Instituto de Biociências

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

EFEITOS DA RESTRIÇÃO PROTEICA MATERNA SOBRE O PADRÃO VASCULAR E EXPRESSÃO DE PROTEÍNAS NO EPIDÍDIMO DE RATOS WISTAR MACHOS EM DIFERENTES FASES DO DESENVOLVIMENTO PÓS-NATAL

MARILIA MARTINS CAVARIANI

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração em Biologia Celular Estrutural e Funcional.

Raquel Fantin Domeniconi

BOTUCATU – SP
2019

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Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, como requisito para obtenção do título de Doutora no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração em Biologia Celular, Estrutural e Funcional.

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Epígrafe

Seu trabalho vai preencher grande parte da sua vida e a única maneira de ficar realmente satisfeito é fazer o que você acredita ser um ótimo trabalho.

Steve Jobs

SUMÁRIO

Resumo

O estado nutricional materno desempenha papel crucial na saúde e no bem-estar do feto. Alterações testiculares, prostáticas e espermáticas foram observadas em animais adultos, cujas mães sofreram restrição de proteína. No entanto, não há trabalhos com este modelo experimental que enfoquem o desenvolvimento, padrão de vascularização e seus reflexos na expressão de proteínas no epidídimo. O objetivo deste estudo é investigar o padrão das respostas morfológicas, imuno-histoquímicas e expressão de proteínas do epidídimo da prole de ratos *Wistar* de mães submetidas à restrição proteica no período de gestação e de lactação. Fêmeas prenhes foram alocadas nos grupos experimentais normoproteico (NP) e hipoproteico (HP) que receberam, respectivamente, dietas contendo 17% e 6% de proteínas durante gestação e lactação. Após o desmame, os ratos receberam dieta padrão para roedores até as idades de 21, 44 e 120 dias pós-natais (DPN) quando foram eutanasiados. Os epidídimos foram coletados e processados segundo técnicas histológicas, imuno-histoquímicas e de *western blotting*. Nos filhotes HP, o tamanho reduzido e baixo peso observados ao nascimento se mantiveram até o DPN 120, acompanhados da redução dos órgãos do sistema genital masculino para todas as idades analisadas. A dieta hipoproteica materna diminuiu os níveis séricos de testosterona nos animais no DPN 44, aumentou os níveis de aldosterona nos animais no DPN 21 e não alterou os níveis de estradiol em nenhuma das idades. Nos animais HP, a expressão de AR foi reduzida em todo epidídimo no DPN 44, enquanto a expressão de ERα mostrou-se aumentada no corpo e cauda nos DPNs 21 e 44 e a expressão de ERβ foi reduzida no segmento inicial e cabeça no DPN 120. A imunolocalização destes receptores hormonais foi observada no núcleo e citoplasma das células epiteliais epididimárias e nas células do interstício do órgão em todas as idades. Apesar da diminuição na expressão de AQP9 no segmento inicial e cabeça assim como o aumento na expressão de AQP1 e AQP9 no corpo e cauda terem sido significativos somente para os animais HP no DPN 44, essas alterações também foram observadas nos animais HP nos DPNs 21 e 120. Para animais NP e HP de todas as idades, a imunolocalização de AQP1 apareceu nas células endoteliais de canais vasculares ao longo de todo órgão, enquanto a imunolocalização de AQP9 foi observada nos estereocílios das células principais do epidídimo. A Expressão do VEGFa não foi diferente entre os animais NP e HP em nenhuma das idades. Já a expressão do seu receptor (VEGFR-2) foi menor no segmento inicial e cabeça dos animais HP no DPN 21. A restrição proteica materna aumentou a expressão da Src 416 em todo epidídimo no DPN 21, enquanto diminuiu a expressão desta proteína no segmento inicial e cabeça no DPN 44 e no corpo e cauda no DPN 120. Já a expressão da Src 527 foi diminuída no segmento inicial e cabeça dos animais HP no DPN 21. Observou-se o aumento da expressão da Cldn-1 somente no corpo e cauda epididimários dos animais HP no DPN 44. O índice de

densidade microvascular (DMV) e o índice de DMV/estroma dos animais HP nos DPNs 21 e 44 foram menores do que nos animais NP nestas idades. Mesmo que algumas das alterações observadas para os filhotes HP não tenham se mantido até a vida adulta, a oferta insuficiente de proteínas no início da vida alterou a estrutura e o funcionamento do epidídimo em períodos importantes de seu desenvolvimento pós-natal.

Palavras chaves: epidídimo, restrição proteica, desenvolvimento pós-natal, programação fetal

Abstract

Maternal nutrition status plays a crucial role in the health and well-being of the fetus. Changes of testes and prostate as well as spermatic disorders were observed in adult animals whose mothers were subjected to protein restriction. However, there are no studies with this experimental model that focus the development and the vascular pattern of epididymis, as well as their reflexes in the expression of proteins of the epididymis. The aim of this study is to investigate the pattern of morphometric, immunohistochemical and protein expression of the epididymis of the Wistar rat offspring whose mothers were subjected to a low-protein diet during gestation and lactation. Pregnant females were divided into normoprotein (NP) group and low-protein (LP) group that received, respectively, diets containing 17% and 6% of protein during gestation and lactation. After weaning, the LP and NP male pups received the standard diet for rodents until the ages of 21, 44 and 120 days (PND), when they were euthanized. The epididymides were collected and processed according to histological, immunohistochemical and western blotting techniques. In the LP offspring, the smaller body size and low weight observed at birth remained until the PND 120, as well as the reduction of the male genital system organs weight for all analyzed ages. Maternal low-protein diet decreased testosterone serum levels in animals at PND 44, increased aldosterone serum levels in animals at PND 21 and did not altered estradiol serum levels in any age. In LP animals, the AR expression was decreased throughout epididymis at PND 44, whereas ERα expression was increased in epididymis corpus plus cauda at PND 21 and 44, and ERβ expression was reduced in epididymis initial segment plus caput at PND 120. Immunolocalization of these hormone receptors were observed in the nucleus and cytoplasm of epididymis epithelial cells and in the interstitial cells of this organ at all ages. The decrease of AQP9 expression on the initial segment plus caput as well as the increase of AQP1 and AQP9 expression on the corpus plus cauda were significant only in LP animals at PND 44. However, these alterations were also observed in LP animals at PND 21 and 120. The immunolocalization pattern of AQP1 and AQP9 was similar between LP and NP animals at all ages, in with AQP1 staining appearing on endothelial cells of vascular channels throughout the organ, while the immunolocalization of AQP9 was observed in the stereocilia of epididymis principal cells. The VEGFa expression in the epididymis was not different between NP and LP animals in none of the analyzed ages. However, the expression of its receptor (VEGFr-2) was lower on epididymis initial segment plus caput of LP animals at PND 21. Maternal protein restriction increased the Src 416 expression throughout epididymis at PND 21, whereas decreased the expression of this protein on epididymis initial segment plus caput at PND 44 and on epididymis corpus plus cauda at PND 120. Conversely, Src 527 expression was decreased in epididymis initial segment plus caput in LP animals at PND 21.

Cldn-1 expression was increased only in the epididymis corpus plus cauda of LP animals at PND 44. The microvascular density (MVD) and the MDV/stroma index of LP animals at PND 21 and 44 were lower than those of NP animals at these ages. Although some of the changes observed for LP pups were not maintained until adulthood, we observed that the insufficient supply of proteins in early life altered the structure and the functioning of the epididymis in important periods of postnatal epididymal development.

Keywords: epididymis, protein restriction, postnatal development, fetal programming

Introdução

Programação Fetal

Um dos primeiros conceitos a surgir na literatura, relacionando as condições do meio intrauterino às alterações no desenvolvimento da progênie, foi o da "origem fetal das doenças da idade adulta"(Barker, 1995a). A hipótese da "origem fetal" propõe que alterações na nutrição e no estado hormonal fetais resultam em adaptações durante o desenvolvimento capazes de alterar a estrutura, fisiologia e metabolismo do indivíduo em formação, predispondo-o, assim, a doenças cardiovasculares, metabólicas e endócrinas na vida adulta (Barker, 1995b; 2007).

As alterações e adaptações do concepto podem ocorrer de forma permanente e, nesse caso, surge o conceito de "programação fetal", que consiste em um processo mais geral por meio do qual um estímulo ou insulto aplicado em um período crítico ou sensível do desenvolvimento intrauterino, pode levar a alterações permanentes na estrutura ou no funcionamento de órgãos e/ou funções metabólicas do organismo em formação (Lucas, 1991).

Eventos decorridos no início da vida podem gerar consequências a longo prazo através de danos diretos (como por exemplo, perda de um membro devido a acidente vascular), por indução, deleção ou desenvolvimento prejudicado de estrutura somática ou ainda por meio de alteração fisiológica, sendo os dois últimos provocados por um estímulo ou insulto precoce ocorrido em período crítico do desenvolvimento, com consequências a longo prazo para a função do organismo. Dessa maneira, o termo "programação" se aplica aos dois últimos casos, nos quais o estímulo programador possui um efeito duradouro ou permanente (Lucas, 1998).

Hales e Barker (1992) investigando a correlação entre o baixo peso ao nascer e na infância associados com maior risco de desenvolvimento de diabetes *mellitus* tipo 2 e síndrome metabólica na vida adulta, propuseram a "hipótese do fenótipo poupador", afirmando que uma condição nutricional intrauterina adversa é capaz de programar uma série de adaptações no feto em desenvolvimento constituindo um fenótipo "econômico", que além de aumentar suas chances de sobrevivência imediata, também conferem vantagens em um ambiente pós-natal de escassez nutricional.

Dessa maneira, o feto interage de maneira dinâmica com o ambiente materno e prevê o meio em que provavelmente nasça, se adaptando para obter uma futura vantagem competitiva (Gluckman e Hanson, 2006). Se os ambientes pré e pós-natais forem compatíveis, o fenótipo será normal, no entanto, se houver incompatibilidade entre o ambiente previsto e o real na vida pós-natal, a programação fetal tornará o indivíduo inapto, aumentando a suscetibilidade do

organismo à obesidade e a doenças metabólicas (Armitage *et al.*, 2005; Martin-Gronert e Ozanne, 2010; Qasem *et al.*, 2012).

Atualmente, a hipótese de que as condições ambientais intrauterinas são capazes de influenciar o estabelecimento de doenças na idade adulta é conhecida como *Developmental Origins of Health and Disease Hypothesis* ou "hipótese das origens desenvolvimentistas da saúde e doença" (DOHaD) (Jazwiec e Sloboda, 2019).

Uma das características mais interessantes e significativas da programação fetal é a evidência de que as adaptações ao ambiente intrauterino podem ser passadas ao longo de várias gerações, com os descendentes nunca tendo sido expostos diretamente ao fator estressor, principalmente através das chamadas alterações epigenéticas (Zambrano, 2009; Sebert *et al.*, 2011).

Os eventos epigenéticos estão relacionados com a plasticidade do organismo perante estímulos adversos, capazes de alterar permanentemente a estrutura e a função específica de sistemas e órgãos, desempenhando, portanto, papel importante no desenvolvimento e sendo essenciais para a programação correta da expressão dos genes (Rothhammer e Bosserhoff, 2007). Processos epigenéticos, como a metilação do DNA e modificações de histonas, podem induzir mudanças hereditárias na expressão gênica sem que tenha havido alteração na sequência genética. Assim, o epigenoma pode ser considerado um registro molecular de eventos que se acumulam ao longo da vida de um organismo (Bird e Macleod, 2004; Godfrey *et al.*, 2015).

Restrição Proteica Materna e a Programação Fetal

Durante a gravidez, o feto em desenvolvimento depende totalmente da mãe e do ambiente materno para suprir suas necessidades nutricionais. Portanto, parece quase inquestionável afirmar que a nutrição materna é capaz de influenciar a saúde fetal e o resultado da gravidez (Mcardle *et al.*, 2006; Lindsay *et al.*, 2019).

Neste sentido, diversos estudos demonstram que os componentes e a qualidade da dieta materna, durante períodos críticos do desenvolvimento, podem remodelar o genoma da prole no útero. E que alterações epigenéticas induzidas no início da vida podem alterar permanentemente o fenótipo do organismo adulto, tornando-o susceptível a uma série de doenças (Masuyama e Hiramatsu, 2012; Jiang *et al.*, 2014; Fleming *et al.*, 2015).

A "fome holandesa" foi um período de extrema escassez alimentar que teve início com a invasão dos Aliados (Estados Unidos, Reino Unido, União Soviética, França, China e Polônia)

na Holanda durante a segunda guerra mundial. Como retaliação, os alemães proibiram o transporte de alimento para o país até o início de novembro de 1944, quando esse embargo foi suspenso e o transporte de alimentos pela água foi novamente permitido. No entanto, nesta época a maioria dos canais e vias fluviais estava congelada devido ao inverno rigoroso, tornando impossível a entrada de mantimentos na Holanda, de modo que os estoques de alimentos do país se esgotaram rapidamente (Roseboom *et al.*, 2011).

Durante a "fome holandesa", as rações diárias oficiais para a população adulta do país diminuíram abruptamente de aproximadamente 1.400 calorias por dia, em outubro de 1944, para menos de 1000 calorias, no final de novembro de 1944. No auge da fome, entre dezembro de 1944 e abril de 1945, as rações diárias oficiais em Amsterdã variavam entre apenas 400 e 800 calorias (Carpinello *et al.*, 2018). Apesar de trágico em termos humanitários, essas características proporcionam uma oportunidade única de estudar os efeitos de um período curto, porém grave, de desnutrição materna durante os diferentes estágios da gestação na prole (Painter *et al.*, 2005).

Grande parte dos conhecimentos sobre programação fetal resultou do estudo de modelos de desnutrição materna e sua influência na saúde da prole. Estudos descritos a partir das décadas de 1970 e 1980 passaram a associar a restrição nutricional materna e, principalmente, a restrição proteica, não só com o retardo no crescimento intrauterino da prole, mas, principalmente, com a maior predisposição para o desenvolvimento de doenças crônicas na vida adulta, em particular a hipertensão, obesidade e a diabetes *mellitus* tipo 2 (Imdad e Bhutta, 2012; Grissom *et al.*, 2014).

Durante a gravidez, recomenda-se o aumento da ingestão de proteínas para suprir a demanda adicional de nitrogênio exigida tanto pela mãe, quanto pelo indivíduo em formação. Nessa fase há também um aumento do *turnover* proteico para satisfazer as exigências de crescimento rápido do embrião (Jolly *et al.*, 2004). Além disso, a ingestão insuficiente de proteínas entre grande parte da população humana, por motivos culturais ou econômicos, é uma preocupação global. Fazendo do modelo de restrição proteica um dos modelos de restrição precoce do crescimento mais caracterizados e estudados até hoje (Fleming *et al.*, 2015; Semba, 2016; Herring *et al.*, 2018).

Algumas áreas de particular interesse têm sido as investigações dos efeitos a longo prazo de dietas maternas com baixa concentração de proteína sobre a função e estrutura de órgãos e tecidos sensíveis à insulina, funções cardíacas, hipertensão, obesidade e função renais da prole (Stocker *et al.*, 2005; Le Clair *et al.*, 2009; Hales e Barker, 2013; Herring *et al.*, 2018).

A restrição proteica materna leva a diminuição da vascularização das ilhotas pancreáticas, da massa de células beta e do tamanho e conteúdo de insulina das ilhotas em seus descendentes, tornando o processamento da glicose insuficiente e culminando com o aparecimento da diabetes *mellitus* tipo 2 (Bertin *et al.*, 2002; Martin-Gronert e Ozanne, 2007; Berends *et al.*, 2013). Disfunção cardíaca patológica, remodelação diastólica e aumento da sensibilidade à lesão isquêmica durante a vida adulta também são observadas em fetos cujas mães sofreram restrição de proteína durante a gestação e lactação (Meyer e Zhang, 2007). Muitos estudos relatam ainda, que a restrição proteica materna resulta em alterações na estrutura e funcionamento renal (redução no número de néfrons) dos fetos (Woods *et al.*, 2001; Zimanyi *et al.*, 2006; Mino *et al.*, 2010; Sene Lde *et al.*, 2013), além de produzir proles com restrição de crescimento ao nascer e posterior desenvolvimento de obesidade precoce (*catch-up growth*) (Ozanne e Hales, 2004; Ozanne e Nicholas Hales, 2005; Berends *et al.*, 2013).

A nutrição durante a gravidez é, portanto, um fator ambiental capaz de ativar interações fisiológicas entre mãe e concepto, mediadas por de diversos mecanismos, incluindo a sinalização hormonal, podendo causar alterações epigenéticas em genes reguladores dos tecidos-alvos desses hormônios. Tais interações podem modificar a eficiência placentária, o crescimento e o caráter metabólico fetal, estabelecendo as bases para diversas doenças quando a disponibilidade de nutrientes gestacional e pós-natal são discordantes (Fleming *et al.*, 2015).

Restrição Proteica Materna e Aspectos Reprodutivos Masculinos

Embora alguns efeitos da restrição proteica sejam consequências diretas das alterações na disponibilidade de substrato, vários outros são mediados por efeitos hormonais. Estes podem alterar o desenvolvimento de tecidos fetais específicos durante os períodos sensíveis do desenvolvimento, ou levar a mudanças duradouras na secreção hormonal ou na sensibilidade do tecido ao hormônio (Godfrey e Barker, 2000; Console *et al.*, 2001; Peixoto-Silva *et al.*, 2011; Rinaldi *et al.*, 2018).

Ratos submetidos a condições adversas no balanço energético, como na restrição proteica materna durante os períodos de gestação e lactação, apresentam aumento ou diminuição da secreção dos hormônios luteinizante (LH), folículo estimulante (FSH), testosterona, estradiol e aldosterona, o que pode ter impacto significativo sobre órgãos e funções do sistema genital (Zambrano *et al.*, 2005; Guzman *et al.*, 2006; Gao *et al.*, 2012; Otani *et al.*, 2012; Sathishkumar *et al.*, 2012).

Estudos epidemiológicos e experimentais, humanos e animais, relacionados aos efeitos da restrição proteica materna sobre aspectos reprodutivos, sobretudo os masculinos, são relativamente escassos em comparação àqueles que abordam aspectos da síndrome metabólica. No entanto, dados da literatura indicam que este modelo experimental provocou alterações testiculares, prostáticas, espermáticas e de instalação da puberdade em animais adultos, cujas mães sofreram restrição de proteína (Zambrano *et al.*, 2005; Toledo *et al.*, 2011; Rodriguez-Gonzalez *et al.*, 2012; Rodriguez-Gonzalez *et al.*, 2014; Colombelli *et al.*, 2017; Santos *et al.*, 2018).

Com relação às alterações espermáticas, foi observado que a restrição proteica materna provocou alterações associadas, principalmente, às funções do epidídimo, como a motilidade, viabilidade e concentração espermáticas, além de aumentar o número de espermatozoides com alterações morfológicas (principalmente na cauda e peça intermediária) e presença de gota citoplasmática (Toledo *et al.*, 2011; Rodriguez-Gonzalez *et al.*, 2014). Dessa maneira, apesar dos trabalhos mostrarem efeitos da restrição proteica associados com as funções epididimárias, as causas dessas alterações ainda não foram esclarecidas. Ou seja, não há informações na literatura que esclareçam como a restrição proteica, em fase inicial do desenvolvimento, afeta a função deste órgão.

O Epidídimo

O epidídimo é um órgão do sistema genital masculino formado por um único ducto altamente enovelado, cuja função primária é transportar os espermatozoides que chegam do testículo, via ductos eferentes, até o ducto deferente. Durante este trajeto o epidídimo desempenha sua função principal, sendo responsável pela estocagem, imunoproteção, concentração e maturação destes gametas (Cosentino e Cockett, 1986; Hermo, L.; e Robaire, B. , 2002; Gatti *et al.*, 2004; Junqueira, 2011).

O ducto epididimário apresenta muitas regiões citologicamente distintas, sendo constituído por epitélio colunar pseudo-estratificado composto por seis tipos celulares: células estreitas, apicais, claras, basais, halo e principais, que desempenham diferentes funções necessárias para o bom funcionamento deste órgão (Figura 1) (Hermo, L.; e Robaire, B. , 2002; Robaire B.; Hinton, 2015).

De maneira geral, o epidídimo do rato é dividido anatomicamente em quatro regiões distintas: segmento inicial, cabeça, corpo e cauda, com cada segmento sendo responsável por

funções características como: secreção, endocitose, absorção e acidificação, que levam à constituição de meio intraluminal específico e adequado para o amadurecimento espermático (Figura 1) (Gatti *et al.*, 2004; Franca *et al.*, 2005; Robaire *et al.*, 2006; Robaire B.; Hinton, 2015).

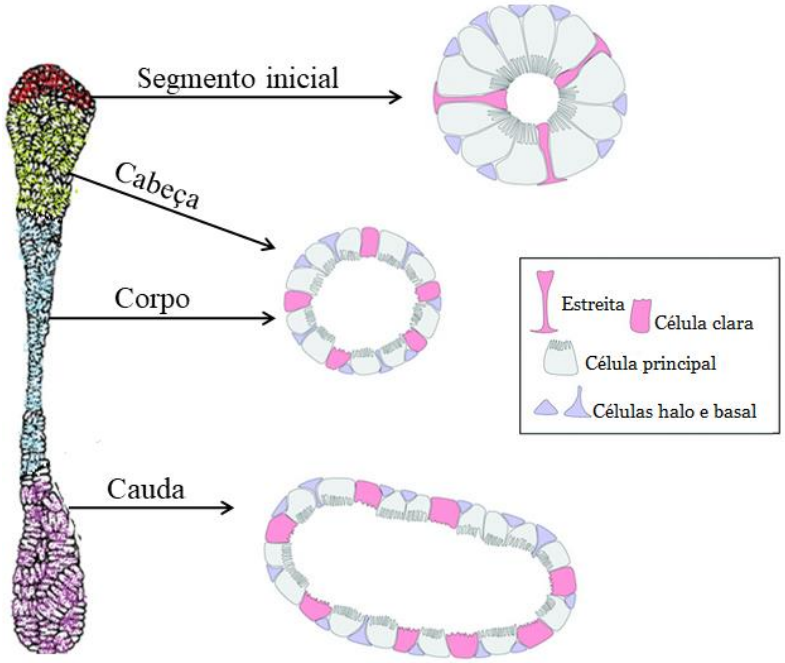

Figura 1 – Representação esquemática do epidídimo de rato evidenciando as quatro regiões em que o órgão é dividido: segmento inicial, cabeça, corpo e cauda. E representação das células que compõem o epitélio epididimário em cada uma dessas regiões. Fonte: adaptado de Shum *et al.* (2009).

No início de seu desenvolvimento o epidídimo consiste em um órgão epitelial tubular simples. A determinação de como os processos de alongamento, expansão e dobramento levarão à constituição do epidídimo com um complexo órgão enovelado é um tema que vem atraindo cada vez mais o interesse de pesquisadores (Joseph *et al.*, 2009).

A parte proximal do ducto de Wolffian dá origem ao epidídimo. Em humanos, somente a partir da oitava semana do desenvolvimento fetal, por influência da testosterona, este ducto adquire aspecto enovelado (Moore *et al.*, 2016). Nesse período há considerável remodelação extracelular, que ocorre de maneira assimétrica e pode ser observada apenas no local onde o *looping* ou enrolamento acontece. Assim, a interação ente epitélio e mesênquima é necessária para que ocorra o enovelamento do órgão (Hinton *et al.*, 2011).

O desenvolvimento pós-natal epididimário nos mamíferos em geral é dividido em três períodos: período indiferenciado, período de diferenciação e período de expansão (Hinton *et al.*, 2011).

O período indiferenciado corresponde ao intervalo do DPN 1 até o DPN 15, nele inicia-se a segmentação anatômica do epidídimo e o epitélio epididimário é composto por células colunares (Hermo, L. e Robaire, B., 2002; Rodríguez, 2002).

O período de diferenciação abrange os dias entre o DPN 16 e o DPN 44 e seu início é marcado pelo aparecimento das células halo. Aos 21 dias de idade, ocorre pico da atividade proliferativa das células do segmento inicial além da completa formação da barreira hemato-epididimária (Rodríguez, 2002).

Após o DPN 44 o epidídimo inicia o período de expansão, marcado pelo crescimento contínuo do órgão. No DPN 49 os primeiros espermatozoides começam a aparecer no lúmen epididimário, atingindo sua concentração máxima no órgão por volta do DPN 90 (Hermo, L. e Robaire, B., 2002; Rodríguez, 2002).

Aquaporinas e o Epidídimo

Uma das mudanças mais importantes no fluido luminal é induzida pela reabsorção de água, que ocorre principalmente nos ductos eferentes e região proximal do epidídimo, continuando em proporções menores, mas com importância funcional significativa, ao longo deste órgão. A absorção da água promove o aumento da concentração espermática e proteica do ambiente luminal, fatores estes associados às primeiras modificações dos espermatozoides, tais como a migração da gota citoplasmática, início do batimento do flagelo e ligação à zona pelúcida (Dacheux e Dacheux, 2014).

As aquaporinas (AQP) compreendem uma família composta por 13 proteínas hidrofóbicas integrais de membrana, organizadas como tetrâmeros e com massa molecular baixa que varia de 25 a 37 kDa (Preston e Agre, 1991; King *et al.*, 2004; Roche e Tornroth-Horsefield, 2017), que atuam como canais de água aumentando a permeabilidade da membrana celular. Até o momento, seis membros desta família de proteínas têm sido identificadas no epidídimo de rato: AQP1, AQP2, AQP3, AQP5, AQP9 e AQP11 (Agre, 2004; Da Silva *et al.*, 2006; Domeniconi *et al.*, 2008; Schimming *et al.*, 2015; Yeste *et al.*, 2017).

A AQP2 é encontrada na cauda epididimária de ratos machos jovens, mas não em animais adultos, sugerindo sua participação não somente na dinâmica de reabsorção/secreção

no órgão, mas também no desenvolvimento epididimário pós-natal (Da Silva *et al.*, 2006; Arrighi *et al.*, 2010). AQP3 está presente no epidídimo de maneira célula-específica, uma vez que pode ser observada nas células basais epididimárias, mas não nas células principais, claras e estreitas do órgão (Hermo *et al.*, 2004). A AQP5 pode ser observada na membrana apical das células principais epididimárias nas regiões de corpo e cauda, mas encontra-se ausente na região da cabeça do órgão (Da Silva *et al.*, 2006). Em ratos jovens e adultos, a AQP11 é encontrada nos estereocílios das células principais de maneira irregular nas regiões de segmento inicial e cabeça, ausente na região de corpo e de maneira mais uniforme na região distal da cauda do órgão (Hermo *et al.*, 2008).

Com relação à dinâmica de reabsorção/secreção que ocorre no epidídimo, crescentes evidências sugerem as AQP1 e AQP9 como as principais envolvidas no transporte de solutos que ocorre no órgão (Pastor-Soler *et al.*, 2001; Da Silva *et al.*, 2006). A AQP1 é ausente nas células epiteliais do epidídimo, mas é expressa no músculo liso adjacente e em células endoteliais de canais vasculares do órgão (Huang *et al.*, 2006; Domeniconi *et al.*, 2008; Arrighi *et al.*, 2010; Teixeira *et al.*, 2012). Já a AQP9 está expressa na região apical das células principais do segmento inicial, cabeça, corpo e cauda do epidídimo (Pastor-Soler *et al.*, 2001; Badran e Hermo, 2002; Domeniconi *et al.*, 2008; Hermo *et al.*, 2008) (Figura 2). No epidídimo, a água é transportada a partir do lúmen via AQP9, expressa nos estereocílios das células principais, e então removida do espaço intertubular através da ação das AQP1, expressas nas células endoteliais dos canais vasculares ao longo de todo órgão (Badran e Hermo, 2002).

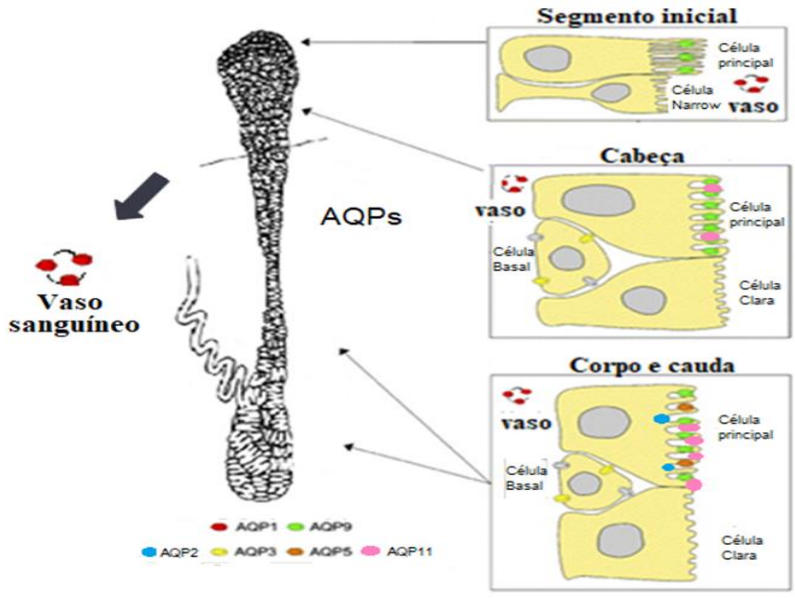

Figura 1 – Representação esquemática da localização das AQPs no epidídimo do rato. Fonte: adaptada de Da Silva *et al.* (2006) e Guo *et al.* (2007).

Foi demonstrado que a expressão de AQP9 é modificada pela subnutrição no início da vida. Filhotes cujas mães sofreram restrição alimentar durante a gestação e lactação, apresentaram diminuição na imunomarcação desta proteína nos estereocílios das células principais e completa ausência de imunomarcação nas células claras da cauda de seus epidídimos, prejudicando suas funções e, possivelmente, comprometendo a fertilidade destes animais (Arrighi *et al.*, 2010).

Dessa maneira, investigar os efeitos, especificamente, da restrição proteica materna sobre o padrão de expressão da AQP1 e AQP9 no epidídimo da prole ao longo de seu desenvolvimento é extremamente relevante e promissor.

Hormônios e o Epidídimo

No sistema genital masculino, testículo e epidídimo são os principais alvos da ação androgênica, com a testosterona e seu principal metabólito, a di-hidrotestosterona (obtida pela ação da enzima 5α-redutase), sendo críticas para a manutenção da espermatogênese e da função secretora das células epiteliais epididimárias (Sharpe *et al.*, 1994; Robaire e Hermo, 1998). O fluido testicular que alcança o lúmen epididimário é rico em testosterona e apesar da alta concentração deste hormônio em todo órgão, as concentrações de di-hidrotestosterona são muito maiores no lúmem da cabeça epididimária, indicando maior sesibilidade desta região à possíveis variações na concentração de andrógenos (Turner, 2002).

O *Androgen Receptor* (AR) é membro da super família de receptores esteroidais com peso molecular de 110 kDa que desempenha papel chave na ação dos hormônios andrógenos e seus metabólitos (Chang *et al.*, 1995). A própria expressão de AR é regulada por estes hormônios, e apesar de vários estudos demonstrarem que a regulação androgênica sobre a expressão de AR ocorre de forma tecido e tipo celular específica, os mecanismos pelos quais esta regulação se dá ainda não estão completamente compreendidos (Bentvelsen *et al.*, 1995; Zhu *et al.*, 2000; Ezer e Robaire, 2002). No entanto, sabe-se que a expressão deste receptor ocorre ao longo de todo sistema genital masculino, durante seu desenvolvimento até a idade adulta (Williams *et al.*, 2001).

Zhu *et al.* (2000) através do tratamento com Azalina (antagonista do hormônio liberador do hormônio luteinizante) observaram uma queda da testosterona sérica acompanhada da menor expressão de AR na cabeça, corpo e cauda do epidídimo de ratos. Da mesma maneira, Liu e Wang (2005) observaram que em ratos diabéticos a concentração de testosterona e a expressão

de AR epididimária apresentaram-se diminuídas em relação à ratos saudáveis, demonstrando que a diminuição da testosterona sérica está positivamente relacionada com a diminuição da expressão de AR neste órgão.

Apesar da testosterona ser considerada o principal hormônio regulador das funções epididimárias, o estrogênio também é produzido no macho, sendo encontrado em grandes concentrações no sêmen e desempenhando diversas funções, que incluem a regulação e manutenção das funções epididimárias, principlamente relacionadas à atividade de reabsorção que ocorre neste órgão (Hess *et al.*, 2002).

Em machos imaturos, a principal fonte de estrogênio são as células de Sertoli enquanto que nos adultos, as células germinativas apresentam maior expressão da enzima aromatase p450, responsável pela conversão da testosterona em estradiol, sendo a maior fonte deste esteroide no sistema genital masculino (Nitta *et al.*, 1993; Hess *et al.*, 1995; Janulis *et al.*, 1998).

O estrogênio atua através de dois tipos de receptores nucleares, o *Estrogen Receptor* α *(*ERα) e *Estrogen Receptor* β (ERβ) de aproximadamente 66 e 59 kDa respectivamente, ambos presentes no epidídimo (Kuiper *et al.*, 1996; Pelletier *et al.*, 2000; Zaya *et al.*, 2012).

Dados da literatura revelam que a localização de ERα no epidídimo de ratos ainda é bastante controvérsia. Alguns estudos demonstram que apesar de estar presente em todo epidídimo, a região da cabeça deste órgão expressa quantidades mais significativas deste receptor, indicando maior sensibilidade desta região aos hormônios estrogênicos, principalmente em relação à reabsorção mais intensa que ocorre na cabeça epididimária (Zhou *et al.*, 2001; Hess *et al.*, 2002; Kolasa *et al.*, 2003; Schon *et al.*, 2009).

Diferente de ERα, que é mais encontrado na região de cabeça do epidídimo, a expressão de ERβ aparece distribuída de forma mais homogênea neste órgão (Hess *et al.*, 1997; Hess *et al.*, 2002; Yamashita, 2004; Schon *et al.*, 2009). Sua função no sistema genital masculino permanece alvo de investigações, já que camundongos ERβ *knockout* são férteis e apresentam testículos e epidídimos normais (Krege *et al.*, 1998; Couse e Korach, 1999).

Por muitos anos, a aldosterona, sintetizada pelas glândulas adrenais, foi considerada um "hormônio renal", uma vez que desempenha sua principal função no córtex deste órgão promovendo a retenção do íon sódio (Na^+) e a eliminação do íon potássio (K^+), atuando na manutenção do equilíbrio hídrico, eletrolítico e da pressão sanguínea (Nguyen Dinh Cat e Jaisser, 2012; Harvey e Thomas, 2017). No entanto, atualmente se sabe que este mineralocorticoide também desempenha papel na indução do estresse oxidativo, disfunção endotelial, inflamação e fibrose no coração e vasos sanguíneos além de infuenciar a função adipócita e a resistência à insulina (Nguyen Dinh Cat e Jaisser, 2012).

Em relação ao papel da aldosterona no sistema genital masculino, mais especificamente no epidídimo, diversos trabalhos demonstram a influência deste hormônio na absorção de líquidos do lúmen epididimário através da captação de Na^+ contra o gradiente eletroquímico, principalmente na cabeça, mas também ao longo de todo órgão, contribuindo assim para uma das principais funções epididimárias: a concentração dos espermatozoides (Wong e Lee, 1982; Turner e Cesarini, 1983; Pearce *et al.*, 1986). Além disso, recentemente foi verificado que a aldosterona circulante também tem participação ativa na regulação da secreção de prótons pelas células claras da cabeça do epidídimo. Identificando o sistema renina-angiotensina-aldosterona (RAAS) como um importante modulador da acidificação luminal na região proximal do órgão (Roy *et al.*, 2013).

Em roedores, a restrição proteica materna é o modelo mais utilizado para investigar os efeitos da programação fetal no desenvolvimento de doenças crônicas na vida adulta, em particular a hipertensão (Griffin *et al.*, 2001), e um crescente corpo de evidências indica que a aldosterona tem grande contribuição no estabelecimento da pressão elevada na prole (Rocha *et al.*, 1998; Griffin *et al.*, 2001; De Lima *et al.*, 2013). Nesse sentido, trabalhos têm observado e correlacionado a hipertensão e o baixo peso ao nascer com o aumento da aldosterona circulante em modelos de restrição proteica materna (Reynolds *et al.*, 2009; Otani *et al.*, 2012).

Uma vez que a aldosterona está envolvida na absorção de líquidos do lúmen do epidídimo e que a restrição proteica se mostrou eficiente em aumentar os níveis circulantes deste mineralocorticoide, é interessante observar a relação entre a restrição proteica materna com a concentração de aldosterona e seus possíveis efeitos no meio intraluminal epididimário em diferentes estágios do desenvolvimento deste órgão.

Fator de Crescimento Endotelial Vascular e o Epidídimo

Nas glândulas adrenais, o fator de crescimento endotelial vascular (VEGF) sérico é capaz de aumentar a disponibilidade de aldosterona na circulação diretamente, através de sua ação sobre as células epiteliais da zona glomerular, ou indiretamente, através da camada endotelial, ambos de maneira renina-independente (Gennari-Moser *et al.*, 2013). Além disso, dados da literatura comprovam a modulação da aldosterona sobre o VEGF, sendo capaz de aumentar ou diminuir sua expressão ou concentração sérica dependendo do tipo celular ou tecido analisado (Ladage *et al.*, 2011; Walczak *et al.*, 2011; Hsu *et al.*, 2015). No entanto, não

existem estudos referentes a influência deste mineralocorticoide sobre a expressão de VEGF no epidídimo e suas consequências para o padrão vascular deste órgão.

No processo de maturação espermática, a regulação do transporte de metabólitos, hormônios e nutrientes, assim como a secreção e reabsorção de fluido luminal no ducto epididimário são muito importantes para manter a especificidade do microambiente deste órgão, requerendo uma vasculatura altamente ramificada para proporcionar suprimento sanguíneo adequado (Setchell *et al.*, 1993; Setchell, 2006). O tratamento do tecido epididimário com VEGF provocou o aumento de vesículas transcitóticas, canais transendoteliais, fenestração dos capilares e indução de lacunas transendoteliais. Tais alterações morfológicas têm sido associadas com o aumento da permeabilidade capilar, sugerindo o VEGF como um importante regulador da permeabilidade vascular no epidídimo, já que este órgão não é local de angiogênese ativa durante a vida adulta (Ergun *et al.*, 1998).

A superexpressão de VEGF, em camundongos transgênicos, resultou em epidídimos alargados e inchados especialmente nas regiões de cabeça e corpo. Além disso, todos os animais transgênicos exibiram hiperproliferação epitelial, dividindo o ducto em vários sublúmens estreitos na região da cauda proximal do epidídimo, sendo que em alguns animais observou-se hiperplasia também na região da cabeça deste órgão (Korpelainen *et al.*, 1998).

A superexpressão do VEGF no epidídimo foi, portanto, associada com o aumento da permeabilidade vascular neste órgão. O aumento da permeabilidade epididimária, especialmente na região da cabeça, pode ter consequências importantes, já que uma porção significativa do fluido luminal do testículo é normalmente reabsorvida pelo epitélio desta região, que se apresenta rodeada por uma densa rede capilar (Setchell, 2006). O inchaço observado no ducto epididimário pode ter sido resultado da alteração na reabsorção do fluido, ou ainda uma consequência da oclusão do ducto pela hiperproliferação do epitélio da região caudal do órgão (Korpelainen *et al.*, 1998).

Tendo tudo isso em mente, levantamos a hipótese de que no modelo de restrição proteica materna, se for comprovado o aumento da aldosterona circulante, e este for acompanhado do aumento do VEGF, estes poderiam ser fatores determinantes para o estabelecimento do padrão vascular e para as alterações funcionais epididimárias, que prejudicam o amadurecimento e a concentração espermática da prole. Além disso, se a restrição proteica provocar mudanças no padrão de expressão das AQP1, em células endoteliais de canais vasculares, e AQP9, as condições do ambiente intraluminal epididimário podem ser alteradas, com impacto sobre a fertilidade desses animais.

Src, Claudinas e o Epidídimo

Foi mencionado anteriormente, que alguns efeitos da restrição proteica são consequências diretas das alterações na disponibilidade de substrato (Godfrey e Barker, 2000). E que durante a gravidez, é recomendado o aumento da ingestão de proteínas devido à necessidade de suprir a demanda adicional de nitrogênio exigida pelo aumento do *turnover* proteico para satisfazer as exigências de crescimento rápido do embrião (Jolly *et al.*, 2004). Nesse sentido, seria interessante também avaliar os efeitos da restrição proteica materna sobre o desenvolvimento do epidídimo através da análise da expressão das proteínas Src e da integridade da barreira hemato-epididimária.

As Src são proteínas quinases não receptoras que atuam em múltiplos ambientes celulares, desempenhando papel chave na regulação da transdução de sinal por um conjunto diverso de receptores de superfície celular (Parsons e Parsons, 2004). Com relação ao epidídimo, as Src se destacam como reguladoras do desenvolvimento epididimário, principalmente da região de segmento inicial. Além disso, essas proteínas desempenham papel importante relacionado às alterações sofridas pelos espermatozoides durante seu processo de maturação no epidídimo (Krapf *et al.*, 2012; Xu *et al.*, 2016).

A atividade da Src é regulada pela fosforilação da tirosina em dois sítios diferentes com efeitos opostos. A fosforilação do Y416 no loop de ativação do domínio quinase promove a ativação da enzima, enquanto a fosforilação de Y527 na cauda carboxi-terminal torna a enzima menos ativa (Xu *et al.*, 1997).

Por fim, a formação e a integridade da barreira hemato-epididimária são essenciais para a manutenção da especificidade do ambiente intraluminal epididimário (Hoffer e Hinton, 1984; Dufresne e Cyr, 2007). Neste contexto, as claudinas (Cldn), que constituem uma família de proteínas transmembranas, são componentes essenciais das junções de oclusão que integram esta barreira (Gregory e Cyr, 2006; Cyr *et al.*, 2007).

Diversas Cldns compõem as junções de oclusão, no entanto, estudos demonstram a presença da Cldn-1 em todas as regiões do epidídimo de rato, não somente compondo a barreira hemato-epididimária como também ao longo de todas as interfaces das células epiteliais adjacentes e em toda extensão da membrana plasmática basal, sugerindo o papel de molécula de adesão para Clnd-1 (Gregory *et al.*, 2001; Dufresne e Cyr, 2007; Kim e Breton, 2016). Além disso, camundongos *knockout* para Cldn-1 morrem de desidratação logo após o nascimento devido a falta da barreira epidérmica, demonstrando assim a imprescindibilidade da Cldn-1 para

a sobrevivência, não podendo ser substituída por nenhuma das outras proteínas de junção de oclusão (Furuse *et al.*, 2002).

Assim, levando ainda em consideração os efeitos diretos da restrição proteica materna, com relação às alterações na disponibilidade de substrato, e as possíveis alterações na expressão de VEGF, AQP1, AQP9 e aumento da permeabilidade vascular do epidídimo, investigamos os efeitos deste modelo experimental sobre a integridade da barreira hemato-epididimária, utilizando como parâmetro de análise o padrão de expressão das Cldn-1 no epidídimo.

Justificativa

A ingestão insuficiente de proteínas entre grande parte da população humana, por motivos culturais ou econômicos, é uma preocupação global. Fazendo do modelo de restrição proteica um dos modelos de restrição precoce do crescimento mais caracterizados e estudados até hoje. É durante a passagem pelo epidídimo que os espermatozoides adquirem motilidade e capacidade fértil. Para isso, é essencial que o desenvolvimento deste órgão aconteça de maneira adequada, permitindo seu pleno funcionamento e desempenho de funções características como: secreção, endocitose, absorção e acidificação, que levam à constituição de um meio intraluminal específico e adequado para o amadurecimento espermático. Em animais adultos, cujas mães sofreram restrição de proteína, já foram observadas a diminuição da viabilidade, motilidade e da concentração espermática, assim como alterações na morfologia destes gametas, parâmetros intimamente relacionados à funcionalidade do epidídimo. Dessa maneira, apesar dos trabalhos mostrarem efeitos da restrição proteica associada a funções epididimárias, as causas dessas alterações ainda não foram esclarecidas. Ou seja, não há informações na literatura que esclareçam como a restrição proteica, em fase inicial do desenvolvimento, afeta a função deste órgão bem como seu desenvolvimento e padrão vascular, relacionando possíveis alterações na expressão de proteínas associadas à manutenção do ambiente epididimário especializado e específico, justificando-se o desenvolvimento deste trabalho.

Objetivos

Objetivos Gerais

Avaliar os efeitos da restrição proteica materna, durante o período de gestação e lactação, sobre o desenvolvimento, padrão vascular e expressão de proteínas relevantes para o pleno funcionamento e desempenho de funções características do epidídimo de ratos machos em diferentes estágios do desenvolvimento pós-natal.

Objetivos Específicos

- Avaliar os efeitos da restrição proteica materna durante gestação e lactação sobre parâmetros biométricos (distância ano genital, distância crânio caudal, peso corpóreo, peso dos órgãos do sistema genital masculino) da prole masculina ao nascimento e nas idades de 21, 44 e 120 dias pós-natais.
- Avaliar os efeitos da restrição proteica materna sobre a imunolocalização e o padrão de expressão da AQP1 e AQP9 no epidídimo da prole nas idades de 21, 44 e 120 dias pós-natais.
- Avaliar a relação entre a restrição proteica materna com a concentração sérica de aldosterona, a expressão do VEGF e seus possíveis efeitos no meio intraluminal e no padrão vascular epididimário nas idades de 21, 44 e 120 dias pós-natais.
- Avaliar os efeitos da restrição proteica materna sobre o padrão de expressão da Src e sobre a integridade da barreira hemato-epididimária utilizando como parâmetro de análise o padrão de expressão de Cldn-1 nas idades de 21, 44 e 120 dias pós-natais.
- Observar os efeitos da restrição proteica materna, durante o período de gestação e lactação, sobre a imunolocalização e expressão de AR, ERα, ERβ e sobre a expressão das enzimas 5α-redutase e aromatase p450 nas idades de 21, 44 e 120 dias pós-natais.

Materiais e Métodos

Animais

Inicialmente foram utilizados 20 ratos machos e 38 ratas fêmeas da linhagem *Wistar* com 45 dias de idade provenientes do Biotério Central da Universidade Estadual Paulista – UNESP – Campus de Botucatu/SP.

Os animais foram mantidos no Biotério de Pequenos Mamíferos do Departamento de Anatomia do Instituto de Biociências da UNESP – Campus Botucatu, em gaiolas de polietileno (43x30x15cm) com substrato de maravalha autoclavada, em condições controladas de luminosidade (12 horas de luz/ 12 horas de escuro) e temperatura (média de 22°C). Os animais receberam água e ração para roedores *ad libitum*. Os procedimentos experimentais foram realizados de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foram aprovados junto à Comissão de Ética em Experimentação Animal (CEEA) do Instituto de Biociências de Botucatu sob o número 797-CEUA (anexo 1).

Rações utilizadas

As rações utilizadas foram preparadas e adquirida pela Pragsoluções Biociências (Jaú - SP). Ambas as rações seguiram os valores nutricionais da dieta padrão para gestantes AIN-93G, no entanto, na ração hipoproteica a concentração de proteína caseína foi reduzida de 17 para 6% enquanto a concentração de amido de milho, dextrina e sacarose foram aumentados para manutenção da isocaloria entre as rações (Tabela 1).

Tabela 1 – Composição da ração fornecida aos animais no período gestacional e de lactação.

Componentes*	Normoproteica (17% de proteína)	Hipoproteica (6% de proteína)
Caseína (84% de proteína)**	202,00	71,50
Amido de Milho	397,00	480,00
Dextrina	130,50	159,00
Sacarose	100,00	121,00
Óleo de Soja	70,00	70,00
Fibra (microcelulose)	50,00	50,00

Mistura de Minerais ***	35,00	35,00
Mistura de Vitaminas ***	10,00	10,00
L – cistina	3,00	1,00
Cloreto de Colina	2,50	2,50

*Dieta para a fase de gestação em roedores - AIN-93G
** Valores corrigidos de acordo com o conteúdo de proteína na caseína
*** Segundo AIN-93G

Delineamento Experimental

Quando os animais atingiram a idade de 95 dias, começaram os procedimentos para obtenção de fêmeas prenhes, para tanto duas fêmeas e um macho foram alojados em caixas maternidade durante a noite para pareamento. Nas manhãs seguintes foram realizados esfregaços vaginais, utilizando soro fisiológico e *swab* estéril, seguidos de análise das lâminas em microscópio de luz, onde a presença de espermatozoides indicou prenhez do animal, marcando assim o dia 0 da gestação (DG 0). As fêmeas não prenhes foram novamente alocadas com machos até que a prenhez fosse confirmada. Após a detecção da prenhez as fêmeas foram aleatoriamente transferidas para caixas individuais para formação dos grupos maternos experimentais: grupo normoproteico (NP): 19 mães alimentadas durante a gestação e lactação com dieta normoproteica *ad libitum* (17%); grupo hipoproteico (HP): 19 mães alimentadas durante a gestação e lactação com dieta hipoproteica *ad libitum* (6%).

As dietas normoproteica e hipoproteica foram administradas até o dia do desmame da prole (DPN 21). Após o DPN21, as proles dos grupos experimentais NP e HP receberam dieta padrão para roedores até o momento das coletas nos DPNs 44 e 120 (Figura 1).

As idades de 21, 44 e 120 dias foram baseadas em três fases diferentes do desenvolvimento epididimário pós-natal: no DPN 21 ocorre o pico da diferenciação celular; o DPN 44 corresponde ao período final da diferenciação do epidídimo e início de sua expansão; por fim, no DPN 120 o epidídimo é considerado bem diferenciado e plenamente funcional.

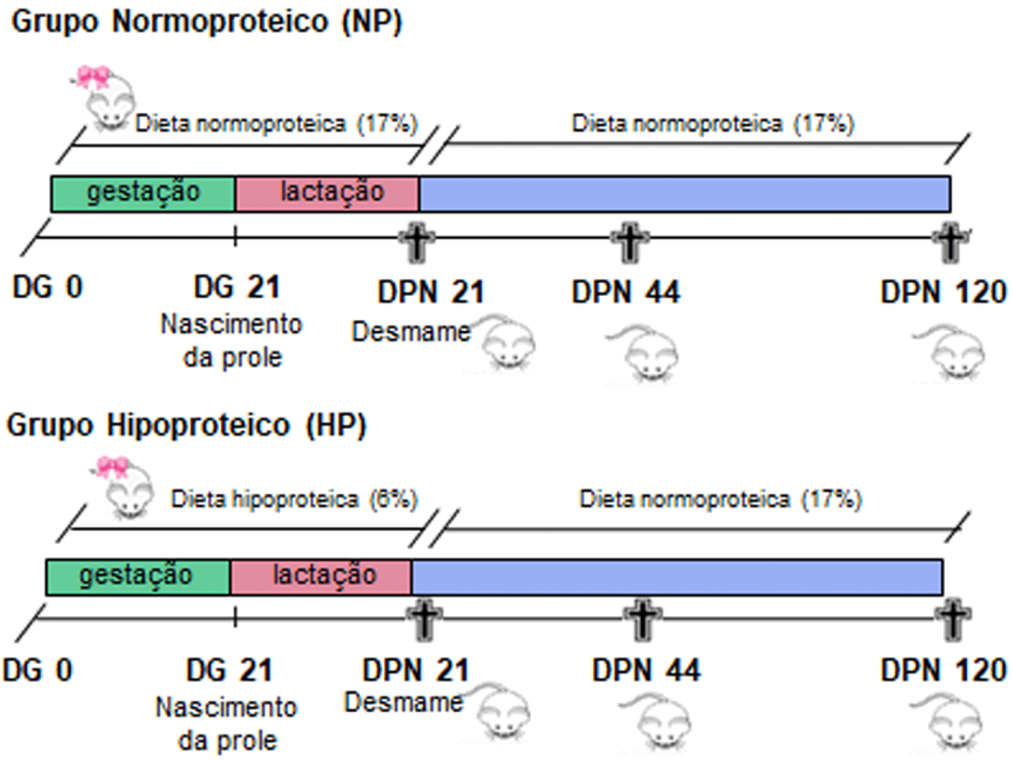

Figura 1. Delineamento Experimental. Fêmeas grávidas foram alimentadas com dieta hipoproteica (grupo HP) ou normoproteica (grupo NP) *ad libitum* durante a gestação e lactação (do GD 0 até DPN 21). Após o desmame, filhotes machos de ambos os grupos receberam dieta padrão para roedores até as idades de 21, 44 e 120 dias.

Parâmetros maternos

Durante toda gestação e até o DPN 14 foi realizado o controle semanal de ração consumida pelas ratas mães, com esses valores sendo divididos por sete para estimar o consumo diário de cada animal. Optou-se por não realizar o consumo diário de ração efetuando a pesagem da mesma todos os dias a fim de evitar o estresse materno, além disso, o consumo de ração não foi monitorado na última semana de lactação (DPN 14 ao 21) porque os filhotes das mães NP já estavam se alimentando também com ração, comprometendo o resultado das pesagens. Os pesos maternos foram monitorados durante toda gestação, com as ratas sendo pesadas sempre nos dias gestacionais (DGs) 0, 7, 14 e 20.

No dia do desmame, as mães foram pesadas, eutanasiadas por decapitaçãoe tiveram os pesos de suas gorduras viscerais, uterinas, retro-peritoniais, rins direito e esquerdo e fígados aferidos. Além disso, os rins esquerdos e fragmento dos fígados maternos foram coletados e estocados a -80°C para futuras análises metabólicas.

Parâmetros relacionados à prole

No DPN 1, os filhotes machos e fêmeas de cada ninhada foram separados, pesados individualmente e tiveram as distâncias ano-genital (DAG) e crânio-caudal (DCC) registradas com o uso de paquímetro digital (Western®). Para cada ninhada NP e HP, foram contabilizados o número de filhotes machos e fêmeas, além do número total de filhotes.

Para assegurar a disponibilidade de alimento igualitária entre todos os componentes da prole, foram mantidos oito filhotes por rata mãe, dando preferência pela prevalência de machos. Os filhotes permaneceram com as mães em caixas individuais até o desmame (DPN 21) ou até o momento da eutanásia, no caso dos animais coletados com 21 dias de idades (NP=17/grupo; HP=23/grupo). Após o desmame, os ratos receberam dieta sólida padrão para roedores e água filtrada *ad libitum* até as idades de 44 (NP=12/grupo; HP=10/grupo) e 120 (NP=13/grupo; HP=12/grupo) dias, quando foram eutanasiados.

Nos dias das coletas, os animais foram pesados e tiveram novamente suas DAG e DCC aferidas. Em seguida foram anestesiados por narcose induzida em câmara de CO_2 e mortos por decapitação, procedendo-se a coleta do sangue a partir do rompimento dos vasos cervicais.

O epidídimo esquerdo, gordura epididimária esquerda, testículo esquerdo, próstata ventral, glândula seminal e fígado de cada animal foram coletados, dissecados e pesados. Posteriormente calculou-se o peso relativo dos órgãos segundo a fórmula: Peso do órgão avaliado/Peso corpóreo final X 100 (dado expresso em g ou mg/100g de peso corporal). Os epidídimos esquerdos depois de pesados foram imediatamente acondicionados em criotubos, submersos em nitrogênio líquido e armazenados a -80°C até o momento da extração de proteínas para realização da técnica de *Western blotting*. Os epidídimos direitos, por sua vez, foram coletados, dissecados e fixados em formol tamponado a 10% (tampão fosfato 0,1M e pH 7,3) por 24 horas, em seguidas foram lavados em água corrente por 24 horas e armazenados em álcool 70%.

Dosagens hormonais

As amostras de sangue foram submetidas à centrifugação (14.000 rpm, 4°C por 20 minutos) para a obtenção de soro. As concentrações séricas de testosterona, estradiol e aldosterona foram dosadas por quimioluminescência no laboratório VITAE – Cromatografia

Líquida em Análises Clínicas (Rua Borges Lagoa, n. 1231 – conjunto 72 – Vila Clementino, São Paulo – SP), responsável técnico: Marcos Cesar Carvalho (CPF: 126.742.528-80).

Para as dosagens de testosterona e estradiol foram utilizados kits específicos fornecidos pela "Beckman Coulter, Inc." (Brea, CA, EUA), enquanto para a dosagem de aldosterona utilizou-se o kit LIAISON® Aldosterone fornecido pela "DiaSorin Inc." (Stillwater, MN, EUA). Os limites inferiores de detecção da testosterona e do estradiol foram de 10 ng/dL e 1,7 ng/dL, respectivamente, e os limites inferior e superior de detecção da aldosterona foram de 0,97 ng/dL e 100 ng/dL, respectivamente.

O número de amostras de soro para cada grupo (NP e HP) foi de no mínimo 7, sendo que para os animais de 21 dias tiveram que ser feitos pools de 2 ou 3 animais para que o volume de amostra fosse suficiente para realização das dosagens. Todas as amostras foram analisadas ao mesmo tempo para evitar a variação inter-ensaio.

Processamento e inclusão dos epidídimos e preparação das lâminas para realização de imuno-histoquímica e análises histológicas

O material estocado em álcool 70% foi submetido a banhos de 1 hora em álcool 80%, 90% e 100%, 2 horas em álcool 100%, e 4 horas em álcool butílico-N seguido de outro banho de álcool butílico-N *overnight*. No dia seguinte, o material foi submetido a um terceiro banho de álcool butílico-N por 6 horas seguindo para os banhos em paraplástico (Paraplast Plus, ST. Louis, MO, USA). No terceiro dia, foi realizado mais um banho de 6 horas em paraplástico seguido de inclusão dos órgãos em blocos.

Depois de devidamente emblocados em paraplástico, os epidídimos dos animais de 21, 44 e 120 dias de idade foram cortados em micrômetro LEICA RM 2165 (Leica Biosystems, Nußloch, Germany) em cortes de 5µm. Os cortes foram acondicionados em cuba de banho-maria (Lupetec BH05, série 1394) para que não enrugassem e em seguida “pescados” com lâminas previamente silanizadas, para que a adesão do material fosse garantida. Foram cortados quatro blocos de cada grupo (HP e NP) em cada idade, sendo que os blocos dos animais de 21 dias foram cortados até o final em cortes seriados e os blocos dos animais de 44 e 120 dias foram cortados até que obtivéssemos de 25 a 30 lâminas de cada em cortes semi-seriados.

A silanização das lâminas foi feita previamente com banhos de cinco minutos em: acetona pura, seguido de banho em solução de acetona com (3-Aminopropyl) triethoxysilane 2% e dois banhos em água destilada.

Análises morfológicas

Para todas as idades (21, 44 e 120 dias), quatro lâminas de cada um dos quatro animais de cada grupo (HP e NP) foram coradas com Hematoxilina e Eosina (H.E.). Primeiramente as lâminas foram desparafinizadas em estufa, em seguida passaram por banhos de 15 minutos em xilol I e xilol II, dois banhos de 5 minutos em álcool 100%, banhos de 5 minutos em álcool 95% e em álcool 70% e por fim permaneceram 10 minutos em água corrente.

Os cortes foram então corados com Hematoxilina de Harris (Dinâmica®- Indaiatuba, São Paulo) por 1 minuto, permaneceram 10 minutos em água de viragem e então foram corados com Eosina (Synth® -Diadema, São Paulo) por 15 segundos. Por fim, as lâminas foram desidratadas em diferentes graduações de álcool, diafanizadas em xilol e posteriormente montadas com Permount.

As lâminas foram escaneadas pelo 3DHistech Pannoramic MIDI, analisadas quanto à integridade de epitélio e interstício e fotografadas utilizando-se o programa Pannoramic Viewer.

Análises morfométricas

Nas análises morfométricas foram aferidos a altura epitelial, diâmetro luminal e diâmetro tubular epididimários. Para tanto, pelo menos 10 secções transversais de túbulos em cada uma das regiões epididimárias (segmento inicial, cabeça, corpo e cauda) de 4 lâminas de 4 animais/grupo nas idades de 21, 44 e 120 dias foram medidas com uso do programa Pannoramic Viewer (Serre e Robaire, 1998).

Imuno-histoquímica

Para a técnica de imuno-histoquímica utilizamos quatro lâminas de epidídimos de animais diferentes para cada grupo de cada uma das idades de 21, 44 e 120 dias.

Primeiramente, as lâminas foram desparafinizadas em estufa a 60ºC por 40 minutos, passaram por banhos de 15 minutos em xilol I e xilol II, dois banhos de 5 minutos em álcool 100%, banhos de 5 minutos em álcool 95% e em álcool 70% e por fim permaneceram 5 minutos em água. Em seguida, foram feitas as recuperações e exposições dos epítopos: para AR e ERα, nas lâminas dos animais de 21 e 44 dias, a recuperação foi feita em banho-maria na panela

elétrica com as lâminas mergulhadas em tampão Tris-EDTA (Trizma base, EDTA e Tween 20%, pH 9,0); para AR e ERα, nas lâminas dos animais de 120 dias, assim como para ERβ, AQP1, e AQP9 em todas as idades, a recuperação foi feita em tampão Citrato (Ácido cítrico e Citrato de sódio, pH 6,0); na pressão para AR e ERα (DPN 120) e ERβ ou em 4 ciclos de 5 minutos no micro-ondas para AQP1 e AQP9.

Após a recuperação, foi feito o bloqueio das reações inespecíficas (bloqueio da peroxidase), e as lâminas foram cobertas com solução de peróxido de hidrogênio (H_2O_2, 0,3% em metanol) ao abrigo da luz durante 15 minutos. Em seguida realizou-se o bloqueio proteico em leite Molico® 3% diluído em PBS (fosfato de sódio monobásico 0,1M, Fosfato de sódio dibásico 0,1M e NaCl, pH 7,4) por uma hora.

As lâminas foram incubadas com os anticorpos primários AR (Milipore – USA) (1:150), ERα (Milipore – USA) (1:100), ERβ (Milipore – USA) (1:100), AQP1 (Milipore– USA) (1:200) e AQP9 (Alpha Diagnostic – EUA) (1:200) todos diluídos em BSA 1% e mantidas *overnight* em repouso (Tabela 2).

Tabela 2 – Características dos anticorpos.

Anticorpos	***Especificidade***	***Concentração***	***Fonte***
Primário			
AR	Receptor de andrógeno	1:1000	Milipore - USA
ERα	Receptor de estrógeno α	1:200	Milipore – USA
ERβ	Receptor de estrógeno β	1:300	Milipore – USA
AQP1	Aquaporina 1	1:800	Milipore - USA
AQP9	Aquaporina 9	1:500	Alpha Diagnostic – USA
Src 416	Fosforilação Y416 domínio quinase	1:500	Cell Signaling – USA
Src 527	Fosforilação Y527 cauda carboxi-terminal	1:1000	Cell Signaling – USA
Cldn-1	Proteína transmembrana de junção de oclusão	1:1000	Thermo Fisher Scientific – USA
B-actina	Proteína endógena	1:800	Santa Cruz – USA
Secundário			
	Anti-Rabbit	1:2000/1:5000/ 1:15000	Sigma – USA

	Anti-Goat	1:6000	Sigma – USA

Características dos anticorpos utilizados para realização da técnica de *western blotting*.

No dia seguinte, foram realizadas 3 lavagens de 5 minutos com PBS, e incubação em anticorpo secundário Anti-Rb conjugado com peroxidase (Sigma – USA) (1:200 para AR, ERα no DPN 21 e 44 e AQP 1 em todas as idades) e Anti-Rb biotinilado (Sigma – USA) (1:200 para AR, ERα no DPN 120 e ERβ e AQP9 em todas as idades) diluídos em BSA 1% por 2 horas (Tabela 2). No caso das lâminas que utilizaram anticorpos biotinilados, após a incubação com anticorpo secundário, as mesmas foram incubadas com complexo ABC (ABC kit, Vectastain, da Vector®- CA, USA) por 45 minutos, e posteriormente submetidas a mais 3 lavagens de 5 minutos com PBS.

Os componentes imunorreativos foram revelados a partir de contato com 3,3'-Diaminobenzidina e as lâminas foram contra-coradas com Hematoxilina de Harris (Dinâmica®-Indaiatuba, São Paulo), desidratadas em banhos de álcool 95%, álcool 100%, álcool/xilol e xilol e posteriormente montadas com Permount.

Por fim, as lâminas foram escaneadas pelo 3D Histech Pannoramic MIDI e analisadas e fotografadas utilizando-se o programa Pannoramic Viewer.

Análise da densidade microvascular (DMV)

O índice de densidade microvascular foi determinado adaptando os procedimentos descritos por Colombelli *et al.* (2017).

Resumidamente, seções do epidídimo de animais HP e NP (n = 4/grupo para todas as idades) imunocoradas para anticorpos monoclonais anti-AQP1 foram avaliadas através de análise estereológica (Weibel *et al.*, 1966) para estimar o número de microvasos no segmento inicial juntamente com a cabeça epididimária e no corpo juntamente com a cauda do órgão. Os dados foram expressos como porcentagem de microvasos por área total analisada.

Extração e quantificação de proteínas e *Western blotting*

Os epidídimos esquerdos de cinco animais de cada grupo (NP e HP) das idades de 21, 44 e 120 dias foram divididos em segmento inicial e cabeça (SI+CB) e corpo e cauda (CP+CD).

Apenas no caso dos animais do grupo HP de 21 dias foram feitos pools, devido ao tamanho reduzido do órgão.

As amostras foram devidamente identificadas e processadas a 4°C em tampão RIPA (BioRad®, BioRad Laboratories, USA; 30mg de tecido/100µl de tampão de extração) e coquetel de inibidores de proteases (Sigma-Aldrich®, USA) em homogeneizador do tipo Tureaux em 3 ciclos de 10 segundos. O homogeneizado foi centrifugado a 14.000 rpm a 4ºC por 20 minutos e o sobrenadante coletado e estocado em -80ºC até o momento da quantificação.

A quantificação de proteínas foi realizada em placas de ELISA com 96 poços, em leitor de ELISA (Epoch Microplate Spectrophotometer, BioTek®-VT, USA) pelo método de Bradford (Bradford, 1976).

As alíquotas foram tratadas com tampão fosfato de sódio 1M pH 7,0 contendo azul de bromofenol 0,1%, glicerol 50%, SDS 10% e ditiotreitol 100mM (Laemmli, 1970) e aquecidas a 95°C por 5 minutos em banho seco. Em seguida, 70 µg/µL das proteínas foram separadas por eletroforese vertical (Mini-Protean, BioRad®) em gel de SDS-poliacrilamida a 4-15% utilizando-se protocolo de 70V por quinze minutos, seguido de 120V por mais uma hora e meia (exceto para Cldn-1, que permaneceu à 120V por apena 25 minutos).

A eletro-transferência do gel para membrana de nitrocelulose foi feita em sistema úmido a 350ma. Ligações inespecíficas de proteínas foram bloqueadas através da exposição das membranas ao Leite Molico® 3% (para AR, ERβ, 5α-redutase, aromatase p450, AQP1, AQP9, Src 146, Src 527, Cldn-1 e β-actina) e 1% (para ERα) em tampão TBS-T (Tris base 1M, Nacl 5M e Tween 20) por 1 hora em temperatura ambiente. Em seguida, as membranas foram incubadas com os anticorpos primários: AR (Milipore – USA) (1:1000), ERα (Milipore – USA) (1:200), ERβ (Milipore – USA) (1:300), 5α-redutase (Biorbyt – USA) (1:500), aromatase p450 (Milipore – USA) (1:500), AQP1 (Milipore – USA) (1:800), AQP9 (Alpha Diagnostic – USA) (1:500), Src 146 (Cell Signaling – USA) (1:500), Src 527 (Cell Signaling – USA) (1:1000), Cldn-1 (Thermo Fisher Scientific – USA) (1:1000), e β-actina (Santa Cruz – USA) (1:800), todos diluídos em TBS-T, e mantidas *overnigth* sob agitação (Tabela 3).

Tabela 2 – Características dos anticorpos.

Anticorpos	***Especificidade***	***Concentração***	***Fonte***
Primário			
AR	Receptor de andrógeno	1:1000	Milipore - USA
ERα	Receptor de estrógeno α	1:200	Milipore – USA
ERβ	Receptor de estrógeno β	1:300	Milipore – USA

5α-redutase	Conversora de testosterona em di-hidrotestosterona	1:500	Milipore - USA
Aromatase p450	Conversora de testosterona em estradiol	1:500	Biorbyt - USA
AQP1	Aquaporina 1	1:800	Milipore - USA
AQP9	Aquaporina 9	1:500	Alpha Diagnostic – USA
Src 416	Fosforilação Y416 domínio quinase	1:500	Cell Signaling – USA
Src 527	Fosforilação Y527 cauda carboxi-terminal	1:1000	Cell Signaling – USA
Cldn-1	Proteína transmembrana de junção de oclusão	1:1000	Thermo Fisher Scientific – USA
B-actina	Proteína endógena	1:800	Santa Cruz – USA
Secundário			
	Anti-Rabbit	1:2000/1:5000	Sigma – USA
	Anti-Goat	1:6000	Sigma – USA

Características dos anticorpos utilizados para realização da técnica de *western blotting*.

Na manhã seguinte, foram realizadas 3 lavagens de 15 minutos com TBS-T, incubação em anticorpo secundário Anti-Rb (Sigma – USA) (1:2000 para AR, ERα, ERβ, 5α-redutase e aromatase p450), (1:5000 AQP1, AQP9, Src 416 e Src 527) e Anti-Goat (Sigma – USA) (1:6000 para β-actina) diluídos em TBS-T por 2 horas, e posteriormente mais 3 lavagens de 15 minutos com TBS-T.

Os componentes imunorreativos foram revelados a partir de substratos quimioluminescentes (ECL), e as bandas obtidas foram submetidas à análise semi-quantitativa por densitometria óptica (Image J for Windows ®). Os valores obtidos para cada banda de AR, ERα, ERβ, 5α-redutase, aromatase p450, AQP1, AQP9, Src 416, Src 527 e Cldn-1 e foram normalizados pelos valores das bandas de β-actina, proteína endógena que permanece inalterada mesmo em condições adversas.

Análises estatísticas

Inicialmente foram retirados os *outliers* com o uso do software GraphPad Prism® (versão 5.00, Graph Pad, Inc., San Diego, CA, EUA), e posteriormente realizou-se teste de normalidade Shapiro Wilk (Sdittami.Altervista. Web version). As comparações entre os grupos HP e NP em todas as idades analisadas foram realizadas utilizando o Teste t de Student para dados paramétricos e o teste de Mann-Whitney para dados não paramétricos. Os dados são apresentados como médias ± E.P.M. e as diferenças foram consideradas estatisticamente significantes quando $p < 0,05$. O software GraphPad Prism® (versão 5.00, Graph Pad, Inc., San Diego, CA, EUA) foi usado para realizar as análises estatísticas.

Capítulo I

A primeira parte deste estudo deu origem ao artigo "***Maternal Protein Restriction Differentially Alters the Expression of AQP1, AQP9 and VEGFr-2 in the Epididymis of Rat Offspring***", recentemente publicado no volume especial "*Aquaporins: Water Channels Essential for Living Organisms 2.0*" do periódico internacional "*International Journal of Molecular Sciences*", v. 20 (n. 3), 2019. Fator de impacto 4.183.

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Article

Maternal Protein Restriction Differentially Alters the Expression of AQP1, AQP9 and VEGFr-2 in the Epididymis of Rat Offspring

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Abstract: Background: Maternal protein restriction causes sperm alterations in the offspring, most of which are associated with epididymal functions. Because fluid reabsorption/secretion dynamics in the epididymal environment play important roles in the process of sperm maturation and concentration, we investigated the effects of maternal protein restriction on the expression of aquaporins (AQP1 and AQP9), vascular endothelial growth factor (VEGFa), and its receptor VEGFr-2 in different stages of postnatal epididymal development. Methods: Pregnant rats were divided into groups that received normoprotein (17% protein) and low-protein diets (6% protein) during gestation and lactation. After weaning, male rats only received the standard diet and were euthanized at the predetermined ages of 21, 44 and 120 days. Results: Maternal protein restriction decreased AQP1 and AQP9 expression in the initial segment and caput epididymis compared to the increased expression of these proteins observed in the corpus and cauda at all ages. Although protein restriction reduced the microvasculature density (MVD) on postnatal day (PND) 21 and 44, the MVD was unaltered on PND 120. Conclusions: Maternal protein restriction changed the structure or function of the offspring's epididymis, specifically by affecting fluid dynamics and vasculogenesis in important stages of epididymis development.

Keywords: epididymis; protein restriction; AQP1; AQP9; VEGFr-2; testosterone; aldosterone

1. Introduction

The developing fetus depends on the mother and the maternal environment to supply their nutritional needs. The components and quality of the maternal diet during critical developmental periods have been reported to influence the offspring genome in the uterus [1,2]. The epigenetic changes occurring in early life can permanently alter the phenotype of the adult organism, making it susceptible to a range of diseases that characterize metabolic syndromes, such as type 2 diabetes, hypertension, and coronary heart disease [3–8].

During pregnancy, adequate protein intake is recommended to ensure that the additional nitrogen demands of both mother and fetus are met since an increase in protein turnover to meet the requirements for rapid embryo growth occurs at this stage [9]. Based on these findings, the protein restriction model is one of the most well-characterized models of early growth restriction [6,10,11].

Relatively few epidemiological and experimental studies have examined the effects of a maternal low-protein diet on the male reproductive function compared to studies of the metabolic disturbances. However, this experimental model causes sperm alterations in male offspring, most of which are associated with epididymal functions, such as sperm motility, viability, and concentration [12–15].

The epididymis is a convoluted duct whose primary function is to transport the spermatozoa from the testis to the vas deferens. The main function of this organ occurs during sperm transit through the highly specialized lumen of epididymis; it is responsible for the concentration, maturation, storage, and protection of spermatozoa [16–19]. Important changes in the epididymal luminal fluid are induced by water reabsorption, which increases sperm and protein concentrations in the intraluminal environment and is possibly associated with the first modifications of the spermatozoa [20].

Aquaporins (AQP) are transmembrane proteins involved in the fluid reabsorption/secretion dynamics in the epididymal intraluminal environment, playing a pivotal role in the process of sperm maturation and concentration [21]. Six members of this family of proteins have been identified in the epididymis, including AQP9 and AQP1 [22,23]. AQP9 is continuously expressed in the apical region of the principal cells of the initial segment, caput, corpus, and cauda epididymis, while AQP1 is absent in epithelial cells of the epididymis and is expressed at high levels in the adjacent smooth muscle and endothelial cells of the organ's vascular channels [23–26].

In addition to AQPs, aldosterone also influences the absorption of liquids from the epididymal lumen by promoting Na^+ uptake against the electrochemical gradient throughout the epididymis [27–29]. The mechanisms regulating metabolite, hormone, and nutrient transport, as well as the secretion and reabsorption of luminal fluid in the epididymal duct, are crucial to maintaining the specificity of the epididymal microenvironment and require a highly branched vasculature to provide adequate blood supply [30,31]. Vascular endothelial growth factor (VEGF) and its receptor isoform (VEGFr-2) affect the microenvironment of the epididymis and sperm maturation [32]. In addition, treatment of epididymal tissue with VEGF causes morphological changes associated with increased capillary permeability, suggesting that this factor is an important regulator of vascular permeability in the epididymis [33]. The mechanisms by which protein restriction at an early stage of development affects the epididymal function remain to be identified. Therefore, this study investigated the effects of maternal protein restriction on serum testosterone and aldosterone levels in rat offspring, as well as the expression of AQP1, AQP9, vascular endothelial growth factor (VEGF)a, and VEGFr-2 at different stages of postnatal epididymal development.

2. Results

2.1. A Maternal Low-Protein Diet Promotes Changes in Testosterone and Aldosterone Levels in an Age-Dependent-Manner

Maternal protein restriction during gestation and lactation caused a slight increase in testosterone levels on PND 21 (111%; $p > 0.05$; Figure 1A(I)). Meanwhile, at PND 44, the animals exhibited a significant decrease in circulating testosterone levels (a 0.43-fold reduction compared with the normoprotein (NP) group; Figure 1A(II)) and the PND120 animals exhibited a nonsignificant decrease in the levels of this steroid hormone (0.81-fold reduction compared with the NP group) (Figure 1 A(III)).

A maternal low-protein diet significantly increased serum aldosterone levels in 21-day-old animals (a 3.55-fold increase compared with the NP group; Figure 1B(I)). Otherwise, PND 44 and 120 animals showed a gradual decrease in the levels of this hormone compared with low-protein (LP) animals at PND 21 (LP PND 44: 0.16-fold decrease compared with LP PND 21; LP PND 120: 0.09-fold decrease compared with LP PND 21). After comparing the LP and NP groups at the same ages, we observed that maternal protein restriction increased serum aldosterone levels at PND 44 (1.38-fold increase compared with the NP group; $p > 0.05$) and decreased aldosterone levels at PND 120 (0.47-fold decrease compared with the NP group; $p > 0.05$), but the difference was not significant (Figure 1B(II,III)).

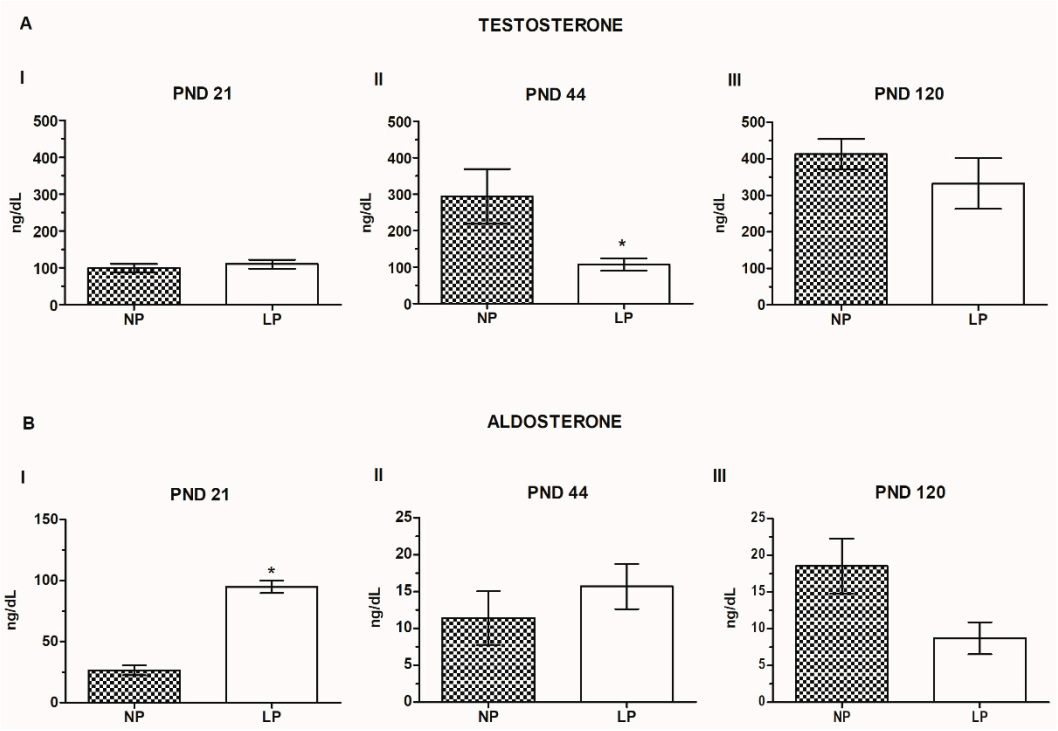

Figure 1. Serum hormones levels. (**A**) Plasma testosterone levels (ng/dL) at postnatal day (PND) 21 (**I**), PND 44 (**II**) and PND 120 (**III**), * $p < 0.05$ compared with the normoprotein (NP) group. (**B**) Plasma aldosterone levels (ng/dL) at PND 21 (**I**), PND 44 (**II**) and PND 120 (**III**), * $p < 0.05$ compared with the NP group. T-tests and the Mann-Whitney tests were used to assess the significance of the differences in parametric and nonparametric data, respectively.

2.2. VEGFr-2 but Not VEGFa Expression is Altered by Maternal Protein Restriction

VEGFa is a growth factor that is secreted as a dimer and plays a variety of functions in angiogenesis and vascular permeability mainly through VEGFr-2. Although VEGFa binds to VEGRr-1 with high affinity, this binding induces only limited downstream signaling. Thus, VEGRr-1 limits the availability of VEGFa to VEGFr-2, restricting the action of this growth factor [34,35]. Furthermore, the VEGFa/VEGFr-2 axis is able to affect the epididymal microenvironment and sperm maturation as the main regulator of angiogenesis in the organ [32]. Therefore, we investigated the effects of maternal protein restriction on VEGFa and VEGFr-2 expression in the epididymis of the offspring.

Although maternal protein restriction during gestation and lactation increased serum aldosterone levels in 21-day-old animals (Figure 1B(I)), the epididymal expression of VEGFa was unchanged at all of the analyzed ages (Figure 2A–C). Notably, we observed a significant decrease in VEGFr-2 expression in the initial segment plus caput (IS+CP) epididymis of PND 21 animals (0.42-fold decrease compared with the NP group) (Figure 2A(I)).

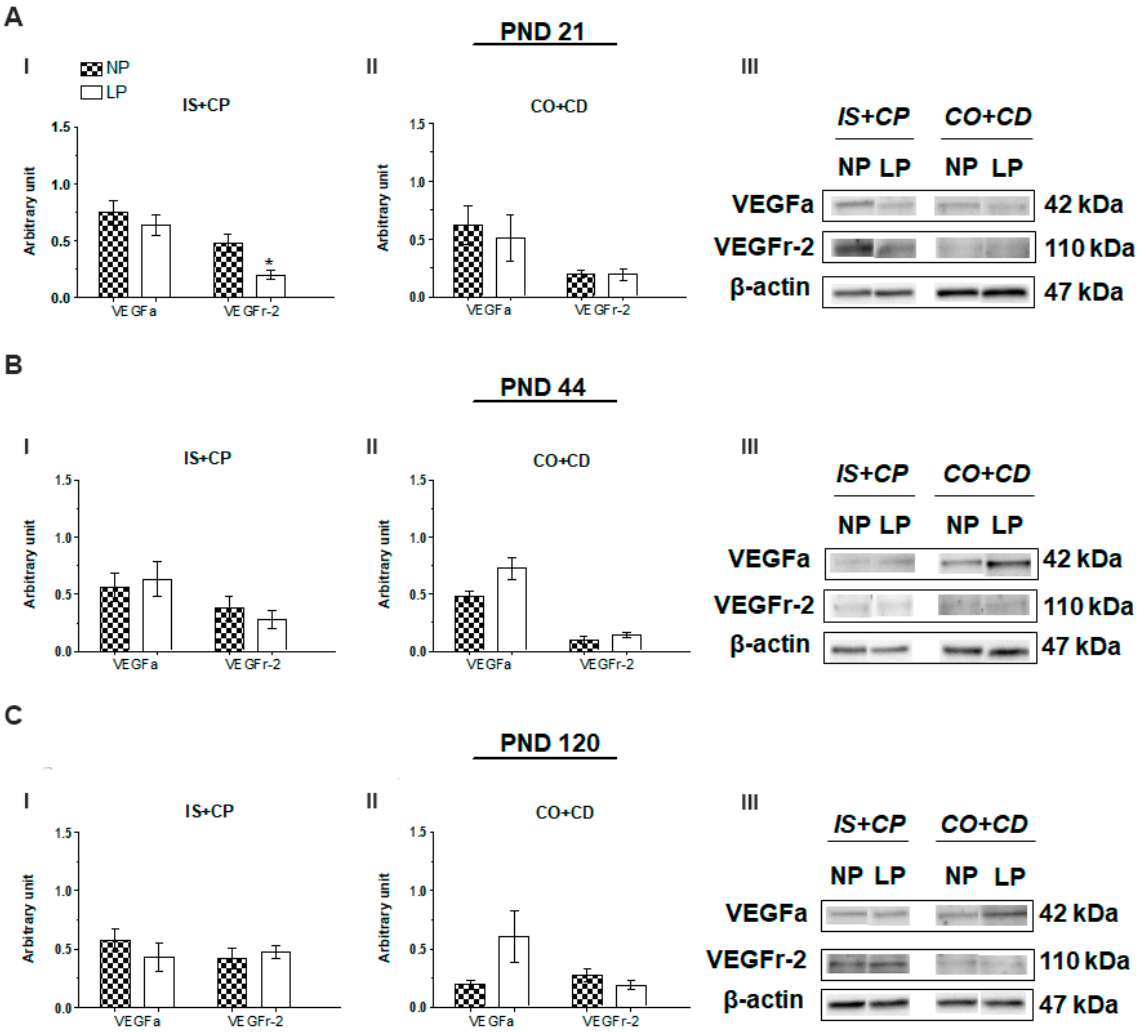

Figure 2. The VEGFa and VEGFr-2 immunoblots. (**A**) Levels of VEGFa and VEGFr-2 in the IS+CP **(I)** and corpus plus cauda (CO+CD) epididymis (**II**) of NP and low-protein (LP) animals on PND 21. Representative blots showing the levels of the VEGF, VEGFr-2 and β-actin proteins (70 µg of protein) in 21-day animals (**III**). (**B**) Levels of VEGFa and VEGFr-2 in the IS+CP (**I**) and CO+CD epididymis (**II**) of NP and LP animals on PND 44. Representative blots showing the levels of the VEGF, VEGFr-2, and β-actin proteins (70 µg of protein) in 44-day animals (**III**). (**C**) Levels of VEGFa and VEGFr-2 in the IS+CP (**I**) and CO+CD epididymis (**II**) of NP and LP animals on PND 120. Representative blots showing the levels of the VEGF, VEGFr-2 and β-actin proteins (70 µg of protein) in 120-day animals (**III**). Data are presented as means ± S.E.M. * $p < 0.05$, Mann-Whitney test.

2.3. A Maternal Low-Protein Diet Decreases the Microvascular Density (MVD) on PND 21 and 44

The MVD index and the MVD/Stroma index were used to analyze the epididymis microvasculature; these values revealed a significant reduction in the blood supply of the IS+CP (the MVD index was decreased 0.71-fold decreased compared with the NP group; the MVD/Stroma index was decreased 0.72-fold compared with the NP group) and corpus plus cauda (CO+CD) (MVD index: 0.66-fold decrease compared with the NP group; MVD/Stroma index: 1.7-fold decrease compared with the NP group) in LP animals compared to the NP group on PND 21 (Figure 3A(I,II)). This reduction was maintained until PND 44 (IS+CP MVD index: 0.72-fold decrease compared with the NP group; IS+CP MVD/Stroma index: 0.83-fold decrease compared with the NP group; CO+CD MVD index: 0.7-fold decrease compared with the NP group; CO+CD MVD/Stroma index: 0.73-fold decrease compared with the NP group; Figure 3B(I,II)). However, this difference was no longer observed at

PND 120, and the MVD index and the MVD/Stroma index of LP animals were similar to animals whose mothers consumed the normoprotein diet (Figure 3C(I,II)).

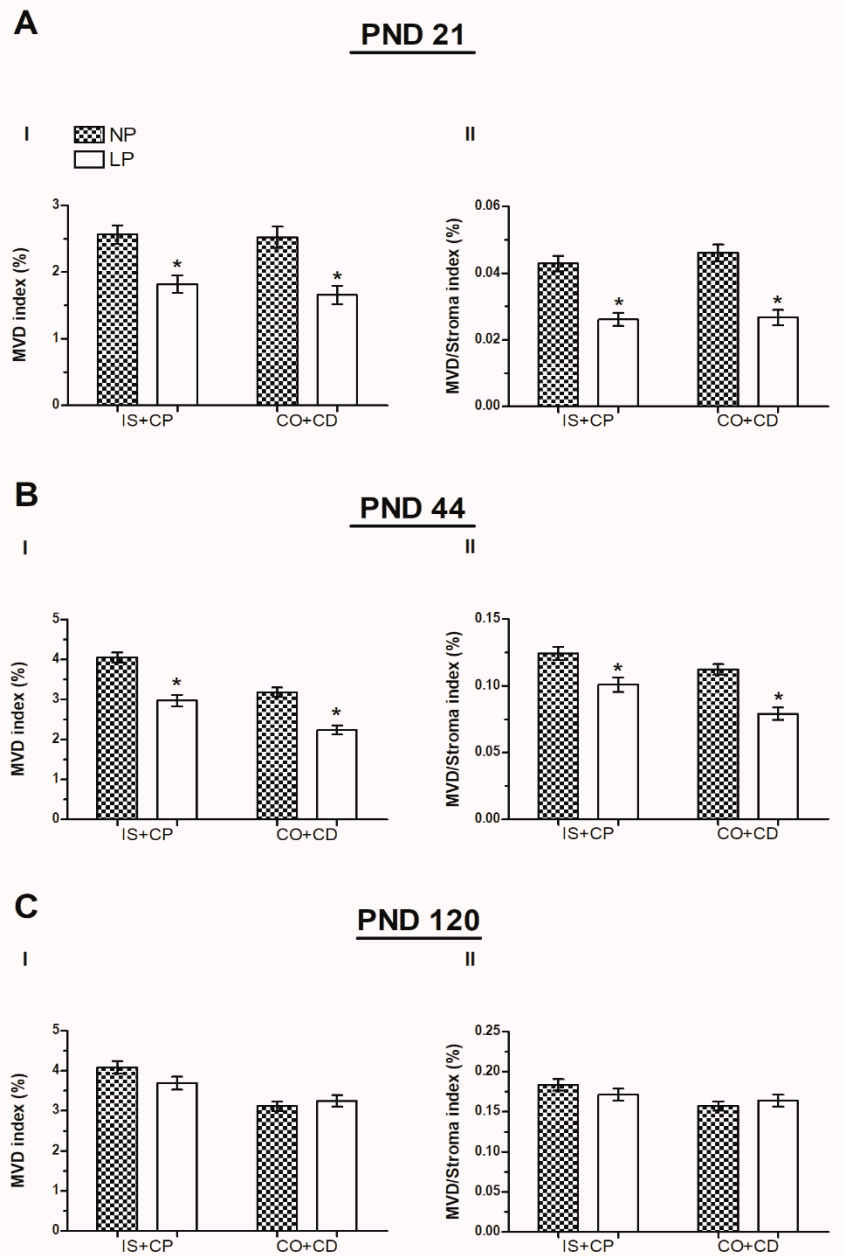

Figure 3. The microvascular densities. (**A**) microvasculature density (MVD) index (**I**) and MVD/Stroma index (**II**) in the IS+CP and CO+CD of 21-day-old animals. (**B**) MVD index (**I**) and MVD/Stroma index (**II**) in the IS+CP and CO+CD of 44-day-old animals. (**C**) MVD index (**I**) and MVD/Stroma index (**II**) in the IS+CP and CO+CD of 120-day-old animals. Data are presented as means ± S.E.M. * $p < 0.05$, Mann-Whitney test.

2.4. The Impact of the Maternal Low-Protein Diet on AQP1 and AQP9 Immunolocalization

No studies have performed AQP1 immunostaining in the epididymis of young animals. Therefore, this study is the first to assess AQP1 immunolocalization in the epididymis of 21- and 44-day-old rats, as well as AQP9 immunolocalization at PND 44. The immunolocalization pattern of AQP1 was the same in PND 21 and 44 animals, and no differences were observed between the NP and LP groups. In these animals, AQP1 staining appeared on endothelial cells of vascular channels throughout the

epididymis (Figures 4A and 5A, respectively). At PND 120, AQP1 was observed in endothelial cells of vascular channels throughout the epididymis in both the NP and LP groups (Figure 6A), as well as in the peritubular muscle cells of the initial segment (Figure 6A(I,II)); these findings are consistent with previous studies investigating the role and immunolocalization of aquaporins in the epididymis of adult rats [22,25,36–39]. However, in the PND 120 animals whose mothers were subjected to protein restriction, AQP1 was also observed on smooth muscle cells adjacent to the epididymal duct in the cauda epididymis (Figure 6AV(III)).

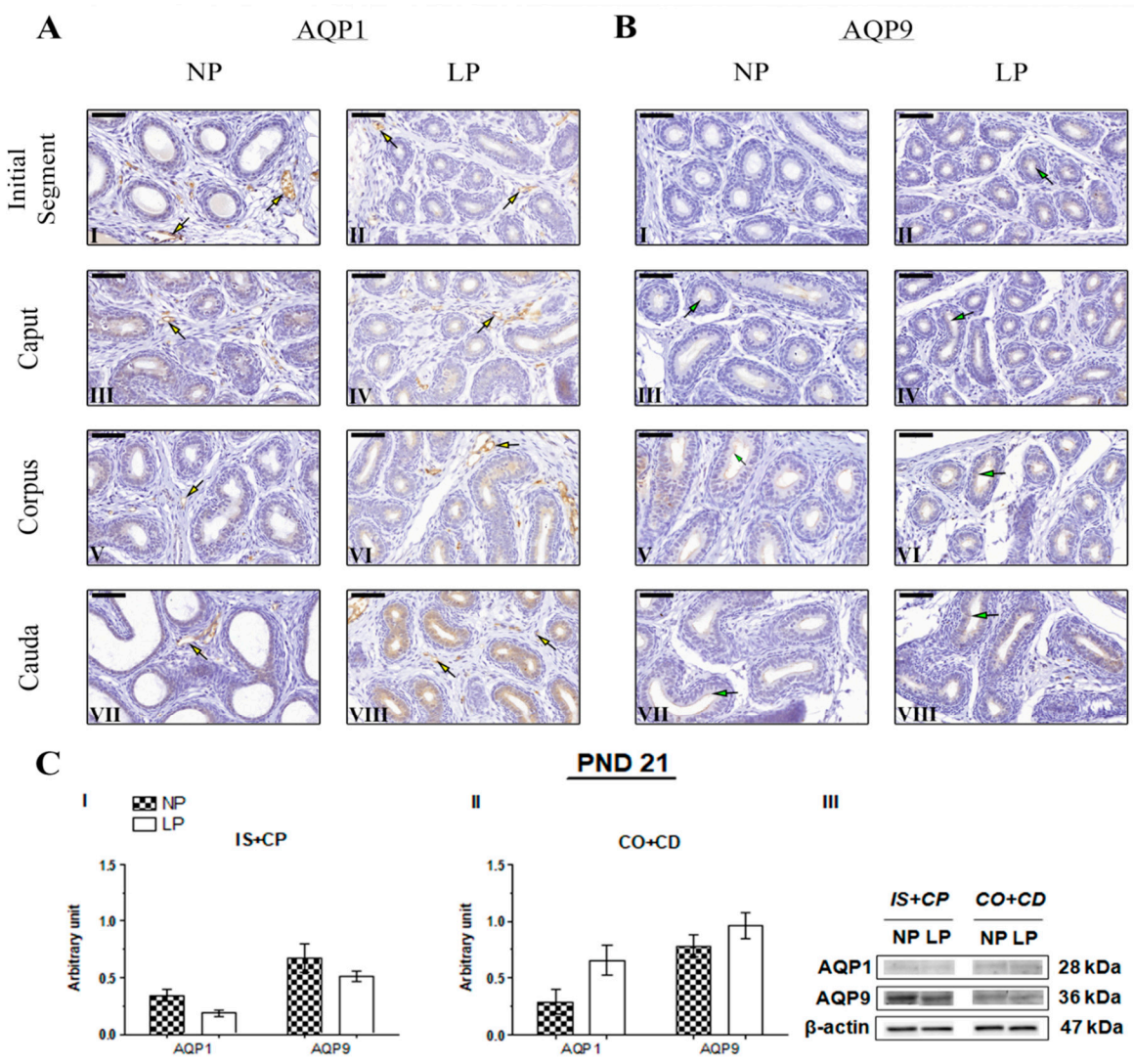

Figure 4. Expression and immunolocalization of aquaporins (AQP)1 and AQP9 in the epididymis of 21-day-old animals. (**A**) Immunoreactivity for AQP1 in endothelial cells of vascular channels in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**), and cauda (**VII** and **VIII**) of NP and LP animals (yellow arrows). (**B**) Immunoreactivity for AQP9 in the stereocilia of epididymis principal cells in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**), and cauda (**VII** and **VIII**) of NP and LP animals (green arrows). Bar = 50 μm. (**C**) Extracts obtained from individual animals were used for a densitometry analysis of the levels of proteins in the initial segment plus caput (**I**) and corpus plus cauda (**II**) following normalization to the housekeeping protein β-actin. Representative blots showing the levels of the AQP1, AQP9 and β-actin proteins (**III**, right panel). Data are presented as means ± S.E.M. and were analyzed using the Mann-Whitney test.

Immunolocalization of AQP9 was observed in the stereocilia of epididymis principal cells in NP and LP animals at all investigated ages. AQP9 immunostaining appears weak in the initial

segment, weak to moderate in the caput, and intense in the corpus and cauda epididymis of 21-day-old animals [36,40]. No AQP9 immunoreactivity was observed in the initial segment of the epididymis of NP animals on PND 21 (Figure 4B(I)). However, in both groups, the intensity of AQP9 staining was weak in the epididymis caput (Figure 4B(III,IV)), strong in the corpus (Figure 4B(V,VI)), and moderate in the cauda at this age (Figure 4B(VII,VIII), thereby revealing a segment-dependent function. The intensity of AQP9 staining was slightly higher in the epididymal cauda of LP animals compared with NP animals (Figure 4B(VIII)).

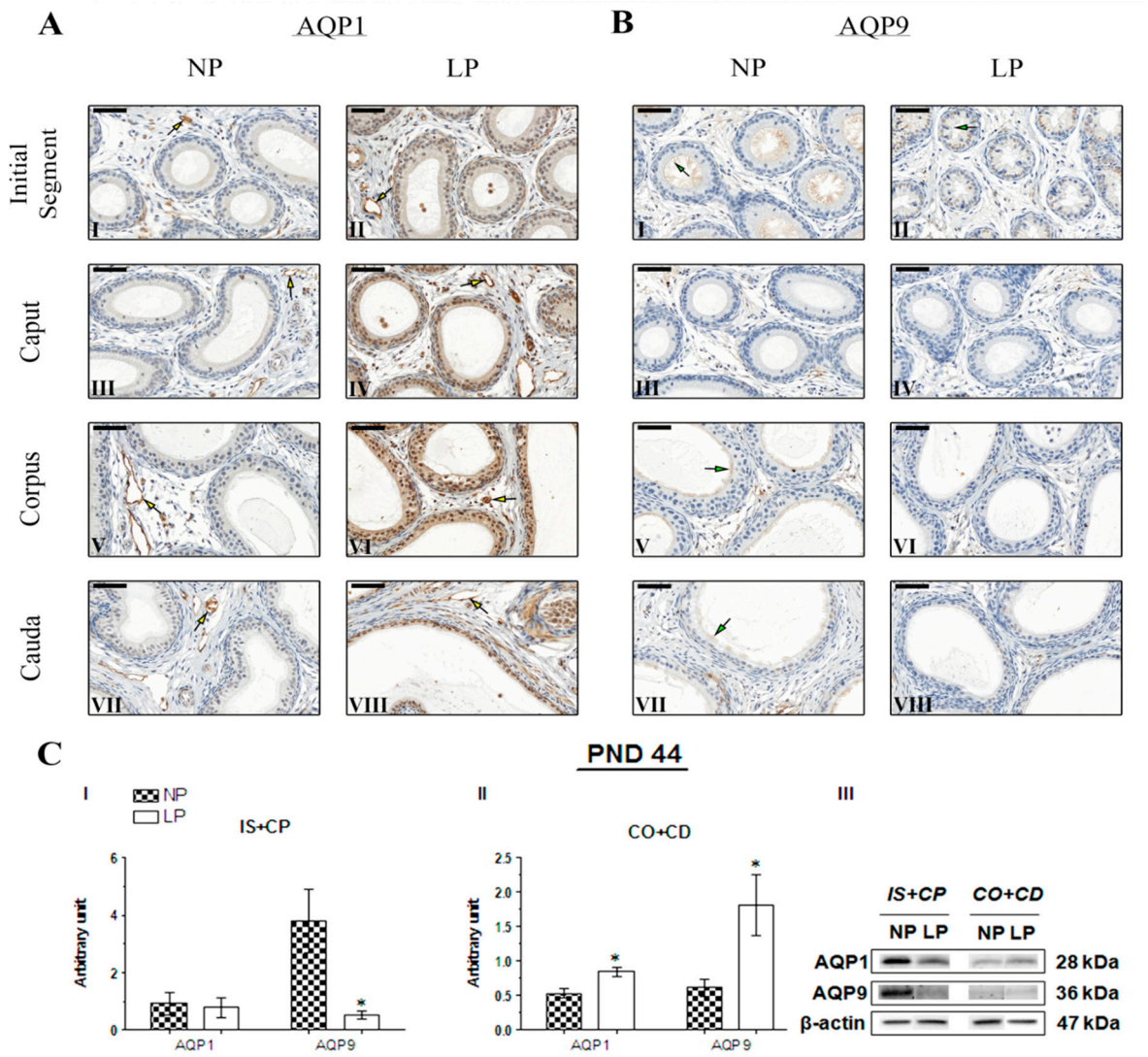

Figure 5. Expression and immunolocalization of AQP1 and AQP9 in the epididymis of 44-day-old animals. (**A**) Immunoreactivity for AQP1 in endothelial cells of vascular channels in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**) and cauda (**VII** and **VIII**) of NP and LP animals (yellow arrows). (**B**) Immunoreactivity for AQP9 in the stereocilia of epididymis principal cells in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**) and cauda (**VII** and **VIII**) of NP and LP animals (green arrows). Bar = 50 µm. (**C**) Extracts obtained from individual animals were used for a densitometry analysis of the levels of the proteins in the initial segment plus caput (**I**) and corpus plus cauda (**II**) following normalization to the housekeeping protein β-actin. Representative blots showing the levels of the AQP1, AQP9 and β-actin proteins (**III**, right panel). Data are presented as means ± S.E.M. * $p < 0.05$, Mann-Whitney test.

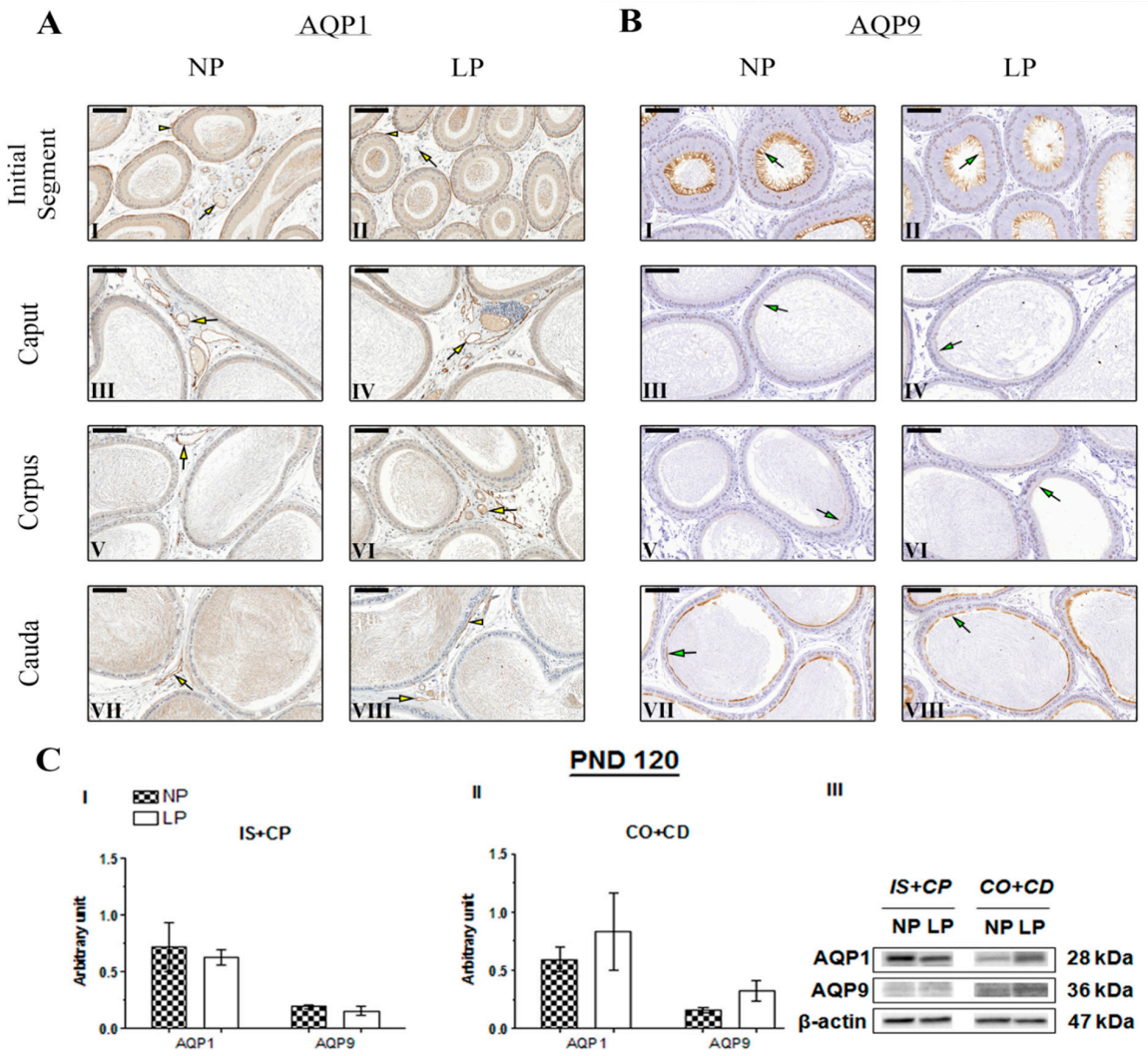

Figure 6. Expression and immunolocalization of AQP1 and AQP9 in the epididymis of 120-day-old animals. (**A**) Immunoreactivity for AQP1 in endothelial cells of vascular channels in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**), and cauda (**VII** and **VIII**) of NP and LP animals (yellow arrows). AQP1 immunostaining was also observed in the peritubular muscle cells of the initial segment in NP and LP animals (**I** and **II**) and in the peritubular muscle cells of the cauda in LP animals (**VIII**) (yellow arrowheads). (**B**) Immunoreactivity for AQP9 in the stereocilia of epididymis principal cells in the initial segment (**I** and **II**), caput (**III** and **IV**), corpus (**V** and **VI**), and cauda (**VII** and **VIII**) of NP and LP animals (green arrows). Bar = 50 μm. (**C**) Extracts obtained from individual animals were used for a densitometry analysis of the levels of the proteins in the initial segment plus caput (**I**) and corpus plus cauda (**II**) following normalization to the housekeeping protein β-actin. Representative blots showing levels of the AQP1, AQP9, and β-actin proteins (**III**, right panel). Data are presented as means ± S.E.M. and were analyzed using the Mann-Whitney test.

In the 44-day-old NP animals, strong labeling for AQP9 was observed in stereocilia of principal cells in the initial segment (Figure 5B(I)). In the caput epididymis, the staining disappeared (Figure 5B(III)), and was only intensified in the corpus and cauda of the organ (Figure 5B(V,VII)). In PND 44 LP animals, slightly less intense AQP9 staining was observed in the initial segment than in the NP group (Figure 5B(II)), and staining was not observed in caput, corpus, and cauda epididymis (Figure 5B(IV, VI and VIII)). However, AQP9 expression in these regions was also observed and quantified using Western blotting, probably due to the higher sensitivity of the technique.

At PND 120, NP animals showed intense AQP9 staining in the initial segment and cauda epididymis (Figure 6B(I,VII)), corroborating the data from literature [25,37–40]. At PND 120, slightly

less intense AQP9 staining was observed in the initial segment of LP animals than in NP animals (Figure 6B(II)), whereas no differences were observed in the intensity of AQP9 staining in the caput, corpus, and cauda epididymis between NP and LP animals (Figure 6B(III–VIII)).

2.5. The Maternal Low-Protein Diet Changes AQP1 and AQP9 Expression in the Epididymis of the Offspring

Maternal protein restriction during gestation and lactation decreased AQP1 and AQP9 expression in the IS+CP and increased the levels of these proteins in CO+CD epididymis at all ages analyzed. However, these results were only significant for AQP9 in the IS+CP (0.14-fold decrease compared with the NP group) and CO+CP (2.94-fold increase compared with the NP group) and for AQP1 in the CO+CD (1.6-fold increase compared with the NP group) on PND 44. At this age, AQP1 expression was only slightly decreased in the IS+CP (0.84-fold decrease compared with the NP; $p > 0.05$) (Figure 5C(I,II)).

Importantly, although the difference was not significant, AQP1 and AQP9 expression decreased in the IS+CP (0.56-fold decrease in AQP1 levels compared with the NP group; 0.76-fold decrease in AQP9 levels compared with the NP group) and increased in the CO+CD (2.28-fold increase in AQP1 levels compared with the NP group; 1.23-fold increase in AQP9 levels compared with the NP group) at PND 21 (Figure 4C(I,II)). This same pattern of AQP1 and AQP9 expression was observed in 120-day-old animals, both in the IS+CP (0.88-fold decrease in AQP1 levels compared with the NP group; 0.79-fold decrease in AQP9 levels compared with the NP group) and CO+CD (1.42-fold increase in AQP1 levels compared with the NP group; 2.00-fold increase in AQP9 levels compared with the NP group) (Figure 6C(I,II)).

3. Discussion

Nutrition during pregnancy is a factor that is capable of activating physiological interactions between the mother and fetus through several mechanisms, including hormonal signaling, causing epigenetic alterations and the regulation of the target tissues of these hormones. These interactions may modify placental efficiency and body growth, metabolism, and fetal organ function, providing the basis for various diseases when the availability of gestational and postnatal nutrients is discordant [6].

Although some effects of protein restriction may be a consequence of direct changes in substrate availability, several others are mediated by hormonal effects. These changes may alter the development of specific fetal tissues during critical periods of development or induce long-lasting changes in hormone secretion or in tissue sensitivity to these hormones [41–43].

In the male genital system, the testis and epididymis are the main targets of androgen action, as testosterone is essential for the maintenance of the epididymal structure and functions [44,45]. Furthermore, higher androgen concentrations are detected in the epididymal lumen than in the circulation [46]. In the present study, a low-protein diet decreased the serum testosterone level on PND 44 without significantly altering the level of this hormone on PND 21. Importantly, the maintenance of testosterone levels on PND 25, followed by a significant decrease in the concentration of this hormone on PND 70 in animals whose mothers were subjected to protein restriction during pregnancy and lactation, has been reported [12], confirming the role for protein deficiency at the beginning of development in the decreased activity of the pituitary-gonadal axis throughout the animal's life.

For many years, aldosterone, which is synthesized by the adrenal glands, was considered a "renal hormone", since has important functions in the cortex of the organ [47,48]. However, aldosterone also exerts extrarenal effects. In the male genital system, more specifically in the epididymis, several studies have documented the influence of aldosterone on the absorption of fluids from the epididymal lumen, mostly in the epididymis caput but also throughout the organ. Thus, aldosterone contributes to one of the main epididymal functions—the concentration of spermatozoa [27–29].

Maternal protein restriction is the most widely used model to investigate the effects of fetal programming on the development of chronic diseases in adulthood, particularly hypertension [49]. Based on accumulating evidence, aldosterone substantially contributes to the establishment of high

blood pressure in the offspring whose mothers consumed a low-protein diet during gestation [49–51]. In this context, studies have observed and correlated hypertension and low birth weight with increased circulating aldosterone levels in maternal protein restriction models [52,53]; these findings are consistent with the increased aldosterone levels observed in LP animals on PND 21 (data on pups' weights at birth are shown in Table S1: Body weights of male and female offspring at birth).

Considering the adrenal glands, serum VEGF can increase aldosterone availability in the circulation through its action on epithelial cells in the glomerular zone or throughout the endothelial layer, both of which occur in a renin-independent manner [54]. Furthermore, aldosterone is able to modulate VEGF expression or serum concentrations, depending on the cell type or tissue analyzed [55–57]. To date, no studies have examined the influence of this mineralocorticoid on the expression of VEGF in the epididymis and its consequences on the vasculature of this organ.

The decrease in VEGFr-2 expression induced by increased aldosterone concentrations has already been observed in rat progenitor endothelial cells [55] and in human umbilical vein endothelial cell cultures [58]. Thus, the increased levels of this mineralocorticoid observed in 21-day-old LP animals might be related to the decrease in VEGFr-2 expression in the IS+CP epididymis of these animals. Additionally, the caput epididymal region is more responsive to the actions of aldosterone [27–29], consistent with the lower expression of VEGFr-2 observed in the IS+CP, but not in the CO+CD epididymis.

The lower VEGFr-2 expression observed in the IS+CP epididymis and the decreased epididymal MVD index observed in 21-day LP animals revealed that a maternal low-protein diet altered the epididymal structure and function in the offspring without altering VEGF expression, even if the difference in the vascular pattern was not maintained until adulthood. The epididymis microvascular pattern was influenced in a period of epididymal postnatal differentiation in which the peak of proliferative activity of the initial segment cells occurs, in addition to the complete formation of the blood-epididymal barrier [59]. At this age, adequate blood supply is imperative to promote the regulated transport of metabolites, hormones, and nutrients that reach the epididymis during its postnatal development to maintain the specificity of the microenvironment [30,31,60,61].

More recently, lower levels of testosterone have been associated with increased levels of Nestin, a class VI intermediate filament protein related to neovascularization, and this negative correlation increases the epididymal vasculature in rats [62]. In the present study, despite the reduced MVD index in LP animals at PND 44, we observed a significant decrease in serum testosterone levels that might be a first sign of the substantial increase in the microvasculature of protein-restricted animals, thus nearing the MVD values of LP and NP animals on PND 120.

AQP9 is the predominant aquaporin in the epididymis, playing an important role in the dynamics of reabsorption/secretion and solute transport throughout the organ [25,36,63,64]. Castrated rats show a decrease in epididymal AQP9 expression that is reversed by the administration of testosterone [65], confirming the roles of androgens, such as testosterone and its main metabolite, dihydrotestosterone, in modulating AQP9 expression in the epididymis, particularly in the initial segment [37]. Testicular fluid reaching the epididymal lumen is enriched in testosterone and, despite the high level of this hormone in the whole organ, substantially higher concentrations of dihydrotestosterone are observed in the caput epididymis, indicating the greater sensitivity of this region to variations in androgen concentrations [66]. Because AQP9 expression is modulated by testosterone and the caput epididymal region is more sensitive to changes in the levels of this hormone [65,67], the lower expression of AQP9 observed in the IS+CP epididymis of LP animals at PND 44 might be related to the decreased serum testosterone levels observed in LP animals at this age.

A marked increase in sperm and protein concentrations occurs during spermatozoa transit through the epididymis, indicating an elevation in fluid reabsorption, particularly in the initial segment and caput epididymis. The concentration of proteins in the luminal fluid increases from 4 mg/mL in the initial segment to a maximum of 50–60 mg/mL in the distal cauda [20,25,68,69]. The decrease in AQP9 expression observed in the IS+CP of LP animals on PND 44 may have resulted in lower

water uptake in this region, allowing more water to reach the corpus and cauda epididymis. In the epididymal cauda, water reabsorption still occurs to provide more efficient space for the action of imobiline, a protein secreted by epididymis principal cells, whose main function is to immobilize the spermatozoids while they are stored in this region [36,70]. We postulate that the increased AQP9 expression observed in the CO+CD of PND 44 LP animals represents a compensatory mechanism to remove the excess water from the lumen in an attempt to preserve epididymal functions and the balanced intraluminal environment.

The increased AQP9 expression observed in the CO+CD may drain the excess water that was not absorbed by IS+CP and result in increased water in the intertubular space, leading to the appearance of edema in the epididymal cauda. In the epididymis, water is transported from the lumen via AQP9, which is expressed in the stereocilia of principal cells, and then removed from the intertubular space by the action of AQP1, which is expressed on the endothelial cells of the vascular channels throughout the organ [36]. Therefore, the increase in AQP1 expression observed in the CO+CD of PND 44 LP animals might be a physiological strategy to eliminate the excess water from the whole organ.

Although the decrease in serum testosterone levels was not significant in LP animals at PND 120, it seems to have been quite sufficient enough to directly or indirectly alter the expression of aquaporins in the epididymides of these animals in the same manner to the LP animals on PND 44. In PND 21 LP animals, testosterone levels were slightly increased by maternal protein restriction, indicating that animals at this age are responsive to the alterations in the expression of AQP1 and AQP9 in a hormone-independent manner, since the expression of AQP9 does not appear to exclusively depend on the higher levels of androgens that are achieved during and after sexual maturation in rats [65].

Some studies have shown that both aldosterone injections and aldosterone replacement in adrenalectomized rats are able to decrease AQP1 expression in the cochlea of guinea pig [71,72] and in the renal inner medulla of rats [73], respectively. As the caput epididymal region is more responsive to the actions of aldosterone [27–29], the increase of the aldosterone level in LP animals at PND 21 and 44 may be involved in the lower AQP1 expression observed in the IS+CP of these animals. In PND 120 LP animals, aldosterone levels were decreased by maternal protein restriction, thereby indicating that the alterations in the expression of AQP1 occurred in an aldosterone-independent manner in animals at this age.

Notably, although the decrease in AQP9 expression observed in the IS+CP together with the increase in AQP1 and AQP9 expression observed in the CO+CD were only significant for LP animals at PND 44, these alterations were also observed in PND 21 and PND 120 animals whose mothers were subjected to protein restriction during gestation and lactation. Our data support the hypothesis that the low-protein diet altered the expression of these proteins in the epididymis of the offspring, which led to the alterations in epididymal functions related to sperm motility, viability, and concentration, as previously observed by other authors using this experimental model [13,15].

4. Materials and Methods

4.1. Animals

Twenty male and thirty-eight female Wistar rats (45 days old) were purchased from the Central Biotherium, Institute of Biosciences/Campus of Botucatu, UNESP—São Paulo State University (Botucatu, Sao Paulo, Brazil). All animals were maintained in polyethylene cages (43 × 30 × 15 cm) with an autoclaved pine shaving substrate under controlled light (12-h light/dark cycle) and temperature (22 ± 1 °C) conditions. The animals were provided with filtered water and a solid diet for rodents ad libitum. The experimental procedures were performed in accordance with the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee on Animal Experimentation (EAEC) of the Institute of Biosciences of Botucatu with number 797-CEUA (01/22/2016).

4.2. Experimental Design

At an age of 95 days, two receptive females were placed in maternity boxes with one male rat overnight for mating. Early the next morning, vaginal smears were collected to evaluate the presence of spermatozoa, confirming the pregnancy; this time point was designated gestational day (GD) 0. Pregnant females were randomly divided into two groups (n = 19): A low-protein (LP) group, in which mothers were fed a low-protein diet (6%) ad libitum during gestation and lactation, and a normoprotein (NP) group, in which mothers were fed a normoprotein diet (17%) ad libitum during gestation and lactation. Normoprotein and low-protein diets were offered to the indicated groups from GD 0 until offspring were weaned at PND 21. Only eight pups per litter, preferably males, were maintained with each mother to ensure equal availability of nourishment. After weaning, the LP and NP male pups received the standard diet for rodents until the ages of 21 (NP, n = 17; LP, n = 19), 44 (NP, n = 12; LP, n = 10) and 120 (NP, n = 13; LP, n = 12) days, when they were euthanized and the blood and epididymides were collected (Figure 7). The ages of 21, 44, and 120 days were based on three different phases of postnatal epididymal development: The peak of cell differentiation, the final period of epididymis differentiation, and the beginning of its expansion in a well-differentiated adult epididymis, respectively.

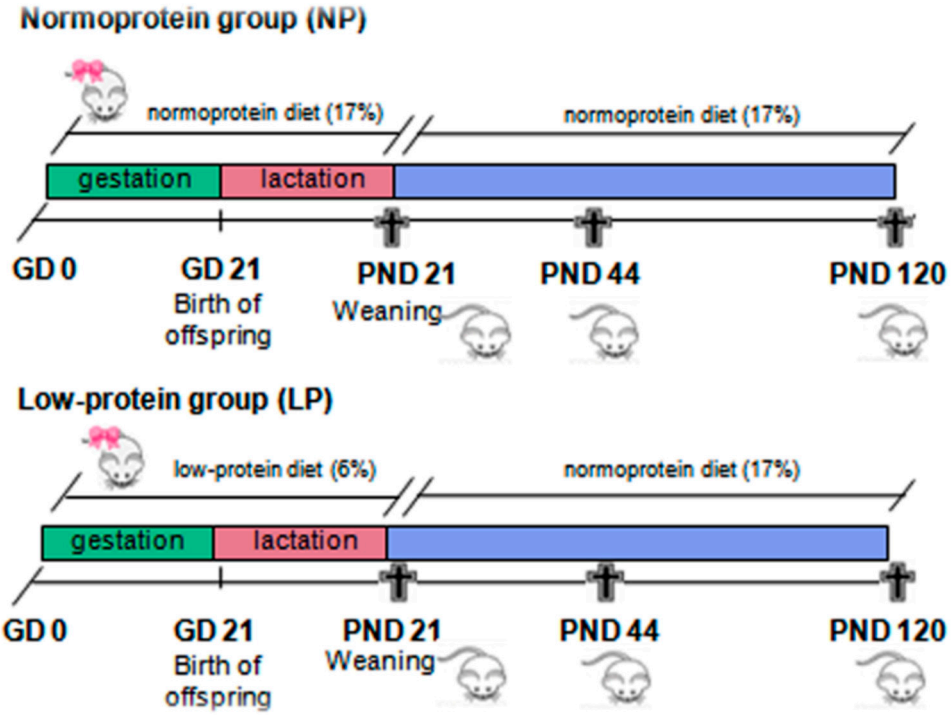

Figure 7. The experimental design. Pregnant females were fed with low-protein diet (LP group) or normoprotein diet (NP group) ad libitum during gestation and lactation from GD 0 until PND 21. After weaning, male pups from both groups received the standard diet for rodents until the ages of 21, 44, and 120 days.

4.3. Hormonal Assay

Animals were anesthetized by induced narcosis in a CO2 chamber, euthanized by decapitation, and the blood was collected by cervical vessel rupture. Blood serum was obtained by centrifugation at 14,000 rpm for 20 min at 4 °C. Serum samples were assayed for testosterone and aldosterone levels using chemiluminescence with specific kits provided by "Beckman Coulter, Inc." (Brea, CA, USA) and "DiaSorin Inc." (Stillwater, MN, USA), respectively. The lower limit of detection for testosterone was 10 ng/dL and the lower and the higher limits of detection for aldosterone was 0.97 ng/dL and 100 ng/dL, respectively. All samples were assayed at the same time to prevent interassay variation.

4.4. Immunohistochemistry

After euthanasia, the right epididymides were collected, dissected, fixed with 10% buffered formalin (0.1 M phosphate buffer, pH 7.3) for 24 h, then washed, dehydrated in a graded series of ethanol solutions, diaphanized in N-butyl alcohol, and embedded in paraplastic (Paraplast Plus, St. Louis, MO, USA). The epididymides were cut into 5-µm-thick sections using a LEICA RM 2165 micrometer (Leica Biosystems, Nußloch, Germany). Four blocks were cut from each group (LP and NP) at each age; the blocks from 21-day-old animals were cut into serial sections, while the blocks from 44- and 120-day-old animals were cut into semi-serial sections.

For immunohistochemistry, epididymal sections from LP and NP animals (n = 4 animals/group at each age) were deparaffinized in the oven (40 min at 60 °C), followed by a 15 min incubation in xylene and hydration with decreasing concentrations of ethanol. Antigen retrieval was performed in 0.01 M sodium citrate buffer, pH 6.0, in a microwave for 20 min (4 × 5 min). Afterward, endogenous peroxidases were blocked (H_2O_2, 0.3% in methanol) and then the tissues were incubated with 3% BSA for 1 h. The epididymal sections were incubated overnight at 4 °C with a 1:200 dilution of the following primary antibodies in 1% BSA: Anti-AQP1 (Millipore, Temecula, CA, USA) and anti-AQP9 (Alpha Diagnostic, San Antonio, TX, USA). On the next morning, sections were washed with PBS and then incubated with 1:200 dilutions of a peroxidase-conjugated anti-Rb secondary antibody (Sigma, St. Louis, MO, USA) for AQP1 and biotinylated anti-Rb secondary antibody (Sigma, St. Louis, MO, USA) for AQP9 both in 1% BSA for 2 h. The sections labeled with biotinylated antibodies were incubated with the ABC complex (ABC Vectastain® kit, Burlingame, CA, USA) for 45 min and subsequently washed with PBS buffer. The immunoreactive components were reacted with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) and the slides were counterstained with hematoxylin. Finally, the slides were scanned using a 3D Histech Pannoramic MIDI and analyzed and photographed using the Pannoramic Viewer program.

4.5. Western Blot

The left epididymis of five animals from each group (LP and NP) at the ages of 21, 44 and 120 days were divided into initial segment plus caput (SI+CP) and corpus plus cauda (CO+CD). Samples were homogenized at 4 °C with RIPA lysis buffer (Bio-Rad, Hercules, CA, USA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The homogenate was then centrifuged at 14,000 rpm for 20 min at 4 °C to remove the cell debris, and the supernatant was collected. Total protein concentrations were quantified using the Bradford colorimetric method [74]. Then, aliquots of 70 µg of protein were treated with 1.5× Laemmli buffer and the proteins were then separated by 4–15% polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 120 V. Following electrophoresis, proteins were electrotransferred to a nitrocellulose membrane in a wet system at 350 mA. Nonspecific protein binding was blocked with 3% milk (Molico®) in TBS-T buffer for 1 h at room temperature. Membranes were incubated overnight at 4 °C with the following primary antibodies diluted in TBS-T: Anti-AQP1 (1:800 dilution; Millipore, Temecula, CA, USA), AQP9 (1:500 dilution; Alpha Diagnostic, San Antonio, TX, USA), VEGFa (1:1000 dilution; Santa Cruz, Santa Cruz, CA, USA), VEGFr-2 (1:1000 dilution; Santa Cruz, Santa Cruz, CA,USA) and β-actin (1:800 dilution; Santa Cruz, Santa Cruz, CA, USA). On the next morning, membranes were washed with TBS-T buffer and incubated with an anti-Rb secondary antibody for VEGFa (1:2000 dilution; Sigma, St. Louis, MO, USA), AQP1 and AQP9 (1:5000 dilution; Sigma, St. Louis, MO, USA), anti-Ms secondary antibody for VEGFr-2 (1:5000 dilution; Abcam, Cambridge, UK) and anti-goat secondary antibody (1:6000 dilution; Sigma, St. Louis, MO, USA) diluted in TBS-T for 2 h, and then washed with TBS-T buffer. Thereafter, the immunoreactive bands were revealed with a luminescence kit (Amersham ECL™ Western Blotting Detection Reagent Select) from GE Healthcare® and subjected to semiquantitative analysis with optical densitometry using ImageJ analysis software for Windows. The values obtained for each band of AQP1, AQP9, VEGFα, and VEGFr-2 were normalized to the β-actin density and the data are presented as means ± S.E.M. The optical densitometry index (% band intensity) was used to represent immunoblotting data.

4.6. Microvascular Density (MVD) Determination

The MVD was determined by adapting the procedures described by Reference [75]. Briefly, epididymal sections from LP and NP animals (n = 4 animals/group at all ages) that had been immunostained with the monoclonal anti-AQP1 antibody were evaluated using a stereological analysis [76] to estimate the number of microvessels in the initial segment plus caput and corpus plus cauda epididymis. Data are presented as the percentage of microvessels per total area analyzed.

4.7. Statistical Analysis

Comparisons between LP and NP groups at all analyzed ages were performed using the Student's t-test for parametric data and the Mann-Whitney test for nonparametric data. Data are presented as means ± S.E.M., and differences were considered statistically significant when $p < 0.05$. GraphPad Prism® software (version 5.00, Graph Pad, Inc., San Diego, CA, USA) was used to perform the statistical analyses.

5. Conclusions

The low-protein diet increased aldosterone levels and decreased VEGFr-2 expression in the initial segment and caput epididymis during an important period of postnatal epididymal development, thereby influencing the epididymal vascularization pattern and fluid transport and potentially interfering with the structure and function of the organ. Furthermore, the pups whose mothers had limited protein intake showed a decrease in AQP9 expression in the initial segment and caput and an increase in AQP1 and AQP9 expression in the corpus and cauda, which potentially led to alterations in epididymal functions related to sperm motility, viability, and concentration. Based on our findings, maternal protein restriction during gestation and lactation permanently altered hormone levels and the expression of proteins important for the correct development and proper function of the epididymis in the developing organism.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/3/469/s1. Table S1: Body weight of male and female offspring at birth.

Author Contributions: M.M.C., T.d.M.S., D.N.P., and R.F.D. participated actively in the elaboration of the main idea of the study, collected, analysis and interpretation of the data and wrote the manuscript. L.G.d.A.C., P.F.F.P., and W.R.S. contributed to the acquisition of material, with the experimental design and analysis and interpretation of the data. All authors critically reviewed the paper and approved its final version.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

NP	Normoprotein
LP	Low-protein
PND	Postnatal day
AQP	Aquaporins
VEGFa	Vascular endothelial growth factor
VEGFr-2	Vascular endothelial growth factor receptor 2
MVD	Microvasculature density
IS+CP	Initial segment plus caput
CO+CD	Corpus plus cauda

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Capítulo II

A segunda parte deste estudo deu origem ao artigo "**Maternal Protein Restriction Alters the Expression of Proteins Related to the Structure and Functioning of the Offspring Epididymis in an Age-Dependent Manner**", a ser submetido ao periódico internacional "*The Journal of Nutritional Biochemistry*". Fator de impacto 4,49.

MATERNAL PROTEIN RESTRICTION ALTERS THE EXPRESSION OF PROTEINS RELATED TO THE STRUCTURE AND FUNCTIONING OF THE OFFSPRING EPIDIDYMIS IN AN AGE-DEPENDENT MANNER

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Abstract: Nutrition is an environmental factor able to activate physiological interactions between the fetus and mother. Maternal protein restriction is able to alter sperm parameters associated with epididymal functions. Since correct development and functioning of the epididymides are fundamental for mammalian reproductive success, this study investigated the effects of maternal protein restriction on epididymal morphology and morphometry in rat offspring as well as on the expression of Src, Cldn-1, AR, ER, aromatase p450 and 5α-reductase in different stages of postnatal epididymal development. For this purpose, pregnant females were allocated to normal-protein (NP – 17% protein) and low-protein (LP – 6% protein) groups that received specific diets during gestation and lactation. After weaning, male offspring was provided only normal-protein diet until the ages of 21, 44 and 120 days, when they were euthanized and their epididymides collected. Maternal protein restriction decreased genital organs weight as well as crown-rump length and anogenital distance at all ages. Although the low-protein diet did not change the integrity of the epididymal epithelium, we observed decreases in tubular diameter, epithelial height and luminal diameter of the epididymal duct in 21-day-old LP animals. The maternal low-protein diet changed AR, ERα, ERβ, Src 416 and Src 527 expression in offspring epididymides in an age-dependent manner. Finally, maternal protein restriction increased Cldn-1 expression throughout the epididymides at all analyzed ages. Although some of these changes did not remain until adulthood, the

insufficient supply of proteins in early life altered the structure and functioning of the epididymis in important periods of postnatal development.

Keywords: maternal protein restriction; epididymis; hormone receptors; Src; Cldn-1, postnatal development

1. Introduction

One of the first concepts to emerge in the literature relating the conditions of the intrauterine environment to changes in progeny development was the "fetal origin of diseases" theory [1]. The "fetal origin" hypothesis proposes that changes in fetal nutrition and hormonal status result in adaptations during development capable of altering the structure, physiology and metabolism of the embryo, thus predisposing it to cardiovascular, metabolic and endocrine diseases in adult life [2, 3]. Currently, the hypothesis that intrauterine environmental conditions are able to influence the establishment of adulthood diseases is known as the Developmental Origins of Health and Disease hypothesis (DOHaD) [4].

It is well established that maternal nutrition has a significant impact on development and fetal health, since during gestation and lactation, the fetus depends exclusively on the mother to supply its nutritional requirements [4-6]. Therefore, nutrition during pregnancy is an environmental factor able to activate physiological interactions between the fetus and mother that are often mediated through hormonal signaling and may cause epigenetic alterations in genes that regulate the target tissues of these hormones. These interactions may modify fetal growth and metabolic character, establishing the basis for several diseases when there is inconsistency between gestational and postnatal nutrient availability [7].

Hormonal signaling changes during sensitive periods of development may alter the development of specific fetal tissues, lead to long-lasting changes in tissue sensitivity to hormones or even change hormone secretion [8-11]. Rats subjected to maternal protein restriction during intrauterine development show changes in testosterone, estradiol and aldosterone concentration that result in significant impacts on organs and functions of the genital system [12-15].

The epididymis is an androgen-dependent organ anatomically divided into the initial segment, caput, corpus and cauda. Each epididymal region is responsible for characteristic functions such as secretion, endocytosis, absorption and acidification,

which lead to the establishment of a specific intraluminal environment suitable for the concentration and maturation of the spermatozoa produced by the testes [16-19].

A maternal low-protein diet during gestation and lactation is able to induce testicular, prostatic and sperm changes in adult animals [12-14, 20-22]. Regarding sperm alterations, it has been observed that maternal protein restriction caused alterations mainly associated with epididymal functions such as sperm motility, viability and concentration [20, 22]. However, although studies have shown effects of protein restriction associated with epididymal functions, the causes of these alterations have not yet been entirely clarified.

During gestation, an increase in protein turnover occurs to enable rapid embryo growth; therefore, adequate intake of protein at this stage is recommended [23]. Although some effects of protein restriction are mediated by hormonal effects, several others are direct consequences of changes in substrate availability.

Srcs are nonreceptor protein kinases that act in multiple cellular environments, playing key roles in the regulation of signal transduction through several cell surface receptors [24]. In the epididymis, Srcs stand out as regulators of epididymal development in addition to playing important roles in spermatozoa changes that occur during epididymal transit and sperm maturation within the organ [25, 26].

The structure and integrity of the blood–epididymal barrier are essential for the maintenance of epididymal intraluminal environment specificity [27, 28]. In this context, the claudins (Cldns) are a family of transmembrane proteins that are essential components of the tight junctions that compose this barrier [29, 30]. Although several claudins integrate tight junctions, studies have demonstrated the presence of Cldn-1 in all regions of the rat epididymis. In addition, Cldn-1 appears throughout all interfaces of adjacent epithelial cells and throughout all basal plasma membrane extensions, suggesting that Cldn-1 plays a role as an adhesion molecule [28, 31, 32]. Cldn-1 knockout mice die of dehydration soon after birth due to lack of the epidermal barrier, thus demonstrating that Cldn-1 is indispensable for survival and cannot be replaced by any of the other tight junction proteins [33].

Insufficient protein intake in a large proportion of the human population due to cultural or economic reasons is a global concern. For this reason, the model of protein restriction remains one of the most characterized early growth restriction models studied

until now [7, 34, 35]. Maternal protein restriction is able to alter sperm parameters associated with epididymal function, such as sperm motility, viability and concentration. Since the correct development and functioning of the epididymis are fundamental for mammalian reproductive success, this study investigated the effects of maternal protein restriction on epididymal morphology and morphometry in rat offspring as well as on the expression of Src, Cldn-1, AR, ERα, ERβ, aromatase p450 and 5α-reductase in different stages of postnatal epididymal development.

2. Materials and Methods

2.1. Animals and experimental design

Wistar rats 45 days in age (male n= 20; female n= 38) were obtained from the Central Biotherium, Institute of Biosciences/Campus of Botucatu, UNESP – São Paulo State University. The animals were housed in polyethylene cages (43x30x15 cm) lined with an autoclaved pine sawdust substrate under controlled conditions of temperature and light (12-h light/dark cycle). The animals were maintained with free access to water and commercial solid food for rodents.

For mating, two sexually receptive females and one breeder male rat at 95 days of age were kept in maternity boxes overnight. The next morning, pregnancy was confirmed by the presence of spermatozoa in vaginal smears; this day was designated gestational day (GD) 0.

Pregnant females were housed individually in standard rat cages and divided into a low-protein (LP) group (n=19) and a normal-protein (NP) group (n=19). The LP mothers were fed a low-protein diet (6%), while the NP mothers were fed a normal-protein diet (17%), both provided from Pragsoluções Biociências (Jaú, SP, Brazil). Both groups received their respective diets *ad libitum* (Table 1).

Table 1 – Composition of diets offered to animals during gestation and lactation.

Components *	Normoprotein diet (17%)	Low-protein diet (6%)
Casein (84% of protein)**	202.00	71.50
Cornstarch	397.00	480.00
Dextrin	130.50	159.00
Sucrose	100.00	121.00
Soy oil	70.00	70.00

Fiber (microcellulose)	50.00	50.00
Mineral Blend ***	35.00	35.00
Vitamin Blend ***	10.00	10.00
L-cystine	3.00	1.00
Choline chloride	2.50	2.50

* Diet for the gestation phase in rodents - AIN-93G
** Corrected values according to protein content in casein
*** According to AIN-93G

The normal-protein and low-protein diets were offered to the indicated groups during gestation and lactation, from GD 0 until the offspring were weaned at postnatal day (PND) 21. To ensure equal availability of nourishment, only eight pups per litter (preferably males) were maintained with each mother. After weaning, the LP and NP male offspring received the standard diet for rodents until the age of 21 (NP, n = 17; LP, n = 19), 44 (NP, n = 12; LP, n = 10) and 120 (NP, n = 13; LP, n = 12) days, when they were decapitated and their blood and epididymides collected (Figure 1). The ages of the animals at euthanasia were chosen based on three different phases of postnatal epididymal development: at 21 days of age, peak epididymal cell differentiation occurs; at 44 days of age, the final period of epididymal differentiation and the beginning of epididymal expansion occurs; and at 120 days of age, the epididymides are well differentiated, and the animals are considered sexually mature [36].

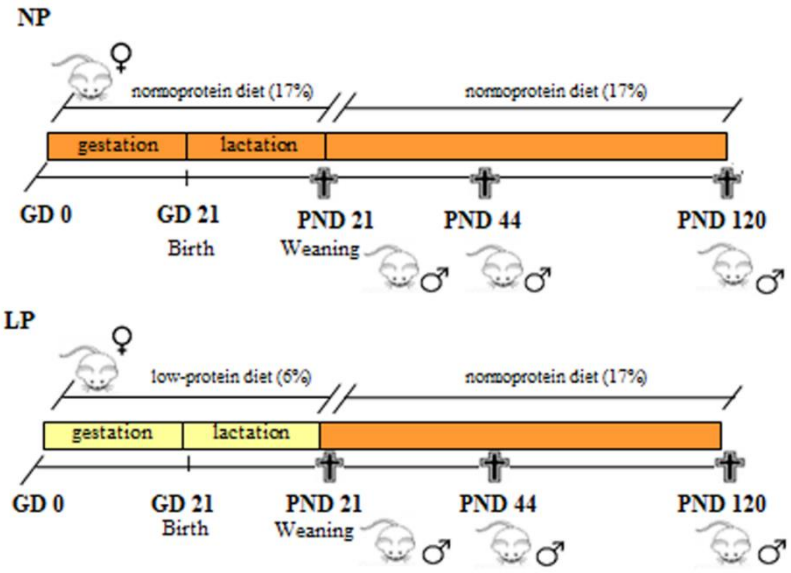


Figure 1. Experimental design. Pregnant rats received a low-protein diet (LP group) or a normal-protein diet (NP group) ad libitum from GD 0 until PND 21 (during gestation and lactation). From weaning, male pups from both groups received a standard diet for rodents until PNDs 21, 44 and 120.

The experimental procedures were approved by the Ethics Committee on Animal Experimentation (EAEC) of the Institute of Biosciences of Botucatu (number 797-CEUA) in addition to being in accordance with the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2. Hormonal assay

Through narcosis induced in a CO_2 chamber, the animals were anesthetized and then decapitated (for cervical vessel rupture), and the blood was collected. Blood serum was obtained by centrifugation at 21952 rcf for 20 min at 4°C. Serum samples were assayed for estradiol levels using chemiluminescence with a specific kit provided by Beckman Coulter, Inc. (Brea, CA, USA). The lower and higher limits of detection for estradiol were 0.017 ng/dL and 6.9 ng/dL, respectively. All samples were assayed at the same time to prevent interassay variation.

2.3. Morphological and morphometrical analyses of the epididymis

Following euthanasia, the right epididymides were individually collected, immediately fixed in 10% buffered formalin (0.1 M phosphate buffer, pH 7.3) for 24 h and then washed in running water for 24 h. Then, the epididymides were dehydrated in a graded series of ethanol solutions, diaphanized in N-butyl alcohol and embedded in Paraplast (Paraplast Plus, St. Louis, MO, USA). Epididymal sections 5 μm thick were made using a LEICA RM 2165 micrometer (Leica Biosystems, Nußloch, Germany). Four blocks were cut for the NP and LP groups at each age; the blocks from the animals at PND 21 were cut into serial sections, while the blocks from the animals at PNDs 44 and 120 were cut into semiserial sections.

For all ages (21, 44 and 120 days), four slides from each of the four animals of each group (HP and NP) were stained with hematoxylin and eosin (H&E). The slides were scanned with a 3DHistech Pannoramic MIDI, analyzed for epithelial and interstitial integrity and then photographed using the Pannoramic Viewer program.

Morphometric analyses were conducted by adapting the procedures described by Serre and Robaire [37]. For this technique, the same slides used for analysis of epididymal morphology were analyzed. Briefly, the epithelial height, luminal diameter and tubular epididymal diameter was measured in at least 10 transverse sections of epididymal tubules in each of the epididymal regions using the Pannoramic Viewer program.

2.4. Immunohistochemistry

For immunohistochemistry, sections of epididymides from LP and NP animals (n = 4 animals/group at each age) were deparaffinized (40 min in the oven at 60°C), incubated in xylene and hydrated with decreasing concentrations of ethanol. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) in a water bath for AR and ERα at PNDs 21 and 44 and in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker for AR and ERα at PND 120 and for ERβ at all ages. After endogenous peroxidase was blocked (H_2O_2, 0.3% in methanol), the tissues were incubated with 3% BSA for 1 h. The sections of epididymis were incubated overnight at 4°C with 1:100 dilutions of the following primary antibodies in 1% BSA: anti-AR (Millipore, Temecula, CA, USA), anti-ERα (Millipore, Temecula, CA, USA) and anti-ERβ (Millipore, Temecula, CA, USA). Early the next morning, the sections were washed with PBS buffer and then incubated with 1:200 dilutions of a peroxidase-conjugated anti-Rb secondary antibody (Sigma, St. Louis, MO, USA) for AR and ERα at PNDs 21 and 44 and a biotinylated anti-Rb secondary antibody (Sigma, St. Louis, MO, USA) for AR and ERα at PND 120 and for ERβ at all ages in 1% BSA for 2 h. The sections labeled with biotinylated antibodies were incubated for 45 min with an ABC complex (ABC Vectastain® kit, Burlingame, CA, USA) and subsequently washed with PBS. The immunoreactive components were reacted with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA), and counterstaining was performed with hematoxylin. Finally, a 3D Histech Pannoramic MIDI was used to scan the slides, which were photographed and analyzed using the Pannoramic Viewer program. Negative controls were obtained from each reaction using 1% BSA and omitting the primary antibody in the overnight incubation step.

2.5. Western blot analysis

The left epididymides from NP and LP animals at the ages of 21, 44 and 120 days (n = 5) were divided into the initial segment plus caput (SI+CP) and the corpus plus cauda (CO+CD). The samples were homogenized with RIPA lysis buffer (Bio-Rad, Hercules, CA, USA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Afterwards, the homogenate was centrifuged at 14,000 rpm for 20 min to remove the cell debris, and the supernatant was then collected. Total protein concentrations were measured using the Bradford colorimetric method [38]. Afterwards, 70 µg of protein was added to 1.5X Laemmli buffer, and the individual proteins were then separated by 4-15%

polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 120 V. Following electrophoresis, the proteins were electrotransferred to nitrocellulose membranes in a wet system at 350 mA. The membranes were blocked with TBS-T solution containing 3% milk (Molico®) for 1 h at room temperature. The membranes were incubated overnight at 4°C with the following primary antibodies diluted in TBS-T: anti-AR (1:1000 dilution; Millipore, Temecula, CA, USA), anti-ERα (1:200 dilution; Millipore, Temecula, CA, USA), anti-ERβ (1:300 dilution; Millipore, Temecula, CA, USA), anti-Src 416 (1:500 dilution; Cell Signaling, Danvers, MA, USA), anti-Src 527 (1:1000 dilution; Cell Signaling, Danvers, MA, USA), anti-Cldn-1 (1:1000 dilution; Thermo Fisher Scientific, Rockford, IL, USA) and anti-β-actin (1:800 dilution; Santa Cruz, Santa Cruz, CA, USA). Early the next morning, the membranes were washed with TBS-T solution and then incubated with a 1:2000 dilution of an anti-Rb secondary antibody for AR, ERα, ERβ and Cldn-1 (Sigma, St. Louis, MO, USA), a 1:5000 dilution of an anti-Rb secondary antibody for Src 416 and Src 527 (Sigma, St. Louis, MO, USA) and an anti-goat secondary antibody (1:6000 dilution; Sigma, St. Louis, MO, USA) diluted in TBS-T for 2 h before being washed with TBS-T solution. Subsequently, the immunoreactive bands were developed using a chemiluminescence kit (Amersham ECL™ Western Blotting Detection Reagent Select) from GE Healthcare® and analyzed semiquantitatively by optical densitometry with ImageJ analysis software for Windows. The values obtained for each band of AR, ERα, ERβ, Src 416, Src 527, and Cldn-1 were normalized to the β-actin density, and the data are presented as the mean ± S.E.M. The immunoblotting data are presented as optical densitometry index (% band intensity).

2.6. Statistical analysis

GraphPad Prism® software (version 5.00, Graph Pad, Inc., San Diego, CA) was used to perform the statistical analyses. At all analyzed ages, comparisons between the LP and NP groups were performed using the Mann-Whitney test for nonparametric data and Student's t-test for parametric data. All data are presented as the mean ± S.E.M., and statistical significance was set at $p < 0.05$.

3. Results

3.1. A maternal low-protein diet promotes changes in gestational performance as well as genital organ weight in male offspring

Gestational performance is an important parameter in studies that use maternal malnutrition as an experimental model. This parameter is assessed on the day of offspring birth and presents relevant information regarding gestation and offspring development.

Maternal protein restriction during gestation and lactation did not alter the number of male pups but significantly decreased the body weight of the pups at birth (0.91-fold decrease in the LP group compared with the NP group) (Table 2).

Table 2 - Body weight, crown-rump length and absolute and relative anogenital distance of males at birth.

Parameters	***NP***	***LP***
Number of male pups	5.29 ± 0.43	5.47 ± 0.52
Body weight (g)	6.33 ± 0.12	5.79 ± 0.10*
Anogenital distance (mm)	2.99 ± 0.03	2.59 ± 0.05*
Crown-rump length (mm)	49.13 ± 0.32	47.46 ± 0.21*
Relative anogenital distance (mm)	0.061 ± 0.01	0.055 ± 0.01*

NP, n = 17 litters/group. LP, n = 19 litters/group. The values are expressed as the mean ± S.E.M. * $p < 0.05$. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

With regard to the other parameters analyzed on the day of offspring birth, this experimental model caused a significant reduction in crown-rump length (CRL) (0.97-fold reduction in the LP group compared with the NP group) (Table 2) and absolute anogenital distance (AGD) (0.87-fold reduction in the LP group compared with the NP group) (Table 2), as well as in relative AGD (0.9-fold reduction in the LP group compared with the NP group) (Table 2).

At PNDs 21 and 44, AGD remained significantly reduced in LP animals compared with NP animals (PND 21: 0.59-fold reduction in the LP group compared with the NP group; PND 44: 0.74-fold reduction in the LP group compared with the NP group) (Figure 2).

Although CRL remained significantly reduced in LP animals at PNDs 21 and 44 (PND 21: 0.67-fold reduction in the LP group compared with the NP group; PND 44: 0.8-fold reduction in the LP group compared with the NP group) (Supplementary Table 1), there was no significant difference in relative AGD between the groups at both ages (PND

21: 0.9-fold reduction in the LP group compared with the NP group; PND 44: 0.9-fold reduction in the LP group compared with the NP group) (Supplementary Table 1).

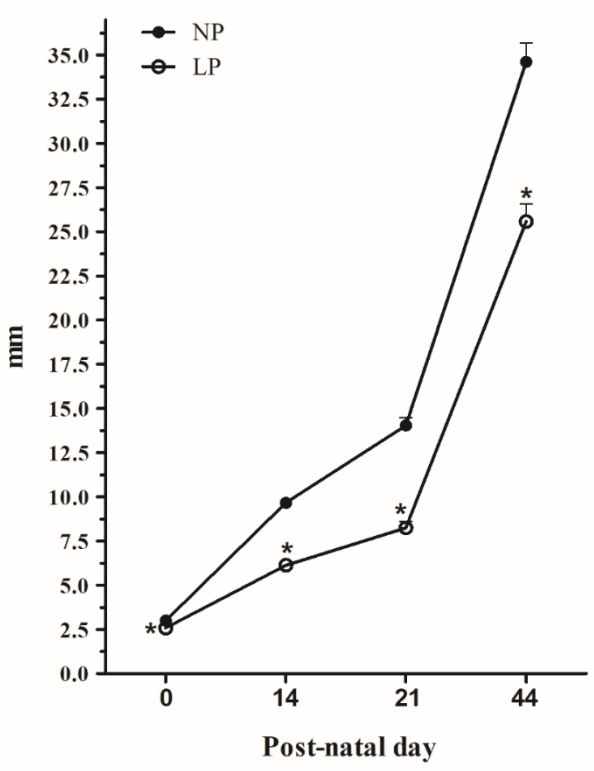


Figure 2. Evolution of anogenital distance. PND 0: NP, n = 17, LP, n = 19; PND 14: NP, n = 19, LP, n = 26; PND 21: NP, n = 17, LP, n = 22; PND 44: NP, n = 12, LP, n = 10. Evolution of AGD in animals whose mothers received normal-protein and low-protein diets during gestation and lactation. The values are expressed as the mean ± S.E.M. *p <0.05. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

Regarding genital organ weight, the low-protein diet caused a significant decrease in the absolute weights of the testes and epididymides at all analyzed ages (testis weight: 0.41-fold decrease in the LP group compared with the NP group at PND 21; 0.55-fold decrease in the LP group compared with the NP group at PND 44; 0.79-fold decrease in the LP group compared with the NP group at PND 120. Epididymis weight: 0.52-fold decrease in the LP group compared with the NP group at PND 21; 0.51-fold decrease in the LP group compared with the NP group at PND 44; 0.84-fold decrease in the LP group compared with the NP group at PND 120) (Table 3).

Ventral prostate and empty seminal vesicle absolute weights were significantly reduced in LP animals at PNDs 21 and 44 (ventral prostate weight: 0.38-fold decrease in the LP group compared with the NP group at PND 21; 0.43-fold decrease in the LP group compared with the NP group at PND 44. Empty seminal vesicle weight: 0.42-fold decrease in the LP group compared with the NP group at PND 21; 0.40-fold decrease in the LP group compared with the NP group at PND 44) (Table 3), but there was no significant difference at PND 120 (0.83-fold decrease in ventral prostate weight in the LP group compared with the NP group; 1.12-fold increase in empty seminal vesicle weight in the LP group compared with the NP group) (Table 3).

Table 3 - Body weight and absolute and relative genital organs weight at PND day 21, 44 and 120.

Parameters	***PND 21***		***PND 44***		***PND 120***	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***
Body weight (g)	58.63 ± 1.59	19.29 ± 0.74*	219.30 ± 4.99	122.50 ± 4.84*	492.00 ± 13.96	399.90 ± 7.33*
Testes (mg)	110.40 ± 2.79	45.30 ± 2.39*	944.10 ± 35.77	516.10 ± 27.03*	1802 ± 68.05	1415 ± 39.23*
Testes (mg/100g)	189.50 ± 5.00	235.2 ± 8.80*	412.30 ± 21.29	420.20 ± 12.65	375.80 ± 14.87	355.30 ± 12.94
Epididymis (mg)	17.29 ± 0.82	8.93 ± 0.40*	141.70 ± 8.58	72.52 ± 4.27*	826.40 ± 34.26	697.40 ± 21.20*
Epididymis (mg/100g)	28.65 ± 0.82	46.50 ± 1.62*	61.98 ± 2.06	58.87 ± 1.67	172.40 ± 7.87	175.40 ± 2.94
Ventral prostate (mg)	21.49 ± 1.57	8.08 ± 0.59*	103.70 ± 11.36	44.40 ± 5.70*	928.50 ± 79.31	769.80 ± 63.45
Ventral prostate (mg/100g)	39.34 ± 3.42	41.67 ± 2.44	46.85 ± 4.45	36.32 ± 4.42	189.10 ± 12.97	190.70 ± 13.48
Seminal vesicle (empty) (mg)	11.28 ± 0.38	4.70 ± 0.29*	100.90 ± 10.94	39.31 ± 3.95*	1472 ± 104.00	1647 ± 104.80
Seminal vesicle (empty) (mg/100g)	18.77 ± 0.88	24.46 ± 1.30*	45.43 ± 4.34	31.80 ± 2.69*	302.40 ± 27.27	424.40 ± 22.97*

PND 21: NP, n = 17, LP, n = 22; PND 44: NP, n = 12, LP, n = 10; PND 120: NP, n = 10, LP, n = 10. The values are expressed as the mean ± S.E.M. * $p < 0.05$. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

The relative weights of genital organs were significantly elevated in LP animals at PND 21 (1.24-fold increase in relative testis weight in the LP group compared with the NP group; 1.62-fold increase in relative epididymis weight in the LP group compared with the NP group; 1.06-fold increase in relative ventral prostate weight in the LP group

compared with the NP group; 1.30-fold increase in relative empty seminal vesicle weight in the LP group compared with the NP group) (Table 3).

At PND 44, the relative testis weight was slightly elevated in LP animals (1.02-fold increase in the LP group compared with the NP group) (Table 3), while for the epididymis and ventral prostate, the relative weights were slightly reduced in LP animals (0.95-fold decrease in relative epididymis weight in the LP group compared with the NP group; 0.78-fold decrease in relative ventral prostate weight in the LP group compared with the NP group) (Table 3). The relative empty seminal vesicle weight was significantly reduced in LP animals (0.70-fold decrease in the LP group compared with the NP group) (Table 3).

Finally, at PND 120, the relative weights of the epididymis and ventral prostate showed practically no differences between NP and LP animals (Table 3). The relative testis weight was slightly reduced in LP animals (0.95-fold decrease in the LP group compared with the NP group) (Table 3), and the relative empty seminal vesicle weight was significantly elevated in animals whose mothers received a low-protein diet (1.40-fold increase in the LP group compared with the NP group) (Table 3).

3.2. Maternal protein restriction did not change the integrity of the epididymal epithelium or the organ interstitium but changed the tubular diameter, epithelial height and luminal diameter of the epididymal duct

Both the NP and LP groups at PNDs 44 and 120 presented initial segment regions with small tubular diameters and organized epithelia containing principal cells, basal cells, narrow cells and few apical cells (Figure 3 B, I and V; C, I and V). The epididymal caput also had a well-organized epithelium with the presence of principal cells, basal cells and clear cells. In this region, the tubular diameter was slightly greater than that in the initial segment region (Figure 3 B, II and VI; C, II and VI). With age advancement, the epididymal corpus showed principal cells with slightly more cubic shapes than the principal cells of the initial segment and caput regions in addition to presenting clear cells and basal cells (Figure 3 B, III and VII; C, III and VII). In the cauda region, the epithelial height did not differ greatly from the epithelial height of the caput region between the groups of animals at 21 and 44 days but was much reduced in the animals at 120 days. On PNDs 44 and 120, principal cells, basal cells and a greater number of clear cells were

observed in the caudal region compared to the other epididymal regions (Figure 3 B, IV and VIII; C, IV and VIII).

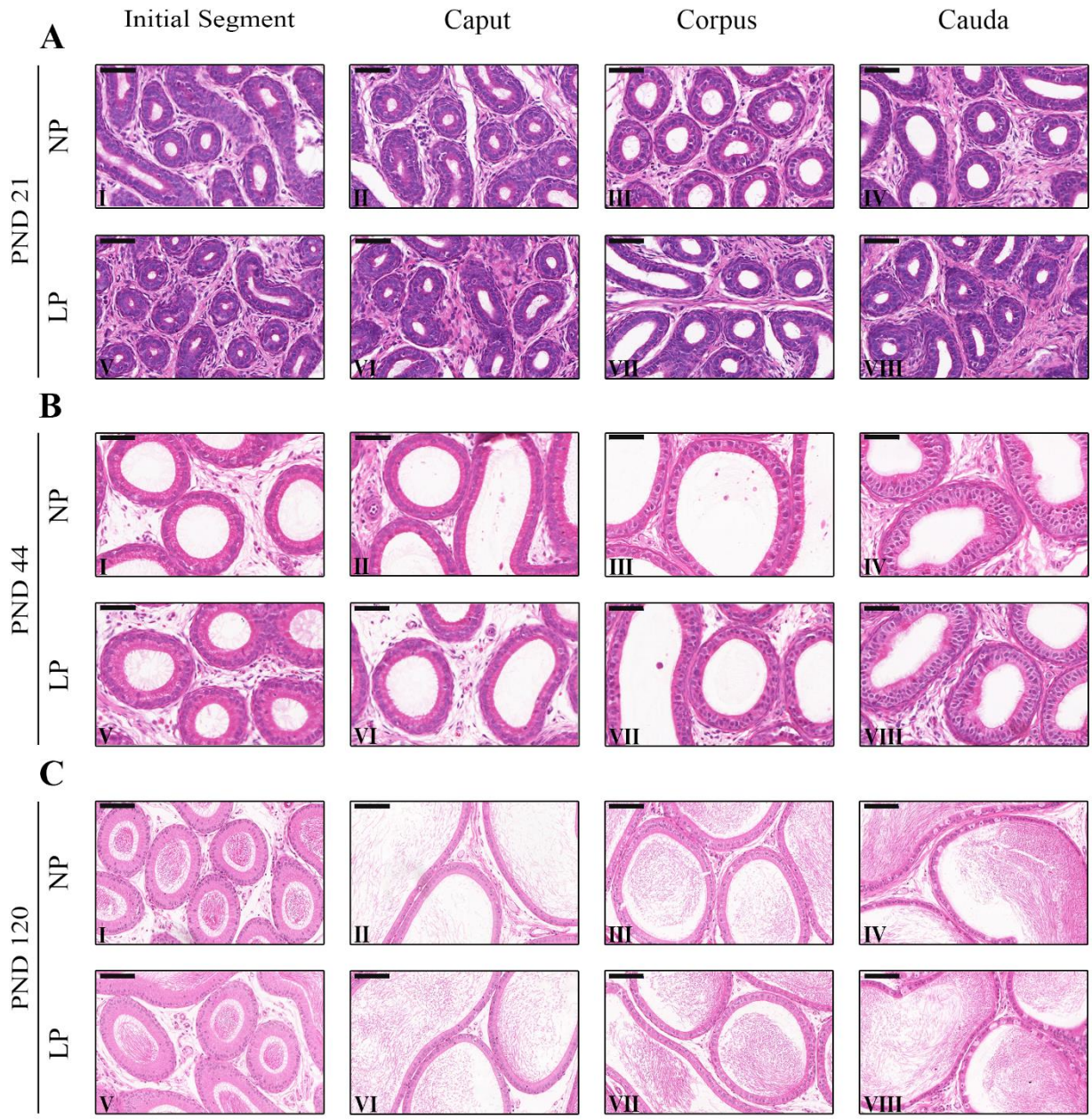


Figure 3. Morphology of the epididymides. (A) H&E staining of the epididymides of NP and LP animals at PND 21 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions. Bar = 50 µm. **(B)** H&E staining of the epididymides of NP and LP animals at PND 44 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions. Bar = 50 µm. **(C)** H&E staining of the epididymides of NP and LP animals at PND 120 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions. Bar = 100 µm.

LP animals at PND 21 presented reductions in tubular diameter, epithelial height and luminal diameter in all epididymal regions. However, the decrease in tubular diameter

in the cauda and the decreases in epithelial height in the initial segment and cauda were not significant at this age (Table 4).

At PND 44, the tubular diameter and luminal diameter were decreased nonsignificantly in all epididymal regions of animals whose mothers received a low-protein diet during gestation and lactation. Conversely, the LP animals presented a slight increase in epithelial height in the initial segment, caput and corpus, while in the cauda region, a significant decrease in epithelial height was observed (Table 4).

Table 4 – Morphometry of epididymis; tubular diameter, epithelium height and luminal diameter at PND 21, 44 and 120.

Parameters (μm)								
PND 21	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***	***NP***	***LP***
Tubular diameter	40.88 ± 1.59	30.61 ± 0.45*	48.34 ± 2.86	36.64 ± 0.85*	60.60 ± 2.33	44.66 ± 1.45*	58.57 ± 4.86	48.96 ± 4.15
Epithelium height	12.22 ± 0.05	9.73 ± 0.19	13.12 ± 0.78	10.93 ± 0.32*	16.18 ± 0.63	12.27 ± 0.24*	13.57 ± 1.09	14.15 ± 1.47
Luminal diameter	17.68 ± 1.08	10.65 ± 0.29*	21.99 ± 2.32	13.72 ± 0.12*	26.81 ± 1.56	18.36 ± 1.54*	34.45 ± 4.10	20.59 ± 2.34*
PND 44	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***	***NP***	***LP***
Tubular diameter	100.8 ± 4.36	97.91 ± 0.78	131.3 ± 12.69	114.1 ± 7.63	188.1 ± 14.56	147.0 ± 12.50	118.5 ± 2.49	113.1 ± 3.82
Epithelium height	19.44 ± 0.33	19.96 ± 0.47	15.67 ± 0.67	15.86 ± 0.68	17.91 ± 1.42	20.01 ± 1.50	24.63 ± 0.59	22.78 ± 0.25*
Luminal diameter	61.54 ± 4.63	58.51 ± 0.99	99.11 ± 14.26	84.58 ± 7.41	152.6 ± 17.42	108.1 ± 16.98	64.97 ± 1.46	63.94 ± 3.38
PND 120	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***	***NP***	***LP***
Tubular diameter	172.8 ± 11.26	173.9 ± 7.68	320.5 ± 23.68	318.9 ± 8.31	326.1 ± 8.95	298.4 ± 11.37	289,7 ± 6,12	312,3 ±19,11
Epithelium height	29.54 ± 0.40	33.38 ± 3.72	22,07 ± 1,31	21,63 ± 0,01	23.02 ± 1.12	23.95 ± 1.15	19.13 ± 1.23	18.85 ± 1.53
Luminal diameter	107.5 ± 5.52	99.77 ± 6.55	276.6 ± 21.32	273.3 ± 9.77	280.2 ±10.65	252.2 ± 10.54	239.8 ± 10.86	274.4 ± 21.86

NP, n=4; LP, n=4. The values are expressed as the mean ± S.E.M. * $p < 0.05$. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

In 120-day-old animals, maternal protein restriction had slightly decreased the tubular diameter in the caput and corpus regions and slightly increased this parameter in

the initial segment and cauda regions. Conversely, epithelial height was increased nonsignificantly in the caput and corpus regions but decreased nonsignificantly in the initial segment and cauda region. Finally, at this age, the luminal diameter was decreased nonsignificantly in all epididymal regions of LP animals, except for the cauda region, in which a slight increase in luminal diameter was observed (Table 4).

3.3. Maternal protein restriction did not alter estradiol serum levels in male offspring

In 21-day-old animals, only a slight increase in circulating estradiol was observed in rats whose mothers received a low-protein diet (1.90-fold increase in the LP group compared with the NP group). At PND 120, LP animals showed a slight decrease in this steroid hormone (0.87-fold reduction in the LP group compared with the NP group) (data not shown).

It was not possible to measure serum estradiol levels in 44-day-old animals because the values of this steroid hormone were below the lower limit of detection of the chemiluminescence technique (0.017 ng/dL). This occurred for both LP and NP animals, impeding comparison between the groups.

In male rats, the period between PNDs 42 and 55 corresponds to the peripubertal period. At this time, the levels of estradiol are very low, while the testosterone levels reach their peak [39, 40]. Therefore, although the lack of estradiol concentration measurements at PND 44 impaired the evaluation of maternal protein restriction effects on animals at this age, the low levels were expected.

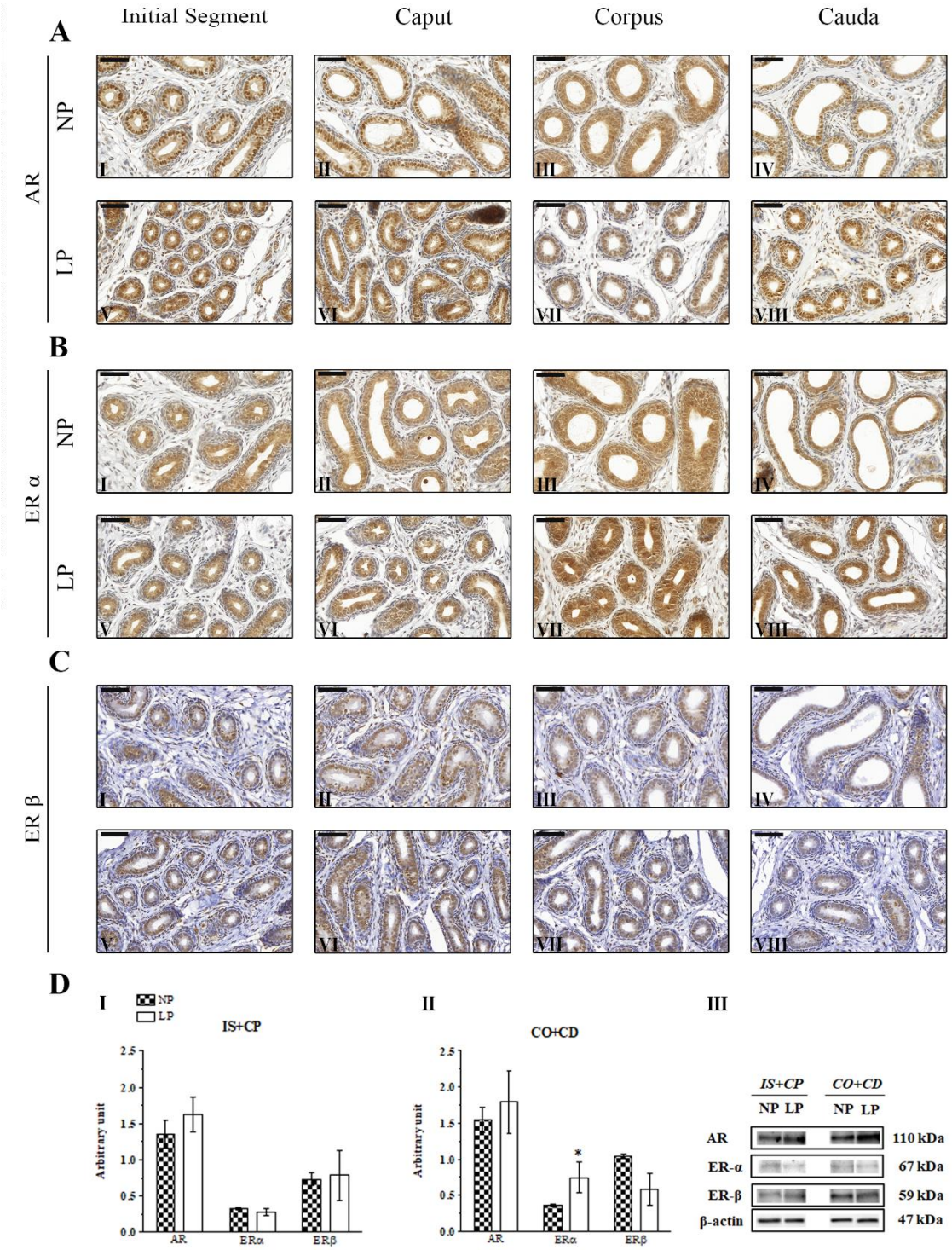
3.4. Impact of the maternal low-protein diet on AR, ERα and ERβ immunolocalization

Immunolocalization of AR, ERα and ERβ was observed in the nuclei of epididymal epithelial cells and in epididymal interstitial cells at all analyzed ages. Furthermore, in the 44- and 120-day-old animals, these receptors were also labeled in peritubular smooth muscle cells.

3.4.1. AR immunolocalization

In both groups at PND 21, the AR-labeling pattern appeared more intense and uniform in epididymal epithelial cells, whereas in mesenchymal cells, it was less intense and more heterogeneous; these findings are consistent with those of previous studies investigating the immunolocalization of AR in the epididymides of young rats [41, 42]. However, the AR-labeling intensity was slightly increased in the epididymal caput and

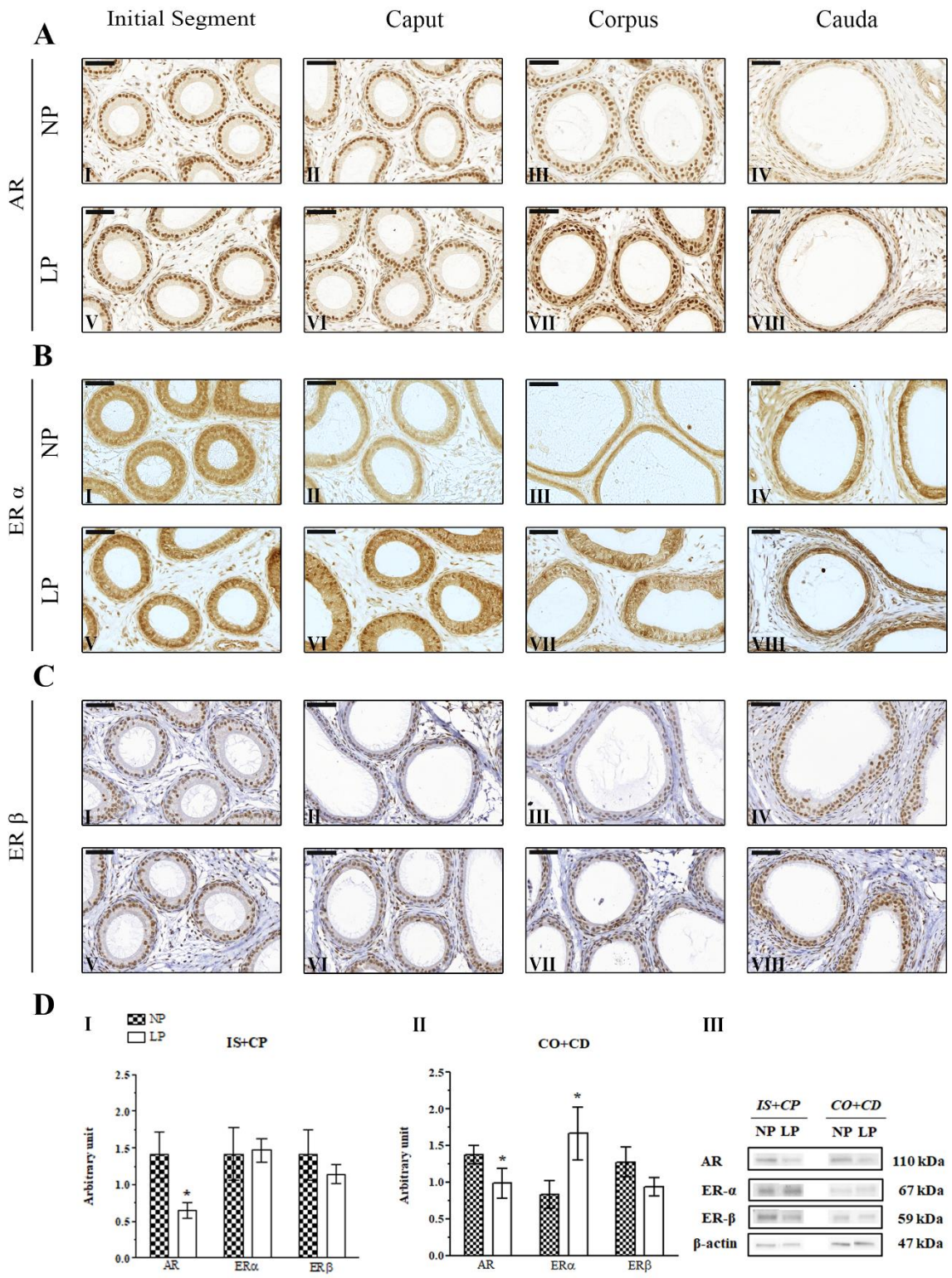
cauda in LP animals of this age compared to NP animals of this age (Figure 4 A, VI and VIII).



4. Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 21-day-old animals. (A) Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals.

(B) Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(C)** Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. Bar = 50 µm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the proteins in the initial segment plus caput **(I)** and the corpus plus cauda **(II)** regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

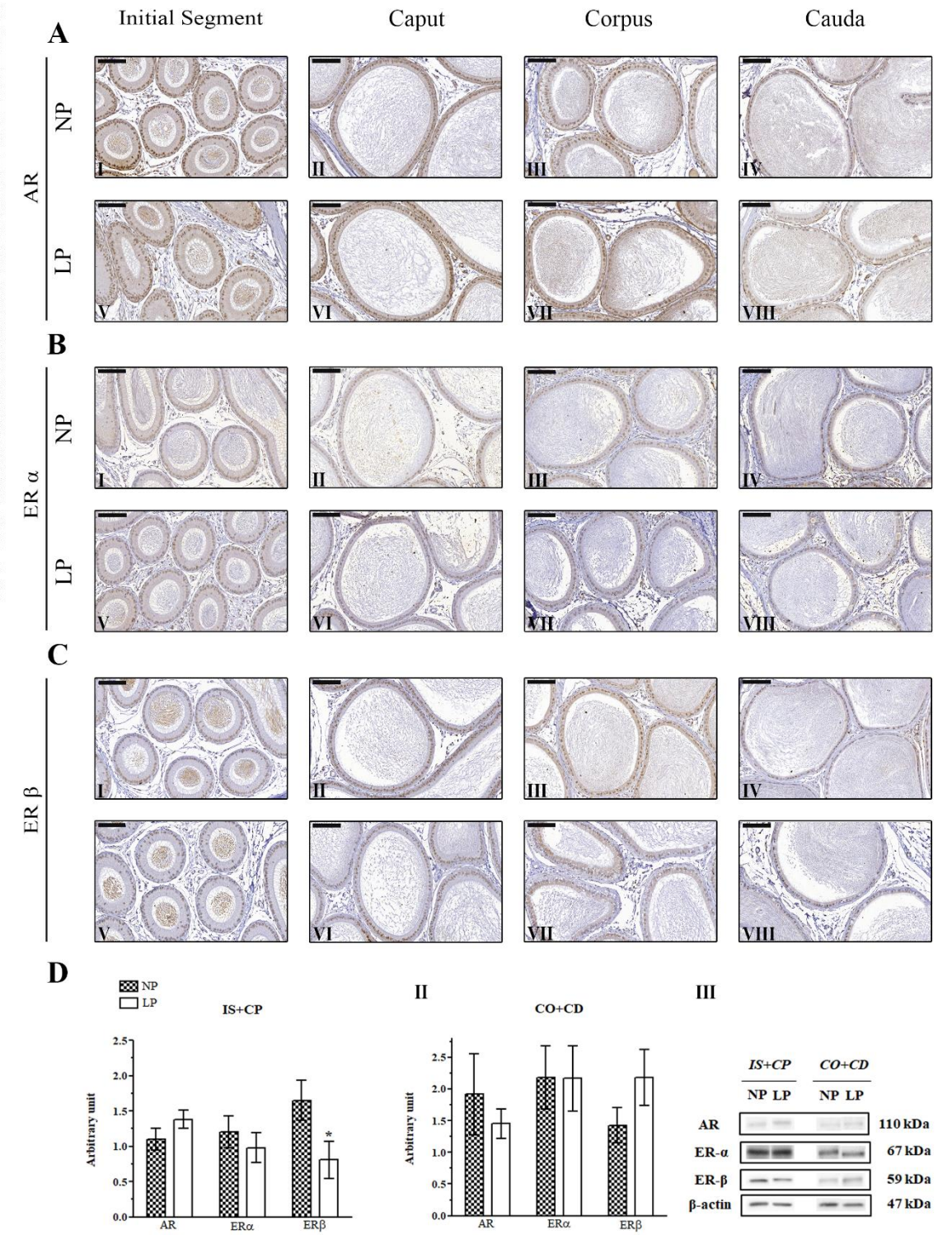
In the 44-day-old NP animals, AR immunolocalization was observed in the nuclei of epididymal epithelial cells, with slightly more intense staining in the caput and cauda regions than in the other regions of the epididymis, corroborating the findings of Yamashita [43], Perobelli, Patrao [44] and Leite, Rosa Jde [45] (Figure 5 A, I and IV). Although we observed significant reductions in AR expression in the epididymal IS+CP and CO+CD regions of LP animals at PND 44 (Figure 5, C, I and II), there were no differences in the labeling pattern for this receptor between the NP and LP groups at this age (Figure 5 A).



5. Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 44-day-old animals. (A) Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(B)** Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(C)**

Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. Bar = 50 µm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the proteins in the initial segment plus caput **(I)** and corpus plus cauda **(II)** regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

In the epididymal epithelia of 120-day-old NP and LP animals, the nuclear staining of AR was more intense and homogeneous in the principal cells than in the other cells throughout the organ (Figure 6 A, I-VIII). Clear cells of the cauda region showed quite heterogeneous staining ranging from very discrete nuclear labeling to absent labeling in these cells [42, 46-48] (Figure 6 A, IV and VIII).



6. Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 120-day-old animals. (A) Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(B)** Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(C)** Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. Bar = 100 µm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the

proteins in the initial segment plus caput **(I)** and corpus plus cauda **(II)** regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

3.4.2. ERα immunolocalization

At PNDs 21 and 44, NP animals showed mesenchymal cells with ERα labeling that was heterogeneous, moderate and only nuclear, while in the epithelial cells, the staining for this receptor appeared in a homogeneous way in the nucleus and cytoplasm throughout the epididymis. In differentiated clear cells, ERα labeling was only nuclear [42] (Figure 4 B, I-IV; Figure 5 B, I-IV). The ERα immunolocalization pattern observed in 21- and 44-day-old LP rats did not differ from that observed for NP rats at these ages. However, the intensity of cytoplasmic labeling in the cauda region was more intense in LP animals than in NP animals of both ages, staining even the clear cell cytoplasm of this region (Figure 4 B, VIII; Figure 5 B, VIII).

No studies have performed ERα and ERβ immunostaining in the epididymides of 44-day-old animals. Therefore, this study is the first to assess ERα and ERβ immunolocalization in the epididymides of rats at PND 44.

No differences were observed in the ERα immunolocalization pattern between NP and LP animals at PND 120. In both groups, nuclear and cytoplasmic ERα labeling was observed in epididymal epithelial cells, mainly in principal cells, in all regions of the organ [49, 50]. Nuclear labeling for this receptor was also observed in interstitial and peritubular smooth muscle cells throughout all regions of the epididymis [42, 49, 51] (Figure 6 B, I-VIII).

3.4.3. ERβ immunolocalization

The same pattern of ERβ immunolocalization was observed for NP and LP animals at PND 21. The intensity of nuclear and cytoplasmic ERβ labeling in epididymal epithelial cells varied considerably, and some of these cells showed a complete absence of immunoreactivity. A decreasing gradient was observed in the nuclear ERβ staining intensity from the initial segment and caput to the corpus and cauda of the organ. Immunostaining was also observed in the mesenchymal cells surrounding epididymal ducts [42, 52] (Figure 4 C, I-VIII).

There was no difference in the pattern of ERβ labeling in LP animals compared to NP animals at PND 44. In both groups, epithelial cells, mainly principal cells, showed homogeneous nuclear immunostaining and discrete cytoplasmic labeling throughout the epididymis, with elevated nuclear staining intensity in the cauda region. In addition, heterogeneous nuclear labeling was observed in mesenchymal and peritubular smooth muscle cells in all regions of the organ (Figure 5 C, I-VIII).

The 120-day-old NP and LP animals showed the same pattern of ERβ immunostaining. Nuclear and cytoplasmic labeling of this hormone receptor was observed in epithelial cells throughout the epididymis. Principal cells presented homogeneous nuclear staining, while the other epididymal epithelial cells, peritubular smooth muscle cells and interstitial cells showed heterogeneous labeling [42, 43, 50, 53] (Figure 6 C, I-VIII). Nuclear labeling of epithelial cells in the caput region was weak in LP animals compared to NP animals (Figure 6 C, IV, VIII).

3.5. A maternal low-protein diet changes AR, ERα and ERβ expression in the epididymides of offspring in an age-dependent manner

A low-protein diet during gestation and lactation significantly decreased AR expression in the IS+CP (0.50-fold decrease in the LP group compared with the NP group) and CO+CD (0.72-fold decrease in the LP group compared with the NP group) at PND 44 (Figure 5 D, I and II). In 21-day-old animals, maternal protein restriction slightly increased AR expression in the IS+CP (1.20-fold increase in the LP group compared with the NP group) and CO+CD (1.17-fold increase in the LP group compared with the NP group) (Figure 4 D, I and II). In addition, at PND 120, this experimental model resulted in a nonsignificant reduction in the AR levels in the CO+CD region of the epididymis (0.76-fold decrease in the LP group compared with the NP group) (Figure 6 D, II).

The low-protein diet significantly increased ERα expression in the CO+CD region at PNDs 21 and 44 (PND 21: 2.10-fold decrease in the LP group compared with the NP group; PND 44: 2.01-fold decrease in the LP group compared with the NP group) (Figure 4 D, II; Figure 5 D, II). ERα expression was only slightly reduced in the IS+CP region of LP animals at PND 21 (0.85-fold decrease in the LP group compared with the NP group) and in the IS+CP and CO+CD regions of LP animals at PND 120 (IS+CP: 0.82-fold decrease in the LP group compared with the NP group; CO+CD: 0.99-fold decrease in the LP group compared with the NP group) (Figure 4 D, I; Figure 6 D, I and II).

There was no difference in ERβ expression between NP and LP animals at PNDs 21 and 44. Only in 120 day-old animals was a significant decrease in ERβ expression observed in the IS+CP region (0.50-fold decrease in the LP group compared with the NP group) (Figure 6 D, I).

3.6 Aromatase p450, but not 5α-reductase, expression is altered by maternal protein restriction

The cytochrome P450 aromatase enzyme is responsible for the aromatization of testosterone to 17β-estradiol, one of the main endogenous estrogens, and is thus a major source of this steroid in the male genital system [54-58]. The enzyme 5α-reductase mediates the conversion of testosterone to dihydrotestosterone, which is five to ten times more potent than testosterone and has a higher affinity for AR [59-61].

A recently published study by our research group showed that maternal protein restriction during gestation and lactation significantly decreased serum testosterone levels in 44-day-old animals [15]. However, the epididymal expression of 5α-reductase was unchanged at all of the analyzed ages (Figure 7). Notably, we observed a significant increase in aromatase p450 expression in the CO+CD region of the epididymis in PND 21 animals (2.64-fold increase in the LP group compared with the NP group) (Figure 7 A, II).

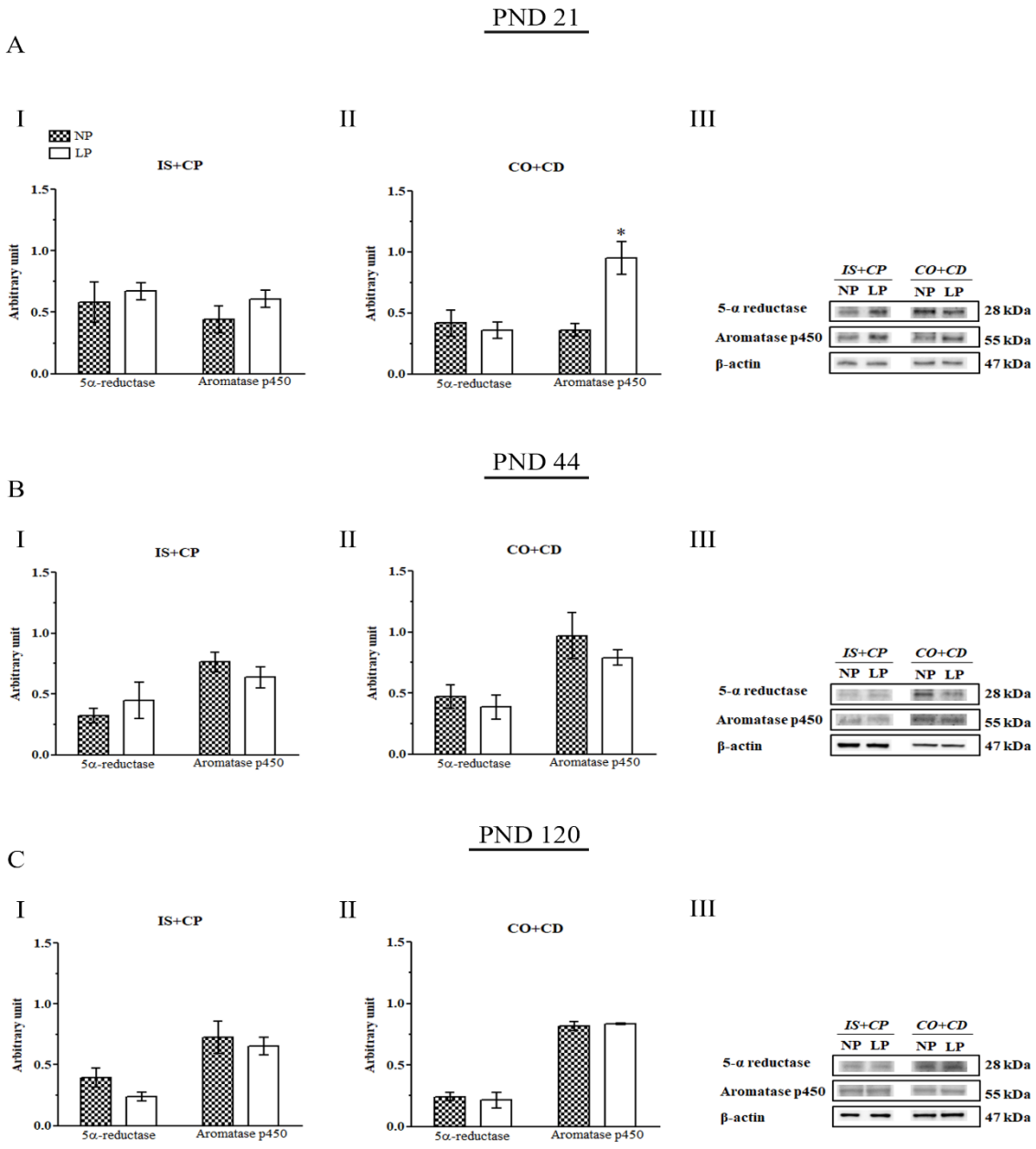


Figure 7. 5α-reductase and aromatase p450 immunoblots. (A) Levels of 5α-reductase and Aromatase p450 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of 5α-reductase, aromatase p450 and β-actin (70 µg of protein) in 21-day-old animals **(III)**. **(B)** Levels of 5α-reductase and aromatase p450 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of 5α-reductase, aromatase p450 and β-actin (70 µg of protein) in 44-day-old animals **(III)**. **(C)** Levels of 5α-reductase and Aromatase p450 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of 5α-reductase, aromatase p450 and β-actin (70 µg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

3.7. Maternal protein restriction changed both Src 416 and Src 527 expression in an age-dependent manner

The low-protein diet increased Src 416 expression in the IS+CP (2.35-fold increase in the LP group compared with the NP group) and CO+CD (2.38-fold increase in the LP group compared with the NP group) regions of 21-day-old animals. In addition, at this same age, the Src 416 increase appeared to be accompanied by a decrease in Src 527 expression in the IS+CP region (0.33-fold decrease in the LP group compared with the NP group) in LP animals (Figure 8 A, I, II).

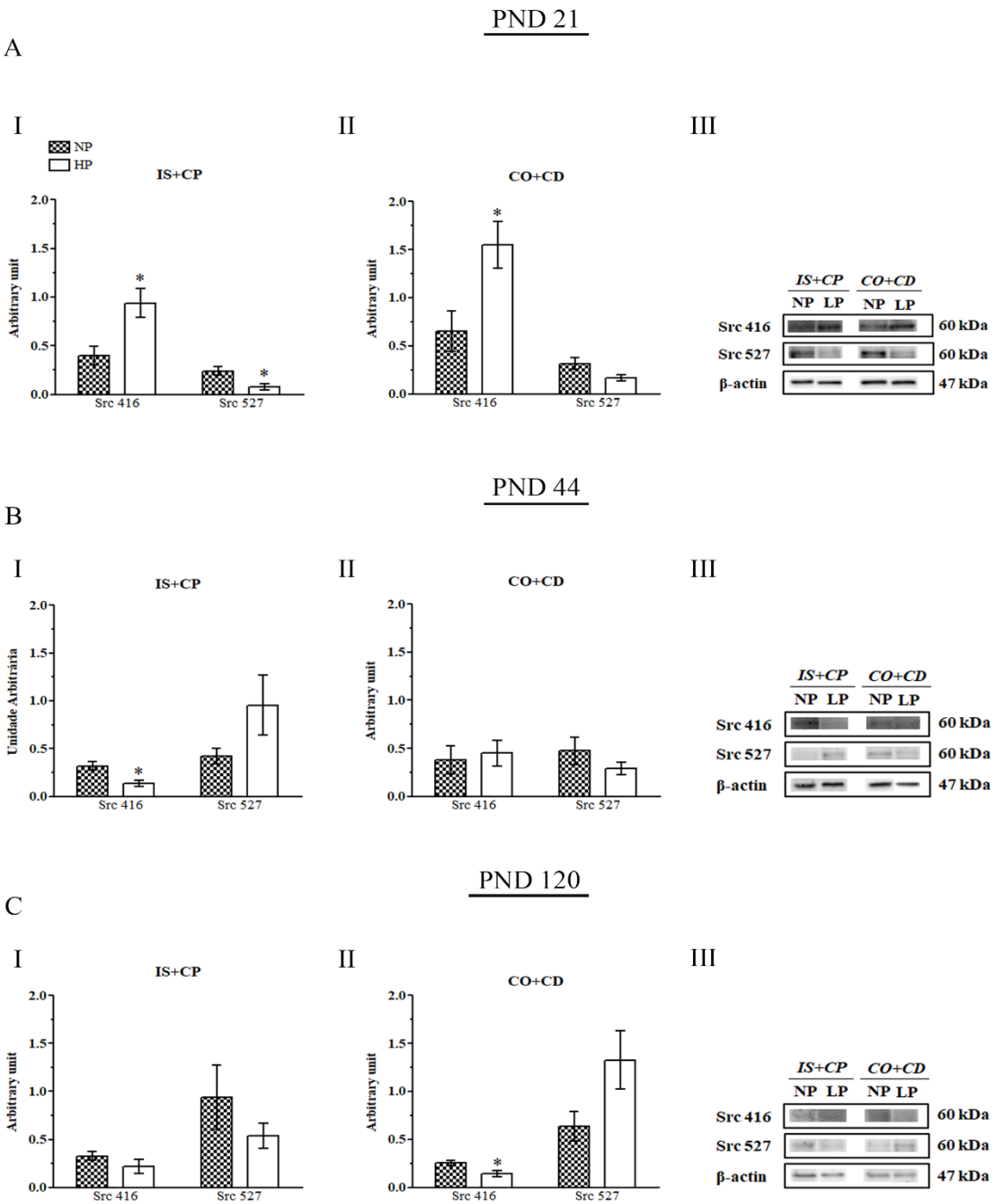


Figure 8. Src 416 and Src 527 immunoblots. (A) Levels of Src 416 and Src 527 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 µg of protein) in 21-day-old animals **(III)**. **(B)** Levels of Src 416 and Src 527 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 µg of protein) in 44-day-old animals **(III)**. **(C)** Levels of Src 416 and Src 527 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 µg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

At PND 44, LP animals presented a significant decrease in Src 416 expression in the IS+CP region (0.44-fold decrease in the LP group compared with the NP group), while Src 527 expression was increased nonsignificantly in this region (2.26-fold increase in the LP group compared with the NP group) (Figure 8 B, I).

In adulthood (PND 120), we also observed a significant decrease in Src 416 expression accompanied by a nonsignificant increase in Src 527 expression in animals whose mothers received a low-protein diet during gestation and lactation (0.56-fold decrease in Src 416 levels in the LP group compared with the NP group; 2.06-fold increase in Src 527 levels in the LP group compared with the NP group). However, for PND 120, these results were observed in the CO+CD epididymal region of LP rats (Figure 8 C, II).

3.8. The low-protein diet increased Cldn-1 expression throughout the epididymis

Maternal protein restriction during gestation and lactation increased Cldn-1 expression in the IS+CP and CO+CD epididymal regions at all ages analyzed. However, these results were only significant for Cldn-1 in the CO+CP region (2.17-fold increase in the LP group compared with the NP group) on PND 44. At this age, Cldn-1 expression was only slightly increased in the IS+CP region (1.3-fold decrease in the LP group compared with the NP group) (Figure 9 B, I and II).

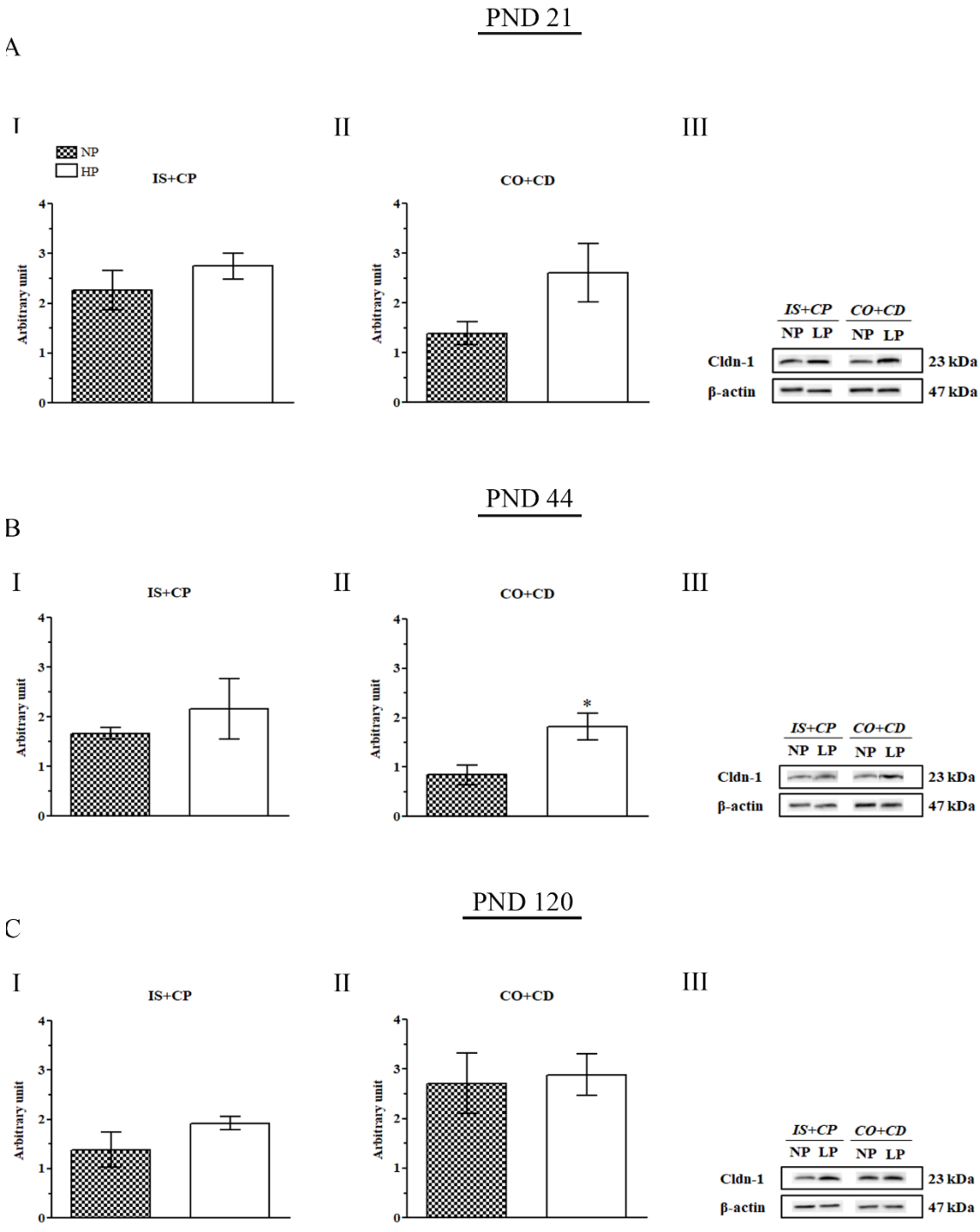


Figure 9. Cldn-1 immunoblot. (A) Levels of Cldn-1 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of Cldn-1 and β-actin (70 µg of protein) in 21-day-old animals **(III)**. **(B)** Levels of Cldn-1 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of Cldn-1 and β-actin (70 µg of protein) in 44-day-old animals **(III)**. **(C)** Levels of Cldn-1 in the IS+CP **(I)** and CO+CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of Cldn-1 and β-actin (70 µg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. *p <0.05, Mann-Whitney test.

Importantly, although the difference was not significant, Cldn-1 expression was increased in the IS+CP (1.22-fold increase in the LP group compared with the NP group) and CO+CD (1.88-fold increase in the LP group compared with the NP group) regions at PND 21 (Figure 9 A, I and II). This same pattern of Cldn-1 expression was observed in 120-day-old animals, both in the IS+CP (1.39-fold increase in the LP group compared with the NP group) and CO+CD (1.06-fold increase in the LP group compared with the NP group) regions (Figure 9 C, I and II).

4. Discussion

Low birth weight is an important sign of malnutrition during pregnancy and a crucial indicator of slow fetal growth [62]. Adverse intrauterine nutritional conditions are able to program a series of adaptations in the developing fetus constituting an "economic" phenotype in order to increase the chances of immediate survival of the fetus and to grant advantages in a postnatal environment of nutritional scarcity [63]. Therefore, the fetus interacts with the maternal environment dynamically in an attempt to predict the environment in which it is likely to be born, adapting for future competitive advantage [64].

Some studies have shown that maternal protein restriction does not alter offspring weight at birth, whereas others have demonstrated that insufficient protein intake during gestation and lactation significantly reduces the birth weights of both male and female pups [65-72]. The lower birth weight observed in the offspring whose mothers were fed a low-protein diet could be related to adaptation to a protein-deficient intrauterine environment, preparing the pups' bodies to survive in a postnatal environment where the protein supply would also be low. Furthermore, during pregnancy, increased protein intake is recommended to attend the additional demand for nitrogen required by both mother and fetus [23]. With reduced protein intake, the pregnant rats of the LP group could not provide their developing offspring enough protein to reach a size similar to that of the offspring of rats fed a normal-protein diet. The CRLs of LP pups were also significantly lower than the CRLs of NP pups, consistent with their lower birth weights and with the results of other studies using this experimental model [13, 73].

AGD is a marker of sexual differentiation that reflects the action of androgenic hormones during the formation of the genital system in the uterus, being on average twice as large in males as in females [74-76]. Anogenital distance is usually regulated by

testosterone produced by fetal testicles and is also affected by maternal androgens via the placenta [74]. However, AGD reduction in LP pups could be correlated to the size of the pup independently of its intrauterine environment, since lighter and smaller animals tend to have significantly shorter AGDs than larger animals [74, 77, 78].

The difference in AGD and in CRL between NP and LP animals was maintained until PND 44, showing that maternal protein restriction during gestation and lactation delays the development of male offspring. However, when we observed the value of the AGD normalized by the CRL value, this difference no longer appear, indicating that at PNDs 21 and 44, the increase in AGD was proportional to the increase in CRL in LP animals, causing the ratio of these values to approach that of the values found for NP animals.

There is a maximum potential growth of anogenital distance programmed in utero [76, 79]. In male rats, AGD lengthens until PND 38, remaining constant from that period, and responds to hormonal stimuli during pubertal development, being negatively modulated by high doses of antiandrogens and testosterone [76]. Thus, the maintenance of AGD reduction found in LP animals could be a consequence of protein restriction-induced programming during intrauterine life in addition to being a response to the slight increase in serum testosterone levels observed in 21-day-old animals. The decrease in AGD was maintained until PND 44, even with the significant reductions in testosterone levels observed in LP animals at this age (data related to testosterone levels are included in a recently published study from our research group, Cavariani, de Mello Santos [15]).

Maternal protein restriction is able to produce offspring with growth restriction at birth and subsequent catch-up growth [80, 81]. However, in several studies in which pregnant rats were fed a low-protein diet, the pups had reduced body weights at birth that were maintained up to one year of age [12, 72, 73, 82-84]. Consistent with these results, we observed lower body weights of the male offspring of the LP group compared to the NP group at birth and at all analyzed ages. These findings show that the components and quality of the maternal diet during critical periods of development may alter the development of offspring in the uterus and permanently modify the phenotypes of the adult organisms.

Few epidemiological and experimental studies have addressed the effects of maternal protein restriction on offspring reproductive aspects, especially male

reproductive aspects. Regarding the weight of genital system organs, the literature is controversial; studies have yielded results ranging from reductions in this parameter to no alterations in this parameter in animals whose mothers received a low-protein diet during gestation and lactation [12, 20-22].

The testes are the organs where gamete production occurs, while the epididymides are responsible for the storage, protection, concentration and maturation of these gametes. Therefore, the survival of mammals depends on these organs being fully functional [16-18] As previously mentioned, some effects of protein restriction are direct consequences of the alteration in the availability of substrate, and during pregnancy, increased protein intake is recommended to supply the requirements of rapid embryo growth [8, 23]. In this context, the increased relative weight of these organs in 21-day-old LP animals could be an attempt to preserve their full functionality despite the poor nutritional environment to which the animals were exposed during their development and early postnatal life. However, even though the epididymal weight in relation to body weight was higher in LP animals at this age, the epididymal morphometry showed that the diameter of the epididymal duct, the diameter of the epididymal lumen, and the height of the epithelium were significantly smaller in LP animals compared to NP animals.

According to the "thrifty phenotype hypothesis", adverse intrauterine nutritional conditions are able to program adaptations in the developing fetus to increase its chances of immediate survival and to confer advantages for its long-term survival in a postnatal environment of nutritional scarcity [63]. The phenotype of an organism will tend to be normal if there is similarity between the pre- and postnatal environments. However, if the postnatal environment is incompatible with the predicted environment, fetal programming will make the organism susceptible to metabolic diseases [85-87]. Indeed, several studies have shown that a maternal low-protein diet during gestation and lactation can lead to permanent metabolic changes in offspring, even if the offspring have access to normal-protein diets after weaning [12-15, 88]. In the current study, animals from both groups were fed a normal-protein diet after weaning (PND 21). The increase in protein intake by the animals of the LP group seems to have been enough to increase the weight of these animals in relation to the weight of their organs and to increase epididymal diameter and epithelial height, since the increases in the relative weights of the testes, epididymides, and seminal glands and the decreases in epididymal morphometry observed for LP animals at PND 21 no longer appeared in these animals at PNDs 44 and

120. However, we cannot affirm that this restoration was enough to prevent changes in the functions of these organs.

At 44 days, the absolute and relative weights of the empty seminal glands were lower in LP animals than in NP animals. As these organs are very sensitive to testosterone, these decreases could be directly related to the lower concentrations of this hormone observed in these animals [15]. Rats at PND 120 are sexually mature, and their seminal glands are full of a fluid that contributes to the coagulation of ejaculated semen [89]. The increase in empty seminal gland weight in 120-day-old LP animals could have been due to a higher concentration of this fluid in animals of this group, independent of their seminal gland size.

AR is a member of the steroid receptor superfamily that plays a key role in the action of androgenic hormones [90]. Increases in testosterone levels appear to be accompanied by increased expression of AR [91, 92] while decreases in testosterone levels are followed by reductions in the expression of this hormone receptor [47, 93]. Thus, the nonsignificant increases in the expression of AR in the epididymal IS+CP and CO+CD regions observed in 21-day-old LP animals could have been a consequence of a nonsignificant increase in serum testosterone levels in these animals [15]. Similarly, the reductions in AR expression in both the IS+CP and the CO+CD regions of the LP animal epididymides at PND 44 could have been related to the decreases in the levels of this androgenic hormone observed in these animals [15].

In the 120-day-old LP animals, the nonsignificant decrease in serum testosterone levels appeared to be accompanied by a nonsignificant decrease in AR expression in the CO+CD region, consistent with the results obtained for the LP animals at PND 44. However, in the epididymal IS+CP regions of these animals, the AR expression was slightly increased, suggesting possible nonandrogenic regulation of this receptor. Although several studies have demonstrated a positive effect of testosterone on AR expression, the mechanisms by which this regulation occurs are still not completely understood [47, 91, 94-96]. Furthermore, in addition to testosterone, AR can be transactivated by Src kinase through Tyr 543 phosphorylation, thereby triggering an extensive set of AR-dependent genes [97, 98]. Moreover, prostate samples of men with castration-resistant prostate cancer present increased Src pathway activity in tumors with low AR activity, suggesting that Src activity probably has a strong negative correlation

with AR activity [99]. The slight increase in AR expression observed in the IS+CP region in LP animals at PND 120 could therefore have been a response to the slight decreases in both Src 416 and Src 527 expression found in this epididymal region.

Several studies have shown the existence of crosstalk between Src and AR [97, 98, 100]. Src is able to mediate AR phosphorylation, resulting in nuclear translocation and AR-responsive transcription [97, 98]. Recently, an inverse AR and Src regulatory network has been supported in which AR may act on Src through microRNA (miR) expression modulation, thus regulating Src expression in a posttranscriptional way [101-103]. AR transcribes and regulates miR-1 and miR-203, which in turn decrease Src expression; therefore, AR negatively regulates Src through miRs [102, 103].

Due to the slight increase in AR expression in LP animals at PND 21, we expected to find decreased Src expression. However, this decrease was noticed only for Src 527, while Src 416 was significantly increased. Src activity is regulated by tyrosine phosphorylation at two different sites with opposite effects. Phosphorylation of Y416 in the activation loop of the kinase domain promotes enzyme activation, whereas phosphorylation of Y527 in the carboxy-terminal tail renders the enzyme less active [104]. The increase in Src 416 expression in LP animals at PND 21 could have been an attempt to preserve epididymal development and functionality in the face of the poor nutritional conditions to which these animals were exposed during their early postnatal life. This increase in Src 416 expression could also have been related to the increased relative epididymis weight observed in these animals.

After weaning, both NP and LP animals were fed normal-protein diets. The increase in protein supply appeared to have been enough to meet the epididymal growth needs at PND 44 as well as the epididymal maintenance needs in adulthood, as we observed decreased Src 416 expression in the IS+CP region in 44-day-old LP animals and in the CO+CD region in 120-day-old LP animals. In addition, the decreased Src 416 appeared to be accompanied by a nonsignificant increase in Src 527 expression and by a decrease in AR expression in those same regions. Importantly, Src is incorporated into sperm during sperm maturation in the epididymides, being essential for sperm motility and for sperm function in fertilization [105]. Therefore, the decrease in Src 416 expression in the CO+CD regions of LP animals at PND 120 could have been related to sperm alterations previously observed by other authors using this experimental model [20, 22],

which could compromise the fertility of adult animals whose mothers were subjected to protein restriction during gestation and lactation.

Although testosterone is the main regulating hormone of epididymal functions, estrogen is also produced and acts in males, regulating the functions of the epididymides, particularly those related to the reabsorption activity that occurs in these organs [106, 107]. In immature males, the main sources of estrogen are Sertoli cells, while in adults, germ cells show elevated expression of the enzyme aromatase, being the major sources of this steroid in the male genital system [54-56]. Estrogen acts through ERα and ERβ nuclear receptors, both of which are present in the epididymides [42, 108, 109].

Administration of estradiol to adult male rats results in increased ER expression in the genital systems of these animals [48, 110, 111]. Similarly, antiestrogenic substances can reduce ER expression, demonstrating the ability of estrogens to regulate the expression levels of their own receptors [112]. In addition, androgenic regulation also has an impact on the expression of ERα and aromatase p450, with this type of receptor and this enzyme, respectively, being positively modulated by testosterone in organs of the male genital system [111, 113, 114]. A recently published study by our research group showed that maternal protein restriction during gestation and lactation significantly decreased serum testosterone levels in 44-day-old animals [15].

Thus, the discrete increase in estradiol levels accompanied by the slight increase in serum testosterone levels in 21-day-old LP animals [15] could have been responsible not only for the increased expression of the aromatase p450 throughout the epididymides but also for the increase in ERα expression observed in the CO+CD epididymal regions of these animals.

Serum estradiol levels in the 44-day-old animals were below the detection level of the chemiluminescence technique and therefore could not be quantified. However, a recent study published by our research group showed that, at this age, testosterone levels are significantly lower in animals whose mothers have received a low-protein diet than in animals whose mothers have received a normal-protein diet [15]. The increase in ERα expression in the CO+CD region in LP animals at PND 44 could represent a compensatory mechanism given the reduced serum concentrations of testosterone. In adulthood, a protease removes DNA from the binding portion of ERα in the epididymides, showing that the action and influence of estrogen on these organs changes with age and

that its action is greater during epididymal development than during adulthood [115, 116]. This mechanism may explain the fact that ERα expression was increased in the CO+CD regions of LP animals at PNDs 21 and 44 but did not remain altered at PND 120.

Regarding ERβ expression, no differences were observed between NP and LP animal epididymides at PNDs 21 and 44. Estrogens have the ability to differentially regulate the expression of the two ER types [112]. Thus, the decrease in ERβ expression observed in the IS+CP epididymal region in LP animals at PND 120 could have been a direct result of the slight decreases in serum estradiol levels observed in these animals without alteration of ERα expression.

Cldn-1 is a transmembrane protein that integrates the blood–epididymal barrier, whose structure and integrity are crucial for maintenance of the specific epididymal intraluminal environment [27-30]. It was observed that a low-protein diet increased Cldn-1 expression in the epididymides of the animals at all analyzed ages, although this increase was significant only in the CO+CD epididymal region at PND 44. Data regarding the expression patterns of AQPs 1 and 9 in a study using this same experimental model demonstrated that despite the fact that a decrease in AQP9 expression in the IS+CP region and increases in AQP1 and AQP9 expression in the CO+CD region were significant only for LP animals at PND 44, these changes were also observed in LP animals at 21 and 120 days of age. The decrease in AQP9 expression in the IS+CP region could have resulted in reduced water absorption; consequently, a greater amount of water could have been present in the epididymal lumen of this region and could have reached the epididymal CO+CD region, which could have led to the appearance of edema in the epididymides [15]. Therefore, the increase in Cldn-1 expression observed in the epididymides of LP animals could have been an attempt to preserve the structure and conformation of the organ until AQP9 drained the excess water that was not absorbed by the IS+CP region, which would then be removed from the epididymal intertubular space by AQP1, preventing edema appearance and keeping the intraluminal environment balanced.

In summary, in the offspring whose mothers had limited protein intake during gestation and lactation, the reduced size and low weight observed at birth remained until adulthood. In addition, decreases in the weights of male genital system organs were observed in rats of all analyzed ages, demonstrating the importance of maternal diet quality for offspring and showing that changes in the components of this diet can

permanently change the phenotypes of the adult organisms. Maternal protein restriction was able to alter the structure and functioning of the developing epididymides, since it altered the expression of proteins important for the regulation, development and maintenance of the organs as well as the hormone receptors AR, ERα and ERβ, Src 416 and Src 527 and Cldn-1. Therefore, although some of these changes did not remain until adulthood, insufficient supply of proteins in early life altered the structure and functioning of the epididymides in important periods of postnatal development, which may have contributed to the appearance of spermatic changes related to sperm motility, viability and concentration that could compromise the fertility of adult animals.

Author contributions

MMC, TMS, DNP, RFD: contributed actively to the elaboration of the study main idea, participated to the animal care, collected, analysis and interpretation of the data and wrote the manuscript. LGAC, PFFP: contributes to the acquisition of material, with the experimental design and analysis and interpretation of the data. All authors critically reviewed the paper and approved its final version.

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Conflicts of Interest

The authors declare no conflict of interest.

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Conclusão

Nos filhotes cujas mães tiveram aporte de proteínas limitado, o tamanho reduzido e baixo peso observados ao nascimento se mantiveram até o último dia de coleta (DPN 120), acompanhados da redução dos órgãos do sistema genital masculino para todas as idades analisadas. Demonstrando a importância da qualidade da dieta materna para a prole, e evidenciando que alterações nos componentes desta dieta podem mudar permanentemente o fenótipo do organismo adulto.

A restrição proteica materna mostrou-se capaz de levar a alterações na estrutura ou no funcionamento de órgãos e/ou funções metabólicas do organismo em formação, uma vez que alterou os níveis séricos de testosterona e aldosterona da prole masculina e a expressão de proteínas importantes para o bom funcionamento do epidídimo como os receptores hormonais AR, ERα e ERβ, a enzima aromatase p450, as AQP1 e AQP9, o VEGFr-2, as Src 416 e Src 527 e da Cldn-1, além de alterar o padrão vascular do órgão.

Mesmo que algumas destas mudanças não tenham se mantido até a vida adulta, a oferta insuficiente de proteínas no início da vida alterou a estrutura e o funcionamento do epidídimo em períodos importantes do desenvolvimento epididimário pós-natal, o que poderia levar à alterações das funções epididimárias relacionadas à motilidade, viabilidade e concentração espermáticas, contribuindo para o aparecimento de alterações espermáticas previamente observadas por outros autores utilizando este modelo experimental.

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Apêndices

Parâmetros maternos

O desenvolvimento normal da prole, *in útero* ou no período pós-natal inicial que corresponde à amamentação, está diretamente relacionado ao estado nutricional materno adequado. Por esse motivo, a maior parte dos trabalhos envolvendo programação fetal utiliza como modelo experimental a desnutrição materna, seja na forma de restrição proteica, calórica ou alimentar (Grissom *et al.*, 2014; Carolan-Olah *et al.*, 2015; Lee, 2015; Schagdarsurengin e Steger, 2016).

O monitoramento de parâmetros maternos durante a realização de um experimento cujo objetivo é verificar o impacto da dieta materna no desenvolvimento da prole, é de extrema relevância para ajudar a entender os resultados obtidos, uma vez que o feto em desenvolvimento depende totalmente da mãe e do ambiente materno para suprir suas necessidades nutricionais. Assim, muitos dos trabalhos que tem a restrição proteica materna durante gestação e lactação como modelo experimental, se preocupam em avaliar não só os aspectos relacionados às proles, mas também os relacionados ao ganho de peso materno e ao seu consumo alimentar (Gao *et al.*, 2015; Batista *et al.*, 2016; Gonzalez *et al.*, 2016).

No presente estudo, não foi observada diferença entre os pesos corpóreos maternos dos animais dos grupos NP e HP durante a gestação (figura 2).

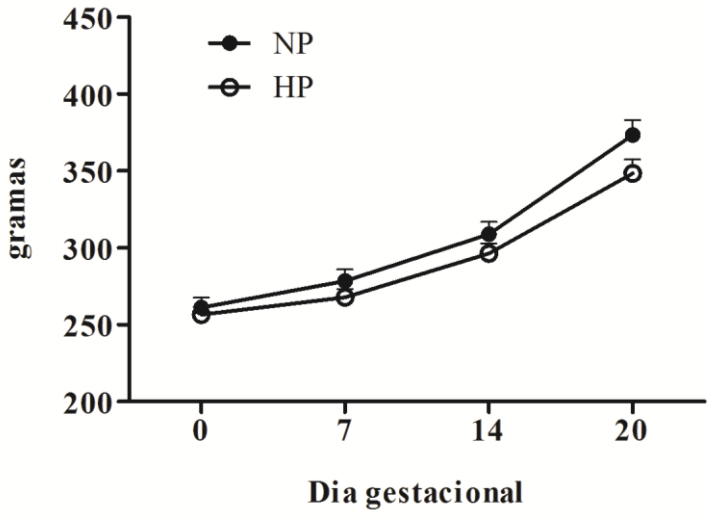

Figura 2. Evolução do peso materno. Evolução do peso corpóreo materno das fêmeas tratadas com ração normoproteica (NP) e hipoproteica (HP) nos DG 0, 7, 14 e 20. Dados expressos em média ± E. P. M. Teste t.

Na literatura, dados referentes ao efeito da dieta hipoproteica sobre a evolução do peso corporal de ratas prenhes durante a gestação variam desde nenhuma diferença, até significativa redução quando comparadas a ratas que receberam ração normoproteica (Zeman, 1967; Rees *et*

al., 1999; Parimi *et al.*, 2004; Whitaker *et al.*, 2012; Gao *et al.*, 2015; Batista *et al.*, 2016; Gonzalez *et al.*, 2016).

Quanto ao consumo de ração, foi observado que entre a primeira e a terceira semanas de tratamento, período que corresponde à gestação, a restrição proteica resultou no aumento da ingestão alimentar pelas mães. No entanto, ao observarmos o consumo alimentar durante a quarta e quinta semanas, período que corresponde à lactação, nota-se uma queda significativa do consumo de ração por parte das mães do grupo HP quando comparadas ao grupo NP (figura 3).

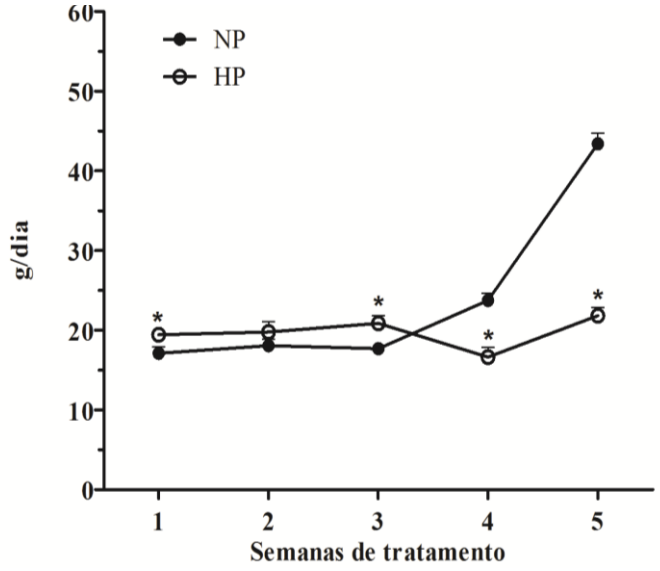

Figura 3. Consumo alimentar materno. Consumo de ração diária por semana das fêmeas tratadas com ração normoproteica (NP, n=19) e hipoproteica (HP, n=19) do DG 0 ao DPN 14 (1-5 semanas de tratamento). Dados expressos em média ± E. P. M. *p<0,05. Teste t.

O aumento do consumo de ração pelas mães alimentadas com dieta hipoproteica durante a gestação já foi observado em estudos conduzidos por Zeman (1967), Desai *et al.* (1996), Ballen *et al.* (2009) e Gao *et al.* (2015). A explicação para esse resultado pode estar no fato de que o metabolismo proteico durante a gestação normal em ratos está dividido em fases anabólicas, quando a fêmea prenhe acumula reservas de nutrientes e catabólicas, quando a mãe mobiliza estas reservas para suprir o rápido crescimento fetal (Naismith e Morgan, 1976). Dados da literatura demonstram que indivíduos submetidos a dietas deficientes em algum nutriente, tendem a aumentar a ingestão alimentar na tentativa de compensar a deficiência nutricional (Widdowson e Mccance, 1975). Desta maneira, as fêmeas do grupo HP poderiam ter aumentando seu consumo alimentar no período gestacional, principalmente na fase anabólica, na tentativa de suprir a deficiência proteica da ração. Esses resultados são condizentes com os resultados obtidos para o peso corporal, onde as mães do grupo HP

mantiveram peso corpóreo semelhante ao grupo NP apesar do consumo de ração ter sido significativamente maior neste grupo. Assim, o aumento da ingestão de ração, das ratas do grupo HP, não foi determinante para o aumento de peso corpóreo e, portanto, deve ter sido de fato uma tentativa de compensar a deficiência nutricional desse grupo.

Além disso, a gordura armazenada durante a gravidez contribui de forma significativa para suprir os custos energéticos deste período e também da lactação que está por vir, além disso, o leite de ratas submetidas à dieta hipoproteica possui concentração de gordura significativamente maior do que o leite de ratas que receberam dieta normoproteica (Naismith *et al.*, 1982; Pine *et al.*, 1994). Assim, mesmo ingerindo uma quantidade maior de ração, as fêmeas do grupo HP precisaram mobilizar grande parte de suas reservas energéticas durante a gestação (em grande parte na forma de gordura e proteína) para produção de leite, o que resultou em peso semelhante deste grupo quando comparado ao grupo NP (Torres *et al.*, 2010).

A queda significativa no consumo de ração por parte das ratas do grupo HP durante a lactação, poderia ser explicada pelo ligeiro aumento de sacarose, utilizada como fonte compensatória de carboidrato, na dieta hipoproteica. Estudo realizado previamente, em ratos, constatou que a sacarose em dietas com disponibilidade de proteína reduzida aumenta a aversão dos animais à comida durante a gestação e a lactação (Wilson, 1997). Além disso, dados da literatura demonstram que a restrição proteica materna e modelos de restrição de crescimento intra-uterinos durante a gestação e lactação diminuem o peso e o tamanho dos filhotes ao nascimento e alteram a composição e o volume do leite produzido (Pine *et al.*, 1994; O'dowd *et al.*, 2008; Ballen *et al.*, 2009; Rebelato *et al.*, 2013; Claycombe *et al.*, 2015; Gonzalez *et al.*, 2016). Filhotes menores apresentam comportamento de amamentação alterado, o que, por sua vez, diminui o estímulo de sucção, que influencia o apetite materno durante a lactação (Zeman, 1967; Cherala *et al.*, 2006). Assim, o fato das mães estarem usando suas próprias reservas para produção de leite (fase catabólica, além de não mais estarem fornecendo aporte direto para o desenvolvimento dos filhotes), somado a possível aversão à comida decorrente da sensibilidade à sacarose, e a diminuição do estímulo de sucção por parte da prole, poderia explicar a queda do consumo de ração nas fêmeas lactantes do grupo HP observada neste estudo.

No DPN 21, juntamente do desmame das proles, foi realizada pesagem e eutanásia das mães, que tiveram os pesos de suas gorduras viscerais, uterinas, retroperitoneais, rins direito e esquerdo e fígados aferidos (Tabela 2).

Tabela 2 - Peso corpóreo e peso absoluto e relativo dos órgãos maternos.

Parâmetros	***NP***	***HP***
Peso corpóreo (g)	305,60 ± 8,14	219,70 ±4,82*
Gordura visceral (g)	2,84 ±0,18	2,24±0,15*
Gordura visceral (g/100g)	0,93 ±0,05	0,97 ±0,06
Gordura uterina (g)	5,76 ± 0,44	4,61 ±0,42
Gordura uterina (g/100g)	1,86 ±0,14	2,02 ± 0,17
Gordura retroperitoneal (g)	2,29 ±0,19	1,95 ± 0,18
Gordura retroperitoneal (g/100g)	0,76 ± 0,07	0,84 ± 0,07
Rim direito (g)	1,00 ± 0,02	0,66 ± 0,01*
Rim direito (g/100g)	0,33 ±0,01	0,30 ± 0,01*
Rim esquerdo (g)	0,97 ± 0,03	0,63 ± 0,01*
Rim esquerdo (g/100g)	0,31 ± 0,01	0,28 ± 0,01*
Fígado (g)	16,36± 0,62	14,00± 0,40*
Fígado (g/100g)	5,35 ± 0,16	6,44 ±0,23*

NP e HP, N=19/grupo. Valores expressos em Média±E.P.M. *p<0,05 (teste t para dados paramétricos e Mann-whitney para dados não-paramétricos).

O peso corpóreo materno nos animais do grupo HP foi significativamente menor do que o peso dos animais do grupo NP. Este registro foi feito ao final do período de lactação, no qual o consumo de ração dos animais submetidos à dieta hipoproteica foi menor do que o dos animais que receberam ração normoproteica. Dessa maneira, a diminuição do peso materno no grupo HP concorda com a menor ingestão alimentar observada nestes animais durante a lactação.

Essa diferença de peso não pode ser atribuída à diferença no acumulo de gordura corporal, uma vez que quando observamos o peso das gorduras maternas notamos que este foi semelhante em ambos os grupos. Apenas a gordura visceral apresentou diferenças estatísticas entre os animais NP e HP, mas esta diferença desapareceu ao calcularmos o peso relativo da gordura em relação ao peso corporal dos animais.

Sabe-se que ratas alimentadas tanto com dietas normo quanto hipoproteicas mobilizam cerca de 60% de sua gordura corporal total durante a lactação. No entanto, neste período, fêmeas submetidas à ração hipoproteica apresentam uma perda de proteínas e de água associada, muito maior do que ratas que receberam dieta com nívéis proteicos adequados (Naismith *et al.*, 1982). Dessa maneira, o menor peso encontrado em fêmeas HP ao final da lactação deve estar associado à diminuição da ingestão de alimento e à maior perda proteínas e de água a elas associadas.

Uma série de trabalhos demonstra a eficiência da dieta hipoproteica, durante a gestação, em diminuir o peso do fígado materno, assim como foi observado no presente estudo (Ballen *et al.*, 2009; Torres *et al.*, 2010; Rebelato *et al.*, 2013). No entanto, quando observamos o peso relativo deste órgão, notamos que este se encontra aumentado nas mães do grupo HP.

A dieta hipoproteica utilizada neste estudo apresenta aporte de carboidratos aumentado em substituição às proteínas para mantê-la isocalórica. Sabe-se que o aumento na oferta de carboidratos provoca um aumento da atividade hepática, e consequentemente no peso do órgão, principalmente relacionado à síntese de ácidos graxos, o que pode levar ao desenvolvimento de esteatose hepática não alcoólica (Ooi *et al.*, 2013; Basaranoglu *et al.*, 2015; Softic *et al.*, 2016). Assim, pode ser que o aumento do peso relativo dos fígados das mães HP tenha sido resultado de maior atividade hepática como consequência do aumento de carboidratos presentes em suas dietas (Ballen *et al.*, 2009; Kwon *et al.*, 2012).

Sabe-se que a dieta hipoproteica oferece benefícios para indivíduos portadores de doença renal crônica. Os mecanismos através dos quais a restrição proteica atrasa o desenvolvimento desta enfermidade ainda não foram completamente elucidados, mas a dieta deficiente em proteína reduz bastante a carga metabólica renal tanto em ensaios clínicos quanto em experimentais (Meireles *et al.*, 1999; Kozlowska *et al.*, 2004; Gao *et al.*, 2011; Aparicio *et al.*, 2012). A redução no peso dos rins de animais tratados com dieta hipoproteica já foi observada por Griffin *et al.* (2003), Slomowitz *et al.* (2004), Gao *et al.* (2010) e De Miguel *et al.* (2011). Estes dados corroboram com a redução no peso renal absoluto e relativo encontrada nas mães do grupo HP, que pode ter acontecido devido à redução do metabolismo renal nestes animais.

Anexos

Anexo 1

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Efeitos da restrição proteica materna sobre o padrão vascular e expressão de proteínas no epidídimo de ratos Wistar machos em diferentes fases do desenvolvimento pós-natal", Protocolo nº **797-CEUA**, sob a responsabilidade de **Raquel Fantin Domeniconi**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Vigência do Projeto:	*Início: 02/02/2016*	*Término: 30/07/2018*
Espécie/linhagem:	*Rato/Wistar*	
Nº de animais:	*130*	
Peso:	*200 a 400g*	*Idade: 14 a 120 dias*
Sexo:	*Masculino e Feminino*	
Origem	*Biotério central da UNESP*	

Botucatu, 22 de janeiro de 2016.

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

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