UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

FABIANA OLIVEIRA DOS SANTOS GOMES

Avaliação dos efeitos do inibidor de fosfodiesterase-5 (Sildenafil) em um modelo de prostatite experimental

RECIFE

2015

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Federal de Pernambuco para a obtenção do Título de Doutora em Ciências Biológicas na área de concentração Biologia Celular e Molecular.

Orientadora: Dra. Christina Alves Peixoto

RECIFE

2015

Catalogação na fonte

Elaine Barroso

CRB 1728

Gomes, Fabiana Oliveira dos Santos

Avaliação dos efeitos do inibidor de fosfodiesterase-5 (Sildenafil) em um modelo de protastite experimental/ Fabiana Oliveira dos Santos Gomes– Recife: O Autor, 2015.

156 folhas : il., fig., tab.

Orientadora: Christina Alves Peixoto

Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Ciências Biológicas. Ciências Biológicas, 2015.

Inclui referências e anexo

1. Prostatite 2. Sildenafil I. Peixoto, Christina Alves (orientadora) II. Título

616.65 CDD (22.ed.) UFPE/CCB-2016-094

UNIVERSIDADE FEDERAL DE PERNAMBUCO

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AGRADECIMENTOS

"*A Deus, que nos concede, a cada dia, uma página de vida nova no livro do tempo. Aquilo que colocarmos nela, corre por nossa conta*".

Aos meus pais, Ivan e Irleide, pelo amor, dedicação, apoio e incentivo.

A minha irmã, Taciana e ao meu cunhado, Alexandro, pela confiança e por me ensinarem o dom de sempre servir, acompanhados de amor de carinho.

A minha filha, Maria Eduarda, com quem aprendi a essência da vida e que a cada dia me faz sentir mais forte e determinada para alcançar meus objetivos.

Ao grande incentivador e colaborador de tudo isso, meu marido Amaro Gomes, pela compressão, paciência, cumplicidade, dedicação e acima de tudo amor para suportar os momentos conturbados durante esse processo.

A minha avó, Isaura e ao meu tio Omir Freitas (*in memoriam*), á quem devo todo meu respeito e admiração, por estar ao meu lado durante a grande jornada em minha vida.

A minha "grande" família, tios, tias, primos, primas e sobrinhas pelo carinho e apoio.

A minha orientadora, Christina Peixoto, pela oportunidade, dedicação, carinho e pela contribuição na minha formação profissional e pessoal. Agradeço a confiança que depositou em mim ao longo dos anos e pelo laço de amizade que criamos.

A Amanda Costa, pela dedicação, paciência, incentivo e companheirismo durante toda a jornada. Admiro seu esforço contínuo em permanecer ao meu lado nos final de semana de experimentação e até tarde no laboratório.

As "Rebeldes e ex-rebelde da Ultraestrutura" Mariana, Ingrid e Shyrlene, por tornarem o dia exaustivo de trabalho mais leve, pelos momentos de descontração e apoio. Agradeço, sobretudo, a Edlene, Amanda e Bruna pelo laço de amizade, paciência, cumplicidade, dedicação e por estar ao meu lado nos feriados, finais de semanas e festas comemorativas.

A toda equipe do Laboratório de Ultraestrutura, em especial a Gabriel, pelo apoio e pelas discussões científicas ou não, ao qual aprendi muito.

Aos meus eternos e melhores amigos, “o grupo” Suzanne Brito, Jefferson José, Alexandre Trajano, Otoniel Rodrigues e Karina Wacemberg.

Aos amigos de Antibióticos, Anekécia e Antônio Sérgio, pelo apoio e carinho.

As minhas amigas do Aggeu Magalhães, Maria Carolina e Andresa Oliveira, pelos bons momentos de descontração, generosidade e conselhos.

Aos novos e antigos amigos, Micheline Miranda, Juliana Cruz, Taci, Amanda, Karla, Lívia (ex-chefa), Renato, Stephany, Jana, Cássia, Bruno, Juliana e Carol, pelas saídas para descontrair, conversas e incentivo.

Ao Centro de Pesquisa Aggeu Magalhães e a pós-graduação em Ciências Biológicas da UFPE, pela oportunidade e apoio. Em especial a Adenilda, pela atenção e dedicação.

A FACEPE pelo suporte financeiro.

“Era ele que erguia casas

Onde antes só havia chão.

Como um pássaro sem asas

Ele subia com as casas

Que lhe brotavam da mão.....

.....De fato, como podia

Um operário em construção

Compreender por que um tijolo

Valia mais do que um pão?”

(*Vinícius de Morares)*

“Superar é preciso. Seguir em frente é essencial. Olhar pra trás é perda de tempo. Passado se fosse bom era presente”.

(Clarice Lispector)

“Se temos de esperar, que seja para colher a semente boa que lançamos hoje no solo da vida. Se for para semear, então que seja para produzir milhões de sorrisos, de solidariedade e amizade”.

(Cora Coralina)

RESUMO

O Sildenafil é um inibidor potente e seletivo da fosfodiesterase-5 (PDE5). Este fármaco foi aprovado para uso terapêutico na disfunção erétil e, atualmente, vem sendo usado também no tratamento da hipertensão pulmonar. Embora mantenha um excelente nível de segurança e perfil de tolerabilidade, poucos estudos avaliaram os possíveis efeitos colaterais do tratamento crônico com Sildenafil sobre o sistema reprodutor masculino, especialmente na próstata visando o relaxamento da uretra e alívio dos Sintomas do Trato Urinário Inferior (STUI). Desta forma, avaliamos o efeito do tratamento, em camundongos C57Bl/6, através de análise morfológica, ultraestrutural e expressão molecular de guanilato ciclase solúvel-sGC, Óxido nítrico sintase endotelial-eNOS, Antígeno prostático específico-PSA e Fator de crescimento transformador beta-TGF-β no tecido prostático. Foi-se observado que o tratamento com Sildenafil não induz danos evidentes na próstata. Além disso, tem sido demonstrado que Sildenafil tem eficácia terapêutica em doenças inflamatórias crônicas, podendo apresentar uma eficácia terapêutica potencial em diferentes doenças. Entre elas, uma atenção especial tem sido dada para as patologias relacionadas ao trato urogenital masculino, como a Hiperplasia Prostática Benigna (HPB), Câncer de próstata e Prostatites. A inflamação tem sido considerada como um fator etiológio da HPB e STUI. Assim, foi proposto um modelo de lesão prostática com injeção intrauretral de LPS (1mg/ml), em camundongos machos Swiss e C57Bl/6, desenvolvido durante 3, 7, 10 e 14 dias. A análise dos resultados mostrou que a indução intrauretral com lipopolisacarideo-LPS atua como importante agente da HPB, além de promover o aumento de fatores de crescimento (FGF-7 e FGF-β), α-actina e citocinas pró-inflamatórias (IL-1, IL-6, IL-17), tanto no estroma como no epitélio. Uma vez que o Sildenafil tem potencial anti-inflamatório, este estudo se propôs a analisar a ação do Sildenafil em um modelo de lesão prostática experimental, induzido por injeção intrauretral de LPS em camundongos C57BL/6. O tratamento com Sildenafil (25mg/kg) dos animais com prostatite apresentaram redução significativa de α-actina, COX-2, NFK-κB, IL-6, IL-17 e FGF-7. Por não induzir danos na próstata , o Sildenafil, por não induzir a longo prazo danos evidentes na próstata pode representar uma estratégia farmacológica para o tratamento de doenças inflamatórias crônicas do trato urogenital.

Palavra chave: Sildenafil, prostatite, LPS, PDE-5

ABSTRACT

Sildenafil is a potent and selective inhibitor of phosphodiesterase-5 (PDE5). This drug has been approved for therapeutic use in erectile dysfunction and currently has been also used to treat pulmonary hypertension. While maintaining an excellent level of safety and tolerability profile, few studies have evaluated the possible side effects of chronic treatment with Sildenafil on the male reproductive system, especially in prostate aimed at relaxing the urethra and relief of symptoms of Lower Urinary Tract (LUTS). Therefore, we assessed the treatment effect in C57Bl/6 mice, using morphological analysis, ultrastructural and molecular expression of soluble guanylate cyclase-sGC, endothelial nitric oxide synthase, eNOS, prostate-specific antigen PSA and transforming growth factor beta TGF-β in prostate tissue. It is observed that the treatment with sildenafil does not induce apparent damage to the prostate. Furthermore, Sildenafil has been shown to have therapeutic efficacy in chronic inflammatory diseases, which have a potential therapeutic efficacy in various diseases. Among them, special attention has been given to the pathologies related to the male urogenital tract, such as Benign Prostatic Hyperplasia (BPH), prostate cancer and Prostatitis. Inflammation has been considered as a factor etiológio of BPH and LUTS. Thus, it has been proposed a prostatic intraurethral injection injury model of LPS (1mg/ml) in male Swiss mice and C57Bl /6 developed for 3, 7, 10 and 14 days. The results showed that the intraurethral induction with lipopolysaccharide-LPS acts as an important agent of HPB, and to promote the increase of growth factors (FGF-7 and FGF-β), α-actin and pro-inflammatory cytokines (IL- 1, IL-6, IL-17), both in the stroma and the epithelium. Since Sildenafil has potential anti-inflammatory, this study aimed to analyze the action of Sildenafil in an experimental prostate injury model induced by intraurethral injection of LPS in C57Bl/6 mice. Treatment with sildenafil (25 mg/kg) of animals with prostatitis showed a significant reduction of α-actin, COX-2 NFK-kB, IL-6, IL-17 and FGF-7. By not induce damage to the prostate, Sildenafil, not to induce long term damage evident in the prostate may represent a pharmacologic strategy for the treatment of chronic inflammatory diseases of the urogenital tract.

Keyword: Sildenafil, prostatitis, LPS, PDE5.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AMP	**Monofosfato de Adenosina**
AMPK	**Protein Kinase Activated by AMP/ Proteína Quinase Ativada por AMP**
APC	**Antigen-Presenting Cell/ Célula Apresentadora de Antígeno**
AR	**Androgen Receptor/ Receptor de Andrógeno**
ARA 70	**Androgen Receptor Associated to Protein 70/ Receptor de Andrógeno associado a proteína 70**
ATP	**Adenosine Triphosphate/ Trifosfato de Adenosina**
AUR	**Acute Urinary Retenction/ Retenção Urinária Aguda**
BKCa	**Large Conductance Calcium Activated Potassium Channels/ Canais de Potássio de Grande Condutância Ativados por Ca^{2+}**
BPH	**Benign Prostatic Hyperplasia/ Hiperplasia Prostática Benigna**
cAMP	**Cyclic Adenosine Monophosphate/ Adenosina Monofosfato Cíclico**
CBP	**Chronic Bacterial Prostatitis/ Prostatite Bacteriana Crônica**
CD	**Cardiovascular Disease/ Doença Cardiovascular**
cGKI	**cGMP-dependent Protein Kinase Type I/ Proteína Quinase Dependente de GMPc**
cGMP	**Cyclic Guanosine Monophosphate/ Monofosfato de Guanosina Cíclico**
COX-2	**Cyclooxygenase/ Ciclooxigenase**
DHT	**Di-hidrotestosterona/ Diidrotestosterona**
DNA	**Deoxyribonucleic Acid/ Ácido Desoxirribonucleico**
ED	**Erectil Disfunction/ Disfunção Erétil**

EMT	**Mesenchymal Epithelial Transition/ Epitélio Mesenquimal de Transição/**
eNOS	**Endothelial Nitric Oxide Synthase/ Óxido Nítrico Sintase Endotelial**
FGF	**Fibroblast Growth Factor/ Fator de Crescimento de Fibroblastos**
FGFR1	**Fibroblast Growth Factor Receptor 1/ Receptor Específico de FGF 1**
GC	**Guanylate Cyclase/ Guanilato Ciclase**
GCp	**Guanylate Cyclase Transmembrane/ Guanilato Ciclase Transmembrana**
GMP	**Guanosine Monophosphate/ Monofosfato de Guanosina**
GTA	**General Transcription Apparatus/ Aparato de Transcrição Geral**
HSPs	**Heat Shock Proteins/ Proteínas de Choque Térmico**
ICAM 1	**Intercellular Adhesion Molecule 1/ Molécula de Adesão Intercelular 1**
IFN-γ	**Interferon γ**
IGF	**Insulin Growth Factors/ Fatores de crescimento semelhantes à insulina**
IKB-α	**Regulatory Protein that Inhibits NF-κB/ Proteína Inibitória do NF-κB**
IL	**Interleukin/Interleucina**
iNOS	**Inducible Nitric Oxide Synthase/ Óxido Nítrico Sintase Induzível**
IPSS	**International Prostate Symptom Score/ Pontuação Internacional de Sintomas Prostáticos**
KATP	**ATP-Sensitive Potassium Channel/ Canais de Potassio Sensíveis ao ATP**
LPS	**Lipopolysaccharide/ Lipopolissacárideos**
LUTS	**Low Urinary Tract Symptoms/ Sintomas do Trato Urinário Inferior**

MET	**Microscopia Eletrônica de Transmissão**
MetS	**Metabolic Syndrome/ Síndrome Metabólica**
MO	**Microscopia Óptica**
NF-κB	**Nuclear Factor kappa B/ Fator Nuclear Kappa B**
nNOS	**Neuronal Nitric Oxide Synthase/ Óxido Nítrico Sintase Neuronal**
NO	**Nítric Oxide/ Oxido Nítrico**
PCa	**Prostate Cancer/ Câncer de Próstata**
PDE	**Phosphodiesterase/ Fosfodiesterase**
PGE- 2	**Prostaglandin- 2/ Prostaglandina-2**
PKA	**Protein Kinase A/ Proteína Quinase A**
PKG	**Protein Kinase G/ Proteína quinase G**
PSA	**Prostate Specific Antigen/ Antígeno prostático especifico**
RNS	**Reactive nitrogen Species/ Espécies Reativas de Nitrogênio**
ROS	**Reactive Oxigen Species/ Espécies Reativas de Oxigênio**
sGC	**Soluble Guanylate Cyclase/ Guanilato Ciclase Solúvel**
SHBG	**Sex Hormone-Binding Globulin/ Globulina Transportadora do Hormônio Sexual**
SNC	**Sistema Nervoso Central**
TGF-β	**Transformation Growth Factor β/ Fator de Transformação do Crescimento β**
TLR	**Toll-Like Receptor/ Receptor do Tipo Toll**
TNF- α	**Tumor Necrosis Factor α/ Fator de Necrose Tumoral α**
VCAM 1	**Vascular Cell Adhesion Molecule 1/ Molécula de Adesão Celular Vascular 1**
VEGF	**Vascular Endothelial Growth Factor/ Fator de Crescimento Endotelial**

SUMÁRIO

1 INTRODUÇÃO

Doenças do trato geniturinário representam morbidades comuns em homens acima de 50 anos. No decorrer das últimas décadas a comunidade científica tem despertado interesse nas doenças prostáticas, especialmente Hiperplasia prostática benigna e Câncer de próstata. A próstata é uma glândula acessória do sistema reprodutor masculino e auxilia na produção do fluído espermático.

A próstata é um órgão sensível a andrógenos que necessita da sinalização adequada entre andrógenos e seus receptores para o desenvolvimento normal. Os andrógenos envolvidos na homeostase prostática são testosterona e diidrotestosterona, a testosterona é convertida a diidrotestosterona pela ação da enzima 5 α-redutase. A ligação da diidrotestosterona ao receptor de andrógeno induz a fosforilação, dimerização, e na translocação do complexo para o núcleo, que por sua vez ativa a transcrição de genes específicos necessários para o crescimento e desenvolvimento das células.

As Fosfodiesterases (PDEs) formam uma grande família e estão subdivididas de acordo com a ordem de descoberta, sequência de aminoácidos e características catalíticas e regulatórias. Estas enzimas hidrolisam os nucleotídeos cíclicos, AMPc e GMPc, às suas formas inativas 5´monofosfatos. Estes nucleotídeos, por sua vez, controlam múltiplas vias de sinalização intracelular que podem ser alterada em muitas patologias, como câncer, inflamação, neurodegeneração e estresse oxidativo.

O Sildenafil é um inibidor potente e seletivo da PDE5, presente em vários tecidos tais como o vascular e o muscular liso. Este fármaco tem sido amplamente usado no tratamento da disfunção erétil e da hipertensão pulmonar. Estudos clínicos e experimentais têm demonstrado que Sildenafil tem eficácia terapêutica em doenças inflamatórias crônicas, tais como disfunção endotelial, colites e doenças neuroinflamatórias.

Além disso, estudos recentes têm demonstrado que o uso diário com inibidores de PDE-5 melhora os sintomas da Hiperplasia Prostática Benigna/Sintomas do Trato Urinário Inferior (BPH/LUTS), possivelmente como resultado de sua ação relaxante via mecanismos do Óxido Nítrico (NO), e inibindo a proliferação das células estromais prostáticas. Uma vez que o Sildenafil tem potencial anti-inflamatório, é possível que a melhora o quadro clínico da prostatite crônica e do LUTS após tratamento com Sildenafil, deva-se não somente ao efeito relaxante da musculatura lisa da uretra e

próstata, mas também ao seu efeito anti-inflamatório devidos aos níveis aumentados de GMPc.

Diante do exposto, o presente estudo se propõe a analisar a ação do Sildenafil, na concentração de 25mg/kg, em um modelo de lesão prostática experimental.

2 OBJETIVOS

2.1 Geral

Caracterizar os efeitos do Sildenafil em um modelo de lesão prostática experimental.

2.2 Específicos

a) Estudar o efeito do tratamento a longo prazo com Sildenafil (25mg/kg) na próstata, através da modulação via NO/cGMP;
b) Avaliar o papel do LPS durante 3, 7, 10 e 14 dias na remodelação tecidual e na expressão de citocinas pró-inflamatórias;
c) Analisar o efeito do Sildenafil, em modelo de lesão prostática experimental sobre o dano tecidual e a resposta inflamatória.
d) Desenvolver um modelo de lesão prostática experimental induzida por LPS.

3 REVISÃO DA LITERATURA

3.1 Aspectos gerais da glândula prostática

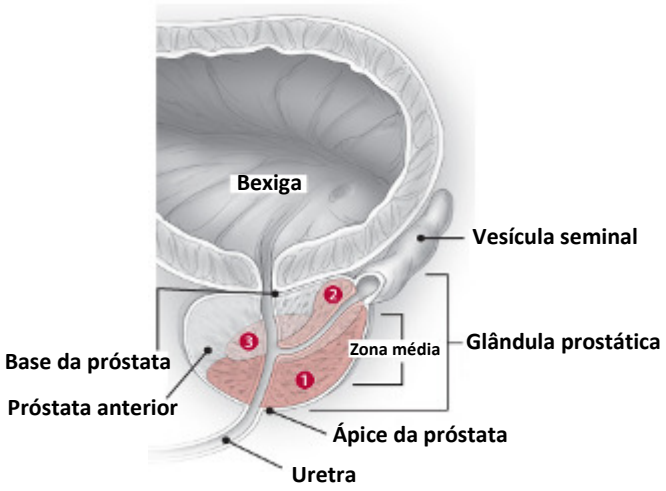
A maioria dos homens, eventualmente, irá desenvolver algum tipo de patologia prostática. As doenças mais comuns são a hiperplasia prostática benigna (BPH), prostatite, e câncer, que apresentam taxa de incidência aumentada com o envelhecimento (SCHRÖDER et al. 2012; KUMAR et al. 2013).

A próstata é uma glândula do sistema reprodutor masculino, que produz um fluido incolor e ligeiramente alcalino (NETTER, 2010), constituindo 10-30% do volume do fluido seminal, que ajuda o sêmen a transportar o esperma durante o orgasmo masculino (OMABE & EZEANI, 2011). Ela apresenta tamanho e forma semelhante a uma noz, localizada na frente do reto, logo abaixo da bexiga e envolve a uretra (ELLEM & RISBRIDGER, 2007).

O desenvolvimento das próstatas de humanos e roedores é completo durante a fase de crescimento púbere que ocorre em resposta ao aumento da produção de testosterona pelas células de Leydig nos testículos (CUNHA et al., 1987). Semelhanças anatômicas entre a próstata de roedores e humanos têm colaborado na aplicação de modelos animais para estudos de alterações moleculares que acompanham o desenvolvimento e a progressão de diferentes patologias prostáticas (SHAPPELL et al., 2004).

A próstata de mamíferos é composta por três zonas distintas e importantes, central; transição e periférica (Figura 1A). Na zona central, as glândulas são maiores, com contornos irregulares e projeções intraluminares salientes, estroma denso, enquanto na zona periférica são vistos ácinos pequenos, redondos e simples em meio a estroma frouxo (SALDANHA et al. 2000; ZYNGER e PARWANI, 2014). Os tipos de lesão proliferativa são diferentes em cada zona. Estudos comprovam que a maior incidência de hiperplasia prostática benigna ocorre na zona de transição, enquanto que os carcinomas prevalecem na zona periférica (OMABE & EZEANI, 2011; SCHILLER & PARIKH, 2011).

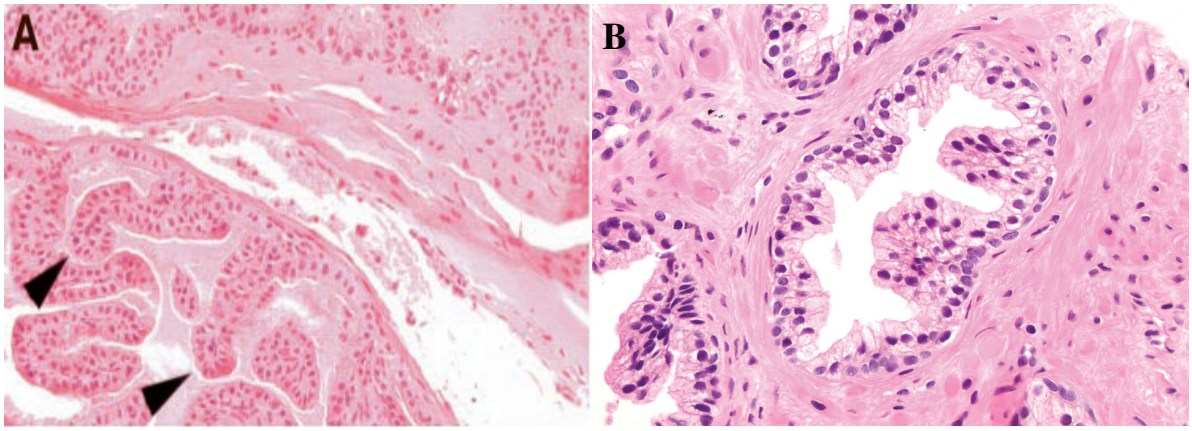
Figura 1 - Representação esquemática das três zonas prostática humana: zona periférica ❶ ; a zona central ❷; e a zona de transição❸ .



Fonte: Annual Report on Prostate Diseases (2011);

A próstata humana é composta 70% de região epitelial glandular e 30% estroma fibromuscular (NETTER, 2010; SCHILLER & PARIKH, 2011). O epitélio glandular é composto por três tipos de células: basais, luminais secretoras diferenciadas e uma pequena subpopulação de células neuroendócrina. As células basais são consideradas células progenitoras, dando origem às células secretoras que revestem os espaços luminais da glândula e, muito provavelmente, às neuroendócrinas, que são responsáveis pela produção de substâncias com efeito parácrino (Figura 2). As células basais tendem a ser orientada paralelamente à membrana basal, nem sempre bem visível por microscopia de luz, e pode ser difícil distingui-las entre as células do estroma. O estroma por outro lado, é constituído por fibroblastos, células dendríticas, células endoteliais, mastócitos, linfócitos e células nervosas. As células estromais são responsáveis pela produção do fator de crescimento (FELDMAN & FELDMAN, 2001; KELLY & YIN, 2008; MAITLAND & COLLINS, 2008). O abundante estroma fibromuscular denso em torno das glândulas encontrado na próstata humana, não está presente em camundongos (SHAPPELL et al., 2003).

Figura 2 – Histologia da próstata de camundongo e humano. (A) Arquitetura luminal da próstata de camundongo, mostrando dobras salientes nos lúmenes da glândula (seta). (B) Próstata normal de humanos, a camada externa de células basais é caracterizada por células cubóides achatada com núcleos orientados paralelamente a membrana basal.



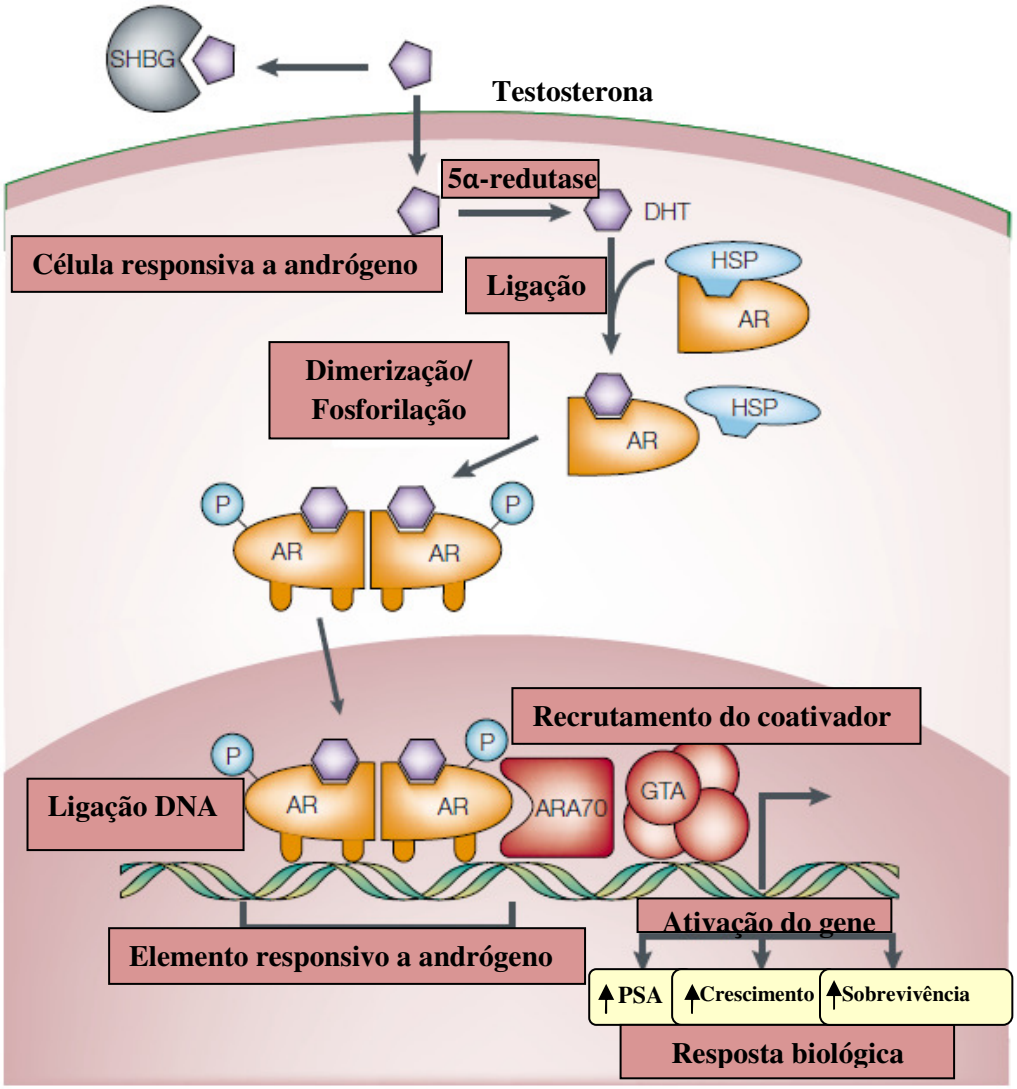
Fonte: Shappell et al. (2004); Zynger e Parwani (2014).

A próstata é um órgão sensível a andrógenos que necessita da sinalização adequada entre andrógenos e seu receptor de andrógenos (AR), para o desenvolvimento normal (WEN et al. 2014). Na próstata, o AR é expresso em ambos os tecidos epiteliais e estromais. As células luminais expressam receptores de andrógenos, E-caderina e integrinas, que se liga ao andrógeno (OMABE e EZEANI, 2011).

O AR é um membro da superfamília de receptores nucleares que pode ser ativado e translocado do citoplasma para núcleo após ligação a testosterona ou diidrotestosterona (DHT), tendo mais afinidade por este último (ANDERSON & LIAO, 1968). A testosterona circula no sangue ligado a albumina e globulina de hormônios sexuais de ligação (SHBG) e uma pequena fração dissolvida livremente no soro (Figura 3). A testosterona livre entra nas células da próstata e é convertida em DHT pela enzima 5α-redutase. A ligação da DHT ao AR induz a dissociação das proteínas de choque térmico (HSPs) e a fosforilação do receptor. O AR dimeriza e pode ligar-se a elementos responsivos aos andrógenos nas regiões promotoras de genes alvo. Os coativadores, tais como ARA 70, e correpressores também se ligam ao complexo AR, facilitando ou prevenindo, sua interação com o aparato de transcrição geral (GTA). A ativação ou repressão de genes alvo leva a respostas biológicas, incluindo o crescimento, sobrevivência e a produção de antígeno específico da próstata (PSA), que é um

marcador de diagnóstico e prognóstico de câncer de próstata (FELDMAN e FELDMAN, 2001).

Figura 3 – Diagrama esquemático da ligação entre receptor de andrógeno e andrógeno



Fonte: Feldman & Feldman (2001).

A transativação do AR no núcleo pode então funcionar através modulação de vários genes alvo, influenciando no desenvolvimento e manutenção da próstata. Além de influenciar diretamente no crescimento celular, epitelial e estromal. O AR influencia no desenvolvimento da próstata através da transição epitélio-mesenquimal (EMT) (WEN et al. 2014).

EMT é um processo pelo qual as células epiteliais perdem a adesão célula-célula e ganham propriedades migratórias para se tornar em células mesenquimais e/ou tronco

mesenquimais, que leva à perda ou expressão reduzida dos marcadores de células epiteliais, como a e-caderina, e ao aumento da expressão de marcadores mesenquimais, como a n-caderina e vimentina, assim como o aumento da expressão do fator de transcrição. Estas células mesenquimais podem, depois, diferenciarem-se em diferentes tipos de células e influenciar a progressão da BPH e câncer (KHAN et al. 2004; MAGDALENA et al. 2009; IWATSUKI et al. 2010; WEN et al. 2014). A EMT pode ser induzida por componentes de matriz extracelular e fatores de crescimento, como o fator de transformação de crescimento beta (TGF-β), responsável pela regulação da diferenciação e proliferação celular, migração e apoptose (MIYAZONO 2009).

A sinalização estromal andrógenos/AR pode ocorrer através da modulação de diferentes fatores de crescimento, incluindo fator de crescimento fibroblastos (FGFs), fator de crescimento semelhante à insulina (IGF), e fator de crescimento endotelial vascular (VEGF), que promove o crescimento e diferenciação do epitélio (LEVINE et al. 1998; KWABI-ADDO et al. 2004; LAI et al. 2012; KATSUNO et al. 2013).

3.2 Comunicação celular na fisiologia prostática: o sistema mensageiro

Os nucleotídeos cíclicos monofosfatados, AMP e GMP, são importantes mediadores intracelulares de várias moléculas de sinalização, e regulam inúmeros processos intracelulares, tais como motilidade do músculo liso, homeostase de eletrólitos, sinais neuroendócrinos, fototransdução retinal e respostas inflamatórias (UCKERT et al., 2006; CHUNG, 2006). AMPc e GMPc ativam alvos intracelulares, tais como canais iônicos, fatores de transcrição, proteínas quinases G (PKG) e via Rho quinase (ROCK2), que são importantes na regulação do tônus da musculatura lisa da próstata (LIU et al., 2007).

O AMPc é sintetizado a partir do ATP por uma proteína transmembrana multipasso chamada adenilil ciclase, e é degradado rápida e continuamente por uma ou mais fosfodiesterases (PDEs) de AMPc, que o hidrolisa à sua forma inativa, adenosina 5'monofosfato. Este nucleotídeo cíclico exerce seus efeitos através da interação com uma proteína receptora intracelular, a proteína quinase dependente de AMPc (PKA), que catalisa a transferência do grupo fosfato terminal do ATP para serinas ou treoninas de determinadas proteínas-alvo, regulando suas atividades. A via de sinalização AMPc/PKA regula o metabolismo, atividade gênica, crescimento e divisão celular,

diferenciação celular, esteroidogênese, motilidade de espermatozóides, bem como a condutividade de canais iônicos (HANSSON et al., 2000).

O GMPc, por sua vez, é produzido pela enzima guanilil ciclase (GC) e solúvel (GCs) e é degradado pela fosfodiesterase tipo 5 (PDE5). É reconhecido como um importante segundo mensageiro de sinais extracelulares provenientes do óxido nítrico (NO) e peptídeos natriuréticos. O NO ativa a GCs resultando a formação de GMPc, o aumento intracelular de GMPc ativa a proteína quinase dependente de GMPc (PKG). Estudos têm mostrado que ativação de PKG pode levar à abertura dos canais de potássio de grande condutância ativados por Ca^{2+} (BKCa) ou canais de potássio sensíveis ao ATP (KATP) (LIU et al., 2007). A Proteína quinase dependente de GMPc (cGKI) tem sido identificada como o principal alvo da sinalização do GMPc. Duas isoformas (cGKI α 76 kDa, e cGKIβ, 78 kDa) estão presentes em músculo liso. Estudos sugerem que o relaxamento muscular mediado por cGKIα está envolvido com a diminuição do cálcio citosólico e modulação da miosina fosfatase, já a cGKIβ, possivelmente, está mais relacionada com a proliferação celular. Tais mecanismos estão diretamente relacionados à manifestação de doenças prostáticas, entretanto dados experimentais ainda são incipientes quanto seus mecanismos de modulação via NO/cGMP/cGKI (WALDKIRCH et al., 2007).

Os efeitos fisiológicos deste nucleotídeo cíclico são determinados pelas atividades de três tipos de receptores intracelulares: proteínas quinases dependentes de GMPc (PKG), canais iônicos regulados por GMPc e PDEs reguladas por GMPc. O GMPc também pode ativar vias do AMPc através de sua ligação aos receptores intracelulares de AMPc, tal como a proteína PKA. Desta forma, o GMPc pode alterar a função celular através da ativação ou inativação de proteínas por fosforilação. Algumas funções fisiológicas mediadas pelo GMPc têm sido descritas como fatores regulatórios clássicos no relaxamento do músculo liso, inibição da agregação plaquetária, degranulação de neutrófilos, transdução de sinal visual, entre outras (LINCOLN & CORNWELL, 1993; CORBIN & FRANCIS, 1999).

O GMPc exerce um importante papel como mediador das ações do NO. O NO é um radical livre gasoso que atua como um importante sinalizador de processos intra e extracelulares (DUSSE et al., 2003). Esse gás é sintetizado intracelularmente por três isoformas da enzima óxido nítrico sintase (NOS): i) As formas constitutivas dependentes de Ca^{2+}, consistindo na forma endotelial (e-NOS) e a neuronal (n-NOS); ii) A forma induzível independente de Ca^{2+} (i-NOS), presente, entre outras células, em

macrófagos e células gliais, que produzem NO após estímulo imunológico (i.e., interferon-γ (IFN-γ), fator de necrose tumoral-α (TNF-α), Lipopolissacarídeo (LPS) (PUZZO et al., 2008). Há controvérsias em relação à presença da forma induzível da NOS (iNOS) no tecido prostático normal, porém, há indícios de que a enzima é expressa em hiperplasia prostática e câncer (KLOTZ et al., 1998; BALTACI et al., 2001). Na próstata humana, a eNOS está relacionada à manutenção da perfusão vascular local, enquanto que a nNOS está envolvida no controle do tônus da musculatura lisa e função glandular, incluindo a proliferação de células epiteliais e subepiteliais. No passado, o NO tinha sido identificado como um importante neurotransmissor não-adrenérgico e não-colinérgico no trato urinário inferior. No entanto, atualmente o NO parece estar envolvido no processo de micção, através da inibição de neurotransmissores da uretra e pela modulação dos nervos aferentes da bexiga (ANDERSSON e PERSSON, 1995). Muitos estudos relatam o papel fisiológico do NO sobre o controle da função da próstata em homens e mamíferos, incluindo a regulação do tônus do músculo liso, função secretora e fluxo sanguíneo (HEDLUND, 2005; ANDERSSON, 2007).

Estudos mostram que a via da RhoA/Rho-quinase (ROCK2) está envolvida em várias funções fisiológicas importantes, incluindo a contração do músculo liso, proliferação celular, adesão celular, migração celular, e várias respostas inflamatórias (RIENTO et al., 2003). Em particular, a proteína quinase associada à Rho 2 (ROCK2) desempenha papéis importantes na modulação da contração do músculo liso em vários tecidos, incluindo corpos cavernoso, próstata e bexiga (WIBBERLEY et al., 2003; REES et al., 2003).

Por outro lado, tem sido demonstrado que o acúmulo de AMPc e GMPc pode inibir a inflamação (ZHU et al., 2001; KHOSHAKHLAQH et al., 2007), prevenindo os tecidos contra danos causados por esse processo. Esse papel modulador da inflamação exercido pelo AMPc e GMPc é uma ferramenta potencial para interferir na evolução de doenças nas quais o processo inflamatório exerce um papel central, como, por exemplo, a prostatite aguda e crônica, sintomas do trato urinário inferior (LUTS), hiperplasia prostática benigna (BPH) e câncer de próstata .

3.3 Inibidores de fosfodiesterase tipo 5: Sildenafil

As Fosfodiesterases (PDEs) de mamíferos formam uma grande família e estão subdivididas de acordo com a ordem de descoberta, sequência de aminoácidos e características catalíticas e regulatórias. Estas enzimas hidrolisam os nucleotídeos cíclicos, AMPc e GMPc, às suas formas inativas 5´monofosfatos (UCKERT et al., 2006; CHUNG, 2006). Estes nucleotídeos, por sua vez, controlam múltiplas vias de sinalização intracelular que podem estar alteradas em muitas patologias, como câncer, inflamação, neurodegeneração e estresse oxidativo (ISERI et al., 2009).

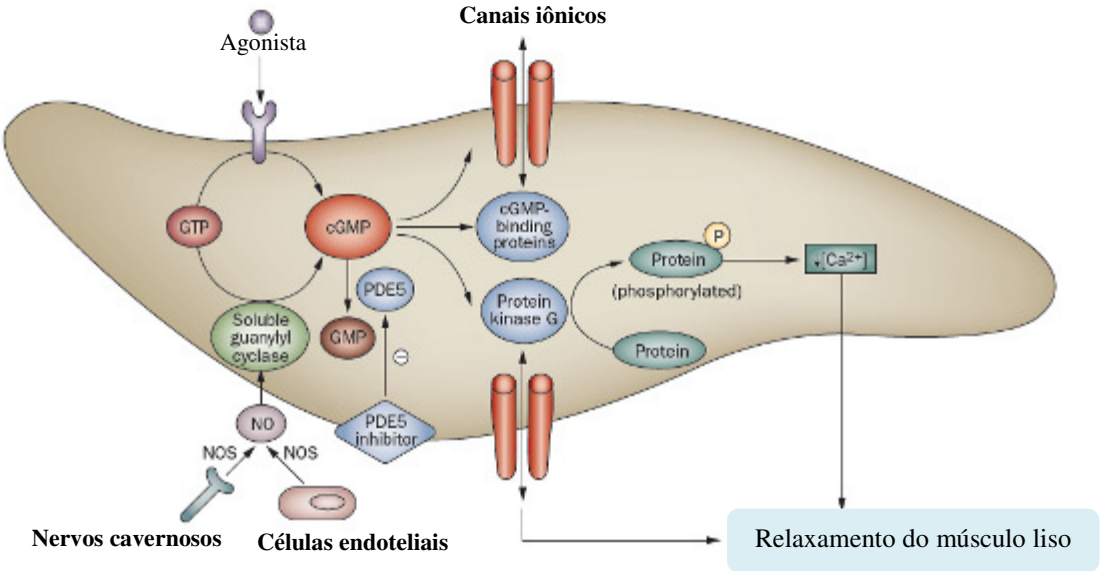
O Sildenafil, designado quimicamente como 1-[4-etoxido-3-(6,7-dihidro-1-metil-7-oxo-3-propil-1H-pirazolo[4,3-d]pirimidina-5-il)fenilsulfonil]-4-metilpiperazina citrato, é um inibidor potente e seletivo da PDE5, presente em vários tecidos tais como o vascular e o muscular liso. Este fármaco foi resultado de um programa que iniciou em 1985, na Sede de Investigação Européia Pfizer em Sandwich, UK, com finalidade de desenvolver um inibidor de PDE5 para estimular a via NO-GMPc para o tratamento de *angina pectoris* em pacientes com doença arterial coronária. Este fármaco foi patenteado em 1996, e aprovado para o uso terapêutico na disfunção erétil em 1998 (SCHWARZ et al., 2007; NAYLOR et al., 1996; BALLARD et al., 1996). Atualmente, ele vem revolucionando o tratamento da hipertensão pulmonar, que é uma doença vascular agressiva e com baixa expectativa de vida. Seu mecanismo de ação atenua os sinais e sintomas da doença por aumentar o suprimento sanguíneo aos pulmões. A pressão sistólica, a hipertrofia ventricular e a muscularização das artérias pulmonares são reduzidas após o esquema terapêutico com Sildenafil (RUBIN et al., 2011; WATANABE et al., 2011).

A PDE5 hidrolisa especificamente o GMPc, contribuindo para a modulação intracelular deste nucleotídeo. Esta enzima é um homodímero, e cada monômero é uma proteína quimérica composta por um domínio regulatório (R) e um catalítico (C) que transforma o GMPc em 5´-GMP. O domínio R contém vários subdomínios funcionais, incluindo sítios de fosforilação, sítios alostéricos de ligação de GMPc e contatos de dimerização. A ligação do GMPc ao sítio alostérico da PDE5 ativa a fosforilação da enzima pela proteína quinase dependente de GMPc (PKG), resultando em uma maior afinidade deste nucleotídeo cíclico ao domínio R e aumento de sua taxa de hidrólise pelo sítio catalítico. Este efeito está envolvido na regulação por feedback negativo dos níveis celulares de GMPc. O Sildenafil é um inibidor competitivo da catálise pela

PDE5, e representa um análogo não hidrolisável pelo sítio catalítico da enzima. Sua administração promove um efeito de feedback positivo, representado pela inibição da degradação e acumulação do GMPc através da interação do Sildenafil com o domínio C. (BLOUNT et al., 2007; CORBIN et al., 2003).

Este fármaco tem sido amplamente utilizado no tratamento da disfunção erétil. As causas orgânicas mais comuns desta condição clínica estão associadas a fatores vasculares, neurológicos e farmacológicos. A ereção normal ocorre em resposta à estimulação sexual, que promove a liberação de NO a partir de neurônios e do endotélio dos corpos cavernosos do pênis. O NO ativa a guanilato ciclase solúvel (GCs), levando à formação de GMPc, que causa relaxamento do músculo liso por reduzir a concentração de cálcio intracelular (Figura 4). Os espaços lacunares do tecido são preenchidos com sangue e a pressão intracavernosa aumenta, tornando o pênis ereto. Em resposta ao estímulo sexual, o Sildenafil promove a ereção através da inibição seletiva da PDE5. Logo, a inibição desta enzima eleva a concentração de GMPc e, com isso, potencializa o relaxamento do músculo liso dos corpos cavernosos. A dose oral recomendada de Sildenafil é de 50mg em uma dose única, administrada aproximadamente uma hora antes da atividade sexual, e com freqüência de uma vez ao dia (LANGTRY, 1999; PFIZER, 1998).

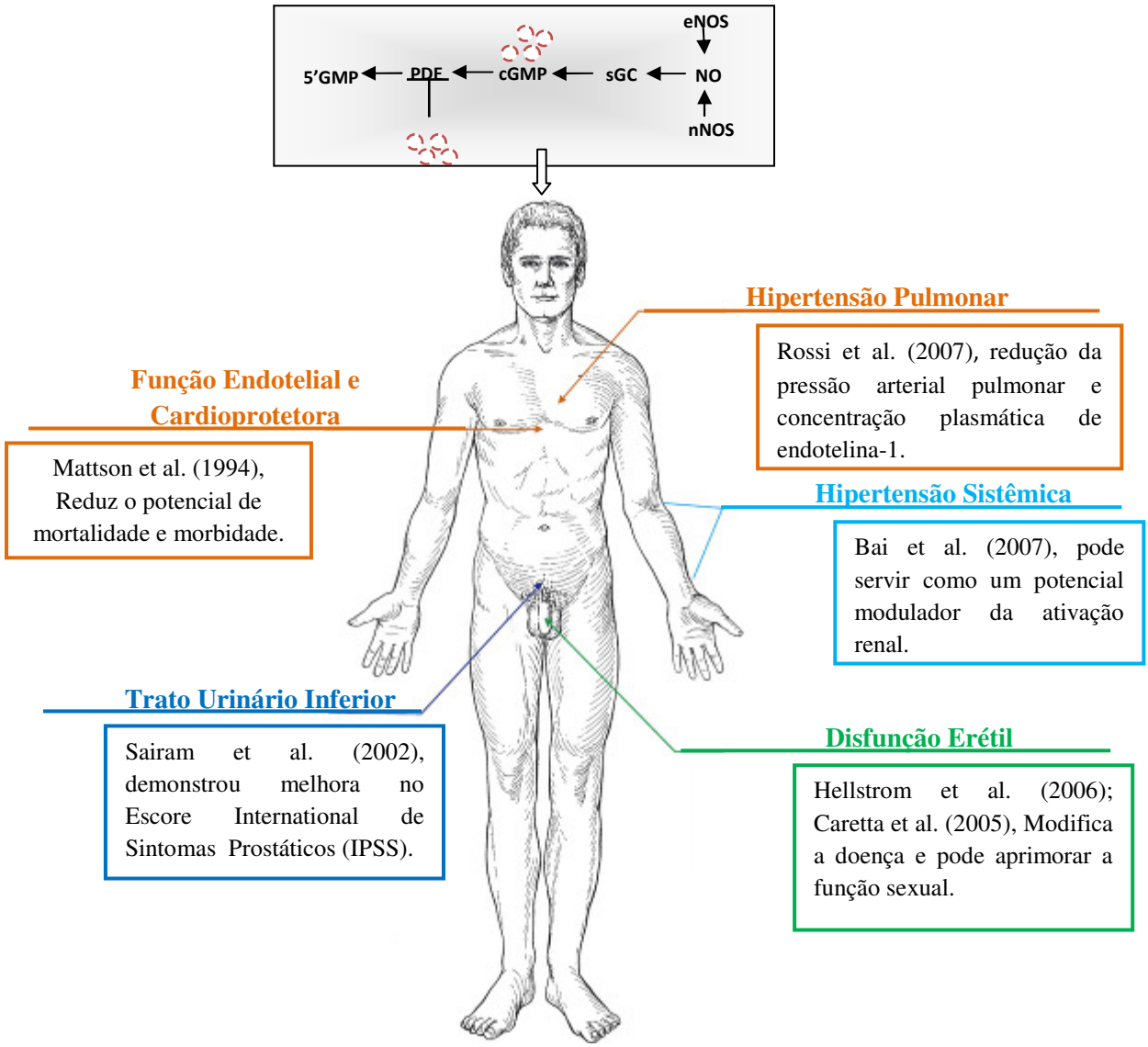
Figura 4 – Mecanismo de ação do inibidor de PDE5 (Sildenafil)



Fonte: Magheli & Burnett (2009).

O reconhecimento do importante papel das enzimas PDEs, atrelados com a alteração dos níveis intracelular de nucleotídeo cíclico tem despertado interesse no meio cientifico. Além de manter um excelente nível de segurança e perfil de tolerabilidade no tratamento da Disfunção Erétil (DE). O Sildenafil também fornece um benefício prolongado para doenças urológicas (LUTS-Sintomas do Trato Urinário Inferior; BPH-Hiperplasia Prostática Benigna; IPSS-Pontuação Internacional de Sintomas Prostático; Ejaculação Precoce) e não-urológicas (Doença Pulmonar Obstrutiva Crônica; Depressão e Ansiedade; Hipertensão Pulmonar e Sistêmica; Cardioproteção e Disfunção Endotelial) (Figura 5) (BELLA et al., 2007).

Figura 5 - Usos terapêuticos dos inibidores de PDE5. Os inibidores de PDE5 bloqueiam a degradação do GMPc (painel superior) levando a um aumento do nível desse segundo mensageiro, que pode exercer sua ação em vários órgãos alvo.



Fonte: Bella et al., (2007); Puzzo et al. (2008).

3.4 Patologia da glândula prostática

3.4.1 Hiperplasia prostática benigna

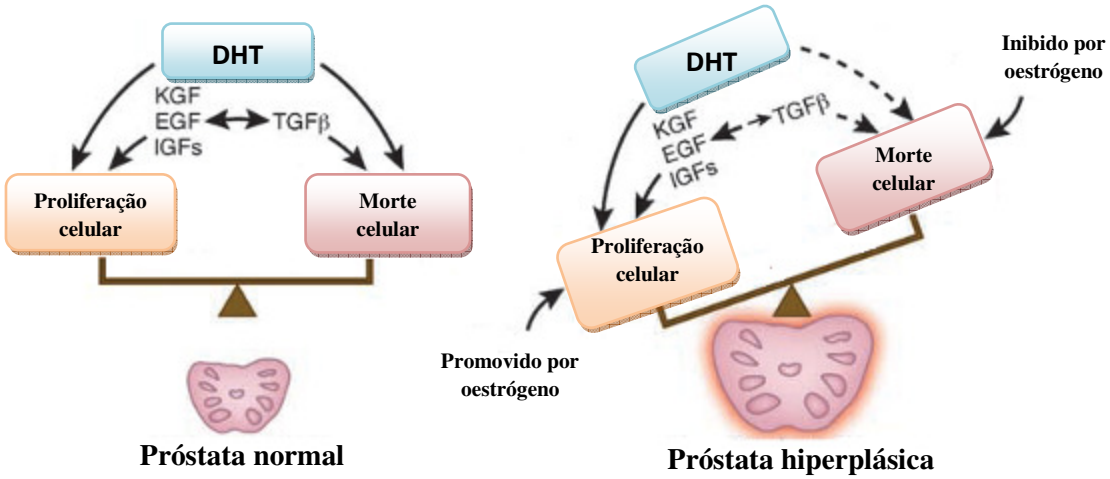
A hiperplasia prostática benigna (BPH) é uma doença crônica progressiva, sendo a neoplasia benigna mais comum no homem, possui prevalência em 50% os que têm 50 anos de idade e 90% com 80 anos, gerando impacto negativo na qualidade de vida dessa população (MC VARY 2006). BPH frequentemente apresenta manifestações clínicas secundárias, como sintomas do trato urinário inferior (LUTS) e retenção urinária aguda (AUR), possivelmente em decorrência de disfunção e hipertrofia da bexiga (ROEHRBORN et al. 2008; KUMAR et al. 2013).

Sua etiologia émultifatorial, e acredita-se que o envelhecimento e níveis elevados de DTH são os principais fatores de risco, uma vez que, a próstata é mais sensível aos andrógenos em homens mais velhos (CRUZ & DESGRANDCHAMPS, 2010), processo que favorece a remodelação prostática no epitélio e estroma fibromuscular, especialmente na zona de transição (BUSHMAN, 2009). Embora o andrógeno testosterona não cause BPH, ele é necessário durante o desenvolvimento prostático na puberdade e no envelhecimento, pois promove proliferação de células prostáticas através da ação intraglandular da DTH, seu metabólito ativo (CARSON & RITTMASTER, 2003).

Além de promover crescimento e diferenciação das células prostáticas através do mecanismo de ligação do receptor de andrógenos, a testosterona e diidrotestosterona, também induz a síntese de fatores de crescimento, que atuam nos compartimentos epiteliais e estromais de forma autócrina e parácrina (LEE & PEEHL, 2004; ROEHRBORN, 2008). O desequilíbrio entre fatores de crescimento celular e apoptose induz aumento do volume prostático (Figura 6), cuja principal alteração ocorre no metabolismo intracelular da célula basal, que se torna hipertrófica. (EMBERTON et al. 2008).

O fator de crescimento de queratinócitos mais presente na próstata são o FGF-2 e FGF-7. FGF-2 é sintetizado ativamente pelas células estromais e epiteliais, que expressam também seu receptor específico FGFR1 (BOGET et al. 2001). Ele age como um indutor autócrino da proliferação estromal, capaz de manter a homeostase mesenquimal na próstata normal e promover a remodelação estrutural na primeira fase da BPH (JANSSEN et al. 2000).

Figura 6 – Balanço entre fatores estimuladores de crescimento e inibidores envolvidos na homeostase celular na glândula prostática.



Fonte: Roehrborn (2008)

Nos últimos anos, a comunidade cientifica e urologistas têm despertado interesse para condições clinicas associada à progressão da BPH e PCa.

A síndrome metabólica (MetS) é um agrupamento de anormalidades metabólicas diretamente associadas ao aumento do risco de desenvolver doenças cardiovascular e metabólica. As características desta síndrome incluem: resistência à insulina, obesidade central, dislipidemia, e hipertensão (DESPRE & LEMIEUX, 2006; VIGNOZZI et al. 2013). Recentemente, vários estudos epidemiológicos, clínicos e histopatológicos têm identificado outras manifestações associadas a MetS, tais como hipogonadismo masculino, hipogonadotrófico, disfunção erétil (ED), BPH e LUTS (CORONA et al. 2009; PARSONS, 2011; 2013; MORELLI et al. 2013).

Embora a associação entre MetS e patologias prostáticas seja geralmente aceita, os mecanismos patogênicos pelos quais a MetS promove a BPH ainda não estão completamente esclarecidos (De NUNZIO et al. 2012). No entanto, alguns fatores da MetS como hiperinsulinemia (VIKRAM et al. 2010; VIGNOZZI et al. 2013), dislipidemia (KIM et al. 2012; VIGNOZZI et al. 2013) e desequilíbrio de esteróides sexuais (ZHOU et al. 2011; VIGNOZZI et al. 2012), tem sido proposto por estimular a inflamação crônica e supercrescimento. Conquanto a inflamação crônica promova hiperproliferação de ambas as células epiteliais, e estromais, uma desregulação autoimune e resposta imune para perfil de citocinas Th1/Th17 pode levar ao

desenvolvimento da remodelação e destruição imunomediada do tecido, como observado na fase inicial da BPH (FIBBI et al. 2010).

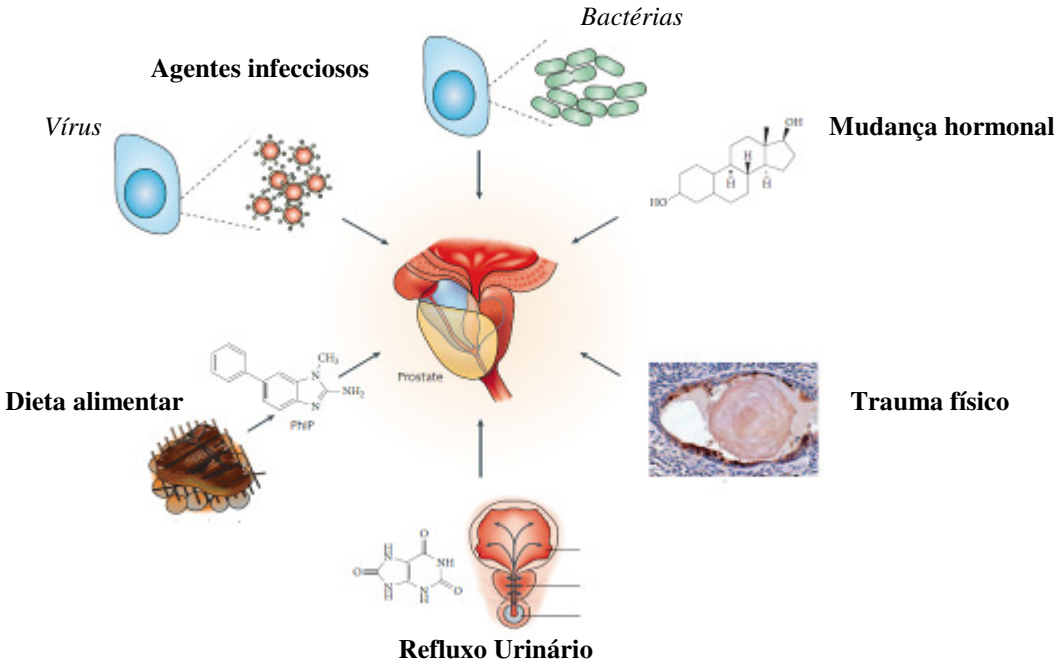
3.4.2 Prostatite

Na maioria dos casos, a causa da inflamação da próstata é incerta. Existem vários fatores potenciais, incluindo a infecção direta, o refluxo da urina, indução química e trauma físico, fatores dietéticos, estrogênios, ou uma combinação de dois ou mais desses fatores (Figura 7). Além disso, qualquer um destes pode levar a uma interrupção na tolerância imunológica e ao desenvolvimento de uma reação auto-imune para a próstata (DE MARZO et al., 2007). O processo inflamatório é caracterizado por aumento do fluxo sanguíneo e da permeabilidade vascular, dilatação venular e recrutamento de células para o sítio inflamatório. Estas alterações bioquímicas e celulares são reguladas por mediadores produzidos por células do sistema imune e células residentes no tecido. Na fase aguda da inflamação também existe o envolvimento de neutrófilos, enquanto que, na fase tardia, monócitos/macrófagos e algumas linhagens de monócitos migram para o sítio inflamatório (HUERRE & GOUNON, 1996). Estes mediadores em questão podem ser definidos como moléculas solúveis e difusíveis, e são representados por produtos da degranulação de mastócitos (histamina e serotonina), componentes do sistema complemento, citocinas e óxido nítrico (SHERWOOD & TOLIVER-KINSKY, 2004).

Por sua vez, a prostatite bacteriana crônica (CBP) é a causa mais comum de recorrência de infecção do trato urinário em homens. A classificação da CBP baseia-se em sintomas do paciente, presença ou ausência de leucócitos e bactérias na secreção prostática. A etiologia da CBP é diversa, assim, seu diagnóstico e tratamento ainda não foram claramente elucidados (CHO et al., 2002; YOON et al., 2010). Diversos organismos patogênicos são responsáveis por causar infecção e induzir uma resposta inflamatória na próstata. Estes incluem microrganismos sexualmente transmissíveis, tais como *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis* e *Treponema pallidum*, e bactérias não sexualmente transmissíveis, como o *Propionibacterium acnes* e aqueles conhecidos por causar prostatite bacteriana aguda e crônica, principalmente Gram-negativos tais como *Escherichia coli* (DE MARZO et al., 2007).

Nas bactérias Gram-negativas, a parede celular é composta por uma camada de peptidioglicano e três outros componentes que a envolvem externamente; lipoproteína, membrana externa e lipopolissacarídeo. O lipopolissacarídeo (LPS) é o maior fator de virulência, determinando efeitos biológicos que resultam na amplificação das reações inflamatórias. Esta endotoxina é um antígeno fraco não específico que é pobremente neutralizado por anticorpos, sendo capaz de ativar a cascata do complemento. Além do mais, ativa plaqueta, mastócitos, basófilos e células endoteliais. O LPS induz os macrófagos a secretarem outras proteínas, as interleucinas (IL-1, IL-6 e IL-8), fator alfa de necrose tumoral (TNF-α), oxigênio reativo, óxido nítrico, interferon, fatores ativadores de plaquetas e prostaglandinas (FULMER e TURNER, 1999; 2000).

Figura 7 – Possíveis causas da inflamação prostática



Fonte: De Marzo et al. (2007).

3.5 Papel da inflamação nas doenças prostáticas

Inflamação é um fenômeno complexo que consiste de componentes moleculares e celular. A resposta imune humoral é mediada por citocinas pró-inflamatórias, que causam e exacerbam a inflamação, e anti-inflamatórias, que atenua o processo inflamatório e promovem a cicatrização. Por sua vez, a resposta celular é mediada por leucócitos, monócitos e macrófagos. Em tecidos normais, as citocinas anti-inflamatórias são sincronicamente regulada após a produção de citocinas pró-inflamatórias, levando ao término da inflamação (SCIARRA et al.; BEGLEY et al. 2008).

O tecido prostático tem resposta imunológica ativa e envolve um amplo espectro de células inflamatórias endógenas, tais como fagócitos, linfócitos T e B, contra os antígenos estranhos. Na inflamação crônica, composta principalmente por células T ativadas e fagócitos mononucleares (monócitos e macrófagos), há persistência de promotores ou uma falha no mecanismo de reparo da inflamação. Este vai liberar mais citocinas pró-crescimento, bem como vários fatores de crescimento e atrair células imunológicas para o local da inflamação, amplificando a resposta inflamatória. As células T diminuem em número com a idade, fator correlacionado com a incidência de prostatite durante o envelhecimento. Células T estimulam a formação da matriz e mitógenos estromais que pode promover proliferação e hiperplasia da próstata (HAMID et al. 2011).

As células T, em tecidos hiperplásicos e carcinogênicos secretam mais citocinas pró-inflamatórias (IL-1, IL-1α, IL-2, IL-6, IL 7, IL-8 e IL-17) e quimiocinas (CXC), quanto comparados com tecidos normal. Essas citocinas induzem a proliferação e crescimento das células epiteliais e do estroma fibromuscular por um mecanismo autócrino/parácrino ou através da indução da expressão de COX-2 (MECHERGUI et al; PENNA et al. 2009).

Macrófagos e neutrófilos proporcionam uma fonte de radicais livres que podem induzir a hiperplasia ou transformação pré-cancerosas através de dano tecidual induzido pelo estresse oxidativo. Neste cenário, tanto o NO como a COX podem desempenhar um papel importante na determinação da associação entre inflamação e crescimento da próstata (SCIARRA et al., 2008).

Na fase crônica da inflamação ocorre produção continua da enzima ciclooxigenase-2 (COX-2). COX-2 aumenta produção de prostaglandina (PGE2), a

expressão da família de genes pró-apoptóticos (e reduz a proteína E-caderina, acarretando perda de adesão célula-célula. Além disso, modula a produção de fatores angiogênicos. Por fim, a COX-2 aumenta o crescimento celular e o potencial carcinogênico das células através da oxidação de pró-carcinógenos a carcinógenos, diminui a apoptose, bem como a resposta imune das células anormais ou cancerosas (WANG et al. 2004; PATHAK et al. 2005).

O processo inflamatório também produz radicais livres, estresse oxidativo, espécies reativas de nitrogênio (RNS) e várias espécies reativas de oxigênio (ROS). Esses fatores pode causar dano no tecido, modificar a estrutura e função das proteínas e induzir mudanças genômicas causando modificações pós-tradução, incluindo aquelas envolvidas no reparo do DNA e apoptoses. Além disso, podem conduzir danos ao DNA tais como, mutações pontuais, deleções ou rearranjos. Estes danos também alteraram a população de células progenitoras, resultando na modulação de um desequilíbrio entre proliferação e a morte celular. O tecido prostático é vulnerável a danos oxidativo no DNA devido à rápida renovação das células e redução das enzimas de reparo do DNA. Sendo assim, o equilíbrio entre estresse oxidativo e componente antioxidante das células têm um papel preponderante no desenvolvimento das patologias da próstata (SANDHU et al. 2008; UEMURA et al. 2008).

A relação entre Óxido Nítrico (NO) e inflamação é complexa porque em altas concentrações o NO é citotóxico e pró-inflamatório, efeitos que são o oposto àqueles induzidos por baixas concentrações de NO que envolvem a via do GMPc. A enzima óxido nítrico sintase induzível (iNOS) é ativada em macrófagos e outras células que produzem NO em concentrações micromolares, induzindo dano oxidativo ao DNA e modificações na função e estrutura de proteínas que podem ocasionar a morte celular. Por outro lado, NO produzido pela enzima óxido nítrico sintase endotelial (eNOS), em concentrações nanomolares, tem efeitos anti-inflamatórios através da via de sinalização do GMPc, podendo inibir diretamente a atividade do NF-κB por ativar sua proteína inibitória, o IKβα, ou indiretamente por ativar a Proteína quinase-A (PKA). Além disso, a eNOS pode inibir a transcrição gênica de NF-κB, limitando o processo inflamatório local (MATTHEWS et al., 1996; SPIECKER et al., 1997; CONNELLY et al., 2003; AIZAWA et al., 2003; GRUMBACH et al., 2005).

A proteína quinase ativada por AMP (AMPK) é conhecida como um regulador do metabolismo energético intracelular, entretanto, atualmente, pesquisas relatam que o AMPK tem um papel anti-inflamatório por ativar a eNOS (MORROW et al., 2003;

CHEN et al., 2003), além disso NO pode atuar como um ativador endógeno do AMPK, sugerindo uma relação de reciprocidade entre AMPK e a eNOS (ZHANG et al., 2008).

Além disso,têm demonstrado que o fator nuclear-κB (NF- κB) desempenha um papel importante na regulação dos genes responsáveis pela geração de mediadores de proteínas inflamatórias, como o fator de necrose tumoral-α (TNF- α), interleucina-1 (IL-1β), Molécula de Adesão Celular Vascular-1 (VCAM-1), Molécula de Adesão Intercelular-1 (ICAM-1). Esses mediadores inflamatórios desempenham um papel regulatório no crescimento, diferenciação e ativação de células imunes (SCHINS et al., 2000). O NF- κB reside no citosol sob a sua forma inativa como um dímero das subunidades RelA e p50. Devido à sua ligação com a sua proteína inibitória IKβ, conhecida também como IKβα, o NF-κB é incapaz de se translocar para o núcleo. Na sua via clássica de ativação, o IKβα é fosforilado e subsequentemente degradado no proteossomo. Desta forma, o NF- κB é liberado para sua migração para o núcleo onde ativará a transcrição de vários genes pró-inflamtórios. As citocinas inflamatórias TNF-α e a IL-1β, bem como as espécies reativas de oxigênio são exemplos de potentes indutores da ativação do NF- κB (VAN BERLO et al., 2010).

3.6 Sildenafil na inflamação

Devido à grande diversidade e ao papel chave no controle da sinalização de nucleotídeos cíclicos, as PDEs tornaram-se alvos terapêuticos atraentes. Nos últimos anos, o potencial terapêutico dos inibidores destas enzimas tem sido bastante explorado.

Santos et al. (2005) mostrou que o Sildenafil tem um efeito protetor contra gastropatia induzida por indometacina em ratos. Outros estudos mostram que o Sildenafil diminui significativamente os níveis de peroxidação lipídica do cólon, além de atenuar a super-regulação de citocinas pró-inflamatórias, TNF-α e IL-1β, que desempenham um papel significativo na patogênese da doença inflamatória intestinal. Esses inibidores possuem atividades sinérgicas induzindo a produção de outras citocinas, moléculas adesão, metabólitos do ácido araquidônico, bem como a ativação de células imunes (RISE et al., 2009).

A administração crônica de Sildenafil a pacientes com diabete tipo 2, reduz os níveis de marcadores inflamatórios vasculares tais como endotelina, proteína-C reativa,

interleucina-6, molécula de adesão intercelular (ICAM) e molécula de adesão vascular (VCAM), além de aumentar os níveis de nitrito/nitratos (AVERSA et al., 2007).

Além disso, o Sildenafil reduz déficits neurológicos, aumenta a recuperação funcional e a neurogênese após derrame em ratos (ZHANG et al., 2002 ; 2006). Outros estudos indicam que este fármaco aumenta os níveis de GMPc no cérebro e exerce efeitos neuroprotetores em doenças inflamatória crônica do SNC (FIRESTEIN et al., 1998).

Recentemente, demonstrou-se que o Sildenafil melhora os sinais clínicos e a neuropatologia em modelo murino de esclerose múltipla. De acordo com Pifarre et al. (2011) o tratamento com Sildenafil previne a perda axonal e promove a remielinização, além de reduzir a infiltração de leucócitos $CD3^+$ e a ativação de micróglia/macrófagos, indicando, portanto, que este fármaco pode ser uma ferramenta terapêutica importante para o tratamento a esclerose múltipla.

Alguns estudos têm contribuído para esclarecer os efeitos do Sildenafil em processos fisiopatológicos na próstata. Além de promover o relaxamento do músculo liso, o inibidor de PDE5 inibiu a proliferação de células do estroma da próstata humana e reduziu *in vivo* os sintomas inflamatórios decorrentes da prostatite, BPH e LUTS (TINEL et al., 2006; WANG, 2010). Além disso, a possível utilização de inibidores de PDE5 para o tratamento de patologias prostáticas é sustentada pela presença da PDE5 na zona de transição da próstata, juntamente com PDE4 e PDE11(UCKERT et al., 2006).

A pesquisa básica e estudos clínicos recentemente têm sugerido que a inflamação pode ser um mecanismo central no desenvolvimento histológico de BPH e progressão de doenças prostáticas (KRAMER et al., 2007; DI SILVERIO et al., 2003). Consistente com esses achados, o estudo clínico REDUZIR (*Estudo de pesquisa clínica para reduzir a incidência de câncer de próstata nos homens que estão em maior risco*) mostrou uma relação entre inflamação e volume da próstata (NICKEL et al., 2008; ROBERT et al., 2011). Agentes anti-inflamatórios têm sido investigados para o tratamento de diversas patologias prostáticas, tais como: esteróides, inibidores da ciclooxigenase-2 (COX-2) e agentes fitoterápicos, cujos efeitos mostraram-se promissores em termos de inibição da proliferação celular (ROBERT et al., 2011).

Evidências sugerem que o processo inflamatório pode contribuir para a carcinogênese da próstata. Estes dados foram comprovados através de estudos

epidemiológicos e clínicos, este estudo sugere que um quinto dos casos de câncer de próstata foi proveniente da inflamação crônica devido a agentes infecciosos ou fatores ambientais (DE MARZO et al., 2007).

Visto que tem sido demonstrado que o Sildenafil pode ter um efeito modulador, reduzindo a inflamação em modelos de doenças inflamatórias (KHOSHAKHLAQH et al., 2007; VISSER et al., 2009 ; HEMNES et al., 2008), esse fármaco torna-se uma alternativa como agente inibidor do processo inflamatório da prostatite aguda e crônica. Portanto, devido às atuais limitações nas estratégias terapêuticas disponíveis para o tratamento da prostatite, o presente projeto propõe investigar os efeitos do Sildenafil em um modelo de prostatite, induzido por LPS.

Effect of chronic Sildenafil treatment on the prostate of C57Bl/6 mice

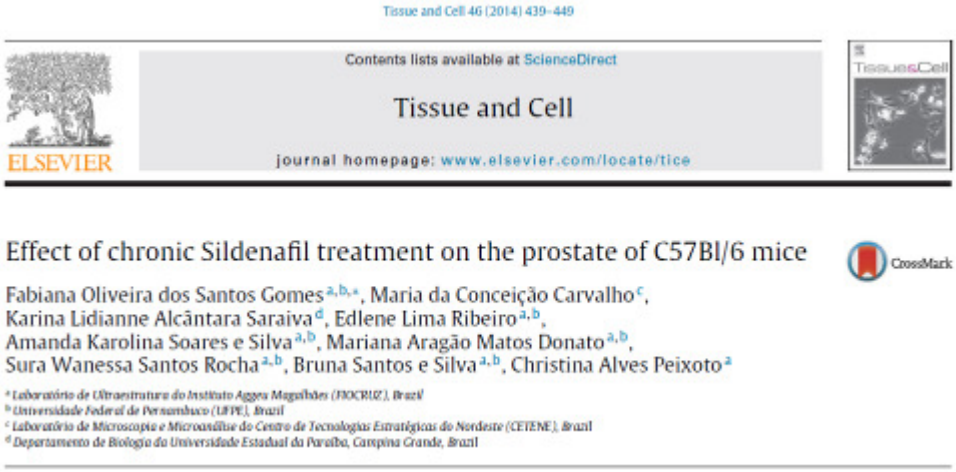
Gomes, F.O.S

Artigo I

4 RESULTADOS E DISCUSSÃO

4.1 Pergunta condutora 1: Qual o possível efeito do tratamento crônico com Sildenafil (25mg/kg) na próstata?

Artigo I


Tissue and Cell 46 (2014) 439–449

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Effect of chronic Sildenafil treatment on the prostate of C57Bl/6 mice

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Summary

Sildenafil is a potent and selective inhibitor of phosphodiesterase-5 (PDE5) and is considered first-line therapy for erectile disfunction. Nowadays, Sildenafil is used extensively throughout the world on patients with pulmonary hypertension. However, few studies have evaluated the possible side effects of chronic Sildenafil treatment on the male reproductive system, specifically in the prostate. In the present study, it was demonstrated via morphological and ultrastructural analysis that chronic treatment with Sildenafil induced an enhancement of the glandular activity of the prostate. In addition, mice treated with Sildenafil showed a significant increase in testosterone serum levels. However, no statistically significant differences were observed in nitric oxide serum levels, or in sGC, eNOS, PSA and TGF-β prostatic expression. In conclusion, the present study suggests that chronic use of Sildenafil does not cause evident prostatic damage, and therefore, can be used pharmacologically to treat a variety of disorders.

Keywords: Sildenafil, inhibitor of phosphodiesterase-5 (PDE5), prostate

1. INTRODUCTION

Inhibitor PDE-5, such as Sildenafil (Viagra), Vardenafil (Levitra), and Tadalafil (Cialis), has been used as a pharmacological vasodilator tool for several non-urological (pulmonary hypertension, systemic hypertension, diabetes, cardioprotection and endothelial function) and urological (erectile dysfunction, lower urinary tract symptoms, benign prostatic hyperplasia, priapism, premature ejaculation, and Peyronie's disease) disorders (Bella et al., 2007).

Phosphodiesterases (PDEs) are enzymes that are widely distributed in the body, hydrolyzing cyclic nucleotides, cAMP and cGMP to their inactive 5'-monofosfatos forms. Experimental studies using immunohistochemical methods have detected PDE isoenzymes 4, 5 and 11 in the fibromuscular prostatic stroma, as well as in the glandular structures of the transition zone of the prostate, suggesting that PDE enzymes play an important role in dynamic activity, secretory function and prostatic tissue proliferation (Uckert et al., 2006).

The PDE-5 enzyme is responsible for the hydrolysis of the GMPc, which restores GMP levels. Endothelial-derived nitric oxide (NO) activates soluble guanylyl cyclase (sGC) in vascular smooth muscle, leading to an increase in intracellular cGMP, which activates cytosolic cGMP-dependent protein kinase (PKG). The ability of Sildenafil to compete kinetically with PDE5 by the cGMP catalystic site makes it a select inhibitor of PDE5 (Francis and Corbin, 1999). The accumulation of cGMP induced by Sildenafil promotes relaxation of the smooth muscle cells, not only in the corpus cavernosum, but also in the bladder neck, urethra and prostate, by decreasing intracellular calcium concentration (Wang, 2010; Liu et al., 2007).

McVary *et al.* (2007) reported satisfactory results in a randomized, double blind, placebo-controlled study of 12-week, once-daily dosing of 50 and 100 mg of Sildenafil

in 369 men with ED and LUTS. Other authors also demonstrated a positive effect on urinary obstruction and irritation symptoms (Ying et al., 2004; Mulhall et al., 2006; Roehrbon et al., 2008; Stief et al., 2008).

Studies support the idea that PDE-5 expression increases in several types of human carcinoma, such as colon adenocarcinoma, bladder squamous carcinoma and lung cancers, suggesting the involvement of these enzymes in the control of cell proliferation and apoptotic mechanisms (Piazza et al., 2001; Moon et al., 2002; Whitehead et al., 2003; Sarfati *et al*., 2003). Additionally, in human prostate cancer cell lines, the increase of intracellular second messengers (cAMP and cGMP) initiates morphologic differentiation, inhibiting the growth and the invasive potential of these cells (Bang et al., 1994; Goto et al., 1999).

Studies have also demonstrated that Sildenafil attenuated pulmonary hypertension by increasing the supply of blood to the lungs reducing the right ventricular systolic pressure, right ventricular hypertrophy, the pulmonary artery muscularization, suggesting that the NO-cGMP pathway contributed to the drug response (Zhao et al., 2001; 2003).

Based on these evidences, in 2005, Sildenafil (Revatio, Pfizer) was approved for the chronic treatment of pulmonary hypertension. Recently, the Food and Drug Administration (FDA) and European Medical Agency (EMA) approved the daily use of the PDE5 inhibitor as a new opportunity for men with BPH/LUTS with coexisting ED.

Therefore, Sildenafil have a potential therapeutic indication for several chronic diseases; however the safety, efficacy and cost-effectiveness need to be ascertained. There is a paucity of data on the long-term effects of chronic PDE-5 inhibitor use on the prostatic function. Since Sildenafil has a vasodilatation action as a result of its effect on NO/sGC/GMPc/PKG pathways, the aim of the present study was to investigate the

effect of chronic Sildenafil treatment on prostate model mice. The following end points were achieved: 1) Prostate histopathology (histology and ultrastructure), 2) Detection of sGC, PSA and TGF-β (immunohistochemical), 3) Nitric oxide (NO) synthesis (nitrite concentration), 4) expression of sGC, eNOS and TGF-β (western blot), 5) Hormonal assays (testosterone dosage).

2. MATERIAL AND METHODS

2.1 Animals

Forty pubertal male C57BL/6 mice (obtained from the Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife, Brazil) aged 25 days and weighing 15–20g were used in all experiments. Mice were examined for health status and acclimated to the laboratory environment, which had a temperature of 23ºC and a 12h light: 12h dark photoperiod. The animals were housed in metal cages and fed a standard diet and water ad libitum. The experimental group was composed of 11 animals, which received a dose of 25 mg/kg body weight of Sildenafil (Pfizer Inc., New York, NY, USA) for 4 weeks, administered through drinking water (Zhao et al., 2003). Body weight was recorded every day and the drug concentration in the water was adjusted to maintain the dose. The control group was also composed of 20 animals, which received only pure water, using the same procedure as described above. All experiments were performed according to ethical guidelines (L-0035/08 – CEUA/ FIOCRUZ). After treatment with Sildenafil, the experimental and control animals were anaesthetized with ketamine (115 mg/ kg, i.m.) and xylazine (10 mg/ kg, i.m.) (Sespo Comércio e Indústria Ltda., Sao Paulo, Brazil), before blood collection by cardiac puncture without anticoagulant. The serum was separated and stored at –70 °C for testosterone hormone radioimmunoassay. The prostates were quickly dissected and fixed for morphological analysis.

2.2 Light microscopy

The prostates were fixed in Bouin's solution for 8 h. Next, they were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections of 5µm were cut using a microtome (Leica RM 2125RT), stained with haematoxylin–eosin and PAS (periodic acid-Schiff), and evaluated with an inverted microscopy (Observer Z1, Zeiss Micro Imaging GmbH) equipped with a camera and 4.7.4 image analysis program (AxionCam MRm Zeiss) at a magnification of 400x.

2.3 Electron transmission microscopy

The fragments of prostate were fixed overnight in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer. After fixation, the samples were washed twice in the same buffer and were then post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone, and embedded in Embed 812. Polymerization was performed at 60°C for 3 days. Ultrathin sections were collected on 300-mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate, and examined using a FEI Morgani 268D transmission electron microscope.

2.4 Immunohistochemical assays for sGC, PSA and TGF-β

Ultrathin sections (5 µm in thickness) of each group were cut and adhered to slides treated with 3-amino-propyl-trietoxi-silane (APES [Sigma, USA]). Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). The sections were heated for 30 minutes in a sodium citrate buffer (0.01 M, pH 6.0) to increase epitope exposure. To minimize endogenous peroxidase activity, the slides were treated with 0.3% (v/v) H_2O_2 in water for five minutes. The sections were washed with

0.01M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1h, at room temperature. The sections were then incubated for 12 hours at 4ºC with rabbit polyclonal antibody against anti-guanylyl cyclase β1 soluble (sGC) (Sigma, USA), polyclonal antibody Prostate-specific antigen (PSA) (ABCAM, CA, USA), and rabbit polyclonal Transforming growth factor βs (TGF-β) (Santa Cruz Biotechnology, Santa Cruz, CA). The optimal concentration used for these antibodies was 1:100. The antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB ® + Kit, Peroxidase) using 3.3-diaminobenzidine as the chromogen. The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the first antibody. Five pictures taken at the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.5 Hormone assays

Serum testosterone was assayed using a solid-phase radioimmunoassay kit in accordance with the manufacturer's instructions (Coat-A-Count Total Testosterone; Diagnostic Products Corporation, Los Angeles, CA, USA). The sensitivity of the testosterone assay was 4 ng/dl and the intra and inter-assay variation coefficients were 4–18% and 5.9–12% respectively. The values were expressed in ng/ml. Data was analyzed using the Mann–Whitney test to compare testosterone levels of the controls and the organisms that underwent Sildenafil treatment (Zar, 1996).

2.6. Measurement of NO

Greiss colorimetric reaction, which detects nitrite (NO_2^-) and oxidation of NO in serum, was used to measure nitric oxide. Blood was obtained by cardiac puncture and

centrifuged at 1000 x g for 10 min. Subsequently serum samples were diluted fourfold with distilled water, and deproteinized by adding 1/20th volume of a zinc sulfate solution (300g/L), to give a final concentration of 15g/L. After 3.500g centrifugation for 10 minutes, 100µL of samples were added to an ELISA plate (96 wells) in duplicate, followed by the same volume of Griess reagent. Griess reagent is composed of 1% sulfanilamide diluted in 2.5% H_3PO_4 (solution A) and N-1-naphtyl-ethtylenodiamina, also diluted in 2.5% H_3PO_4 (solution B). To prepare a standard curve, a solution of sodium nitrite in an initial concentration of 100µM was serially diluted in PBS. After incubation for 10 minutes in the dark, a spectrophotometer reading was taken at 490nm. The absorbance of different samples was compared with the standard curve, and the results expressed as mean ± standard error of the duplicate, using GraphPad Prism software (v. 5.0).

2.7 Western blot for eNOS, sGC and TGFβ

The prostates were quickly dissected and then homogenized in a Wheaton Overhead Stirrer (nº 903475) in an extraction cocktail (10mM Ethylenediamine tetraacetic acid (EDTA), 2mM phenylmethylsulfonyl fluoride (PMSF), 100mM sodium fluoride, 10mM sodium pyrophosphate, 10mM sodium orthovanadate (NaVO4), 10mg of aprotinin and 100mM Tris(hydroxymethyl)aminomethane, pH 7.4). Homogenates were centrifuged at 3000 xg for 10 min and the supernatant was collected and stored at -70º C until used for immunoblotting. Protein levels were determined using the Bradford method taking bovine serum albumin as standard (Bradford, 1970). The proteins (40 mg) were separated in 10% (sGC, eNOS and TGF-β) sodium dodecyl sulfate–polyacrylamide by gel electrophoresis under reduced conditions and were electrophoretically transferred onto nitrocellulose membrane (Bio Rad, CA, USA, Ref. 162-0115). After blocking

overnight at 4ºC with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4), the membranes were incubated at room temperature for 3 h, with rabbit polyclonal antibody against eNOS (1:1000 dilution; BD Transduction Laboratories, USA), sGC (1:200 dilution, Abcam, CA, USA) and TGF-β (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and diluted in buffer solution TBS-T containing 3% non-fat milk. After washing (six times, 10 min each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:80000 (Ref. A6154), diluted in TBS-T with 1% nonfat milk for 1h30min at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to make the labeled protein bands visible and the blots were developed on X-ray film (Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the density of pixels of each band was determined using the Image J 1.38 program (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD). For each protein investigated, the results were confirmed using three sets of experiments. Immunoblot for β-actin was used as a control for the protein blots. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:2000 dilution, Sigma, USA), and protein densitometry was performed.

2.8 Statistical analysis

GraphPad Prism software, version 5 was used for statistical analysis. Data was expressed as mean ± standard deviation. The differences between the control and treated groups were analyzed used Mann–Whitney or T-test. Probability values less than 0.05 were considered significant.

3. RESULTS

3.1 Morphological analysis

Histological analysis of the prostate glands of animals in the control group showed well-preserved acini and ducts composed of a single layer of secretory epithelial cells, characterized by columnar cells. Below the epithelium, there was a continuous layer of basal cells and a basal membrane. The stromal compartment, formed by a subepithelial region and a layer of smooth muscle cells surrounding the tubules, was also observed (Fig. 1A).

After 30 days of treatment with 25mg/kg of Sildenafil, there was a clear difference between the Sildenafil treated and the control groups in the glandular and stromal regions. The secretory epithelium lining showed tall columnar cells in evident cellular proliferation (Fig. 1B), some of which were hypertrophied, with round profile and evident nuclei, while others had an absence of nuclei (Fig. 1C). Moreover, the glandular apical region showed evident secretion vesicles, indicating an increase in glandular activity (Fig. 1D).

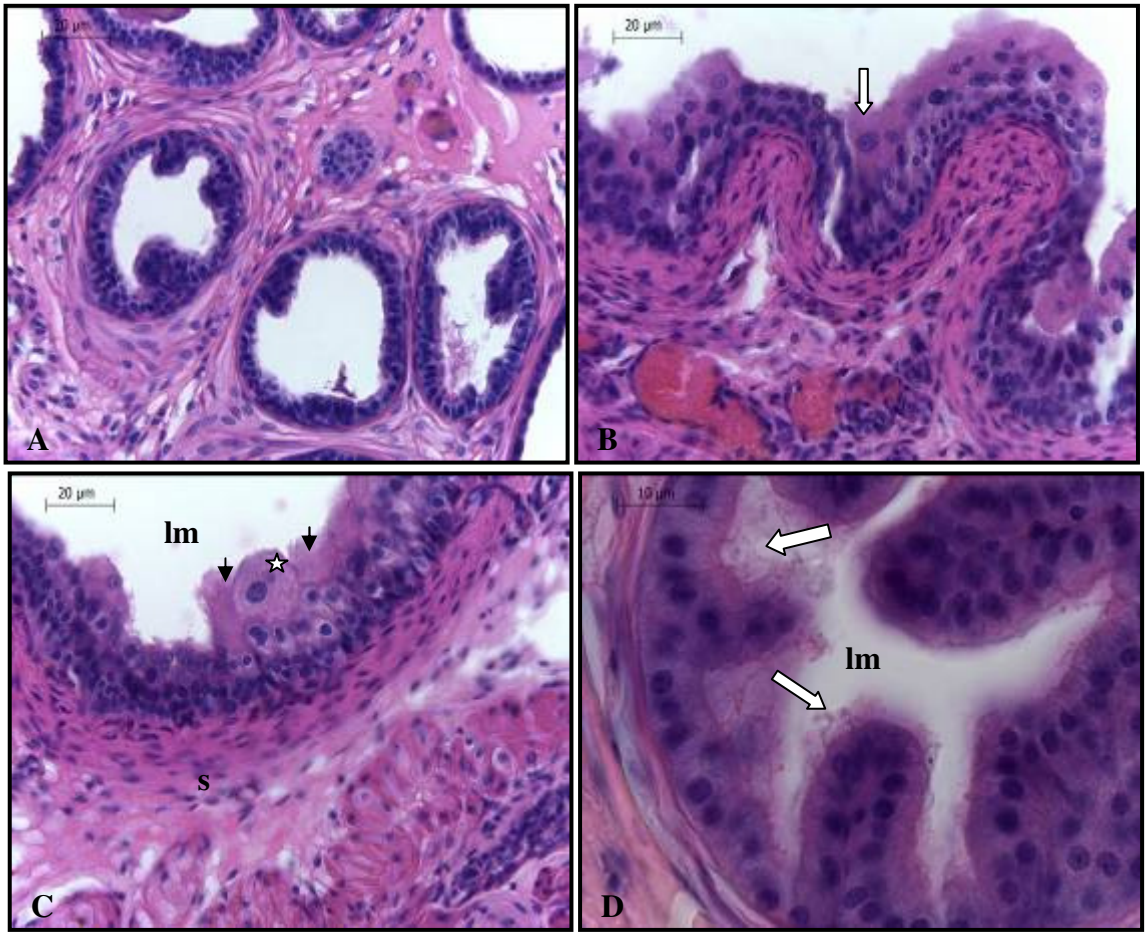
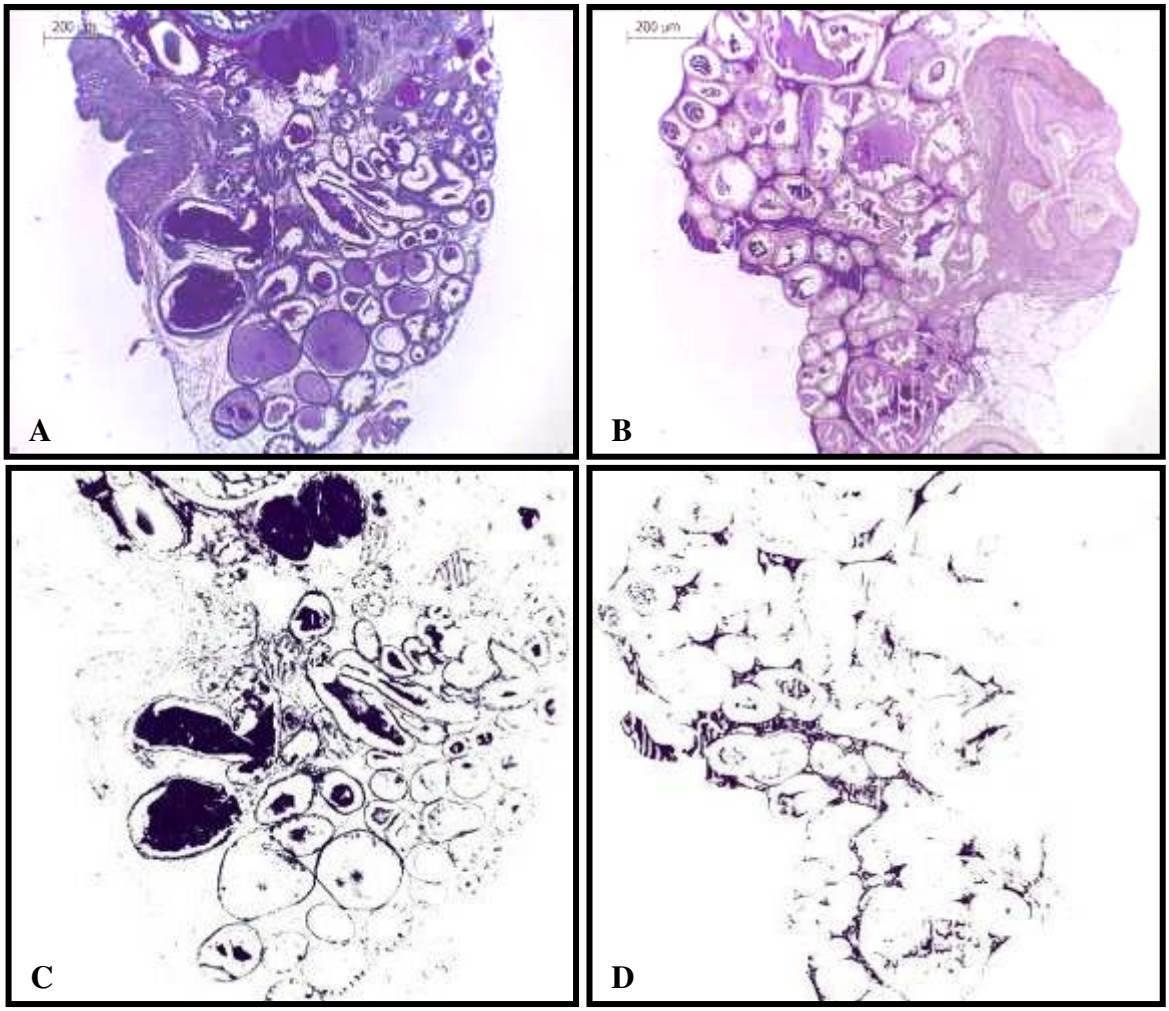


Fig. 1: Histological analysis of mice prostates following Sildenafil treatment. Stained with hematoxylin-eosin. (A) Prostate of the control group showed well-preserved acini and ducts composed of a single layer of secretory epithelial cells. (B) Group treated with 25 mg/kg of Sildenafil showed evident proliferation (arrows), (C) preserved stromal region and round hypertrophied cells (star). The absence of nuclei in some cells (arrowheads) can be observed, (E) Secretion vesicles above the epithelial glandular cells (arrows). (ep, epithelium; lm, lumen; st stroma. n= 20 mice from each group. Scale bar = 20µm and 10µm).

3.1 Carbohydrates

The distribution of carbohydrates was analyzed using the Periodic acid-Schiff technique. In the control group, the presence of carbohydrates was identified in the glandular region (Fig. 2A). Contrastingly, there was increased labeling in the stromal region in the group treated with Sildenafil (Fig. 2B).



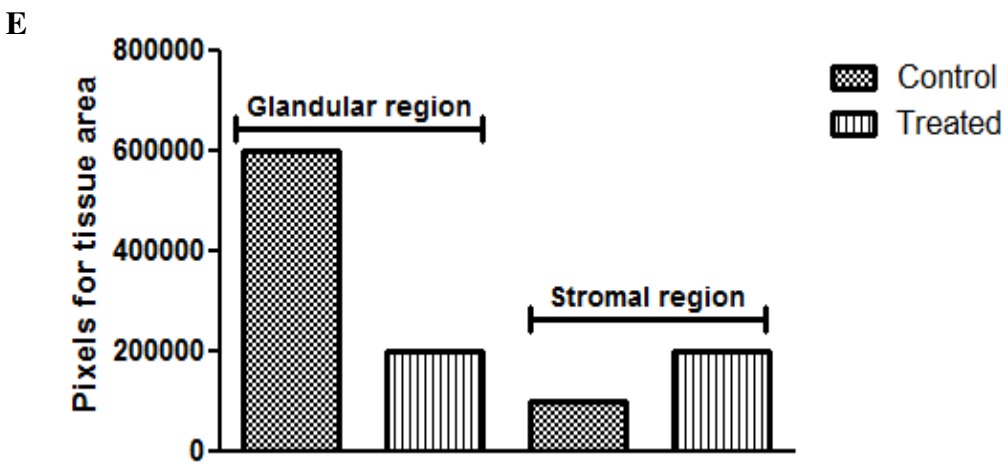


Fig. 2: Effect of Sildenafil treatment on prostatic carbohydrate content - PAS (Periodic acid-Schiff). (A, C) Control group, (B, D) Sildenafil group. n= 20 mice from each group, (E) Quantification of tissue area in pixels. Scale bar = 20 µm.

3.2 Ultrastructural analysis

Ultrastructural analysis of the prostate gland showed a columnar epithelium morphological pattern with evident elliptical nuclei, with rough endoplasmic reticulum (RER), apical Golgi complex and secretory vesicles (Fig. 3A, B). There were a small number of secretion vesicles with electron-lucent content with spherical eletrodense condensations.

Contrastingly, ultrastructural analysis of prostatic cells from the group treated with Sildenafil showed several characteristics of cellular activation, such as hypertrophied rough endoplasmic reticulum (Fig. 3C), dilated cistern of Golgi complex occupying the apical cellular region (Fig. 3D), containing electrodense secretion and numerous secretory vesicles (Fig. 3E) . Exocytocis of the granular content was also observed (Fig. 3F).

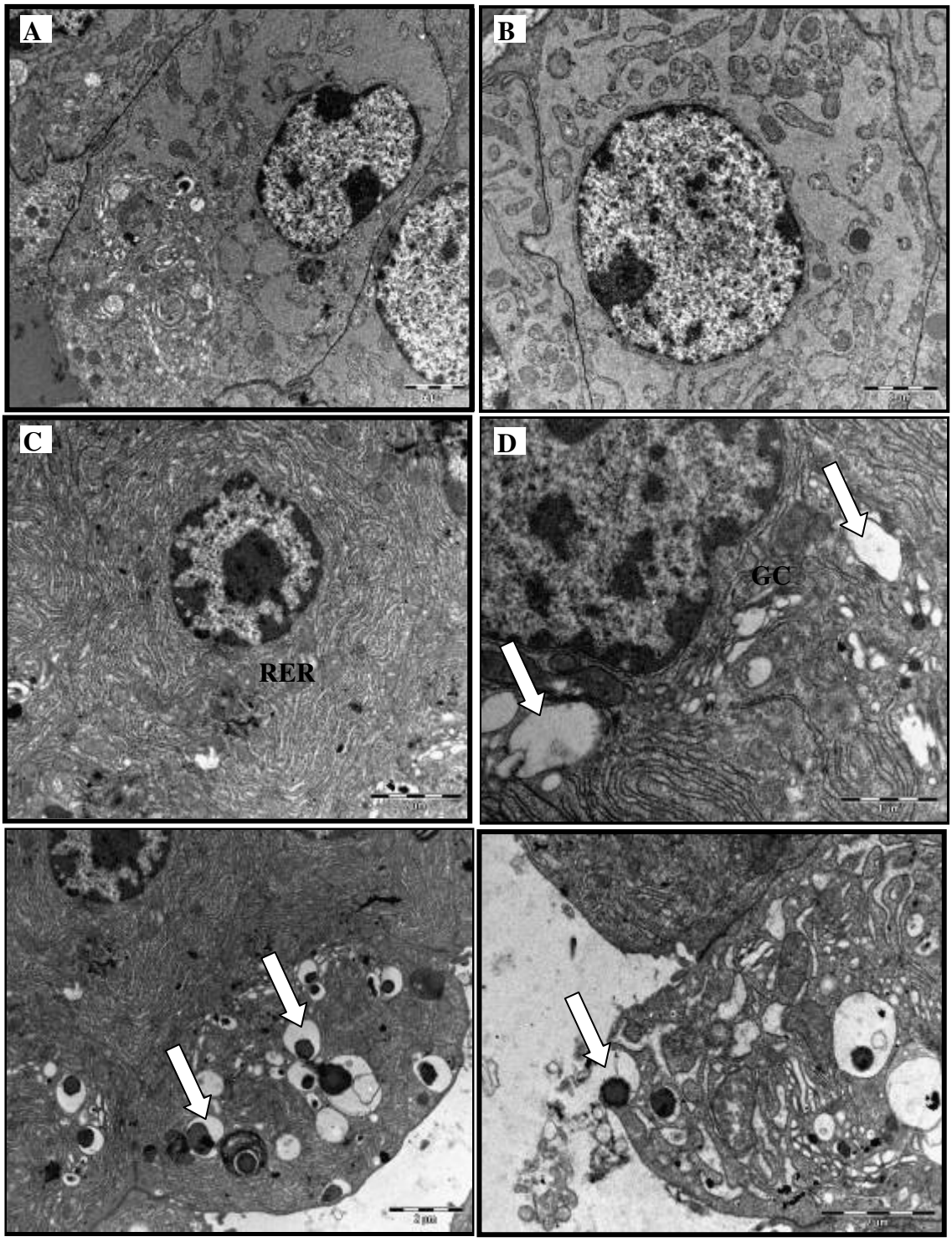


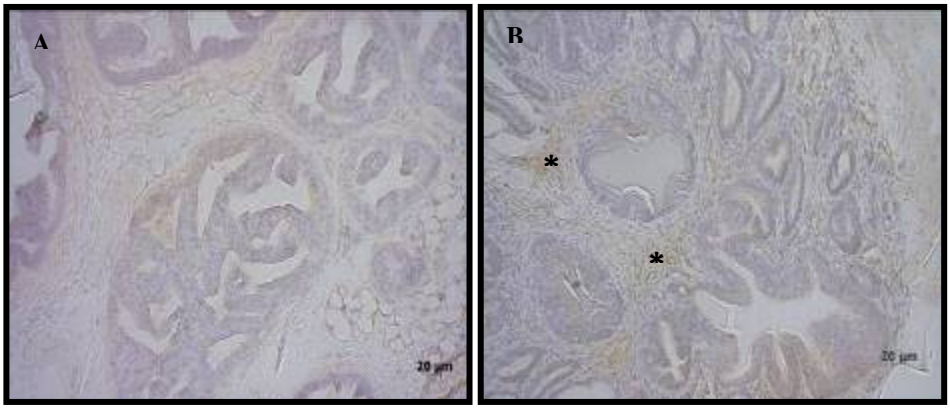
Fig. 3: Transmission electron microscopy. (A, B) Control group showed an epithelial cell morphology pattern with rough endoplasmic reticulum, apical Golgi complex and secretory vesicles. (C, D, E and F). Epithelial prostatic cells from the Sildenafil-treated group (25 mg/kg) had the following characteristics: (C) prominent rough endoplasmic

reticulum (RER), (D) hypertrophied Golgi complex (GC) showing large lacunas (arrows), some of which contained electrodense material (arrowheads); (E) numerous secretory granules were observed in the apical region (sg) with electrodense material (arrows), (F) exocitosis (arrow).

3.3 Immunohistochemical analysis for sGC, PAS and TGF-β

In the present study sGC expression in the prostate tissue was evaluated by immunohistochemical detection. Tissue sections obtained from mice from the control group demonstrated no positive staining for sGC in the stromal and glandular region (Fig. 4A). Slight staining for sGC was found in the stromal region of the prostate of mice treated with Sildenafil 25mg/kg. However, pixel quantification did not indicate statistically significant difference (Fig 4B-C).

Similarly, prostate tissue sections from the control and Sildenafil treated groups showed no significant differences for PSA (Fig. 5A, 5B, 5C, 5D, 5E) and TGF-β (Fig. 6A, 6B, 6C, 6D) in the stromal and glandular regions.



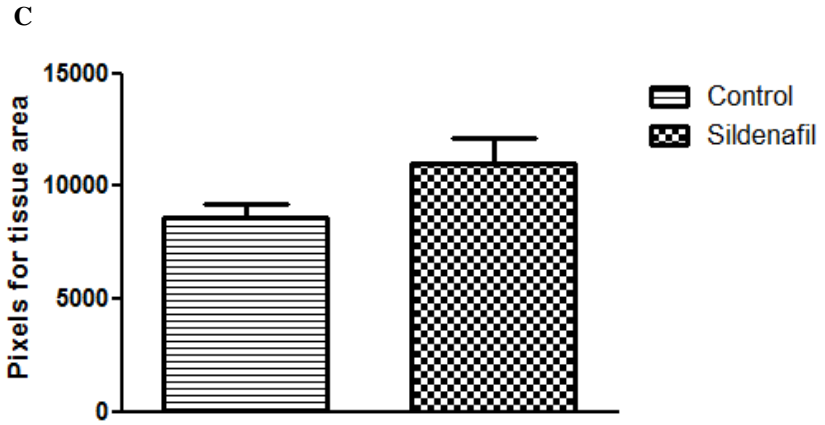
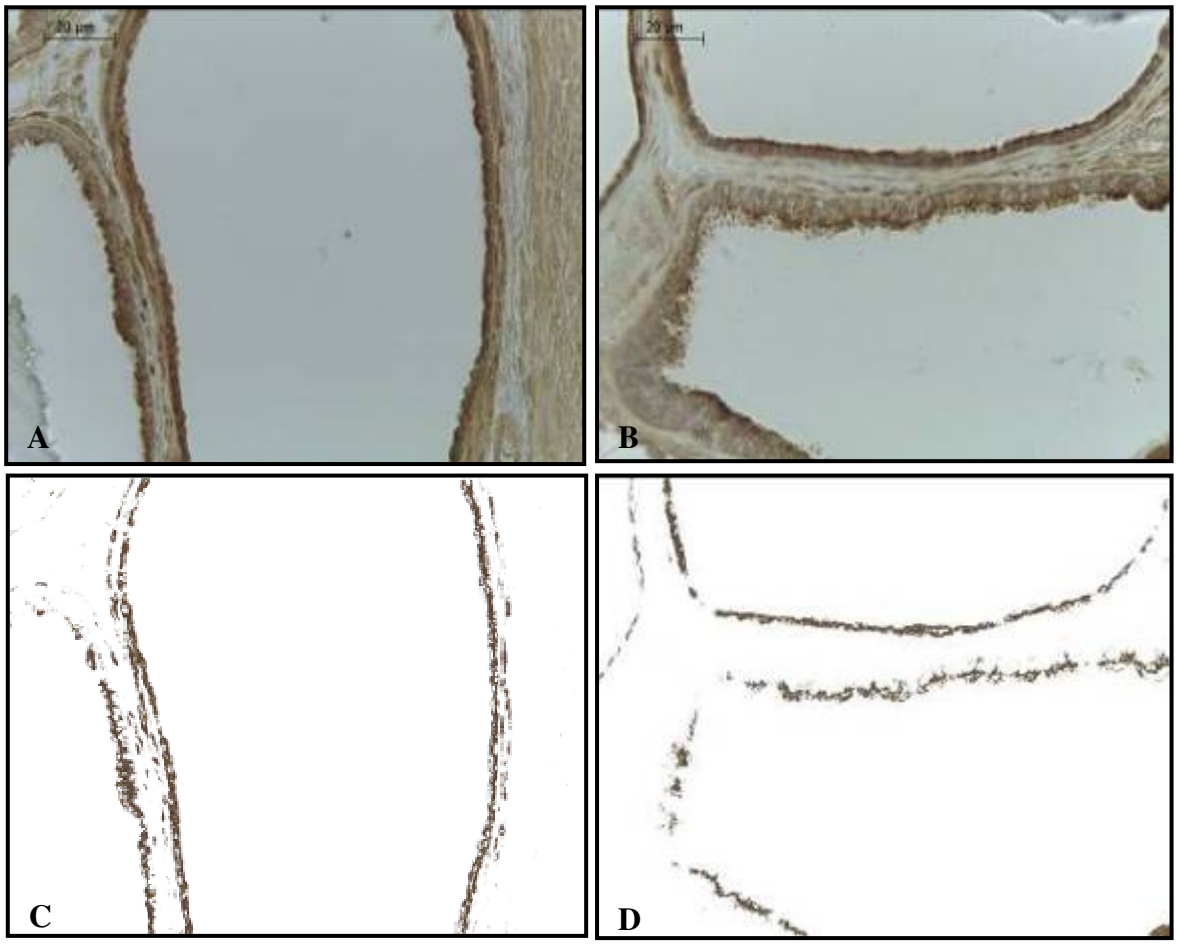


Fig. 4: Effects of Sildenafil on immunohistochemical localization of sGC. (A) Control group with no positive staining for sGC. (B) Treated group with positive staining for sGC when Sildenafil was administered for 30 days. (C) Pixel quantification of tissue area. n = 10 mice from each group. Scale bar = 20 µm.



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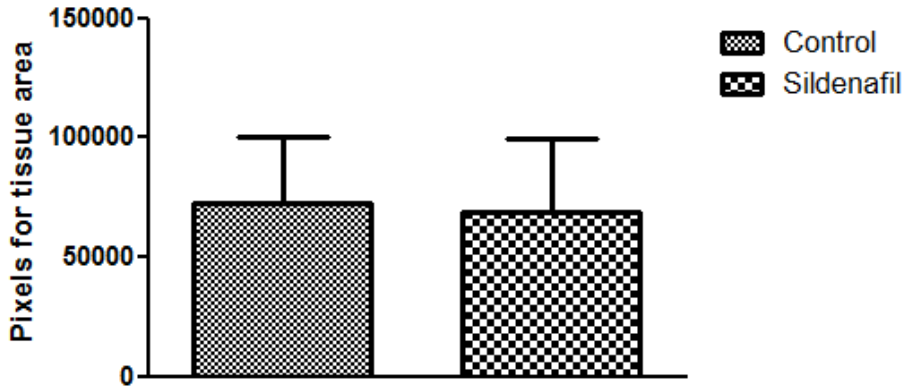
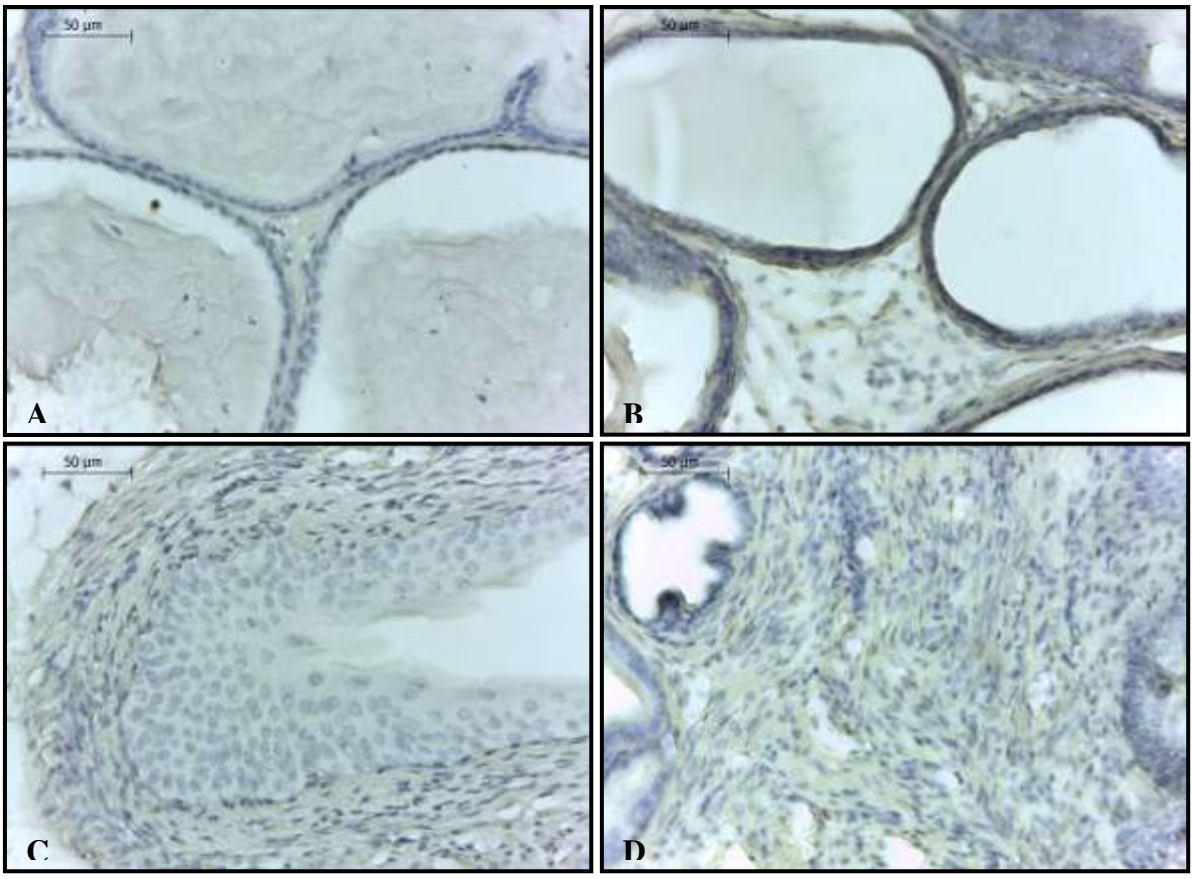


Fig. 5: Effects of Sildenafil on immunohistochemical localization of PSA in glandular region. Control group (A, C). Sildenafil-treated group for 30 days (B, D). Pixel quantification (E). n = 10 mice from each group. Scale bar = 50 µm.



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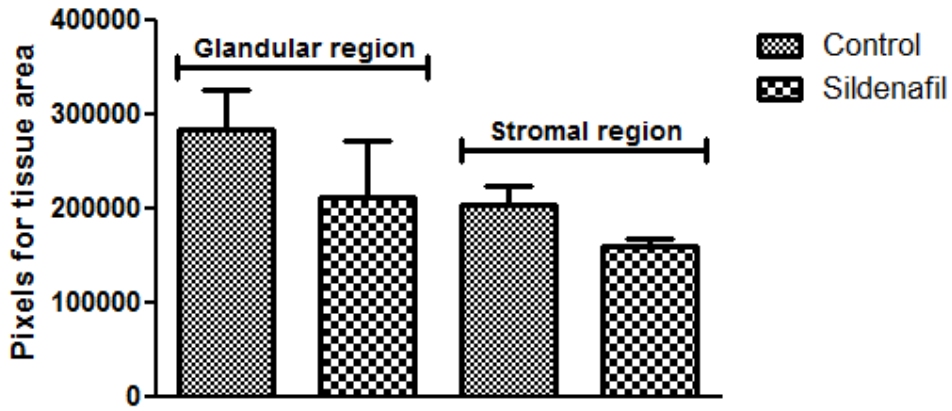


Fig. 6: Effects of Sildenafil on immunohistochemical localization of TGF-β. Control group, glandular (A) and stromal region(C). Sildenafil treated group, glandular (B) and stromal region (D). Pixel quantification (E). n = 10 mice from each group. Scale bar = 50 µm.

3.4 Hormone assay

Serum testosterone levels were significantly higher in 25 mg⁄kg Sildenafil administered mice when compared with animals from the control group (Mann–Whitney, P = 0,0057). The parameters of the two groups are shown in Table 1.

	Serum testosterone levels (ng/ml)					
	N	Mean	Minimum	Maximum	SD	Mann-Whitney
Control	11	0,74	0,19	2,4	0,70	18
Sildenafil 25mg/kg	11	5,74	0,41	11	4,71	

Significant difference between Sildenafil 25 mg⁄kg and control samples, P = 0,0057.

3.5. Measurement of NO

NO levels in serum were analyzed using the Greiss reaction test. NO level was slightly higher in the Sildenafil 25mg/kg group than in the control group, however the difference was not significant (Fig. 7).

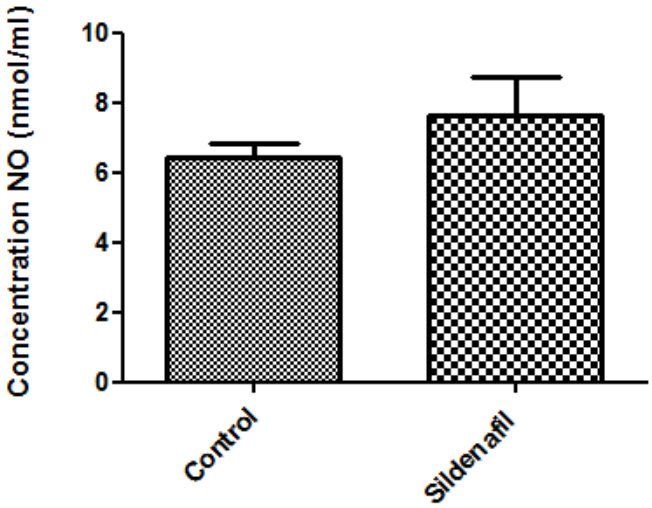
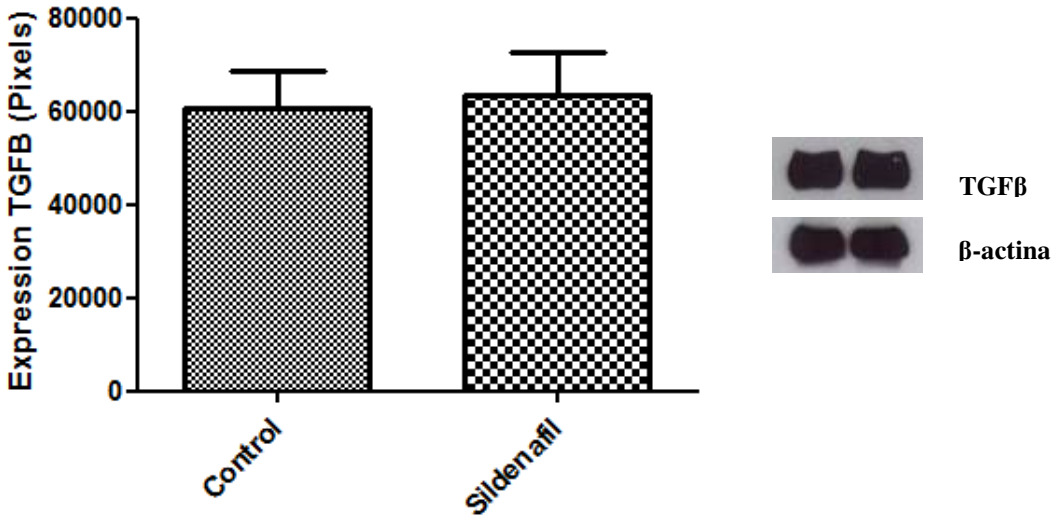
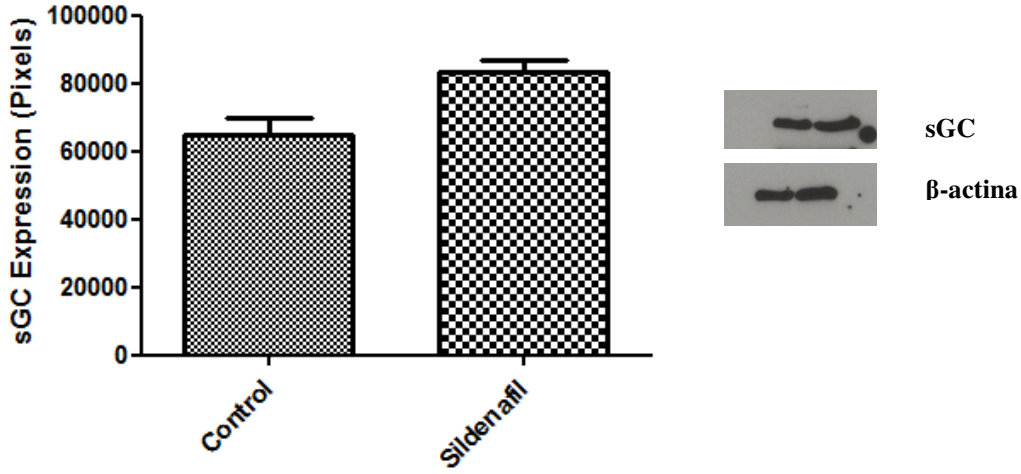


Fig. 7: Effect of Sildenafil on NO production in serum. Nitrite and nitrate levels and quantity of stable NO metabolites were higher in serum after treatment for 30 days, however the difference was not significant.

3.6. Expression of eNOS, sGC and TGFβ

The expression of eNOS, sGC and TGFβ in prostate was analyzed using the Western blot technique. Chronic Sildenafil treatment did not result in a significant difference in sGC, eNOS and TGFβ expression (Fig. 8, 9 and 10).





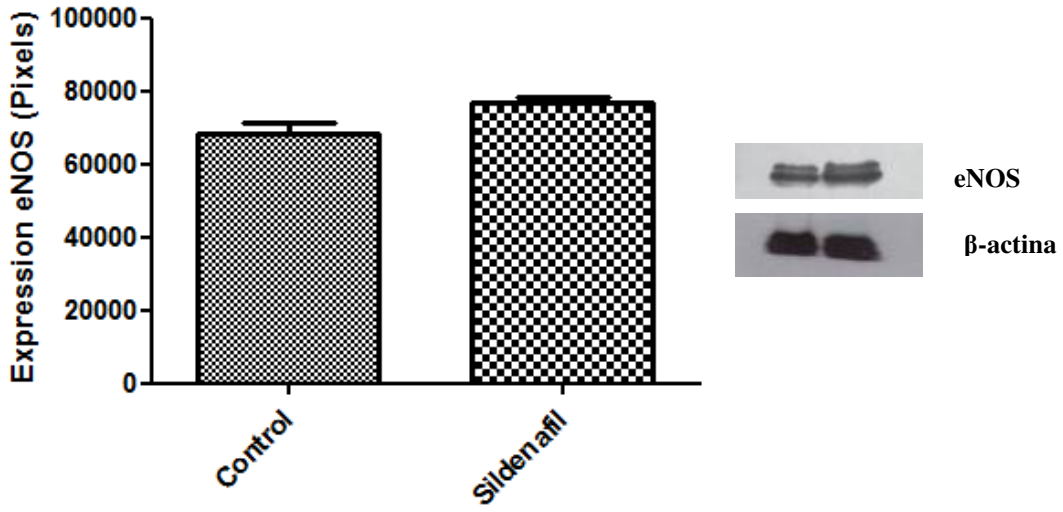


Fig.8: Western blot analysis of sGC, eNOS and TGFβ expression. Content measured by pixel quantification of Western blot bands showed no significant difference between Sildenafil and control groups.

4. DISCUSSION

Sildenafil has been used as a pharmacological strategy in the treatment of several urological and non-urogical disorders. However, there are few detailed studies of the possible effects of chronic treatment with Sildenafil on the male reproductive system.

Saraiva *et al*. (2009) undertook an *in vivo* investigation of the effects of chronic Sildenafil treatment (25mg/kg) on male *Swiss Webster* mice**.** This study demonstrated that Leydig cells had alterations in the smooth endoplasmic reticulum, large vacuoles scattered through the cytoplasm, enlarged mitochondria and cells with intense secretory activity and hormonal production**.** Other *in vitro* studies found evidence of the

antiproliferative effect of PDE inhibitors in smooth muscle cells from human BPH tissue (Wong et al., 2009; Adolfsson et al., 2002; Cook and Haynes, 2004).

The prostatic gland is composed of epithelial and stromal cells. Interactions of these cells with androgens have a fundamental role in the growth, development and differentiation of the prostate (Chung and Davies, 1996; Thomson et al., 2002; Hayward et., 1997; Cunha et al., 2004).

In the present study, the effects of chronic treatment with Sildenafil on the prostrate of C57Bl/6 mice were evaluated. Histological analysis showed no pathological alteration of the glandular and stromal region. However, epithelial cells showed some morphological characteristics of exacerbated activity. This hypothesis was confirmed by ultrastructural analysis as hypertrophied RER, prominent Golgi complex and secretory vesicle formation were identified. Histological glycogen staining using the periodic acid-Schiff (PAS) technique also confirmed that chronic treatment with Sildenafil induced prostatic secretion enhancement.

Differentiated prostatic epithelial cells can directly influence fibroblasts, vascular endothelial and inflammatory cells, to generate a microenvironment favorable to the onset of carcinogenesis (Cano et al., 2007).

The secretory function of the prostate is dependent upon direct stimulation of the prostatic epithelial cells by androgens (Hayward and Cunha, 2000). The importance of the interaction between steroid hormones and prostatic function has been studied in several prostatic pathologies in recent years. Androgen deprivation leads to loss of secretory function and a reduction in glandular size. This regression is caused by widespread apoptosis in the prostate (Kerr et al., 1972). Oliver *et al.* (2010) showed that in lower concentrations of testosterone the cyclic adenosine monophosphate (cAMP) is more active in human cultured prostatic stromal cells (HCPSC). Many authors have

showed the association between testosterone serum levels and the risk of prostate cancer, while others have demonstrated opposing results (Hoffman et al., 2000; Gill et al., 2010).

Prostate-specific antigen (PSA) is a glycoprotein produced by the prostatic epithelial cells that is considered the most useful marker of prostate cancer (Bok and Small, 2002; Vermassen et al., 2012). Its regulation has important clinical implications on cleavage semenogelins and fibronectin in coagulated semen, causing liquefaction, and aiding fertilization (Lilja et al., 1987). The present study showed that chronic treatment with Sildenafil can stimulate prostatic activity possible by elevating testosterone levels. According to data from literature, testosterone can directly influence PSA levels; however, the results of the present study showed that although high testosterone serum levels were detected after chronic Sildenafil treatment, no significant expression of PSA was observed.

Several studies indicate that PSA can accelerate carcinogenesis in the prostate. PSA can directly affect proteolysis components of the basement membrane, which can aide tumor-cell invasion and metastasis (Webber et al., 1995). In the case of advanced prostate cancer, a decrease in PSA level after systemic therapy has been shown to correlate with an improved outcome (Smaal et al., 2001).

PSA is thought to cleave insulin-like growth-factor-binding protein 3 (IGFBP3), thereby liberating insulin-like growth-factor 1 (IGF1), which is a mitogen to the prostatic stromal and epithelial (Cohen et al., 1992; Sutkowski et al., 1999; Djavan et al., 2001). PSA can also activate latent transforming growth factor (TGF)-β, which can stimulate cell detachment and facilitate the spread of tumor-cells (Killian et al., 1993). Based on these observations, the expression of TGF prostatic tissue was

evaluated by western blot. No significant difference was detected after Sildenafil treatment, which is consistent with PSA results.

Metabolic syndrome (MetS) is a complex of clustering metabolic abnormalities and comprises a number of disorders such as insulin resistance, hypertension and obesity, which all act as risk factors for cardiovascular diseases. Recent studies have demonstrated that MetS, BPH/LUTS and prostatic cancer are often comorbid (Hammarsten and Peeker, 2011). Hyperinsulinemia, hyperglycemia and insulin-like growth factor-1 (IGF-1) contribute to the development and progression of BPH / LUTS. Hyperinsulinemia is also associated with increased sympathetic nervous system activity via enhanced glucose metabolism. This process promotes the increase in α-adrenergic receptors leading to increased smooth muscle tone of the male genitourinary tract (Mc Vary, 2006; Ozden et al., 2007).The is a clear association between autonomic neural input and prostate growth rate (Mc Varry et, 1994). Besides, fasting plasma insulin, in particular, has been linked to BPH and lethal prostate cancer (Hammarsten and Peeker, 2011).

Another association between insulin resistance and BPH is related to IGF-1. Since these molecules have similar structure, insulin can bind to IGF-1 receptors and activate the signaling pathway for growth and proliferation of epithelial and stromal prostatic cells (Nunzio et al., 2012).

Chronic inflammation is one of the putative links between MetS and BPH/LUTS. Recently, Vignozzi et al (2013) demonstrated that PDE5 blockade exerts anti-inflammatory effects on myofibroblast prostatic cells, blunting inflammatory and metabolic insults. These authors showed that treatment with tadalafil or vardenafil suppressed IL-8 and IP-10 secretion induced by inflammatory (TNF-α) and metabolic

(oxLDL, AGE and IGF-1) stimuli, also suppressing TNF-α genes related to inflammation or tissue remodeling.

Other studies suggest that PDE5i could be a pharmacological strategy for the treatment of ED and LUTS/BPH by modifying NO/cGMP signaling pathway and improving the RhoA/Rho-kinase (ROCK), besides reducing the hyperactivity of the autonomic nervous system and chronic pelvic ischemia (Gacci et al., 2013).

Nitric oxide is a gas that is synthesized intracellularly by three NOS isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) (Andersson, 2007; Aaltommaa et al., 2000; Uotila et al., 2001; Cronauer et al., 2007; Nanni et al., 2009; Sanli et al., 2009; Sanli et al., 2011; Yu et al., 2013). It has been demonstrated that the isoform eNOS plays a predominant role in tumor growth, metastasis and angiogenesis in human prostate cancer (PC), as well as in maintenance of the vascular tone and mediating vascular endothelial growth factor (VEGF)-induced endothelial cell activation (Ying and Hofseth, 2007; Polytarchou et al., 2009; Ziaei et al., 2013).

The fibromuscular stroma is densely supplied by NO synthase-containing nerve terminals (Burnett et al., 1995). According to data from literature, the NO plays an important role in the control of prostate function in mammals and humans, including the regulation of prostate smooth muscle tone, glandular secretory function and local blood flow (Hedlund, 2005; Andersson, 2007; Kedia et al., 2008).

Activators of the NO/GMPc signaling cascade may interfere with regulation of smooth stromal muscle tone (Waldkirch et al., 2007). Secondary messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are synthesized by activation of adenylyl- and guanylyl-cyclases respectively, and degraded by cyclic nucleotide phosphodiesterases (PDE) (Hall, 1993). Nitric oxide (NO) activates soluble guanylyl cyclase (sGC), leading to an increase in intracellular cGMP,

which activates cytosolic cGMP-dependent protein kinase (PKG). Soluble guanylyl cyclase (sGC) is considered the most important receptor for the signaling molecule NO (Carvajal et al., 2000; Uckert et al., 2006).

To evaluate if chronic Sildenafil treatment could influence the NO/cGMP cascade in prostate, levels of serum NO, immunohistochemistry for sGC and western blot for sGC and eNOS were evaluated. No statistical significant differences were observed in nitric oxide serum level or in sGC and eNOS prostatic expression.

Conclusion

In summary, chronic treatment with Sildenafil (25mg/kg) induced an enhancement of prostatic glandular activity, possibly due to increased testosterone production. However, there was no increase in the expression of PSA and TGF-β. This data suggests that extensive use of Sildenafil in non-urological disorders such as pulmonary hypertension may not damage the prostate.

Acknowledgements

This study was supported by Fundação Oswaldo Cruz (FIOCRUZ) and Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE).

Conflito of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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A new mouse model for prostatitis induced by LPS

Gomes, F.O.S

Artigo II

5.2 Pergunta condutora 2: A injeção intrauretral com LPS durante 3, 7, 10 e 14 dias constitui um modelo experimental eficaz de lesão prostática?

Artigo II (A ser submetido)

Fator de impacto: 1,9

A new mouse model for prostatitis induced by LPS

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Summary

A man will almost inevitably develop some type of prostate pathology during the aging process. In recent years, chronic inflammation has been recognized as having a prominent role in the pathogenesis of benign prostatic hyperplasia (BPH) and cancer. It is believed that chronic inflammation induces prostatic fibromuscular growth. This correlation has been clearly illustrated by both in vivo and in vitro studies; however current experimental models of BPH require complex surgery or hormonal treatment. Therefore, the aim of the present study was to propose a new murine model of BPH/prostatitis induced by intraurethral injection of LPS (1mg/ml), using male Swiss and C57Bl/6 mice, for 3, 7, 10 and 14 days. The results showed that LPS played an important role in the cell proliferation of the prostate. Histological and ultrastructural analysis showed epithelial hyperplasia, clear stromal cells, little inflammatory infiltration and heavy bleeding. Treatment with LPS also promoted the increase of growth factor (FGF-7 and TGF-β), α-actin and proinflammatory cytokines (IL-1, IL-6, IL-17), both in the stroma and epithelium. According to the present findings it can be concluded that the intraurethral administration of LPS promotes tissue remodeling, as well as stimulating the pattern of pro-inflammatory cytokines, and therefore, constitutes an effective experimental model of BPH/inflammation.

Keywords: Inflammation, cytokine, growth factor.

1. INTRODUCTION

In recent decades basic science and preclinical studies have highlighted the role of inflammation in the development and progression of prostatic diseases (Hamid et al., 2011; Omabe and Ezeani, 2011; Bjorling et al., 2011). While the causes of prostatitis are not completely understood, it is believed that extrinsic and intrinsic factors such as bacterial or virus infection, disordered immune response, food diet, tension or physical injury problem, uric acid disorder, and urethral stricture are directly involved (De Marzo et al., 2007; Wang et al., 2015). The relationship between prostatitis and benign prostatic hyperplasia (BPH) and prostate cancer (PCa) is based on the hypothesis that inflammation exerts an important role in the development of such diseases (De Nunzio et al., 2011; Robert et al., 2011; Elkahwaji, 2012; Thapa and Ghosh, 2015). In some types of cancer, inflammatory conditions are present before any malignant change occurs, while in other types of the disease, an oncogenic change induces an inflammatory microenvironment that promotes the development of tumors (Mantovani et al., 2008).

Prostatitis animal models are extremely useful for elucidating the cellular and molecular mechanisms of pathogenesis. They also provide support for new, more rational and effective therapeutic strategies, contributing to an improved quality of life for people with chronic diseases of the urogenital tract. Data in literature features a variety of testing methods, including animal models of bacterial and non-bacterial prostatitis (immune, spontaneous, sex hormone, chemical agent, miscellaneous stress, mechanical, diet and Lipopolysaccharide-LPS) (De Marzo et al., 2007; Vykhovanets et al., 2008; Li et al., 2013). Several experimental models induce prostatic alterations by direct injection of mitogen or other agents into the prostate after laparostomy (Fulmer & Turner, 1999; Mosli et al., 2012). Others use the intraurethral injection of *E. coli* for

prostatitis studies of infection/inflammation (Elkahwaji et al., 2007; 2009; Boehm et al., 2012). While these models are well-established they are not effective for the characterization of potential anti-inflammatory drugs, where co-treatment with antibiotics is indispensable. Therefore, the proposal of a new murine model of non-bacterial prostate inflammation, which is both easy to perform and less invasive, would provide a useful new methodological strategy.

Lipopolysaccharide (LPS), a component of the outer layer of the cell wall of gram-negative bacteria, is a potent activator of the immune system. It induces the subsequent release of tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and IL-6 from macrophages and monocytes. Activated immune cells are major sources of inflammatory mediators, which are responsible for elevated levels of cytokines, growth factors and reactive oxygen species (ROS), all of which contribute to cell proliferation and DNA damage.

Therefore, the present study proposed a new model of BPH/prostatitis induced by intraurethral injection of LPS (1mg/ml) into male Swiss and C57Bl/6 mice over periods of 3, 7, 10 and 14 days. The following end points were determined for inflammatory response: (1) dysplastic changes including focal disruption of the epithelium, polymorphism of epithelial cell nuclei, and increased epithelial proliferation (histology and ultrastructure); (2) expression of IL-1, IL-6, IL-17, TGF-β, FGF-7 and actin-α (immunohistochemistry).

2. MATERIAL AND METHODS

2.1 Animals

Fifty male Swiss and C57Bl/6 mice (obtained from the Centro de Pesquisas Aggeu Magalhães / FIOCRUZ, Recife, Brazil) aged three months and weighing 20–29g were

used in all experiments. Mice were examined for health status and acclimated to the laboratory environment, which had a temperature of 23ºC and a 12h light: 12h dark photoperiod. The animals were housed in metal cages and fed a standard diet and water ad libitum. The experimental groups were composed of 10 animals each, as follows: control – vehicle intraurethral; LPS - LPS 1mg/kg intraurethral for 3, 7, 10 and 14 days. All experiments were performed in accordance with ethical guidelines (28/2011 – CEUA⁄ FIOCRUZ).

2.2 Intraurethral Injection

The experimental animals were placed in the supine position and the genital area was sterilized with 70% alcohol. Then the animals were catheterized per urethra using a lubricated sterile polyethylene catheter (intramedic PE-10). LPS (1mg/ml in saline; Escherichia coli serotype 026:B6; Sigma-Aldrich, Oakville, Ontario, Canada) was injected with a final volume of 100 µl inside the prostatic urethra using insulin syringes. Animals were then sacrificed 3, 7, 10 and 14 days after LPS induction (Figure 1). The prostates were quickly dissected and fixed for morphological analysis.

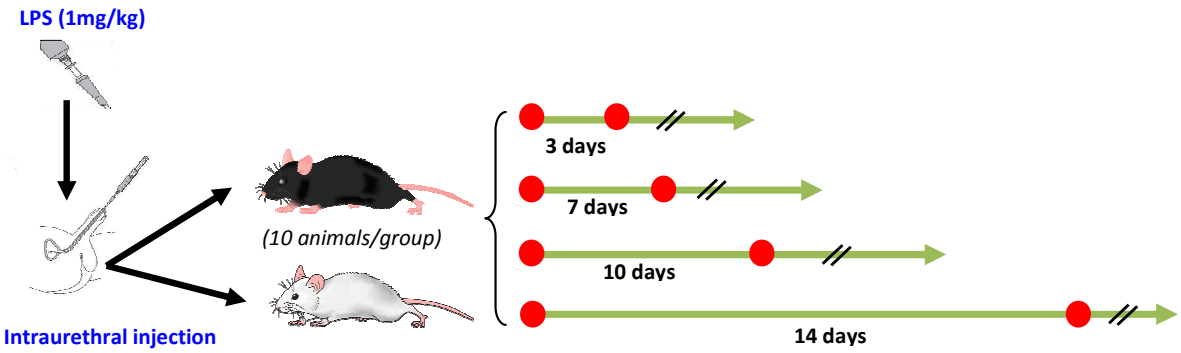

Figure 1: Experimental design – LPS induction of inflammation intraurethral for 3, 7, 10 and 14 days in swiss and C57Bl/6 mice.

2.3 Light microscopy

Prostates were fixed in Bouin solution for 8 h. Next, they were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections of 5µm were cut using a microtome (Leica RM 2125RT), stained with haematoxylin–eosin and evaluated with an inverted microscopy (Observer Z1, Zeiss Micro Imaging GmbH) equipped with a camera and 4.7.4 image analysis program (AxionCam MRm Zeiss) at a magnification of 400x.

2.4 Feulgen Staining

The cuts were stained with Schiff's reagent for DNA stains of the prostate cells. The slides were pre-treated with HCL 1M at 60°c for 8 min, followed by incubation in the same solution for 1 minute at room temperature. Subsequently, the cuts were stained with 1% Schiff's reagent for 1 hr and counter-stained with a solution of 1% light green dye in 1% acetic acid for 40 seconds. After this process, the slides were dehydrated in ethanol at 100%, cleared in xylene and mounted. Five images of the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.5 Electron transmission microscopy

The fragments of prostate were fixed overnight in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer. After fixation, the samples were washed twice in the same buffer and were then post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone, and embedded

in Embed 812. Polymerization was performed at 60°C for 3 days. Ultrathin sections were collected on 300-mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate, and examined using a FEI Morgani 268D transmission electron microscope.

2.6 Immunohistochemical assays for IL-1β, IL-6, IL-17, TGF-β, FGF-7 and α-actin

Ultrathin sections (5 µm in thickness) of each group were cut and fixed to slides treated with 3-amino-propyl-trietoxi-silane (APES [Sigma, USA]). Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol (70 to 100%). The sections were heated for 30 minutes in a sodium citrate buffer (0.01 M, pH 6.0) to increase epitope exposure. The slides were treated with 0.3% (v/v) H_2O_2 in water for five minutes to minimize endogenous peroxidase activity. The sections were washed with 0.01M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1h, at room temperature. The sections were then incubated for 12 hours at 4ºC with rabbit anti-IL17 antibody (Santa Cruz Biotechnology, sc-7927, 1:50); anti-IL1β antibody (GenWay, SanDiego, CA, USA, GWB-BBP232, 1:100); anti-IL6 (ABCAM, CA, USA, ab6672, 1:50); anti-FGF-7 (Santa Cruz Biotechnology, sc-7882, 1:50); anti-TGF-β (ABCAM, CA, USA, ab66043, 1:50) and anti-α-actin (ABCAM, CA, USA, ab5694, 1:50). Antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB ® + Kit, Peroxidase) using 3.3-diaminobenzidine as a chromogen. The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the first antibody. Five pictures taken at the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.7 Statistical analysis

GraphPad Prism software, version 5 was used for statistical analysis. Data was expressed as mean ± standard deviation. The differences between the control and treated groups were analyzed using the Mann–Whitney or T-test. Probability values lower than 0.05 were considered significant.

3 RESULTS

3.1 LPS action on prostatic architecture

Histological analysis of prostatic fragments of the Swiss and C57Bl/6 control groups showed well-preserved architecture with epithelial and stromal cells presenting patterned morphological characteristics and a dense stroma. In the glandular acini there was the presence of various intraluminal with protruding projections (papilliform), which did not occupy the entire space of the gland lumen. The acini were coated by a double cell layer, a basal layer of flat cuboidal epithelium, juxtaposed by cubic secreting cells (Figure 2A, B and 3A, B).

After intraurethral injection of LPS in the Swiss mice for 3 days, the prostate showed minor changes, such as an increase in the number of acini, reduced intraluminal space and cells with pyknotic nuclei (Figure 2C and D). After 7 days of induction, in addition to the histological findings presented in the 3 day group, it was possible to identify areas of hemorrhage (Figure 2E and F). In the 10 and 14 day groups, the prostate showed significant changes including the presence of tissue disruption with several acini within a dense hyperplasic stroma (not shown). In addition, glandular cells were numerous and showed intense mitotic activity (Figure 2G and 2J), although some cells presented pyknotic nuclei (Figure 2H) and little inflammatory infiltration (Figure

2H and 2I). In the 14 day group, the luminal space was reduced by the invasion of the acini (Figure 2I).

In C57Bl/6 animals after 3 and 7 days of induction with LPS, the main changes were cell hyperplasia (Figure 3C, D and E, F), loose stroma (Figure 3H) and intraluminal cellular debris (Figure 3I). The acini presented papilliform projections and basal membrane folding (Figure 3D). After 10 days of LPS induction, the inclusion of lipids inside the stromal region, intraluminal projections, and the presence of pyknotic nuclei were observed (Figure 3G). After 14 days, the prostate showed acutely inflamed foci, hyperplasia of the clear cells and intense secretory activity (Figure 3I and J). Also several hemorrhagic foci were observed (not shown).

Feulgen staining selectively stains DNA, and it was therefore used to confirm cellular proliferation after LPS induction. The control groups (Swiss and C57Bl/6) showed homogenous distribution of cellular labeling (2K and 3K). After 3, 7, 10 and 14 days of LPS treatment, the glandular cells presented intense labeling, confirming cellular mitotic activity (Figure 2L, M, N, O and 3 L, M, N, O).

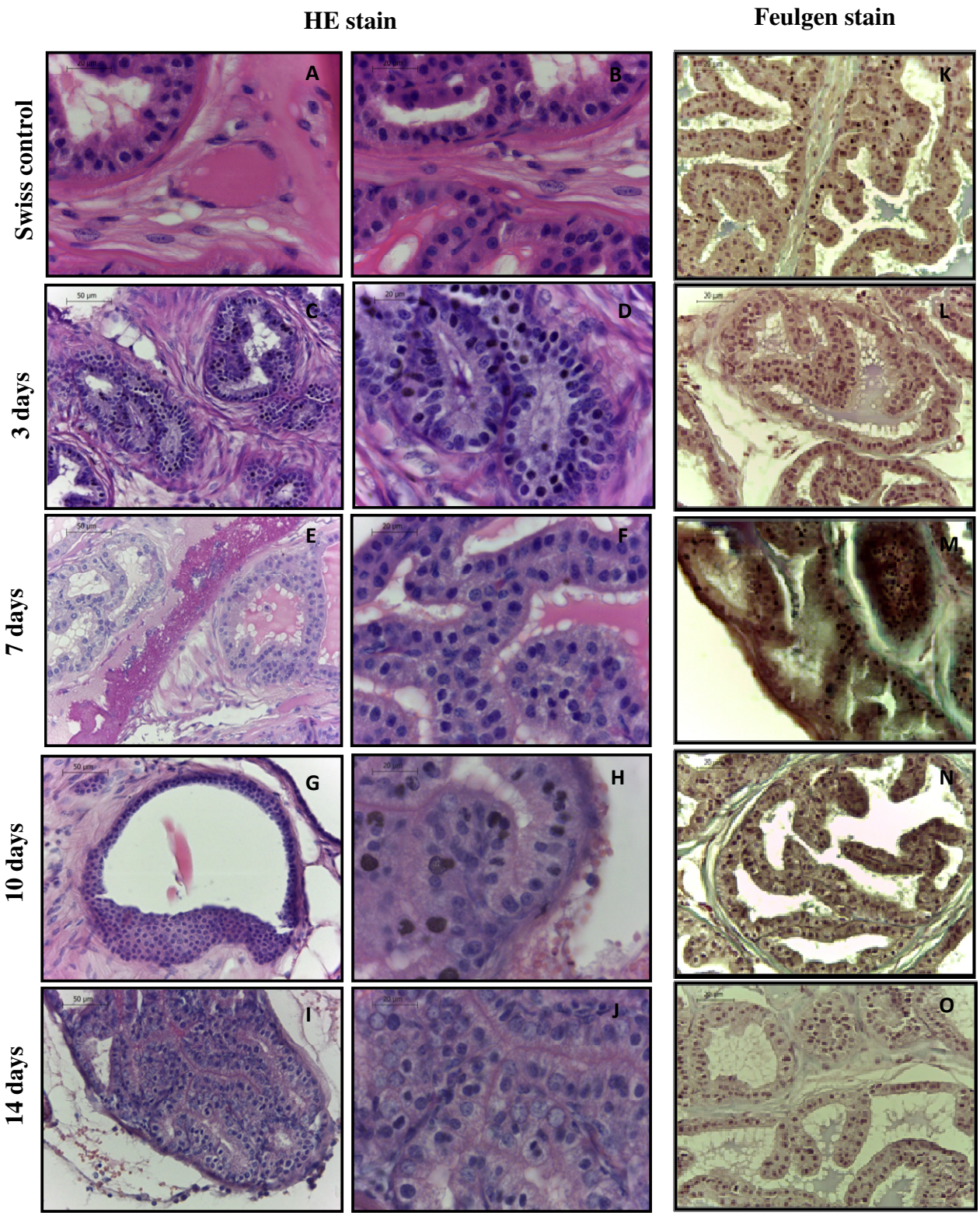

Figure 2: Histological analysis of swiss mice prostates. Stained with hematoxylin-eosin. (A, B) Prostate of the control group showed well-preserved. (C-J) After intraurethral injection of LPS for 3, 7, 10 and 14 days. (2C and D)Showed cells with

pyknotic nuclei . (2E and F) areas of hemorrhage. (2G and 2J) mitotic activity. Scale bar = 20µm (A, B, D, F, H and J) and 50µm (C, E, G and I). Prostatic epithelial cell proliferation induced by LPS. (K) PBS-control tissues show staining for Feulgen. (L-O) Prostate tissues exhibiting a varying degree of cell proliferation at 3, 7, 10 and 14 days after induced for LPS. Scale bar = 20µm

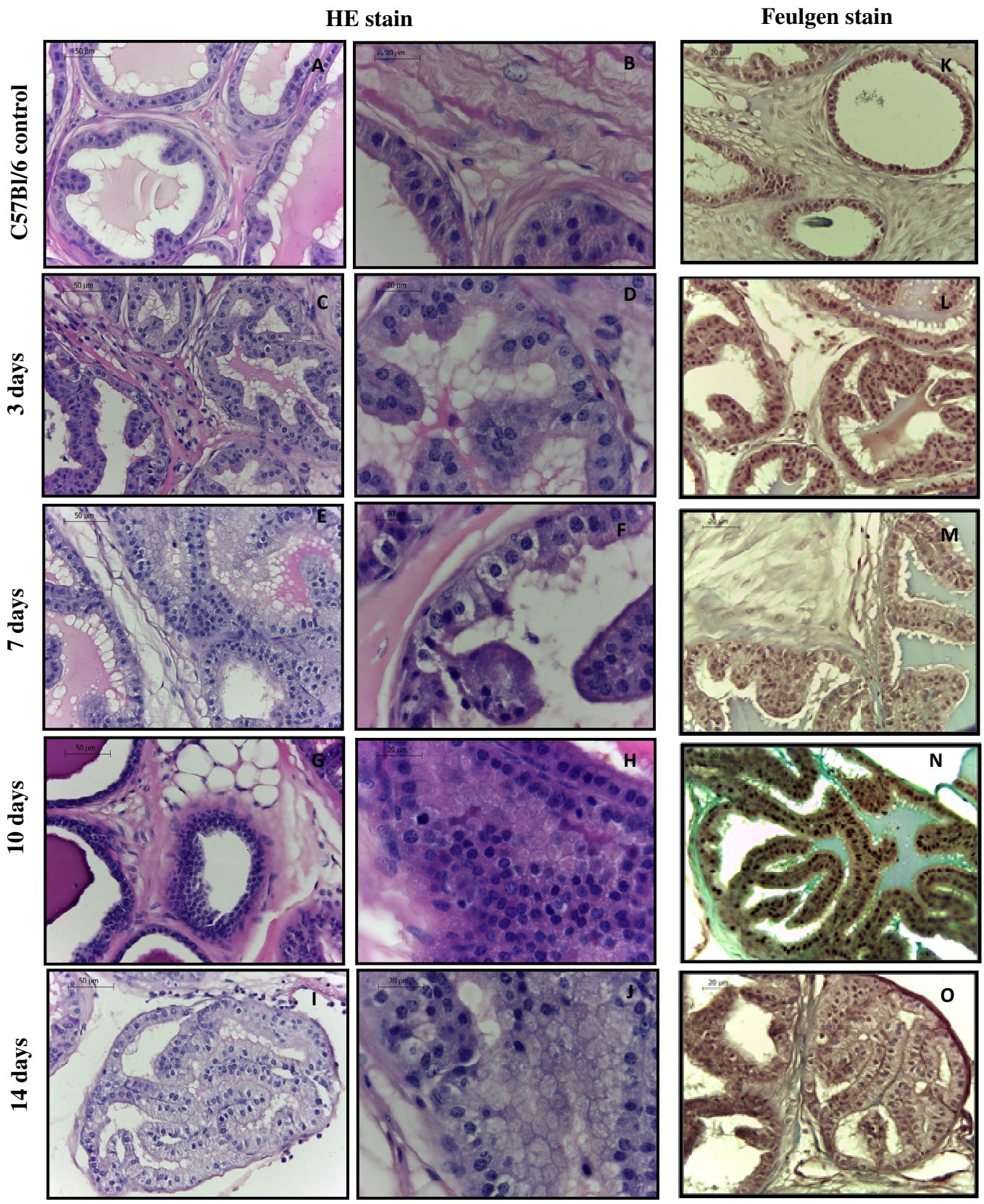

Figure 3: Histological analysis of C57Bl/6 mice prostates. Stained with hematoxylin-eosin. (A, B) Prostate of the control group showed well-preserved. (C-J) After

intraurethral injection of LPS for 3, 7, 10 and 14 days were observed: (3C-F) cell hyperplasia intraluminal cellular debris. (3I) pyknotic nuclei (3G). inflamed foci, hyperplasia of the clear cells and intense secretory activity (3I, J). Scale bar = 20µm (B, D, F, H and J) and 50µm (A, C, E, G and I). Prostatic epithelial cell proliferation induced by LPS. (K) PBS-control tissues show staining for Feulgen. (L-O) Prostate tissues exhibiting cell proliferation at 3, 7, 10 and 14 days after induced for LPS. (N) Intense cell proliferation. Scale bar = 20µm

3.2 Ultrastructural analysis

Ultrastructural analysis of control, Swiss and C57Bl/6 animals showed the following prostate characteristics: intact basement membrane, overlapping columnar cells with preserved elliptical nuclei, the presence of vesicles in the apical cellular region, lysosomes and peroxisomes and numerous mitochondria (Figure 4A, B and 5A, B).

In contrast, the Swiss and C57Bl/6 animals treated with LPS for 3 days showed glandular cells with large secretion vesicles (Figure 4C, D and 5C, D), as well as rough hypertrophic endoplasmic reticulum and dilatation of the cisternae of the Golgi complex (Figures 5C and D).

After 7 days of LPS induction, the Swiss group showed the following alterations: cytoplasmic disorganization, several degenerated mitochondria and intense deposition of collagen fibers, indicative of fibrotic processes (Figures 4E and F). In the C57Bl/6 group, hemorrhagic foci (Figure 5E) and cells in mitosis were observed (Figure 5F).

After 10 days of LPS induction, the Swiss animals presented glandular cell death, characterized by loss of cytoplasmic content, an indented or picnotic nucleus, and

degenerated mitochondria (Figures 4G and 4H). Also, the basal membrane was thick and corrugated (Figure 4G). In contrast, the glandular cells of the C57Bl/6 animals showed cytoplasm disorganization with electron-dense vesicles (Figure 5H). Additionally, some hemorrhagic foci were also observed (Figure 5G).

After 14 days of LPS induction, the Swiss animals showed drastically injured glandular cells (Figures 4I and J), whereas the C57Bl/6 mice had glandular cells with electron-dense vesicles and vacuolization (Figure 5J), some of which also showed loss of cellular content and picnotic nuclei (Figure 5I).

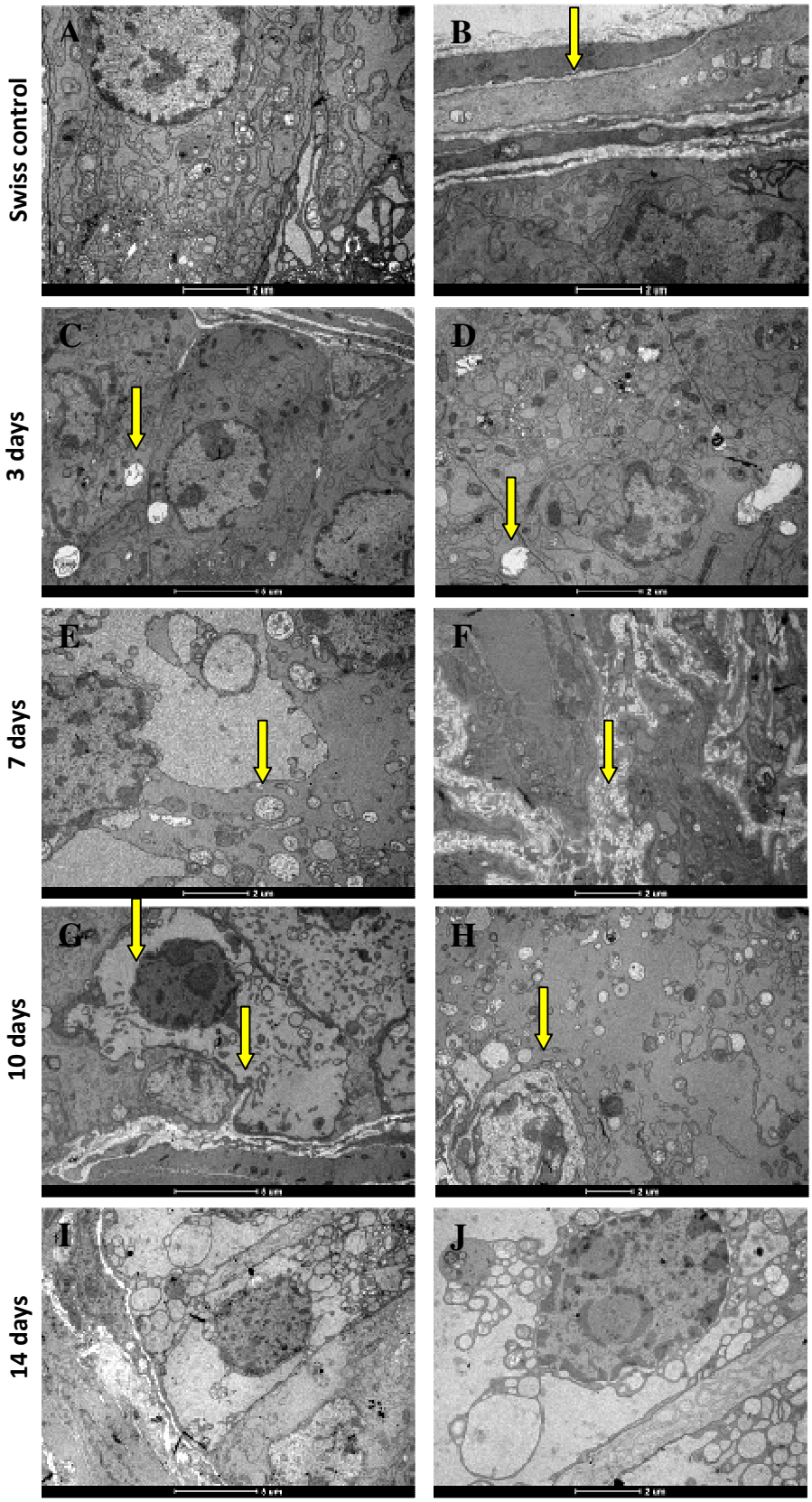

Figure 4: Morphological ultrastructural features of prostate cell by transmission electron microscopy in swiss mice, after 3, 7, 10 and 14 days of LPS induction. Scale bars represent 2 µm (A, B, D, E, F, G and H) and 5 µm (C, I and J).

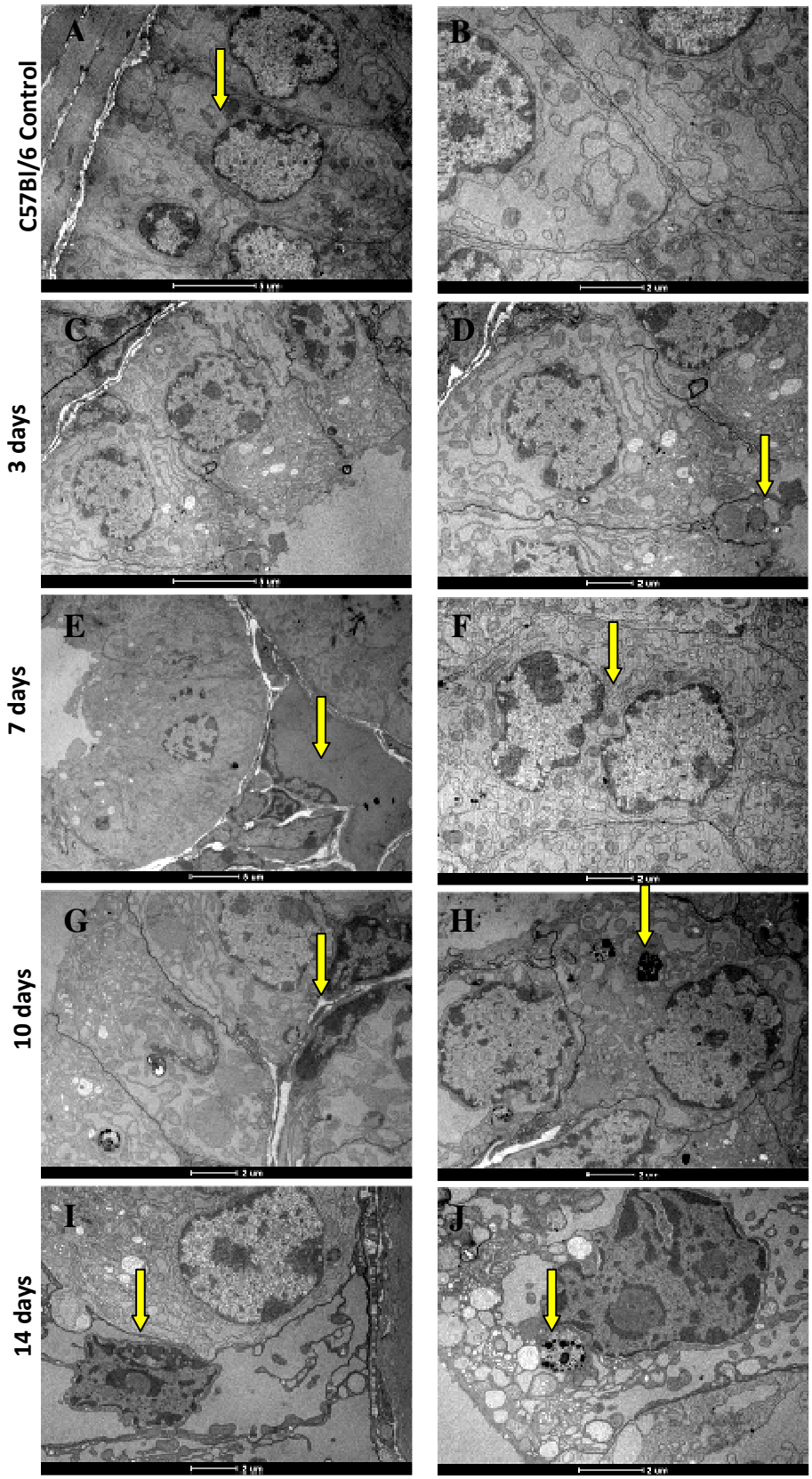

Figure 5: Morphological ultrastructural features of prostate cell by transmission electron microscopy in C57Bl/6 mice, after 3, 7, 10 and 14 days of LPS induction. Scale bars represent 2 µm (5B, D, F, G, H, I, J) and 5 µm (5A, C, E).

3.6. Intraurethral LPS induces expression of pro-inflammatory cytokines, growth and fibrosis markers

IL-17 over-regulates the secretion of other pro-inflammatory cytokines such as IL-1, IL-6 and TGF-β (Steiner et al., 2003; Hamid et al., 2011). The control groups of the Swiss and C57Bl/6 animals had basal levels of IL-17. However after LPS induction, both strains had a significantly higher expression of IL-17 in the stromal and glandular tissues throughout the treatment ($p < 0.05$). The Swiss mice presented the highest expression on the 14th day, whereas the C57Bl/6 mice presented significantly higher expression from the 10^{th} day (($p < 0.05$).

The IL-1 family of cytokines mediates growth in normal development, and exercises a similar activity in inflammatory response (Jerde and Bushman, 2010). The control group showed weak reactivity to IL-1β, both in the Swiss mice and the C57Bl/6 animals in stromal and glandular areas. However, 7 and 10 days after LPS induction, prostatic IL-1β expression was significantly higher in Swiss mice ($p < 0.05$). On the other hand, prostatic IL-1 β expression in C57Bl/6 animals was significantly increased on the 14th day ($p < 0, 05$).

IL-6 is known to be a potent autocrine growth factor for the prostate epithelial and stromal cells (Fibbi, et al., 2009). In both the Swiss and C57BL/6 animals IL-6 expression was significantly increased 3, 7, 10 and 14 days after LPS induction, when compared to the basal levels observed in the control groups ($p < 0.05$). Interestingly, in the prostrates of C57Bl/6 mice IL-6 was significantly reduced on the 14^{th} day when compared to the 7^{th} and 10^{th} days ($p < 0.05$), which was probably related to resolution of inflammation.

FGF-7 is a fibroblast growth factor that acts as a mitogen for prostatic stromal cells (Peehl and Rubin, 1995). The Swiss and C57Bl/6 groups showed significantly

elevated levels of FGF-7 expression in the stromal tissue when compared to the control groups ($p < 0.05$), indicating stromal mitotic activity.

TGF-β is a transforming growth factor that induces a reactive phenotype in the stromal cells, which is a shift from a smooth muscle cell phenotype to a myofibroblast phenotype, expressing actin and vimentin (Liu et al., 2011). The control Swiss and C57BL/6 groups showed low levels of TGF-β expression in stromal tissue. However, from the 3rd day onwards, all groups exhibited a significant expression of TGF-β ($p < 0.05$). Similar results were obtained for α-actin expression in the stromal tissue after intraurethral LPS induction, confirming the reactive phenotype of the stromal cells.

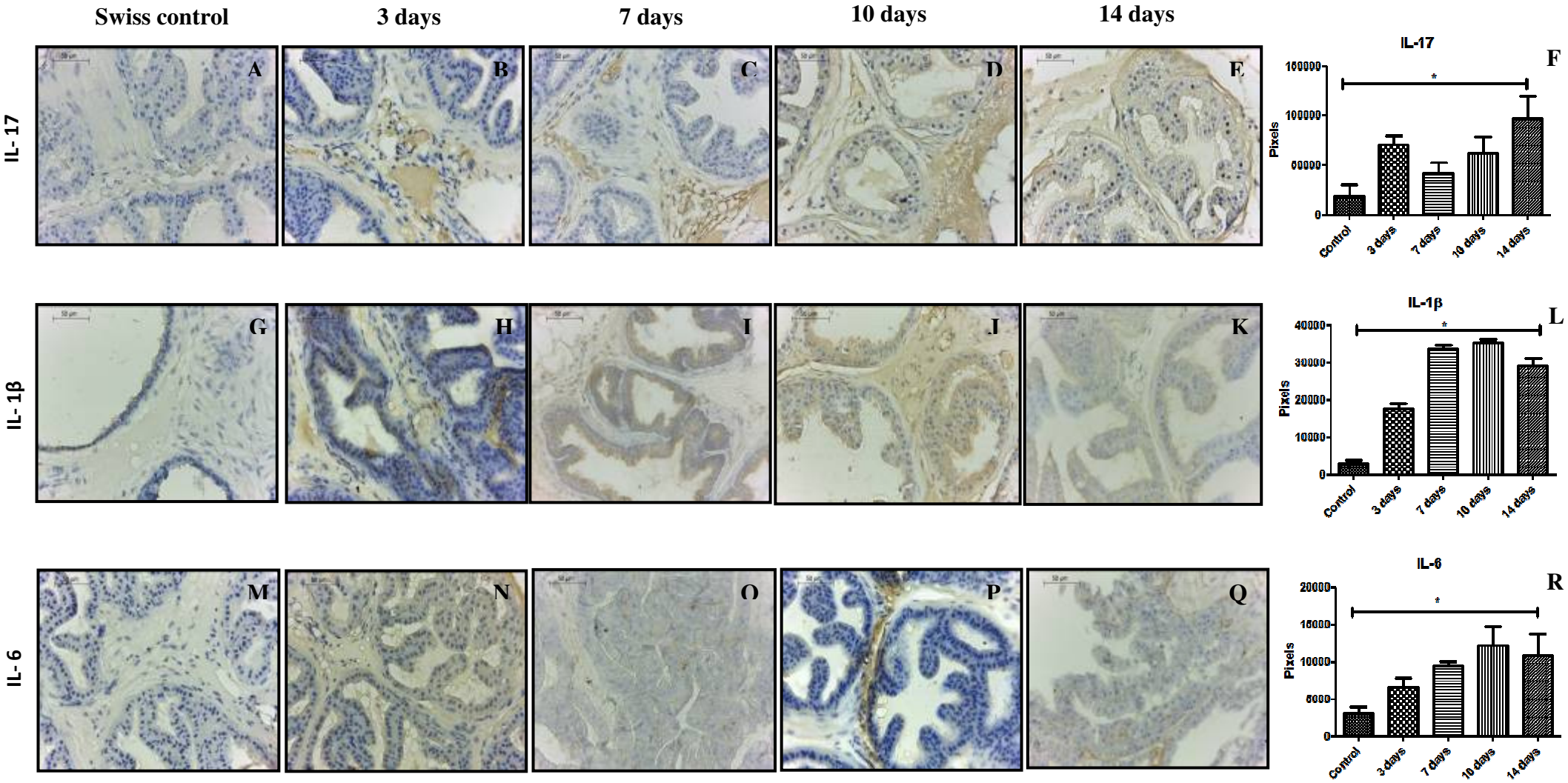

Figure 6: Immunohistochemical localization of cytokines (IL-17, IL-1β, IL-6,) in prostate tissue of the swiss mice, after induced by LPS. (B-E) In tissue, IL-17 positive labeling was detected cytoplasmic and extracellular. (H-K) IL-1β positive labeling was detected cytoplasmic. (N-Q) IL-6 positive labeling was detected extracellular. (A, G and M) the control groups no positive labeling. (F, L and R) data expressed as mean ± SD, *($p < 0.05$).

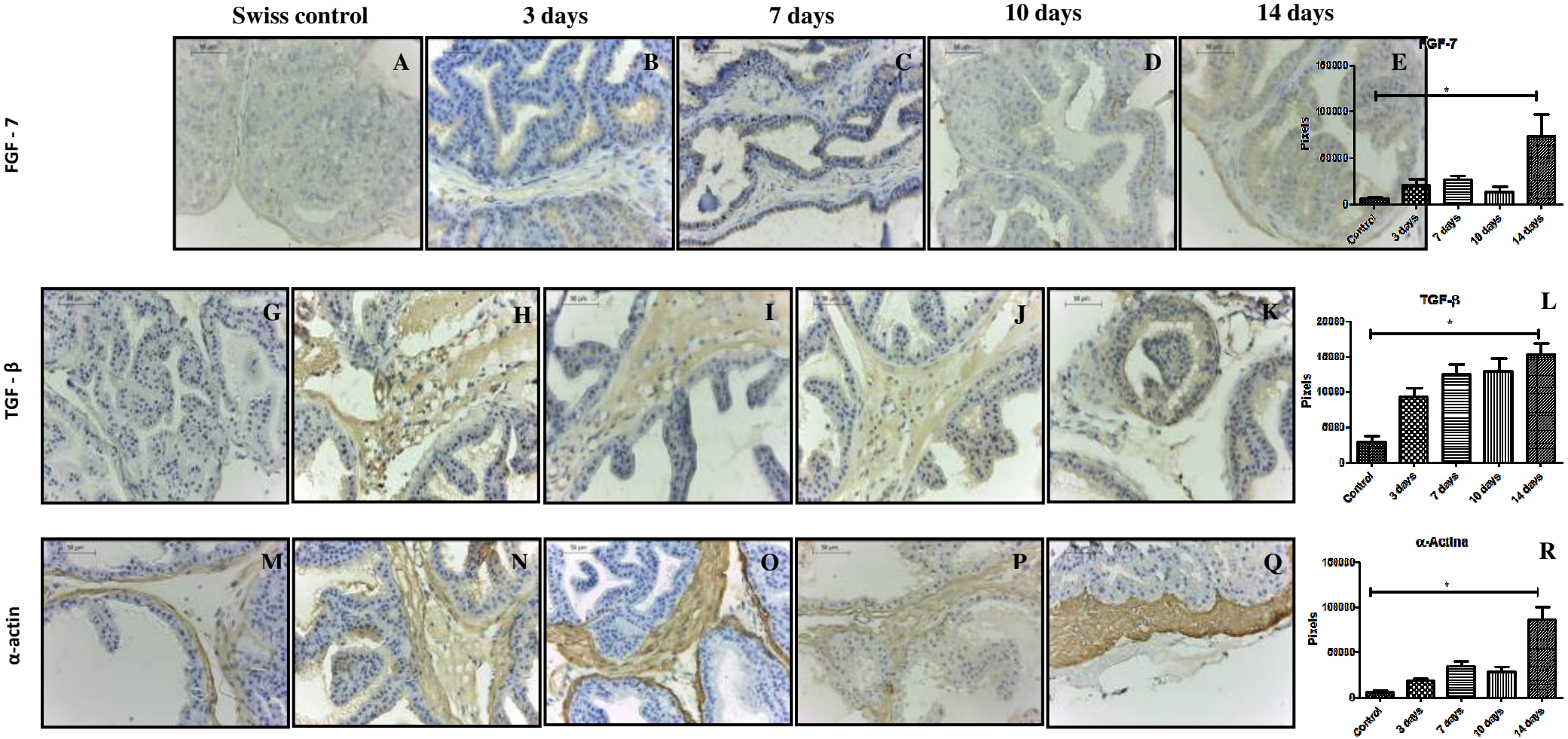

Figure 7: Immunohistochemical localization of cytokines (FGF-7, TGF-β and α-actina) in prostate tissue of the swiss mice, after induced by LPS. (B-E) TGF-7 showing cytoplasmic positive staining of glandular cells. (H-K) TGF-β positive labeling was detected: secreted, extracellular space and extracellular matrix. (N-Q) α-actin positive labeling was detected in stromal region. (A and G) the control groups no positive labeling, (M) α-actin was detected little labeling. (F, L and R) data expressed as mean ± SD, *($p < 0.05$).

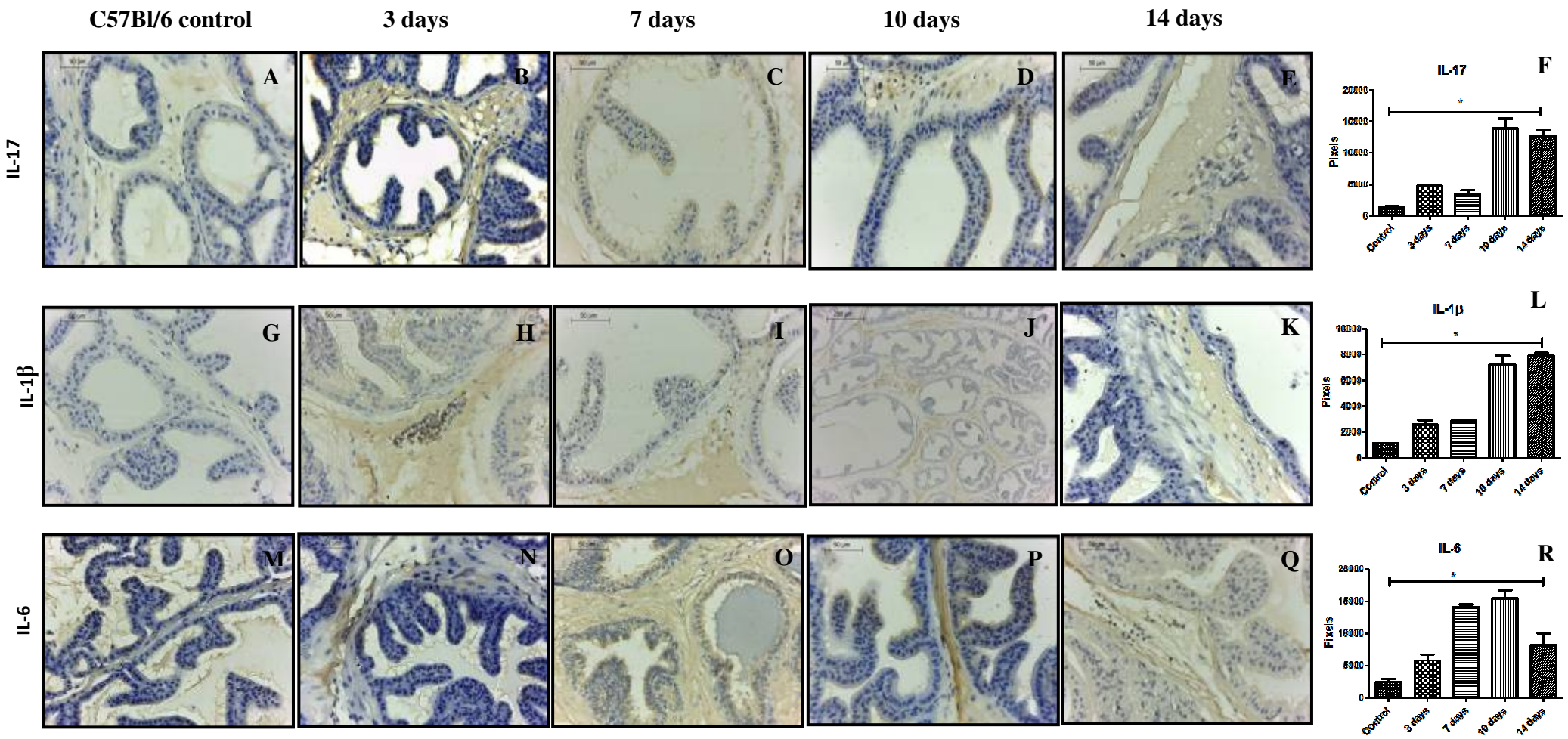

Figure 8: Immunohistochemical localization of cytokines (IL-17, IL-1β, IL-6,) in prostate tissue of the C57Bl/6 mice, after induced by LPS. (B-E) In tissue, IL-17 positive labeling was detected cytoplasmic and extracellular. (H-K) IL-1β positive labeling was detected cytoplasmic. (N-Q) IL-6 positive labeling was detected in epithelial and stromal region. (A, G and M) the control groups no positive labeling. (F, L and R) data expressed as mean ± SD, *($p < 0.05$).

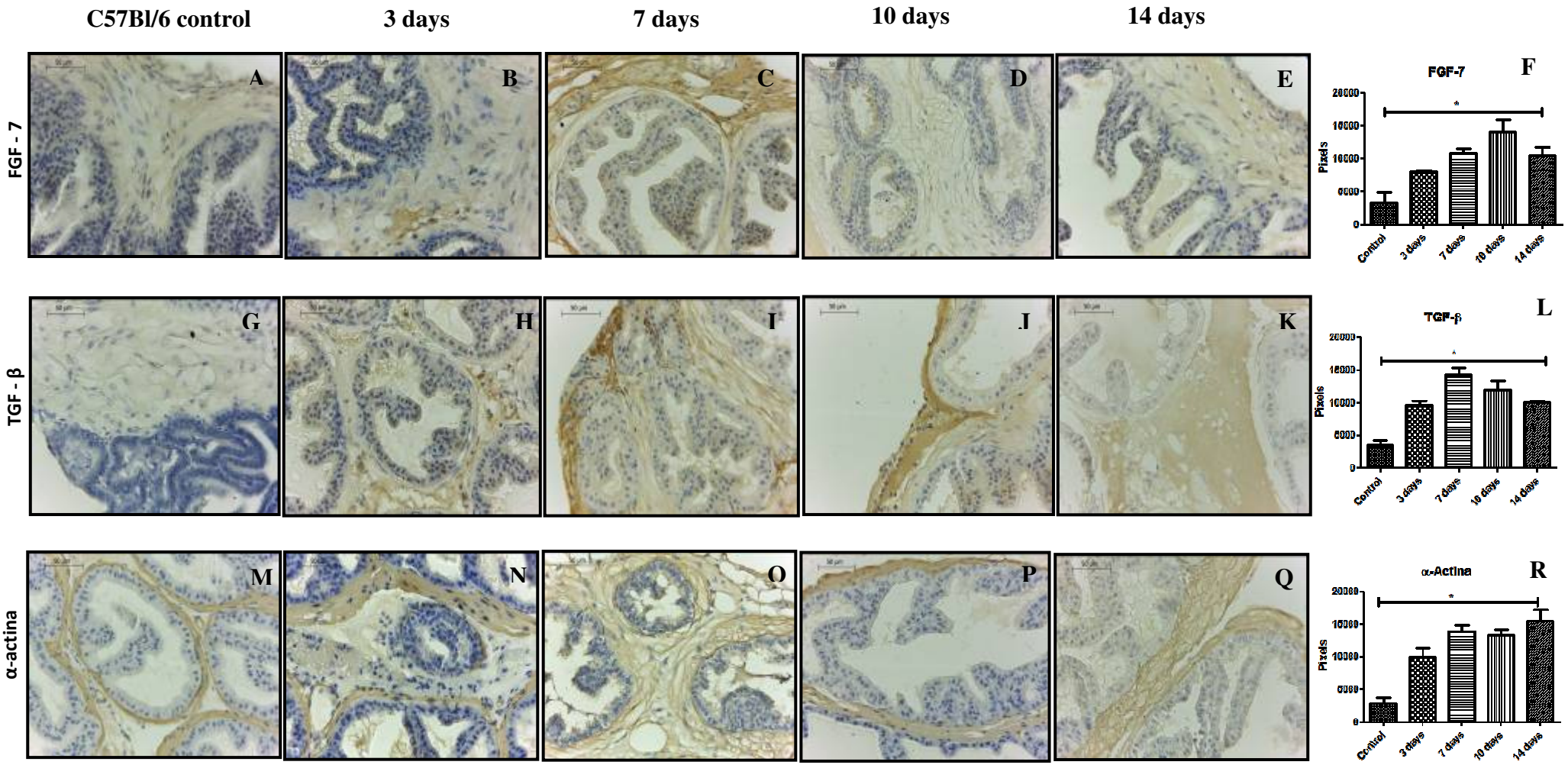

Figure 9: Immunohistochemical localization of cytokines (FGF-7, TGF-β and α-actina) in prostate tissue of the swiss mice, after induced by LPS. (B-E) TGF-7 showing cytoplasmic positive staining of glandular cells. (H-K) TGF-β positive labeling was detected: secreted, extracellular space and extracellular matrix. (N-Q) α-actin positive labeling was detected in stromal region. (A and G) the control groups no positive labeling, (M) α-actin was detected little labeling. (F, L and R) data expressed as mean ± SD, *($p < 0.05$).

4 DISCUSSION

Experimental animal models are necessary to understand the mechanisms of action and the molecular pathways of human disease, allowing the possibility of discovering the signaling pathways associated with various diseases. Although several experimental models of prostatitis currently exist, all have limitations (Vykhovanets et al., 2007). Mouse prostatitis models may be advantageous as there are several strains of knockout mouse available for different genes. The mouse bacterial prostatitis model can induce inflammation after intraurethral injection of $2x10^6$ *E. coli* (Boehm et al., 2012). However, some animals can develop severe acute inflammation in the prostatitis or die of sepsis. In addition, although the bacterial prostatitis model reproduces the human bacterial prostatitis, as it can be caused by urine reflux, it is not useful for analyzing the mechanism of action of anti-inflammatory drugs, where there is a need to test co-treatment with antibiotics. Therefore the bacterial prostatic inflammation model proposed in the present study is an alternative means of evaluating prostatic inflammation without an infectious component.

The action of LPS in the development of inflammation was measured in Swiss and C57Bl/6 mice through the expression of inflammatory markers and stromal and epithelial proliferation. The purpose of evaluating different strains was based on a susceptibility study that evaluated the resistance and susceptibility of animals following infection with *E. coli* in C57Bl/6 animals (Elkahwaji et al., 2005), the results of which showed no significant prostate infection after 5 days of infection. Contrastingly, Boehm et al., (2012) in a study using C57Bl/6 mice showed that transurethral inoculation of uropathogenic *E. coli* produced a significant inflammatory response and induced epithelial proliferation and reactive hyperplasia. The present study assessed both the C57Bl/6 strain and the Swiss strain, as there is no data in literature regarding the use of

the Swiss strain in a prostatitis model. The results showed that while both strains developed non-bacterial prostatitis, the C57Bl/6 strain displayed the advantage of presenting a knockout series for several genes, which allows the function of different cytokines and growth factors in prostatitis development to be evaluated.

Clinical trials have indicated a positive correlation between inflammation and prostate proliferative diseases such as prostate cancer and BPH (Roehrborn et al., 2006; Nickel et al., 2008). Inflammatory infiltration is present in approximately 40% of cases of patients with BPH, who present a significantly higher risk for BPH progression and acute urinary retention (Sciarra et al., 2007; Elkahwaji et al., 2007). In the present study inflammatory infiltration, acinar and vascular changes and interstitial fibrosis were observed, findings similar to those observed in a recent study of bacterial prostatitis induced by *E. coli* (Boehn, et al., 2012). In addition, histopathological and ultrastructural analysis after LPS induction showed a proliferation of epithelial and stromal cells, results which were subsequently confirmed by Feulgen staining, a specific marker for mitotic activity, as well as by FGF-7 expression, which is a fibroblast growth factor that acts as a mitogen for prostatic stromal cells (Peehl and Rubin, 1995). Therefore, the LPS-induced prostatitis promoted inflammation, epithelial and stromal hyperplasia and interstitial fibrosis, and could be an alternative strategy for the study of prostatic inflammation.

Inflammation contributes to the pathogenesis of BPH/LUTS by inducing prostatic fibrosis (Ma et al., 2012; Cantiello et al., 2013). Recent studies have showed that chronic prostatic inflammation induced by the instillation of *E. coli* resulted in a significant increase in prostate collage content, supporting the theory of a role for inflammation in prostatic fibrosis (Wong et al., 2014; 2015). LPS-induced prostatitis

also promoted collagen deposition and fibrosis, observed in ultrastructural analysis, as well as by TGF-β and α-actin expression.

In vivo and *in vitro* studies have shown that LPS activates the immune system by stimulating pro-inflammatory cytokines and growth factors (Lohrer et al., 2000; Ikezoe et al., 2003;Kwark et al., 2005; Iglesias-Gato et al., 2012). In the present study, the influence of LPS in the activation of the expression of cytokines and growth factors in prostatic tissue in a new non-bacterial prostatitis model was analyzed. It was observed that the intraurethral injection of LPS induced the activation of pro-inflammatory cytokines (IL-1β, IL-6, IL-17), growth factors (TGF-β, FGF-7) and α-actin. These cytokines were thought to induce inflammatory response, fibromuscular growth and the proliferation of prostatic stromal or epithelial cells by an autocrine or paracrine loop, or via induction of COX-2 expression (Hamid et al., 2011). IL-17 up-regulates the secretion of other proinflammatory cytokines such as IL-1, IL-8 and IL-6, as well as TGF-β. IL-6 is recognized as a potent growth factor for prostatic epithelial and stromal cells. Interestingly, the features of inflammation associated with BPH are characterized by an abundance of T cells and an increase of cytokines such as IL-17, IL-1, and IL-6 (Krammer et al., 2007; Steiner et al, 2003).

Conclusion

Based on the findings of the present study, it can be concluded that the intraurethral administration of LPS induced inflammation, epithelial and stromal proliferation as well as fibrosis, and therefore, constitutes an efficient experimental model of prostate inflammation.

This mouse model of LPS-induced prostatitis will be useful for additional studies seeking to define new potential therapeutic strategies for prostatic inflammation.

Acknowledgements

This study was supported by the Fundação Oswaldo Cruz (FIOCRUZ) and the Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE).

Conflict of interest

The authors declare that no conflicts of interest exist regarding the publication of this article.

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Avaliação dos efeitos do inibidor (Sildenafil) em um modelo experimental de prostatite

Gomes, F.O.S

Artigo III

5.3 Pergunta condutora 3: O tratamento com Sildenafil reduz a inflamação após indução por LPS?

Artigo III (Manuscrito em fase de redação)

Fator de impacto: 2,68

Avaliação dos efeitos do inibidor (Sildenafil) em um modelo experimental de prostatite

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Resumo

O efeito do inibidor de fosfodiesterase-5, citrato de Sildenafil, na inflamação vem sendo amplamente abordado na literatura. O Sildenafil exerce um papel importante por atenuar a inflamação e o estresse oxidativo através da inibição do NF-kB e ativação da via NO-guanilato ciclase/cGMP. A melhora clínica da hiperplasia prostática benigna e nos sintomas do trato urinário inferior promovida pelo Sildenafil tem sido atribuída ao aumento dos níveis de GMPc/PKG. Devido à ausência de dados na literatura, o presente estudo se propõe a avaliar o efeito do tratamento crônico do Sildenafil em modelo de prostatite induzida por LPS. A inflamação foi evidente em todos os animais que foram submetidos à indução intrauretral por LPS, caracterizada pelo infiltrado inflamatório, atipia celular e acinar. O tratamento com LPS também promoveu o aumento da expressão de α-actina, COX-2, NFK-κβ e citocinas pró-inflamatórias (IL-1β, IL-6, IL-17) e do fator de crescimento tecidual (FGF-7). A próstata dos animais tratados com 25mg/kg de Sildenafil apresentou características morfológicas semelhantes aos dos animais controle, com redução efetiva da inflamação. Da mesma forma, os animais tratados com Sildenafil apresentaram redução significativa de α-actina, COX-2, NFK-κB, IL-1β, IL-6, IL-17 e FGF-7. Desta forma, o Sildenafil pode representar uma estratégia farmacológica para o tratamento de doenças inflamatórias crônicas do trato urogenital, no entanto estudos adicionais são necessários para elucidar o mecanismo de ação do Sildenafil em diferentes vias no processo inflamatório.

Palavra chave: Sildenafil, inflamação crônica, prostatite, citocinas

1 Introdução

Por muitas décadas a inflamação na glândula prostática, prostatite, foi subestimada quanto ao seu potencial patogênico. No entanto, com o advento da biologia molecular, além dos estudos experimentais e clínicos suportam cada vez mais a ideia de que a inflamação desempenha importante papel no desenvolvimento e progressão das doenças prostáticas, especialmente Hiperplasia prostática benigna (BPH) e câncer.

Na maioria dos casos, a etiopatogenia da prostatite não é clara. Existem várias fontes potenciais para o desencadeamento do processo inflamatório, incluindo infecção direta ocasionada pelo refluxo de urina, trauma químico e físico, fatores dietéticos, estrogênios, ou uma combinação de dois ou mais desses fatores (DeMarzo et al., 2007). De acordo com o National Institutes of Health (NIH), a prostatite é classificada em prostatite aguda e crônica bacteriana, prostatite crônica/síndrome da dor pélvica crônica, e prostatite assintomática. A estratégia terapêutica é baseada na etiologia, e as farmacoterapias mais utilizadas são antibióticos, anti-inflamatórios e alfa-bloqueadores (Thakkinstian et al., 2012).

Nos últimos anos, dados da literatura têm sugerido os inibidores de fosfodiesterases como fármacos promissores para o tratamento de doenças inflamatórias (Chong et., 2013; Azam et al., 2014; Song and Tang, 2014, Bäck and Hansson, 2015). Os inibidores de fosfodiesterases-5 (PDE-5) (Vardenafil, Tadalafil e Sildenafil) vêm despertando interesse por atuarem em vias específicas com relevância para as doenças do trato geniturinário, tais como, NO/GMPc. Os inibidores de PDE-5 potencializam o efeito do óxido nítrico (NO) sobre o músculo liso e consequentemente promovem a elevação intracelular de cGMP, que por sua vez, inibe a via de sinalização RhoA/Rho-quinase, mediando o relaxamento das células musculares lisas, e promovendo a melhora da perfusão sanguínea, bem como reduzindo a hiperatividade autônoma dos nervos aferentes da bexiga e próstata (Kedia et al., 2008; Morelli et al., 2009; Behr-Roussel et al., 2010, Minagawa et al., 2012; Zhang & Park, 2015).

Estudos experimentais têm demonstrado os efeitos benéficos do tratamento crônico com Sildenafil no remodelamento vascular pulmonar e vasodilatação, justificando a sua investigação na hipertensão arterial pulmonar (Wang et al., 2014). Embora desde 2007, estudos clínicos tenham relatados efeitos benéficos do Sildenafil dos Sintomas do Trato Urinário Inferior (LUTS) e BPH, há poucos estudos sobre os

efeitos em longo prazo avaliando a segurança, eficácia e influência sobre a progressão dessas doenças (Gacci et al., 2013).

Devido ao papel da inflamação no desenvolvimento e progressão BPH/LTS, o presente estudo se propõe a avaliar o efeito do Sildenafil em modelo de prostatite abacteriana sobre o dano tecidual e a resposta inflamatória através das análises histológicas, imunohistoquimicas IL-1(GenWay, SanDiego, CA, USA, GWB-BBP232, 1:100) e α-actina (ABCAM, CA, USA, ab5694, 1:50), e de marcadores inflamatórios (COX-2, NFK-κB, IL-1β, IL-6 e IL-17) e do fator de crescimento tecidual (FGF-7).

2 Metodologia

2.1 Indução de prostatite abacteriana (LPS), em C57Bl/6 camundongos

O procedimento foi realizado conforme adaptado de Boehm et al., 2012. Os animais foram anestesiados, a assepsia da área foi feita com álcool 70% e, posteriormente, a inflamação foi induzida durante 30 dias com 200µl de LPS liofilizado reconstituído em PBS estéril (LPS de E. coli, o sorotipo 026-D6; Sigma Chemical Company, St. Louis, MO), via uretral com tubo de polietileno 0,28mm de diâmetro interno e 0,61mm de diâmetro externo (Intramedic™ PE-10).

Os animais dos grupos controle e Sildenafil foram submetidos à cateterização uretral (tubo de polietileno Intramedic™ PE-10) com salina, de modo que todos os animais passassem pelo mesmo estresse decorrente ao uso do cateter. Após 30 dias do esquema terapêutico, os animais foram eutanasiados, e as próstatas foram removidas e processadas para posterior análise. Todos os experimentos foram realizados de acordo com diretrizes (28/2011 - CEUA/Fiocruz).

2.2 Solução de Sildenafil

A solução foi composta por água de beber e Sildenafil (Viagra, Pfizer) na concentração de 25mg/kg/dia, administrada através de garrafas de água monitoradas diariamente. A concentração da droga na água foi ajustada para manutenção da dose (ZHAO et al., 2003).

2.3 Efeito do Sildenafil após indução da inflamação com LPS

Camundongos C57Bl/6 machos, três meses de idade, provenientes do Biotério de Experimentação Animal do Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo

Cruz (FIOCRUZ), foram separados em grupos de 20 animais cada, conforme estabelecido: Controle - PBS intrauretral e água de beber; LPS - Indução intrauretral com 200µl de LPS e água de beber; LPS/Sildenafil-20 - Indução com 200µl de LPS e tratamento com Sildenafil na concentração de 25 mg/kg, durante 20 dias.

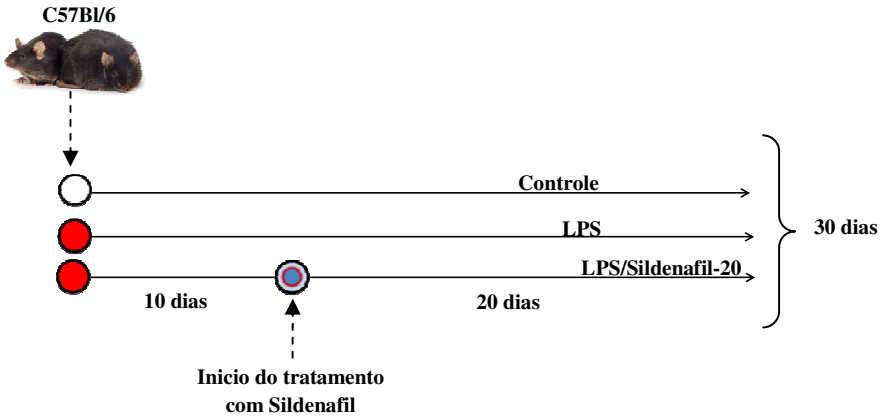

Figura 1: Delineamento experimental. Circulo branco: animal controle; vermelho: administração intrauretral de LPS durante 30 dias; azul: animal tratado com Sildenafil nos últimos 20 dias durante a indução por LPS.

2.4 Análise histopatológica

Os fragmentos foram lavados duas vezes em PBS pH 7.2 e fixados por 8 horas em Bouin (Ácido Pícrico saturado 1%, Formol a 40% e Ácido acético glacial), desidratados em série crescentes de etanol, diafanizados em Xilol, incluídos e emblocados em parafina purificada (VETEC). A seguir, as amostras foram cortadas em Micrótomo (Leica RM2125RT) e coradas com Hematoxilina e Eosina..

2.5 *Localização de IL-1β e α-actina*

Para a análise imunohistoquímica foram utilizadas quatro próstatas para cada grupo (controle, LPS e LPS/Sildenafil20). Após fixação, as amostras foram incluídas e emblocadas em parafina purificada (VETEC). Para aumentar a exposição dos epítopos, os cortes foram aquecidos durante 30 minutos em tampão citrato de sódio (0,01 M, pH 6,0). A atividade da peroxidase endógena foi minimizada através da incubação com 0,3% (v/v) de H_2O_2 em água por cinco minutos. Os cortes foram lavados com PBS 0,01 M (pH 7,2) e, em seguida, bloqueados com BSA 1%, 0,2% Tween 20 em PBS, por 1h

em temperatura ambiente. Posteriormente, os cortes foram incubados overnight a 4 ºC com os anticorpos primários (anti-IL-1β e anti α-actina) na concentração 1:100. A reação antígeno-anticorpo foi visualizada com avidina-biotina peroxidase (Dako LSAB ® + Universal Kit, peroxidase) utilizando 3,3-diaminobenzidina como cromógeno. As lâminas foram então contra-coradas com hematoxilina.

2.6 Análise da expressão dos marcadores inflamatórios COX-2, IL-17, TGF-β, e NF-κB e do fator de crescimento de fibroblatos FGF-7

Para análise da expressão das proteínas por Western blot, o processamento foi feito segundo CRUZ-HÖFLING et al. (2009): oito próstatas de cada grupo foram homogeneizadas em um coquetel de extração (10mM de EDTA, 2 mM de PMSF, 100 mM de NaF, 10mM de pirofosfato de sódio, 10 mM de NaVO4, 10 mg de aprotinina/ml e 100mM de Tris, pH 7,4). Os homogenatos foram centrifugados à 3000xg por 10 min e o sobrenadante foi coletado e estocado à -70ºC até o momento do uso para imunoblotting. As proteínas (40µg) foram separadas em géis de duodecil sulfato de sódio-poliacrilamida, nas concentrações conforme o peso da proteína de interesse, por eletroforese em gel, sob condições reduzidas, e foram transferidas eletroforeticamente para uma membrana de nitrocelulose (BioRad, CA, USA). Após bloqueio overnight à 4ºC com 5% leite desnatado em TBS-T (Tampão Trissalina 0,1% adicionado com Tween20, pH 7,4), as membranas foram incubadas à temperatura ambiente, por 4 h, com os anticorpos policlonais, produzidos em coelho, contra: COX-2, IL-17, , TGF-β, FGF-7, diluídos em solução tampão TBS-T, contendo 3% de leite desnatado. Após lavagens em TBS-T, as membranas reagirão com anticorpos secundários anti-coelho IgG, conjugado com Labeled peroxidase (H+L) 1:5000, Sigma, diluído em TBS-T com 1% de leite desnatado, por 1 h, à TA. Um agente revelador quimioluminescente foi usado para tornar as bandas protéicas visíveis e os blots foram revelados em filme de raio-X (Fuji Medical, Kodak). Para quantificação, a densidade de pixels de cada banda foi determinada usando o programa Image J 1.44. Para cada proteína investigada, os resultados foram confirmados em triplicata. Foi feito imunoblot para β-actina, como controle da expressão das proteínas estudadas. Para isso, após visualização das bandas com o reagente qumioluminescente (Luminol), as proteínas foram removidas das membranas, as quais foram submetidas ao anticorpo policlonal contra β-actina (1:250; Sigma, USA) e, em seguida, realizada a densitometria.

2.7 Análise Estatística

Os dados foram analisados através do ANOVA one-way, seguido de um teste a posteriori de Dunnet e Tukey usando o Graph Pad Prism v.05.

3 Resultados

3.1 Análise histopatológica e da imunoexpressão de IL-1β e α-actina

A inflamação prostática em animais do grupo LPS foi comparada com controles e co os que receberam tratamento com Sildenafil nos últimos 20 dias do inicio da indução. A próstata dos camundongos do grupo controle apresentou morfologia carcterística, tais como: células epiteliais cúbicas e células estromais fusiformes distribuídas de forma homogênea, e região glandular com ácinos apresentando projeções papiliformes (Figura 2A). Os animais induzidos com LPS apresentaram infiltrados inflamatórios, hiperplasia, focos hemorrágicos, atipia estromal e epitelial, alterações acinares, e presença de estroma denso, caraterizando fibrose (Figura 2D). Por outro lado, os animais tratados com 25mg/kg de Sildenafil apresentaram redução significativa da inflamação, com características morfológicas semelhante aos dos animais do grupo controle (Figura 2 G).

A α-actina é uma proteína estrutural característica de miofibroblastos (Liu et al., 2011). O grupo controle apresentou níveis basais α-actina. Por outro lado, o grupo induzido com LP apresentou aumento significativo da expressão α-actina na região estromal. A expressão de α-actina foi significativamente diminuída no grupo tratado com Sildenafil (Figura 2 B, E, H e J).

A IL-1β é uma das principais citocinas pró-inflamatórias na próstata (Jerde and Bushman, 2010). O grupo controle apresentou baixa reatividade para IL-1β nas células epiteilais e estromais. Por outro lado, o grupo induzido com LPS, apresentou aumento significativo da imunoexpressão de IL-1β na região estromal e glandular. O tratamento com Sildenafil reduziu significativamente a marcação prostática para IL-1β (Figura 2 C, F, I e K).

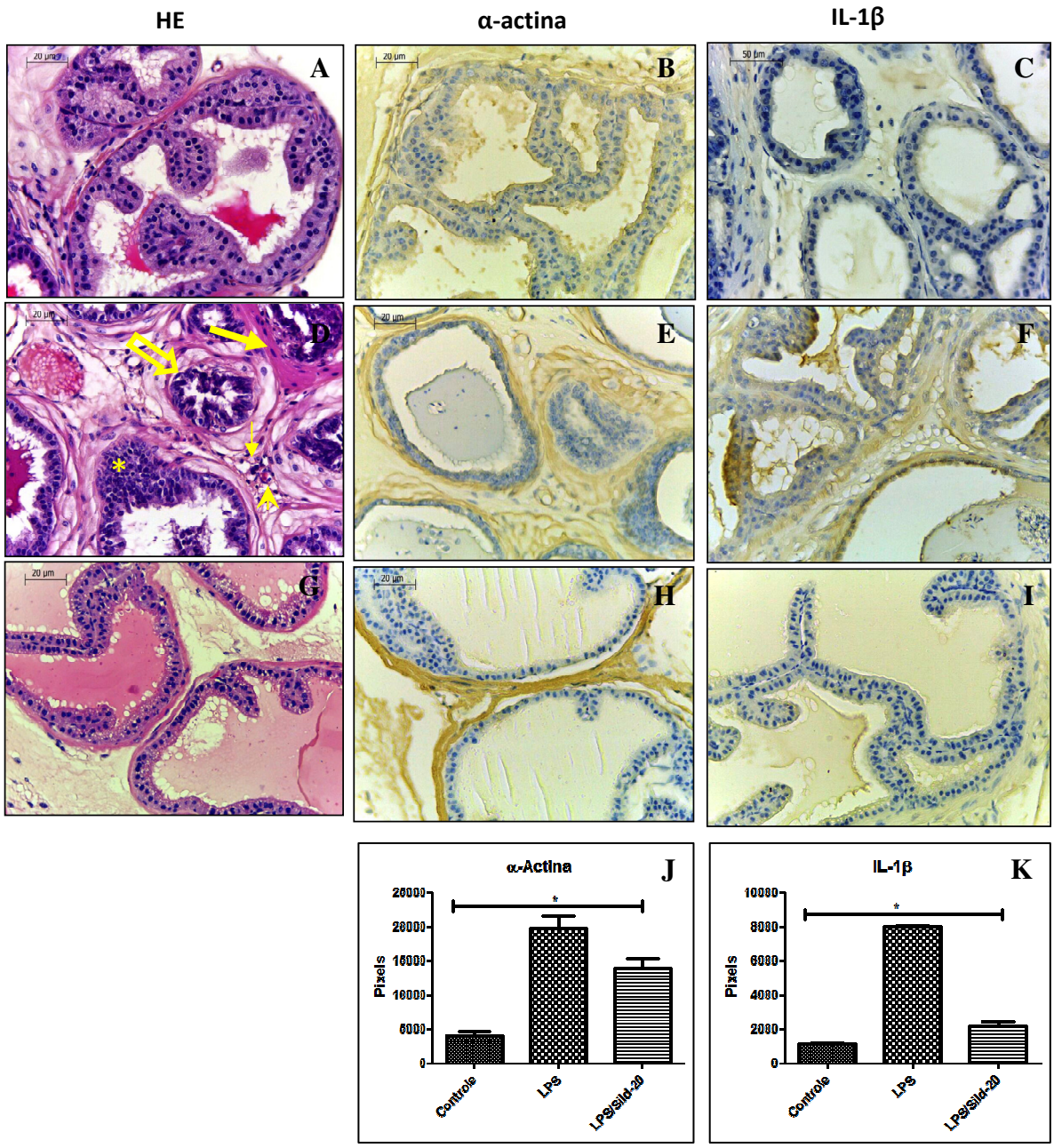

Figura 2: Análise histológica e imunohistoquímica da próstata de camundongos C57Bl/6, após indução intrauretral com LPS. HE – controle (A); LPS (D) e Sildenafil (G). (D) área com infiltrado inflamatório focal (seta curta); foco hemorrágico (ponta-de seta); desorganização acinar (seta aberta); proliferação celular (asterisco); estroma extremamente denso, indicando um processo fibrótico (seta longa). Imuhistoquímica para α-actina (B, E, H) e quantificação da marcação (J). Imuhistoquímica para IL-1 (C, F, I) e quantificação da marcação (K). Os resultados são expressos como média ± SD, *($p<0,05$), ANOVA com teste de Tukey post hoc.

3.2 Ação do Sildenafil sobre a imunoexpressão de COX-2, IL-17, IL-1, FGF-7 e NF-κB em modelo de hiperplasia/prostatite induzido por LPS.

A via ciclooxigenase 2 (COX-2) é responsável pela síntese de prostaglandinas, prostaciclinas e tromboxano. Na inflamação crônicas níveis elevadosde Cox-2 sãoproduzidos continuamente (Sciarra et al., 2008; Krammer et al., 2002). A próstata dos animais controle apresentou níveis basais de COX-2, enquanto que os animais com inflamação prostática induzida por LPS, apresentou níveis significativamente elevados. Por outro lado, animais do grupo LPS/Sildenafil-20 reduziu os níveis de COX-2 de forma significativa quando comparado com os grupos LPS e controle (Figura 3A).

O fator de transcrição nuclear κB (NF-κB) é um elemento-chave na regulação das citocinas pró-inflamatórias. A indução da inflamação prostática com LPS induziu uma redução da expressão de NF-κB quando comparado com os grupos controle e LPS/Sildenafil-20, entretanto esses dados não foram estatiscamente significativos. Da mesma forma, o grupo LPS/Sildenafil-20 reduziu os níveis de NF-κB quando comparado com o grupo com LPS e o grupo, embora de forma não sifginificativa (Figura 3B).

IL-17 regula a secreção de outras citocinas pró-inflamatórias, tais como IL-1β, IL-6 e TGF-β (Steiner et al., 2003; Hamid et al., 2011). A próstata dos animais do grupo controle apresentou níveis basais de IL-17. No entanto, após a indução por LPS, ocorreu uma expressão significativa de IL-17. A próstata dos animais do grupo LPS/Sildenafil-20 apresentaram redução dos níveis de IL-17, quando comparado com os grupo LPS, sendo entretanto, semelhante ao grupo controle (Figura 3C).

TGF-β é um fator de crescimento importante para o desenvolvimento da fibrose intersticial. Os animais do grupo LPS apresentaram uma redução da expressão de TGF-β quando comparados com o grupo controle. Por sua vez o grupo LPS/Sildenafil-20 reduziu os níveis de TGF-β quando comparado com os demais grupos (Figura D).

FGF-7 é um fator de crescimento de fibroblastos que atua como um agente mitogênico para células do estroma prostático (Peehl e Rubin, 1995). A expressão de FGF-7 dos animais do grupo LPS aumentou significativamente em relação aos grupos controle e LPS/Sildenafil-20. O tratamento com Sildenafil reduziu os níveis de FGF-7 quando comparado com o grupo LPS (Figura 3E).

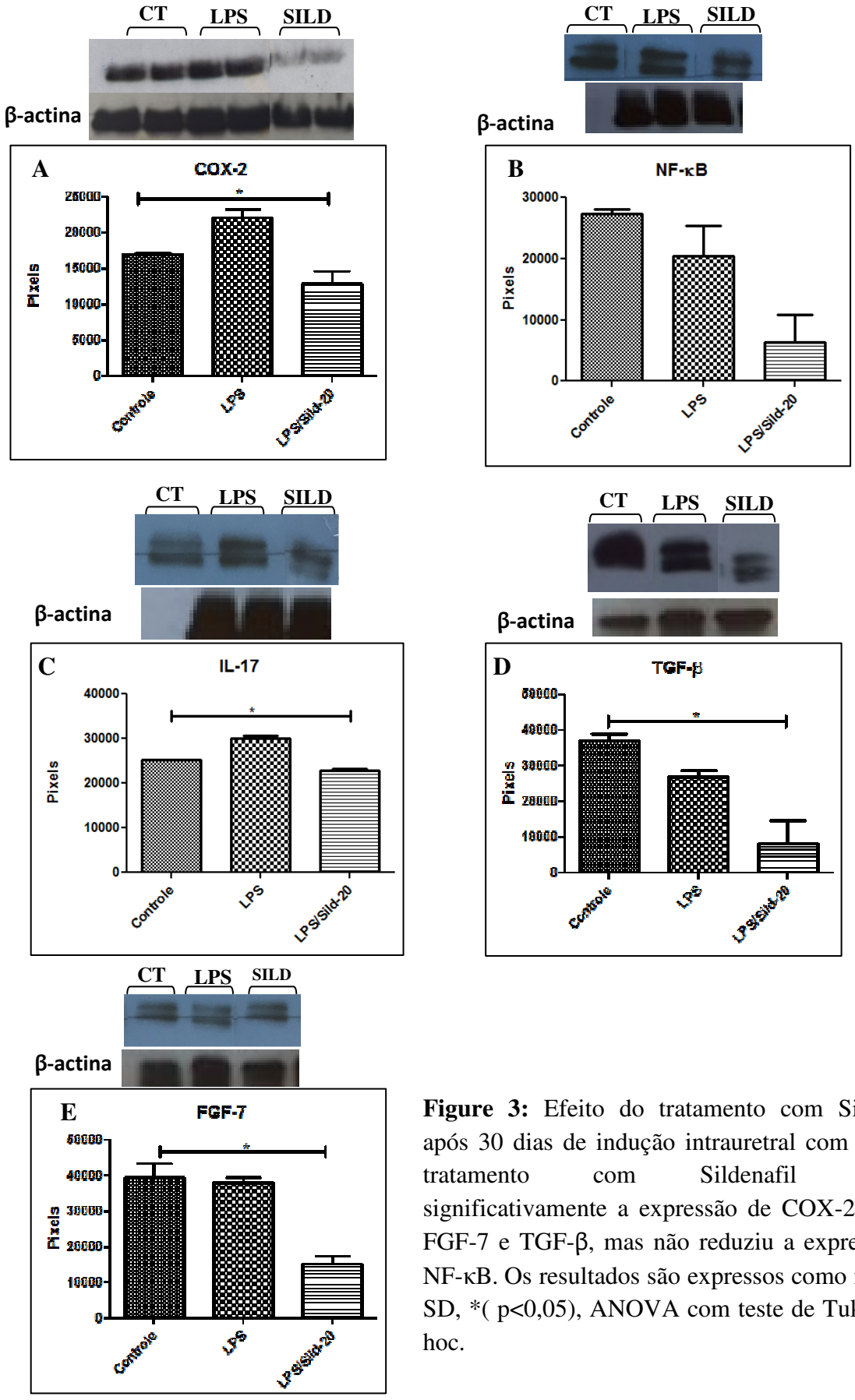

Figure 3: Efeito do tratamento com Sildenafil após 30 dias de indução intrauretral com LPS. O tratamento com Sildenafil reduziu significativamente a expressão de COX-2, IL-17, FGF-7 e TGF-β, mas não reduziu a expressão de NF-κB. Os resultados são expressos como média ± SD, *(p<0,05), ANOVA com teste de Tukey post hoc.

4 Discussão

A ciência básica e estudos pré-clínicos demonstraram que a inflamação é um fator etiológico importante para o desenvolvimento de doenças prostáticas. O papel da inflamação em doenças prostáticas é sugerido pela presença de células inflamatórias na próstata de pacientes com hiperplasia e câncer (Novara et al., 2006; Nickel, 2007; Delongchamps et al., 2008).

O lipopolissacárido (LPS) é um componente da parede celular de bactérias gram-negativas (Ikezoe et al., 2003). Estudos têm mostrado que o LPS é um potente ativador do sistema imune, por induzir a libertação de fator de necrose tumoral (TNF-α), interleucinas (IL-1 e IL-6), fator nuclear (NF-κB), macrófagos e monócitos (van Deventer et al., 1990; Lawrence et al., 2001; Beutler, 2002). O presente estudo utilizou um novo modelo de prostatite não-bacteriana recentemente caracterizado por Gomes et al., (a ser submetido). Confirmando dados anteriores, a análise histopatológica demonstrou que a prostatite induzida por LPS apresentou infiltrados inflamatórios, focos hemorrágicos, hiperplasia estromal e epitelial, bem como fibrose intersticial. Da mesma forma, a injeção intrauretral de LPS induziu a ativação dos fatores pró-inflamatórios (IL-1β COX-2, IL-17, NF-kB), fatores de crescimento (TGF-β,FGF-7) e α-actina. Esses fatores são reconhecidos por induzir uma resposta inflamatória, crescimento fibromuscular e proliferação das células do estroma ou células epiteliais através de resposta autócrina ou parácrina ou através da indução da COX-2 (Hamid et al., 2011).

Na próstata, a COX-2 pode aumenta o potencial carcinogênico das células através da oxidação de pró-carcinogênicos a carcinogênicos, aumento do crescimento celular, diminuição da apoptose, bem como a diminuição da resposta imune nas células anormais ou cancerosas (Pathak et al., 2005; Sarkar et al., 2007). Hsu et al., mostrou que as células epiteliais prostáticas normais não expressam níveis significativos de COX-2 (Hsu et al., 2000). Muitos estudos têm mostrado que a expressão de COX - 2 no câncer de próstata, em comparação em HBP (Konig et al., 2004; Wang et al., 2004). Níveis elevados de COX-2 foram induzidos pela injeção intrauretral de LPS, confirmando o estabelecimento da resposta inflamatória. Por sua vez, o tratamento com sildenafil reduziu de forma evidente a expressão de COX-2.

O NF-kB é conhecido como um fator de transcrição importante no processo inflamatório, altamente ativo em macrófagos. A translocação do NF-kB para o núcleo ativa genes responsáveis pela resposta imunológica, inflamação, proliferação e

migração celular, e apoptose. NF-kB pode potencializar a amplificação da resposta inflamatória no ambiente tumoral (Sarkar et al., 2007; Nunez et al., 2008). Além disso, a desregulação da transcrição fator NF-kB tem sido proposta como um mecanismo molecular que leva à inflamação crônica e câncer (Wong et al., 2009).

A contribuição da IL-17 na patogênese da próstata não está totalmente elucidada. Recentemente, um estudo mostrou que a desregulação da produção de IL-17 observado nas células T senescentes pode contribuir diretamente para o aumento do risco de câncer de próstata em idosos (De Angulo et al., 2015). Em modelo murino, Zhang et al. relataram que a IL-17 promove a formação e crescimento do adenocarcinoma da próstata (Zhang et al., 2012). Além disso, a IL-17 pode indiretamente promover a diferenciação de macrófagos M2, através da estimulação da via COX-2/PGE2 nas células cancerígenas (Li et al., 2014). IL-17 pode também aumentar a expressão de moléculas de adesão, incluindo molécula de adesão intercelular-1 (ICAM-1), VCAM-1 e E-selectina em células endoteliais (Roussel et al, 2010;. Xing et al, 2013.). Finalmente, IL-17 regula a secreção de outras citocinas pró-inflamatórias, tais como IL-1, IL-8 e IL-6, bem como o fator de crescimento TGF-β. Interessantemente, as citocinas IL-8 e IL-6 são fatores de crescimento importantes para células epiteliais e do estroma prostático (Novara et al., 2006; Lucia e Lambert, 2008).

A interleucina-1 (IL-1) estimula a sinalização durante o desenvolvimento da próstata e a resposta hiperplásica na inflamação (Jerde e Bushman, 2009). IL-1β é um mediador importante da inflamação relacionada com o câncer, secretada por células imunológica, estromais e tumorais (Germano et al., 2008), e regula a expressão de genes importantes na inflamação e câncer (Kasza et al., 2013). Além disso, a IL-1β induz a regulação de NF-kB na próstata de camundongos que pode ser responsável pelo extravasamento de leucócitos no estroma da próstata (Vykhovanets et al., 2009). Da mesma forma, foi demonstrado que a inibição de IL-1β reduz o crescimento do tumor de forma estável, limitando a inflamação e induzindo a maturação de células mielóides imaturas em macrófagos M1 (Carmi et al., 2013). A prostatite induzida por LPS induziu elevada expressão de IL-17 e IL-1β, confirmando dados anteriores (Gomes et al., 2015). Por outro lado, o tratamento com sildenafil reduziu de forma significativa a expressão de ambas as citocinas, confirmando a ação anti-inflamatória deste fármaco descrita em outras patologias (Karakoyun et al., 2011; Wang et al., 2014).

O TGF-β apresenta um importante papel na sinalização mediada entre células estromais e epiteliais na próstata, sendo também crítico na remodelação do músculo liso

prostático (Alonso-Madalena et al., 2009). No câncer de próstata, TGF-β pode estar envolvido na alteração do metabolismo de esteróides (Gray et al., 2009), na proliferação de células (BaoHan et al., 2013), apoptose (Yang et al., 2010), e também na indução de um fenótipo reativo das células estromais, que mudam seu fenótipo de células musculares para um fenótipo de miofibroblastos que expressam actina e vimentina (Liu et al., 2011), e também componentes da matriz extracelular que secretam o colagéno I e fibronectina (Heitzer e DeFranco, 2007). O FGF-7, por sua vez, atua como mediador do estroma andrógeno com um efeito parácrino mitogênico do epitélio. A desregulação dos fatores de crescimento tem sido sugerido no desenvolvimento de BPH e câncer de próstata em homens idosos (Smith et al., 2004).

a indução da inflamação prostática por LPS por 30 dias não induziu um aumento a expressão de TGF-β e FGF-7, o que pode ser explicado por um efeito tempo dependente, cujos níveis elevados foram atingidos durante os 15 dias iniciais da indução, conforme obervado previamente. A elevada imunomarcação para α-actina no grupo LPS, confirma que em algum momento da indução por LPS houve produção destes fatores de crescimento. Por sua vez, o tratamento com sildenafil foi capaz de reduzir a expressão de destes fatores de crescimento e de α-actina, confirmando que este fármaco foi eficiente em reduzir a fibrose prostática.

Conclusivamente, os resultados do presente estudo demonstram que o sildenafil pode representar uma alternativa farmacológica para o tratamento inflamação prostática e seu consequente desenvolvimento e progressão BPH/LTS, contribuindo para uma melhor qualidade vida destes paciente.

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The Role of Phospodiesterase-5 Inhibitors in Prostatic Inflammation: a Review

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Artigo IV

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Peixoto and Gomes *Journal of Inflammation* (2015) 12:54
DOI 10.1186/s12950-015-0099-7

REVIEW Open Access

The role of phosphodiesterase-5 inhibitors in prostatic inflammation: a review

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Abstract

Clinical and basic experimental evidence indicates that chronic inflammation is the greatest factor in benign prostatic hyperplasia (BPH) progression, which is the most common cause of Lower Urinary Tract Symptoms (LUTS). The use of anti-inflammatory agents such as steroids, cyclooxygenase-2 (COX-2) and phytotherapics have been investigated as forms of treatment for various prostate diseases. Recent evidence has demonstrated that PDE5 inhibitors (PDE5Is) improve symptoms of BPH / LUTS, possibly as a result of the relaxing of the smooth muscle fibers of the bladder and prostate by NO/cGMPc signaling, or by improving RhoA/Rho-kinase (ROCK), and reduction of the hyperactivity of the autonomic nervous system. However, some results have suggested that besides vasodilatation and their anti-proliferative effect, PDE5Is exert a direct anti-inflammatory effect, by raising cGMP. Given that inflammation is major factor in benign prostatic hyperplasia (BPH) progression, PDE5Is could act also restore prostatic function as they act as potent anti-inflammatory drugs. This review aims to provide a comprehensive summary of the use of phosphodiesterase-5 inhibitors to treat prostatic inflammation.

Keywords: phosphodiesterase-5 inhibitors; BPH; LUTS; inflammation

1 Introduction

The cyclic monophosphate nucleotides cAMP and cGMP are synthesized by the guanylyl and adenylyl cyclase enzymes, respectively. They are recognized as important second messengers of extracellular signals. Through cyclic dependent protein kinases, PKA and PKG control physiological functions, such as retinal phototransduction, smooth muscle relaxation, cardiac contractility, neuroendocrine signals, inhibition of platelet aggregation, degranulation of neutrophils, and myelogenic inflammatory response [1-5]. The critical role of cAMP and cGMP in intracellular signaling pathways has identified them as potential therapeutic targets [6,7].

Mammalian phosphodiesterases (PDEs) comprise a large group of enzymes that hydrolyze cAMP and cGMP to their inactive forms 5'-GMP and 5´-AMP [8,9]. Eleven PDE families have been described in order of discovery, amino acid sequence and catalytic and regulatory characteristics. Some PDEs, such as PDE4 and PDE7 are highly specific for cAMP hydrolysis, whereas PDE5, PDE6 and PDE9 are specific for cGMP. Some PDEs, such as PDE1, PDE2 and PDE3 hydrolyze both nucleotides [10]. Therefore, inhibitors of PDE (PDEIs) can prolong the intracellular action of cAMP and cGMP. There are potent non-selective PDEi, such as theophiline, and others that are specific, such as Rolipram for PDE4, and Sildenafil, Tadalafil and Vardenafil for PDE5 [7].

Potent and selective PDE5 inhibitors have been approved for therapeutic use for the condition of erectile dysfunction [11] and are currently also being used in the treatment of pulmonary hypertension [12,13] and Raynaud's phenomenon [14].

cGMP accumulation may inhibit inflammation, and as such it is a potential tool against the evolution of diseases in which inflammation plays a central role [15,16,8,17-19].

Chronic prostatitis is characterized by pelvic and genitourinary pain and lower urinary tract symptoms (LUTS) that affect frequency and urgency and causes dysuria. Its precise aethiology remains unclear, although some possibilities include undetectable infection, trauma and immunological origin. Therapeutic management includes antibiotics and α-adrenergic blockers, but until now, no definitive treatment has been identified [20].

In this way, phosphodiesterase-5 inhibitors may not only mediate smooth muscle relaxation, but also can directly reduce prostatic inflammation by increasing cGMP levels.

2. Phosphodiesterase-5 and Inflammation

The nitric oxide (NO) is a highly reactive molecule with diverse physiological functions [21]. This messenger plays important roles in the modulation of vascular tone [22], neurotransmission [23,24] and the immune system [25,26]. NO is formed from L-arginine by NO synthases (NOS). In addition to the constitutive forms of the enzyme, endothelial (eNOS or NOS3) and neuronal (nNOS ou NOS1), the inducible form (iNOS or NOS2) is found in activated macrophages and other immune cells that produce NO in the micromolar range [27]. At these concentrations, NO induced oxidative DNA damage and modified protein structure and function, which can lead to cell death. Controversially, eNOS produces NO at nanomolar concentrations, which have been documented to have anti-inflammatory actions [28], which appear to be related directly or indirectly to the inhibition of the key transcription factor Nuclear Factor κB (NF-κB) [29,30].

NO is also the main activator of soluble guanylyl cyclase (sGC), an enzyme that synthesizes cGMP. The level of cGMP is regulated by phosphodiesterases type 5 (PDE5s) enzymes, which break down the phosphodiesteric bond of cGMP. Several PDE5 inhibitors (PDE5Is) have been developed and used as therapeutic agents, as they increase cyclic nucleotide levels by blocking PDE function, enhancing NO-cGMP signalization [7].

Current concepts based on clinical and experimental data support a link between endothelial dysfunction and inflammation, manifested as deficiencies in the production of NO and prostacyclin [31]. The chronic consequence of endothelial dysfunction is initiation of vascular diseases. Sildenafil, the selective PDE5 inhibitor, widely used for treatment of erectile dysfunction in humans (Viagra®, Pfizer) has been shown to improve NOS activation of endothelial cells through ERK signaling [32]. In addition to increasing NO production by eNOS activation, Sildenafil also reduces the oxidative stress induced by hyperglicaemia and insulin resistance conditions [33]. Sildenafil also stimulates eNOS mRNA transcription in cardiomyocytes, resulting in increased expression of eNOS, elevated NO generation, guanylyl cyclase activation and enhanced GMPc formation [34]. Therefore, Sildenafil can elevate cGMP in two alternative ways: inhibiting PDE5 enzymes and/or inducing mRNA expression of eNOS.

Interestingly, acute and short-term administration of Sildenafil improves endothelial function in men with Type 2 diabetes [35], whereas chronic administration of Sildenafil, besides significantly improving endothelial function, can also reduce inflammatory markers (nitrite/nitrate levels, C-reactive protein, IL-6, ICAM-1 and VCAM-1) in patients with Type 2 diabetes [36]. In addition to improving endothelial function in patients with coronary arterial disease and diabetes, and reducing oxidative

stress in many tissues [37,38], Sildenafil can also normalize endothelial function and decrease plaque deposition in the aorta in experimental models of atherosclerosis [39].

Sildenafil has also been shown to be of potential benefit in the early phases of inflammation and vascular remodeling in a pulmonary arterial hypertension (PAH) experimental model. The administration of Sildenafil following Monocrotaline-induced PAH significantly reduced the severity of inflammation in the acute stage of the disease and prevented pulmonary arterial remodeling. These results suggest that in addition to its vasodilatation and anti-proliferative effects, Sildenafil has a direct anti-inflammatory effect [40].

The beneficial effect of the phosphodiesterase-5 inhibitor has been demonstrated in experimental inflammatory bowel disease (IBD), a relapsing and remitting disease appearing as a form of ulcerative colitis or Crohn's disease with a non-well-known etiology. Treatment of experimental IBD with Sildenafil reduced markers of oxidative stress such as myeloperoxidase (MPO) and lipid peroxidation product (TBARS) significantly, thereby modulating the inflammatory response [18]. Recent studies extended and confirmed the anti-inflammatory effect of PDE5 inhibitor in a rat model of colitis [41]. Other inhibitors of the PDE superfamily have been also proposed for IBD treatment, and PDE4, PDE5 and PDE7 inhibitors seem strong candidates for the next generation of effective drugs [42].

In caecal ligation and puncture (CLP), a model of polymicrobial sepis, PDE5 markedly attenuated injury in vital organs such as the kidney and lungs by inhibiting proinflammatory cytokine response and ROS generation [43].

Increased intracellular cGMP levels also lead to the suppression of colon tumor cell growth and the induction of apoptosis by activating cGMP dependent protein kinase (PKG). The COX inhibitory metabolite of sulindac, sulindac sulfide, as well as several

other NSAIDs, such as indomethacin, meclofenamic acid and celecoxib, also inhibit PDE5 activity, which is closely associated to tumor cell growth [44,45]. Studies involving human clinical specimens which reported higher PDE5 levels in colorectal, bladder, lung, and breast carcinomas than with normal epithelium [46-49] corroborate these results.

Myeloperoxidase (MPO) level in the blood can be considered as a marker of endothelial dysfunction and could be a predictor of cardiovascular disease risk. The oxidation of LDL by MPO (MoxLDL) leads to a specific induction of the inflammatory response, increasing the release of cytokines such as interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) by endothelial cells and monocytes respectively [50]. According to Roumeguère et al (2010) among the three available specific PDE5-Is for treatment of erectile dysfunction (ED), only tadalafil decreased the inflammatory response on endothelial cells stimulated by myeloperoxidase-modified low-density lipoprotein (Mox-LDLs) or tumor necrosis factor alpha. Mox-LDLs have been found in the atherosclerotic plaque but also in the corpus cavernosum of patients with ED of vascular origin [51].

Varma et al., (2012) proposed that PDE5is have cardioprotective effects and also could be used to treat insulin resistance and inflammation. These authors provided evidence that tadalafil therapy reduced circulating inflammatory cytokines in diabetic animal model while improved fasting glucose levels and reduced infarct size after ischemia-reperfusion injury in the heart [52].

cAMP and cGMP may play a protective role in the modulation of some inflammatory cell activities of allergic disorders. Sildenafil inhibits inflammation and airway reactivity in animal models of airway diseases (asthma and chronic obstructive pulmonary disease), the effectiveness of which does not appear to be dependent on

endogenous NO levels [53]. Similarly, vardenafil mimics the effect of NO by increasing GMPc levels with a subsequent reduction of histamine release and mast-cell-mediated allergic reactions [54]. Other studies reported that selective PDE4 inhibitors suppressed hematological and immunological markers of inflammation and were also effective in reducing specific airway resistance [55,56].

The PDE5 inhibitors have also muscle and neuroprotective activities. The administration of Sildenafil after bilateral cavernosal nerve resection preserves penile corporal smooth muscle and ameliorates fibrotic degeneration by down-regulating genes related to fibrosis and up-regulating genes related to smooth muscle preservation [57]. At the level of the pelvic ganglia, Sildenafil exerts a neuroprotective effect by activating neurotrophic factors involved in neuronal survival and regeneration [58].

Garcia et al., (2014) demonstrated that Sildenafil can attenuate inflammation and oxidative stress in damaged cavernosal nerves by modulating cytokine expression and promoting a neuroprotective environment that favors neuron survival. According to such authors, initiation of the treatment immediately after surgery or even before radical prostatectomy would produce a better outcome, by promoting regeneration and functional recovery of the peripheral nerves [59].

Other studies have shown that the selective PDE5 inhibitors raise cGMP levels in the brain and offer protective effects, such as improvement of cognition and memory [60], reduction of neuronal cell death in ischemic cerebrovascular injury [61], reduction of white matter damage and regulation of inflammatory responses in Multiple Sclerosis models [62]. Interestingly, Alzheimer's disease (AD) has been highly associated with cGMP signaling dysfunction and an ongoing inflammatory process. Zhang et al., (2013) demonstrated that Sildenafil prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in APP/PS1 transgenic mice, an AD

experimental model [63]. On the other hand, Garcia-Barroso showed that tadalafil cross the blood-brain barrier and inhibits PDE5 present in the hippocampus, and that tadalafil was more effective than Sildenafil in attenuating the phenotypic traits of a mouse model of AD [64].

Results obtained in our laboratory using a multiple sclerosis (MS) model demonstrated that Sildenafil exerts an effective anti-inflammatory action, greatly reducing levels of IFN-γ, TNF-α, IL-1β, IL-2 and cycloxygenase-2 (COX-2), as well as protecting myelin structure. Therefore, the oral administration of Sildenafil can be a possible therapeutic tool for individuals with MS and other neuroinflammatory/neurodegenerative diseases, providing additional benefits to those of current treatments [4].

2.**Phosphodiesterases and Prostatic Inflammation**

Benign prostatic hyperplasia (BPH) is a common cause of Lower Urinary Tract Symptoms (LUTS), which include poor urinary stream, urinary hesitancy, a feeling of incomplete bladder emptying, urgent and/or frequent urination, and urge incontinence. Approximately 40% of men will have BPH by the age of 50, and 80% by the age of 80 [65].

Clinical and basic experimental evidence indicates that chronic inflammation is the major factor in benign prostatic hyperplasia (BPH) progression. Recognition of prostate secretion products by autoreactive T cells and animal models on experimental prostatitis demonstrate an autoimmune component to chronic inflammation. The close association between activated T cells and stromal cells suggests that these T cells trigger abnormal growth in the prostate as a result of their specific cytokine production. In BPH, these T cell-derived growth factors are strongly up-regulated and have been documented as

stimulating stromal growth, matrix formation and angiogenesis [66]. Consistent with these findings, the REDUCE clinical trial has shown a relationship between prostatic inflammation and prostate volume, and the severity of LUTS [67].

A link between inflammation and prostate proliferative diseases such as BPH and Prostate Cancer (PCa) has also been suggested. Inflammatory infiltration is present in approximately 40% of cases of patients with BPH, who have a significantly higher risk of BPH progression and acute urinary retention. Evidence from genetic studies supports the hypothesis that prostate inflammation may be a cause of PCa development [68].

In several types of human carcinoma, such as colon adenocarcinoma, bladder squamous carcinoma and lung cancers, PDE-5 expression is elevated, suggesting that these enzymes play a role in controlling cellular proliferation and apoptosis mechanisms [49,69]. Additionally, in human prostate cancer cell lines, studies suggested that the increase of cAMP and cGMP initiates morphologic differentiation, inhibiting the growth and the invasive potential of these cells [70,71].

Anti-inflammatory agents, whose effects are promising in terms of inhibition of cell proliferation [72], have been analyzed for the treatment of various prostate diseases such as steroids, cyclooxygenase-2 (COX-2) and phytotherapics. In vitro studies also found evidence of the antiproliferative effect of PDE inhibitors in smooth muscle cells from human BPH tissue [73,74].

Preclinical and clinical studies have provided evidence that PDE5 inhibitors improve symptoms of Benign Prostatic Hyperplasia / Symptoms of Upper Urinary Tract (BPH / LUTS), possibly as a result of their relaxing action via NO mechanisms, and inhibition of prostatic stromal cells proliferation [75-77]. The possible use of PDE5 inhibitors for the treatment of prostate diseases is supported by the presence of PDE5 in

the transition zone of the prostate, together with PDE4 and PDE11 [8], as well as the presence of PDE5 in blood vessels and in the muscular fibers of the bladder and urethra [78].

Several randomized, double-blind, placebo-controlled, multinational trials have investigated the efficacy and safety of tadalafil [79-87] or Sildenafil [88,89,79,90-92] in the treatment of BPH-LUTS, as well as in the treatment of men with ED and with BPH-LUTS, leading to regulatory approval in the USA and Europe.

Nonsystematic and systematic reviews have tried to analyze the role of combined PDE5Is and α-blocker therapy, and have reported a significant improvement in urinary symptoms [76,92-95]. The most remarkable outcome from the first systematic review was that the combination of PDE5Is and α-adrenergic blockers can significantly improve maximum urinary flow rate, compared with only α-adrenergic blockers, whereas PDE5Is only did not increase Qmax, compared with placebo [92].

Similarly, a recent systematic review and network meta-analysis comparing the effectiveness of oral drug therapies for BPH/LUTS revealed that of all the available drug treatments, combination therapy with α1-adrenoceptor antagonists and PDE5 inhibitor ranked highest in efficacy for decreasing the International Prostate Symptom Score (IPSS) total score, storage subscore and voiding subscore. PDE5 inhibitors used alone also had a promising effect, except on maximum flow rate (Qmax). The results suggested that this combination therapy is the most efficient treatment of LUTS/BPH [96].

In 2010, Eryildirim et al. evaluated the effectiveness of Sildenafil citrate on lower urinary system symptoms (LUTS) by using symptom score scales, and by analyzing whether or not the presence of asymptomatic inflammatory prostatitis altered the

symptom scores. Patients were classified as category IV prostatitis (asymptomatic inflammatory prostatitis) by the presence of significant leukocytes (or bacteria or both) in secretion extracted by prostate massage and urine obtained after the massage. In cases of LUTS and ED without asymptomatic inflammatory, Sildenafil citrate had an improving effect on LUTS as well as on ED. However, in cases with asymptomatic inflammatory prostatitis, Sildenafil citrate did not lead to an improvement in LUTS [88]. In addition to the limitation of the study, which did not include a placebo group, was not randomized, and had a small sample size, the absence of results could be explained by the low number of PDE5Is doses, which were restricted to 50 mg Sildenafil citrate administered twice a week for 30 days, ideal for ED treatment but not for chronic inflammation therapy.

Grimsley et al., proposed a hypothesis to explain the mechanism of action of Sildenafil when ameliorating prostatitis symptoms. According to the authors these effects can be explained by the relaxation of the prostatic duct smooth muscle increasing washout of prostatic reflux products [20].

Cantoro et al. (2013) evaluated the effectiveness of tamsulosin (α-adrenergic blocker) monotherapy versus tamsulosin plus Sildenafil combination therapy on erectile dysfunction (ED) in young patients with type III chronic prostatitis, by using symptom score scales. They observed that tamsulosin monotherapy, as well as a combination therapy (tamsulosin plus Sildenafil) had an improving effect on symptoms and on ED in patients with type III prostatitis [89].

Whether PDE5Is an effective prostatitis treatment or not remains controversial. However, it is important to highlight that until today pre-clinical and clinical studies have featured doses and short-term treatment, ideal for ED and BPH/LUTS treatment,

not for chronic inflammation therapy. Although several experimental and clinical studies have found evidence of their possible benefits, no chronic treatment with PDE5Is has been performed to evaluate their effects on the human prostatitis.

It is important also to point out that PDE5Is have been chronically used as a pharmacological strategy for several non-urological disorders, such as pulmonary hypertension, Raynaud's phenomenon and altitude sickness [76]. Although PDE5Is are considered safe drugs with few side effects, long-term studies are needed to evaluate their effects on the normal male reproductive system, specifically on the prostate. The ultrastructural and molecular analysis realized by our group demonstrated that chronic treatment of C57Bl/6 mice with Sildenafil 25mg/kg for 4 weeks enhanced prostatic glandular activity, however, no differences were observed in sGC, eNOS, PSA and TGF-β expression [97]. These results suggested that the chronic use of Sildenafil does not cause evident prostatic damage, and therefore, seems safe for the treatment of a variety of disorders.

Recent studies have demonstrated that BPH/LUTS, prostatic cancer and metabolic syndrome (MetS) are often associated with one another [98]. Metabolic syndrome (MetS) is a complex of clustering metabolic abnormalities and comprises a number of disorders such as insulin resistance, hypertension and obesity, which all act as known risk factors for erection dysfunction (ED).

Interestingly, some studies have shown that treatment with PDE5Is, in addition to relaxing the muscular wall, may positively affect low urinary tract blood perfusion, restoring function and causing morphologic changes in the bladder and prostate induced by chronic pelvic ischemia caused by MetS or hypertension [99,100]. In spontaneous hypertensive rats, chronic treatment with tadalafil or other PDE5Is counteracted all

LUTS alterations, most likely through increased blood perfusion and oxygenation [99-101].

Hypertension, obesity, and hyperinsulinaemia have all been associated with increased sympathetic activity via enhanced glucose metabolism. This process promotes the activity of α-adrenergic receptors, increasing the smooth muscle tone of the male genitourinary tract [102,103]. Insulin-like growth factor-1 (IGF-1) contributes to the development and progression of BPH/LUTS. Since these molecules have a similar structure, insulin can bind to IGF-1 receptors and activate the signaling pathway for the growth and proliferation of epithelial and stromal prostatic cells [104]. Therefore, PDE5Is could be used as pharmacological tools for the treatment of ED, LUTS/BPH and chronic pelvic ischemia by smooth muscle relaxation via cGMP-dependent RhoA/Rho-kinase (ROCK) signaling inhibition [105-108], and possibly by reducing autonomic hyperactivity, which is a component of the metabolic syndrome [109].

Moreover, chronic inflammation has also been claimed to be one of the putative links between MetS and BPH/LUTS. Recently, Vignozzi et al. (2013) demonstrated that the PDE5 blockade exerts anti-inflammatory effects on human myofibroblast prostatic cells, blunting inflammatory and metabolic insults. These authors showed that treatment with tadalafil or vardenafil suppressed IL-8 and IP-10 secretion induced by inflammatory (TNF-α) and metabolic (oxLDL, AGE and IGF-1) stimuli. PDE5Is also inhibited the expression of inflammatory, fibroblast-to-myofibroblast transdifferentiation and tissue remodelling marker genes, most likely via the activation of cGMP/PKG signaling [110].

3.Conclusion

PDE5Is are therapeutical tools used for several urological and non-urological disorders, and experimental evidence suggest that their chronic use does not induce cellular and molecular prostatic alterations. The mechanisms involved in improvements observed in BPH/LUTS possibly include relaxation of the smooth muscles of the bladder and prostate by NO/cGMPc signaling or via improving RhoA/Rho-kinase (ROCK), and by reduction of the hyperactivity of the autonomic nervous system. PDE5Is can also direct and indirectly down-regulate prostatic inflammation/BPH/LUTS by inducing high levels of cGMP. In conclusion, since inflammation is a major factor in benign prostatic hyperplasia (BPH) progression, PDE5Is could also restore prostatic function, as they act as potent anti-inflammatory drugs.

Competing interests

All the authors declare to have no competing interests.

Authors' contributions

The authors were involved in drafting the article or revising it critically for important intellectual content, and both authors approved the final version to be published.

Acknowledgements

The study was supported by Fundação Oswaldo Cruz (FIOCRUZ). C.A.P. is supported by research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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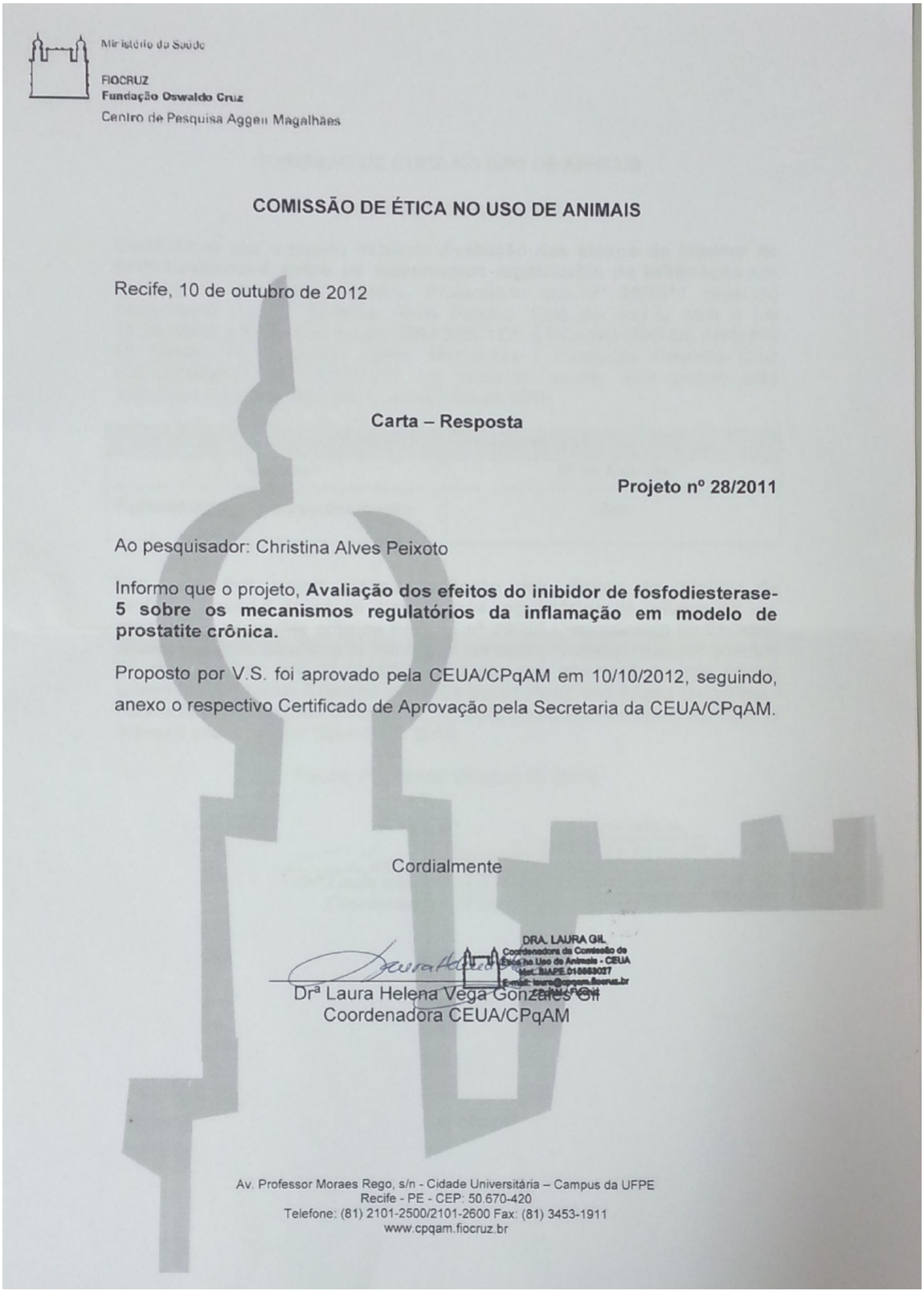
Anexos

6 ANEXOS



Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Recife, 10 de outubro de 2012

Carta – Resposta

Projeto nº 28/2011

Ao pesquisador: Christina Alves Peixoto

Informo que o projeto, **Avaliação dos efeitos do inibidor de fosfodiesterase-5 sobre os mecanismos regulatórios da inflamação em modelo de prostatite crônica.**

Proposto por V.S. foi aprovado pela CEUA/CPqAM em 10/10/2012, seguindo, anexo o respectivo Certificado de Aprovação pela Secretaria da CEUA/CPqAM.

Cordialmente

DRA. LAURA GIL
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