LARISSA AKEMI KIDO

"TERAPIAS ANTIANGIOGÊNICAS, USO DE FINASTERIDA E RESPOSTA HORMONAL NA PRÓSTATA DE CAMUNDONGOS SENIS"

Campinas, 2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestra em Biologia Celular e Estrutural, na área de Anatomia.

Orientadora: Profa. Dra. Valéria Helena Alves Cagnon Quitete

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DEDICATÓRIA

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"A sabedoria é árvore de vida para os que dela tomam, e são bem aventurados todos os que a retêm".

Provérbios 3:18

ÍNDICE

Índice de Abreviaturas

CK: citoqueratinas
CD: antígenos de superfícies
PSA: antígeno específico da próstata
PAP: fosfatase ácida prostática
AR: receptor androgênico
DHT: dihidrotestosterona
LNCap: linhagem celular de adenocarcinoma de próstata dependente de andrógenos
VEGF: fator de crescimento do endotélio vascular
ER-α: receptor estrogênico α
ER-β: receptor estrogênico β
CaP: câncer prostático
HBP: hiperplasia benigna da próstata
IL-6: interleucina 6
NIP: neoplasia intraepitelial prostática
AIP: atrofia inflamatória proliferativa
IGF: fatores de crescimento homólogo à insulina
FGF: fatores de crescimento fibroblásticos
TGF: fatores de crescimento transformadores
HIF: fatores indutores de hipóxia
DMSO: dimetilsulfóxido
CEUA: Comissão de ética no uso de animais
TBS-T: *Tris-buffered saline with tween*
H.E: Hematoxilina Eosina
T.M.: Tricrômico de Masson
DAB: Diaminobenzidina
RIPA: *Radio- Immunoprecipitation Assay*
SDS: Dodecil sulfato de sódio
BSA: *bovine serum albumin*
ANOVA: análise de variância
T: testosterona
PC-3: linhagem celular de adenocarcinoma de próstata independente de andrógenos
U87: linhagem celular de gliomablastoma

1. RESUMO

A senescência está associada a mudanças significativas no ambiente hormonal, sendo fator causador de alterações morfofuncionais na próstata. Os diferentes processos biológicos que ocorrem na próstata são regulados por polipeptídeos, dentre esses os fatores de crescimento do endotélio vascular (VEGF) e Endostatina, relacionados a angiogênese. Além disso, inibidores da enzima 5α redutase-II, como a finasterida, tem papel importante no combate as doenças prostáticas. Assim, os principais objetivos desse estudo foram avaliar os efeitos estruturais e moleculares das terapias antiangiogênicas e da finasterida sobre a próstata ventral de camundongos durante a senescência. Noventa camundongos machos FVB de 18 e 52 semanas de idade foram divididos nos seguintes grupos: Jovem (JV) e Senil (SEN), os quais receberam injeções de Solução Fisiológica 0,9% (5 mL/Kg/dia s.c.); Finasterida (FIN): injeções de Finasterida (20 mg/Kg, s.c.); SU5416 (SU): SU5416 (6 mg/Kg, i.p.); TNP-470 (TNP): injeções de TNP-470 (15 mg/Kg, s.c.), e SU5416 + TNP-470 (SU+TNP): os mesmos tratamentos dos grupos SU e TNP. Após 21 dias de tratamento, amostras do lobo ventral da próstata foram coletadas e submetidas às análises morfológicas, imunohistoquímicas e Western Blotting. Os resultados demonstraram alterações moleculares e estruturais no microambiente prostático durante a senescência, como atrofia, presença de células inflamatórias, e lesões proliferativas, as quais foram interrompidas e ou bloqueadas através dos tratamentos com as drogas antiangiogênicas e pela finasterida. Os resultados moleculares revelaram no grupo senil a diminuição das reatividades para AR e Endostatina, e aumento para ER-α, ER-β e VEGF, quando comparados aos camundongos jovens. Os camundongos dos grupos tratados com finasterida, SU5416 e SU5416+TNP-470, quando comparados aos do

grupo senil, demonstraram de forma geral diminuição das reatividades de VEGF e ER-α e aumento de ER-β. Já o tratamento com TNP-470 foi marcado principalmente pela redução da reatividade e dos níveis protéicos de AR e ER-β, quando comparado aos grupos jovem e senil. Desta maneira, conclui-se que a senescência favoreceu a ocorrência de alterações estruturais e/ou funcionais que sugerem o aparecimento de lesões malignas, em virtude do desequilibrio na sinalização entre epitélio e estroma. O tratamento com finasterida, SU5416 e SU5416+TNP-470 mostraram-se mais ativos na regulação dos processos proliferativos através da via estrogênica.

2. ABSTRACT

Senescence is associated with significant changes in the hormonal environment and is a cause of morphological and functional changes in the prostate. The different biological processes that occur in the prostate are influenced by different factors such as vascular endothelial growth factor (VEGF) and Endostatin, related to angiogenesis. Also, 5α-reductase inhibitors, such as finasteride, play an important role in treatment of prostatic diseases. Thus, the aims of this study were to evaluate the structural and molecular effects of antiangiogenic therapies and finasteride on the ventral prostate of mice during senescence. Ninety 52 and 18 week old male FVB mice, were divided into groups: Young (YNG) and Senile (SEN) groups, which received 0.9% saline (5 mL/kg/day sc) injections; Finasteride (FIN) group: Finasteride (20 mg/kg, sc); SU5416 (SU) group: SU5416 (6 mg/kg, ip) injections; TNP-470 (TNP) group: TNP-470 (15 mg/kg, sc) injections and SU5416+TNP-470 (SU+TNP470) group: The same treatment as the SU and TNP-470 groups. After 21 days of treatment, samples of the ventral lobe of the prostate were collected and analyzed for morphological, immunohistochemical and Western Blotting analyses. The results demonstrated structural and molecular changes in the prostatic microenvironment during senescence, such as atrophy, inflammatory cells, and proliferative lesions, which were interrupted and/or blocked by treatment with antiangiogenic drugs and finasteride. The molecular results revealed decreased reactivity for AR and Endostatin, and an increase for ER-α, ER-β and VEGF in the senile group, when compared to young mice. The mice in the groups treated with finasteride, SU5416 and SU5416 + TNP-470, when compared to the senile group, showed in general decreased VEGF and ER-α reactivities and increased ER-β reactivity. The treatment with TNP-470 however, was marked mainly by

reduced AR and ER-β reactivity and protein levels, when compared to young and senile groups. Thus, it can be concluded that senescence contributed to the occurrence of structural and/or molecular alterations that suggest the onset of malignant lesions, due to the imbalance in the signaling between the epithelium and stroma. Treatments with finasteride, SU5416 and SU5416+TNP-470, were active in the regulation of proliferative processes by means of the estrogen pathways.

3. INTRODUÇÃO

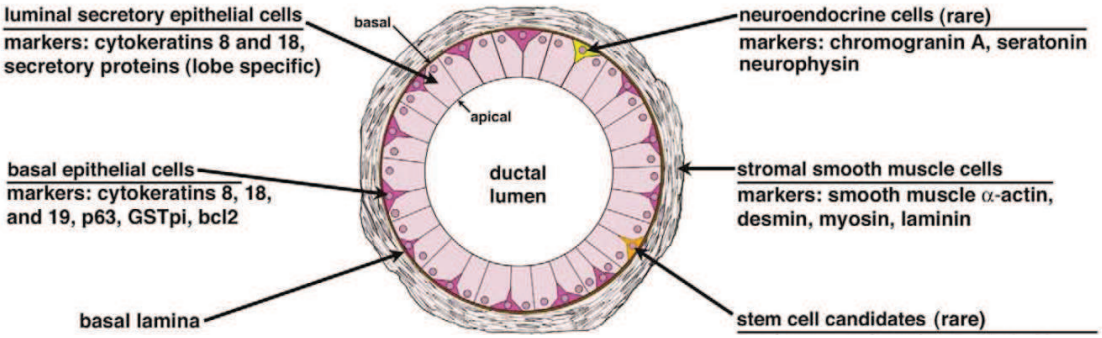
3.1. Morfofisiologia e Hormônios Esteroides na Próstata.

A próstata é uma glândula sexual acessória masculina que secreta diversos nutrientes que compõem o líquido seminal, fluido esse essencial para a nutrição e motilidade dos espermatozóides (Taylor & Risbridger, 2008). A secreção prostática é formada por diferentes constituintes como ácido cítrico, ácido siálico, espermina e prostaglandinas, enzimas como amilase, fibrinogenase, aminopeptidase, transglutaminase, fosfatases ácida e alcalina e zinco, os quais são importantes para a maturação e sobrevivência dos espermatozóides (Lin & Bissell, 1993; Bull et al., 2001).

Nos roedores, a próstata divide-se em três pares de lobos: ventral, lateral e dorsal de acordo com a localização ao redor da uretra prostática, e um par de glândulas coaguladoras ou próstata anterior localizadas na face côncava das vesículas seminais (Marker et al., 2003; Untergasser et al., 2005). Os lobos prostáticos estão conectados à uretra por uma série de ductos e diferem entre si quanto à morfologia, aos tipos de produtos secretados e à resposta hormonal (Jesik, 1982; Costello & Franklin, 1994). De maneira geral, os lobos prostáticos são compostos por um conjunto de estruturas túbulo-alveolares, onde o epitélio secretor simples encontra-se envolvido pelo estroma (Aumüller & Adler, 1979). O lobo ventral é frequentemente utilizado nos estudos sobre doenças prostáticas devido à sua dependência androgênica primária, muito embora não apresente homologia direta com a próstata humana (Slayter et al., 1994). A próstata é composta por diferentes tipos celulares, os quais podem ser distintos pela expressão diferencial de citoqueratinas (CK) e antígenos de superfície (CD) e/ou a expressão conjunta desses, distribuídos nos compartimentos epitelial e basal (Berry et al., 2008). O epitélio secretor é constituído por duas camadas morfologicamente definidas, as

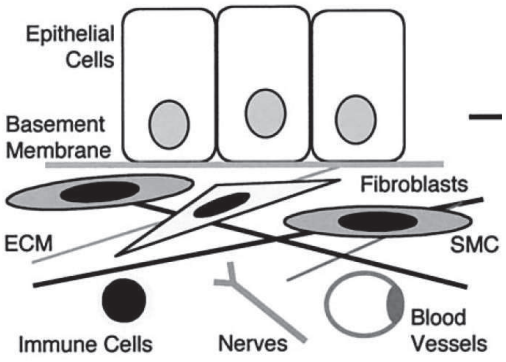
camadas luminal e basal, e três tipos celulares, células basais, luminais ou secretoras e neuroendócrinas (Miki, 2009).

As células luminais epiteliais representam o mais freqüente tipo celular, constituindo o compartimento exócrino da próstata, o qual secreta proteínas como o antígeno específico da próstata (PSA) e fosfatase ácida prostática (PAP) nos humanos (De Marzo et al., 1999) e prostateína e probasina no rato (Berry et al., 2008). As células luminais expressam receptor androgênico (AR) e são andrógeno-responsivas e andrógeno-dependentes (Taylor & Risbridger, 2008). Já as células neuroendócrinas são identificadas pela expressão de neurofisina, cromogranina A, sinaptofisina e calcitonina, mas não expressam PSA e/ou AR. Este tipo celular também é diferenciado, mas não-responsivo a andrógenos (Berry et al., 2008). Estas populações de células diferenciadas podem tornar-se modificadas no câncer de próstata apresentando alterações do número, morfologia e função, sugerindo papel regulatório nessa doença (Nelson et al., 2007; Ruscica et al., 2007). Já as células basais são relativamente indiferenciadas não demonstrando atividade secretora, e formam o compartimento basal da glândula (McNeal, 1988). As células basais são andrógeno-independentes, mas andrógeno-responsivas, ou seja, são independentes de andrógenos para sua manutenção e sobrevivência, crescimento e diferenciação, mas podem ser estimuladas por ação androgênica via fatores de crescimento derivados do estroma, os quais agem sobre as células basais para o repovoamento do compartimento celular luminal (De Marzo et al., 1999; Taylor & Risbridger, 2008). A expressão de AR em células basais é relativamente baixa se comparada às células luminais (Leav et al., 1996), por outro lado, expressam focalmente os receptores estrogênicos e podem proliferar quando submetidas à terapia com estrógenos (Collins & Maitland, 2006).



Marker, et al., 2003

O estroma prostático é formado por um arranjo complexo de células estromais e matriz extracelular associado a fatores de crescimento, moléculas reguladoras e enzimas de remodelação, as quais provêm sinais biológicos gerais e exercem influências mecânicas sobre as células epiteliais (Tuxhorn et al., 2001; Cunha & Matrisian, 2002). Também, vasos sanguíneos, terminações nervosas e células do sistema imune constituem partes integrais do estroma (Tuxhorn, et al., 2001). Os fibroblastos e as células musculares lisas são importantes tipos celulares do estroma prostático, tendo como principal função sintetizar componentes estruturais e reguladores da matriz extracelular. Esta, por sua vez, é composta de uma rede de proteínas fibrilares, glicoproteínas adesivas e proteoglicanos (Kreis & Vale, 1999; Tuxhorn, et al., 2001), sendo reservatório de fatores de crescimento ativos e latentes (Tuxhorn, et al., 2001).



Tuxhorn *et* al., 2001

A morfogênese, a manutenção da atividade funcional e da morfologia, a proliferação e a diferenciação das células da próstata são reguladas por andrógenos (Leav et al., 2001; Cunha et al., 2002; Imamov et al., 2005). A testosterona e a dihidrotestosterona (DHT) são os principais andrógenos a induzir a diferenciação prostática (Hsing, 2001; Toorians et al., 2003), A DHT é resultante da conversão da testosterona pela enzima 5α-redutase (Toorians et al., 2003). Dois tipos de 5α-redutases são identificados nos tecidos, a 5α-redutase tipo I encontrada na maioria dos tecidos, e a tipo II encontrada nos órgãos genitais incluindo a próstata (Hsing, 2001; Marker et al., 2003). Embora a testosterona e a DHT utilizem o mesmo receptor de andrógeno (AR) para atuarem no tecido prostático, essas ações parecem estar associadas a diferentes funções teciduais (Prins et al., 1991; Toorians et al., 2003). Tanto a testosterona quanto a DHT são capazes de manter a atividade prostática, porém a DHT é 10 vezes mais potente que a testosterona, devido a sua dissociação do receptor de andrógeno ser mais lenta (Droller, 1997).

Desde 1990, inibidores das 5α-redutases, como a finasterida que atua sobre a 5α-redutase-II, tem sido utilizados para tratamentos de sintomas lesivos no trato urinário inferior em humanos. Considera-se que os andrógenos intra-prostáticos tem papel importante como agente redutor para a DHT, hormônio esse de papel decisivo na manutenção das funções prostáticas e desenvolvimento e progressão de lesões (Thompson et al, 2009). Diversos trabalhos tem estudado os efeitos biológicos da finasterida na prevenção de câncer de próstata como agente redutor do risco de ocorrência dessa doença (Thompson et al., 2009; Attia & Ederveen, 2012; Kjellman et al., 2012). Golbano et al., (2008) verificaram a presença de um mecanismo pró-apoptótico específico quando administraram finasterida em cultura de células da linhagem LNCap (adenocarcinoma de próstata humano), demonstrando perda da

viabilidade celular e aceleração da apoptose. De acordo com Pareek et al. (2003), a administração de finasterida foi capaz de levar a diminuição da expressão de VEGF causando a inibição da angiogênese e diminuição significativa da densidade da microvasculatura no tecido prostático suburetral. Paralalemente, estudos tem relacionado o uso da finasterida com o aumento do risco de diagnóstico de lesões prostáticas de alto grau (Kjellman et al., 2012). Várias explicações têm sido dadas para esse fenômeno, entre os quais o fato de que ao reduzir a formação de DHT os níveis circulantes de testosterona aumentam, resultando em aumento dos níveis de estrógenos via conversão da enzima aromatase, podendo assim induzir a tumorigênese prostática (Thompson et al., 2003).

Embora a próstata seja primariamente regulada por andrógenos, o seu desenvolvimento é sensível a outros hormônios, como os estrógenos, que atuam sinergicamente à testosterona, influenciando tanto as funções normais do órgão quanto às alterações lesivas (Weihua et al., 2001; Cunha et al., 2002). A biossíntese de estrógenos ocorre a partir de um substrato androgênico, através de sua aromatização pela enzima aromatase (O' Donnell et al., 2001). Os efeitos estrogênicos na próstata são resultados da ligação desses hormônios a receptores estrogênicos específicos α e β (ERα, ERβ), os quais são predominantemente expressos no estroma e no epitélio, respectivamente (Cunha et al., 2002). O papel dos estrógenos na próstata é complexo e caracteriza-se tanto pelos efeitos positivos quanto negativos sobre a morfologia e fisiologia glandular (Attia & Ederveen, 2012).

3.2. Doenças Prostáticas

A morfologia e a fisiologia da próstata tem sido examinadas com particular atenção devido às lesões que acometem esse órgão, tais como a hiperplasia benigna (HBP) e o câncer prostático (CaP) (Leav et al., 2001). Cerca de 90% dos homens aos 80 anos de idade apresentam sintomas de hiperplasia benigna e 23% desenvolvem câncer de próstata (Rizzo et al., 2005). Dados estatísticos vem demonstrando que o câncer de próstata é agora o tipo de câncer mais frequente diagnosticado entre os homens que vivem em países desenvolvidos, com os mais altos índices pertencendo aos norte americanos, seguidos pelos australianos e neo-zelandeses (Parkin et al., 2005; Siegel et al., 2012). Segundo o Instituto Nacional do Câncer foram esperados para o ano de 2012 cerca de 60180 novos casos de câncer de próstata A HBP caracteriza-se por uma predominante proliferação estromal e, embora um aumento substancial do epitélio também ocorra, a integridade regional da glândula é mantida (Droller, 1997; Medina & Moore, 1999), e sua ocorrência está diretamente associada com o envelhecimento (Krieg et al., 1993). O câncer de próstata, ao contrário, se origina no compartimento epitelial da próstata (Hodgson et al., 2012). Não estão claros quais são os primeiros eventos oncogênicos que levam à transformação neoplásica, mas devido à heterogeneidade de CaP há provavelmente numerosos fatores relacionados a deflagração da lesão (Hodgson et al., 2012). Muito embora exista algum componente hereditário associado à incidência CaP, o principal fator de risco para o desenvolvimento desta doença maligna é o avanço da idade, havendo duas fundamentais características distintivas da senescência em homens: a diminuição dos níveis de testosterona e os elevados níveis de citocinas inflamatórias, incluindo interleucina-6 (IL-6) (Hodgson et al., 2012).

Outras lesões da próstata tem sido frequentemente reportados em diferentes tipos de estudos, como a neoplasia intraepitelial prostática (NIP), considerada uma lesão que precede o carcinoma prostático, e a atrofia inflamatória proliferativa (AIP). A AIP, descrita inicialmente por De Marzo et al. (1999), é tipicamente associada com inflamação prostática e considerada uma possível precursora da NIP de alto grau e do CaP (Nelson et al., 2003). As lesões da AIP tendem a ocorrer na periferia da próstata e emergem como conseqüência da proliferação regenerativa das células epiteliais, podendo este evento ser resposta a uma injúria causada por infecção, trauma celular resultante de dano oxidativo, hipóxia e autoimunidade (Nelson et al., 2003). Além disso, as lesões da AIP são frequentemente observadas adjacentes à neoplasia intraepitelial (NIP) de alto grau e estágios iniciais do CaP (De Nunzio et al., 2011).

3.3. Fisiologia prostática, Angiogênese e Senescência

Na próstata os diferentes processos biológicos tais como regulação da proliferação e diferenciação celular, atividade mitogênica, processos secretores e crescimento tumoral, são regulados e/ou influenciados por diferentes polipeptídeos como os fatores de crescimento homólogos a insulina (IGF), fatores de crescimento fibroblásticos (FGF), fatores de crescimento transformadores (TGF) (Takahashi-Konno et al., 2003; Zhao et al., 2004; Marszalek et al., 2005), fatores indutores de hipóxia (HIF), fatores de crescimento do endotélio vascular (VEGF) e a Endostatina (Abdollahi et al., 2003; Berry et al., 2008). As células estromais produzem diversos fatores de crescimento sob a influência de andrógenos, e na ausência desses, alguns dos fatores de crescimento podem ativar o AR e induzir diferentes respostas celulares (Berry et al., 2008). Em associação, células estromais e matriz extracelular criam um microambiente que regula o crescimento e diferenciação funcional das células

adjacentes. (Cornell et al., 2003). Os fatores de crescimento produzidos no estroma exercem influência parácrina sobre as células epiteliais adjacentes, através da ligação com seus receptores específicos. O epitélio, por sua vez, é estimulado a secretar polipeptídios que irão promover o crescimento estromal (Reynolds & Kyprianou, 2006). Assim, os fatores de crescimento e seus receptores atuam como mediadores da interação bidirecional entre epitélio e estroma, sendo esta interação primordial na manutenção da estrutura, função e crescimento da próstata (Zhao et al., 2002).

Com base em aspectos morfológicos, funcionais e embriológicos, a interação epitélio-estroma pode ser considerada como única unidade funcional, sendo a membrana basal o seu ponto de união e responsável por oferecer suporte mecânico e fisiológico ao epitélio secretor (Cunha & Hayward, 2000). Além disso, sabe-se ainda que o desequilíbrio desta interação na glândula favorece a formação do carcinoma prostático (Cunha et al., 2002). As células estromais associadas às células tumorais respondem aos andrógenos e fatores de crescimento, levando a interrupção da homeostase epitélio-estroma, o que desencadeia processos de crescimento, migração, angiogênese, apoptose e metástases tumorais (Wong et al., 2000; Cornell et al., 2003)

A angiogênese é definida como o desenvolvimento de novos vasos sanguíneos a partir de vasos preexistentes e possui um papel importante no crescimento e desenvolvimento dos órgãos, cicatrização de feridas e na tumorigênese (Shibuya & Claesson-Welsh, 2006). O conceito de angiogênese tem sido apontado como uma nova estratégia anticancer, considerando que a neovascularização é necessária para sustentar o crescimento de tumores sólidos (Aragon-Ching et al., 2010).

Os inibidores da angiogênese têm sido desenvolvidos para atingir as células endoteliais vasculares e bloquear a angiogênese do tumor, uma vez que essas células são geneticamente estáveis e, portanto, menos propensas a acumular mutações que lhes permitiria desenvolver resistência a fármacos (Abdollahi et al., 2003; Folkman, 2005). Segundo Folkman et al., (1998) os inibidores da angiogênese são divididos em duas classes: os inibidores diretos e os indiretos. Os inibidores diretos da angiogênese atuam nas células endoteliais vasculares impedindo-as de responder a vários estímulos pró-angiogênicos. Por outro lado, os inibidores indiretos da angiogênese interferem na comunicação pró-angiogênica entre as células tumorais e as células endoteliais. Este efeito inibitório indireto pode ser alcançado por: inibição da expressão de fatores angiogênicos como VEGF e FGF no tumor; bloqueio da expressão ou da ativação dos receptores dos fatores pró-angiogênicos nas células endoteliais, como o bloqueio do receptor para VEGF.

O VEGF é um dos mais importantes fatores indutores da angiogênese, sendo produzido por uma ampla variedade de tipos celulares, e atua como um mitógeno específico das células endoteliais (Kamath et al., 2009; Zhu et al., 2009). Todavia, em condições normais está presente apenas em níveis muito baixos, inclusive na próstata (Van Moorselaar & Voest, 2002; Reynolds & Kyprianou, 2006). Dentre os receptores tirosina-quinase para o VEGF, o principal mediador de suas ações sobre o endotélio vascular é o VEGF-2, cuja via de sinalização leva à proliferação, diferenciação e migração das células endoteliais, além de promover aumento da permeabilidade vascular (Van Moorselaar & Voest, 2002; Delongchamps et al., 2006. Segundo Joseph et al. (1997) a castração levou a um decréscimo significativo dos níveis de VEGF no lobo ventral da próstata de ratos, enquanto que a reposição hormonal com andrógeno exógeno promoveu recuperação parcial do conteúdo desse

fator angiogênico no tecido prostático desses animais. Ainda, destacaram-se as ações dos inibidores indiretos da angiogênese como a inibição seletiva dos receptores de tirosina quinase e a inibição de proteínas reguladoras do crescimento celular (Aragon-Ching et al., 2010).

O SU5416 é um pequeno inibidor sintético lipofílico da tirosina quinase (TKI) que inibe a fosforilação do receptor para VEGF, e demonstrou potentes propriedades antiangiogênicas em estudos pré-clínicos (Fong et al., 1999; Abdollahi et al., 2003; Christensen, 2007). Estudos demonstraram que o SU5416 inibiu a migração de células endoteliais humanas *in vitro* e que a administração dessa droga em modelos *in vivo* enxertados com diferentes linhagens de câncer levou à inibição dos processos de angiogênese, metástases e proliferação tumoral, além de promover o aumento da apoptose de células tumorais e endoteliais (Fong et al., 1999).

O TNP-470, O-(chloroacetylcabomoyl) fumagillol, é um inibidor direto de angiogênese, análogo à fumagilina, que também apresenta importantes propriedades antitumorais e potente ação citostática em células endoteliais (Masiero, et al.,1997). O mecanismo de ação do TNP-470 não está totalmente esclarecido, mas sabe-se que ele inibe especificamente a síntese de DNA nas células endoteliais, indicando que a molécula afeta uma ou várias etapas do ciclo celular e tem ação anti-angiogênica direta (Castronovo & Belotti, 1996; Figg et al., 2002). Ainda, sabe-se que este composto semi-sintético mostrou-se 50 vezes mais potente na inibição do crescimento vascular e menos tóxico, quando comparado que à fumagilina (Masiero et al., 1997).

É conhecido que o envelhecimento está associado a mudanças significantivas no ambiente hormonal, e que nas diferentes espécies animais é fator causador de alterações morfofuncionais na próstata (Morales, 2002; Roy-Burman et al., 2004). A senilidade

masculina está associada à maior propensão para o desenvolvimento de diversas desordens urológicas, incluindo doenças prostáticas, como a hiperplasia benigna e o câncer (Schulman & Lunenfeld, 2002; Lau et al., 2003). No homem, embora haja variações entre os indivíduos, há um declínio progressivo nos níveis de testosterona e dehidroepiandrosterona (DHEA) circulantes, o qual é acompanhado por um acréscimo na conversão de andrógenos em estrógenos, resultando em um desequilíbrio destes últimos com a idade (Srinivasan et al., 1995; Morales, 2002). O aumento de infiltrado inflamatório também foi reconhecido na próstata durante a senescência (Ellem & Risbridger, 2010), mas ainda especula-se a respeito do seu potencial deflagrador ou intensificador nas lesões prostáticas.

No animal senil, a depleção androgênica levou a involução das células epiteliais prostáticas (Cavazos, 1975). Células inflamatórias e atipia focal foram verificadas no epitélio, além de menor taxa de receptores androgênicos na próstata (Lau et al., 2003; Cordeiro et al., 2008). Segundo Lau et al. (2003), tais alterações evidenciadas na próstata de ratos senis são similares àquelas verificadas na hiperplasia benigna prostática em humanos. Esses mesmos autores sugeriram perda de função prostática na senescência, com diminuição da expressão de genes relacionados à síntese e checagem de proteínas, inibição do crescimento e metabolismo energético, além de transcrição aumentada de genes de sobrevivência celular e evasão apoptótica.

Segundo Sprenger et al. (2008), a senescência celular pode ser capaz de inibir a formação de tumores em indivíduos jovens. Contudo, com o decorrer do tempo há um acúmulo de células senescentes, as quais secretam fatores que comprometem a estrutura e a função tecidual. Assim, cria-se um microambiente extracelular alterado, o qual é permissivo ao desenvolvimento de cânceres epiteliais, como o adenocarcinoma prostático. Zhao et al.

(2002) associaram ao envelhecimento as diferenças na expressão gênica e na imunolocalização de fatores de crescimento na próstata de ratos, sugerindo ainda o envolvimento dessa expressão diferencial na patogênese de doenças relacionadas à idade, como o câncer e a hiperplasia benigna.

4. JUSTIFICATIVA E OBJETIVOS

A próstata é um dos órgãos mais acometidos por alterações estruturais e fisiológicas durante a senescência em diferentes espécies e sua fundamental participação no processo reprodutivo masculino tem motivado estudos sobre a biologia celular, molecular e endocrinológica desse órgão. A interação dos diferentes fatores de crescimento e hormônios esteroides tem sido alvo de estudos durante a senescência devido ao desequilíbrio hormonal que ocorre durante esse período da vida, assim como as variadas terapias de tratamento de doenças prostáticas.

A complexidade do processo de angiogênese em doenças malignas sugere que a combinação de agentes antiangiogênicos diretos e indiretos possa ser mais eficaz do que as terapias envolvendo apenas um único agente. Além disso, na literatura especializada não existem trabalhos correlacionando a dinâmica da atividade dos receptores hormonais e tratamento antiangiogênico durante a senescência. Portanto, a associação entre um inibidor direto da angiogênese e um antagonista do receptor de VEGF (indireto) pode ser considerada uma promissora combinação terapêutica, que certamente indicará importantes sinalizações moleculares no microambiente prostático durante a senescência. Também, inibidores da enzima 5α redutase-II, como a finasterida, tem demonstrado importante papel no combate às doenças prostáticas. Dessa forma, o objetivo geral deste estudo foi avaliar os efeitos das

terapias antiangiogênicas e do uso da finasterida, bem como a distribuição e frequência dos receptores esteroides, destacando os estrogênicos e androgênicos, além de fatores pro-angiogênicos e anti-angiogênicos na próstata de camundongos senis.

Os objetivos específicos foram:

1. Caracterização da morfologia dos compartimentos epitelial e estromal da próstata ventral de camundongos FVB jovens, senis e senis submetidos às terapias antiangiogênicas e ao tratamento com finasterida;
2. Caracterização da distribuição e frequência das imunorreatividades de AR, ER-α, ER-β, VEGF, Endostatina e quantificação dos níveis protéicos de AR, ER-α, ER-β, e VEGF na próstata ventral de camundongos FVB jovens, senis e senis submetidos às terapias antiangiogênicas e ao tratamento com finasterida;
3. Determinação do o índice apoptótico e índice de proliferação celular no lobo ventral da próstata de camundongos FVB senis submetidos às terapias antiangiogênicas e à finasterida.

5. MATERIAIS E MÉTODOS

5.1. Animais e Procedimento Experimental

No presente estudo foram utilizados 90 camundongos machos da linhagem FVB, pesando aproximadamente 25g. Os animais foram divididos em seis grupos experimentais:

Grupo Jovem (JV): camundongos FVB (18 semanas de idade) receberam injeções subcutâneas de 5 mL/Kg/dia de Solução Fisiológica 0,9%, durante 21 dias;

Grupo Senil (SEN): camundongos FVB (52 semanas de idade) receberam injeções subcutâneas de 5 mL/Kg/dia de Solução Fisiológica 0,9% por 21 dias;

Grupo Finasterida (FIN): camundongos FVB (52 semanas de idade) receberam injeções subcutâneas de 20 mg/Kg de finasterida (F1293 – Sigma-Aldrich, St. Louis, MO, EUA) diluídos em dimetilsulfóxido (DMSO), em dias alternados, por 21 dias (modificado de Tutrone et al., 1993 e Gonzales et al., 2008);

Grupo SU5416 (SU): camundongos FVB (52 semanas de idade) receberam injeções intraperitoneais de 6 mg/Kg de SU5416 (Cat. 3037 – Tocris Bioscience, Minneapolis, MN, EUA) diluídos em dimetilsulfóxido (DMSO), em dias alternados, por 21 dias (modificado de Strieth et al., 2006);

Grupo TNP-470 (TNP-470): camundongos FVB (52 semanas de idade) receberam injeções subcutâneas de 15 mg/Kg de TNP-470 (Cat. 3750 – Tocris Bioscience, Minneapolis, MN, EUA) diluídos em dimetilsulfóxido (DMSO), em dias alternados, por 21 dias (modificado de Matsusaka et al., 2000);

Grupo TNP-470 + SU5416 (SU+TNP): camundongos FVB (52 semanas de idade) receberam injeções subcutâneas de 15 mg/Kg de TNP-470 (Cat. 3750 – Tocris Bioscience, Minneapolis, MN, EUA) e injeções intraperitoneais de 6 mg/Kg de SU5416 (Cat. 3037 – Tocris Bioscience, Minneapolis, MN, EUA) diluídos em dimetilsulfóxido (DMSO), em dias alternados, por 21 dias (modificado de Matsusaka et al., 2000; modificado de Strieth et al., 2006);

Todos os animais receberam água e a mesma dieta sólida *ad libitum* (Nuvilab, Colombo, Paraná, Brasil). Após 21 dias de tratamento, todos os animais foram pesados em balança analítica Denver P-214 (Denver Instrument Company, Arvada, CO, EUA), anestesiados com Cloridrato de Xilazina 2% (5mg/kg i.m.; König, São Paulo, Brasil) e

Cloridrato de Cetamina 10% (60mg/kg, i.m.; Fort Dodge, Iowa, EUA) e sacrificados. Este estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA), protocolo 2391-1.

5.2. Microscopia de Luz

Amostras prostáticas de 5 animais de cada grupo foram coletadas e fixadas em solução de Bouin por 24 horas. Após a fixação, os tecidos foram lavados em álcool etílico a 70% e desidratadas. Posteriormente, os fragmentos foram diafanizados em xilol e inclusos em polímeros plásticos (Paraplast Plus, ST. Louis, MO, EUA). Em seguida, os materiais foram seccionados no micrótomo Hyrax M60 (Zeiss, Munique, Alemanha), corados em Hematoxilina-Eosina e Tricrômico de Masson (Junqueira et al., 1979) e fotografados no fotomicroscópio Nikon Eclipse E-400 (Nikon, Tóquio, Japão).

5.3. Imunohistoquímica dos Antígenos: Fator de Crescimento do Endotélio Vascular (VEGF); Endostatina; Ki-67, receptor androgênico (AR), receptores estrogênicos α e β (ER- α e ER-β).

Amostras do lobo ventral da próstata foram coletadas de 5 animais de cada grupo experimental, os mesmos utilizados para microscopia de luz. A seguir, cortes teciduais foram submetidos à recuperação antigênica por incubação dos cortes em tampão citrato (pH 6.0) a 100ºC por 15 minutos em microondas (potência de 750 W) ou tratamento com proteinase K, dependendo das características de cada um dos anticorpos analisados. O bloqueio das peroxidases endógenas foi obtido com H_2O_2 (0,3% em metanol) por 15 minutos. Solução bloqueadora com albumina soro bovino (3%) em tampão TBS-T por 1 hora foi utilizada. Posteriormente, os antígenos, AR, ER-α, ER-β, VEGF, Ki67, e Endostatina, foram localizados

através dos anticorpos: policlonal rabbit (sc-816) (Santa Cruz Biotechnology, CA, USA) para AR, monoclonal mouse (1D5) (Dako Cytomation Inc., CA, USA) para ERα, policlonal rabbit (06-629) (Upstate, USA) para ERβ, monoclonal mouse (sc-53462) (Santa Cruz Biotechnology, CA, EUA) para VEGF, policlonal rabbit (ab9260) (Millipore, MA, USA) para Ki67, monoclonal mouse (ab64569) (Abcam, MA, EUA) para Endostatina, policlonal rabbit (sc-146) (Santa Cruz Biotechnology, CA, EUA) diluídos (1:35-50) em BSA 1% e armazenados overnight a 4 ºC. O kit Envision HRP (Dako Cytomation Inc., CA ,USA) foi usado para detecção dos antígenos, de acordo com as instruções do fabricante. Após lavagem com tampão TBS-T, os cortes foram incubados com anticorpo secundário HRP conjugado proveniente do kit Envision (Dako) por 40 minutos e, posteriormente revelados com diaminobenzidina (DAB). Para a contra-coloração destes, foi utilizado Hematoxilina de Harris. 10 regiões prostáticas de 5 animais de cada grupo experimental foram avaliadas através do precipitado de cor castanho de DAB, o qual indicou a imunorreatividade dos anticorpos. A intensidade da imunorreatividade dos antígenos foi graduada como intensa (+++), moderada (++) e fraca (+), de acordo com a freqüência de distribuição dos antígenos no tecido glandular (modificado de Markopoulos et al., 2000).

5.4. Contagem de Células Ki-67 Positivas e Determinação do Índice Proliferativo

Os índices proliferativos foram obtidos por contagem das células Ki-67 positivas. Amostras prostáticas de 5 animais de cada grupo experimental, os mesmos destinados a imunomarcação para Ki-67 foram utilizadas. A seguir, dez campos de cada espécime foram

avaliados com objetiva de 100x e o índice proliferativo foi determinado dividindo-se o número de células Ki-67 positivas pelo número total de células encontradas no campo microscópico.

5.5. Detecção da Apoptose e Determinação do Índice Apoptótico

Amostras do lobo ventral da próstata de 5 animais de cada grupo experimental foram coletadas e fixadas em paraformoldeído a 4% por doze horas. Após a fixação, as amostras foram inclusas em parafina e polímeros plásticos (Paraplast Plus, ST. Louis, MO, EUA), seccionadas no micrótomo Hyrax M60 (Zeiss, Munique, Alemanha) com espessura de 5µm e submetidas às reações de detecção da fragmentação do DNA. A fragmentação do DNA foi detectada utilizando o sistema de detecção fluorescente para apoptose (Promega, Madison, WI, EUA), de acordo com as instruções do fabricante, e Feulgen. Os núcleos apoptóticos foram identificados e fotografados através do microscópico invertido Olympus IX71-II (Olympus, Califórnia, EUA) equipado com fluorescência (IX2-FL-II, Olympus, CA, EUA).

Para a reação de Feulgen, os cortes foram submetidos à hidrólise com 4 NHCl por 65 minutos e tratados com o reagente de Schiff por 40 minutos. Após lavagem em água, os cortes foram desidratados e montados em lâminas. A seguir, dez campos de cada animal foram analisados com objetiva de 100x e o índice apoptótico foi determinado dividindo-se o número de núcleos apoptóticos pelo número total de núcleos encontrados nos campos microscópicos. Os núcleos apoptóticos foram identificados por características como picnose e ou fragmentação nucleolar.

5.6. Extração de Proteínas e Western Blotting

Amostras do lobo ventral foram coletadas de 5 animais de cada grupo experimental, pesadas e homogeneizadas através do homogeneizador Polytron (Kinematica) em tampão de extração RIPA (Radio-Immunoprecipitation Assay) diluído, e coquetel inibidor de proteases (Sigma-Aldrich, St. Louis, MO, EUA). Os extratos dos tecidos foram obtidos por centrifugação durante 20 minutos a 14000 rpm a 4^{o}C. Uma alíquota de cada amostra foi usada para determinação da concentração de proteínas, usando o reagente de Bradford (Bio-Rad Laboratories, Hercules, CA, EUA). As amostras foram misturadas (1:1) com tampão de amostra 3X (100mM Tris-HCl pH 6.8, 10%β-mercaptoetanol, 4% SDS e 20% glicerol), incubadas em banho seco a 95ºC por 5 minutos. O correspondente a 100 microgramas de proteínas foi aplicado no gel de SDS-poliacrilamida. Após a eletroforese, o material foi transferido eletricamente (Sistema Hoefer) para membranas de nitrocelulose (Amersham) a 70 V por 3 horas. As membranas foram então bloqueadas com 3% BSA diluído em TBS-T por uma hora e incubadas com os anticorpos primários: monoclonal mouse (sc-81178) para β-actina, policlonal rabbit (sc-816) (Santa Cruz Biotechnology, CA, USA) para AR, monoclonal mouse (1D5) (Dako Cytomation Inc., CA, USA) para ERα, policlonal rabbit (06-629) (Upstate, USA) para ERβ, monoclonal mouse (sc-53462) (Santa Cruz Biotechnology, CA, EUA) para VEGF na faixa de diluições entre 1:350-1000. Após lavagem com tampão TBS-T, as membranas foram incubadas por 2 horas com os anticorpos secundários anti-rabbit e anti-mouse HRP conjugados na diluição de 1:2000 em 1% BSA. Para detectar as bandas reativas, as membranas foram expostas à solução de quimiluminescência (Pierce Biotechnology, Rockford, Illinois, USA) por 5 min capturados através do aparelho G-Box

Chemi e o software de aquisição de imagem GeneSnap (Syngene, Cambridge, UK). O anticorpo para β actina foi usado como controle endógeno. A intensidade da marcação obtida nas diferentes situações foi determinada e identificada por densitometria através do programa de análise de imagens NIS-Elements: Advanced Research (USA).

5.7. Análise Estatística

Os parâmetros quantificados nas análises de Western Blotting e Índices Proliferativo e Apoptótico foram analisados estatisticamente para os diferentes grupos. Para a análise estatística foram empregados o Test-T e a análise de variância (ANOVA), seguida pelo teste de Tukey para comparação entre médias. Todas as análises foram realizadas com nível de significância de 5% (Zar, 1999).

6. ARTIGO CIENTÍFICO

"Antiangiogenic and finasteride therapies: responses of the prostate microenviroment in elderly mice"

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6.1. ABSTRACT

The aims of this study were to evaluate the structural and molecular effects of antiangiogenic therapies and finasteride on the ventral prostate of mice during senescence. Ninety male FVB mice were divided into groups: Young (18 month-old) and senile (12 month-old) groups, which received 0.9% saline (5mL/kg/day sc); Finasteride group: Finasteride (20mg/kg, sc); SU5416 group: SU5416 (6 mg/kg, ip); TNP-470 group: TNP-470 (15 mg/kg, sc) and SU5416+TNP-470 group: similar to the SU5416 and TNP-470 groups. After 21 days, prostatic samples were collected for morphological, immunohistochemical and Western Blotting analyses. The results demonstrated atrophy, inflammatory cells, and proliferative lesions in the prostate during senescence, which were interrupted and/or blocked by treatment with antiangiogenic drugs and finasteride. The molecular results revealed decreased reactivity for AR and Endostatin, and an increase for ER-α, ER-β and VEGF in the senile group, when compared to young mice. The mice in the groups treated with finasteride, SU5416 and SU5416 + TNP-470, when compared to the senile group, showed decreased VEGF and ER-α reactivities and increased ER-β reactivity. The treatment with TNP-470 was marked mainly by reduced AR and ER-β protein levels, when compared to young and senile groups. Thus, it can be concluded that senescence favored the occurrence of structural and/or molecular alterations that suggest the onset of malignant lesions, due to the imbalance in the signaling between the epithelium and stroma. Treatments with finasteride, SU5416 and SU+TNP470, were active in the regulation of proliferative processes by means of the estrogen pathways. **Key-words:** prostate; antiangiogenic; finasteride; aging; hormonal receptors.

6.2. INTRODUCTION

The prostate is an androgen-dependent male accessory sex gland that secretes various nutrients into the seminal fluid, which is essential for sperm nutrition and motility (Cunha et al. 1987; Taylor and Risbridger, 2008). The prostate is divided into three pairs of lobes in rodents: ventral, lateral and dorsal, depending on the location around the prostatic urethra, and a pair of coagulating glands or anterior prostate located in the concave face of the seminal vesicles (Marker et al. 2003; Untergasser et al. 2005). The luminal epithelial cells make up the exocrine compartment of the prostate, and are the most common cell type (De Marzo et al. 1999). The basal cells, which are also part of glandular epithelium are androgen-independent and androgen-responsive, in other words they are androgen independent for their maintenance, survival, growth and differentiation, but can be stimulated by androgen action via growth factors derived from the glandular stroma (De Marzo et al. 1999; Taylor and Risbridger, 2008). The prostatic stroma, on the other hand, is formed by a complex arrangement of stromal cells and extracellular matrix associated to growth factors, regulatory molecules, and remodeling enzymes, which lead to general biological signals and exert mechanical influences on the epithelial cells (Cunha and Matrisian, 2002).

Testosterone (T) and dihydrotestosterone (DHT) are the main androgens that induce prostate differentiation (Hsing, 2001; Toorians et al. 2003). DHT is a result of the conversion of testosterone by the 5α-reductase enzyme (Toorians et al. 2003). Testosterone and DHT use the same androgen receptor (AR) and both are able to maintain prostatic activity. However, DHT is 10 times more potent than testosterone due to its slower dissociation from the androgen receptor (Droller, 1997; Toorians et al. 2003). Since 1990, 5α-reductase inhibitors such as finasteride, which acts on 5α-reductase II, has been used for the treatment of harmful

symptoms in the lower urinary tract in humans. (Thompson et al. 2009). Studies have verified the biological effects of finasteride in preventing prostate cancer and as a risk reducing agent of this disease (Thompson et al. 2009).

Although the prostate is primarily regulated by androgens, its development is sensitive to other hormones such as estrogens, which act synergistically to testosterone, influencing both the normal functions of the body as well as the harmful alterations (Weihua et al. 2001; Cunha et al. 2002). The estrogenic action occurs via receptors, and two of these isoforms (ER-α and ER-β) are present in different male reproductive tissues, including the prostate (Pelletier and El-Alfy, 2000).

Angiogenesis blocking has been identified as a new anticancer strategy, considering that neovascularization is required to sustain the growth of solid tumors (Aragon-Ching et al. 2010). According to Folkman et al. (1998) angiogenesis inhibitors are divided into two classes: direct and indirect inhibitors. The direct angiogenesis inhibitors act in the vascular endothelial cells by preventing them from responding to several pro-angiogenic stimuli. Meanwhile, the indirect inhibitors of angiogenesis interfere in the pro-angiogenic communication between the tumor cells and the endothelial cells (Folkman et al. 1998).

SU5416 is a tyrosine kinase lipophilic synthetic inhibitor (TKI) that inhibits the phosphorylation of VEGF receptor, and has potent antiangiogenic properties in preclinical studies (Fong et al. 1999; Abdollahi et al. 2003; Christensen, 2007). On other hand, the direct angiogenesis inhibitor TNP-470, a fumagillin analog, has a potent cytostatic action in endothelial cells and also significant antitumor properties (Masiero et al. 1997; Folkman, 2005).

Senescence is the period of life that is associated with significant changes in the hormonal environment of the different animal species, and is a causative factor of morphofunctional changes in the prostate (Morales, 2002; Roy-Burman et al. 2004). Male senility is associated to higher propensity for the development of various urological disorders, including prostatic diseases, such as benign hyperplasia and cancer (Schulman and Lunenfeld, 2002; Lau et al. 2003). According to Lau et al. (2003), the changes found in the prostate of senile mice are similar to those observed in benign prostatic hyperplasia in humans. Zhao et al. (2002) associated senility to differences in gene expression and in the immunolocalization of growth factors in the prostate of mice, suggesting the involvement of this differential expression in the pathogenesis of age-related diseases, such as cancer and benign hyperplasia.

The prostate is one of organs most affected by structural and physiological changes during senescence in the different species, and its important role in the reproductive process has motivated studies on the maintenance of its structure and function. Moreover, the complexity of the angiogenic process in malignant diseases suggests that the combination of direct and indirect anti-angiogenic agents can be an effective way of treating and or preventing lesions. Thus, the aim of this study was to evaluate the effects of antiangiogenic therapies and the use of finasteride on the molecular and structural biology of the mouse prostate during senescence.

6.3. MATERIALS AND METHODS

6.3.1 - Animals and Experimental Procedure

The present study used 90 male FVB mice, weighing approximately 25g. The animals were divided into six experimental groups:

Young Group (YNG): FVB mice (18 weeks old) received subcutaneous injections of 5mL/kg/day 0.9% saline solution for 21 days;

Senile Group (SEN): FVB mice (52 weeks old) received subcutaneous injections of 5mL/kg/day 0.9% saline solution for 21 days;

Finasteride Group (FIN): FVB mice (52 weeks old) received subcutaneous injections of 20mg/Kg of finasteride (F1293 - Sigma-Aldrich, St. Louis, MO, USA) diluted in dimethylsulfoxide (DMSO) every other day for 21 days (modified from Tutrone et al. 1993; Gonzales et al. 2008);

SU5416 Group (SU): FVB mice (52weeks old) received intraperitoneal injections of 6mg/kg SU5416 (Cat.3037 - Tocris Bioscience, Minneapolis, MN, USA) diluted in dimethylsulfoxide (DMSO), every other day for 21 days (modified from Strieth et al. 2006);

TNP-470 Group (TNP-470): FVB mice (52weeks old) received subcutaneous injections 15mg/kg of TNP-470 (Cat.3750 - Tocris Bioscience, Minneapolis, MN, USA) diluted in dimethylsulfoxide (DMSO) other other day for 21 days (modified from Matsusaka et al. 2000);

TNP-470+SU5416 Group (SU+TNP): FVB mice (52weeks old) received subcutaneous injections 15mg/kg of TNP-470 (Cat.3750 - Tocris Bioscience, Minneapolis, MN, USA) and intraperitoneal injections of 6mg/kg SU5416 (Cat.3037 - Tocris Bioscience, Minneapolis, MN, USA) diluted in dimethylsulfoxide (DMSO), every other day for 21 days (modified from Matsusaka et al. 2000; modified from Strieth et al. 2006).

All the animals received water and the same solid diet *ad libitum* (Nuvilab, Colombo, Paraná, Brazil). After 21days of treatment, all the animals were weighed on an analytical balance DenverP-214 (Denver Instrument Company, Arvada, CO, USA), then anesthetized

with 2% xylazine hydrochloride (5mg/kg i.m.; König, SãoPaulo, Brazil) and 10% ketamine hydrochloride (60mg/kg, IM, Fort Dodge, Iowa, USA) and sacrificed. This study was approved by the Ethics Committee on Animal Use (CEUA), protocol 2391-1.

6.3.2. Light Microscopy

Prostatic samples taken from five animals from each group were collected and fixed in Bouin's solution for twenty-four hours. After fixation, tissues were rinsed in 70% ethanol and dehydrated. After that, the fragments were diaphonized in xylene and embedded in plastic polymers (Paraplast Plus, ST. Louis, MO, USA). Next, the materials were sectioned in a Hyrax M60 microtome (Zeiss, Munich, Germany), then stained with hematoxylin-eosin and Masson Trichrome (Junqueira et al. 1979) and photographed in a Nikon EclipseE-400 light microscope (Nikon, Tokyo, Japan).

6.3.3 Immunohistochemistry of antigens: Vascular Endothelial Growth Factor (VEGF); Endostatin, Ki-67, androgen receptor (AR), α and β estrogen receptors (ER-α and ER-β).

Samples of the ventral lobe of the prostate were collected from five animals in each experimental group, the same used for light microscopy. Next, tissue sections were submitted to antigen retrieval by incubating the sections in citrate buffer (pH 6.0) at 100 °C for 15 minutes in a microwave oven (750 watts) or treatment with proteinase K, depending on the characteristics of each of the antibodies analyzed. The blocking of endogenous peroxidases was obtained with H_2O_2 (0.3% in methanol) for 15 minutes. Nonspecific binding was blocked by incubating the sections in a blocking solution with bovine serum albumin (3%) in TBS-T buffer for 1 hour. After that, AR, ER-α, ER-β, Ki67, VEGF and Endostatin antigens, were

immunolabelled using the following antibodies: polyclonal rabbit AR-N20 (sc-816) (Santa Cruz Biotechnology, CA, USA) for the androgen receptor, monoclonal mouse (1D5) (Dako Cytomation, Inc., CA, USA) for ER α, polyclonal rabbit (06-629) (Upstate, USA) for ERβ, polyclonal rabbit (ab9260) (Millipore, Massachusetts, USA) for Ki67, (sc-53462) (Santa Cruz Biotechnology, CA, USA) for VEGF, monoclonal mouse (ab64569) (Abcam, USA) for Endostatin diluted (1:35-50) in 1% BSA and incubated overnight at 4°C. The HRP Envision Kit (Dako Cytomation, Inc., CA, USA) was used to detect the antigens, according to the manufacturer's instructions. After washing with TBS-T buffer, the sections were incubated with secondary conjugate HRP antibody from the Envision kit (Dako) for 40 minutes and subsequently developed with diaminobenzidine (DAB). Harris' Hematoxylin was used for counter-staining. Ten prostatic regions from 5 animals from each experimental group were evaluated using the DAB brown colored precipitate, which indicated antibody immunoreactivity. The intensity of the immunoreactivity of antigens was graded as intense (+++), moderate (++) and weak (+), according to the distribution and frequency of the glandular tissue antigens (modified from Markopoulos et al. 2000).

6.3.4. Cell Count Positive Ki-67 and Determination of proliferative index

Proliferative index were obtained by counting the Ki-67 positive cells. The prostate samples of five animals (those intended for Ki-67 immunostaining) from each experimental group, were used. Next, ten fields for each specimen were evaluated witha 100x objective lens and the proliferative index was determined by dividing the number of the Ki-67 positive cells by the total number of cells found in the microscopic field.

6.3.5. Detection of Apoptosis and Determination of the apoptotic index

Samples of the prostatic ventral lobe of five animals from each experimental group were collected and fixed in 4% paraformoldehyde for twelve hours. After fixation, the samples were included in paraffin and plastic polymers (Paraplast PlusTS.Louis,MO, USA), then sectioned in the Hyrax M60 microtome (Zeiss,Munich, Germany) with 5μm thickness and submitted to DNA fragmentation reaction detection. The DNA fragmentation was detected using the detection system for fluorescent apoptosis (Promega, Madison, WI, USA) according to the manufacturer's instructions, and Feulgen. Apoptotic nuclei were identified and photographed using the Olympus IX71 inverted-II microscope (Olympus, Califórnia, USA) equipped with fluorescence (IX2-FL-II, Olympus, Califórnia, USA).

The sections were submitted to hydrolysis with 4NHCl for 65 minutes for the Feulgen reaction, and treated with Schiff reagent for 40 minutes. After washing with water, the sections were dehydrated and mounted on slides. Next, ten fields of each animal were analyzed with a 100x objective lens and the apoptotic index was determined by dividing the number of apoptotic nuclei by the total number of nuclei found in the microscopic fields. Apoptotic nuclei were identified by characteristics such as pyknosis and or nucleolar fragmentation.

6.3.6. Protein Extraction and Western Blotting

Samples from the ventral lobe were collected from five animals in each experimental group, weighed, and then homogenized by the Polytron homogenizer (Kinematica) in a diluted extraction buffer RIPA (Radio-Immunoprecipitation Assay) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Tissue extracts were obtained by

centrifugation for 20 minutes at 14000 rpm at 4°C. An aliquot of each sample was used to determine the protein concentration using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA. USA). The samples were mixed (1:1) with 3X sample buffer (100mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 4% SDS and 20% glycerol), incubated in a dry bath at 95°C for 5 minutes. What corresponded to 100 micrograms of protein was applied to the SDS-polyacrylamide gel. After electrophoresis, the material was transferred electrically (Hoefer system) into nitrocellulose membranes (Amersham) at 70 V for 3 hours. The membranes were then blocked with 3% BSA diluted in TBS-T for one hour and incubated with the primary antibodies: monoclonal mouse antibody (sc-81178) for β-actin, polyclonal rabbit (sc-816) (Santa Cruz Biotchenollogy, CA, USA) for AR, 1D5 monoclonal mouse (1D5) (06-629) (Dako Cytomation Inc., CA, USA) for ERα, polyclonal rabbit (Upstate, USA) for ERβ, monoclonal mouse antibody (sc-53462) (Santa Cruz Biotechnology, CA, USA) for VEGF in a dilution range of 1:350-1000. After washing with TBS-T buffer, the membranes were incubated for 2 h with secondary antibodies anti-rabbit and anti-mouse HRP conjugate at a dilution of 1:2000 in 1% BSA. To detect reactive bands, membranes were exposed to chemiluminescence solution (Pierce Biotechnology, Illinois, USA) for 5 minutes captured by device G-Box Chemi and software image acquisition GeneSnap (Syngene, Cambridge, UK). The antibody for β actin was used as endogenous control. The intensity of labeling obtained in different situations and identified was determined by densitometry using the NIS-Elements image analysis program: Advanced Research (USA).

6.3.7. Statistical Analysis

The quantified parameters in the Western Blotting and proliferative and apoptotic indice methods were statistically analyzed for the different groups. The T-Test and variance analysis (ANOVA) followed by Tukey test for comparison of means was used for statistical analysis. All analyzes will be performed with a 5% significance level (Zar, 1999).

6.4. RESULTS

6.4.1. Light microscopy, apoptotic index and proliferative index.

6.4.1.1. Young Group

The acini showed simple epithelium with predominantly columnar cells and basal nuclei. Basal cells were discontinuously localized between the luminal cells in contact with the basal membrane. The acinar mucosa showed extensive folding regions (Figures 1a, 1b, 1c, 1d). The prostatic stroma of young animals showed smooth muscle cells, collagen fibers distributed concentrically around the acini, apart from blood capillaries (Figures 1a, 1b, 1c, 1d). The animals in this group showed 19.8% and 12.7% proliferation and apoptosis indices respectively. The apoptotic index was statistically significant when compared with the other experimental groups (Figure 3; Table 1).

6.4.1.2. Senile Group

The animals in the senile group presented predominantly atrophic luminal epithelium characterized by the general decrease in secretory epithelial cells and acinar mucosa folding. The presence of basal cells interspersed in luminal cells was observed. The basal membrane appeared intact and without discontinuity points. Occasional prostatic intraepithelial neoplasia

points (PIN), with flat and cribriform patterns, characterized by the presence of cells with increased volume and nucleus occupying most of the cytoplasm, apart from the presence of proliferative inflammatory atrophy (PIA) and microacini were observed during senescence (Figure 1e, 1f, 1g, 1h). The prostatic stroma presented hypertrophy with thick layers of smooth muscle cells interspersed with collagen fibers, apart from the presence of frequent and abundant inflammatory cells, particularly lymphocytes (Figure 1e, 1f, 1g, 1h).

Animals from the senile group showed 22% and 21.3% proliferation and apoptosis indices, respectively (Figure 3; Table 1).

6.4.1.3. Finasteride Group

The animals treated with finasteride presented a predominantly atrophic epithelium and intense decrease of the acini mucosa folding. This atrophy is intensified in the glandular tissue when compared to atrophy observed in the senile group. Although the prostatic stroma was rare in this group, some occasional points of hypertrophy and inflammatory foci were observed (Figure 1i, 1j, 1k, 1l).

Animals in the finasteride group presented 24.6% and 17.6% proliferation and apoptosis indices, respectively, showing no statistically significant differences when compared to the indices of the senile group (Figure 3; Table 1).

6.4.1.4. SU5416 Group

The animals treated with the indirect angiogenic inhibitor SU5416 showed variation of the cells that make up the prostatic secretory epithelium, and these ranged from simple columnar to cubic, in other words, atrophic in some regions. Occasional points of epithelial

proliferation, characterizing flat pattern prostatic intraepithelial neoplasia, were also found in this group. Despite the prostatic stroma presenting a tendency to be scarce, a thickening of the smooth muscle cells surrounding the glandular acini was observed, a fact that has characterized stromal hypertrophy. The presence of inflammatory cells was lower when compared to the senile group (Figure 2a, 2b, 2c, 2d).

The SU5416 group animals presented 33.6% and 21% proliferation and apoptosis indices, respectively, showing no statistically significant differences when compared to the indices of the senile group (Figure 3; Table 1).

6.4.1.5. TNP-470 Group

The acini, in the prostate of animals treated with the direct angiogenesis inhibitor TNP-470 were smaller and sometimes their lumen were reduced. The luminal epithelium was predominantly characterized by the presence of cubic cells, demonstrating atrophy in some regions. Furthermore, occasional points of focal atypia indicated by clear halo cells around the nucleus were observed. Intense hypertrophy and hyperplasia were observed in the stroma as a result of the increase of collagen fibers and smooth muscle cells. Also, inflammatory cell reduction was observed in relation to the senile group. When the prostate in mice treated with TNP-470 was compared to that of the SU5416 group of mice, intensified structural changes especially related to the stroma were observed (Figure 2e, 2f, 2g, 2h).

The animals of the TNP-470 group presented proliferation and apoptosis indices of 32% and 20.2%, respectively, showing no statistically significant differences when compared to the indices of the senile group (Figure 3; Table 1).

6.4.1.6. SU5416+TNP-470 Group

The animals treated simultaneously with SU5416 and TNP-470 antiangiogenic inhibitors presented variation of the prostatic secretory epithelium cellular shape, with cells ranging from simple columnar to cubical. Epithelial atrophy and occasional focal atypia were observed, as well as some prostatic intraepithelial neoplasia points. The PIN foci presented cells with increase of nuclear and cytoplasmic volume (Figure 2i, 2k, 2j, 2l).

The prostatic stroma of animals treated with TNP-470 and SU5416 did not change significantly, and was therefore characterized by the presence of concentrically distributed fibrillar elements around the acini, smooth muscle cells and blood capillaries. Reduction of inflammatory cells was observed in this group in relation to individual treatment with antiangiogenic drugs. Generally, significant structural recovery of epithelial and stromal compartments was observed, considering particularly the epithelial proliferations and the increase of smooth muscle cells (Figure 2i, 2k, 2j, 2l).

The animals of the SU5416+TNP-470 group presented proliferation and apoptosis indices of 40.4% and 32.8% respectively. These increases were statistically significant when compared to different experimental groups (Figure 3; Table 1).

6.4.2. Immunohistochemistry and Western Blotting

6.4.2.1. Young Group

AR immunolocalization was intense, especially in the nuclei of epithelial cells and moderate in the stromal cells (Figure 4a; Table 2). Lack of reactivity for ER-α was observed in the nuclei of epithelial cells and moderate in the stromal cells (Figure 4b; Table 2). The immunolocalization for ER-β showed weak reactivity in the nuclei of epithelial cells and

stromal cells (Figure 4c; Table 2). Weak reactivity for VEGF was observed in the apical region of epithelial cells cytoplasm and in the stromal cells (Figure 4d, Table 2). The immunolocalization for the Endostatin was intense and appeared diffusely in the cytoplasm of the epithelial cells and weak in the stromal cells (Figure 4e; Table 2).

The AR, ER-α, ER-β, and VEGF protein levels were 135.4%, 115.3%, 105.1% and 51%, respectively, compared to the standard β-actin in the young group (Figure 6).

6.4.2.2. Senile Group

The immunolocalization for AR was moderate in the nuclei of epithelial cells and weak in the stromal cells (Figure 4f; Table 2). Weak reactivity for ER-α was observed in the nuclei of epithelial cells and intense in the stromal cells (Figure 4g; Table 2). The immunolocalization for ER-β showed moderate reactivity in the nuclei of epithelial cells and weak in the stromal cells (Figure 4h; Table 2). Intense reactivity for VEGF was found in the cytoplasm of epithelial cells and moderate in the stromal cells (Figure 4i; Table 2).The immunolocalization for Endostatin was moderate in the cytoplasm of epithelial cells and stromal cells (Figure 4j; Table 2).

The AR, ER-α, ER-β, VEGF protein levels were 102.2%, 160.4%, 119.5%, 168.5%, respectively, compared to the standard β-actin in the senile group. When compared to the animals in the young group, the decrease of the AR protein level and increased protein levels of the ER-α, ER-β and VEGF molecules were characterized (Figure 6).

6.4.2.3. Finasteride Group

AR immunolocalization was moderate in the epithelial cells and weak in the stromal cells (Figure 4k; Table 2).Weak reactivity for ER-α was observed in the nuclei of epithelial cells and moderate in the stromal cells (Figure 4l; Table 2). The immunolocalization for ER-β was intense in the nuclei of epithelial cells and moderate in the stromal cells (Figure 4m; Table 2). Moderate reactivity for VEGF was found in epithelial cells and weak in stromal cells (Figure 4n; Table 2). The immunolocalization for Endostatin was intense in the cytoplasm of epithelial cells and moderate in the stromal cells (Figure 4o; Table 2).

The AR, ER-α, ER-β, and VEGF protein levels were 72.6%, 142.4%, 275.7%, 92.5%, respectively, compared to the standard β-actin in the finasteride group. Regarding to senile group, there was an increase in ER-β protein level, and a reduction in ER-α and VEGF protein levels (Figure 6).

6.4.2.4. SU5416 Group

The immunolocalization of AR was moderate in the epithelial cells and weak in the stromal cells (Figure 5a; Table 2). Lack of reactivity for ER-α in the nuclei of the epithelial cells and weak reactivity in the stromal cells were observed (Figure 5b; Table 2). The immunolocalization for ER-β was moderate in the nuclei of the epithelial cells and stromal cells (Figure 5c; Table 2).Weak reactivity for VEGF was found in the apical region of the epithelial cells and stromal cells (Figure 5d; Table 2). The immunolocalization for Endostatin was moderate in the apical region of epithelial cells cytoplasm and weak in the stromal cells (Figure 5e; Table 2).

The AR, ER-α, ER-β and VEGF protein levels were 81%, 84.8%, 132.8% and 28.1% respectively, compared to the standard β-actin in the SU5416 group. In relation to the senile group, an increase in the ER-β protein level and a decrease of ER-α and VEGF levels were verified (Figure 6).

6.4.2.5. TNP-470 Group

The AR immunolocalization was weak in both epithelial and stromal cells (Figure 5f; Table 2). Lack of reactivity for ER-α was observed in the nuclei of epithelial cells and there was moderate reactivity in the stromal cells (Figure 5g; Table 2). The immunolocalization for ER-β was weak in the nuclei of the epithelial and lack of reactivity was found in the stromal cells (Figure 5h; Table 2). Intense reactivity for VEGF was found diffusely in the cytoplasm of epithelial cells and moderate in the stromal cells (Figure 5i; Table 2). The immunolocalization for Endostatin was moderate in the epithelial and stromal cells (Figure 5j; Table 2).

The AR, ER-α, ER-β and VEGF protein levels were 37.1%, 117.6%, 86.8%, 160.8% respectively compared to standard β-actin in the TNP-470 group. The protein levels of the molecules listed above decreased in relation to those verified in the senile group, excepting the VEGF (Figure 6).

6.4.2.6. THE SU5416+TNP470 Group

AR immunolocalization was weak in the epithelial and stromal cells (Figure 5k; Table 2). Lack of reactivity for ER-α was observed in the nuclei of epithelial cells and weak reactivity was observed in the stromal cells (Figure 5l; Table 2). ER-β immunolocalization was intense in the nuclei of the epithelial cells and moderate in the stromal cells (Figure 5m;

Table 2). Weak reactivity for VEGF was found in the cytoplasm of epithelial cells, and also in the stromal cells (Figure 5n; Table 2). The immunolocalization for Endostatin was moderate in the epithelial and intense in the stromal cells (Figure 5o; Table 2).

The AR, ER-α, ER-β, and VEGF protein levels were 62% 77.3%, 306.3%, 65.4%, respectively, compared to the standard β-actin in the SU5416+TNP-470 group. Regarding the senile group, AR, ER-α and VEGF protein levels decreased, while the ER-β level increased (Figure 6).

6.5. DISCUSSION

Structural analysis showed the effects of senescence on the prostatic structure such as epithelial atrophy, stromal hypertrophy, increased frequency of inflammatory cells in the prostatic stroma, apart from occasional points of prostatic intraepithelial neoplasia (PIN). The treatment with finasteride led mainly to epithelial atrophy and scarcity of stromal elements as well as reducing the incidence of inflammatory cells.

On the other hand, the antiangiogenic therapies, in general, led to the recovery of structural alterations of the prostate in relation to the senile group, highlighting the decrease in the occurrence of inflammatory cells in the stroma, and a decrease of the epithelial points undergoing cellular proliferation. Stromal hypertrophy and a reduction of the luminal acini occurred particularly in animals treated with the direct inhibitor TNP-470 and these characteristics were more pronounced than those found in the group receiving SU5416.

The combination of antiangiogenic therapies led to the reduction both of the inflammatory cells as well as the regions of cell proliferation, when compared to the other experimental treatments. Although in SU5416+TNP-470 group the proliferation and apoptosis

indices are statistically significant in relation to the other experimental groups, the ratio between these processes decreased indicating the prevalence of proliferative process on the apoptosis

Senescence is associated to molecular, cellular and physiological changes characterized by homeostatic imbalance in the prostate microenvironment, and increased incidence of tumors and other lesions associated with this period of life (Krtolica and Campisi, 2002; Bianchi-Frias et al. 2010; Montico et al. 2011). Studies verified the morphological changes in the ventral prostate during senescence, such as focal atypia, decreased acini projections and stromal hyperplasia (Lau et al. 2003 and Acosta et al., 2004). Also, Bianchi-Frias et al. (2010) also found changes in the stromal compartment during senescence, especially in the increase of inflammatory infiltration and disorganization of the architecture of smooth muscle cells and fibroblasts located around the glandular acini. According to Taniguchi et al. (2009), inflammation has been recognized as an important factor for the progression of symptoms related to benign prostatic hyperplasia (BPH). Furthermore, it is known that epithelial cells depend on the interaction with the stroma to perform their functions in the tissue (Krtolica & Campisi, 2002). Montico et al. (2011) found that the occurrence of hypertrophy and inflammatory cells in the prostatic stroma of senile rats indicated the structural disorganization and hormonal imbalance in the prostate during senescence. Bianchi-Frias et al. (2010) identified increased gene expression associated with inflammation in the prostate of the senile animals, emphasizing the capacity of stromal cells produce and respond to inflammatory signs during this period of life.

Recent studies showed that, despite the beneficial effects of cell senescence, senescent cells may also exert adverse effects on the tissue microenvironment, the most notable being

the acquisition of a "senescent secretory phenotype", which implies in the increased secretion of pro-inflammatory cytokines (Davalos et al. 2010). These changes can lead to phenotypes and lesions associated to aging, including late onset of cancer (Davalos et al. 2010). Unlike previous studies, Banerjee et al. (1998) did not find morphological changes due to senility in the greater part of the prostate ventral lobe.

Thus, from these results it can be concluded that senility promoted structural prostate changes, suggesting impairment of the function of this organ, and a predisposition towards the development of malignant lesions due to a favorable microenvironment. In addition, such changes may suggest impairment of the reproductive function.

The development and maintenance of the prostatic structure is dependent, at least in part, on the action of androgens that possess as one of their functions, the double ability to stimulate proliferation and inhibit cell death (Justulin, et al. 2010). Also, it is known that the prostatic androgenic action is dependent on the conversion of testosterone (T) into dihydrotestosterone (DHT) by 5α-reductase enzyme, that DHT exhibits high affinity to androgen receptors, and that this interaction is necessary for the maintenance of epithelial morphology (Morrissey et al. 2002). Finasteride, a selective inhibitor of the 5α-reductase enzyme blocks the formation of potent prostatic androgen, without interfering with the function of testosterone, and has been used in the preventive treatment of prostatic diseases, including cancer (Azzolina et al. 1997; Andriole et al. 2005).

Prahalada et al. (1998) found that chronic treatment with finasteride resulted in a marked decrease in the weight of the ventral prostate, and a significant reduction of the epithelial and stromal compartments, considering epithelial and stromal cell density. Several authors have also verified the presence of a specific pro-apoptotic mechanism, as a result of

treatment with finasteride (Pareek et al. 2003; Golbano et al. 2008; Thompson et al. 2009). Rittmaster et al. (1995) demonstrated that finasteride caused a significant increase in the number of apoptotic cells in the prostate gland of rats during the first days of treatment. However, from the 14th day of treatment on, there was no significant difference in the number of apoptotic cells (Rittmaster et al. 1995). These same authors also stated that although DHT is dominant in the prostate, the high levels of testosterone induced by finasteride may be sufficient to mitigate the apoptotic process (Rittmaster et al. 1995).

The present results indicated that treatment with finasteride compromised the prostatic structure in senile animals, and that epithelial atrophy and stromal decreased are strong characteristics in the prostate of these animals. However, this same treatment strongly contributed towards reducing the incidence of inflammatory cells, which may possibly have resulted in a stable microenvironment, considering the importance of the inflammatory process and or the occurrence of malignant proliferation in prostatic diseases.

In the last decade, the antiangiogenic agents have been the subject of several studies on the best forms of therapy for the control and treatment of cancer, especially in relation to the control of tumor growth (Burke and DeNardo, 2001). According to Abdollahi et al. (2003), the main challenge for antiangiogenic therapies is to create combined protocols capable of neutralizing the various angiogenic stimuli produced by the tumor and its microenvironment.

The SU5416, indirect inhibitor of angiogenesis, is a synthetic compound that acts as a potent inhibitor of the tyrosine kinase FLK-1/KDR receptor, selective for VEGF. Studies have indicated that this drug has a broad antitumor effect resulting from the blocking mechanism of angiogenesis (Fong et al. 1999; Huss et al. 2003; Aragon-Ching et al. 2010). The action of this drug prevents endothelial cells from responding to different proangiogenic stimuli (Abdollahi

et al. 2003). Huss et al. (2003) reported reduction in the progression of prostatic lesions and the density of microvessels, in addition to significantly increased tumor apoptotic index in transgenic mice for prostate adenocarcinoma, after 6 weeks of treatment with SU5416. Abdollahi et al. (2003), on the other hand, demonstrated that SU5416 was not able to induce significant apoptosis in human prostate cancer cells (PC-3) and gliomablastoma (U87) in vitro. However, the quantitative analysis of proliferation of tumor cells in a xenografic mode (Ki67 positivity %) presented a significant reduction after treatment with SU5416.

The TNP-470 is a synthetic analog of fumagillin and is able to inhibit angiogenesis and suppress tumor growth *in vivo* (Yamaoka et al. 1993; Kato et al. 1994). Yamaoka et al. (1993) found that TNP-470 exhibited potent antitumor activity mainly in human cell linages for prostate (PC-3) and breast cancer (MDA-MB231). Miki et al. (1998) evaluated the effect of TNP-470 in mouse cell lines for prostate cancer (AT6.3) *in vitro* and when implanted into nude mice, and verified the reduction of both tumor growth and mestastasis. Amikura et al. (2006) found that TNP-470 did not suppress metastasic formation in the liver and lung, considering that the antitumor effect of TNP-470 is displayed when there is already a tumor angiogenesis system installed. Furthermore, Amikura et al. (2006) reported that the isolated treatment with TNP-470 is not sufficient to restrain or stop the cancer. Instillation of TNP-470 in urinary bladders stimulated with N-methyl-N-nitrosurea, to induce tumors in mice, presented significant reduction in tumor incidence, suggesting that TNP-470 might also have a chemoprotector effect (Tanaka et al. 1997).

Thus, it can be concluded from these results that treatment with antiangiogenic drugs, as well as the combination of direct and indirect acting drugs, influenced the dynamics of the proliferative and apoptotic process observed in the prostate microenvironment in senile mice,

so the treatment is indicated for this organ. Certainly, the results also showed that the prostate microenvironment of the senile animals favored the occurrence of structural changes, which suggested the appearance of malignant lesions due to the imbalance in epithelium and stroma interaction.

The molecular results showed a decrease in reactivity for AR and Endostatin, and an increase for ER-α, ER-β and VEGF in the senile group, when compared to young mice. The mice in the groups treated with finasteride, SU5416 and SU5416+TNP-470, when compared to the senile group, showed, on the whole, decreased reactivity of VEGF and ER-α and an increase of ER-β. The treatment with TNP-470 however, was marked mainly by reduced reactivity and reduced AR and ER-β protein levels when compared to the young and senile groups. The reactivity of Endostatin in this group was lower than in young animals, and similar to the senile ones. Also, lower reactivity for ER-α was charcterized in the TNP-470 group when compared particularly to senile mice.

Androgens are involved in the development of prostatic diseases; the effects of these hormones are mediated by growth factors which act based on autocrine and paracrine mechanisms, increasing cell proliferation and reducing apoptosis (Gnanapragasam et al., 2000). Most of these factors act as potent mitogens and are upregulated by androgens based on mechanisms wich are still not totally explained (Gnanapragasam et al. 2000). However, androgens *per se* are not enough to induce prostatic diseases, and the estrogen action is also important in the development of these lesions (Ellem and Risbridger, 2009). Different authors found that the prostate microenvironment is less sensitive to the actions of androgens in senile rats due to decreased levels of AR (Cordeiro et al. 2008; Ellem and Risbridger, 2010). Banerjee et al. (2000) using young Brown Norway (4 months) and senile (24 months) rats

found a predominant location for AR in the prostate epithelium of the ventral, lateral and dorsal lobes, with a reduction of this receptor in the ventral prostate of senile rats. Yamashita et al. (2004) also identified androgen receptors predominantly in the ventral and dorsal prostate epithelium in 8 week old mice. According to Cândido et al. (2012), the AR distribution pattern is lobe-specific among the senile rats'accessory sex glands, considering the action of androgens and hormonal imbalance due to this life period.

Estrogenic effects in the prostate are results of the binding of these hormones to specific α and β estrogen receptors (ER-α, ER-β), which are predominantly expressed in the epithelium and stroma, respectively (Cunha et al. 2002). Candido et al. (2012) found increased reactivity for ER-α in the prostate os senile rats submitted to both androgen ablation and to administration of estrogen and testosterone. The same authors also detected enhanced reactivity for ER-β in the prostatic stroma and epithelium of senile rats with high serum levels of testosterone and estrogen, respectively. (Cândido et al. 2012). Ellem and Risbridger (2009) reported the existence of a dichotomy of action between ER-α and ER-β. Adverse estrogenic effects via ER-α, are specifically related to the development of proliferative processes, inflammation and cancer. In contrast, estrogenic effects via ER-β pathways seem to mediate antiproliferative, anti-inflammatory and potentially anti-carcinogenic effects of estrogens (Ellem and Risbridger, 2009). Consequently, these data established the rationale for the potential use of specific ER modulators (ER-α antagonists and ER-β agonists) as new options for the treatment of prostate diseases (Ellem and Risbridger, 2009).

Several studies have suggested that VEGF expression is directly regulated by androgens in both normal tissues and in prostate tumors (Van Moorselaar and Voest, 2002; Delongchamps et al. 2006; Reynolds and Kyprianou, 2006). According to Cai et al. (2011),

the interaction between DHT and androgen receptors induced endothelial cell *in vitro* proliferation by upregulation of VEGF and expression of the gene for cyclin. Lissbrant et al. (2004) reported that testosterone can indirectly stimulate vascular growth in the ventral lobe of the prostate of mice by means of the synthesis of VEGF. These same authors stated that the treatment with testosterone significantly increased the proliferation of endothelial cells, vascular volume, and weight of the prostate (Lissbrant et al. 2004). However, these responses were not completely inhibited by treatment with anti-VEGF (Lissbrant et al. 2004). The androgenic control on VEGF levels is also seen in prostate cancer, where it was reported that androgen deprivation was able to lead to decreased levels of VEGF, as its expression increased in androgen-independent tumors (Cheng et al. 2004). Reed et al., (2007) pointed out that the prostate microenvironment of the senile animal is conducive to tumor development and this could grow efficiently through consistent angiogenic support. Studies with castrated models demonstrated reduced prostate vascularization and induction of apoptosis in the endothelial cells prior to gland involution. However, increase in angiogenesis was verified afters testosterone replacement (Shabisgh et al. 1999). There was also a decrease in VEGF protein levels during castration (Joseph et al. 1997). Different authors evaluated the use of finasteride in patients with benign prostatic hyperplasia (BPH), also verifying the decrease in VEGF expression. (Häggström, et al. 2001; Pareek et al. 2003).

Several studies have confirmed the efficacy of angiogenesis inhibitors on VEGF expression (Zhong et al. 2004; Strieth et al. 2006; Ban et al. 2010) and on vascular growth inhibition (Castronovo and Belotti, 1996; Amikura et al., 2006). It is well known that the antiangiogenic action of Endostatin can influence the signaling pathways of the VEGF and FGF-2 receptors, altering the behavior of these factors in the tissue microenvironment and

possibly their potential inhibition of tumor growth by promoting apoptosis. (Sim et al. 2000). Abdollahi et al. (2003) found that, SU5416 and Endostatin, when combined, were effective, empowering their antiangiogenic action on various cancer cell lines.

Based on these results, it was concluded that senility modified the prostatic microenvironment, with consequent changes in the activation and signaling of androgenic, estrogenic signaling and angiogenic pathways. Although estrogen signaling through ER-α could have led to processes of stromal hyperplasia and hypertrophy, a balance by means of an antiproliferative ER-β pathway was detected in senility. Also it was concluded that treatment with finasteride in senility promoted increased ER-β, characterizing the nature of the antiproliferative drug.

Moreover, the preponderance of antiangiogenic factors on proangiogenic factors was verified, which certainly indicated an unfavorable glandular microenvironment to the process of endothelial and consequently tissue proliferation.

Further, it was found that treatment of senile animals with antiangiogenic SU5416 led to a decrease of the proliferative effects in the prostate by means of the estrogen pathway, considering decreased ER-α, increased ER-β and decreased pro-angiogenic factors. The antiangiogenic treatment with TNP-470, on the other hand, was shown to be markedly active in the androgenic pathway, characterized by decreased protein levels of AR. However, a reduced block of the antiproliferative process by means of ER-β can be suggested considering the decreased levels of this receptor in the treatment with TNP-470. The combined treatment with antiangiogenic agents however, act mainly on the estrogenic pathway, resulting in increased ER-β and decreased ER-α protein levels, thus being conducive to a decrease of the proliferative process.

Finally, antiangiogenic therapies were effective, a fact indicated by the reduction of VEGF immunolocalization, when compared to the young animal group, especially in the combined treatment between SU5416 and TNP-470. The imbalance between proangiogenic and antiangiogenic factors in senile mice showed activation of the angiogenesis process, presenting the prostate microenvironment as being conducive to the occurrence of proliferative tissue processes. Among all treatments performed in this study, it can be suggested, therefore, that treatment with finasteride, SU5416 and SU5416+TNP-470 were more effective in maintaining the structural, hormonal and angiogenic factor balance in the prostate during senility.

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6.7. APPENDICES

Table 1. Proliferative and apoptotic index in the experimental groups.

	Young	Senile	Finasteride	SU5416	TNP-470	SU+TNP
Proliferative Index (%)	19.8 ± 5.1	22 ± 11.2	24.6 ± 6.5	33.6 ± 8.7	32 ± 12.1	40.4 ±13,6 *
Apoptotic Index (%)	12.7 ± 5.4*	21.3 ± 6.9	17.6 ± 5.7	21 ± 7.8	20.2 ± 3.5	32.8 ± 8.1 *

(*) Statistically significant difference ($p<0,05$). Data were expressed as the mean ± standard deviation

Table 2. Immunoreactivity positive frequency of AR, ER-α, ER-β, VEGF, and Endostatin in the experimental groups.

Experimental groups	AR		ER-α		ER-β		VEGF		Endostatin	
	Ep	St	Ep	St	Ep	St	Ep	St	Ep	St
Young	+++	++	-	++	+	+	+	+	+++	+
Senile	++	+	+	+++	++	+	+++	++	++	++
Finasteride	++	+	+	++	+++	++	++	+	+++	++
SU5416	++	+	-	+	++	++	+	+	++	+
TNP-470	+	+	-	++	+	-	+++	++	++	++
SU+TNP	+	+	-	+	+++	++	+	+	++	+++

(+) Weak; (++) Moderate; (+++) Intense; (-) Absent.

Figure 1. Photomicrographs of the ventral lobe of the prostate of the young, senile and finasteride groups: **Young Group (A, B, C, D)**: Glandular acini with folded mucosa. Simple secretory epithelium with columnar cells and basal cells **(Bc) (Inset)**. Prostatic stroma with smooth muscle cells interspersed with collagen fibers. **Senile Group** (**E, F, G, H)**: atrophic secretory epithelium **(Inset)**, epithelial cells tendency to cubic and basal cells. Presence of flat pattern prostatic intraepithelial neoplasia (**arrow**), showing flat **(Inset)** and cribriform patterns. Prostatic stroma with infiltration of inflammatory cells **(*)**. **Finasteride Group (I, J, K, L)**: Acini with few folded mucosa and epithelial atrophy. Cubical aspect luminal cells with stroma scarce. **Ep**: epithelium; **St**: stroma; **L**: lumen. Hematoxylin and Eosin (A, B, E, F, I, J); Masson's Trichrome (C, D, G, H, K, L).

Figure 2.Photomicrographs of the prostate ventral lobe from: SU5416, TNP-470 and SU5416+TNP-470 groups. **SU5416 Group (A, B, C, D)**: Glandular acini with little folded mucosa. Simple epithelium with cells ranging from cubical to columnar ones. Scarce prostatic stroma and smooth muscle cell hypertrophy **(smc*). TNP-470 Group (E, F, G, H):** Glandular acini with reduced lumen. Atrophic secretory epithelium with cubic epithelial cells and basal cells. Regions of focal atypia. Hypertrophic and hyperplastic stroma. **SU5416+TNP-470 Group (I, J, K, L):** Secretory epithelium with cells, varying from cubic to columnar ones with few mucosa folds. Presence of microacini **(m)**. Prostatic stroma with fibrocellular aspect. **Ep**: epithelium; **Es**: stroma; **L**: lumen. Hematoxylin and Eosin (A, B, E, F, I, J); Masson's Trichrome (C, D, G, H, K, L).

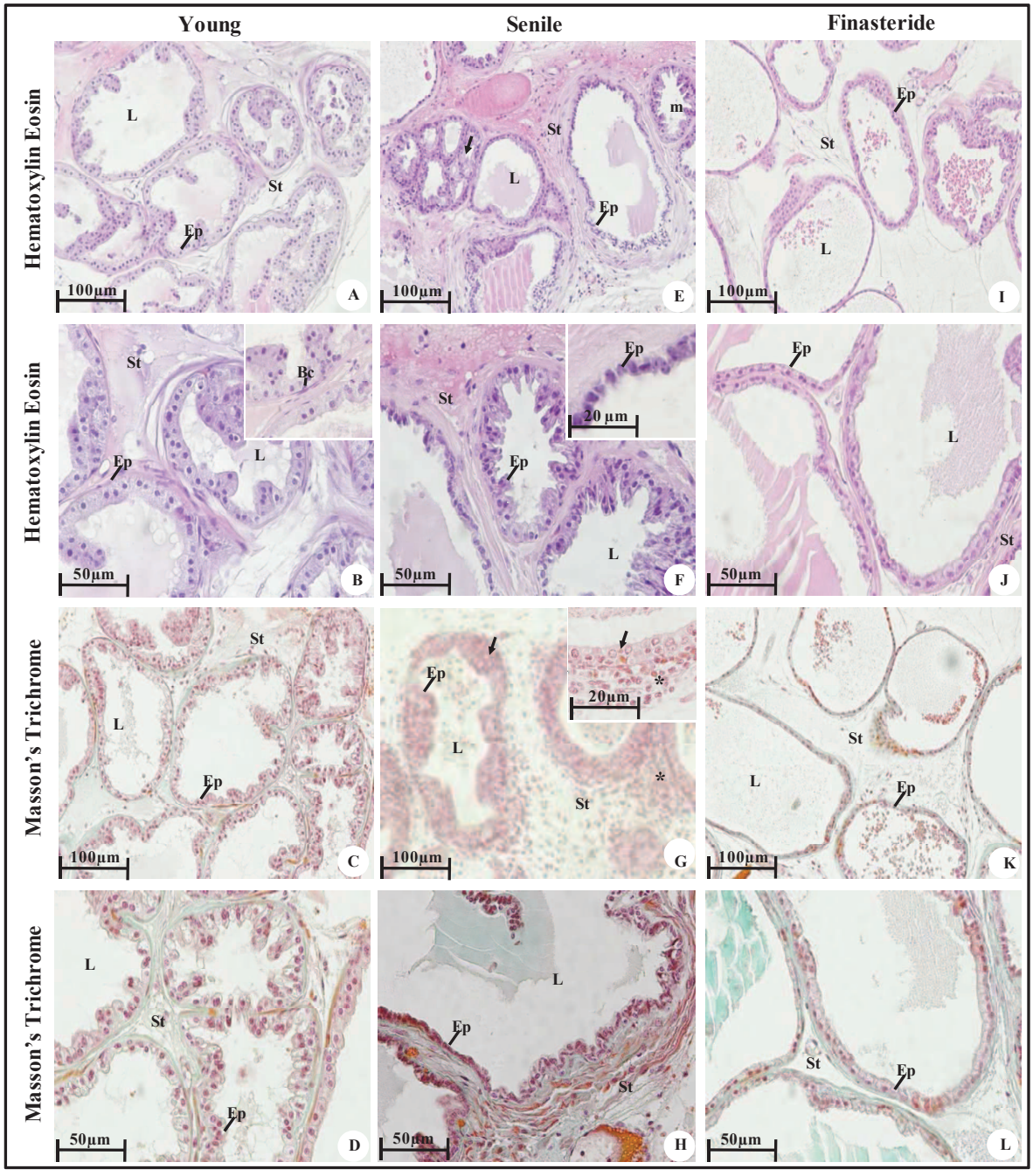
Figure 3. Ki-67 antigen immunolocalization (**A, B, C, G, H, I**) to proliferative index determination (Table 1) and apoptotic index determination, by means of Tunel Technique (**D, E, F, J, K, L**) (Table 1), and Feulgen reaction **(Inset)** to the different experimental groups. **Inset** 

Figure 4. AR, ER-α, ER-β, VEGF, and Endostatin immunoreactivities in the ventral prostate of mice from **Young Group (A, B, C, D, E), Senile Group (F, G, H, I, J)** and **Finasteride Group (K, L, M, N, O**) groups. Epithelial and stromal reactivities were graded as absent (-), weak (+), moderate (++) and intense (+++), according to Table 2.

Figure 5. AR, ER-α, ER-β, VEGF, and Endostatin immunoreactivities in the ventral prostate of mice from **SU5416 Group (A, B, C, D, E), TNP-470 Group (F, G, H, I, J)** and **SU5416+TNP470_Group (K, L, M, N, O)** groups. Epithelial and stromal reactivities were graded as absent (-), weak (+), moderate (++) and intense (+++), according to Table 2.

Figure 6. Representative Western Blotting for AR, ER-α, ER-β and VEGF proteins of the prostate ventral lobe in the six experimental groups. β-actin was used as the endogenous control.

Figure 1.


Young
Senile
Finasteride
Hematoxylin Eosin
Hematoxylin Eosin
Masson's Trichrome
Masson's Trichrome
L
St
Ep
m
Bc
*
100µm
50µm
20µm
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A
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Figure 2.

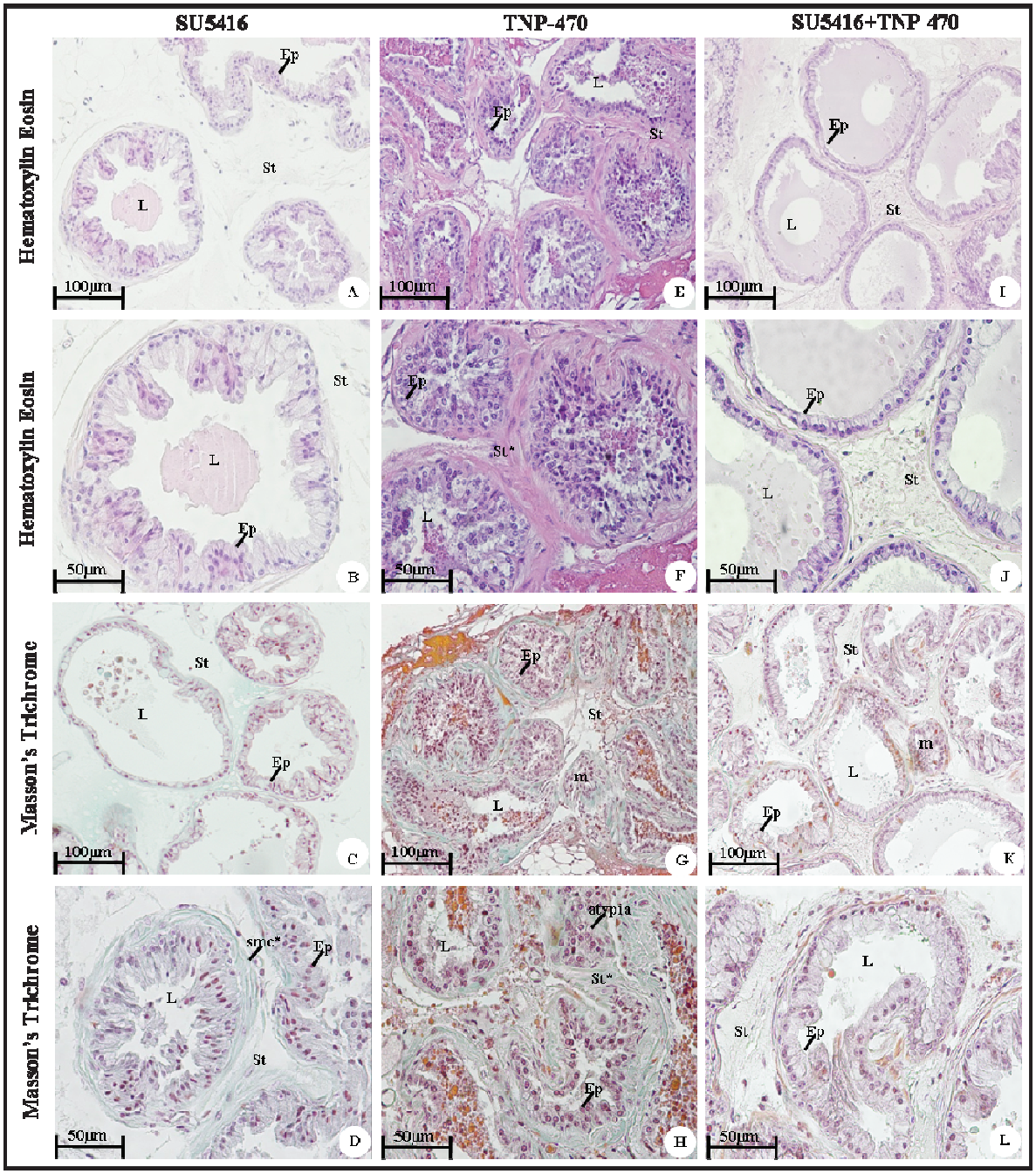

SU5416
TNP-470
SU5416+TNP 470
Hematoxylin Eosin
Hematoxylin Eosin
Masson's Trichrome
Masson's Trichrome
Ep
St
L
100µm
50µm
A
B
C
D
E
F
G
H
I
J
K
L
St*
m
atypia
smc*

Figure 3

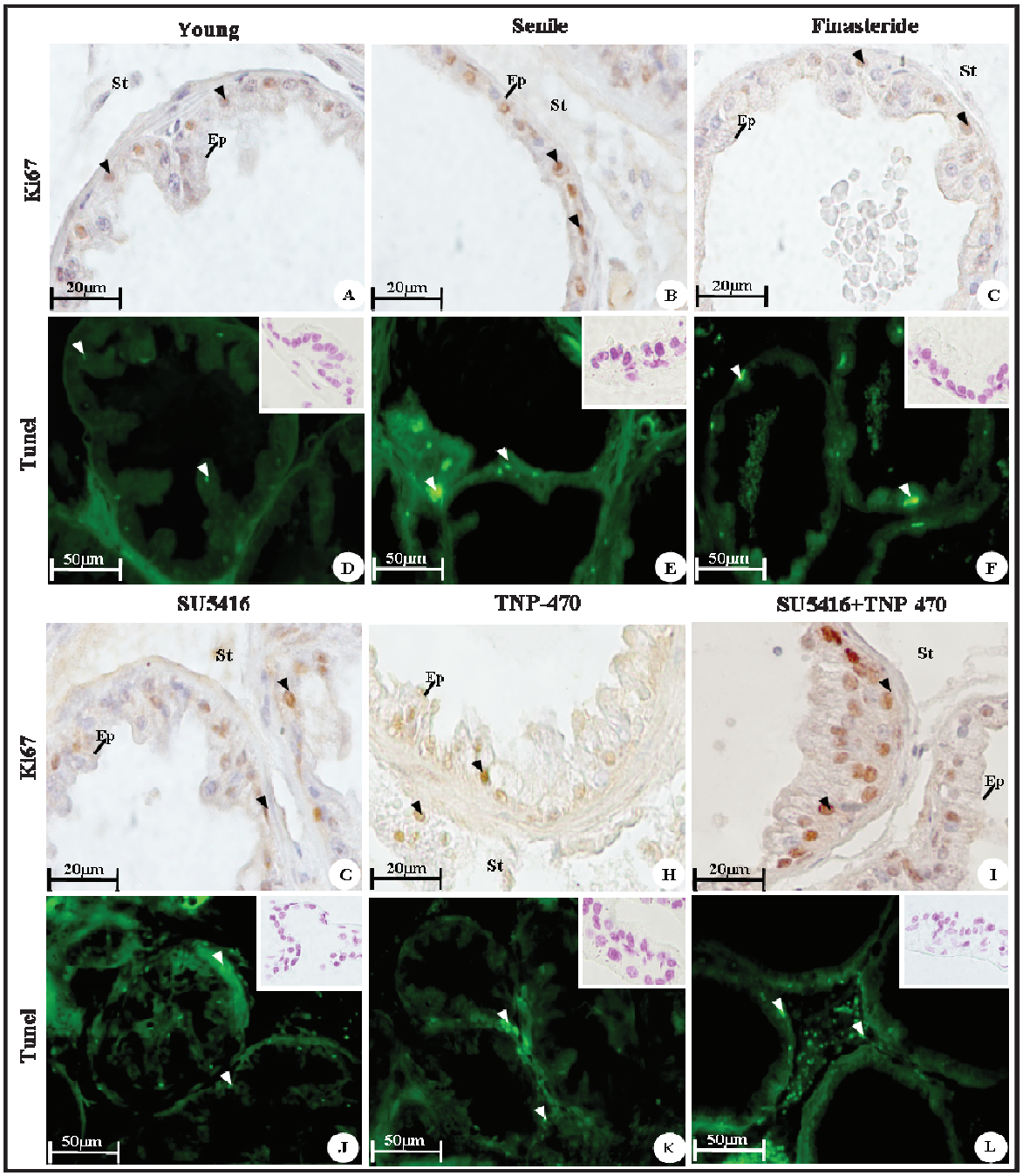
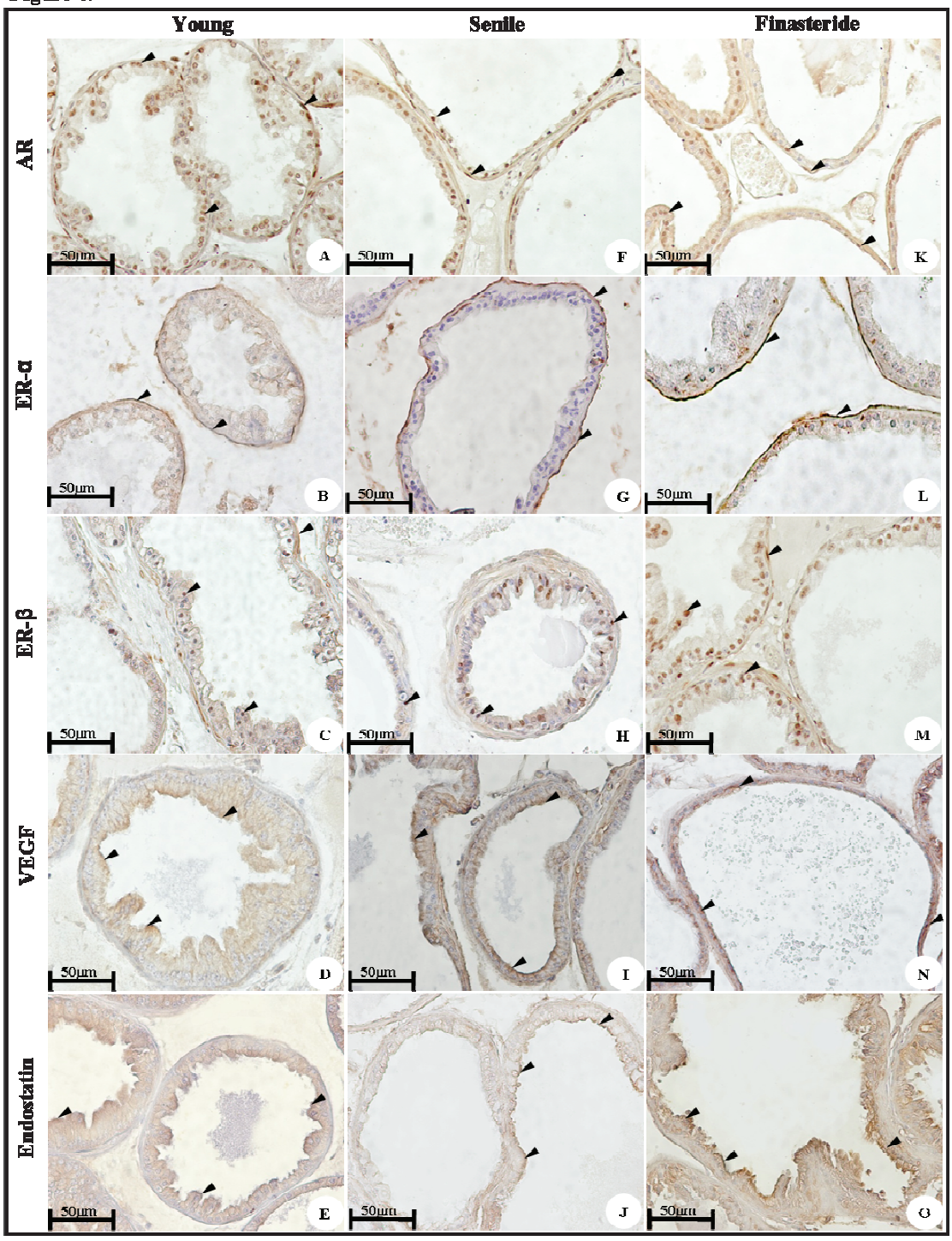

Young
Senile
Finasteride
Ki67
Tunel
St
Ep
20μm
50μm
A
B
C
D
E
F
SU5416
TNP-470
SU5416+TNP 470
G
H
I
J
K
L

Figure 4.


Young
Senile
Finasteride
AR
ER-α
ER-β
VEGF
Endostatin
50μm
A
B
C
D
E
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G
H
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J
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Figure 5.

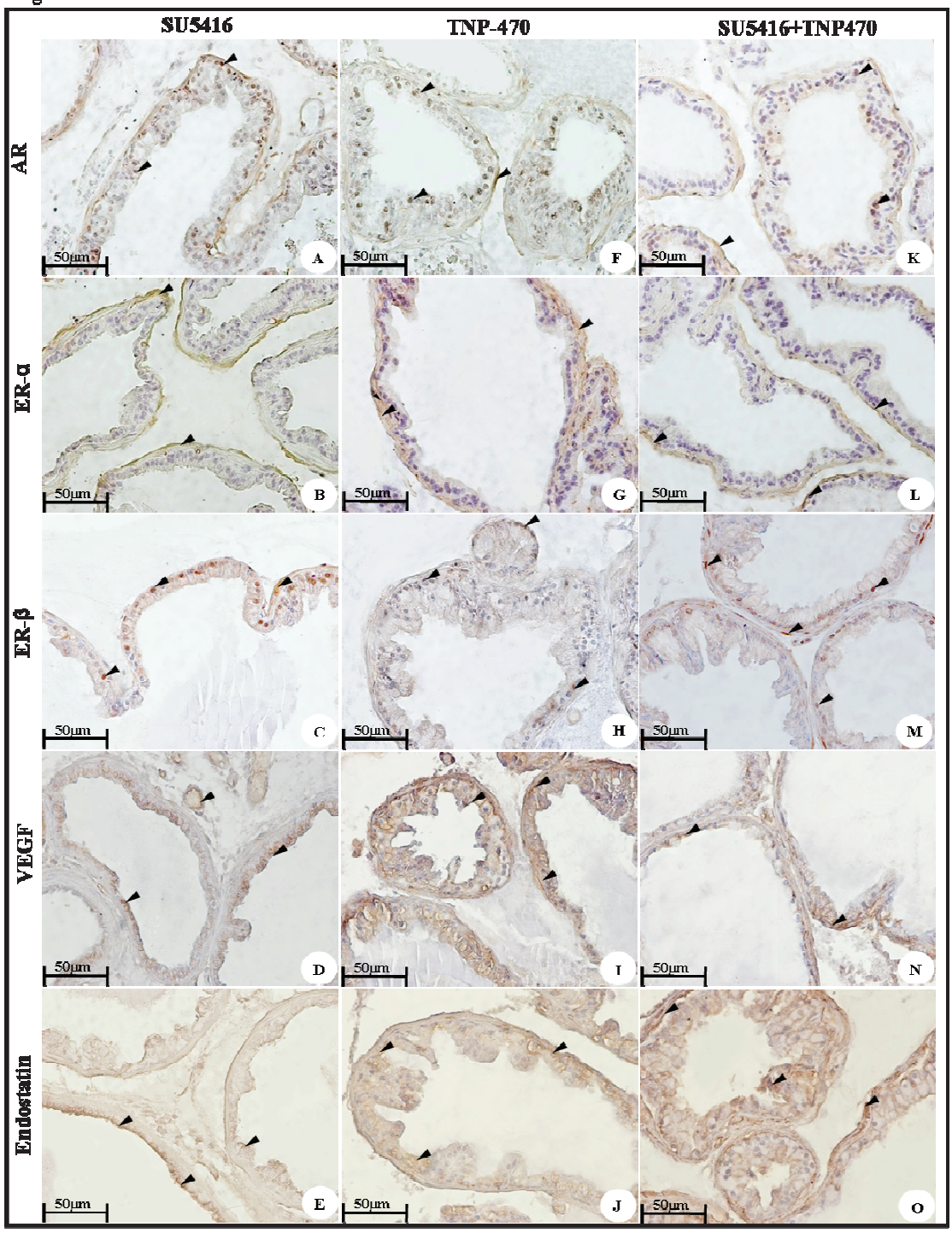
SU5416
TNP-470
SU5416+TNP470
AR
ER-α
ER-β
VEGF
Endostatin
50μm
A
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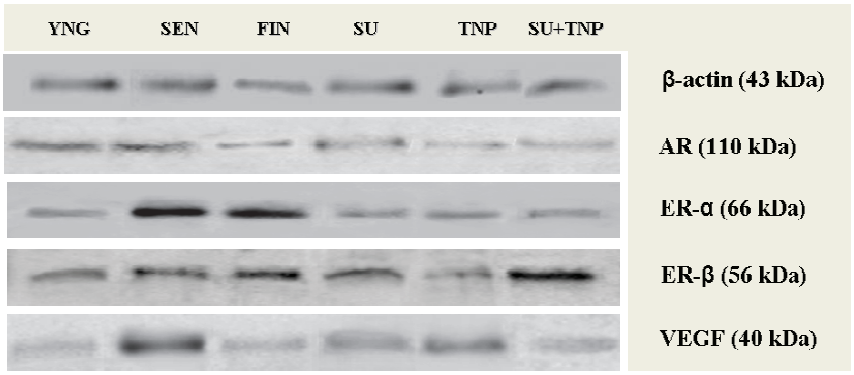
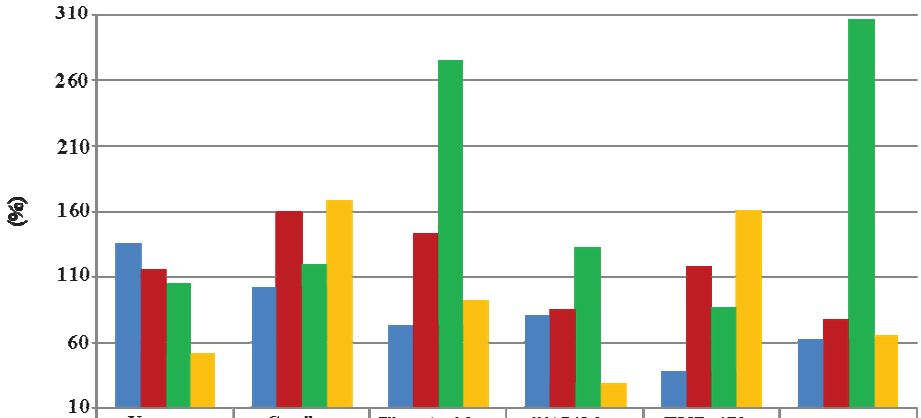
Figure 6





	Young	Senile	Finasteride	SU5416	TNP-470	SU+TNP
AR	135.4 ± 5.4 [a]	102.2 ± 9.2 [b]	72.6 ± 6.9 [b]	61 ± 8.2 [b]	37.1 ± 5.8 [e]	62 ± 6.3 [f]
ER-α	115.3 ± 11.2 [a]	160.4 + 5.1 [b]	142.4 + 7.4 [c]	132.8 ± 7.1 [d]	117.6 ± 8.6 [a]	77.3 ± 4.4 [d]
ER β	105.1 ± 9.8 [a]	119.5 ± 8.5 [b]	275.7 ± 10.3 [c]	84.8 ± 3.8 [d]	86.8 ± 2.7 [e]	306.3 ± 12.2 [f]
VEGF	51 ± 5.6 [a]	168.5 ± 4.8 [b]	92.5 ± 5.2 [c]	28.1 ± 4.7 [d]	160.8 ± 9.1 [b]	63.4 ± 7.3 [a]

Different lowercase letters indicate statistically significant differences (P>0.05) after Tukey's test

7. CONSIDERAÇÕES E CONCLUSÕES GERAIS

- A partir das análises de microscopia de luz pode-se concluir que a senilidade promoveu alterações estruturais do lobo ventral da próstata, sugerindo o comprometimento da função desse órgão, e predispondo ao aparecimento de lesões malignas em decorrência do microambiente favorável. Isto posto, considerando-se o desequilibrio da interação epitélio-estroma do lobo ventral da próstata. Além disso, tais mudanças podem sugerir o comprometimento da função reprodutiva.

- Os presentes resultados também indicaram que o tratamento com finasterida comprometeu a estrutura do lobo ventral da próstata de animais senis, sendo a atrofia epitelial e a escassez estromal características marcantes na próstata desses animais. Por outro lado, este mesmo tratamento contribuiu de forma marcante para diminuição da ocorrência de células inflamatórias, o que possivelmente resultou na estabilidade do microambiente, considerando-se a importância do processo inflamatório e ou ocorrência de proliferação malignas em doenças prostáticas.

- Os tratamentos com as drogas antiangiogênicas, bem como a união das drogas de ação direta e indireta, influenciaram no dinâmica dos processos proliferativo e apoptótico observadas no microambiente prostático em camundongos senis, sendo indicativo no tratamento deste órgão.

- A partir das análises moleculares concluiu-se que a senilidade modificou o microambiente do lobo ventral da próstata, com consequente alterações das vias de ativação e sinalização androgênica, estrogênica e de fatores angiogênicos. Embora a sinalização estrogênica, através ER-α, possa ter levado a processos de hiperplasia e hipertrofia estromal, o equilibrio com a via antiproliferativa do ER-β foi detectado no lobo ventral da próstata de animais senis. O

tratamento com finasterida na senilidade promoveu aumento de ER-β, caracterizando o caráter antiproliferativo do fármaco. Além disso, verificou-se a preponderância de fatores antiangiogênicos sobre fatores proangiogênicos, o que certamente indicou microambiente glandular desfavorável ao processo de proliferação endotelial e consequentemente tecidual.

- Ainda, concluiu-se que o tratamento de animais senis com o antiangiogênico SU5416 levou à minimização dos efeitos proliferativos na próstata através da via estrogênica, considerando a diminuição de ER-α , aumento de ER-β e diminuição de fatores pró-angiogênicos. Já o tratamento antiangiogênico com TNP-470 mostrou-se atuante de forma marcante na via androgênica, caracterizado pela diminuição dos níveis proteicos de AR. Contudo, pode-se sugerir no tratamento com TNP-470 menor eficácia do processo antiproliferativo através da ativação estrogênica via ER- β, visto os níveis proteicos diminuídos deste. Já o tratamento combinado com os agentes antiangiogênicos agiu preponderantemente sobre via estrogênica, resultando no aumento dos níveis proteicos de ER- β e a redução ER- α, sendo assim favorável à diminuição do processo proliferativo.

- Finalizando, as terapias antiangiogênicas foram efetivas, fato indicado pela redução da imunolocalização de VEGF quando comparado aos animais do grupo jovem, sobretudo no tratamento combinado entre SU5416 e TNP-470. O desbalanço entre fatores proangiogênicos e antiangiogênicos nos camundongos senis indicou a ativação do processo da angiogênese, apontando o microambiente prostático como sendo propício a ocorrência de processos proliferativos teciduais. Dentre todos os tratamentos realizados neste estudo, pode-se sugerir, portanto, que os tratamentos com finasterida, SU5416 e SU5416+TNP-470 mostraram-se mais efetivos e ou favoráveis para a manutenção do equilibrio estrutural, hormonal e de fatores angiogênico da próstata durante período da senescência.

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