Instituto de Biociências de Botucatu

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

PG-BGA

Relativa influência da prolactina, sua inibição e combinação com testosterona sobre a próstata de ratos castrados: Comparação entre recrescimento glandular, diferenciação celular e atividade secretora.

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Geral e Aplicada do Instituto de Biociências, Campus de Botucatu, UNESP, como requisito para a obtenção do grau de Mestre, Área de concentração Biologia celular Estrutural e Funcional.

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A minha Família e Amigos...

Dedico

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota." (Madre Teresa de Calcuta)

SUMÁRIO

RESUMO

A próstata é uma glândula do sistema genital masculino que possui grande importância na fertilidade. Estudos clássicos evidenciaram que para que se inicie o desenvolvimento prostático é necessário a presença de andrógeno produzido pelos testículos fetais. Porém, além da estimulação androgênica, estudos apontam para outros hormônios que também atuam na próstata como a Prolactina (PRL). PRL é um hormônio que é principalmente secretado por células lactotróficas da hipofise anterior e está envolvido em muitos processos biológicos, incluindo lactação e reprodução. Assim, investigamos se a modulação da sinalização PRL altera a morfofisiologia da próstata ventral (VP) em ratos castrados. Os ratos Sprague Dawley adultos (n = 6) foram castrados e após 21 dias divididos em 10 grupos experimentais: Castrado Controle (CC): animais castrados que não receberam tratamento; Castrado+testosterona (T): animais castrados que receberam T (4mg / kg); Castrado+PRL (PRL): animais castrados recebendo PRL (0,3 mg / kg); Castrado+T+PRL (TPRL): animais castrados que receberam associação de T e PRL; e Castrado+BR (BR): animais castrados que receberam Bromocriptina (BR) (0,4mg / kg). Grupo Controle: animais intactos (CTR). Os animais foram tratados durante 3 ou 10 dias consecutivos. Ao fim dos tratamentos, os animais foram anestesiados, eutanizados e a prostata ventral (VP) foi removida, pesada e processada para análise histológica e Western blot. O peso corporal não se alterou entre os grupos experimentais. A atrofia glandular foi induzida pela castração e a administração de andrógenos induziram o recrescimento. Não houve diferença entre o peso da VP dos grupos T e TPRL em ambas as idades. PRL ou BR não teviveram efeito sobre o peso glandular. A castração levou a uma diminuição na proliferação celular, na expressão de AR, STAT3 e 5, enquanto T ou TPRL induziram resposta proliferativa, maior expressão de AR, STAT3 e 5. O tratamento com BR durante o dia 3 ou PRL durante 10 dias aumentou STAT3. O tratamento com PRL e BR não alterou a proliferação celular ou a expressão de AR, mas aumentou a expressão da proteína do receptor de PRL (PRLR). A administração de T e TPRL não alterou este parâmetro. A prostateína diminuiu em todos os grupos tratados durante 3 e 10 dias, excepto nos animais dos grupos T10 ou TPRL10. Embora observássemos a ativação da sinalização de PRL, esta não exerceu efeito anabólico na VP de ratos castrados. A administração de PRL a 0,3 mg/kg administrada durante 3 ou 10 dias consecutivos não foi suficiente para superar a deficiência de andrógeno na próstata. Da mesma forma, não há involução glandular adicional no grupo castrado BR. Conclui-se que, apesar de estimular a via de sinalização a jusante, a PRL não atua sobre VP na ausência ou na presença de níveis supra-fisiológicos de testosterona.

Capitulo 1.

INTRODUÇÃO

1.1- PRÓSTATA

A próstata é uma glândula exócrina acessória do sistema genital masculino cujo desenvolvimento e homeostasia encontram-se sob controle androgênico (CUNHA et al., 1985). Ela secreta um complexo proteolítico composto por fosfatase ácida, ácido cítrico, fibrinolisina, enzimas específicas entre outros componentes do fluido seminal (MARKER et al., 2003). Esta secreção é fundamental para o sucesso reprodutivo, pois liquefaz o ejaculado, além de possuir enzimas, nutrientes e gradientes iônicos que alcalinizam o canal vaginal e garantem a motilidade dos espermatozoides (AUMÜLLER; SEITZ, 1990).

No homem adulto, a próstata localiza-se no compartimento subperitoneal, anterior ao reto e inferior a bexiga (SCHAUER; ROWLEY, 2011). Estruturalmente a próstata humana é encapsulada por uma fina camada de tecido fibroelástico que lhe confere uma aparência não lobulada. Contudo estudos mostram que ela possui três zonas morfologicamente distintas: (1) zona periférica (região com maior acometimento de câncer), (2) zona de transição e (3) zona central (Figura1A) (LOWSLEY, 1912).

Já a próstata de roedores é formada por quatro lobos: lobo anterior (AP), lobo ventral (VP), lobo dorsal e lobo lateral, sendo os dois últimos também chamados de lobo dorsolateral (PDL) (Figura 1B). Cada um desses lobos apresenta características histológicas distintas como: extensas pregas epiteliais na AP, dobras menores na DLP e pregas mínimas na VP (CUNHA et al., 1987). Cada lobo é localizado em posições específica em torno da uretra (LOWSLEY, 1912) (Figura 2A).

Em termos de homologia com a próstata humana, o lobo anterior é homólogo a zona central da glândula, enquanto que o lobo dorsolateral é considerado homólogo a zona periférica da glândula da próstata humana. O lobo ventral dos roedores, apesar de não apresentarem homologia com nenhuma zona da próstata humana, é o mais estudado, principalmente por ser o lobo de rápido e fácil acesso durante a coleta, além de ser o lobo maior e por geralmente não desenvolver prostatite, já que este não é o foco do trabalho (ROY-BURMAN et al., 2004).

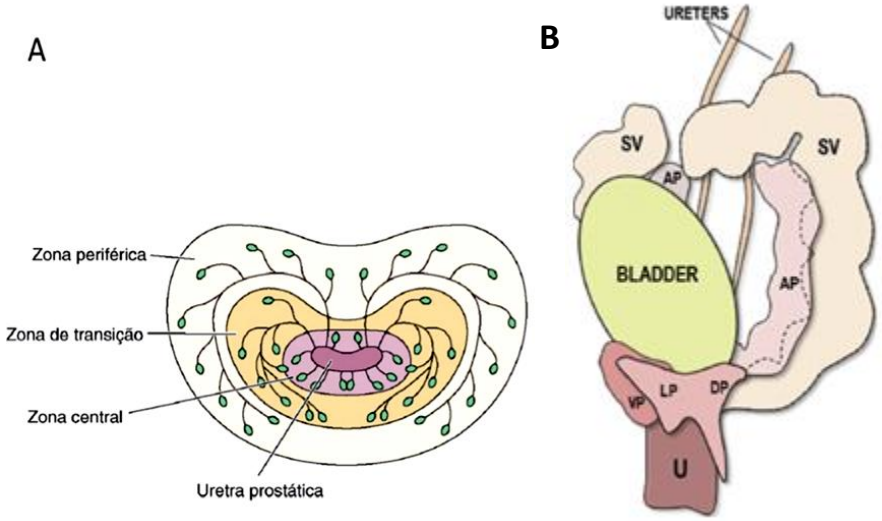

Figura 1. Organização anatômica da próstata humana (A) e da próstata de roedores (B). SV: vesícula seminal; AP: Próstata anterior; VP: Próstata ventral; LP: Próstata Lateral; DP: Próstata dorsal; U: Uretra Adaptado (JUNQUEIRA L. C.; CARNEIRO J., 2013) e (OLIVEIRA et al., 2016)

A VP é constituída de dois lóbulos (direito e esquerdo) que tem sua origem a partir da uretra formando uma estrutura tubular não ramificada, após o seu completo desenvolvimento é possível observar diferentes regiões dos ductos, dos segmentos proximal, intermediário e distal, de acordo com a sua posição em relação à uretra (LEE et al., 1990; SHABSIGH et al., 1999). Enquanto na região distal e intermediária, as células epiteliais são colunares altas, na região proximal, estas células são cúbicas e baixas. Células em proliferação são mais frequentes no epitélio da região distal, células apoptóticas são mais abundantes na região proximal e células secretoras são mais diferenciadas na região intermediária (NEMETH; LEE, 1996). Em relação ao estroma, tanto na região intermediária como na proximal, o tecido fibroso está presente no espaço entre os ductos, ocasionalmente intercalando-se com a camada de células musculares lisas que é contínua na região intermediária e torna-se descontínua na região distal (LEE et al., 1990). Independentemente da região prostática, todo o estroma é rico em elementos da matriz extracelular (MEC)(AUGUSTO; FELISBINO; CARVALHO, 2008).

A próstata apresenta uma complexa rede de ductos que se organizam de forma a dar a característica histológica de glândula túbulo alveolar composta, o seu epitélio usualmente é colunar alto, porém pode haver alteração dependendo do estado funcional da glândula. (DERMER, 1978; ICHIHARA et al., 1985). Ela possui diferentes tipos celulares que desempenham papeis importantes e específicos para a viabilidade do tecido e podem ser dependentes de andrógeno ou não (JUNG-TESTAS et al., 1981) Essas células se distribuem pela próstata de maneira semelhante entre o homem e o rato. Em ambas as espécies, o lúmen

é revestido por células epiteliais secretoras, que possuem polaridade apical-basal e são responsáveis pela síntese e secreção de proteínas e fluidos para o lúmen prostático. Uma diferença da próstata entre humanos e roedores, é que em humanos, as células epiteliais basais formam uma camada continua, enquanto que nos ratos as células estão distribuídas de maneira descontinua. Além das células epiteliais basais e luminais, também são encontradas células neuroendócrinas, porém são células raras, existindo poucos estudos a respeito, assim são escassas as informações sobre essas células (MARKER et al., 2003) (Figura 2B).

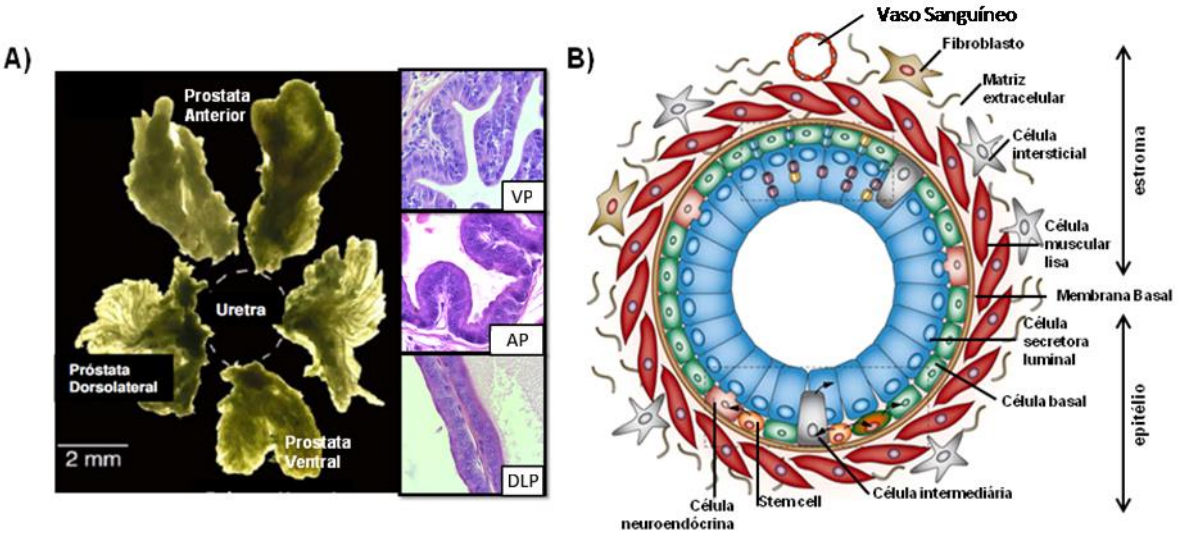

Figura 2. Imagem dos lobos prostáticos e sua organização celular. A) Aparência macroscópica e organização microscópica (coloração em H.E.) dos pares de lobos prostáticos de camundongo- modificado de (MARKER et al., 2003). B) Esquema da organização celular do epitélio e estroma prostático do homem adulto - modificado de(CZYŻ; SZPAK; MADEJA, 2012). PA, próstata anterior; PDL, próstata dorsolateral; VP, próstata ventral.

O componente estromal engloba todos os elementos celulares e extracelulares que estão fora da lâmina basal epitelial como a camada fibromuscular que cerca a glândula. Além da camada muscular, o estroma também apresenta fibroblastos, vasos sanguíneos e os pericitos associados, células imunitárias, terminações nervosas e vasos linfáticos, os quais são incorporados em uma matriz extracelular colagenosa. (AUMÜLLER, 1983).

Além de sua importância para a fertilidade, nos últimos anos a próstata tem despertado grande interesse médico-científico pela alta incidência de distúrbios, principalmente a hiperplasia prostática benigna (HPB) e o câncer de próstata (PCa) (DASGUPTA; SRINIDHI; VISHWANATHA, 2012), que no Brasil, é a segunda causa de óbitos por câncer entre homens. Estimativas do Instituto Nacional do Câncer apontam para aproximadamente 69 mil novos casos diagnosticados em 2016, com número de mortes próximo de 13 mil indivíduos (INCA, 2016).

1.2 – TESTOSTERONA, DESENVOLVIMENTO PROSTÁTICO E CASTRAÇÃO

A testosterona (T) é um hormônio lipídico sintetizado e secretado pelas células de Leydig que se localizam na região intersticial dos Tubulos seminiferos. A sua secreção é controlada através do feedback negativo no eixo hipotálamo-hipófise. A T após ser produzida pode ter ação local (parácrino) ou sistêmica (endócrina) de forma que este hormônio é liberado na corrente sanguínea e pode atuar em outros tecidos alvos. No entanto a T circula no sangue ligada a albumina e a globulina de ligação ao hormônio sexual (SHBG). Além disso dependendo do tecido alvo a T acaba não sendo o androgênio utilizado, esta é convertida em di-hidrotestosterona (DHT), processo que ocorre no citoplasma celular através da enzima 5α-redutase, e a DHT é mais potente que a T (COSTANZO, 2014).

Portanto a T e a DHT são hormônios responsáveis pelo desenvolvimento do sistema genital masculino e pelas características sexuais secundarias.(MACLEAN et al., 1993) A ação de ambos é mediada via receptor de andrógeno (AR), um fator de transcrição nuclear dependente do ligante. (CHANG et al., 1995) Na ausência do ligante o AR é citoplasmático e está associado a proteínas heat-shock (HSPs), com a ligação dos andrógenos o AR dissocia-se das proteínas, é fosfolilaro e se dimerisa. O complexo AR-Andrógeno desloca-se para o núcleo para assim modular a transcrição dos genes alvos. (DAVEY; GROSSMANN, 2016; EDER et al., 2001; FELDMAN; FELDMAN, 2001) (Figura 3)

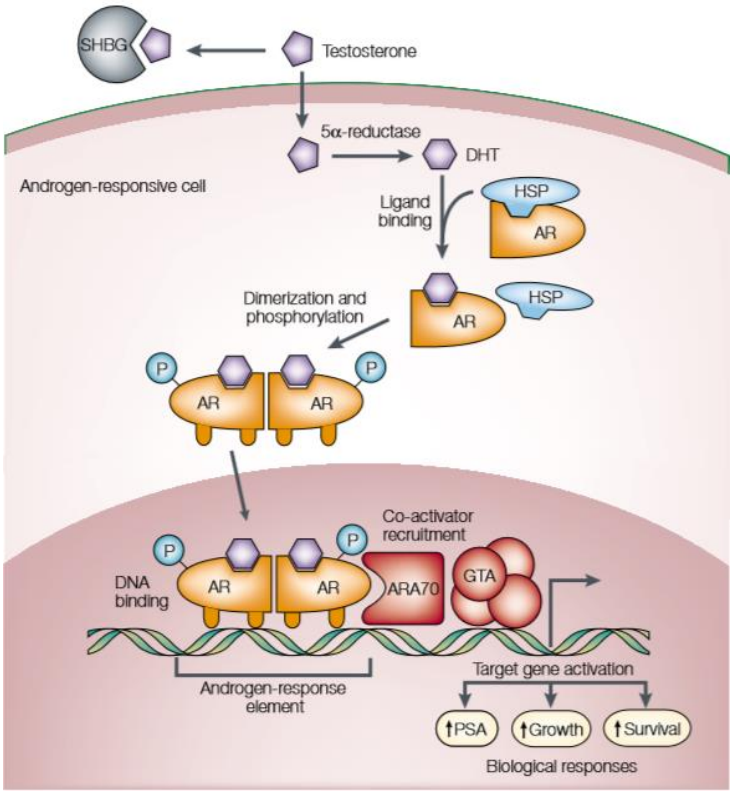

Figura 3 – Ação andrógena. A testosterona circula no sangue ligado à albumina (não mostrada) e à globulina de ligação aos hormônios sexuais (SHBG) e trocas com testosterona livre. A testosterona livre entra nas células da próstata e é convertida em dihidrotestosterona (DHT) pela enzima 5α-redutase. A ligação de DHT ao receptor de androgénio (AR) induz a dissociação de proteínas de "heat-shocke" (HSPs) e a fosforilação do receptor. A AR dimeriza e liga-se aos elementos de resposta androgénica nas regiões promotoras dos genes alvos. Adaptado de (FELDMAN; FELDMAN, 2001) Imagem ilustrativa.

Assim, Tanto o desenvolvimento prostático (pré e/ou pós-natal), como o recrescimento prostático induzido por T em ratos castrados tem sido extensivamente utilizado com o objetivo de avaliar os mecanismos moleculares e cascatas de sinalização durante a morfogênese e recrescimento glandular (CUNHA et al., 1987; JUSTULIN et al., 2010; PENG et al., 2013). O início do desenvolvimento prostático se dá por volta do 17º dia de gestação em camundongos, 18º dia em ratos e entre a 9-10ª semana em humanos (WELSH et al., 2008).

A próstata se desenvolve em diferentes espécies por meio de um processo altamente conservado, denominado "morfogênese de ramificação", onde pequenos brotos se projetam a partir do epitélio do seio urogenital (UGS). Enquanto em humanos a maior parte do desenvolvimento da próstata ocorre no período intra-uterino, nos roedores a próstata é rudimentar ao nascimento, sendo a maior parte do desenvolvimento, durante os primeiros 15

dias de vida pós-natal (TIMMS; MOHS; DIDIO, 1994). Em ambos, esse processo se estende até que a maturidade sexual seja atingida com a puberdade (MARKER et al., 2003) (Figura 4).

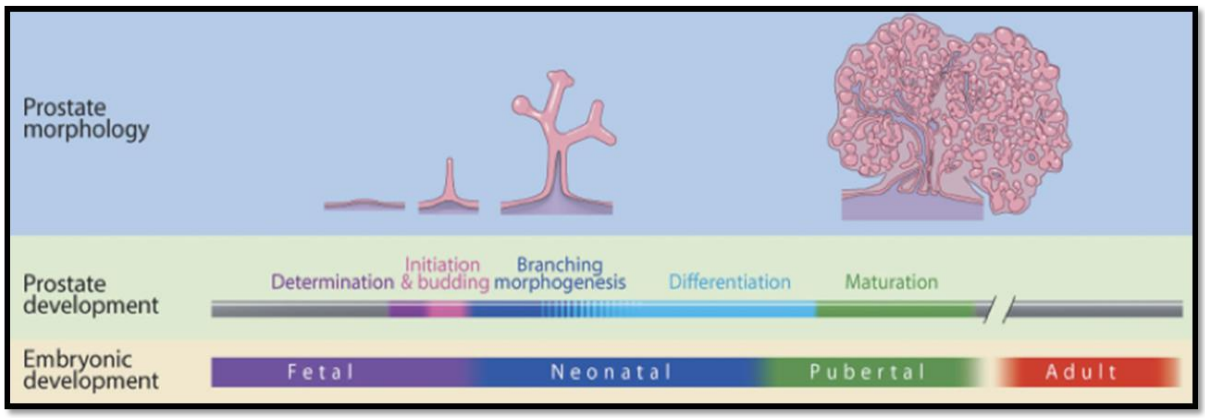

Figura 4: Estágios do desenvolvimento da próstata do rato - adaptado (PRINS; PUTZ, 2008).

Estudos clássicos evidenciaram que a presença de andrógeno produzido pelos testículos fetais é necessário para o início do desenvolvimento prostático (TAKEDA; CHANG, 1991). Particularmente no rato, a síntese de T pelos testículos fetais tem início entre os dias 13-14 da gestação (DG) (WILSON et al., 1983). Em resposta aos andrógenos, o epitélio do UGS passa a expressar Sonic hedgehog (Shh), que por sua vez estimula expressão do gene homeobox *Nkx3.1* no DG15.5, dois dias antes de surgirem os primeiros brotos prostáticos (WILHELM; KOOPMAN, 2006). Depois do nascimento, ocorre alongamento dos brotos e início da morfogênese com a formação dos ductos glandulares (PRINS et al., 1992).

O padrão de ramificação é complexo e lóbulo-específico, sendo que este processo se inicia na VP no dia pós-natal (DPN) 3-5 e dois dias depois na PDL. Nesse período, também ocorre início da diferenciação das célula tronco progenitora em células basais e luminais, com expressão diferencial de citoqueratinas (CK) e do receptor de andrógeno (AR), acompanhada do início da formação do lúmen, que atinge as extremidades distais dos ductos no DPN12 (PRINS; BIRCH, 1995) (Figura 2).

Estudos clássicos demonstram que a homeostasia prostática depende da manutenção de níveis fisiológicos de andrógenos (SUGIMURA; CUNHA; DONJACOUR, 1986). Neste contexto, após a castração cirúrgica (retirada dos testículos) ou química (administração de antiandrogênicos) ocorre um intenso processo de involução prostático, com eliminação de células epiteliais por apoptose, levando a uma rápida queda no conteúdo glandular de RNA, DNA e proteínas de secreção e remodelação do estroma periductal. Por outro lado, a administração de andrógeno a animais castrados estimula o recrescimento glandular, com completa recuperação após 7-10 dias de reposição hormonal (JUSTULIN et al., 2006, 2010;

SUGIMURA; CUNHA; DONJACOUR, 1986). Este ciclo de involução/recrescimento pode se repetir por várias vezes com a completa recuperação glandular (HU et al., 2011; MARKER et al., 2003; SCHRAMEK et al., 2010). Este recrescimento acontece através de ativação de processos proliferativos das células epiteliais que resistiram à condição de privação de andrógeno. (DE CARVALHO; VILAMAIOR; TABOGA, 1997; JUSTULIN et al., 2010; VILAMAIOR et al., 2000; YAMASHITA et al., 1996). Assim, estes dados sugerem a existência de uma população de células progenitoras na próstata de animais adultos, capazes de sobreviver à condição de privação androgênica e com capacidade de proliferar e se diferenciar para recompor os ductos prostáticos observados em um animal adulto normal (SHI et al., 2014).

1.3 PROLACTINA

O desenvolvimento prostático é dependente de andrógeno, porém existem outros hormônios esteroides ou não esteroides que também afetam a próstata. Estudos demonstraram que a prolactina PRL exerce importante papel tanto para o desenvolvimento como para a manutenção glandular de animais adultos (GOFFIN et al., 2005). Neste sentido, tem sido proposto que a modulação da via de sinalização da PRL poderia ser um alvo promissor para o tratamento do PCa (HASSAN et al., 2009; HERRERA-COVARRUBIAS et al., 2015). A PRL secretada de forma autócrina e/ou parácrina vem sendo sugerida para a contribuição da tumorgenese prostática. Camundongos com superexpressão de PRL na próstata desenvolvem hiperplasia, neoplasia intra-epitelial e até adenocarcinoma prostático (ROUET et al., 2010; WENNBO et al., 1997). A presença de PRL e STAT-5 fosforilado em tumores prostáticos está relacionada com o aumento da agressividade da doença (LI et al., 2004).

A PRL é um hormônio polipeptídio secretado pelas células secretoras lactotrofo da hipófise, porém estudos demonstram que este hormônio também possui fonte extra-hipofisaria, como a próstata (SACKMANN-SALA; GOFFIN, 2015). Foi descoberta aproximadamente há 90 anos atrás, conhecida como o hormônio da lactação, ela atua na fisiologia (reprodução, lactação, crescimento, metabolismo e transporte eletrolítico) e na patologia (imunidade e carcinogênese) (BEN-JONATHAN; LAPENSEE; LAPENSEE, 2008; BOLE-FEYSOT et al., 1998; GOFFIN et al., 2002) da glândula mamária (LEE et al., 2013; SCHRAMEK et al., 2010), prostática (SACKMANN-SALA; GOFFIN, 2015), além

do cérebro (LARSEN; GRATTAN, 2012; SHINGO et al., 2003), da medula óssea (OGUETA et al., 2002), do folículo capilar (RAMOT et al., 2010), células hematopoiéticas (WELNIAK et al., 2000), do hipocampo (WALKER et al., 2012).

O gene da PRL é único nos mamíferos e está localizado no cromossomo número 6 em humanos, possuindo 6 exons e 4 introns, (TRUONG et al., 1984; YOSHIKI et al., 1991). Apresenta peso molecular de 23Kda, possuindo em sua forma madura 199 aminoácidos (FREEMAN et al., 2000; GREGERSON, 2006) e é caracterizado por apresentar uma estrutura 3D composto por 4 α hélices antiparalelas (HORSEMAN; YU-LEE, 1994). Embora esta forma seja a mais encontrada, existem outras isoformas da PRL, muitas resultam de alterações pós-trascricionais como glicolisação, fosforilação e sulfatação (FREEMAN et al., 2000). Assim existem hormônios maiores com peso aproximado de 100 KDa com uma atuação mínima no organismo (GIBNEY; SMITH; MCKENNA, 2005; JOSEPH MCKENNA, 2009; SULIMAN et al., 2003) e também existem PRL com peso molecular menores, com 14 KDa, 16KDa e 22KDa.

A PRL com peso molecular de 16 KDa é inicialmente uma proteína de 23KDa clivada por uma metaloproteinase ou pela catepsina D (CLAPP et al., 2006), esta clivagem pode ocorrer dentro das células ou em capilares sanguíneos. Por ela possuir apenas uma ponta N-terminal perde a habilidade de se ligar ao seu receptor (CLAPP; WEINER, 1992; MACOTELA et al., 2006), no entanto é capaz de se ligar a células endoteliais (CLAPP; WEINER, 1992) e tem propriedades anti-angiogênicas (CLAPP et al., 1993).

A ação da PRL é mediada por um receptor transmembrana membro da superfamília de citocinas hematopoiéticas, o receptor de PRL (PRLR). (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). A conformação deste receptor é única, contendo dois domínios extracelulares, sendo um portador de duas pontes dissulfetos (essenciais para a interação com o ligante), e outro importante para o deslocamento e dobramento do receptor (GOFFIN; FERRAG; KELLY, 1998). Além dos dois domínios extracelulares, PRLR possui também um único domínio intermembranar e outro domínio intracelular que apresenta duas regiões importantes, BOX1 e BOX2, responsáveis pela transdução do sinal (KELLY et al., 1991).

Este receptor apresenta apenas um gene em todas as espécies, sendo que no humano este gene é localizado no cromossomo número 5 e em ratos no cromossomo número 15. O gene dos mamíferos apresenta 10 exons, que podem sofrer *splicing* alternativos, o que faz com que haja uma variedade de isoformas deste receptor. Estas apresentam um idêntico domínio extracelular, o que difere é o comprimento do domínio intracelular que pode ser grande, médio

ou curto (BOLE-FEYSOT et al., 1998; HU; MENG; DUFAU, 2001; KLINE; ROEHRS; CLEVENGER, 1999). Em humanos encontramos a forma longa, já em ratos temos uma forma longa e outras três formas curtas já descritas (CLARKE; LINZER, 1993; DAVIS; LINZER, 1989). A ligação com este receptor não é exclusivo da PRL, podendo se ligar também, lactogênio placentário e hormônio do crescimento, o que dificulta determinar o efeito específico da PRL (BROOKS, 2012).

A PRL possui 2 sítios de ligação, o primeiro é composto pelas α hélices 1 e 4, e o segundo pelas 2 e 3. O primeiro sítio de ligação da PRL se liga ao domínio extracelular na região dissulfeto do receptor, este é o pré-requisito para que o segundo sítio possa se ligar (GOFFIN et al., 1996). Como o RPLR apresenta apenas um sitio de ligação é necessário outro RPLR que irá fazer a ligação então com o segundo sítio de ligação da PRL (BOLE-FEYSOT et al., 1998).

Estudos mostram que a região do domínio intracelular é fosforilada bem na região do BOX1 onde fica acoplado a proteína JAK2 (Janus Kinase 2), assim duas JAK2 são fosforiladas. Com a JAK2 fosforilada membros citoplasmáticos desta via também são fosforilados como o STAT (sinal transdutor e ativador de transcrição) família que representa a canônica via JAK-STAT (Figura 5) (GOFFIN et al., 2002).

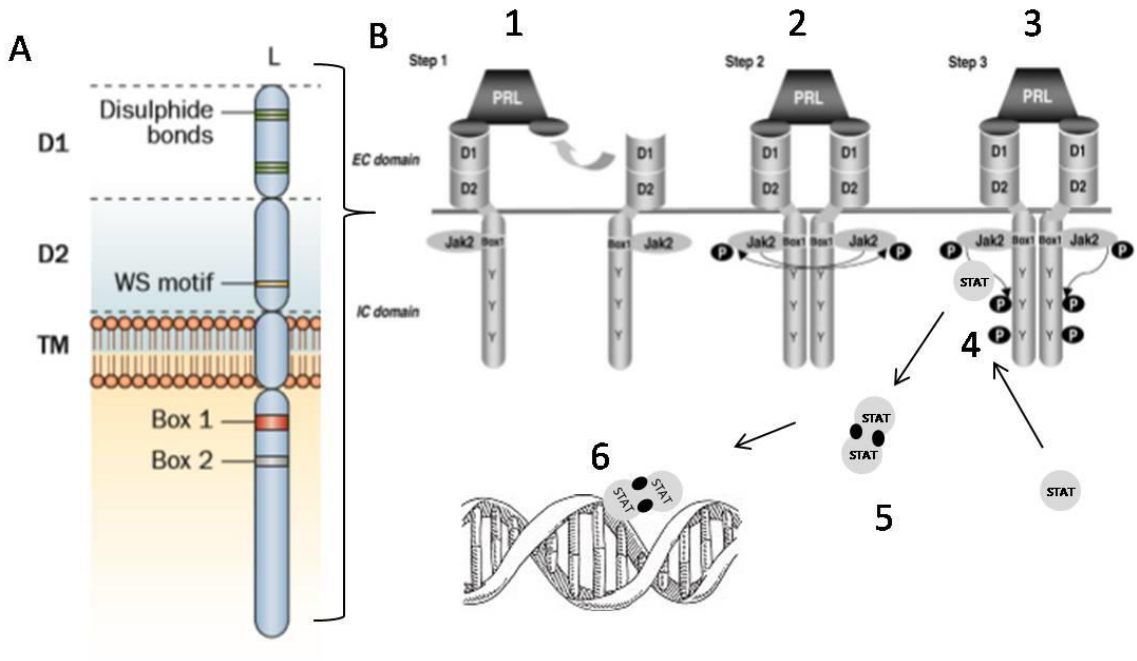

Figura 5 – A) Molécula do receptor de PRL com seus dominios. B) 1-ativação do receptor pela PRL, 2-fosforilação de JAK2, 3/4-fosforilação do STAT, 5-formação do dimero de STAT5 e 6- chegada desse dimero no DNA. Adaptado de (BERNARD et al., 2015; FREEMAN et al., 2000) Imagem ilustrativa.

A ativação do PRLR e da JAK2 cria um local de acoplamento para o STAT, que por sua vez é recrutado para o receptor e vem ser fosforilado com um único resíduo de tirosina no domínio SH2. STATs ativados dissociam-se do receptor e se ligam formando um dímero de STAT, (BOLE-FEYSOT et al., 1998) translocam-se para o núcleo, onde este dímero interage com o seu local de reconhecimento nos promotores de genes alvo (Figura 4) (BOLE-FEYSOT et al., 1998; CARTER-SU; SMIT, 1998).

STATs são uma família de fatores de transcrição que medeiam respostas de citocinas e fatores de crescimento (LEVY; DARNELL, 2002). Dentro da família dos STATs temos o Stat5 a/b que é um mediador primário de efeitos de PRL no epitélio da próstata; ele está presente na progressão do câncer da próstata, e a inibição desta via leva a apoptose disseminado (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). O STAT-5 regula funções como proliferação, progressão do ciclo celular, apoptose, angiogênese e respostas do sistema imune, além de exercer efeitos nos Câncer *Stem Cells* (SIVEEN et al., 2014).

O processo de síntese de PRL é controlado por fatores estimulatórios e fatores inibitório. O efeito de inibição da PRL é mediado por receptores dopaminérgicos da subclasse D2, responsáveis por regular negativamente a produção de PRL pela hipófise, e reduzir os níveis de PRL circulantes, sendo a Bromocriptina (BR) um agonista pioneiro desse efeito (GOFFIN; TOURAINE, 2015). A próstata de rato tem receptores dopaminérgicos localizados em ambas as células estromais e epiteliais, a interação de PRL e BR em tecidos da próstata podem ser relevantes para a manutenção e função da glândula (AMENTA; CAVALLOTTI; AMENTA, 1987).

Estudos mostram que pacientes com câncer de mama e próstata apresentam seus níveis de PRL normalizados quando tratado com BR (LISSONI et al., 2000). Assim como a supressão de PRL causado através do tratamento com BR, em ratos no período de lactação, consegue provocar várias alterações metabólicas influenciando a capacidade reprodutiva desses ratos machos (CARVALHO et al., 2015).

Neste contexto, estudos que enfoquem o efeito da administração de PRL ou de sua inibição podem auxiliar no entendimento das respostas celulares em condições de privação androgênica e de reposição hormonal após a castração, melhorando o entendimento da biologia prostática.

REFERÊNCIAS

Referências desta revisão encontram-se junto com as referências do artigo (página 41).

Capitulo 2

Manuscript

Impact of prolactin administration on the ventral prostate of castrated rats submitted to testosterone replacement

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Abstract

Prolactin (PRL) is a hormone that is mainly secreted by lactotroph cells of the anterior pituitary gland, and is involved in many biological processes including lactation and reproduction. Thus, we investigated whether the modulation of the PRL signaling alters the ventral prostate (VP) morphophysiology in castrated rats. Adult *Sprague Dawley* rats (n = 6) were castrated for 21 days and after divided into 10 experimental groups: Castrated Control (CC): castrated animals that did not receive treatment; Castrated+testosterone (T): castrated animals that received T (4mg/kg); Castrated+PRL (PRL): castrated animals receiving PRL (0.3 mg/kg); Castrated+T+PRL (TPRL): castrated animals that received association of T and PRL; and Castrated+BR (BR): castrated animals that received Bromocriptine (BR) (0.4mg / kg). Control group: intact animals (CTR). The animals were treated for 3 or 10 consecutive days. At the end of experiment, the animals were anesthetized, euthanized and the VP lobes were removed, weighed and processed for histological and western blot analyzes. Body weight did not change between the experimental groups. Castration induced glandular atrophy and androgen administration induced glandular regrowth. There was no difference between VP weight from T and TPRL groups in both ages. PRL or BR had no effect on glandular weight. Castration led to a decrease in cell proliferation, AR and STAT3 and 5 expressions, whereas T or TPRL replacement induced proliferative response, AR and STAT3 and 5 expressions. Treatment with BR for day 3 or PRL for 10 days increased STAT3, respectively. PRL and BR treatment did not alter cell proliferation or AR expression, but increased PRL receptor (PRLR) protein expression. Administration of T and TPRL did not alter this parameter. Prostatein decreased in all groups treated for 3 and 10 days, except in the animals of the T10 or TPRL10 groups. Thus, the administration of PRL at 0.3 mg/kg administered for 3 or 10 consecutive days do not exert anabolic effect on the VP of castrated rats. Although we observed activation of PRL signaling, this was not enough to overcome the prostatic androgen deficiency. Likewise, there is no additional glandular involution in BR castrated group. We conclude that despite stimulating downstream signaling pathway, PRL does not act on VP in the absence or in the presence of supra physiologic levels of testosterone.

1. INTRODUCTION

The prostate is an accessory exocrine gland of the male genital system, whose function is essential for reproductive success in mammals, since it enriches the semen with Zn2 +, enzymes and citrate, essential molecules that synchronize the ejaculatory stimulus (MARKER et al., 2003). In addition to the reproductive function, in the last years the prostate has aroused great medical-scientific interest due to the high incidence of diseases, mainly benign prostatic hyperplasia (BPH) and prostate cancer (PCa) (DASGUPTA; SRINIDHI; VISHWANATHA, 2012). In Brazil, PCa is the second cause of cancer deaths among men. The National Cancer Institute (INCA) estimates approximately 61.000 new cases diagnosed in 2016, with a total of 13.000 death (http://www.inca.gov.br).

The development and prostatic homeostasis are under androgenic control (CUNHA et al., 1985; EISENBERG, 2015; GRAY; SELTZER; TALBERT, 2015). During aging, the hormonal imbalance between androgen/estrogen is considered the major risk factor for the development and progression of BPH and PCa (UNTERGASSER et al., 1999; VERZE; CAI; LORENZETTI, 2016). In this context, androgen ablation has been used for treatment of PCa, mainly due to the induction of androgen-dependent cell death. However, after androgen deprivation, approximately 20% of patients develop more aggressive androgen-independent tumors (CHANDRASEKAR et al., 2015). This is due to the fact that androgen deprivation, despite eliminating androgen-dependent tumor cells, selects a set of less differentiated cells, independent androgens, that use other molecular pathways for their survival and growth (ZENZMAIER; UNTERGASSER; BERGER, 2008).

In recent years, studies have demonstrated the pleiotropic role of prolactin (PRL) on the prostate in men and rodents, both in normal and pathological conditions (OJO et al., 2015). PRL is a polypeptide hormone secreted by the pituitary gland, whose functions are mediated by a specific receptor, the PRL receptor (PRLR) (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). Experimental studies using rodents (PÉREZ-VILLAMIL; BORDIÚ; PUENTE-CUEVA, 1992) and in primates, (ARUNAKARAN; ARULDHAS; GOVINDARAJULU, 1987) demonstrated that, under normal conditions, PRL acts in synergism with androgens, stimulating cellular proliferation, secretory activity and prostatic growth. However, epidemiological data have associated increased serum PRL levels

(hyperprolactinemia) to the development and PCa aggressiveness, especially in those classified as androgens independent tumor (BERINDER et al., 2011).

Studies using transgenic adult mice (which overexpress PRL under the control of probasin) demonstrated that the intraprosthetic increase of PRL induced amplification of progenitor stem cells at the basal compartment of the glandular epithelium. However, these authors concluded that stem cells are not direct target of PRL and they have suggested a paracrine activation pathway where luminal or stromal cells would respond to PRL stimulation, producing growth factors that act on stem cells (SACKMANN-SALA; GOFFIN, 2015).

Inhibition of secretion activity of PRL is controlled by stimulatory and inhibitory factors, and Bromocriptine (BR) is a pioneer agonist in its inhibition (GOFFIN; TOURAINE, 2015). Studies have shown that patients with prostate cancer present their levels of PRL normalized when treated with BR (LISSONI et al., 2000). As well as the suppression of PRL caused by BR treatment in rats during the lactation period, it can provoke several metabolic alterations influencing the reproductive capacity of these male rats (CARVALHO et al., 2015).

In this context, studies focusing on which cell types or prostatic compartment respond to PRL could help the understanding the prostatic biology under PRL influence. The castration-induced prostate involution, followed by glandular regrowth after androgen replacement has been extensively used not only to evaluate the dynamics of prostatic regrowth, but also to assess the influence of non-steroidal hormones and/or growth factors on prostate cancer (DE CARVALHO; VILAMAIOR; TABOGA, 1997; JUSTULIN et al., 2010). Considering the involvement of PRL on prostate carcinogenesis, here we aimed to investigate the impact of PRL administration on VP morphology and functional activity in a model of testosterone (T) ablation by castration. Moreover, we also investigated the VP response followed the combination of T and PRL administration or the inhibition of circulating PRL by BR.

2. MATERIALS AND METHODS

2.1 Animals and treatments

Sprague Dawley (n = 6) male rats were obtained from the Central stock breeder at the State University of Campinas (CEMIB / UNICAMP). The animals were maintained under light and temperature controlled (photoperiod 12h and 22º to 25ºC, respectively) and relative

humidity (55%). The animals had free access to water and chow. After castration, the animals were housed in individualized cages until the end of the experiment. The experimental protocol followed the Ethical Principles in Animal Research and the Brazilian legislation established by the Brazilian Council of Control in Animal Experimentation and was approved by the Ethics Committee for Animal Use from the Institute of Biosciences of Botucatu/ UNESP (CEUA protocol 767).

For castration, the animals were anesthetized with intraperitoneal injection of Thiopentax (30mg/kg body weight) and only then bilateral orchiectomy was performed to remove the testicles. After 21 days of castration, the animals were divided into 9 groups. Castrated Control (CC): castrated animals that did not receive treatment; Castrated+T: castrated animals that received T (4mg/kg, subcutaneously injected) (JUSTULIN et al., 2010) for 3 or 10 days (T3 and T10). Castrated+PRL: castrated animals receiving PRL (0.3 mg/kg, subcutaneously injected) (STOKER; ROBINETTE; COOPER, 1999) for 3 or 10 days (PRL3 and PRL10); Castrated+T+PRL: castrated animals that received association of T and PRL for 3 or 10 days (TPRL3 and TPRL10); and Castrated+BR: castrated animals that received BR (0.4mg / kg, subcutaneously injected) (CARON; JAHN; DEIS, 1994) for 3 or 10 days (BR3 and BR10). In addition to castrated animals, a group of animals that did not undergo castration was used as control (CTR) (Figure 1).

After the experimental period, animals from the different experimental groups were euthanized by Thiopentax (30mg / kg body weight), injected intraperitoneally and followed by decapitation. The rats were weighed; the pairs of ventral prostatic lobes were dissected out, weighted, and processed for the analysis described below. The VP was chosen because it was more responsive to androgenic modulation. (PRINS et al., 2001).

2.2 Quantification of Serum Prolactin

For hormonal quantification, blood samples (n = 6) were transferred to an Eppendorf, centrifuged for 20 mim at 4000 rpm and 4°C. Serum PRL levels were determined by ELISA immunoenzymatic assay, as instructed by the manufacturer (Kit Mouse/Rat Prolactin, CALBIOTECH).

2.3 Histological Procedure

Samples of VP from different experimental groups (n = 6) were fixed for 4 h in Methacarn (70% methanol + 20% chloroform + 10% acetic acid) (PUCHTLER et al., 1970), diaphanized in xylol and included in Paraplast (Sigma Co, Saint Louis, MO). Sections of 5 μm were produced on a rotating microtome and collected on silanized slides.

2.4 Morphological, Morphometric and Stereological Analysis

The slides were stained by Hematoxylin-Eosin (HE) for general view of the glandular structure and to perform the morphometric-stereological analyzes. The epithelial height was determined in 10 microscopic fields of 6 different VP per group at 40X magnification. The stereological analyzes were performed to determine the volume (absolute volume) of the lumen, epithelium and stromal compartment of the VP (WEIBEL; KISTLER; SCHERLE, 1966), where 168 points were counted in each field. For that, 10 random microscopic fields of each animal (n=06) was analyzed at 20X magnification. According to (DEKLERK; COFFEY, 1978), 1 mg fresh prostate tissue has a volume of approximately 1 mm 3. Consequently, the VP weight (mg) can be considered as volume equivalent (mm^3). Thus, for the absolute volume calculation of each compartment, the relative volumes of lumen, epithelium and stroma were multiplied by the mean moist weight of the VP of the respective group.

All measurement was made using a Leica DMLB 80 microscope connected to a Leica DC300FX camera. The digitalized images were analyzed using Leica Q-win software Version 3 for Windows.

2.5 Immunohistochemistry

For immunohistochemistry, sections of Paraplast-embedded prostates (5 μm) were collected on silanized glass. Antigen retrieval was performed using a Decloaker and 10 mM citrate buffer pH 6.0 for 20 min. After washing, the slides were blocked with 3% hydrogen peroxide in methanol for 10 min followed by incubation with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Slides were then incubated with anti-Ki-67 antibody (cellular proliferation marker) (concentration 1:150; AB-16667- Abcam®, Cambridge) and anti-androgen receptor (AR) antibody (clone sc2030, dilution 1:300, Santa Cruz Biotechnology, CA, USA). After washing with PBS, the slides were incubated for 1 h at room temperature with a goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, CA) diluted 1:200 in 1% BSA in PBS, respectively. Chromogen color

development was performed with 3,30-diaminobenzidine tetrahydrochloride, and slides were counterstained with Harris's hematoxylin. A negative control was generated by omitting the primary antibody incubation step (data not showed).

2.6 Determination of Ki-67 index

The number of epithelial cells that showed positive immunolabeling for Ki-67 was counted in 10 random fields at 40X magnification from 06 different VP lobes for each treatment and expressed as a percentage (%; mean ± SD) of the total counted cells. All image acquisition and quantitative measurements were performed with the investigators blind to both the animal identity and experimental condition.

2.7 Protein Extractions and Western Blotting

VP samples (n=06/group) were mechanically homogenized (Turrax type homogenizer) in RIPA buffer (Thermo Fisher, Illinois, USA) with protease inhibitor (BioRad®, USA), centrifuged at 4,000 rpm at 4°C for 20 minutes. The total protein quantification was performed by Bradford method (BRADFORD, 1976). Samples (70 µg/slot) were analyzed by electrophoresis using SDS-PAGE. After electrophoresis, the proteins were trans-bloted onto to a Hybond ECL nitrocellulose membranes (Amersham, Little Chalfont, UK). Blots were blocked with 3% nonfat milk diluted in TBS-Tween for 1h and incubated with the different primary antibodies: anti-PRLR (concentration 1: 1000 AB2772-Abcam®, Cambridge, USA); Anti-prostatein (concentration: 1: 1000, 7821-1009-Abd Serotec®, Raleigh, USA); AR (concentration 1: 500; sc-7805 Santa Cruz, USA); Stat5 A+B (concentration: 1:1000, ab194898-Abcam®, Cambridge, USA); Stat3 (concentration 1:1000, ab68153-Abcam®, Cambridge, USA). After washing in TBS-Tween, the membranes were incubated with peroxidase-conjugated secondary antibodies specific for each target, diluted in TBS-Tween for 2h. The membranes were washed again and the reaction was developed with a chemiluminescent kit (Amersham ™ ECL Select Western Blot Detection Reagent) and band quantification was performed by optical densitometry by ImageJ® software for Windows® and normalized by the values obtained for the β- Actin (endogenous control) (concentration 1:800; SC1615-Santa Cruz®).

2.8 Statistical analysis

Statistical analyzes were performed on GraphPadPrism® software (version 5.00; GraphPad, Inc., San Diego, CA). Initially the results were submitted to analysis of variance - ANOVA, followed by "Tukey-Kramer". The results were expressed as mean±standard deviation and were considered statistically significant differences when $p < 0.05$.

3. RESULTS

3.1 Biometric Parameters

The body weight of the animals did not present statistical difference throughout the experimental period in all groups analyzed (Table 1). The VP weight from CC groups were lower than CTR, T, T+PRL treated groups for both periods. The VP weight in the T and T+PRL groups increased progressively from day 3 until the end of treatment (day 10), with statistically difference been detected after 3 days of T administration. After 10 days of treatment, the values observed in T and T+PRL were similar those from control group (CRT). There were no differences between prostate weight in the PRL and BR treated animals in both period of treatment (Table 1). Moreover, PRL administrated in association with T did not induce an increment in the VP weight (Table 1).

3.2 Morphological and Stereological Analysis of the VP

Histologically, the prostates from CTR animals presented a characteristic morphology, with acini lined by columnar cells, ample luminal compartment and surrounded by a thin stromal layer. The prostatic morphology of CC group was characterized by cubic epithelium lining acini with reduced lumen surrounded by thick stromal layer. Three days of T replacement elicited a glandular regrowth, with increase in epithelial height, acinar lumen and protein secretion. After 10 days of T treatment, the prostate morphology was reestablished and is similar to the CTR group. The simultaneous administration of T+PRL did not cause additional prostatic response in both period of treatment. PRL alone or BR administration for 3 or 10 days did not cause any effects on prostate in castrated animals and the glandular structure remained similar to the CC groups (Figure 2). Stereological data corroborate the morphological results described above (Table 2).

3.3 Serum Prolactin Measurement

The serum concentration of PRL, as determined by ELISA, showed that in the CTR group, the levels were 1.57 ng/ml. The groups treated with BR at both ages did not present detectable levels of serum PRL, confirming the inhibitory effect of BR on PRL secretion. The castration induced a significant reduction of these values to 0.76 ng/ml, being statistically different from the CTR, T3 and BR3 groups. In the PRL3 group, the value was 1.04 ng/ml, being different only from the BR3 group. PRL levels in the T3 group were 1.69 ng/ml, statistically different from the CC, PRL3 and BR3 groups. The TPRL group had a value of 1.23 ng/ml, statistically different from the BR3 group. In the groups treated for 10 days, there was no significant difference between the experimental groups (Table 2).

3.4 Immunohistochemistry

In the CTR group, there is few proliferative cells stained by Ki-67. Castration induced a decrease in the number of proliferating labeled cells. On the other hand, in the T3 and TPRL3 the number of proliferative cells was significantly increased compared to CTR, CC, PRL and BR. Although T or T+PRL for 10 days maintained the higher proliferative levels of epithelial cells, this number was lower than the observed in the groups treated for 3 days. PRL or BR did not induce an increase in the number of proliferative epithelial cells (Figure 3A). The Ki-67 index confirmed the results of immunohistochemistry visualization (Figure 3B).

Immunohistochemistry for AR demonstrated an intense reaction in the nuclei of epithelial cells in the CTR group. The number of stained cells and the intensity of reaction were reduced in castrated animals. Treatment with T progressively restored the AR labeling in the prostate from T3 and TPRL3 groups. In the T10 and TPRL10 groups the AR staining was restored to the levels observed in CTR group. The PRL or BR treatments did not presented effects on AR intensity, which remained similar to the observed in the CC group (Figure 4).

3.5 Western Blot

Protein levels of PRLR observed by western blot showed that castration induced increase in PRLR expression. The treatment with T for 3 or 10 led to a reduction in the PRL

expression in relation to those observed in CC group. Treatment with PRL for 3 days increased PRLR levels in relation to the CTR, T3 and TPRL3 groups. On the other hand, the administration of PRL for 10 days raised PRLR expression significantly compared to all groups of the same treatment period, except for the CC group. The association of T+PRL did not induce significant changes in PRLR expression in relation to the other experimental groups at both treatment periods. Treatment with BR for 3 days led to a significant increase in expression when compared to the CTR group, and at 10 days, this effect was reversed (Figure 5A and B).

Protein levels of STAT3 decreased in castrated rats compared to CTR group. The administration of T, PRL and T+PRL for 3 or 10 days increased the STAT3 expression, excepted for PRL 03, which presented a tendency to increase in STAT3 expression, but without statically difference. Blockage of PRL secretion by BR resulted in an acute increase of STAT3 expression after 3 days of treatment, returning to the low levels observed in CC group with 10 days the treatment (Figure 5A and B). The STAT5 expression was also reduced in CC group. Treatment with T or T+PRL for 3 or 10 days increased the expression of STAT5 compared to CC group. However, the STAT5 was not affected by PRL administration to castrated rats in both period of treatment. In the same way, BR did not provoke alteration in the STAT5 expression in castrated animals (Figure 5 A and B)

Confirming the data observed in the immunohistochemistry reactions, western blotting for AR showed that castration decreased the expression levels in CC group. The administration of T or association of T+PRL for 3 days resulted in increase of AR expression compared to CC group. After 10 days, the AR levels recovery the values observed in the CTR (Figure 6 A and B)

The protein expression of prostatatein (an androgen-regulated secretory protein used to evaluate prostatic function) was reduced by castration. Only the T10 and TP10 groups were able to restore the secretory function to the levels observed in the CTR group (Figure 6 A and B).

4. DISCUSSION

PRL is a hormone that is mainly secreted by lactotroph cells of the anterior pituitary gland, and is involved in many biological processes including lactation and reproduction (BERNARD et al., 2015). It has been recognized that PRL plays a key role in both prostatic development and secretory function (GOFFIN et al., 2005). PRL can acts on the prostate

secretion, regulating the uptake of zinc, citrate synthesis, and the expression of the androgen receptor synergistically with androgens (LIU; FRANKLIN; COSTELLO, 1997).

Although the pituitary is the major source of PRL, it has been demonstrated extra-pituitary synthesis of PRL, such as the prostate (SACKMANN-SALA; GOFFIN, 2015). In our study is that, although castration lead to increased expression of PRLR in rat VP, there is no activation of this downstream pathway. The administration exogenous PRL did not present anabolic effect on VP of castrated animals, although it activated the signaling pathways responsive to PRL. Likewise, its inhibition by BR did not lead to greater glandular involution after castration, or alter the molecular pathway involved with PRL signaling. These results demonstrate that, despite activation of the pathway, PRL does not appear to act on VP regrowth either in the absence or in the presence of elevated T levels.

Castration for 21 consecutive days induced a significant decrease in serum PRL levels compared to the CTR group. There are conflicting data on the literature about the effects of castration on serum PRL levels. EUKER; MEITES; RIEGLE, (1975) demonstrated that serum PRL was increased in the adult castrate rats. On the other hand, SHAAR et al., (1975) observed reduction in PRL levels in both young and old castrated rats. Although the divergence results on the effect of castration on serum PRL, overall, our results pointed to down regulation of serum PRL and PRL signaling pathway.

On the other hand, 3 days of T administration restored the PRL levels observed in the CTR group. The PRL isolated or in combination with T (T+PRL) for 3 days also tend to increase serum PRL, but without statistical difference. As expected, treatment with BR decreased serum PRL to undetectable levels. The increase in PRL in T treated animals can be explained by the indirect effect of T on increasing serum PRL levels by a T aromatization pathway and conversion to estrogen that stimulates the pituitary to increase PRL synthesis (GILLERAN et al., 2003). The administration of PRL alone or in combination with T does not seem to alter circulating levels of PRL, although it was observed a tendency of PRL decreasing compared to the group treated with T alone. Our result can be explained by a short loop feedback, whereby PRL itself acts in the brain to stimulate production of dopamine and thereby inhibit its own secretion (GRATTAN, 2015; ROZENBOIM et al., 2004). In the group of animals treated for 10 days, no significant differences were observed between the experimental groups. Although the variability observed among samples, overall, our results demonstrated that exogenous PRL administered alone down regulates pituitary PRL secretion, whereas T appears stimulate this event.

There was no change in the rat body weight among the experimental groups. Other studies in our group also showed that castrated animals submitted to T replacement the same results (DE CARVALHO; VILAMAIOR; TABOGA, 1997; JUSTULIN et al., 2010; OLIVEIRA et al., 2007). Administration of PRL alone or in combination with T or BR also did not alter this parameter. In a recent study, our group demonstrated that treatment of 23-day postnatal (DPN) mice for 23 days (DPN35) led to an increase in body weight (unpublished data). Previous studies have reported a somatotrophic effect of exogenous PRL administered to male rats during the period from 21 to 60 days of age, leading to body weight gain (PÉREZ-VILLAMIL; BORDIÚ; PUENTE-CUEVA, 1992). These differences may be due to the short treatment period in our study, in addition to the different animal's age.

The prostate development and secretory function are under androgenic control (MARKER et al., 2003). Surgical or chemical castration leads to a prostatic involution by decreasing T and consequently apoptosis of androgen-dependent cells (DENMEADE; LIN; ISAACS, 1996). For this reason, chemical castration is still used as an initial treatment for androgen-dependent PCa, (BACIARELLO; STERNBERG, 2016). Our study also demonstrated that castration for 21 days led to a significant reduction in prostate weight, which resulted in a significant reduction in the height of the secretory epithelial cells and the absolute volume of the glandular compartment (epithelium and lumen). Despite an evident condensation of the stroma compartment, absolute stromal volume values did not change after castration. Other authors have also demonstrated the same effects of castration in different experimental models (AUGUSTO; FELISBINO; CARVALHO, 2008; JUSTULIN et al., 2010; ROCHEL-MAIA et al., 2011).

The androgenic replacement induces glandular regrowth by inducing proliferation of epithelial cells that have resisted the castration (DE CARVALHO; VILAMAIOR; TABOGA, 1997; JUSTULIN et al., 2010; VILAMAIOR et al., 2000; YAMASHITA et al., 1996). This involution/re-growth model has been extensively used to study the kinetics of glandular growth, cell proliferation and stromal remodeling. Three days of T replacement elicits the recovery of glandular morphology and prostatic weight. During this period, T treatment caused intense proliferative activity of the epithelium, as well as increased epithelial cell height and prostatic secretory function, leading to both epithelial and luminal compartment regrowth and complete recovery of glandular morphology after 10 days T replacement. Our results are in accordance with the literature, where the total recovery of prostatic morphology occurs between 7 and 10

days of androgen replacement (JUSTULIN et al., 2006; KLEDZIK et al., 1976; SMITH et al., 1985).

In spite of the castration or T replacement effects on prostate are well know (DE CARVALHO; VILAMAIOR; TABOGA, 1997; JUSTULIN et al., 2010; VILAMAIOR et al., 2000) in our study, these results were used to evaluate the effects of PRL administration alone or in combination with T on prostate morphophysiology. The PRL administration to castrated rats for 3 or 10 days did not induce prostate growth or changes in glandular morphology. Other authors also demonstrated that castration followed by PRL administration did not induce prostatic regrowth (KLEDZIK et al., 1976; VAN COPPENOLLE et al., 2001). On the other hand, other studies have shown that supplementation of PRL to non-castrated adult rats increases cell proliferation, leading to VP growth (GOFFIN et al., 2011; HERRERA-COVARRUBIAS et al., 2015). These results highlighted that, although PRL is considered an anabolic hormone for prostate, in an absence of T, PRL administration was not able to induce glandular regrowth.

It has been demonstrated that active sexual life produces a constant increase in serum PRL, which is associated with the produce of quantity and quality of prostatic semen to ensure the ovum fertilization (PASCUAL-MATHEY et al., 2016; ROJAS-DURÁN et al., 2015). In an experimental model of hyperprolactinemia induced by intraperitoneal injection of PRL or adenohypophysis transplantation under the renal capsule, PASCUAL-MATHEY et al. (2016) demonstrated sustained activation of PRL downstream signaling pathway, involving its receptors, p-Stat3 and MAPK pathway in VP to ensure an increase in the synthesis and secretion of the prostatic fluid for the maintenance of the quantity of semen. Although we did not evaluate sexual behavior or semen quality, we also demonstrated association between increase in PRLR and STAT3 protein expression in both castrated rats and PRL treated for 3 and 10 days. The STAT5 also showed a tendency to increase in both PRL groups, but without statistically difference, as observed by (PASCUAL-MATHEY et al., 2016). Overall, these results reinforce the important role of STAT3 on the response to PRL in theVP.

Androgen ablation is the principal treatment for patients with locally PCa. However, with the androgen blockade, the prostatic cells respond to other hormone and growth-factors to stimulate survive and growth (SACKMANN-SALA et al., 2014). In a model of castration-induced prostate involution, the androgen suppression also induces activation of the alternative pathway to sustain glandular morphophysiology (VIKRAM; JENA, 2011). In this sense, the

increase in PRLR observed in castrated animals can represent an adaptive response to the low levels of androgen after castration (SACKMANN-SALA; GOFFIN, 2015).

In our study, castration for 21 days leads to a downregulation of both STAT 3 and 5. This result is consistent with reduction in serum PRL and glandular involution in castrated rats. Although it has been demonstrated increase in STAT signaling during PCa progression to androgen-independent tumor (WERTZ, 2009; ZHOU et al., 2015), we believe that, contrary of androgen-independent cells, in normal prostate this effect do not occur, mostly because the massive epithelial cell death after castration. In the T3 group, we observed the initiation of glandular recovery. However, without increase in PRLR expression. The same result was observed in the T10 group. Although we did not observe changes in PRLR in T treated rats, the STAT 3 and 5 protein expression was increased in both T3 and T10 groups. Although there is evidences that PRL acts synergistically with T to stimulate prostate growth (LIU; FRANKLIN; COSTELLO, 1997), our results demonstrate that T does not directly regulate PRLR expression. Moreover, the combined administration of T+PRL do not affect PRLR expression, suggesting that androgenic signaling overlap the PRL pathway in the prostate regrowth. These results are consistent with previous studies that demonstrated androgenic-induction of STAT signaling in the renal cells (LIU et al., 2013). Overall, our data highlighted that T-induced prostate regrowth recruits other pathways than the canonical AR signaling.

Our results on AR expression in castrated prostate or after androgen replacement corroborates the previous published data (JUSTULIN et al., 2006; MARKER et al., 2003; VILAMAIOR et al., 2000). The AR expression in the VP from castrated rats was also not altered by PRL administration. Moreover, there is no additional effect on AR expression with the combination of T+PRL. Studies on VP organ cultures demonstrated that PRL an increase in nuclear uptake of [3H]DHT (JOHANSSON, 1976). Barañao et al.(1981) also observed an increase in nuclear androgen receptor content in prostate of immature non-castrated rats receiving PRL injections during early puberty. On the other hand, Prins et al (1987) demonstrated increase in AR expression in lateral prostate of castrated adult rats that underwent pituitary implants and T replacement showed greater in the lateral prostate, with no effect on AR expression in theVP. Gomés et al.(2009) showed increase in AR positive cells after treatment with PRL in non-castrated rats. Although the divergent data in the literature, our results pointed that in an absence of T, PRL *per si* is not able to modulate AR expression. Additionally, there is no changes in prostatic AR expression in castrated rats treated with BR,

corroborating neither PRL administration or PRL physiologically levels interfere with AR expression in the reduced levels of circulating androgen.

In order to if PRL interferes prostate secretory function in castrated animals, we evaluated the prostatein expression, an androgen-dependent protein secreted by VP (SANTOS et al., 2014). In our study, castration significantly reduced the prostatein expression, whereas the T replacement restores the prostatein synthesis after 10 days of treatment. Similar results were observed by (FUJIMOTO et al., 2009) in the VP of castrated rats submitted to T replacement. The PRL administration to castrated rats had no effect on PRL expression. This result demonstrates that PRL do not exert stimulatory effect on prostate secretory function in the absence of T. On the other hand, Reiter and colleagues (REITER et al., 1995) demonstrated that the administration of PRL (10 μg) for 7 days to castrated and hypophysectomized adult rats induced increased prostate expression of the prostate in the VP. With these results, these authors emphasize that PRL regulates the gene expression of prostatic secretory proteins independently of androgen. In our study, we did not evaluate the gene expression of Prostatein, however, our set of results demonstrate that PRL administered to castrated rats has no effect on prostate secretory function.

5. CONCLUSION

The administration of PRL at 0.3 mg/kg administered for 3 or 10 consecutive days to castrated rats do not exert anabolic effect on the VP. Although we observed activation of PRL signaling, this was not enough to overcome the prostatic androgen deficiency. Likewise, there is no additional glandular involution in BR castrated group. Thus, we conclude that despite stimulating downstream signaling pathway, PRL does not act on VP in the absence or in the presence of supra physiologic levels of T.

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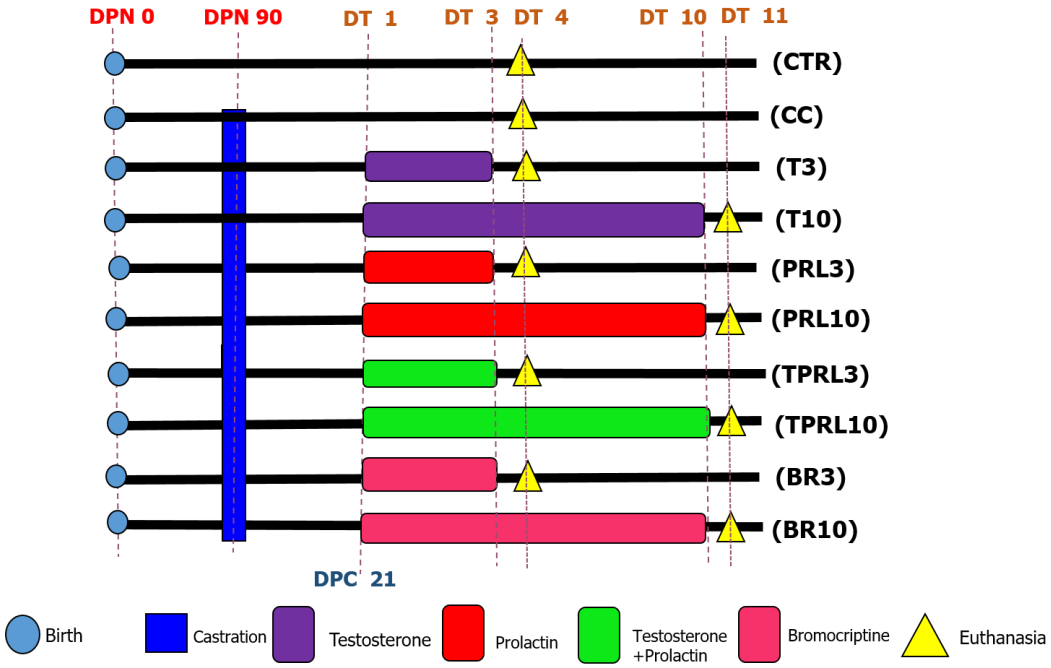

FIGURE 1: Experimental design; CTR: control, CC: castrated animals; ; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.TLD: postnatal day; DT: Day of treatment; DPC: day after castration.

Table 01- Biometric data from the different experimental groups.

	CTR	CC	T3	T10	PRL3	PRL10	TPRL3	TPRL10	BR3	BR10
Body weight 3 months (g)	461,4 ± 47,5	471,7 ± 87,4	488,4 ± 47,0	424,5 ± 74,5	476,5 ± 52,3	447,1 ± 33,1	439,5 ± 76,1	461,6 ± 34,6	449,0 ± 52,9	491,3 ± 54,8
Body weight 21 days castration (g)	514,3 ± 17,8	491,3 ± 70, 1	473,3 ± 39,0	459,0 ± 57,6	497,5 ± 45,7	473,6 ± 37,8	462,0 ± 54,2	465,4 ± 41,6	489,8 ± 32,6	497,2 ± 40,1
Body weight 3 days of treatment (g)	526,7 ± 18,8	496,4 ± 67,5	491,1 ± 36,7	466,0 ± 57,5	497,9 ± 39,3	457,5 ± 44,5	490,8 ± 55,0	493,8 ± 40,0	491,9 ± 34,5	507,9 ± 42,1
Body weight 10 days of treatment (g)				482,3 ± 63,0		451,6 ± 50,8		500,8 ± 43,5		514,3 ± 45,1
Weight of the prostate (g)	0,696 ± 0,2 ^{A}	0,087 ±0,03^{B}	0,1867 ± 0,04^{B}	0,846 ± 0,2 ^{A}	0,0834 ± 0,03^{B}	0,0757 ± 0,02^{B}	0,1309 ± ,07^{B}	0,6589 ± 0,12 ^{A}	0,0955 ± 0,08^{B}	0,0688 ± 0,01^{B}

Values expressed as mean ± standard deviation. Different letters showed significant difference between the groups with p <0.05. . CTR: Control animals; CC: Castrated animals; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.

Table 2 –Stereological, Morphological Data of the Ventral Prostate and Serological

Parameters	**Groups**									
	CTR	**CC**	**T3**	**T10**	**PRL3**	**PRL10**	**TPRL3**	**TPRL10**	**BR3**	**BR10**
Absolute volume (mn^{3})										
Epithelium	8.91 ± 5.4^{A}	1.96 ± 0.33^{B}	5.66 ± 0.88^{C}	15.10 ± 3.65 ^{A}	2.06 ± 0.15^{B}	2.00 ± 0.36 ^{B}	4.14 ± 0.63^{C}	15.00 ± 2.35 ^{A}	2.44 ± 0.32^{B}	1.72 ± 0.29 ^{B}
Lumen	56.37 ± 8.9^{A}	1.43 ± 1.35 ^{B}	7.39 ± 1.41 ^{B}	61.81 ± 7.07 ^{A}	1.36 ± 1.03 ^{B}	1.26 ± 1.00 ^{B}	3.25 ± 1.20 ^{B}	44.02 ± 4.33 ^{A}	1.08 ± 0.65 ^{B}	0.65 ± 0.38 ^{B}
Stroma	5.18 ± 3	4.66 ± 1.07	5.66 ± 0.43	7.67 ± 4.30	4.69 ± 0.98	4.01 ± 0.74	5.14 ± 0.94	7.54 ± 0.80	6.05 ± 0.90	4.48 ± 0.60
Epithelium height (µm)	8.84 ± 0.53^{A}	3.99 ± 0.56^{B}	7.62 ± 0.39^{A}	9.11 ± 2.39^{A}	4.26 ± 0.54^{B}	4.33 ± 0.28^{B}	8.06 ± 1.73^{A}	11.31 ± 0.74^{A}	4.55 ± 0.51^{B}	4.16 ± 0.44^{B}
Serum prolactin (ng/ml)	1.578 ± 0.24^{Ac}	0.768 ± 0.48^{B}	1.6995 ± 0.34^{c}	0.888 ± 0.64	1.0415 ± 0.12^{AB}	0.465 ± 0.95	1.2345 ± 0.16^{ABC}	1.373 ± 0.28	0 ± 0	0 ± 0

Data were expressed as mean ± standard deviation. Different letters show statistical difference with p <0.05. CTR: Control animals; CC: Castrated animals; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.

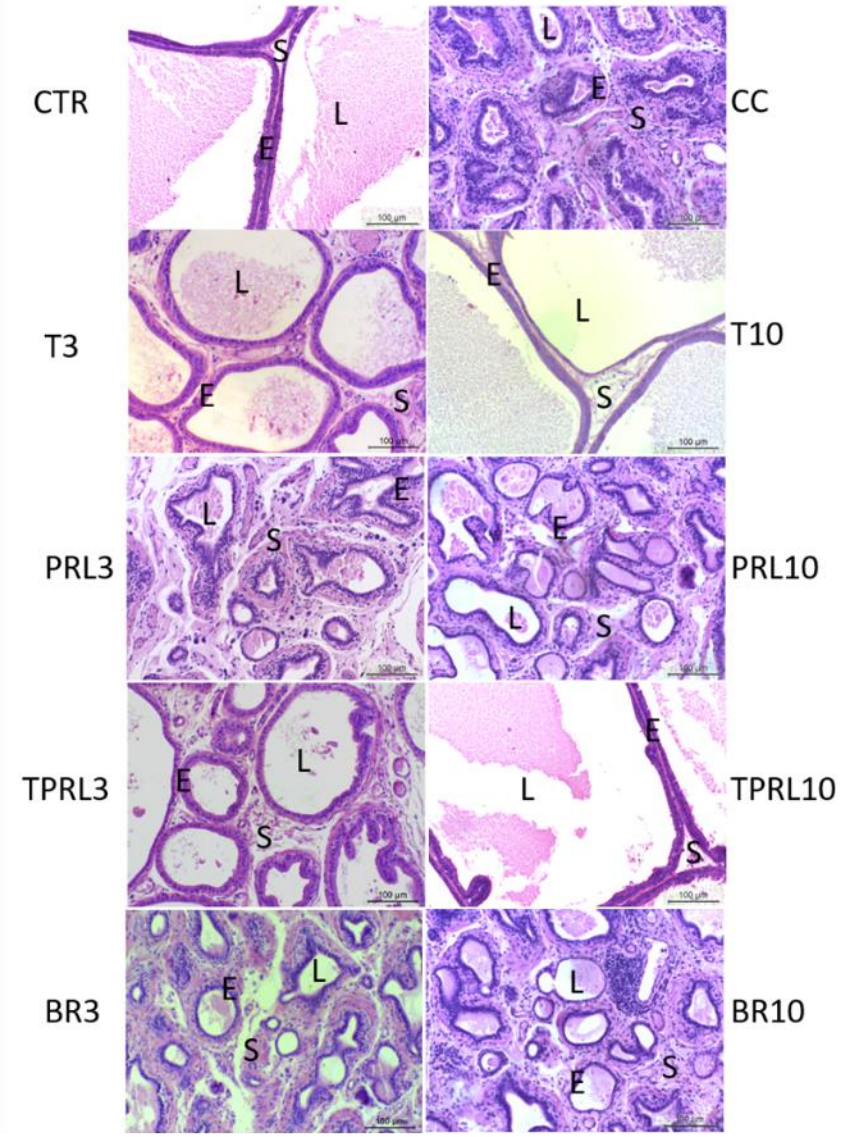

Figure 2: Representative micrograph of PV. All groups were stained with hematoxylin and heosin. L: Lumen; S: Stroma; E: Epithelium; CTR: Control animals; CC: Castrated animals; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.

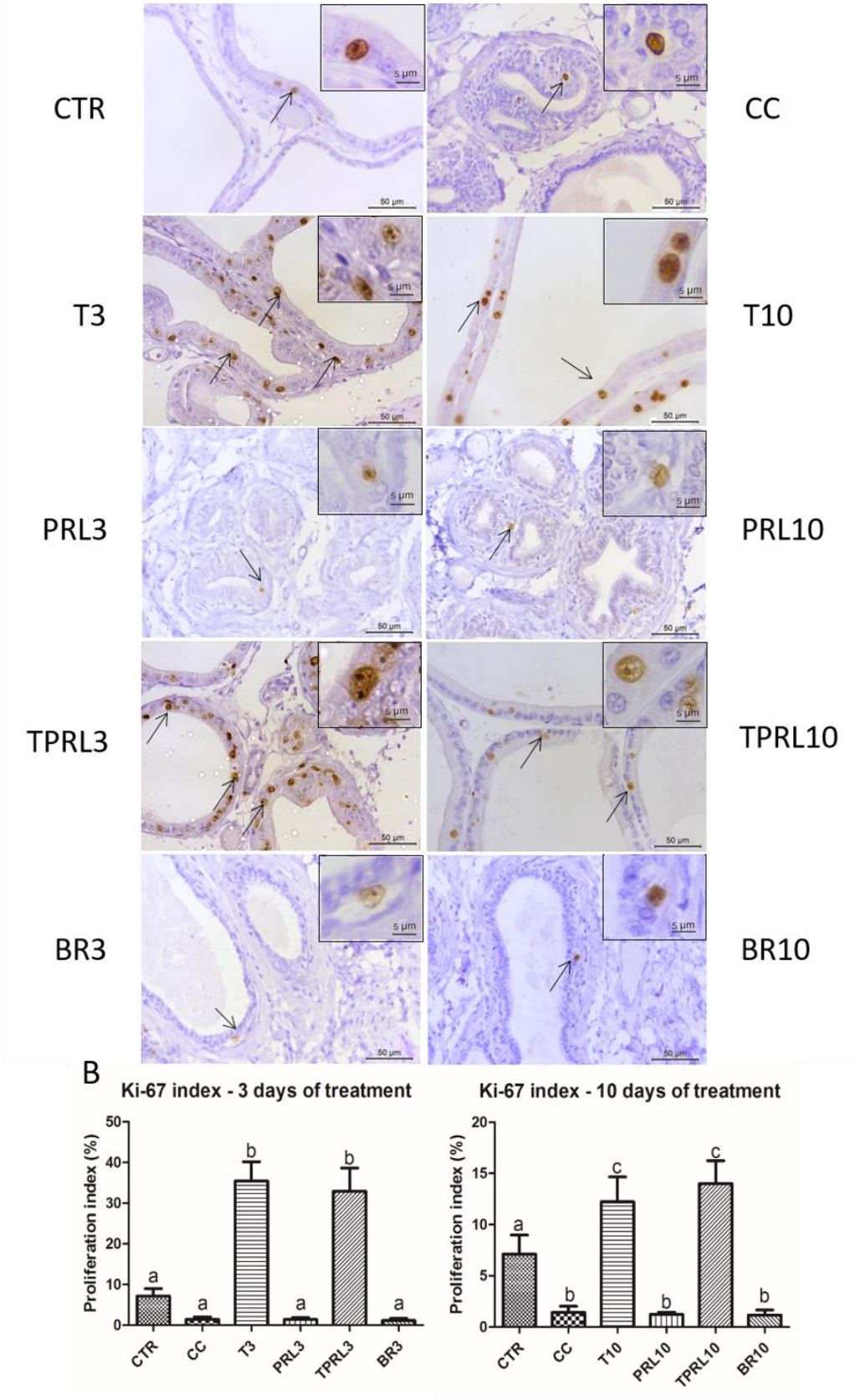

Figure 3 – A) Histological sections representative of histochemical reactions for Ki-67 in the ventral prostate of the different experimental groups. The arrows point to marked epithelial cells. B) Graphics represent the percentage of positive cell for each marker in the VP from all experimental groups. CTR: Control animals; CC: Castrated animals; T3: Animals treated with Testosterone for 3 days; T10: Animals treated with Testosterone for 10 days; PRL3: Animals treated with Prolactin for 3 days; PRL10: Animals treated with Prolactin for 10 days; TPRL3: Animals treated with Testosterone and Prolactin for 3 days; TPRL10: Animals treated with Testosterone and Prolactin for 10 days; BR3: Animals treated with bromocriptine for 3 days; BR10: Animals treated with bromocriptine for 10 days.

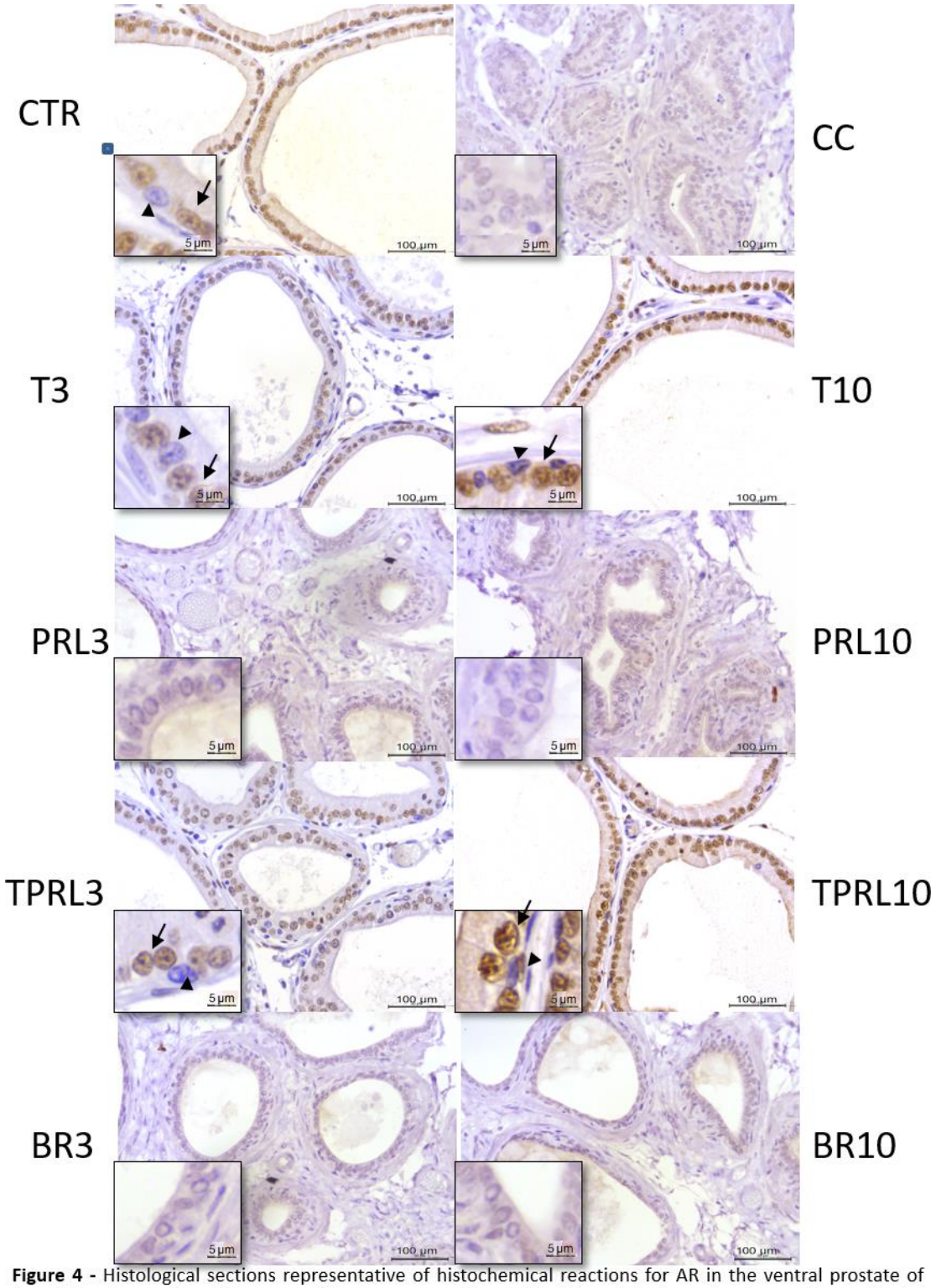

Figure 4 - Histological sections representative of histochemical reactions for AR in the ventral prostate of the different experimental groups. Filled arrow: positive epithelial nuclei; Arrowhead: negative epithelial nuclei. CTR: Control animals; CC: Castrated animals; T3: Animals treated with Testosterone for 3 days; T10: Animals treated with Testosterone for 10 days; PRL3: Animals treated with Prolactin for 3 days; PRL10: Animals treated with Prolactin for 10 days; TPRL3: Animals treated with Testosterone and Prolactin for 3 days; TPRL10: Animals treated with Testosterone and Prolactin for 10 days; BR3: Animals treated with Bromocriptine for 3 days; BR10: Animals treated with Bromocriptine for 10 days.

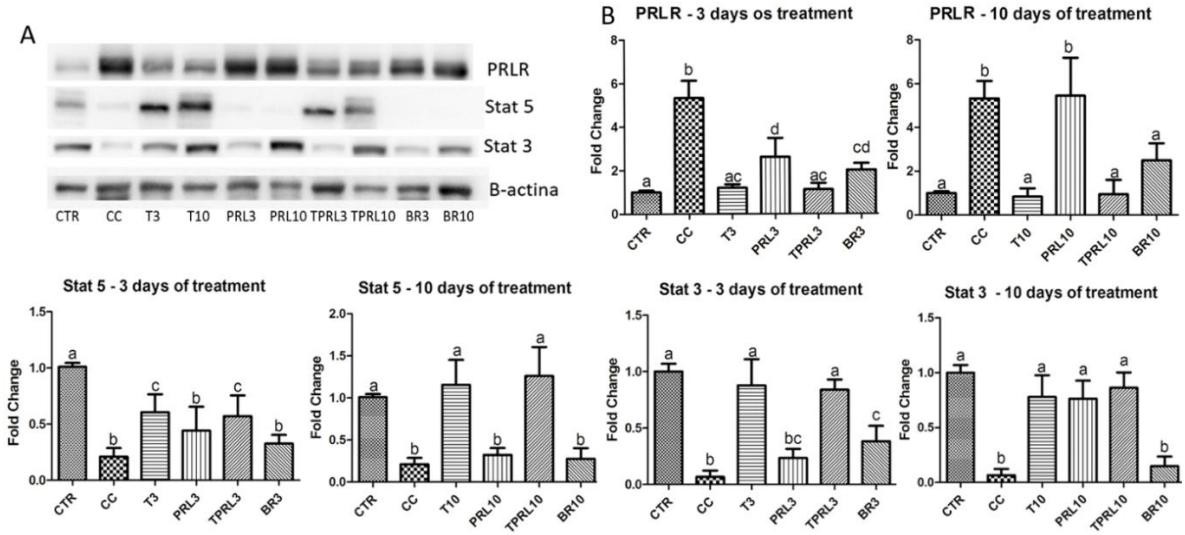

Figure 5. A) Representative western blot reactions for PRLR in the different experimental groups (n = 6 / group) B) Graphics represent western blotting quantification. The target bands were quantified and normalized by the β-actin value. The values were expressed as variation in relation to CTR (fold change), being considered statistically different when p value <0.05. CTR: Control animals; CC: Castrated animals; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.

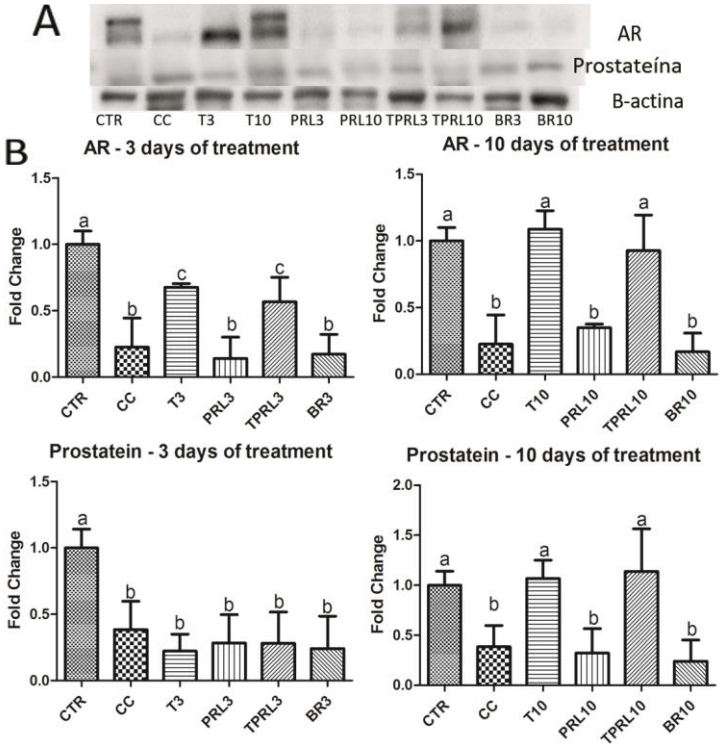

Figure 6. A) Representative western blot reactions for AR and Prostatein in the different experimental groups (n = 6 / group). B) Graphics represent western blotting quantification The target bands were quantified and normalized by the β-actin value. The values were expressed as variation in relation to CTR (fold change), being considered statistically different when p value <0.05. CTR: Control animals; CC: Castrated animals; T3: Castrated animals treated with Testosterone for 3 days; T10: Castrated animals treated with Testosterone for 10 days; PRL3: Castrated animals treated with Prolactin for 3 days; PRL10: Castrated animals treated with Prolactin for 10 days; TPRL3: Castrated animals treated with Testosterone and Prolactin for 3 days; TPRL10: Castrated animals treated with Testosterone and Prolactin for 10 days; BR3: Castrated animals treated with bromocriptine for 3 days; BR10: Castrated animals treated with bromocriptine for 10 days.

ANEXOS

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

Certificado

Certificamos que o projeto intitulado "Efeitos da administração de prolactina sobre a próstata ventral de ratos castrados: Avaliação da morfologia, dinâmica de proliferação e diferenciação das células epiteliais e do estroma glandular", Protocolo nº **767-CEUA**, sob a responsabilidade de **Luis Antônio Justulin Junior**, 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 "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Vigência do Projeto:	*Início: 20/9/2015*	*Término: 20/1/2016*
Espécie/linhagem:	*Rato Sprague dawley*	
Nº de animais:	*80*	
Peso:	*250g*	*Idade: 3 meses*
Sexo:	*Macho*	
Origem	*Centro de Bioterismo da Universidade Estadual de Campinas – Unicamp – Campinas/SP*	

Botucatu, 2 de setembro de 2015.

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

Instituto de Biociências – Diretoria Técnica Acadêmica
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Artigos publicados durante o mestrado:

Streptozotocin-Induced Maternal Hyperglycemia Increases the Expression of Antioxidant Enzymes and Mast Cell Number in Offspring Rat Ventral Prostate

ANA C. L. CAMARGO, SÉRGIO A. A. DOS SANTOS, JAQUELINE C. RINALDI, FLAVIA B. CONSTANTINO, KETLIN T. COLOMBELLI, WELLERSON R. SCARANO, SERGIO L. FELISBINO, AND LUIS A. JUSTULIN*
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ABSTRACT

Gestational diabetes mellitus (GDM) has increased in recent years. Although the cellular and molecular mechanisms involved in GDM-increased risk factors to offspring remained poorly understood, some studies suggested an association between an increase in oxidative stress induced by maternal hyperglycemia and complications for both mothers and newborns. Here, we investigated the impact of maternal hyperglycemia followed by maternal insulin replacement during lactation on the expression of antioxidant enzymes and mast cell number in offspring ventral prostate (VP) at puberty. Pregnant rats were divided into three groups: control (CT); streptozotocin-induced maternal hyperglycemia (MH); and MH plus maternal insulin replacement during lactation (MHI). Male offspring were euthanized at postnatal day (PND) 60 and the VP was removed and processed for histology and Western blotting analyses. Maternal hyperglycemia delayed prostate maturation, and increased mast cell number catalase (CAT), superoxide dismutase (SOD), glutatione-s-transferase (GST-pi), and cyclooxygenase-2 (Cox-2) expression in the offspring of hyperglycemic dams. Maternal insulin replacement restored VP structure, mast cell number and antioxidant protein expression, except for Cox-2, which remained higher in the MHI group. Thus, an increase in oxidative stress induced by intrauterine hyperglycemia impacts prostate development and maturation, which persists until puberty. The overall improvement of maternal metabolism after insulin administration contributes to the restoration of prostate antioxidant enzymes and secretory function. Taken together, our results highlighted that imbalanced physiological maternal-fetal interaction contributes to the impairment of reproductive performance of the offspring from diabetic mothers. Anat Rec, 300:291–299, 2017. © 2016

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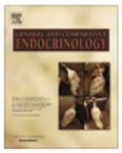

Research paper

Impairment of microvascular angiogenesis is associated with delay in prostatic development in rat offspring of maternal protein malnutrition

Ketlin T. Colombelli, Sérgio A.A. Santos, Ana C.L. Camargo, Flávia B. Constantino, Caroline N. Barquilha, Jaqueline C. Rinaldi, Sérgio L. Felisbino, Luis A. Justulin *

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ABSTRACT

Experimental data demonstrated the negative impact of maternal protein malnutrition (MPM) on rat prostate development, but the mechanism behind the impairment of prostate growth has not been well understood. Male Sprague Dawley rats, borned to dams fed a normal protein diet (CTR group, 17% protein diet), were compared with those borned from dams fed a low protein diet (6% protein diet) during gestation (GLP group) or gestation and lactation (GLLP). The ventral prostate lobes (VP) were removed at post-natal day (PND) 10 and 21, and analyzed via different methods. The main findings were low birth weight, a reduction in ano-genital distance (AGD, a testosterone-dependent parameter), and an impairment of prostate development. A delay in prostate morphogenesis was associated with a reduced testosterone levels and angiogenic process through downregulation of aquaporin-1 (AQP-1), insulin/IGF-1 axis and VEGF signaling pathway. Depletion of the microvascular network, which occurs in parallel to the impairment of proliferation and differentiation of the epithelial cells, affects the bidirectional flux between blood vessels impacting prostatic development. In conclusion, our data support the hypothesis that a reduction in microvascular angiogenesis, especially in the subepithelial compartment, is associated to the impairment of prostate morphogenesis in the offspring of MPM dams.