated with alterations in the cardiovascular system (28,29). Indeed, in our study we observed that the malnutrition triggers weight defect and hypertension. Previous reports evaluating the effects of protein-caloric deprivation in rats have suggested an increased hypothalamic-pituitary-adrenal response and circulating catecholamines (30-32). Then, the increase in heart rate and blood pressure might be related to increased sympathetic nervous system activity (11-14, 31). The same way, we observed an increase in heart rate in PWM group.

Sawaya et al. [4] have demonstrated that in malnutrition, children might have increased diastolic blood pressure. Therefore, because the diastolic blood pressure reflects, in part, the peripheral vascular resistance, vascular reactivity might be altered in malnutrition conditions. Indeed, our results demonstrated an increase in diastolic blood pressure in PWM group. Tennant et al (2014) [19] demonstrated that several acute malnutrition survivors had smaller cardiac output when compared with controls, yet markedly elevated peripheral resistance. Moreover, we previously demonstrated (5) that the post-weaning malnutrition increased diastolic blood pressure and increases the vascular reactivity in resistance arteries. Therefore, the increase of cardiac after load could

induce to changes on heart contractile machinery. Furthermore, we aimed investigated the effects of post-weaning malnutrition in isometric force on isolated papillary muscles.

Our results showed that post-weaning protein malnutrition reduces isometric force and time derivatives (dF/dt_{max} e dF/dt_{min}) of papillary muscles. Murça et al. [34] demonstrated that postnatal protein restriction for 5 weeks causes cardiac adaptation characterized by an early overworking heart, which was due, at least in part, mediated by an increase in the efferent sympathetic fibers to the heart. The discrepancy between our results and those of Murça et al. (2012) [34] can most likely be accounted for by the difference in the length of malnutrition and the type of diet that was used to induce malnutrition in these two studies.

The observed reduction of isometric force and time derivatives could be accounted for by reduces in the sarcolemmal membrane calcium influx and calcium uptake by the SR. In fact, SR function, assessed through PRP with 15-, 30-, and 60-s pauses, did exhibit changes in the isolated papillary muscles of the rats from the PWM group relative to the controls. In addition, cardiac SERCA-2a and PLB or its phosphorylated subunits protein expression were reduced in the PWM group.

One further relevant aim of our study was to investigate whether post-weaning malnutrition could modify the inotropic response of isolated papillary muscles. For this purpose, isoproterenol and calcium concentration-response curves were plotted. The results of these protocols showed that the inotropic intervention induced by isoproterenol did not alter the increase in force between the PWM and CT groups. The studies about the beta-adrenergic heart responsiveness are contradictory. Ransnäs et al. (1989) [35] verified that the β-adrenergic receptor number or antagonist affinity are not influenced by malnutrition, although the β-adrenergic receptors displayed a considerably increased affinity towards isoproterenol (35). The same way, Jayarajan et al. (1985) [36] demonstrated that malnutrition induces an increased sensitivity and reactivity to

catecholamines (36). However, other report showed that the number of alpha and beta-adrenoceptors and the receptor affinity in ventricular membranes were not reduced by protein-calorie malnutrition (37). The inconsistency between these results might occur probably by the difference in the length of malnutrition and the type of diet that was used to induce malnutrition in these studies.

Differently that was observed in inotropic response induced by isoproterenol, the increment of force induced by an increased extracellular calcium concentration was lower in the isolated papillary muscles of the PWM rats compared with the controls. These findings suggest that the cardiomyocytes from PWM group might have lower permeability to calcium or that contractile proteins might have a lower affinity for calcium ions (26). To assess whether the lower inotropic response to increased extracellular calcium concentrations could be related to alterations of the calcium influx through L-type channels, we induced PRCs in the presence and absence of verapamil, which is a blocker of such channels (26). The results showed that the PRCs were similar in the isolated papillary muscles of the animals from the two groups.

One further possible explanation for the malnutrition-induced reduction of the inotropic response to calcium might be related to the alteration of contractile proteins. Therefore, to perform an indirect assessment of the contractile proteins and calcium influx in the isolated papillary muscles, they were subjected to tetanic contractions following the protocol formulated by Leite et al. (1995) [26]. Tetanic contractions induced through inhibition of the SR by means of caffeine or ryanodine are used to induce maximum activation of the contractile machinery in the intact myocardium (38). Caffeine acts by depleting the SR calcium store (26). The results of the present study revealed a similar reduction of the tetanic force in both the presence and absence of verapamil in both groups. These findings once again suggested that chronic malnutrition did not alter the calcium influx. As SR activity is blocked, the tetanic contractions depend only on the

calcium influx and the myosin ATPase activity. Thus, our results suggested that although chronic malnutrition did not alter the calcium influx, it might have modified the function of the contractile proteins.

Cardiac force is also regulated by Na^+K^+,ATPase activity (39). It has been suggested that α1 and α2 isoforms of Na^+K^+,ATPase have different physiological roles within the cardiac myocytes. In the rat heart, approximately 75% of Na^+K^+,ATPase protein is the α-1 isoform; however, α2 and/or α3 are consistently found in strategic sites defined as plasmerosome region adjacent to sarcoplasmic reticulum (39). Different Na^+K^+,ATPase isoforms may function differently, depending on specific membrane localization. The α2 isoforms of the Na^+/K^+-ATPase has been described acting as regulators of cardiac contractility (40). Hearts with genetically reduced NKA-α2 levels are hypercontractile as a result of larger Ca^{2+} transients, whereas hearts with reduced levels of NKA-α1 are hypocontractile (41). Therefore, the reduction of α2 isoforms of Na^+K^+,ATPase activity could elevate local [Na^+] and, via NCX, [Ca^{2+}] augments adjacent sarcoplasmic reticulum Ca^{2+} stores and thereby amplifies Ca^{2+} signaling without elevating bulk [Na^+](cyt). Thus, it is possible that the observed changes in α2 isoforms expression reflect compensatory mechanisms to decreased force in isolated papillary muscle from PWM group. However, this is clearly a speculative model and likely an oversimplification.

In addition, we observed an increased in superoxide anion in hearts from PWM group. Reactive oxygen species might induces deleterious effects when excessively produced causing disturbed balance in Na^+ and Ca^{2+} handling, which, in turn, could result in Na^+ and Ca^{2+} overload, sarcoplasmic reticulum Ca^{2+} loss and contractile dysfunction (42). In fact, the increase of reactive oxygen species seems to decrease SERCA activity that could induces a decrease in cardiac force (43-45). The same way, in our study, we observed a reduction in SERCA protein expression and activity.

Therefore, our results used a qualitative malnutrition model with low-protein to show an increase in blood pressure in the post-weaning protein malnutrition group, which was accompanied *in vitro* by a decrease in cardiac force due to changes in the uptake of Ca^{2+} by the sarcoplasmic reticulum. Superoxide anion production seems to be involved in this response.

Acknowledgements

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Legends of figures

Figure 1. (A) Isometric force, (B) time to peak tension and (C) time to 90% relaxation and positive (dF/dt +) and negative (dF/dt -) force derivatives (D, E) of papillary muscles from control (Ct) and post-weaning protein malnutrition (PWM) rats. The results represent the mean ± SEM. *P< 0.05 vs. Ct, n = 12 (Student's t- test).

Figure 2. Relative potentiation (ratio of post-rest contractions to steady-state contractions) obtained after pauses of 15, 30, and 60 s in the isometric contractions of papillary muscles from control (Ct) and post-weaning protein malnutrition (PWM) rats. The results represent the mean ± SEM (two-way ANOVA, repeated measures), n = 8.

Figure 3. Inotropic effect of inotropic interventions induced by isoproterenol (A) and by different (0.62, 1.25, and 2.5 mM) calcium concentrations (B) on the isometric contractions of papillary muscles from control (CT) and post-weaning protein malnutrition (PWM) rats. The results represent the mean ± SEM. *p < 0.05 vs. Ct (two-way ANOVA, repeated measures), n = 8.

Figure 4. (A) Post-rest contractions (PRCs) obtained after 10 min without stimulation or in a calcium-free solution containing 5 mM caffeine in the absence or presence of verapamil (1 µM) in papillary muscles from control (Ct) and post-weaning protein malnutrition (PWM) rats. (B) PRC delta from the % reduction of the force before and after verapamil. The results represent the mean ± SEM, n = 10.

Figure 5. Relative tetanic plateau force (A) and relative peak plateau force (B) before and after verapamil treatment in papillary muscles from control (Ct) and post-weaning protein malnutrition (PWM) rats. The results represent the mean ± SEM. *P< 0.05 vs. Ct (Student's t- test), n = 8.

Figure 6. Densitometric analysis of the western blot for (A) SERCA-2a, (B) phospholamban (PLB), (C) phospho-Ser^{16}-PLB (PLBp)**,** (D) ratio phospho-Ser^{16}-PLB/PLB, (E) NCX and α2 isoform of Na^+,K^+,ATPase (E) from control (Ct) and post-weaning protein malnutrition (PWM) rats. Results represent mean ± SEM. Student's t- test. *p < 0.05 vs CT, n = 9.

Figure 7. Upper: Representative fluorescent photomicrographs of confocal microscopic heart sections labeled with the oxidative dye hydroethidine. Below: Heart superoxide anion

quantification from control (Ct) and post-weaning protein malnutrition (PWM) rats. Results represent mean ± SEM. Student's t- test. *P < 0.05 vs Ct, n = 10.

Table 1. Body weight (g) measurements of control (CT) and post-weaning protein malnutrition (PWM) offspring.

	CT **(N=10)**	**PWM** **(N=10)**
Birth	6.31 ± 0.7	6.45 ± 0.6
Weaning (21st day)	59.6 ± 2.9	58.2 ± 1.40
90st day	376 ± 7.3	131 ± 9.7*

Values are expressed as the mean ± SEM. * $P < 0.05$ Control *vs.* PWM, Student´s *t*-test.

Table 2. Systolic (SBP, mmHg) and diastolic (DBP, mmHg) blood pressure and heart rate (HR, bpm) determination in the control (CT) and post-weaning protein malnutrition (PWM) adult rats.

	CT **(N=10)**	**PWM** **(N=10)**
SBP (mmHg)	102 ± 4.8	118 ± 4*
DBP (mmHg)	54 ± 4	72 ± 5.8*
HR (bpm)	302 ± 9	328 ± 11*

Values are expressed as the means ± SEM. * $P < 0.05$ Control *vs.* PWM, Student *t*-test.

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Figure 1

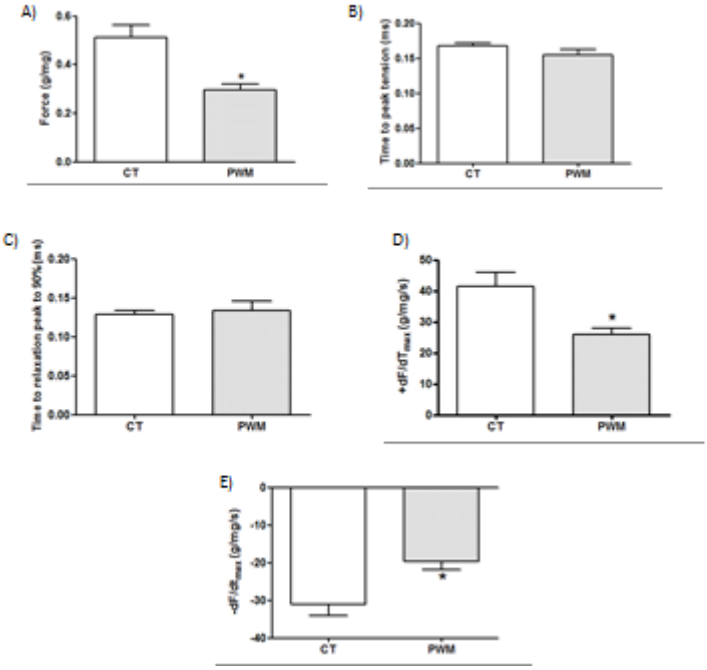


Figure 2

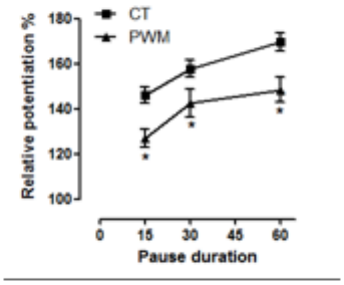


Figure 3

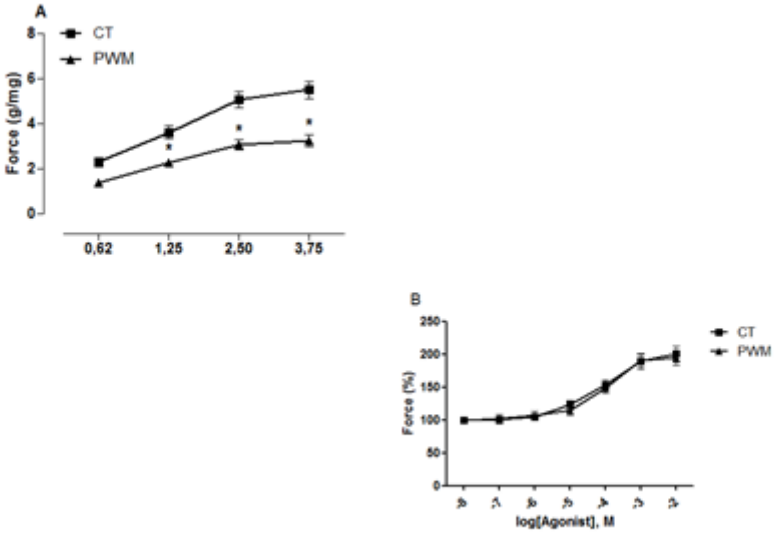


Figure 4

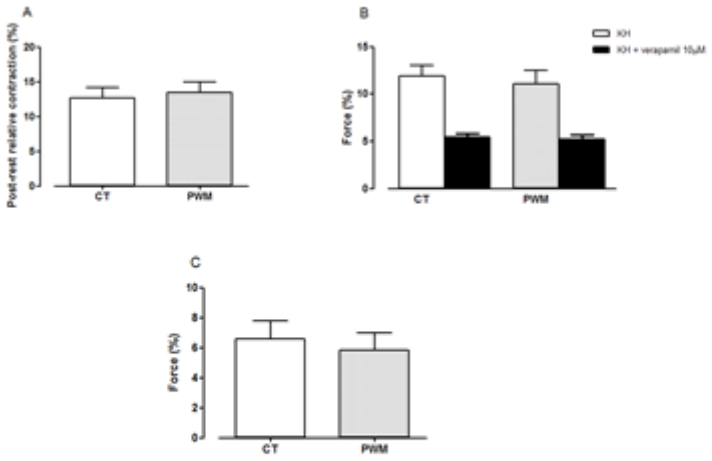


Figure 5

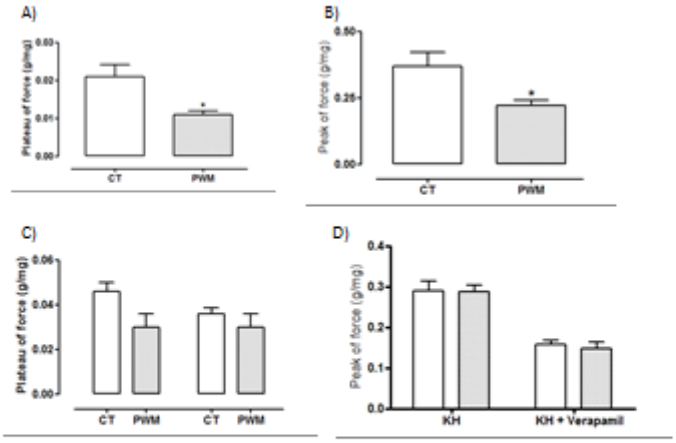


Figure 6

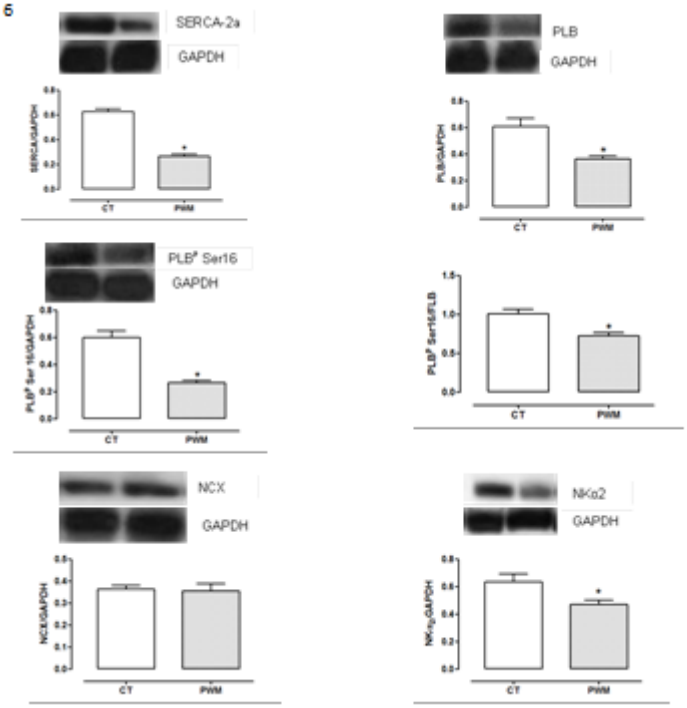
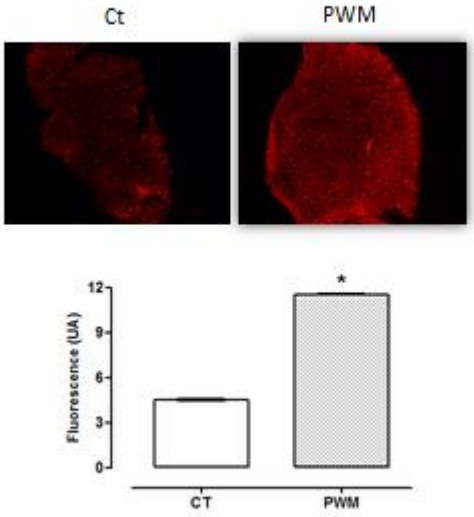


Figure 7



ANEXO III

Artigo 3 – **Publicado** - Post-Weaning Protein Malnutrition Increases Blood Pressure and Induces
Endothelial Dysfunctions in Rats

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PLoS one

Post-Weaning Protein Malnutrition Increases Blood Pressure and Induces Endothelial Dysfunctions in Rats

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Abstract

Malnutrition during critical periods in early life may increase the subsequent risk of hypertension and metabolic diseases in adulthood, but the underlying mechanisms are still unclear. We aimed to evaluate the effects of post-weaning protein malnutrition on blood pressure and vascular reactivity in aortic rings (conductance artery) and isolated-perfused tail arteries (resistance artery) from control (fed with Labina®) and post-weaning protein malnutrition rats (offspring that received a diet with low protein content for three months). Systolic and diastolic blood pressure and heart rate increased in the post-weaning protein malnutrition rats. In the aortic rings, reactivity to phenylephrine (10^{-10}–3.10^{-4} M) was similar in both groups. Endothelium removal or L-NAME (10^{-4} M) incubation increased the response to phenylephrine, but the L-NAME effect was greater in the aortic rings from the post-weaning protein malnutrition rats. The protein expression of the endothelial nitric oxide isoform increased in the aortic rings from the post-weaning protein malnutrition rats. Incubation with apocynin (0.3 mM) reduced the response to phenylephrine in both groups, but this effect was higher in the post-weaning protein malnutrition rats, suggesting an increase of superoxide anion release. In the tail artery of the post-weaning protein malnutrition rats, the vascular reactivity to phenylephrine (0.001–300 µg) and the relaxation to acetylcholine (10^{-10}–10^{-4} M) were increased. Post-weaning protein malnutrition increases blood pressure and induces vascular dysfunction. Although the vascular reactivity in the aortic rings did not change, an increase in superoxide anion and nitric oxide was observed in the post-weaning protein malnutrition rats. However, in the resistance arteries, the increased vascular reactivity may be a potential mechanism underlying the increased blood pressure observed in this model.

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Introduction

Proper nutrition in terms of quality and quantity is essential for the growth and development of organisms, including humans. Furthermore, nutritional deficiencies due to a decrease in or absence of the consumption of macro and micronutrients in food causes malnutrition in which the degree depends on the type of diet, age and the length of decrease consumption. Malnutrition is the result of an interaction of socioeconomic, political, cultural and environmental factors that more strongly affect children who live in extreme poverty [1].

Previous reports have shown that not only intrauterine malnutrition but also its occurrence during childhood might be a risk factor for the development of hypertension [2,3]. Malnutrition is accompanied by increased blood pressure, and this increase could be due in part to an impairment of endothelial function and/or increased sympathetic activity [4,5]. Some authors have shown that, during the period of malnutrition, the levels of circulating catecholamines increase [6,7]. This could be one of the hypertensive mechanisms induced by malnutrition. However, other mechanisms, such as changes in endothelial function, could contribute to the development and/or maintenance of hypertension induced by malnutrition. Previous reports have shown that in rats intrauterine malnutrition increases the generation of superoxide anion by increasing NADPH oxidase activity, which is possibly induced by angiotensin II [5,8]. However, these studies were conducted under intrauterine malnutrition. There are few studies that have investigated endothelial function in chronically malnourished rats. Moreover, there are also few studies evaluating models of malnutrition based on results of food consumption in populations.

In northeast Brazil, nutritional studies on the food consumption of the population allowed for the development of an experimental multideficient diet model, the regional basic diet (RBD) [9]. This diet is similar, in terms of quality, quantity and protein content to the diet that causes childhood malnutrition in Northeast Brazil [9]. Also, according to Teodosio et al. (1990), it is an unbalanced diet and low in some nutrients, mainly proteins. Both essential and non-essential amino acids are extremely limited in this diet, in addition to calories, fat, vitamins and minerals.

However, the factors that promote the development of hypertension associated with malnutrition are still unclear. Therefore, we evaluated the impact of the post-weaning malnutrition in rats on blood pressure, vascular reactivity of the rat tail vascular bed (resistance vessels) and aortic rings (conductance vessels).

Materials and Methods

Animals and experimental groups

The studies were performed on male Wistar rats. All experiments were conducted in compliance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Experimentation of the Biological Sciences Center of the Federal University of Pernambuco (Process Number 23076.008507/2007-16). All rats had free access to water and were fed rat chow *ad libitum*.

Animals were divided into two experimental groups: control (CT) and post-weaning protein malnutrition over 3 months. In the control group, the mothers were fed a chow diet during the pre-mating, mating, pregnancy and lactation. After weaning (21 days), the offspring had the same diet as their mothers (Labina®). In the post-weaning protein malnutrition group, mothers were fed a Labina® diet during the pre-mating, mating, pregnancy and lactation phases. The offspring received the RBD ration post weaning for 3 months.

Diets

Two types of diets were used for this study. The diet used to induced malnutrition was the RBD (Table 1) as described previously [9–12]. The RBD consisting of beans (*Phaseolus vulgaris*), sweet potatoes (*Ipomea batatas*), jerked beef and manioc flour (*Manihot esculenta*). These components were macerated, molded into "pellets" and heated to 50°C. The RBD pellets providing a total of (g/g%): protein 9, carbohydrates 78, lipids 1.1, fiber 7, minerals 4, sodium chloride 0.17 and Kcal/cic 356. No vitamin supplement was added. Moreover, part of the diet was supplemented with 0.2% (g/g) sodium chloride. The control diet was a commercial diet (Labina®). The standard diet contains the following content (g/g%): protein 23, carbohydrates 41, lipids 2.5, fibers 9, minerals 8, sodium chloride 0.37 and Kcal/cic 278 [10].

Hemodynamic measurements

At the end of treatment of malnutrition (3 months), the rats were anesthetized with urethane (1.2 g/kg, ip.), and a polyethylene catheter (PE50) filled with heparinized saline (50 U/mL) was introduced into the carotid artery to record arterial systolic (SBP) and diastolic blood pressure (DBP). Recordings were performed over 30 min with a pressure transducer (TSD 104A- Biopac) and with an interface and software for data collection (MP 30 Biopac Systems, Inc.; CA). The heart rate (HR) was determined from the intra-beat intervals.

"In Vitro" Experiments

Isolated rat aorta preparation. After the hemodynamic measurements, rats were killed by exsanguination. The thoracic aortas were carefully dissected and separated from the connective tissue. For the reactivity experiments, the aortas were divided into cylindrical segments 4 mm in length. To analyze the isoforms of endothelial and inducible nitric oxide synthase protein expression levels, the arteries were rapidly frozen in liquid nitrogen and kept at −70°C until the day of analysis.

Segments of thoracic aorta were mounted in an isolated tissue chamber containing Krebs–Henseleit solution: 118 mM NaCl; 4.7 mM KCl; 23 mM $NaHCO_3$; 2.5 mM$CaCl_2$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 11 mM glucose and 0.01 mM EDTA, gassed with 95% O_2 and 5% CO_2, and maintained at a resting tension of 1 g at 37°C as a previously described [13]. The isometric tension was recorded using an isometric force transducer (TSD125C, CA, U.S.A.) connected to an acquisition system (MP100 Biopac Systems, Inc., CA, U.S.A.).

After a 45 min equilibration period, the aortic rings were initially exposed twice to 75 mM KCl once to check their functional integrity and a second time to assess the maximal tension. Afterwards, the endothelial integrity was tested by adding acetylcholine (10 μM) to segments previously contracted with phenylephrine (1 μM). A relaxation equal to or greater than 90% was considered to be demonstrative of functional integrity of the endothelium. After a 45-min washout, concentration-response curves to phenylephrine were determined. Single curves were performed in each segment. The effects of the following drugs were evaluated: (1) a nonspecific NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 μM), (2) a non-selective cyclooxygenase (COX) inhibitor (indomethacin, 10 μM), (3) an NADPH oxidase inhibitor (apocynin, 0.3 mM). These drugs were added to the bath 30 min before performing the phenylephrine curve.

The influence of the endothelium on the response to phenylephrine in the post-weaning protein malnutrition and control groups were investigated after performing a mechanical removal by rubbing the lumen with a needle. The absence of endothelium was confirmed by the inability of 10 μM acetylcholine to produce relaxation.

Western blot analyses. Proteins from homogenized arteries (80 μg) were separated by 10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes that were incubated with mouse monoclonal antibodies for endothelial nitric oxide synthase (eNOS, 1:250; Transduction Laboratories, Lexington, UK). After washing, the membranes were incubated with anti-mouse (1:5000, StressGen, Victoria, Canada) immunoglobulin antibody conjugated to horseradish peroxidase. After thorough washing, the immunocomplexes were detected using an enhanced

Table 1. Composition (g/g %) of the Regional Basic Diet (RBD) and the control diet.

Diets	Protein	Carbohydrates	Lipids	Vitamin Supplement	Minerals	Sodium	Fiber	Kcal/100
RBD[1]	9	78	1.1	No	4	0.17	7	356
Control[2]	23	41	2.5	Yes	8	0.37	9	278

[1] According to the Laboratory of Experimentation and Analysis of Food (LEAL), Nutrition Department, Federal University of Pernambuco.
[2] As indicated by the manufacturer (Purina Agribrand, Paulínia, SP, Brazil).
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horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International, UK) and film (Hyperfilm ECL International). Signals on the immunoblot were quantified with the National Institutes of Health Image V1.36 computer program. The same membrane was used to determine α-actin expression using a mouse monoclonal antibody (1:5000, Sigma, USA).

Isolated rat tail artery preparation. Isolated rat tail arteries were used in this study as previously reported [14]. Briefly, after the hemodynamic measurements, the rats were heparinized (500 UI, *i.p.*). Ten minutes after the administration of heparin, 1 cm of the tail artery was dissected free and cannulated with an intracath (Nipro 24G ¾, Sorocaba, SP, BR) near the base of the tail. The vascular bed was flushed with Krebs-Henseleit buffer (KHB in mM) (NaCl: 120, KCl: 5.4, $MgCl_2$: 1.2, $CaCl_2$: 1.25, NaH_2PO_4: 2.0, $NaHCO_3$: 27, glucose: 11, and EDTA: 0.08) and bubbled with 5% CO_2 −95% O_2 at 36±0.5°C. The tail artery was then severed from the body and placed in a tissue bath and perfused with KHB at a constant flow of 2.5 mL/min with a peristaltic pump (Milan, Colombo, PR, BR).

After a 45-min equilibration period, the experimental protocol was initiated. The mean perfusion pressure (MPP) was measured by using a pressure transducer (TSD 104A, BIOPAC Systems, Inc., USA), and the data were recorded using an interface and software for data acquisition (model MP100A, BIOPAC Systems, Inc.). Because a constant flow was used, changes in the perfusion pressure represented changes in vascular resistance.

The following protocols were used to investigate the effects of post-lessning malnutrition on vascular reactivity to phenylephrine, acetylcholine and sodium nitroprusside. After a 45-min stabilization period, increasing doses of phenylephrine (0.001–300 µg as bolus injections of 100 µL) were administered into the perfusion medium in preparations from the controls and post-weaning protein malnutrition groups. The arteries were then maintained for a 30-min stabilization period. Then, the vasodilatation induced by acetylcholine (10^{-10}–10^{-3} M) was evaluated in arteries previously contracted with potassium chloride (65 mM) added to the perfusion fluid. Finally, after a 30-min stabilization period, the dilatory responses to sodium nitroprusside (10^{-10}–10^{-3} M) were determined in arteries previously contracted with potassium chloride (65 mM) added to the perfusion fluid.

Statistical analysis

All values are expressed as mean ± S.E.M. The contractile responses to phenylephrine of the aortic rings are expressed as a percentage of the maximum response produced by 75 mM KCl added into the bath. Relaxation response to Acetylcholine (10 µM) was expressed as the percentage of relaxation of the maximal contractile response to phenylephrine (1 µM).

Results regarding the perfusion pressure measurements for the tail artery experiments are presented as the changes in the mean perfusion pressure after subtraction of the peak pressure from the baseline pressure. The concentration-responses curves to acetylcholine or sodium nitroprusside are expressed as the percentage of relaxation of the maximum contractile response to KCl 65 mM.

For each concentration-response curve or dose-response curve, the maximum effect (E_{max}) and the concentration of agonist that produced 50% of the maximal response ($\log EC_{50}$) were calculated using non-linear regression analysis (GraphPad Prism Software, San Diego, CA). The sensitivity of the agonists is expressed as pD_2 ($-\log EC_{50}$). To compare the effects of endothelium denudation or drug incubation on contractile responses to phenylephrine, some results were expressed as the differences of the area under the concentration-response curves (dAUC) in both experimental groups. These values indicate whether the magnitude of the effect of endothelial denudation or drug incubation were different in the control and post-weaning protein malnutrition groups.

For protein expression, the data are expressed as the ratio between signals on the immunoblot corresponding to the studied protein and α-actin. The results are expressed as the means ± SEM of the number of rats studied. Differences were analyzed using Student's *t*-test or one-way ANOVA followed by a Tukey test. $P<0.05$ was considered to be significant.

Drugs and reagents

L-phenylephrine hydrochloride, L-NAME, indomethacin, apocynin, acetylcholine chloride, urethane and sodium nitroprusside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Salts and reagents used were of analytical grade from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Results

No difference in body weight between the groups was observed either at birth or 21 days after birth (Table 2). However, 90 days after birth, the body weight was lower in the post-weaning protein malnutrition rats compared to the controls (Table 2). These results demonstrated that the RBD was able to induce a loss in weight gain.

In order to verify if the loss in weight gain induced by hypoproteic diet produces arterial pressure alterations, blood pressure and heart rate were measured. At the end of treatment of malnutrition (90 days), significant increases in systolic and diastolic arterial blood pressure and heart rate were observed in the post-weaning protein malnutrition group compared to the controls (Table 3). Therefore, these results demonstrated that the post-weaning protein malnutrition induces a weight defect and hypertension.

Blood pressure changes could occur by an increase of cardiac output or/and by increased vascular resistance. Therefore, to further investigate the underlying mechanisms that produced the increase in arterial pressure, the effect of post-weaning malnutrition on vascular reactivity was investigated in isolated aortic rings (conductance arteries) and in the tail vascular bed (resistance arteries). In aortic rings, malnutrition did not affect the maximal response to 75 mM KCl in segments with endothelium (CT: 2.74±0.12 g vs. post-weaning protein malnutrition: 2.38±0.06 g, n = 10) and after endothelium removal (CT: 2.62±0.12 g vs. post-weaning protein malnutrition: 2.30±0.20 g, n = 10). These results demonstrated that the maximal tension that developed in aortic rings of post-weaning protein malnutrition group compared to the controls were not different. After that, vasodilatation and vasoconstriction mechanisms were investigated in aortic rings from post-weaning malnutrition and controls rats.

Table 2. Body weight (g) measurements of control (CT) and post-weaning protein malnutrition (Malnutrition) offspring.

	CT (N = 10)	MALNUTRITION (N = 10)
Birth	6.31±0.7	6.45±0.6
Weaning (21st day)	59.6±2.9	58.2±1.4
90th day	376±7.3	131±9.7*

Values are expressed as the mean ± SEM.
*$P<0.05$ Control vs. Malnutrition, Student's *t*-test.
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Table 3. Systolic (SBP, mmHg) and diastolic (DBP, mmHg) blood pressure and heart rate (HR, bpm) determination in the control (CT) and post-weaning protein malnutrition (Malnutrition) adult rats.

	CT (N = 10)	MALNUTRITION (N = 10)
SBP (mmHg)	102±4.0	118±6*
DBP (mmHg)	54±4	72±5.0*
HR (bpm)	302±9	328±11*

Values are expressed as the means ± SEM.
*$P<0.05$ Control vs. Malnutrition, Student *t*-test.
doi:10.1371/journal.pone.0034876.t003

The endothelium-dependent relaxation induced by acetylcholine in phenylephrine-contracted arteries was similar in aortic rings isolated from controls and post-weaning protein malnutrition (Rmax - CT: 28.86±2.7 g vs. post-weaning protein malnutrition: 100.05± 1.12 g, n = 8), suggesting that the oxide nitric release induced by acetylcholine was not modified. The contractile response induced by phenylephrine did not change in aortic rings from post-weaning protein malnutrition rats compared to the control group (Figure 1A). The influence of the endothelium on the response to phenylephrine was investigated after its mechanical removal. Endothelium removal leads to hyper contraction and produced a left-shifted concentration-response curve to phenylephrine in aortic segments from both groups (Figure 1B and 1C), but this effect was not different, as shown by the differences of the area under the concentration-response curves (Figure 1D).

To investigate whether the nitric oxide release in response to phenylephrine in aortic rings from post-weaning protein malnutrition was altered, rings were incubated with L-NAME. L-NAME (100 μM) induces a hyper contraction and left-shifted the concentration-response curve to phenylephrine in the aortic segments from both groups (Figures 2A and 2B). However, these effects were greater in preparations from the post-weaning protein malnutrition rats compared to controls, as shown by the differences of the area under the concentration-response curves (Figure 2C). In accordance with these findings, eNOS was overexpressed in aortic rings from the post-weaning protein malnutrition rats (Figure 3). These results suggested that the post-weaning protein malnutrition increased the release of nitric oxide, probably by eNOS overexpression.

Hypertension might be associated with increased oxidative stress. To investigate the role of superoxide anion generated by

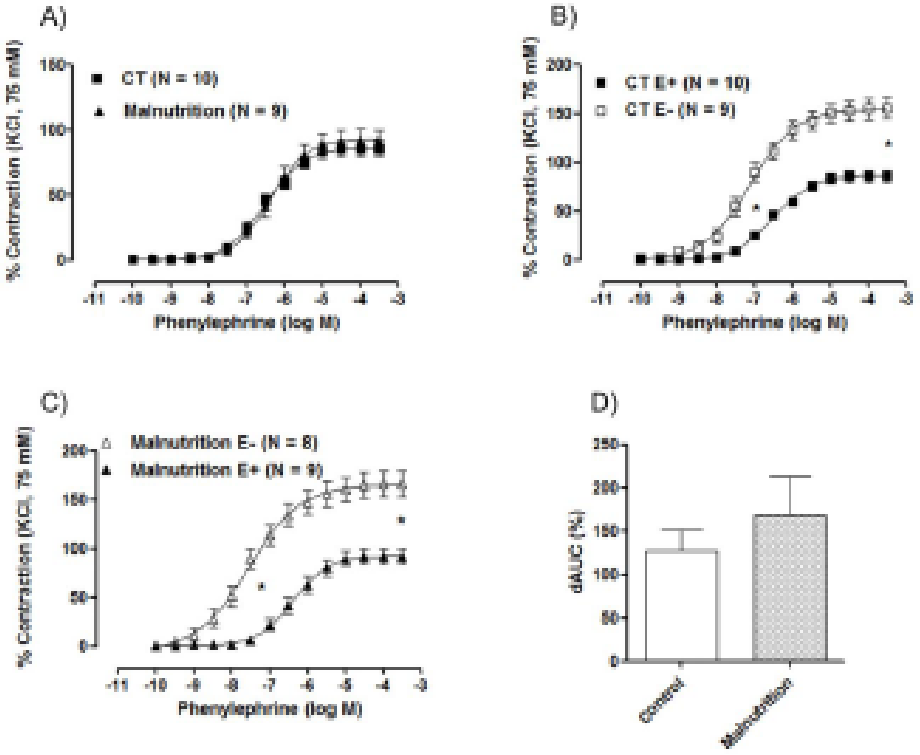

Figure 1. The effects of post-weaning protein malnutrition on the concentration-response curves of phenylephrine. (A) Concentration-response curve to phenylephrine in isolated aortic rings from control (CT) and post-weaning protein malnutrition (Malnutrition) groups. (B, C) Concentration-response curves to phenylephrine in isolated aortic rings from control (CT) and post-weaning protein malnutrition (Malnutrition) groups before (E+) and after removal of endothelium (E−). (C) Percent difference of the area under the curve (% dAUC) in vessels with endothelium intact (E+) and denuded (E−). The number of preparations is indicated in parenthesis. The results are expressed as the means ± SEM. *$P<0.05$ for pD_2 and R_{max}: CT E+ vs. CT E−; pD_2: Malnutrition E+ vs. Malnutrition E− and dAUC% - CT vs. Malnutrition. Student's *t*-test.
doi:10.1371/journal.pone.0034876.g001

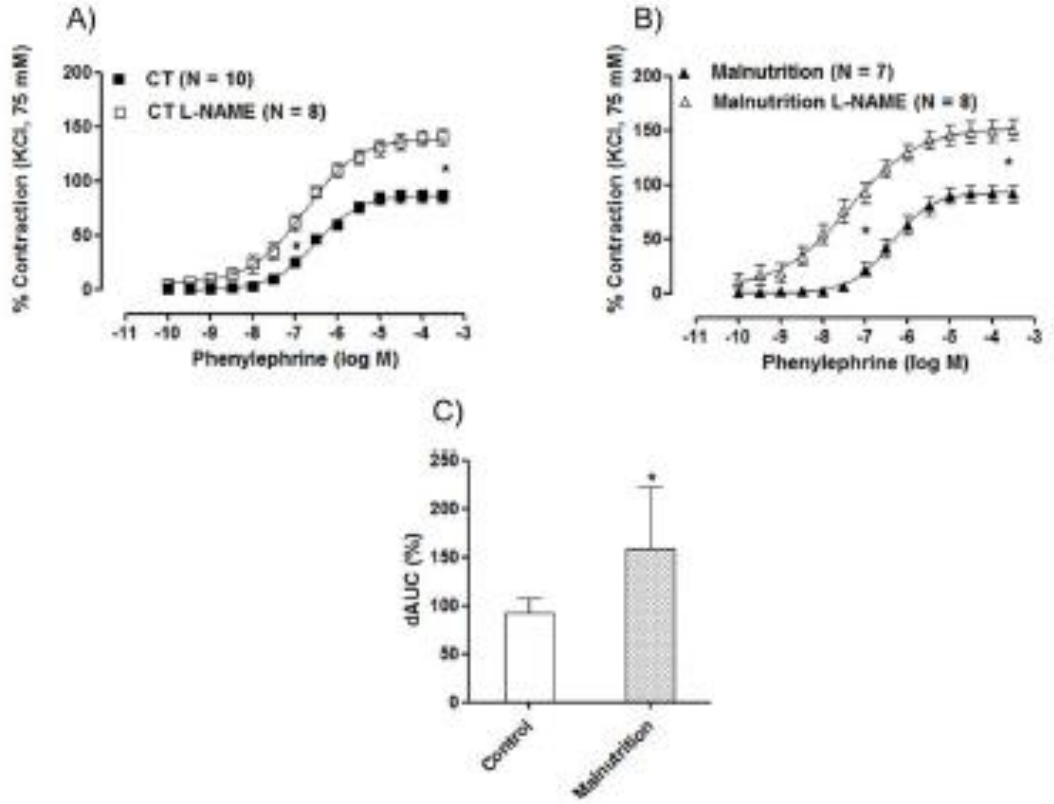

figure 2. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) (A, B) on the concentration-response curve to phenylephrine in isolated aortic rings from control (CT) and post-weaning protein malnutrition (Malnutrition). (C) Comparison of the percent difference of the area under the curve (% dAUC) in vessels in presence or absence of L-NAME from control (CT) and post-weaning protein malnutrition (Malnutrition) groups. The number of preparations is indicated in parenthesis. The results are expressed as the means ± SEM. * $P<0.05$ for pD2 and R_{max}: CT E+ vs. CT L-NAME; pD2 and R_{max}: Malnutrition E+ vs. Malnutrition L-NAME and dAUC% CT vs. Malnutrition by Student's *t*-test.
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NADPH oxidase activity on vascular reactivity to phenylephrine in post-weaning protein malnutrition rats, the NADPH oxidase inhibitor apocynin (0.3 mM) was used. Incubation with apocynin reduced reactivity to phenylephrine in all groups (Figure 4A and 4B), but the effect was greater in the post-weaning protein malnutrition group, as shown by the differences of the area under the concentration-response curves (Figure 4C). These results suggested that the post-weaning protein malnutrition increased the release of superoxide anion generated by NADPH oxidase.

Indomethacin (10 μM), a cyclooxygenase inhibitor, was used to investigate the putative role of prostanoids on vascular reactivity to phenylephrine in post-weaning protein malnutrition rats. Indomethacin did not alter the phenylephrine responses in aortic segments from both groups (results not shown).

Because vascular reactivity in the conductance arteries was almost unaffected, we also investigated the effect of post-weaning malnutrition on the vascular reactivity of the resistance arteries (tail artery). This protocol was used to obtain information regarding the effects of post-weaning protein malnutrition on the resistance vasculature.

In the endothelium-intact tail vascular bed preparations from the post-weaning protein malnutrition group, the mean perfusion pressure was similar in both groups, as observed from the initial dose of phenylephrine response curve, which are similar (Figure 5A). However, the maximal response to phenylephrine (Figure 5A) was greater (408±29.5 mmHg, $P<0.05$, $n=10$) compared to the CT group (295±32 mmHg, $P<0.05$, $n=10$). In tail arteries previously contracted with KCl 65 mM, the acetylcholine induced-relaxation (Figure 5B) was modestly greater in the post-weaning protein malnutrition group (86.5±3.9%, mmHg, $P<0.05$, $n=10$) compared to the CT group (70.8±4.2%, mmHg, $P<0.05$, $n=10$). The endothelium-independent relaxation induced by sodium nitroprusside was similar in both groups (Figure 5C).

Discussion

This study demonstrated that post-weaning protein malnutrition increased blood pressure, which was accompanied by increased vascular reactivity in resistance arteries (tail artery). However, in conductance arteries (aortic rings), malnutrition did not change the vascular reactivity due to increased basal release of NO, probably resulting from eNOS overexpression, although there was an increased release of free radicals derived from NADPH oxidase. Therefore, this balance prevented apparent changes in the phenylephrine concentration-response curves.

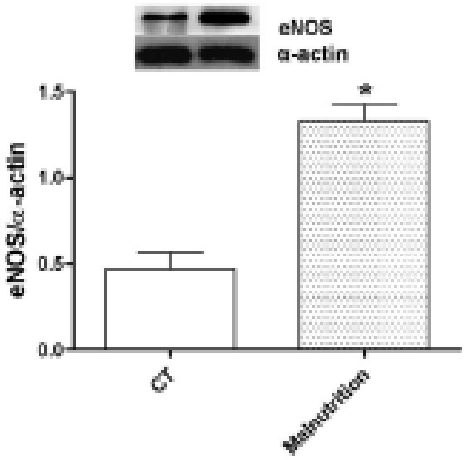

Figure 1. Densitometric analysis of the Western blots for endothelial NO synthase (eNOS) protein expression in the isolated aortic rings from Wistar rats (CT, N = 9) and post-weaning protein malnutrition (Malnutrition, N = 9). The number of preparations is indicated in parenthesis. Each bar represents means ± S.E.M as the ratio between the signal for the eNOS protein and the signal for α-actin. * $P<0.05$ for CT vs. Malnutrition by Student's *t*-test. Representative blots are shown.
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In this study, we investigated the effects of post-weaning protein malnutrition on blood pressure and vascular reactivity of resistance and conductance arteries in rats to elucidate some mechanisms that are correlated with two relevant issues in public health: malnutrition and hypertension. Post-weaning protein malnutrition was induced by a diet that reproduces a regional basic diet (RBD) [9,10–12] widely consumed by inhabitants living in an area of sugar cane plantations along the coast of the State of Pernambuco, Brazil. This diet has been associated both with lower weight gain and the malfunction of various organs, probably because of its low protein content and vitamin and mineral deficiencies [9,10–12]. It is important to mention that this diet is deficient in a qualitative rather than a quantitative nature, the latter of which is used by most researchers and represents the real consumption of this population. Depending on the region and season, populations in northeast Brazil and in other regions of the world might consume a similar diet. Therefore, the importance of knowing its effects on the organism might help to introduce future corrective interventions to minimize the risk of diseases associated with malnutrition.

Animals submitted to post-weaning protein malnutrition showed a marked reduction in weight gain. These results highlight the importance of nutritional status in the growth phase of the animal. They show a greater impact of the post-weaning protein malnutrition period including the stage of growth and development into adulthood. Similar results were previously observed [13] with use of the same diet. This reduction was also found in other studies using a rat experimental model induced by a dietary restriction of 50% [9,16–19] or a low-protein diet with 6% protein [20,21].

An alteration in body weight is an indicator of the nutritional status of the individual and might be associated with alterations in the cardiovascular system [22,23]. Indeed, in our study we observed that the malnutrition triggers weight deficit and hypertension. Several authors that have studied malnutrition induced during pregnancy in rats using quantitative models [18,24–26] have shown that a decrease in body weight could be related to hypertension in the offspring. A previous report [27] has shown that the intrauterine malnutrition with a low protein (8% casein) diet in rats promotes an increase in blood pressure of the offspring at a young adulthood stage. Previous reports evaluating the effects of protein-caloric deprivation in rats have suggested an increased hypothalamic-pituitary-adrenal response and circulating catecholamines [28–30]. Then, the increase in heart rate and blood pressure might be related to increased sympathetic nervous system activity [31].

In accordance to these studies, Sawaya et al. [3] have demonstrated that in malnutrition, children might have increased diastolic blood pressure. Therefore, because the diastolic blood pressure reflects, in part, the peripheral vascular resistance, vascular reactivity might be altered in malnutrition conditions. Indeed, in our experiments, a qualitative model of malnutrition increased systolic and diastolic blood pressure and heart rate. For this purpose, we investigated the vascular reactivity of conductance (aorta artery) and resistance arteries (tail artery).

Malnutrition did not change the vascular reactivity in the isolated aortic rings. However, in the presence of L-NAME, a non-specific inhibitor of NOS, there was a greater vasoconstrictor response to phenylephrine in post-weaning protein malnutrition group, suggesting increased NO production. However, the endothelium-dependent relaxation induced by acetylcholine was similar in aortic rings isolated from controls and post-weaning protein malnutrition, suggesting that the oxide nitric release induced by acetylcholine was not modified.

As mentioned above, we observed that the malnutrition triggers hypertension. Hypertension might be associated with reduced nitric oxide bioavailability, although there are studies that have shown an increase in this vasodilator as a compensatory mechanism [32–34]. Moreover, other studies have shown that the synthesis and/or release of nitric oxide in arterial hypertension is normal, but its bioavailability is reduced due to increased superoxide anion production, mainly by increasing NAD(P)H oxidase activity, which inactivates nitric oxide generating nitrite peroxide [35–37]. We then investigated the role of free radicals by blocking NAD(P)H oxidase with apocynin. Our results suggest an increased release of superoxide anion derived from NAD(P)H oxidase activity in post-weaning protein malnutrition arteries.

Franco et al. [17] also have shown that intrauterine malnutrition increases oxidative stress, suggesting a potential explanation for endothelial dysfunction development. Also, in the same study, they concluded that intrauterine malnutrition induces hypertension in both male and female offspring and hypertension may be more severe in males than females. Franco et al. [17] concluded that malnutrition alters endothelium-dependent responses and endothelial dysfunction is associated with decreased eNOS activity and expression in the aortas of the offspring. However, in our study, we showed increased nitric oxide release, which could result from an increase in eNOS protein expression. It's possible that the low-protein diet used in our study has lower arginine levels. Therefore, the increase of the eNOS protein levels, observed in the aorta, could be an adaptive response to the low arginine, and, consequently, low NO levels [37]. However, although the eNOS activity was not measurement, we observed an increase nitric oxide release in response to phenylephrine in isolated aortic rings. Therefore, in this study we cannot speculate that the increase in eNOS protein levels has relationship with the levels of arginine. In accordance with our findings, we might suggest that the increase

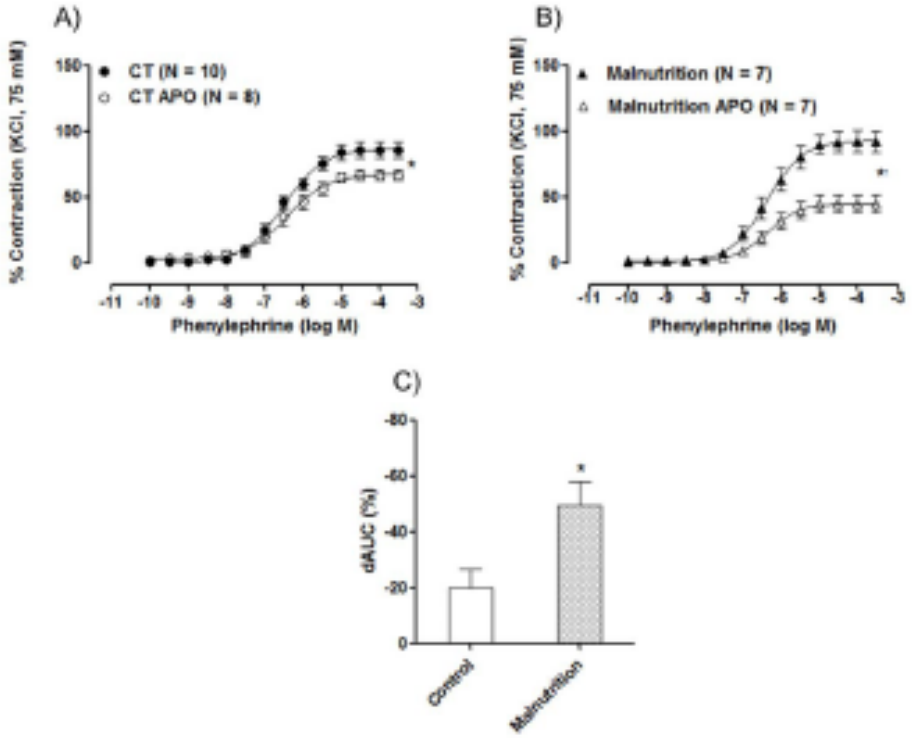

Figure 4. Concentration-response curve to phenylephrine in isolated aortic rings from (A) control (CT) and (B) post-weaning protein malnutrition (Malnutrition) rats before (E+) and after incubation with apocynin (APO). C) Comparison of the percent difference of the area under the curve (% dAUC) in vessels in presence or absence of apocynin from control (CT) and post-weaning protein malnutrition (Malnutrition) rats. The number of preparations is indicated in parenthesis. The results are expressed as the means ± SEM. * $P<0.05$ for R_{max}: CT E+ vs. CT APO; R_{max}: Malnutrition E+ vs. Malnutrition +APO and dAUC% CT vs. Malnutrition by Student's *t*-test.
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of eNOS protein levels and nitric oxide release in response to phenylephrine in isolated aortic rings might be a compensatory mechanism induced by increased blood pressure or oxidative stress.

These results might be a compensatory mechanism induced by increased blood pressure or oxidative stress. The increase in superoxide anion, the most important free radical generated by NADPH oxidase activity, could decrease nitric oxide bioavailability [38]. The main sources of superoxide anion that are implicated in the genesis of endothelial dysfunction are xanthine oxidase and NADPH oxidase [38]. Particularly, the increased NADPH oxidase activity has been reported as a major source of superoxide anion in the vessel wall in experimental hypertension models [38].

Therefore, in order to investigate if the post-weaning malnutrition per se or the increment of blood pressure that was observed after the treatment with RBD could increase the release of free radicals, apocynin was incubated in isolated aortic rings from both groups. Based on the effects of apocynin on reducing phenylephrine reactivity, our findings point towards this possibility. Therefore, we believe that post-weaning protein malnutrition increases oxidative stress that is initially compensated by increased nitric oxide production. However, for a long period, this compensatory response can be lost, and the endothelial dysfunction might increase or maintain hypertension.

Because the vascular reactivity to phenylephrine in conductance arteries was not modified after the post-weaning malnutrition, other endothelium-derived vasoconstrictors are probably released. Therefore, we investigated the prostanoids derived from the cyclooxygenase pathway. In the presence of indomethacin, a COX inhibitor, there was no difference in the vasoconstrictor response to phenylephrine in the post-weaning protein malnutrition and control groups, suggesting that vasoactive prostanoids did not seem to be altered in this artery after malnutrition.

Considering that findings obtained from the aortic rings did not provide a clear explanation for the increased blood pressure, we investigated the effects of malnutrition on a resistance vessel. In fact, we demonstrated that in the tail artery of the post-weaning protein malnutrition group, the vascular reactivity to phenylephrine increased. Therefore, this increase in vascular reactivity might increase vascular resistance, which increases diastolic blood pressure. However, in the same artery, we observed that the relaxation response to acetylcholine increased. This effect could be a compensatory mechanism to the increased vascular reactivity or blood pressure.

Therefore, our results used a qualitative malnutrition model with low-protein to show an increase in blood pressure in the post-weaning protein malnutrition group, which was accompanied in site by an increase in vascular reactivity in the resistance arteries. However, in the conductance arteries, malnutrition did not modify

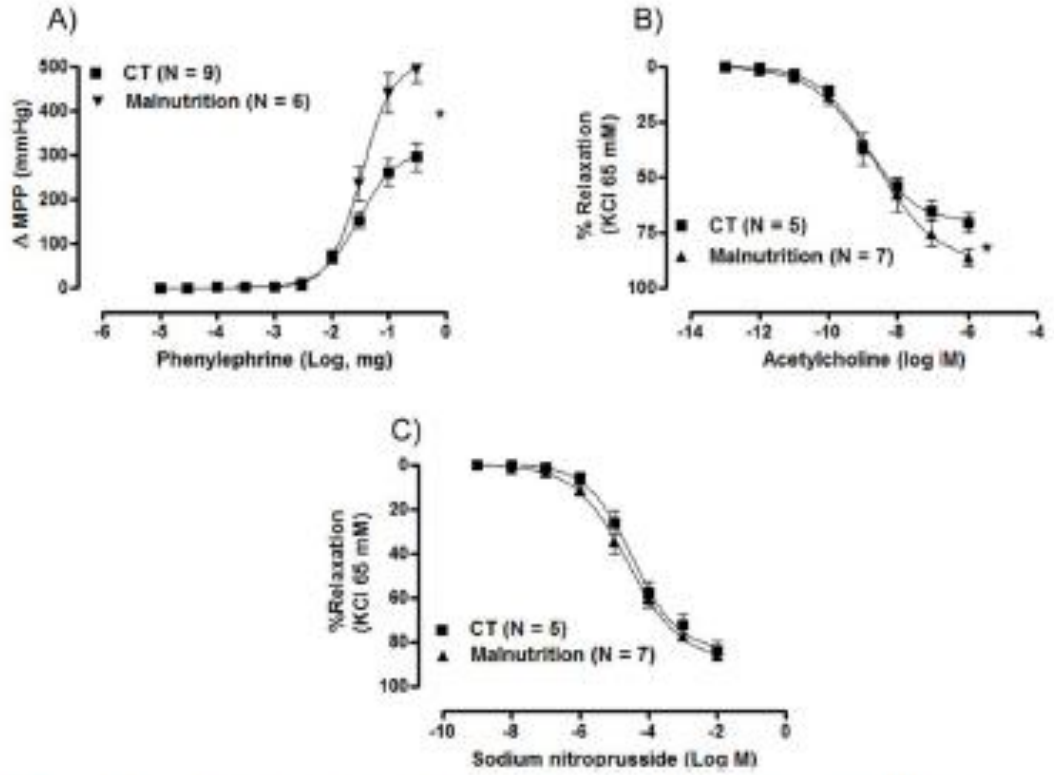

Figure 5. (A) Changes in the mean perfusion pressure (MPP) produced by phenylephrine (PHE) in tail vascular beds from the control (CT) and post-weaning protein malnutrition groups (Malnutrition). (B) Concentration-response curves produced by acetylcholine (ACh) in the tail vascular beds previously contracted with potassium chloride (KCl, 65 mM) (C) Concentration-response curves produced by sodium nitroprusside (SNP) in the tail artery bed previously contracted with with potassium chloride (KCl, 65 mM). The number of preparations is indicated in parentheses. The results are expressed as the means ± SEM. * $P<0.05$ for Rmax CT vs. Malnutrition, Student's *t*-test.
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the vascular reactivity probably due to the increased release of nitric oxide, which balances free radicals and prevents changes in the phenylephrine concentration-response curves. We suggest that the increase in free radicals and nitric oxide in the aortic rings of post-weaning protein malnutrition rats might be a compensatory mechanism to the increase in blood pressure.

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Author Contributions

Conceived and designed the experiments: ACSB JKA TOF DVV ASP. Performed the experiments: ACSB JKA TOF FDMS EAS EPM. Analyzed the data: TOF JKA FDMS ASP DVV. Contributed reagents/materials/analysis tools: CPC DVV ASP. Wrote the paper: ACSB TOF ASP DVV.

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