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

PG-BGA

PAPEL DA PROLACTINA DURANTE DESENVOLVIMENTO PROSTÁTICO EM RATOS: AVALIAÇÃO DA MORFOGÊNESE GLANDULAR, PROLIFERAÇÃO E DIFERENCIAÇÃO DAS CÉLULAS EPITELIAIS

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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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“De todo o amor que eu tenho
Metade foi tu que me deu
Salvando minh'alma da vida
Sorrindo e fazendo o meu eu”
Dona Cila- Maria Gadú

À minhas avós, Magnólia e Maria Auxiliadora...
Dedico

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“Conhecimento não é aquilo que você sabe, mas o que
você faz com aquilo que você sabe. ”
Aldous Huxley

SUMÁRIO

LISTA DE ABREVIATURAS

AR- Receptor de Andrógeno

BR- Bromocriptina

BR21- grupo bromocriptina no DPN21

BR35- grupo bromocriptina no DPN35

PRL21 grupo prolactina no DPN21

PRL35- grupo prolactina no DPN35

CaP- Câncer de próstata

CK- citoqueratinas

CT21- grupo controle no DPN21

CT35- grupo controle no DON35

DHT- Diidrotestosterona

DPN- dia pós-natal

DPN- 21- dia pós-natal 21

DPN- 35- dia pós-natal 35

E2- estrogeno

HE- Hematoxilina e Eosina

HPB- Hiperplasia próstatica benigna

JAK- Janus kinase 2

mRNA- RNA mensageiro

PDL- Próstata dorsolateral

PRL- Prolactina

PRLR- Receptor de prolactina

PV- Próstata ventral

STAT3- transdutor de sinal e ativador de transcrição 3

STAT5- transdutor de sinal e ativador de transcrição 5

T- Testosterona

RESUMO

A próstata é uma glândula acessória do sistema genital masculino e, além da dependência androgênica para o seu desenvolvimento e homeostasia, é influenciada por hormônios não esteróides, entre eles a prolactina (PRL). Estudos em modelos animais demonstraram que a PRL pode atuar com efeito anabólico sobre a glândula prostática. Assim, nosso objetivo foi investigar os efeitos da modulação de PRL sobre o desenvolvimento e maturação da próstata ventral (PV) de ratos. Foram utilizados ratos machos *Sprague Dawley* (DPN 90) divididos em 6 grupos (n=6): Grupo controle (CT): receberam solução salina; Prolactina (PRL): Prolactina (0,3mg/kg); Bromocriptiona (BR): inibidor de PRL (0,4mg/kg). Todos os animais foram submetidos diariamente (via subcutânea) do dia pós-natal 12 (DPN12) até os DPN 21 ou 35. Os animais foram anestesiados, pesados e posteriormente foram eutanasiados. O sangue e os lobos da PV foram coletados, pesados e processados para análises morfológicas, de western blot (WB) e RT qPCR. O tratamento com PRL induziu aumento de peso corpóreo dos animais no DPN35 comparado aos outros grupos. Histologicamente houve maior acumulo de screção no lúmen e diminuição do compartimento epitelial nos grupos PRL e BR comparados ao CT no DPN35. A reação imunohistoquímica para Ki67 demonstrou aumento de células proliferativas nos grupos tratados com PRL. A quantificação de PCNA confirmou estes dados, com aumento significante no grupo PRL35. A intensidade de reação imunohistoquímica para AR foi maior no grupo PRL35. A quantificação de AR por WB confirmou esses dados. A expressão gênica do PRLR aumento no DPN21, enquanto que no DPN35 os grupos PRL e BR diminuíram, em Stat3 aumento nos grupos BR no DPN21 e 35, enquanto que Stat5 não houve diferença nos diferentes grupos. A expressão proteica de PRL aumentou no PRL21, no DPN35 diminuiu em PRL e BR, o PRLR não teve diferença entre os grupos experimentais. A quantificação proteica de STAT3 aumento nos grupos PRL e BR em ambas as idades, no STAT5 AB e prostateína aumentou nos grupos PRL em relação aos outros dois grupos no DPN35. A expressão proteica de CK18 aumentou nos grupos tratados com BR no DPN21 e 35, em NKX3.1 o aumento no PRL em relação aos outro grupo no DPN21. Nossos resultados demonstram que a modulação de PRL interfere com a morfofisiologia prostática, aumentando a resposta proliferativa e atividade secretora, em especial nos animais tratados por período prolongado. Estes dados revelam o importante papel da PRL sobre o desenvolvimento prostático.

ABSTRACT

The prostate gland is an accessory gland of the male genital system and, in addition to androgenic dependence for its development and homeostasis, is influenced by non-steroidal hormones, among them the prolactin (PRL). Studies in animal models have shown that PRL can act with an anabolic effect on the prostate gland. Thus, our objective was to investigate the effects of PRL modulation on the development and maturation of the ventral prostate (VP) of rats. Male Sprague Dawley rats (PND 90) were divided in 6 groups (n= 6): Control group (CT) that received saline solution; Prolactin group (PRL) that received Prolactin (0.3mg/kg); Bromocriptiona group (BR) that received PRL inhibitor (0.4mg/kg). All animals were submitted daily (subcutaneously) from postnatal day 12 (PND12) to PND 21 or 35. The animals were anesthetized, weighed and euthanized. Blood and VP lobes were collected, weighed and processed for morphological analyzes, western blot (WB) and RT qPCR. Treatment with PRL induced body weight gain in PND35 compared to the other groups. Histologically, there was an increase in the luminal compartment and a decrease in the epithelial compartment in the PRL and BR groups compared with CT on PND35. The immunohistochemical reaction for Ki67 demonstrated proliferation of proliferative cells in the groups treated with PRL on PND35. The PCNA quantification confirmed these data, with a significant increase in the PRL35 group. The intensity of reaction for AR was higher in the PRL35 group. Quantification of AR by WB confirmed these data. The gene expression of the PRLR increased in the PND21, whereas on PND35 the PRL and BR groups decreased, in Stat3 increased in the BR groups on PND21 and 35, whereas in the Stat5 groups there was no difference in the different groups. Protein expression of PRL increased in PRL21, on PND35 decreased in PRL and BR, PRLR had no difference between the experimental groups. The protein quantification of STAT3 increased in the PRL and BR groups in both ages, STAT5 AB and prostatein increased in the PRL groups compared with other two groups on PND35. Protein expression of CK18 increased in the groups treated with BR on PND21 and 35, on NKX3.1 the increase in PRL compared to other groups on PND21. Our results demonstrate that PRL modulation interferes with prostate morphophysiology, increasing the proliferative response and secretory activity, especially in animals treated for a long period. These data reveal the relevant role of PRL in prostate development.

Capítulo 1

1- INTRODUÇÃO

1.1 Desenvolvimento Prostático

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 e outros fatores componentes do fluido seminal (MARKER et al., 2003). Esta secreção é fundamental para o sucesso reprodutivo, pois liquefaz o ejaculado além de possuir enzimas e gradientes iônicos que alcalinizam o canal vaginal e nutrientes que garantem a motilidade dos espermatozoides (AUMÜLLER; SEITZ, 1990).

Em homens, é a maior glândula sexual acessória e está situado inferior a bexiga urinária, envolvendo completamente a uretra (BURDEN et al., 2006) (Figura 1A). Macroscopicamente, podemos distinguir três zonas: zona central (equivalente a 5% do tecido prostático), zona de transição, compreendendo cerca de 25% do tecido prostático, e a zona periférica, que corresponde a 70% do volume da glândula e é a principal zona de ocorrência de câncer (Figura 1) (BURDEN et al., 2006).

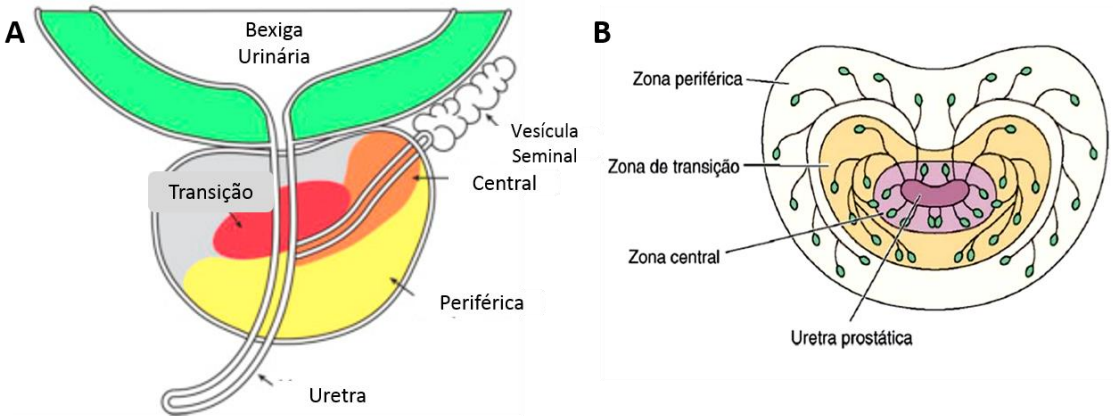

Figura 1. **A**- Localização e organização anatômica da próstata humana, mostrando sua divisão em três zonas, dispostas ao redor da uretra prostática (PENG; JOYNER, 2015) . **B**- Próstata humana é uma glândula compacta, que pode ser dividida em 3 zonas: central, transição e zonas periféricas (JUNQUEIRA; CARNEIRO, 2008).

Em ratos e camundongos a glândula é composta por quatro lobos distintos: anterior ou glândula coaguladora, dorsal, lateral e ventral (Figura 2) (MARKER et al., 2003; SHIRAI et al., 2000). Os lobos dispõem-se ao redor da bexiga urinária, contendo particularidades quanto à ramificação de ductos e produção de secreções proteicas (SUGIMURA; CUNHA; DONJACOUR, 1986). Estudos comparativos do desenvolvimento da próstata em roedores e

humanos demonstraram que a morfogênese ocorre de maneira análoga em ambos os modelos (TIMMS; MOHS; DIDIO, 1994). Os componentes glandulares apresentam características comuns nos diferentes animais, independente do aspecto macroscópico da glândula. Os tipos de populações celulares são similares e, provavelmente, desempenham as mesmas funções, variando somente na distribuição relativa entre as espécies (IMAMOV et al., 2004). Portanto, este fator favorece estudos referentes à homologia morfofuncional entre as diferentes espécies (IMAMOV et al., 2004; KARR et al., 1995; PRICE, 1963).

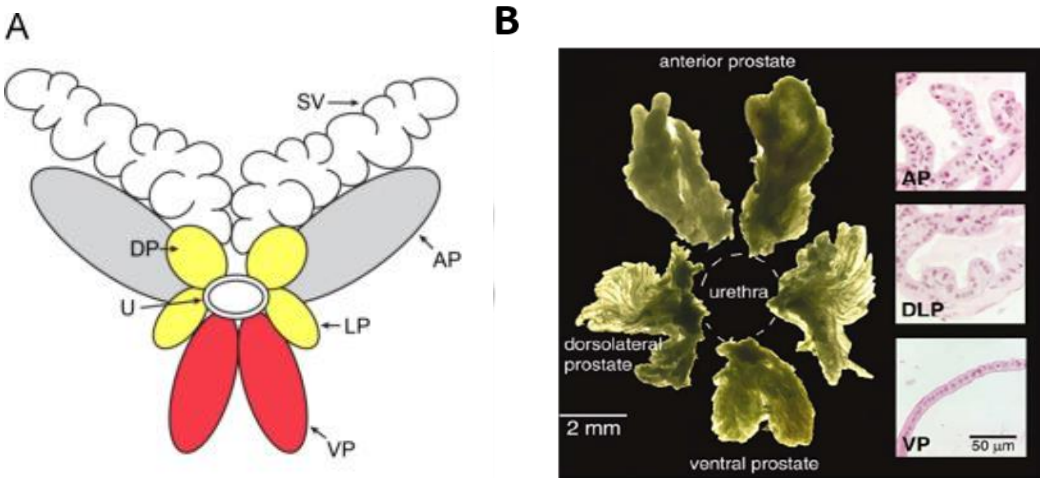

Figura 2- A- Vista anterolateral (lado esquerdo) dos lobos da próstata em relação à bexiga urinária, vesículas seminais e uretra, evidenciando os quatro lobos dispostos ao redor da bexiga urinária. **B**- Aparência macroscópica e organização microscópica (Coloração hematoxilina -eosina H.E.) dos pares de lobos prostáticos do rato adulto (B). (Adaptado de Marker et al., 2003). AP- próstata anterior; DLP- próstata dorsolateral; VP- próstata ventral.

O desenvolvimento prostático (pré e/ou pós-natal) em roedores tem sido extensivamente utilizado com o objetivo de avaliar os mecanismos moleculares e cascatas de sinalização ativados/inibidos durante a morfogênese glandular (CUNHA et al., 1987). 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). Em todas as espécies, inclusive nos humanos, a próstata se desenvolve 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 intrauterino, nos roedores a próstata é rudimentar ao nascimento, sendo a maior parte do desenvolvimento, durante os primeiros 15 dias de vida pós-natal (DPN) (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 3). Os eventos de proliferação e diferenciação celulares são complexos e regulados pela interação parácrina entre o epitélio e o estroma, modulada pela expressão de genes em uma sequência espacial e temporal precisamente coordenada, a qual são moduladas por fatores androgênicos (HUANG et al., 2009).

Durante o desenvolvimento embrionário, os andrógenos atuam via receptor, o Receptor de Andrógenos (AR), no mesênquima urogenital para induzir a proliferação epitelial, a formação de ductos e a diferenciação das células epiteliais que ocorrem em paralelo ao desenvolvimento do estroma (CUNHA et al., 1992; MARKER et al., 2003). A testosterona, um dos principais andrógenos, é produzida por células intersticiais do túbulo seminífero. Na próstata, a testosterona livre, é convertida em diidrotestosterona (DHT), pela 5 α-redutase do tipo II, mais específica para células epiteliais da próstata. E tanto a testosterona, quanto a DHT podem se ligar ao AR e migrar para o núcleo das células epiteliais (TAPLIN; HO, 2001).

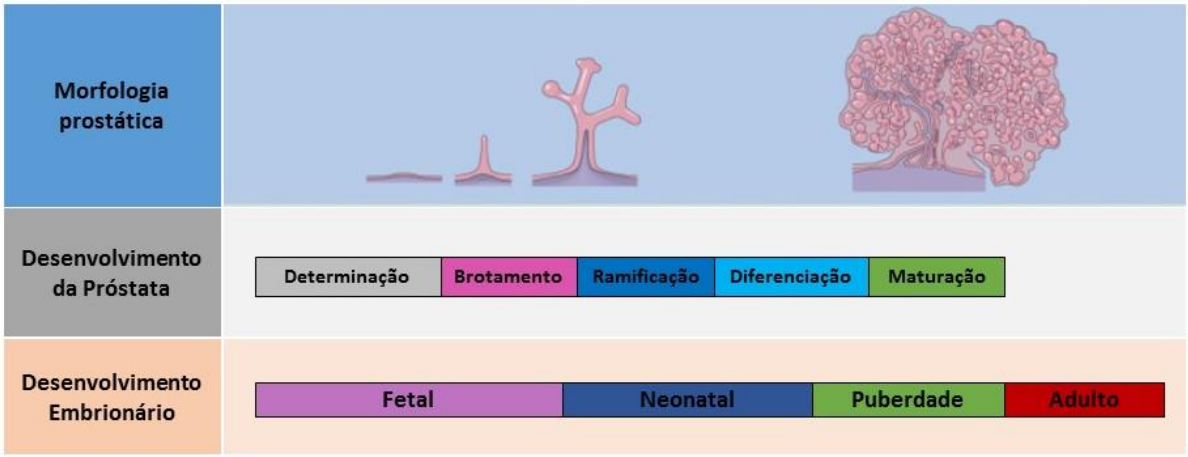

Figura 3. Estágios do desenvolvimento da próstata do rato (adaptado de PRINS; PUTZ, 2008).

A morfogênese prostática é dependente da exposição a andrógenos que são produzidos pelos testículos fetais (CUNHA et al., 1987). Em resposta aos andrógenos, o epitélio do seio urogenital 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 (PRINS, 1992). O padrão de ramificação é complexo e lóbulo-específico, sendo que este processo se inicia na próstata ventral (PV) no DPN3-5 e dois dias depois na próstata dorsolateral (PDL). Nesse período, também ocorre início da diferenciação das células basai/*stem cells* em células basais e luminais, com expressão diferencial de citoqueratinas (CK) e receptor de andrógeno (AR), acompanhada do início da

formação do lúmen, que atinge as extremidades distais dos ductos no DPN12 (PRINS; BIRCH, 1995) (Figura 4). Concomitantemente, as células mesenquimais prostáticas condensam-se ao redor dos brotos em formação, forma-se uma camada periductal de células musculares lisas enquanto as células interductais diferenciam-se em fibroblastos (HAYWARD; ROSEN; CUNHA, 1997). Outro processo importante que ocorre entre os DPN10-15 é a diferenciação funcional das células epiteliais luminais (PRINS; BIRCH, 1995), sendo que em roedores, as proteínas de secreção prostáticas são detectáveis a partir do DPN20 e tornam-se mais abundantes concomitantemente ao aumento dos níveis séricos de testosterona.

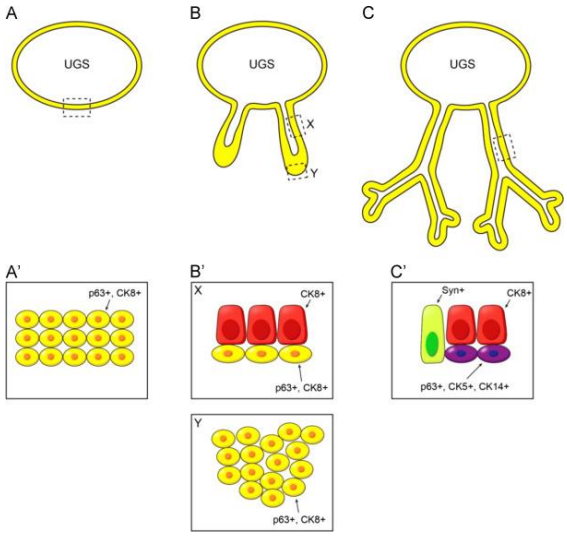

Figura 4. Diagrama ilustrando o desenvolvimento prostático e diferenciação das células epiteliais do camundongo. (A) No dia gestacional 15 (E15), o seio urogenital consiste de um epitélio pseudoestratificado e todas as células coexpressam p63 e CK8. (B) No E17, os brotos prostáticos iniciam sua formação. Na região proximal (próximo à uretra, X), as células epiteliais se distinguem em uma linhagem CK8+ revestindo o lúmen e uma linhagem p63+ CK8+ na base do epitélio. Na região distal que ainda não formou luz (Y), as células epiteliais co-expressam p63 e CK8. (C) No DNP1, e se inicia o processo de elongação e ramificação dos ductos. Sinaptofisina (Syn+) (Adaptado de PENG; JOYNER, 2015).

Além de sua importância para a fertilidade, nos últimos anos a próstata tem despertado grande interesse médico-científico pela alta incidência de doenças, principalmente a hiperplasia prostática benigna (HPB) e o câncer de próstata (CaP) (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 62 mil novos casos diagnosticados em 2016, com número de mortes próximo de 13 mil indivíduos (http://www.inca.gov.br).

1.2 Prolactina

A prolactina (PRL) é um hormônio polipeptídio de aproximadamente 23kDa, pertencente à família das citocinas, sintetizado por células especializadas do lobo anterior da hipófise, os lactotrofos, sua secreção está sob regulação da dopamina ou hormônio inibidor de prolactina, produzido pelo hipotálamo e liberado na eminência média (FREEMAN et al., 2000; GRATTAN; KOKAY, 2008). Sabe-se sobre a interação desse hormônio com a glândula mamária, visto que uma das suas principais funções é estimular o crescimento da glândula e induzir a lactação durante a gestação, porém a prolactina apresenta mais de 300 atividades biológicas, dentre elas, controle do desenvolvimento e funções na reprodução (BEN-JONATHAN et al., 2009; BOLE-FEYSOT et al., 1998).

O gene da PRL tem o tamanho de 10 Kb, composto por 5 éxons e 4 íntrons (COOKE et al., 1981; TRUONG et al., 1984). Duas regiões promotoras independentes são responsáveis pela transcrição do gene, a região proximal 5000 pb dirige a expressão específica de pituitária (BERWAER et al., 1993), enquanto que uma região de promotor mais distante é responsável pela expressão extrapituitário. A molécula é organizada de uma única cadeia de aminoácidos com três pontes dissulfeto intramoleculares entre seis resíduos de cisteína, em humanos consiste em 199 aminoácidos, enquanto que em ratos 197 aminoácidos (BEN-JONATHAN et al., 1996; BOLE-FEYSOT et al., 1998; WENNBO et al., 1997).

A PRL atua pela via endócrina clássica, após sua produção é secretada e transportada pelo sistema circulatório, atuando em células-alvo ou locais periféricos via receptores específicos. Estudos indicam que a atuação da PRL pode ser de forma autócrina ou parácrina (fator de crescimento, neurotransmissor ou imunomodulador), portanto, muitas das ações associada a esse hormônio podem ser ativadas, sem afetar sua concentração circulante (Figura 5) (BEN-JONATHAN et al., 2009; BOLE-FEYSOT et al., 1998; KELLY et al., 1991).

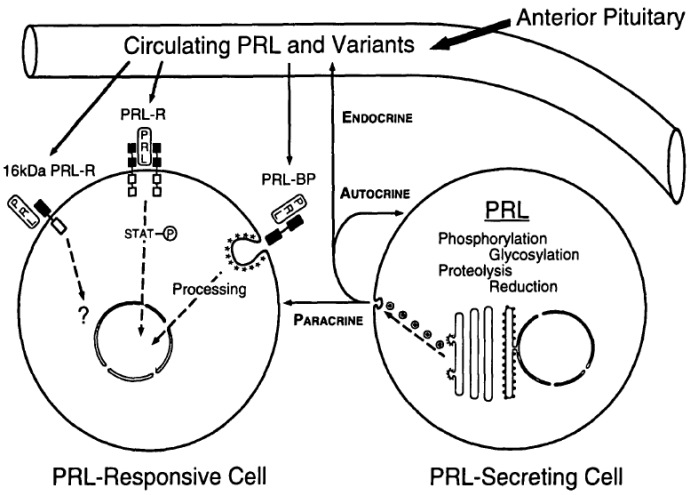

Figura 5. Diagrama mostrando as ações pleiotrópicas da PRL. A PRL circulante e as variantes são derivadas da hipófise anterior. Produzida localmente, a PRL pode afetar tanto as células adjacentes (parácrinas) ou células secretoras da própria PRL (autócrinas). O processamento da PRL pode ocorrer, tanto no local de produção e em tecidos-alvo (Adaptado de Ben-Jonathan et al. 2009)

A PRL, assim como outros hormônios, sua ação acontece na ligação a um receptor transmembrana específico, o receptor de prolactina (PRLR), pertencente à superfamília dos receptores de citocinas de classe I (BOLE-FEYSOT et al., 1998). Ao contrário da PRL, no qual um único transcrito codifica uma proteína madura, estudos apresentam em diversas espécies que o PRLR apresenta múltiplas isoformas resultantes do splincing alternativo do transcrito primário (ALI; PELLEGRINI; KELLY, 1991; BOUTIN et al., 1988; DAVIS; LINZER, 1989). As isoformas do PRLR diferem conforme a composição e o comprimento da cauda citoplasmática, então sendo identificadas como formas curtas (291 aminoácidos), intermediária (393 aminoácidos) ou longa (591 aminoácidos). Além dessas diferentes isoformas, outras solúveis também foram identificadas, a proteína de ligação de PRL (PRLpb), que resulta do *splincing* alternativo do mRNA ou clivagem proteolítica do PRLR ligado a membrana. Em todos os casos, o domínio extracelular de ligação ao ligante, é idêntico em todas as isoformas (Figura 6) (BOLE-FEYSOT et al., 1998; GOFFIN; KELLY, 1997; KELLY et al., 1991).

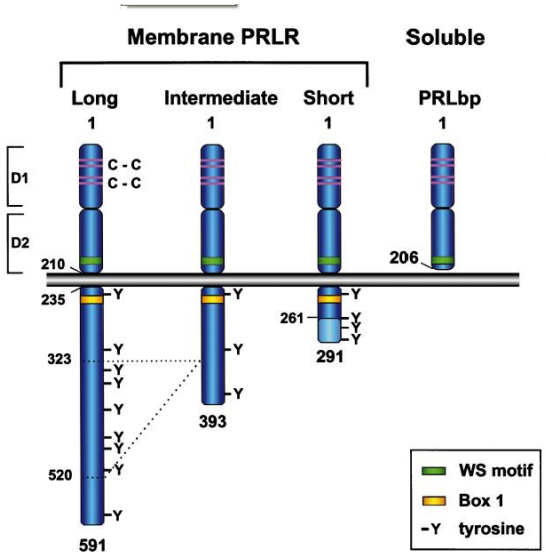

Figura 6. Representação esquemática das isoformas do PRLR. Adaptado de (BOLE-FEYSOT et al., 1998)

A PRL contém duas regiões responsáveis por se ligar no PRLR, região 1 e 2, onde primeiro ocorre a ligação da região 1 no sitio especifico do PRLR, levando à formação de um complexo inativo H1:R1 (um hormônio, um receptor), sendo a formação deste primeiro complexo uma condição prévia para que a região 2 da PRL se ligue ao outro sitio especifico do PRLR, ativando o complexo trimérico H1:R2 (um hormônio, um homodímero do receptor) (Figura 7) (GOFFIN; KELLY, 1997; GOFFIN et al., 1996)

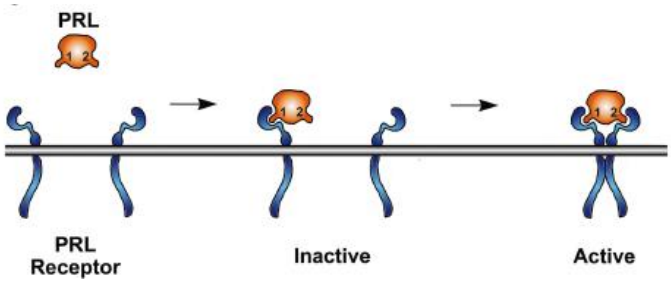

Figura 7. Esquema representando a ativação do PRLR induzida pela molécula de prolactina (PRL).

Todas as ações atribuídas à PRL, resultam da ligação do hormônio ao seu receptor, o que conduz a ativação de uma cascata de eventos celulares. O receptor de membrana clássico, PRLR, dimeriza após ligação a PRL e ativa a via de sinalização, a principal via canônica ativada envolve a quinase de janus kinase 2 (JAK2) e o fator de transcrição STAT5 (transdutor de sinal e ativador de transcrição 5), ambas as quais são ativadas por fosforilação da tirosina. A JAK2 está constantemente associada ao PRLR (LEBRUN et al., 1994). Após a fosforilação, a família de proteínas STAT, é um

importante transdutor na sinalização do receptor de citocina. A família STAT consiste em oito membros, 4 deles (STAT 1, STAT3, STAT 5a e STAT5b) foram identificados como moléculas transdutoras de PRLR. A proteína STAT acoplada ao PRLR é fosforilada pela JAK, então o dímero de STAT se transloca para o núcleo e ativa o gene alvo, iniciando diversos efeitos biológicos associados a PRL. Outras vias de sinalização podem também estar envolvidas com PRLR (Figura 8) (FREEMAN et al., 2000; GROSVENOR; MENA, 1980).

A PRL pode regular uma variedade de respostas celulares, entre elas, proliferação, diferenciação e migração. Assim, cada resposta celular depende do contexto em que o hormônio se encontra, como exemplo, pode proteger as células β-pancreáticas da apoptose (ARUMUGAM; FLEENOR; FREEMARK, 2014; TERRA et al., 2011), como pode estimular a apoptose na hipófise (FERRARIS et al., 2012, 2014)

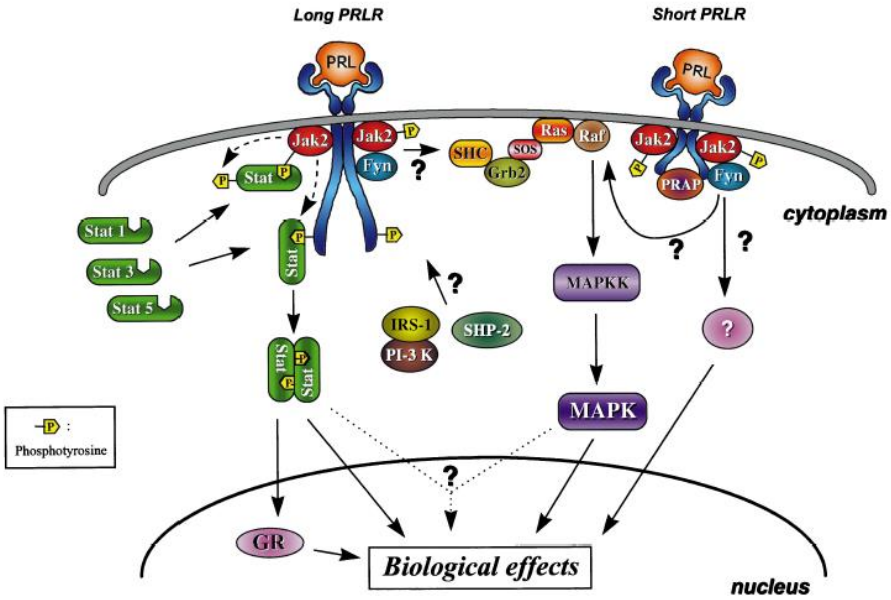

Figura 8. Representação esquemática das vias de sinalização das isoforrmas longa e curta do PRLR, demonstrando as diferentes vias ativadas. Adaptado de (BOLE-FEYSOT et al., 1998)

Normalmente, o alto nível de PRL pode ser denominado como hiperprolactinemia, a qual pode resultar em diversas desordens reprodutivas (GOFFIN et al., 2006). Atualmente, os agonistas da dopamina são os mais utilizados como alternativa terapêutica para a hiperprolactinemia, visto que o a dopamina é o supressor fisiológico primário de produção de prolactina pela hipófise (BEVAN et al., 1992; GOFFIN et al., 2006). A bromocriptina é considerada o agonista pioneiro para o uso nos tratamentos relacionado a prolactina (Figura 9), um agonista da dopamina D2 que inibe a secreção da PRL a partir da hipófise anterior,

assim reduzindo os níveis circulantes de PRL (DORSHKIND, 2000), e é utilizado no tratamento de prolactinomas (SEEMAN; VAN TOL, 1994), para tratar a diabetes do tipo 2 (LIANG et al., 2015) e para a supressão da lactação (BERNARD et al., 2015; VERMA; SHAH; FARIDI, 2006).

Carvalho et. al, (2015) demonstraram que a inibição da prolactina com bromocriptina, durante a fase de lactação, causou na prole diversas alterações metabólicas, como desnutrição. Na idade adulta, observou-se hiperinsulinemia, aumento de glicose e triglicérides, hipotireoidismo e hipoprolactinemia (BONOMO et al., 2007). O efeito catecolaminérgicos da bromocriptina são semelhantes ao modelo de hipertensão em ratos, onde ocorre o aumento da atividade simpática regulando os receptores androgênicos e o desenvolvimento de hiperplasia prostática (GOLOMB et al., 1998; MATITYAHOU; ROSENZWEIG; GOLOMB, 2003).

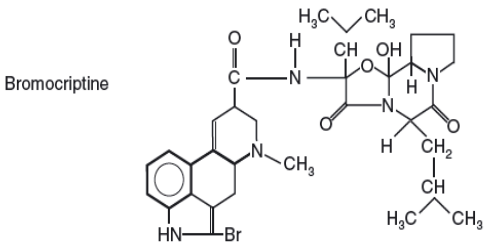

Figura 9. Imagem representativa da molécula de Bromocriptina adaptado de (HOLT; BARNETT; BAILEY, 2010).

Desde 1973 estudos demonstram a associação de altos níveis de PRL circulante com aumento do risco de tumores, assim surgiu um número crescente de estudos envolvendo a ligação entre os tumores prostáticos com excesso de produção de PRL. Em condições normais, os níveis de PRL circulantes são menores em machos que em fêmeas.

Assim, além do papel da PRL em induzir o crescimento mamário e lactação, muitos estudos têm atribuído um papel regulatório sistêmico importante para a prolactina em machos, tais como: modulação do sistema imune, manutenção das células beta na ilhota pancreática e metabolismo de lipídeos (BEN-JONATHAN et al., 2009; DORSHKIND, 2000). A super-expressão de PRL local amplia o pool de células basais/estaminais, tendo sido identificada como originador de câncer da próstata (CaP), demonstrando uma potência oncogênica da PRL no tecido da próstata (GOFFIN et al., 2011; SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). Por isso, nos últimos anos tem aumentado o número de estudos para decifrar os mecanismos celulares e moleculares pelas quais a PRL e PRLR participam na tumorigênese prostática.

1.3 Prolactina e Próstata

De acordo com Cunha et al., estrógenos atuam em sinergia com a testosterona, influenciando não apenas as funções normais da próstata, como as funções associadas a patogênese (CUNHA; HAYWARD; WANG, 2002). Além dos esteróides, outros hormônios não-esteroidais também tem sido caracterizado por atuarem no desenvolvimento e fisiologia prostática, dentre eles a PRL. A resposta prostática à PRL foi primeiramente observada por Flores et al., em próstatas de ratos hipofisectomizados e castrados, submetidos ao tratamento com extrato de hipófise (FLORES; SEGALOFF; STEELMAN, 1956). Posteriormente, vários estudos utilizando modelos animais atribuíram um papel relevante da PRL sobre o desenvolvimento normal, crescimento e função prostática (CARON; JAHN; DEIS, 1994). Este fato ganhou maior importância quando Nevalainen et al., demonstraram que a glândula prostática expressa tanto o transcrito para PRL, como seu receptor. Assim, se postulou que a PRL pode atuar como um fator de crescimento produzido localmente e que atua de forma autócrina ou parácrina, vias que divergem do conceito canônico de sinalização, onde a PRL é produzida pela hipófise e atua sobre alvos distantes do seu local de síntese (NEVALAINEN et al., 1997a).

Em ratos, os níveis de PRL em machos são baixos antes do DPN15, o aumento inicia entre DPN15 e 20, sendo o pico no DPN25. Após o pico de PRL, os níveis diminuem ligeiramente e se tornam constantes entre DPN 30 e 50 (NEGRO-VILAR; OJEDA; MCCANN, 1973). Estudos em seres humanos demonstram a influência dos níveis circulantes de PRL durante a maturação sexual (BARTKE A, KLEMCKE H, 1986). Negro- Vilar et. al, demonstraram um aumento dos níveis de PRL com o início do pico de crescimento para próstata, além disso, os níveis de estradiol em ratos machos são altos ao nascimento, caem logo após o nascimento, tendo o aumento a partir do DPN12 a 19, após esse período, os níveis diminuem imediatamente e estabilizam para a idade adulta (DÖHLER KD, 1975).

Estudos recentes têm buscado estabelecer qual o compartimento tecidual ou tipo celular seria alvo do efeito anabólico da PRL sobre o desenvolvimento da próstata. Sackmann-Sala et al., através de células basais/*stem cells* extraídas de camundongos adultos transgênicos e postas para crescer em cultura (onde as células epiteliais super- expressavam prolactina), demonstraram que o aumento intraprostático da expressão de PRL induziu amplificação dessas células no compartimento basal do epitélio glandular destes animais. Entretanto, estes autores concluíram que as células basais/*stem cells* não são ativadas diretas da PRL e sugeriram que possa ocorrer uma via de ativação parácrina, onde as células luminais ou as células estromais

responderiam à estimulação pela PRL e produziriam fatores parácrinos que atuariam nas células do compartimento basal (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). Para além do efeito sobre as células basais e luminais, a PRL está associada à secreção epitelial, metabolismo energético, e produção de citrato (COSTELLO; FRANKLIN, 1994), culturas de órgãos de explantes de tecido humano ou roedores evidenciaram um papel proliferativo e anti-apoptótico desse hormônio no epitélio da próstata (AHONEN et al., 1999; NEVALAINEN et al., 1997b).

Outros estudos, relacionam o sinergismo da PRL com o receptor de andrógenos, principal andrógenos envolvido no desenvolvimento e homeostasia da glândula, assim, esse sinergismo pode induzir a diferenciação e proliferação da próstata de ratos e humanos, também foi proposto, que a PRL pode aumentar as concentrações de esteróides livres no sangue, bem como a absorção de testosterona (T) em células da próstata (PRINS, 1987; SACKMANN-SALA et al., 2014a; STOKER et al., 1999). Portanto, estudos em modelos animais sugerem a participação da PRL no desenvolvimento normal, crescimento e funções da glândula prostática, a descoberta que a próstata humana expressa PRLR, indicam que está glândula pode ser um alvo direto deste hormônio (NEVALAINEN et al., 1997a).

Atualmente, diversas funções relacionadas ao eixo PRL/PRLR são responsáveis pela coordenação da homeostase de todo o corpo, tanto em estados fisiológicos normais ou respostas adaptativas (DORSHKIND; HORSEMAN, 2000; FERRARIS et al., 2013; TOVAR; DIÉGUEZ, 2014). Embora trabalhos relacionem a expressão da prolactina com o potencial agressivo e metastático do câncer de próstata, pouco se sabe sobre o papel deste hormônio durante o desenvolvimento, em especial durante o processo de proliferação e diferenciação das células basais/*stem cells*, eventos importantes para a morfogênese normal e manutenção da homeostasia tecidual na idade adulta (PRINS; PUTZ, 2008).

2- Justificativa e relevância do tema

Nos últimos anos, estudos evidenciam que a PRL é um hormônio pleiotrópico e versátil que possui várias células/tecidos alvo, podendo induzir muitas, e por vezes, diferentes respostas biológicas. Atualmente, diversos estudos demonstram relação entre tumores e a expressão de prolactina (FREEMAN et al., 2000; GILLERAN et al., 2003; SACKMANN-SALA; GOFFIN, 2015), porém, poucos estudos relacionam o papel da PRL ou sua inibição durante o desenvolvimento ou em neonatos (CARVALHO et al., 2015; GILLERAN et al., 2003). Este fato ganhou importância desde a descoberta de que a próstata humana expressa o receptor para

PRL (PRLR), sendo alvo direto deste hormônio (NEVALAINEN et al., 1997a). Assim, dado a importância do período de desenvolvimento e maturação da próstata, aliado ao fato de não existir, na literatura, estudos sobre o papel da PRL durante a morfogênese da próstata, acreditamos ser extremamente relevante caracterizar o padrão de expressão da PRL e sua via de sinalização durante o desenvolvimento prostático, assim como os efeitos de sua inibição durante a morfogênese tardia da próstata ventral de rato, modelo amplamente utilizado para entendimento da biologia prostática.

3- Objetivos

3.1 Objetivo geral

Este projeto teve por objetivo geral determinar o padrão de expressão da prolactina durante o desenvolvimento prostático, assim como os efeitos de sua inibição sobre a morfogênese glandular em ratos Sprague Dawley.

3.2 Objetivos específicos

- O padrão de expressão da prolactina durante o desenvolvimento da próstata de rato, nos dias pós-natal (DPN) 21 e 35;

- Os efeitos da inibição da via da prolactina utilizando-se inibidor comercial (Bromocriptine mesylate) sobre o processo de ramificação ductal e proliferação do epitélio prostático no DPN 21 e 35;

- Os efeitos da indução da via da prolactina utilizando-se prolactina comercial (Prolactin from sheep pituitary) sobre a ramificação ductal e proliferação do epitélio prostático no DPN 21 e 35;

- Os níveis séricos de prolactina dos diferentes grupos experimentais;

- A expressão de proteínas intracelulares relacionadas à ativação da via da prolactina (STAT3 e Stat5a/b) na próstata ventral;

- A localização de AR nas células epiteliais por imunohistoquímica

- Se os tratamentos alteram o padrão de diferenciação das células epiteliais através da quantificação da expressão proteíca de CK18 por western blotting;

- O padrão de expressão prostático de genes envolvidos nesta via;

- Correlacionar estes resultados a possíveis alterações no padrão de desenvolvimento e diferenciação celular induzidos por PRL com potencial para levar ao surgimento de lesões prostáticas durante o envelhecimento.

REFERÊNCIAS

NOTA: As referências da introdução geral encontram-se juntamente com as referências do capítulo 2.

Capítulo 2: Manuscript

Influence of prolactin signaling modulation on development and maturation of the rat ventral prostate

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Abstract

The prostate gland is an accessory gland of the male genital system and, in addition to androgenic dependence for its development and homeostasis, is influenced by non-steroidal hormones, among them the prolactin (PRL). Studies in animal models have shown that PRL can act with an anabolic effect on the prostate gland. Thus, our objective was to investigate the effects of PRL modulation on the development and maturation of the ventral prostate (VP) of rats. Male Sprague Dawley rats (PND 90) divided into 6 groups (n= 6) were used: Control group (CT): received saline solution; Prolactin (PRL): Prolactin (0.3mg/kg); Bromocriptiona (BR): PRL inhibitor (0.4mg/kg). All animals were submitted daily (subcutaneously) from postnatal day 12 (PND12) to PND 21 or 35. The animals were anesthetized, weighed and euthanized. Blood and VP lobes were collected, weighed and processed for morphological analyzes, western blot (WB) and RT qPCR. Treatment with PRL induced body weight gain in PND35 compared to the other groups. Histologically there was an increase in the luminal compartment and a decrease in the epithelial compartment in the PRL and BR groups compared to CT on PND35. The immunohistochemical reaction for Ki67 demonstrated proliferation of proliferative cells in the groups treated with PRL on PND35. The PCNA quantification confirmed these data, with a significant increase in the PRL35 group. The intensity of reaction for AR was higher in the PRL35 group. Quantification of AR by WB confirmed these data. The gene expression of the PRLR increased in the PND21, whereas at PND35 the PRL and BR groups decreased, in Stat3 increased in the BR groups at PND21 and 35, whereas in the Stat5 groups there was no difference in the different groups. Protein expression of PRL increased in PRL21, on PND35 decreased in PRL and BR, PRLR had no difference between the experimental groups. The protein quantification of STAT3 increased in the PRL and BR groups at both ages, in STAT5 AB and prostatein increased in the PRL groups compared with other two groups on PND35. Protein expression of CK18 increased in the groups treated with BR on PND21 and 35, on NKX3.1 the increase in PRL compared to the other groups on PND21. Our results demonstrate that PRL modulation interferes with prostate morphophysiology, increasing the proliferative response and secretory activity, especially in animals treated for a prolonged period. These data reveal the important role of PRL in prostate development.

Introduction

The prostate gland is an accessory gland of the male genital system, which development and function are androgen dependent. Beside being any essential role for reproductive success in mammals, the prostate has aroused great medical-scientific interest, as it is accessed by prostate cancer (CaP), the second most common type of cancer among men (COLLOCA et al., 2016; ESFAHANI; ATAEI; PANJEHPOUR, 2015). There were estimated 62.000 new cases diagnosed in Brazil, in 2016, with a death rate of 13.000 individuals (INCA, 2016).

Despite the androgenic dependence for its development and homeostasis (CUNHA et al., 1985), other non-steroidal hormones also act on the prostate, among them prolactin (PRL). PRL is a polypeptide hormone secreted mainly by the pituitary, which functions are mediated by a specific receptor, the prolactin receptor (PRLR) (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). Although the pituitary gland being known as the gland responsible for the secretion of PRL, this hormone also possess an extra-pituitary source, being synthesized by the prostate (BOLE-FEYSOT et al., 1998; MARANO; BEN-JONATHAN, 2014). Thus, besides serum PRL, this hormone may act in an autocrine manner on the prostate, regulating the proliferation and apoptosis of epithelial cells in synergism with androgens (AHONEN et al., 1999; NEVALAINEN et al., 1997a). Moreover stimulating the secretory function of both the prostate and the seminal vesicle; (PRINS; BIRCH; GREENE, 1991; PRINS, 1987; TAM; WONG; TANG, 1992).

Studies in animal models suggest that at physiological levels, PRL participates in the control of prostatic homeostasis and that the increase in expression (hyperprolactinemia) especially diagnosed in adult-aged individuals is related to reproductive dysfunctions and aggressiveness of CaP. Transgenic adult mice that overexpress intraprostatic PRL under the control of probasin have a higher incidence of prostatic hyperplasia and intraepithelial neoplasia associated with basal/stem cell amplification (SACKMANN-SALA; GUIDOTTI; GOFFIN, 2015). However, these authors demonstrated that basal/stem cells do not respond directly to PRL, and paracrine stimulation is observed in stem cells, where luminal cells or stromal cells respond to PRL stimulation and synthesize paracrine factors acting on cells in the basal compartment .

Other authors demonstrated that, on normal levels, PRL acts on the hypothalamic-pituitary-gonadal axis, increasing the expression of luteinizing hormone (LH), which acts on Leydig cells, stimulating the testosterone secretion. Oppositely, a long period of exposure to hyperprolactin is related to the deregulation of testicular function, which leads to a lower

synthesis of testosterone and sexual dysfunction (SHAPIRO et al., 1980). Under these conditions, bromocriptine (BR) has been used to normalize serum PRL levels. BR inhibits PRL secretion from the pituitary gland, thus reducing circulating PRL levels (DORSHKIND; HORSEMAN, 2000).

Despite the recognition that PRL influences prostatic morphophysiology, studies on the effects of hyper or hypoprolactinemia on perinatal prostate development are still scarce, a period of fundamental importance for glandular development, where cell proliferation and differentiation processes occur, besides the ductal branch (GILLERAN et al., 2003; PRINS, 1992).

The prostatic morphogenesis begins rats starts on gestational day (DG) 15.5, two days before the first prostatic sprouts appear (WILHELM; KOOPMAN, 2006). After birth, sprout lengthening and morphogenesis begin with the formation of ducts (PRINS, 1992). The branching is complex and lobe-specific, been the beginnig of this process in the ventral prostate (VP) on postnatal day (PND) 3-5. In this period, stem cell differentiation also occurs in basal and luminal cells, reaching the distal ends of the ducts in the PND12(PRINS; BIRCH, 1995). Concomitantly, prostatic mesenchymal cells condense around the forming buds, developing a periductal layer of smooth muscle cells, whereas the intervening cells differentiate in fibroblasts (HAYWARD; ROSEN; CUNHA, 1997). Another important process which occurs between PND10-15 is the functional differentiation of luminal epithelial cells (PRINS; BIRCH, 1995), being that rodents, prostatic secretory proteins are detectable on PND20 and become more abundant concomitantly with serum levels increased of testosterone. In this manner, environmental and/or endocrine factors which cause unbalance at this stage of development may negatively impact the prostate development and maturation, leading to an increase in the incidence of prostatic affections with aging (FREEMAN et al., 2000).

Considering that in rats the process of prostate development and maturation begins late in the fetal period and is completed after birth (PRINS; PUTZ, 2008), the objective of this study was to characterize the impact of prolactin regulation on the development and maturation of the male rats prostate of the Sprague Dawley progeny.

Material and Methods

Animals and experimental design

Sprague Dawley albino rats, male (n=10) and female (n = 36) adults, with age of 90 days, were obtained from the Central Stock breeder at the State University of Campinas (Campinas, SP, Brazil). 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 (Protocol number CEUA 756).

The animals were kept under controlled temperature conditions (22 to 25ºC), relative humidity (55%), and a 12 hr photoperiod. The animals had free access to water and chow. During mating, two females were kept with one male in a polyethylene cages (Harem System). After mating and pregnancy testing, the rats were placed in individual cages and fed standard ration for rats. After birth, offspring of male rats were divided as described in the experiment.

From the postnatal day 12 (PND12) the animals (n= 6) were divided into 6 experimental groups to start the respective treatments, according to Figure 1. Control Group (CT): Received saline solution (vehicle) daily until postnatal day (PND) 21 and 35 (CT21 and CT35). Prolactin (PRL) group: Prolactin from sheep pituitary (Sigma 63103) at a concentration of 0.3 mg/kg diluted in saline solution between PND 21 and 35 (PRL21 and PRL35). Bromocriptine (BR) group: Received prolactin inhibitor (BR, Santa Cruz Biotechnology) at a concentration of 0.4 mg/kg diluted saline solution in the periods of PND21 and PND35 (BR21 and BR35) (CARON; JAHN; DEIS, 1994). In all groups the route of administration was subcutaneous (Fig. 1)

After the treatment period, the males were anesthetized with Thiopental (30mg/kg), weighed and euthanized. Blood sample were collected from ruptured cervical vessels for hormonal quantification. Then, the VP were dissected, weighed and frozen in liquid nitrogen for molecular analyzes or fixed in Methacarn (PUCHTLER et al., 1970) for morphological analyzes.

Hormonal assay

For hormones measurement, blood samples (n = 6/group) from each animal were collected at the time of euthanasia. Serum were obtained after centrifugation (2400g for 20 min) and stored at -20 ºC. The concentration of PRL (Kit Mouse / Rat Prolactin, CALBIOTECH)

was determined by ELISA immunoenzymatic assay, as instructed by the manufacturer. The sensitives of these assays was10ng/mL for PRL.

Biometric, Morphological and Stereological Analyses

Samples of the ventral prostate (VP) of the animals of the PND 21 and 35 of the different experimental groups (n=6/Group) were dissected, weighed and fixed in Methacarn (PUCHTLER et al., 1970) (70% methanol + 20% chloroform + 10% acetic acid) and after 4 hours in the fixative, the material was dehydrated, diaphanized in xylol and included in Paraplast (Sigma). Cuts of 5μm were produced in a rotating microtome, collected on silanized slides.

The slides were stained with Hematoxylin-Eosin (HE) and in these sections the general morphology, height of the secreting epithelial cells and the volume fraction of the luminal, epithelial and prostatic stromal compartments were analyzed according to the dot counting system by Weibel's grid (WEIBEL; KISTLER; SCHERLE, 1966). The measurements were determined in 10 microscopic fields chosen at random (40x objective) from the 6 different animals per group. The slides were documented in Leica DM 2500 microscope coupled to a digital camera and analyzed in Leica Qwin software.

Immunohistochemical of Ki67 and AR

Histological sections of 5 μm thickness of the different experimental groups in the PND 21 and PND35 were dewaxed, hydrated and subjected to antigenic recovery in a pressure cooker by treatment with 10 mM sodium citrate buffer pH 6.0 for 30 minutes. After antigen recovery, endogenous peroxidase blockade were carried out in a solution containing 3% hydrogen peroxide diluted in methanol. Then sections were washed in PBS, followed by treatment with 3% milk diluted in PBS to block unspecific protein-protein interactions. Subsequently, histological sections were incubated overnight at 4 °C with the respective primary antibodies: anti-Ki-67 (for cell proliferation, concentration 1: 150; AB-16667-Abcam®); Anti-androgen receptor (AR, 1: 100 concentration; SC-816-Santa cruz®), all diluted in 1% BSA in PBS. The sections were washed 3 times in PBS for 5 min and incubated with the specific secondary antibodies conjugated with peroxidase, at room temperature. The reactions were developed using diaminobenzidine (DAB) and counterstained with hematoxylin. The slides were

documented in a Leica DM 2500 microscope coupled to a digital camera and analyzed in Leica Qwin software.

Western Blotting Analysis

The VP were homogenized in extraction buffer (50 mM Tris-HCl buffer, 0.25% Triton-X 100) and the protein concentration was determined by Bradford assay (BRADFORD, 1976). The samples (70 mg/line) were analyzed by electrophoresis using SDS-PAGE gel. Western blot analysis were conducted by blotting onto the nitrocellulose membrane (Biorad®). Blots were blocked in 5% non-fat milk diluted in PBS and incubated with the follow primary antibodies: rabbit polyclonal STAT5 AB (ab194898- Abcam®, Cambrigde), rabbit monoclonal STAT3 (ab68153), mouse polyclonal PCNA (SC56-Santa Cruz®, Califórnia), rabbit polyclonal Prostatein (7821-1009- Abd Serotec®, Raleigh- Carolina do Norte), goat polyclonal NKX 3.1 (sc-15022- Santa Cruz®, Califórnia), rabbit polyclonal Cytokeratin 18 (CK18) (SC 28264- Santa Cruz®, Califórnia). After washing in PBS buffer, the blots were incubated with specific secondary antibody for 1 hr, washed with PBS, then developed using ECL kit (Amersham).The expression levels were normalized to the b-actin values and the results were expressed as the mean ± SD.

Analysis of genetic expression

Samples from the different experimental groups collected in PND21 and 35 (n=6) were homogenized in solution containing TRIzol® (Invitrogen Inc., Carlsbad, CA, USA). The homogenate was incubated for five minutes at 37 ° C and then 0.2 mL of chloroform per mL of TRIzol ® used. After three minutes of incubation the material was centrifuged at 12,000g for 15 minutes at 4 ° C. The formed aqueous phase was then separated and precipitated with 0.5 mL of isopropanol for 10 minutes at 37 ° C. After this time, the sample was again centrifuged at 12,000g for 10 minutes at 4ºC. The precipitate formed was washed with 1mL of 75% ethanol and centrifuged at 7,500g for 5 minutes at 4Â°C. The supernatant was carefully removed and the pellet was dried at room temperature for about 5 minutes. Then, overall RNA was dissolved in distilled and autoclaved water, incubated for 10 minutes at 60 °C, and finally stored at -80 °C.

For each sample, an amplification plot was plotted showing an increase of the fluorescent dye reporter (ΔRn) in each PCR cycle. From this graph, the cycle was determined

where the reaction crosses the threshold (CT), based on the variability of the baseline data obtained from the initial PCR cycles. The primer was purchased from Applied Biosystems, the gene analyzed: PRLR 5' ACCGGGGAGCGGAGG/ 3' TCTTTAGTTTTGCCAGGGAGCA; Stat5 5'CATGTACCCACCGAACCCC / 3'GAGTCCAGCGTTCAGGACAA and Stat3 5' CCAGGTAGTGCTGCCCCTTA/ 3' ATGGTATTGCTGCAGGTCGT. The relative quantification of each gene was performed using the 2-ΔΔ CT method (LIVAK; SCHMITTGEN, 2001). The values obtained for all the samples were normalized by the ratio obtained between the target gene and the GUSB reference genes.

Statistical analysis

Statistical analyses were performed on GraphPad Prism® software (version 5.00; Graph Pad, Inc., San Diego, CA). The groups were compared within their respective ages, submitted to analysis of variance - ANOVA, followed by "Tuckey-Kramer". The results were expressed as mean ± standard deviation and were considered statistically significant differences when $p < 0.05$.

Results

- Hormonal, biometric parameters of male offspring in PND 21 and 35

The animals in the PND21 did not present significant differences in body weight among the groups. However, on PND35 animals treated with PRL presented a significant increase in body weight when compared with CT35 and BR35 groups. The PRL35 group showed an increase of 8.14% when compared with CT35, and 12.56% to BR35 (Table 1).

The quantification of serum PRL in different groups demonstrated a tendency to increase serum levels in the groups receiving exogenous PRL and a decrease in the BR groups for both ages (Table 1).

- Morphological and Stereological Analyses of the Ventral Prostate

The animals of the CT21 presented the characteristic morphology of the VP at this age, with dilated and forming acini, broad light and with secretion inside. It is observed covering, a cubic secretory epithelium associated with a delicate fibromuscular stroma. No histological differences were observed in the groups treated with PRL or BR at PND21. Stereological

analyses were corroborated by the histological data, where there were no alterations in the epithelial, luminal and stromal compartments (Table 2). At PND35, the animals of the CT group presented more acini dilated than the PND21, evidencing a continuous glandular growth. A secretory cylindrical epithelium was observed covering the acini, with an evident area of supranulear Golgi apparatus. A fine fibromuscular stroma is among the secretory acini (Figure 2). In the PRL and BR groups there was a reduction in the height of the glandular epithelium of the VP on PND35, as well as dilation of the alveoli compared with CT group (Figure 2). The height of epithelial cells also decreased in the PRL and BR groups regarding to CT (reduction of 54% and 53% respectively) (Figure 2).

The stereological results confirm the changes observed in the histological analyses of the different experimental groups (Table 2).

Immunohistochemistry

The Ki-67 reaction evidenced epithelial cell proliferation in all groups. The PRL treatment did not alter epithelial proliferation in the experimental group at PND21. A higher number of staining cells was observed in the PRL group compared with CT and BR (Fig. 3A) for 35 days.

The androgen receptor (AR) immunostaining was detected mainly in the secretory epithelial cell nucleus in the VP of all experimental groups in different ages. No differences were observed in the intensity of staning in the VPs of the different groups on PND21 (Figure 4). On PND35, the groups treated with PRL presented increase in the intensity of epithelial cell marking. (Fig. 4).

Determination of gene expression by RT-qPCR

The PRLR gene expression was increased in the BR group compared with CT and PRL on PND21. Oppositely, on PND35 there is a reduction in PRLR gene expression in the PRL and BR compared with CT (Fig 5A). The Stat3 gene expression on PND21 increased in the BR group compared with PRL, whereas, on PND35 the group treated with BR the expression increases compared with CT group (Fig. 5B). No statistical difference was observed in Stat5 among the different experimental groups in both ages (Fig. 5C).

Western Blotting

The other cell proliferation marker, the PCNA, the WB reaction for PCNA, confirmed data of staning cell in Ki67, although on DPN21 in different groups the difference was not statistically significant (Fig. 3C). The opposite occurred on PND35, which treatment with PRL on PND35 presented increase expression protein compared with CT and BR group (Figure 3C).

The AR protein expression did not alter among the experimental groups on PND21, but on PND35 the protein expression in PRL group increased compared with CT and BR (Fig. 4B and C).

The PRL protein expression presented a significant increase in PRL21 group compared with BR group. On PND35, the PRL expression decreased in both PRL and BR groups compared with CT (Fig. 6B). Although there was observed differences in PRL expression among the treatment, the PRLR had no significant difference in any experimental group (Fig. 6C).

The STAT3 expression increased with both PRL and BR treatment compared with CT in both ages (Fig. 7B), but STAT5AB did not have alteration in the different experimental groups. The opposite occurred on PND35, where the PRL35 group presented a significant increase in the expression of STAT5AB when compared with CT35. Whereas, the administration of BR reduced the expression of STAT5AB to the levels observed in the CT (Fig. 7C).

The secretory activity was determined by the expression of prostatein, which expression is under the androgenic stimulus. No significant differences were observed in prostatein expression in the different groups on PND21. On PND35, the prostatein expression is lower in BR compared with PRL group (Fig. 8B).

The effects of PRL or BR on epithelial cell differentiation were determined by molecular markers, by the expression of NKX3.1 and CK18. The expression of CK18 was increased in BR compared with groups CT and PRL in both ages, without difference observed in the PRL treated groups (Fig. 8C). NKX3.1 had a significant increase in the PRL group compared with CT and BR on PND21, but no statistical differences were observed on PND35 (Fig. 8D).

Discussion

Studies indicate that PRL plays a crucial role in the normal development and function of the prostate (FREEMAN et al., 2000; GILLERAN et al., 2003; GOFFIN et al., 2005; PRINS et al., 2001). Although the pituitary gland is known as the gland responsible for PRL secretion. This hormone also has an extrahypophyseal source, such as the prostate (BOLE-FEYSOT et al., 1998; MARANO; BEN-JONATHAN, 2014). Thus, besides the serum PRL, this hormone may act in an autocrine manner in the prostate. Therefore, we evaluated in this study the effects of activation or inhibition of the signaling pathway PRL in prostate development and secretory function in rats. In our study, the administration of PRL or its inhibitor, BR, was conducted from PND12 until PND21 or 35. This period was chosen, although prostate development in rodents begins at the end of the gestational period and it continues after birth (PRINS; PUTZ, 2008). Moreover, there is an intense fluctuation of PRL levels in this period (STOKER; ROBINETTE; COOPER, 1999).

In our study, there is a tendency of serum PRL after hormone administration, as well as a decrease after secretory inhibition with BR. However, no statistical differences were detected among the different experimental groups. Previous studies have demonstrated that the exogenous administration of PRL or its inhibition alter their serum levels. While GILLERAN et al. (2003) demonstrated decrease in serum PRL in rats administered with BR from PND15 to 90. ROZENBOIM et al., (2004) presented increase in PRL acts in the brain to stimulate production of dopamine and thereby inhibit its own secretion. Thus, we believe that, although the variability of the sample, PRL modulation affected not only prostate parameters but also corporal structure.

In this sense, the PRL administration induced body weight gain in the animals on PND35, despite a tendency of any increase on PND21. The temporal analysis demonstrated that the statistically difference was established on PND22. Previous studies have reported a somatotrophic effect of exogenous PRL administered to male, newborn and adult rats, leading to body weight gain (PÉREZ-VILLAMIL; BORDIÚ; PUENTE-CUEVA, 1992; RYNÍKOVÁ et al., 1988). This fact can be explain by the relationship between PRL and leptin, where PRL secretion increases leptin levels, leading to a lipogenesis and accumulation of adipose tissue (GUALILLO et al., 1999; ZHANG et al., 2016). Therefore, we can correlate the body weight gain of the animals from PRL35 group with the possible increase of leptin, as proposed by YU et al. (1997).

Oppositely, BR treated animals presented tendency to weight reduction, which could increase with longer treatment periods. Studies have presented beneficial effects of BR on hyperglycemia and hyperlipidemia in animal models leading to weight loss (ARULDHAS et al., 1994; KUMAR V S et al., 2013). These data confirm the influence of PRL in energy balance regulation.

Treatment of PRL from PND12 to PND21 did not alter the weight and morphology of the ventral prostate. However, animals treated with PRL until PND35 presented dilated lumen associated with reduction in cellular height and volume of epithelial compartment. Moreover, an increased cell proliferation was observed in PRL group on PND35. Other authors have demonstrated the anabolic effect of PRL on VP, stimulating cell proliferation in a synergic manner with AR (GOFFIN et al., 2005; HERNANDEZ et al., 2006; HERRERA-COVARRUBIAS et al., 2015). Moreover, we observed an increase in secretory activity in VP after PRL administration as demonstrated by higher expression of prostatein in PRL group on PND35. This result can be linked to the dilation of the lumen and reduction of the height of the epithelial cells in PRL35 group. Reiter and colleagues (1995) also observed that the administration of PRL (10 μg) for 7 days to castrated and hypophysectomized adult rats increased prostatein expression in the VP. Thus, our results highlighted the role of PRL in inducing increased prostatic secretion.

Histologically, treatment with BR for 35 days produced the same result observed in the PRL35 group. However, we believe that the mechanism which cause this result is different from that observed for treatment with PRL. SHAPIRO et al. (1980) demonstrated that BR acts on stromal smooth muscle cells, inducing relaxation, which could explain the presence of more dilated acini in the BR35 group. Moreover, studies have presented that the administration of BR to men leads to a decrease in serum testosterone levels (OSEKO et al., 1991), as well as a decrease in testosterone synthesis in isolated Leydig cells (MARÍN-LÓPEZ et al., 1996). Although we did not evaluate testosterone levels in our study, there is a tendency to the reduction of prostatein expression in BR treated groups, indicating that BR administration could alter testosterone levels in these animals.

In order to evaluate whether PRL administration or inhibition modulates the downstream signaling pathway, the expression of PRLR and STAT was determined. On PND21, the PRLR gene expression was increased in the BR group. We believe that this effect can be a compensatory mechanism due to a low level of circulating PRL after BR treatment. FERRARIS et al., (2012) demonstrated that PRLR antagonist increases the genic expression of

the PRLR in the female rats. Oppositely, on PND 35, the PRLR gene expression was reduced in both PRL and BR groups. In the PRL group, this result can be a response to a long-standing exposure to high levels of serum PRL, while in BR group, the prolonged PRL blockade, down regulated the PRLR gene expression. Whereas in the protein level was not observe changes in PRLR expression, which suggests there is a post-transcriptional mechanism regulating PRLR in these hormonal conditions (SACKMANN-SALA et al., 2014b; SCHAUWECKER et al., 2016).

The Stat3 mRNA was increased in BR groups at PND21 and 35, while PRL treatment did not alter Stat3 expression. As described for PRLR, the reduction of Stat3 can be attributed to a mechanism of feedback, where the reduction of circulating PRL leads to an increase in Stat3 expression (LIU et al., 2013). Similar result was observed by PRINS et. al (1995) for AR mRNA, which demonstrated increase in the transcript early following androgen withdrawal. At protein level, the STAT3 expression was increased in PRL and BR groups at both PND21 and 35. These results highlight that STAT3 expression can be modulate by other pathways than the canonical PRL signaling as observed by LIU et al. (2013). There is no change in Stat5 mRNA expression in all experimental groups, but the protein expression increased in the group treated with PRL at PND35. This result can be attribute to a post-transcriptional regulation of STAT5 expression (LEE et al., 2014). Overall, our results demonstrated a complex regulation involved in the STAT signaling pathway on prostate.

CK18 is used as a molecular marker of luminal cells in prostatic (ZHANG et al., 2013). In our study, it was observed increase of CK18 in animals treated with BR at PND 21 and 35, demonstrating an increase in the dynamic of cell basal cell differentiation into luminal cells. However, animals treated with PRL had an increase in the stem cell marker NKX3.1, a factor responsible for maintaining the undifferentiated characteristic of prostate cells (ABATE-SHEN; SHEN; GELMANN, 2008). Other authors have demonstrated association of PRL overexpression, STAT signaling activation and increase of stem cell number in both normal and pathological conditions (ROBERTSON et al., 2003; SACKMANN-SALA; GOFFIN, 2015; TALATI et al., 2015). Thus, in our experimental model, modulation of PRL signaling interferes with the dynamic of cell proliferation and differentiation.

Conclusion

Our results demonstrate that, despite there were no variation in glandular weight, PRL modulation interferes with prostatic development and secretory function through a mechanism of increasing epithelial proliferative response and dynamic of cell differentiation, especially in animals treated for a long period.

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ANEXOS

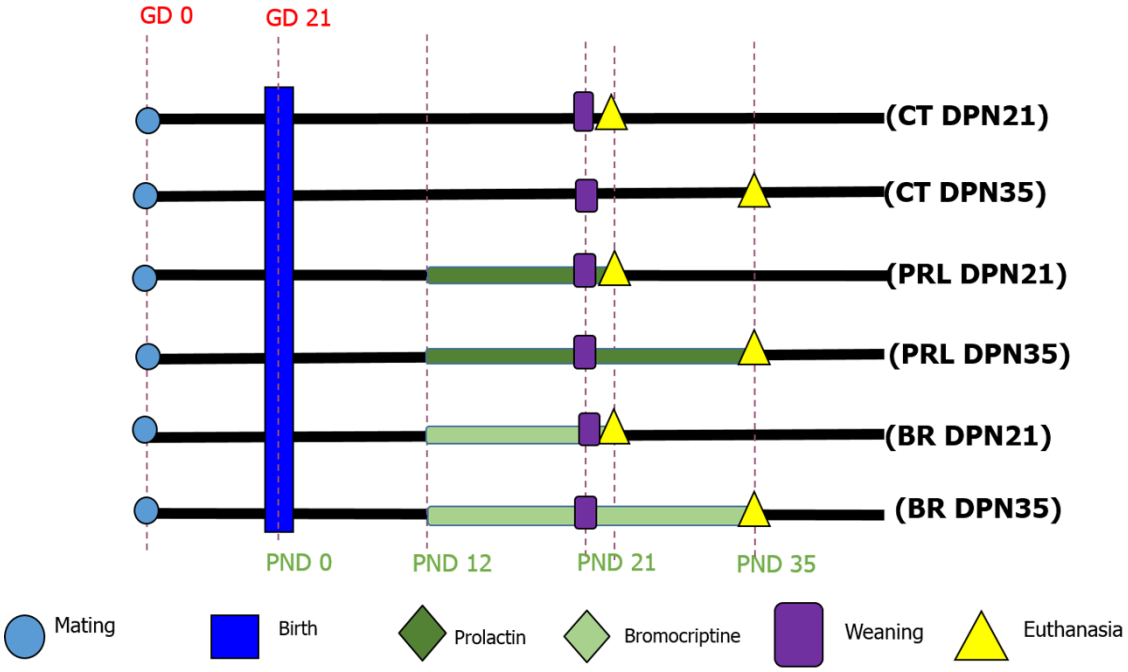

Fig. 1. Illustration of experimental procedures. GD = gestational day, PND = postnatal day, the Offspring CT = Control, PRL = Prolactin, BR = Bromocriptine.

Table 1. Biometric parameters of offspring in postnatal days 21 and 35

Parameters	Experimental Groups					
	CT21	PRL21	BR21	CT35	PRL35	BR35
Body weight(g)	53.35± 4.20	55±5.47	53.06±4.81	135±9.58^{A}	146±10.08^{B}	129.7±12.31^{A}
Próstata Ventral (PV) (n = 6)						
VP absolute weight (mg)	30.34±6.27	37.40±16.6	36.4±0.58	59.8±1.42	60.7±1.34	55.0±0.92
VP relative weight	0.55± 0.12	0.63±0.23	0.67±0.08	0.43±0.10	0.42±0.09	0.43±0.10
Serum levels of Prolactin	1.1±0.0	1.93±1.52	0.76±0.42	0.68±1.1	0.87±0.86	0.11±0.28

Data were expressed as mean ± standard deviation. Different letters show statistical difference with p <0.05. Statistical test: Anova followed by "Tuckey Kramer".

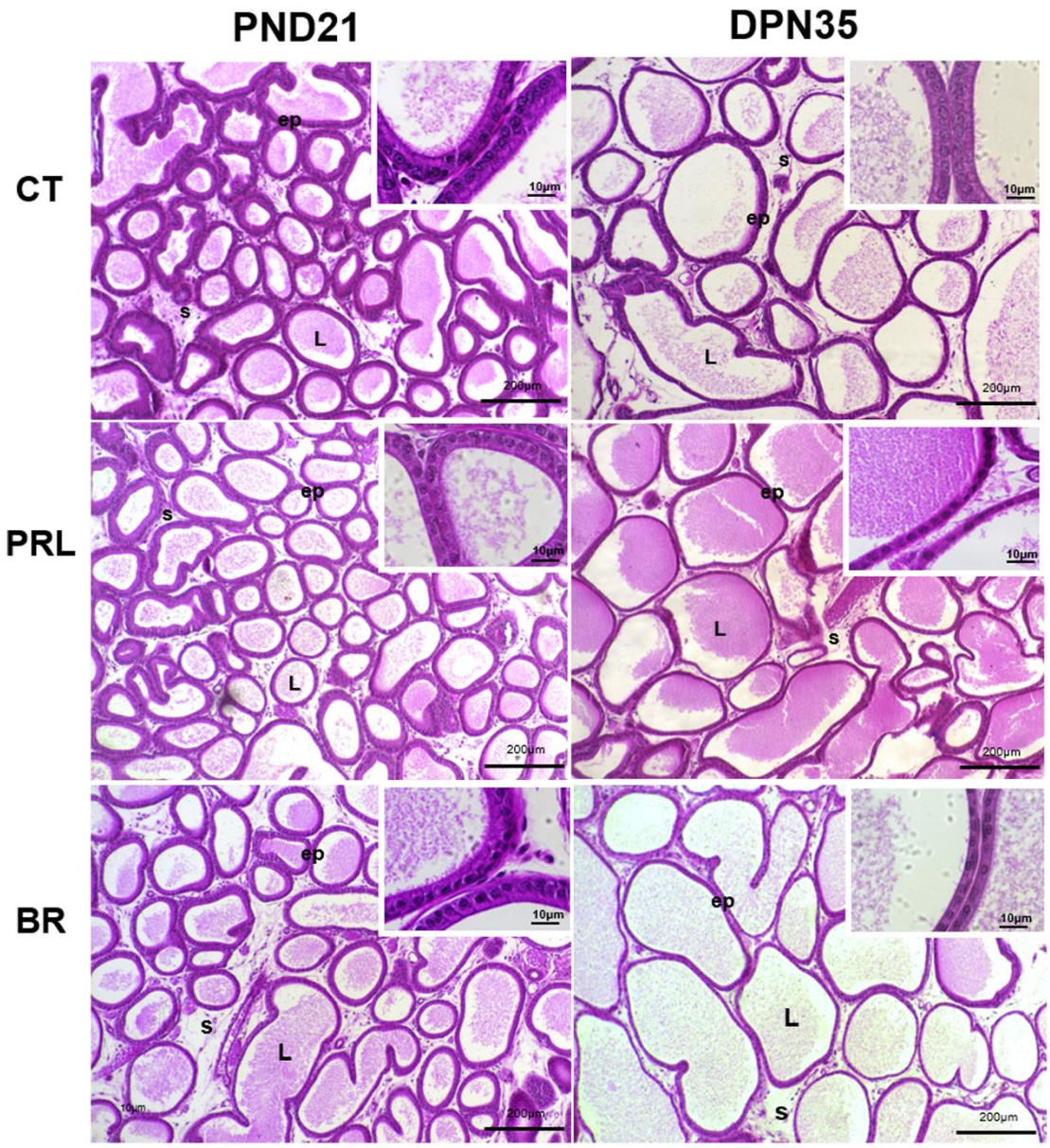

Fig. 2. Histological sections of the ventral prostate lobes from Control (CT). Prolactin (PRL) and Bromocriptine (BR) groups on PND 21 and PND 35 stained with hematoxylin–eosin. ep: epithelium; s: stroma; L: lumen. .

Table 2. Stereological analysis of the prostate of the animals of the different experimental groups

Parâmetros	Experimental Groups					
	CT21	PRL21	BR21	CT35	PRL35	BR35
Relative Frequency PV						
Lumen	51.67±7.52	45.82±7.41	51.19±8.86	55.75±6.26^{A}	66.82±3.50^{B}	66.26±4.44^{B}
Epithelium	28.92±6.97	27.53±2.69	26.63±3.72	26.49±3.91^{A}	15.3±1.11^{B}	16.48±2.11^{B}
Stroma	19.35±4.97	26.65±5.72	22.18±6.12	17.76±6.15	16.65±2.47	18.44±4.96
Height of epithelium (µM)	5.94±0.71	5.687±0.24	5.628±0.55	6.453±0.61^{A}	3.481±0.61^{B}	3.433±0.32^{B}

Data were expressed as mean ± standard deviation. Different letters show statistical difference with $p < 0.05$. Statistical test: Anova followed by "Tuckey Kramer".

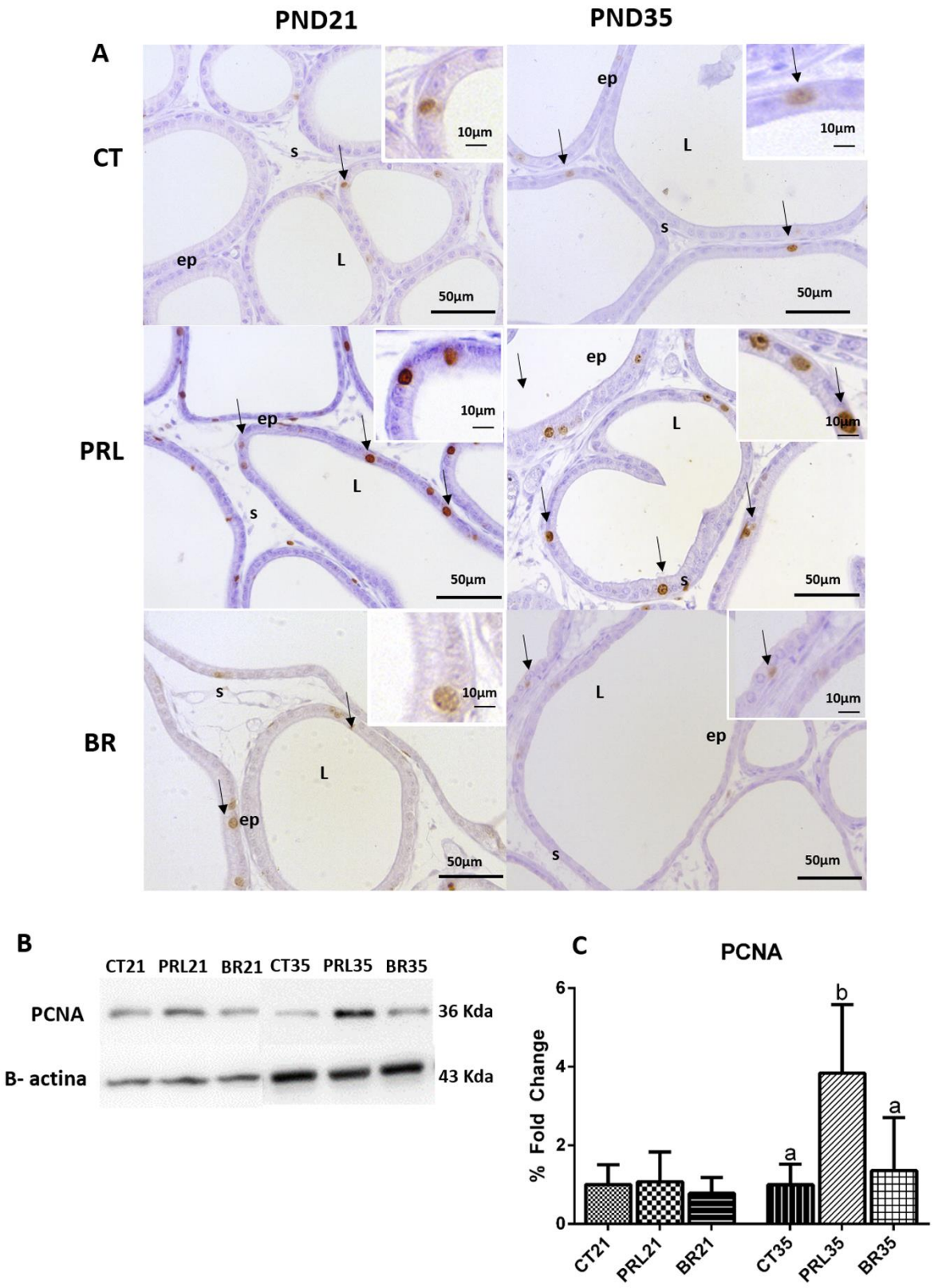

Fig.3. (A) Representative histological section of immunohistochemical reactions for Ki-67 in sections of the ventral prostate lobes from Control (CT), Prolactin (PRL) and Bromocriptine (BR) groups at PND 21 and PND 35, ep- epithelium, s-stroma, L-lumen. Arrows indicate marked cells; **(B and C)** Representative western blotting reactions for PCNA in the different experimental groups; The values were expressed as variation in relation to the CT group (fold change), the different letters show statistical difference with $p < 0.05$ .. Anova test followed by "Tuckey Kramer".

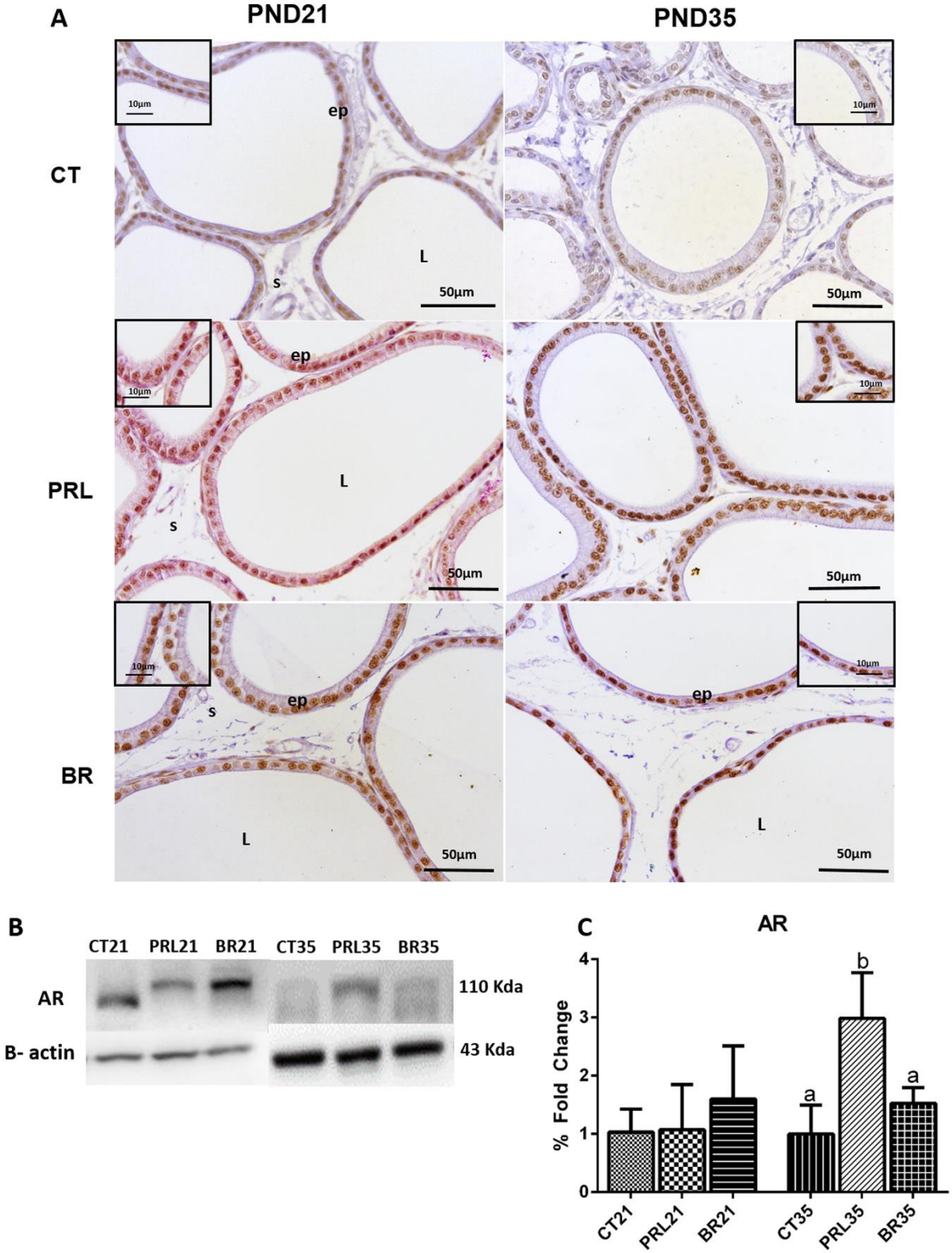

Fig. 4. (A) Representative histological sections of immunohistochemical reactions for AR in sections the offspring rat VP from Control (CT), Prolactin (PRL) and Bromocriptine (BR) groups on PND 21 and PND 35. ep- epithelium. s-stroma. L-lumen. Arrows indicate marked cells; **(B and C)** Representative western blotting reactions for AR in the different experimental groups; The values were expressed as variation in relation to the CT group (fold change). the different letters show statistical difference with $p < 0.05$ .. Anova test followed by "Tuckey Kramer".

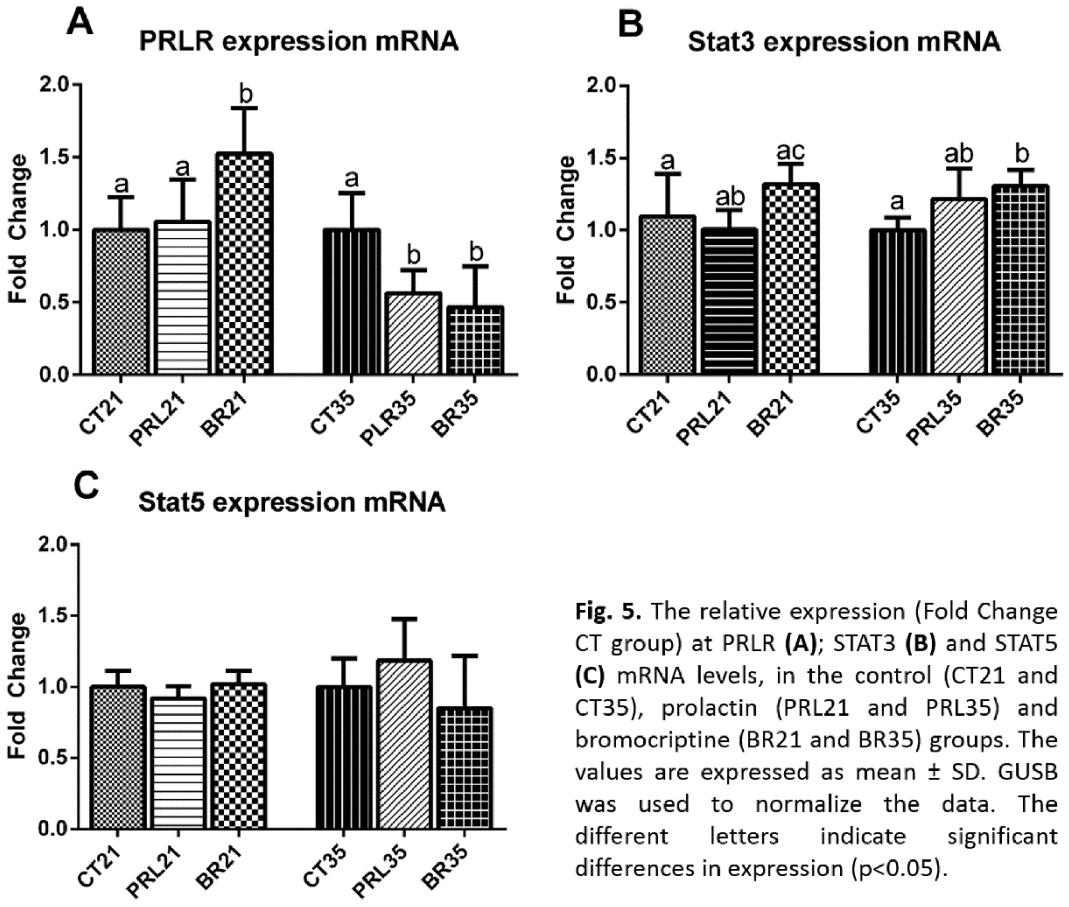

Fig. 5. The relative expression (Fold Change CT group) at PRLR **(A)**; STAT3 **(B)** and STAT5 **(C)** mRNA levels, in the control (CT21 and CT35), prolactin (PRL21 and PRL35) and bromocriptine (BR21 and BR35) groups. The values are expressed as mean ± SD. GUSB was used to normalize the data. The different letters indicate significant differences in expression ($p<0.05$).

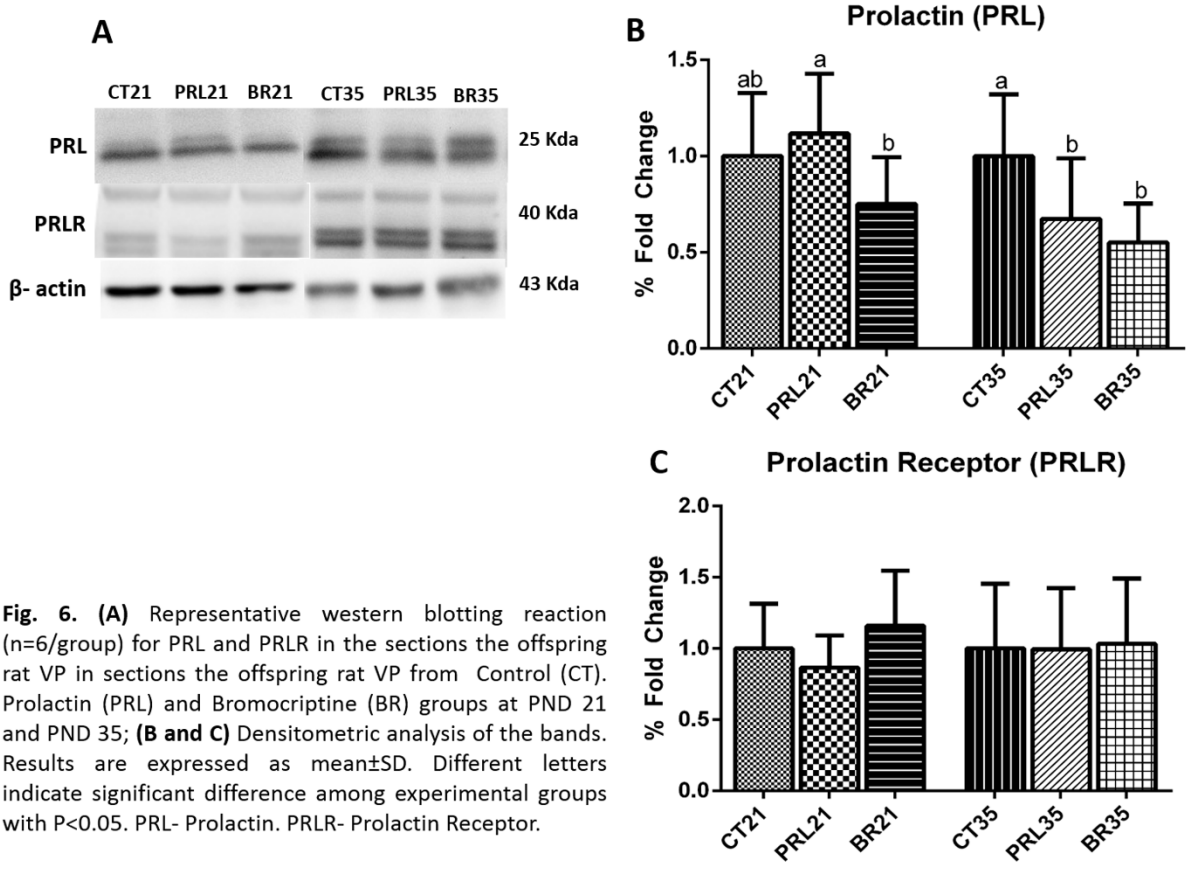

Fig. 6. (A) Representative western blotting reaction (n=6/group) for PRL and PRLR in the sections the offspring rat VP in sections the offspring rat VP from Control (CT). Prolactin (PRL) and Bromocriptine (BR) groups at PND 21 and PND 35; **(B and C)** Densitometric analysis of the bands. Results are expressed as mean±SD. Different letters indicate significant difference among experimental groups with $P<0.05$. PRL- Prolactin. PRLR- Prolactin Receptor.

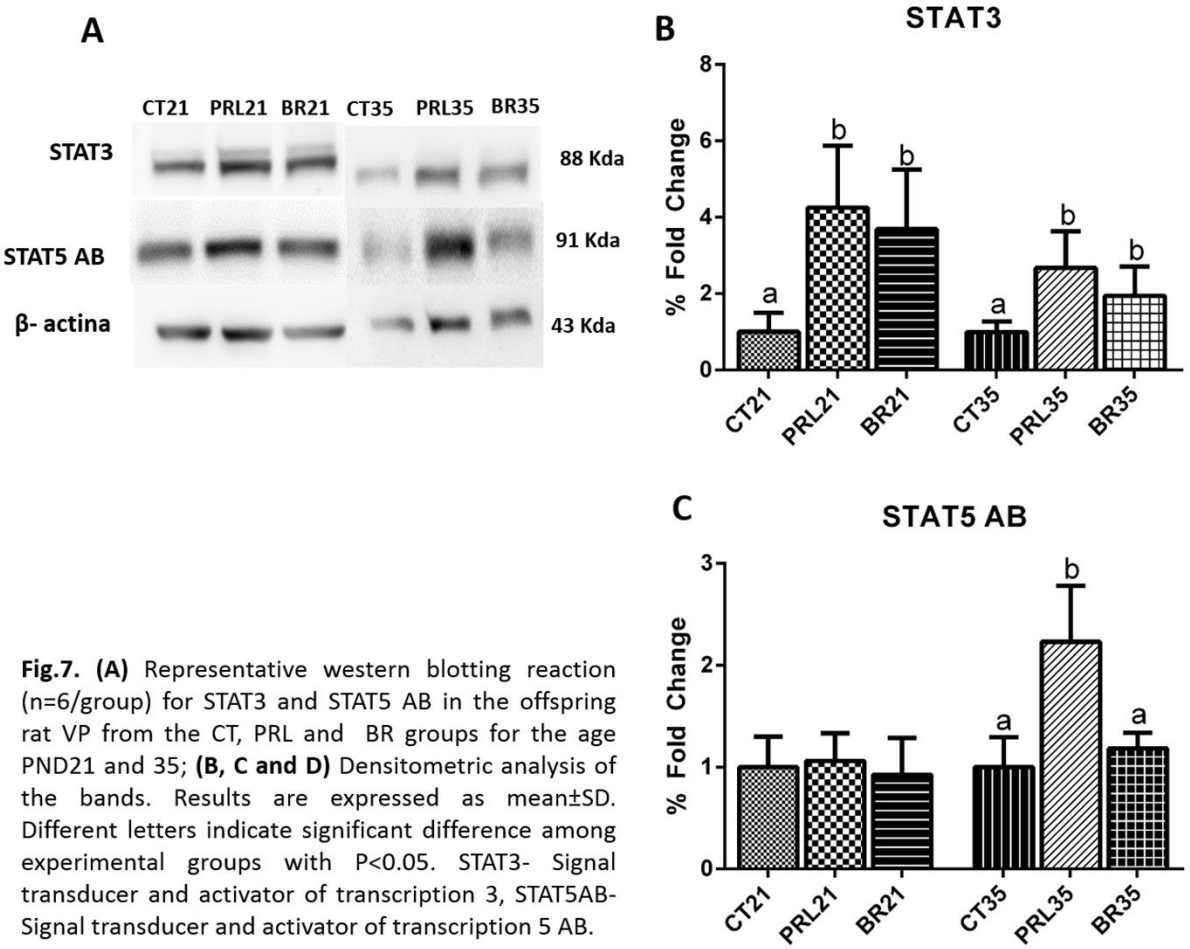

Fig.7. (A) Representative western blotting reaction (n=6/group) for STAT3 and STAT5 AB in the offspring rat VP from the CT, PRL and BR groups for the age PND21 and 35; **(B, C and D)** Densitometric analysis of the bands. Results are expressed as mean±SD. Different letters indicate significant difference among experimental groups with P<0.05. STAT3- Signal transducer and activator of transcription 3, STAT5AB- Signal transducer and activator of transcription 5 AB.

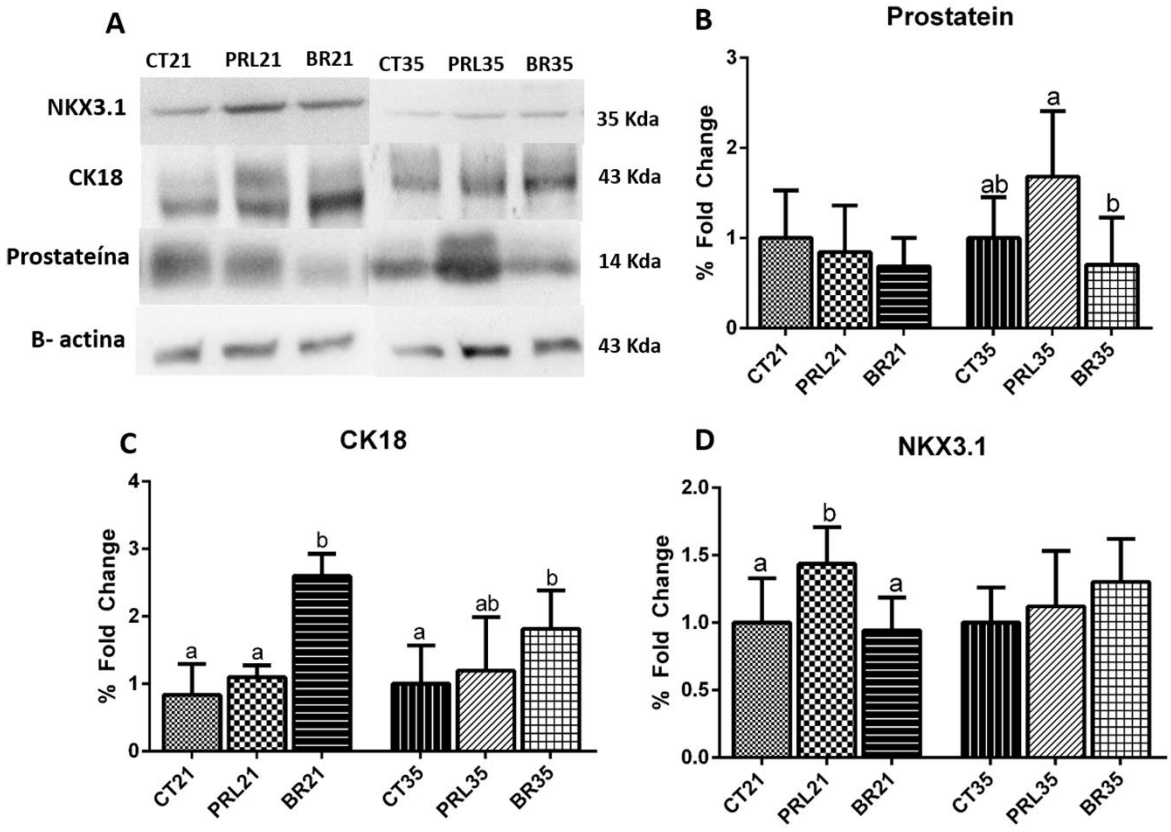

Fig. 8. (A) Representative western blotting reaction (n=6/group) for Prostatein, NKX3. and CK18 in the offspring rat VP from the control (CT21 and CT35), prolactina (PRL21 and PRL35) and bromocriptine (BR21 and BR35) groups; Densitometric analysis of the bands of Prostatein **(B),** CK18 **(C)** and NKX3.1 **(D)**. Results are expressed as mean±SD. Different letters indicate significant difference among experimental groups with P<0.05. Prostatein- major secretory protein of the rat ventral prostate, CK18- Cytokeratin 18; NKX3.1- homeobox gene.

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Certificate

We certify that the protocol nº **756** about "Inhibition of the hormone Prolactin in prostate development in rats: Evaluation of glandular morphogenesis, cell proliferation and differentiation epithelial" agree with Brazilian legislation regulated by the National Council for the Control of Animal Experimentation (CONCEA) and ETHICAL PRINCIPLES IN ANIMAL RESEARCH formulated by the Brazilian Society of Science in Laboratory Animals, and was approved by the BIOSCIENCE INSTITUTE/UNESP ETHICS COMMITTEE ON USE OF ANIMALS (CEUA), in August 26th, 2015.

Botucatu, August 26th, 2015.

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Artigos publicados durante o Mestrado

THE ANATOMICAL RECORD 00:00–00 (2016)

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, SÉRGIO 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, 00:000–000, 2016. © 2016 Wiley Periodicals, Inc.

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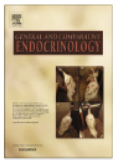

Research paper

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.