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Efeitos da exposição ao β-Cariofileno na próstata ventral de gerbilos adultos após a suplementação pela Testosterona

São José do Rio Preto
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“Que todos os nossos esforços estejam sempre focados no desafio à impossibilidade. Todas as grandes conquistas humanas vieram daquilo que parecia impossível”.

Charles Chaplin

RESUMO

A glândula prostática é muito suscetível às alterações nos níveis dos andrógenos, que podem desempenhar um importante papel no desenvolvimento da hiperplasia prostática benigna (HPB). O uso de compostos naturais no tratamento da HPB vem crescendo e tem demonstrado efeitos benéficos à próstata. O β-Cariofileno (BCP) é um composto fitocanabinoide que está presente em diferentes espécies de plantas e possui efeitos anti-inflamatório, apoptótico, antiangiogênico e pode estar relacionado com a inibição da proliferação de uma variedade de células tumorais, inclusive da próstata. Porém, esses estudos, até o momento, se restringiam aos efeitos do BCP em células em cultura, sendo necessário o conhecimento desses efeitos em modelos animais. Dessa forma, o presente trabalho avaliou as alterações provocadas pela testosterona na próstata ventral e analisou os efeitos do BCP na HPB induzida pela testosterona na próstata ventral de gerbilos adultos. Para tal foram utilizados gerbilos machos adultos submetidos a diferentes condições experimentais: injeções subcutâneas de testosterona (3 mg/kg), em dias alternados, durante 4 semanas e eutanasiados, imediatamente (grupo 1) e 30 dias (grupo 2) após o término do experimento; administração diária de BCP (50 mg/kg/dia), via gavagem, por 4 semanas, em gerbilos intactos (grupo 3) e que receberam a suplementação pela testosterona por 30 dias (grupo 4), além dos animais pertencentes ao grupo controle (grupo 5), que não receberam nenhum tratamento ou suplementação. Foram realizadas análises morfológicas, morfométricas, estereológicas, imuno-histoquímicas, sorológicas e da expressão de proteínas na próstata ventral. Os resultados obtidos demonstraram que a suplementação pela testosterona promoveu o estabelecimento de lesões, de proliferação, aumento da quantidade de células inflamatórias e alterou a proporção entre os compartimentos teciduais prostáticos nos grupos 1 e 2, que receberam somente a testosterona. A administração de BCP em gerbilos com HPB reduziu a frequência de células proliferativas e inflamatórias nos compartimentos epitelial e estromal e de macrófagos no estroma. As análises dos receptores hormonais mostraram o aumento da frequência da 5 α-redutase 2 na próstata de todos os grupos experimentais comparado ao controle, do receptor de andrógeno (AR) nos grupos 1 e 2 e a redução desse receptor nos grupos 3 e 4. Além disso, foi observado o aumento da expressão do receptor de estrógeno do tipo β (ER-β) no grupo 4 comparado ao grupo 2 indicando um possível efeito anti-proliferativo do BCP na hiperplasia prostática. Também foi verificado o aumento das células em

apoptose após o tratamento com o fitocanabinoide em ambos os grupos 3 e 4. Assim, conclui-se que a suplementação pela testosterona foi suficiente para induzir a HPB e essa condição hiperplásica se manteve mesmo após 4 semanas do fim do experimento e, pela primeira vez demonstrado em modelo animal, que o BCP apresentou efeitos positivos para a próstata hiperplásica, como a redução da proliferação e inflamação, indicando resultados promissores no tratamento da HPB.

Palavras-chave: Próstata. Testosterona. Hiperplasia. β-Cariofileno. Gerbilos.

ABSTRACT

The prostate gland is very susceptible to changes in androgen levels, which can play an important role in the development of benign prostatic hyperplasia (BPH). The use of natural compounds in the treatment of BPH has been growing and has shown beneficial effects on the prostate. The phytocannabinoid β-Caryophyllene (BCP) is present in different plant species and has anti-inflammatory, apoptotic, antiangiogenic effects and may be related to the inhibition of proliferation of a variety of tumor cells, including the prostate. However, these studies have been restricted to the effects of BCP in cultured cells and the knowledge of these effects is necessary for the experimental models. Thus, the present study evaluated the changes caused by testosterone in the ventral prostate and analyzed the effects of BCP on testosterone-induced BPH in the ventral prostate of adult gerbils. In order, we used adult male gerbils submitted to different experimental conditions: subcutaneous applications of testosterone (3 mg/kg), on alternate days, for four weeks and euthanized, immediately (group 1) and 30 days (group 2) after the end of the experiment; daily administration of BCP (50 mg/kg/day), via gavage, for four weeks, in intact gerbils (group 3) and that received supplementation by testosterone during 30 days (group 4), and animals from the control group (group 5), which received no treatment or supplementation. Morphological, morphometric, stereological, immunohistochemical, serological, and protein expression analyses were performed on the ventral prostate. The results showed that testosterone supplementation promoted the establishment of lesions, cell proliferation, increased number of inflammatory cells and altered the proportion between prostatic tissue compartments in the groups 1 and 2 that received testosterone alone. BCP administration in gerbils with BPH reduced the frequency of proliferative and inflammatory cells in the epithelial and stromal compartments and macrophages in the stroma. Hormone receptor analyses showed an increase in the frequency of 5 α-reductase 2 in the prostate of all experimental groups compared to the control, of the androgen receptor (AR) in groups 1 and 2, and a reduction of this receptor in groups 3 and 4. In addition, the estrogen receptor type β (ER- β) expression increased in group 4 compared to group 2 indicating a possible anti-proliferative effect of BCP on prostatic hyperplasia. The increase of cells in apoptosis after the phytocannabinoid treatment was also observed in both groups 3 and 4. In conclusion, the testosterone supplementation was sufficient to induce BPH and this hyperplastic condition remained even four weeks after the end of the experiment and, for the first

time demonstrated in an animal model, that BCP showed positive effects for the hyperplastic prostate, such as reduced proliferation and inflammation, indicating promising results in the treatment of BPH.

Keywords: Prostate. Testosterone. Hyperplasia. β-Caryophyllene. Gerbils.

LISTA DE ILUSTRAÇÕES

Introdução

Artigo 1

Artigo 2

Artigo 3

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

5αR	5 α-redutase
AR	receptor de andrógeno
BCP	β-Cariofileno
BCPO	óxido de β-Cariofileno
CB	canabinoides
CML	células musculares lisas
DHEA	dehidroepiandrosterona
DHT	di-hidrotestosterona
(E)-BCP	trans-cariofileno
ER-α	receptor de estrógeno tipo α
ER-β	receptor de estrógeno tipo β
HPB	Hiperplasia Prostática Benigna
LUTS	sintomas do trato urinário inferior
MEC	matriz extracelular
MMP	metaloproteinase de matriz extracelular
NIP	neoplasia intraepitelial prostática
PAP	fosfatase ácida prostática
PSA	antígeno prostático específico
RCB1	receptor de canabinoides tipo 1
RCB2	receptor de canabinoides tipo 2
S-DHEA	sulfato de dehidroepiandrosterona
T	testosterona
TGF-β	fator de crescimento de transformação β
(Z)-BCP	(Z) - β-Cariofileno ou iso-cariofileno

SUMÁRIO

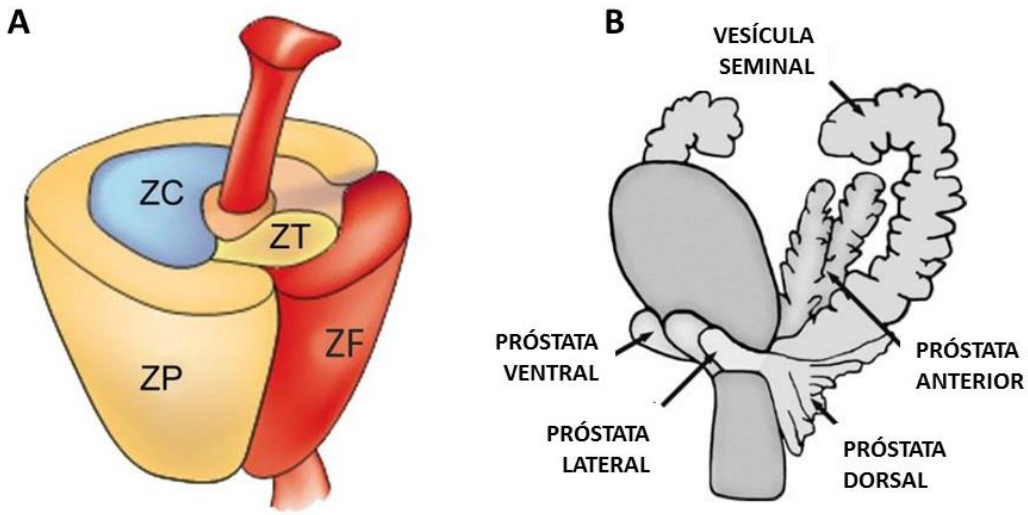
1. INTRODUÇÃO

1.1. A glândula prostática

A próstata é uma glândula acessória do sistema reprodutor de mamíferos e, em machos está localizada abaixo da bexiga urinária (AARON; FRANCO; HAYWARD, 2016; VERZE; CAI; LORENZETTI, 2016). É responsável pela produção de nutrientes, manutenção do gradiente iônico e por propiciar o pH ideal que irão compor o fluido seminal e contribuir com a motilidade e a sobrevivência dos espermatozoides, garantindo o sucesso reprodutivo (UNTERGASSER; MADERSBACHER; BERGER, 2005).

A glândula prostática adulta é composta por uma estrutura túbulo-alveolar (PRICE; D., 1961; ROCHEL et al., 2007). Em humanos, a próstata possui morfologia mais compacta e é diferenciada nas zonas central, de transição, periférica e fibromuscular (Figura 1A) (CROWLEY et al., 2020; DE MARZO et al., 2007; MCNEAL, 1988). Entretanto, a próstata de roedores é dividida em lobos distintos, são eles: anterior ou glândula coaguladora, ventral, lateral e dorsal (Figura 1B), sendo que esses dois últimos podem ser encontrados agrupados em alguns animais, sendo denominados de lobo dorsolateral (CROWLEY et al., 2020; HAYWARD; CUNHA, 2000; SHAPPELL et al., 2004).

Figura 1. Representação esquemática da próstata humana (A) e de roedores (B). Zona central (ZC), zona periférica (ZP), zona de transição (ZT) e zona fibromuscular (ZF).



FONTE**:** Extraído e adaptado de DE MARZO et al., 2007; RISBRIDGER; TAYLOR, 2006.

Histologicamente, a próstata é composta pelos compartimentos luminal, epitelial e estromal (Figura 2). O epitélio prostático é pseudoestratificado e apresenta quatro tipos de células (basais, secretoras luminais, intermediárias e neuroendócrinas) que reagem diferentemente aos hormônios esteroides (RISBRIDGER; TAYLOR, 2006; RUMPOLD et al., 2002). As células secretoras luminais são os tipos mais frequentes no compartimento epitelial e são responsáveis pela síntese e secreção de proteínas, incluindo o antígeno prostático específico (PSA) e a fosfatase ácida prostática (PAP), em direção ao lúmen (MARKER et al., 2003; RISBRIDGER; TAYLOR, 2006).

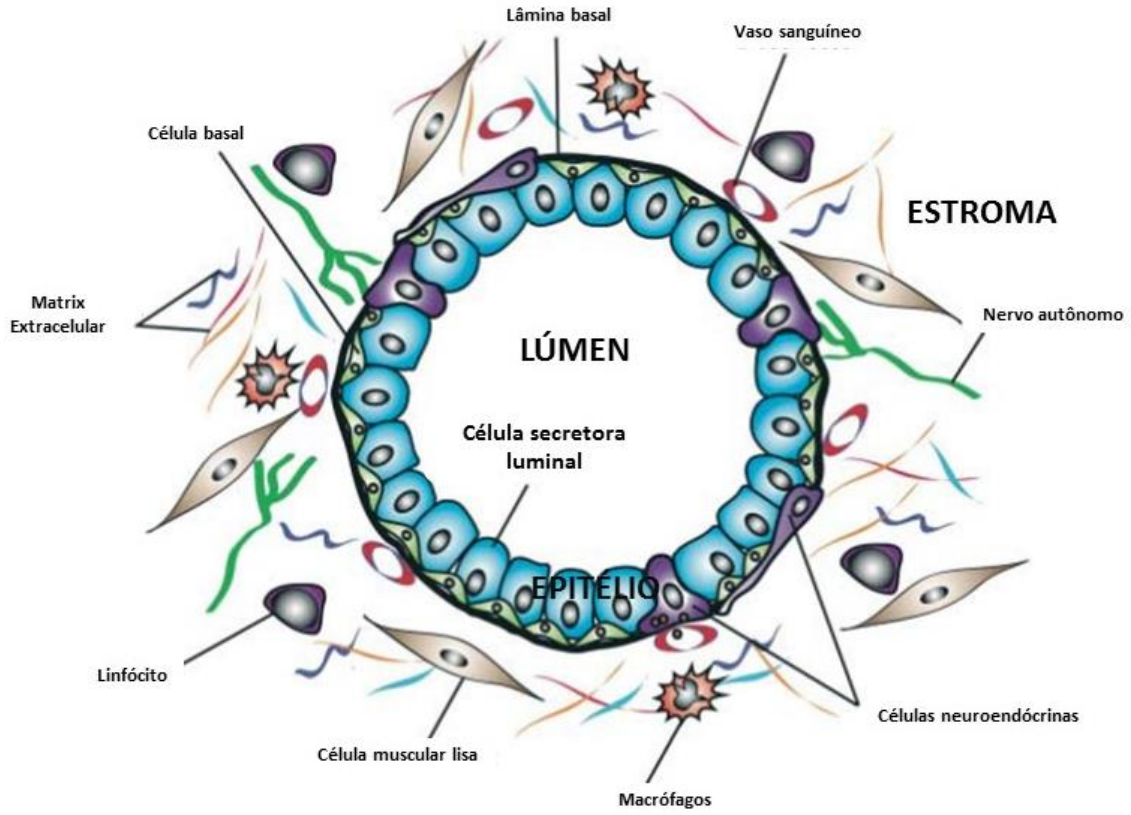
As células epiteliais basais atuam como fonte progenitora das células secretoras (RISBRIDGER; TAYLOR, 2006). Na próstata humana, as células basais se distribuem de modo contínuo entre a membrana basal e as células secretoras. Porém, em roedores são encontradas em menor quantidade e dispersas em uma camada descontínua ao redor dos ductos prostáticos (MARKER et al., 2003). No compartimento epitelial ainda podem ser encontradas as células neuroendócrinas que estão presentes em menor frequência e também, as células intermediárias (MARKER et al., 2003) e outros tipos celulares, como os macrófagos e os linfócitos (CHATTERJEE, 2003; SFANOS et al., 2018).

A lâmina basal faz a separação do epitélio e do estroma (CZYZ; SZPAK; MADEJA, 2012; SCHAUER; ROWLEY, 2011). A interação física e bioquímica entre os constituintes estromais e epiteliais é crucial para a manutenção e o funcionamento da glândula prostática (LEVESQUE; NELSON, 2018). O compartimento estromal é composto por células musculares lisas (CML), fibroblastos, células endoteliais, células nervosas, telócitos e infiltrados celulares como os linfócitos, macrófagos e mastócitos, imersos em uma matriz extracelular (MEC) associada a fatores de crescimento, moléculas reguladoras e enzimas remodeladoras (CHATTERJEE, 2003; CORRADI et al., 2013; CZYZ; SZPAK; MADEJA, 2012; FELISBINO et al., 2019; GANDAGLIA et al., 2013; MARKER et al., 2003; ROY-BURMAN et al., 2004; SFANOS et al., 2018; TUXHORN; AYALA; ROWLEY, 2001).

As CML são o tipo celular mais frequente no estroma e atuam na contração durante a ejaculação, de modo a auxiliar na eliminação da secreção prostática na uretra e, juntamente com os fibroblastos estão relacionadas com a produção de fatores autócrinos e parácrinos que contribuem para a homeostase prostática (MARKER et al., 2003; ROCHEL et al., 2007). Além disso, essas células também

propiciam um microambiente adequado composto por fibras elásticas e colágenas (DE CARVALHO; TABOGA; VILAMAIOR, 1997; DE CARVALHO; VILAMAIOR; TABOGA, 1997; VILAMAIOR et al., 2000), proteoglicanos (KOFOED et al., 1990; LEVESQUE; NELSON, 2018), laminina (DE CARVALHO; LINE, 1996) e metaloproteinases de matriz extracelular (MMPs) (KIANI et al., 2020), que auxiliam na estrutura, na flexibilidade e na permeabilidade da MEC (LEVESQUE; NELSON, 2018; TUXHORN; AYALA; ROWLEY, 2001).

Figura 2. Componentes celulares dos compartimentos da próstata madura.



FONTE: Extraído e adaptado de BARRON; ROWLEY, 2012.

1.2. Regulação hormonal da próstata

Os hormônios esteroides, como os andrógenos e os estrógenos, estão envolvidos na formação, no desenvolvimento e na manutenção da próstata (THOMSON; CUNHA; MARKER, 2008; VICKMAN et al., 2020; WILSON, 2011). A produção de andrógenos é regulada pelo eixo hipotalâmico-hipofisário-gonadal (KLUTH et al., 2014) e a testosterona (T) é o principal andrógeno circulante no

organismo masculino, sendo produzida, em sua maioria, pelos testículos (ROEHRBORN, 2008; WILSON, 2011). As glândulas adrenais também produzem andrógenos circulantes, são esses: dehidroepiandrosterona (DHEA), sulfato de dehidroepiandrosterona (S-DHEA) e androstenediona (HO; HABIB, 2011). Apesar desses andrógenos serem produzidos em menor quantidade, eles podem ser convertidos em metabólitos de esteroides mais ativos e atuar indiretamente no crescimento e na manutenção da glândula prostática (HO; HABIB, 2011; ZONG; GOLDSTEIN, 2013).

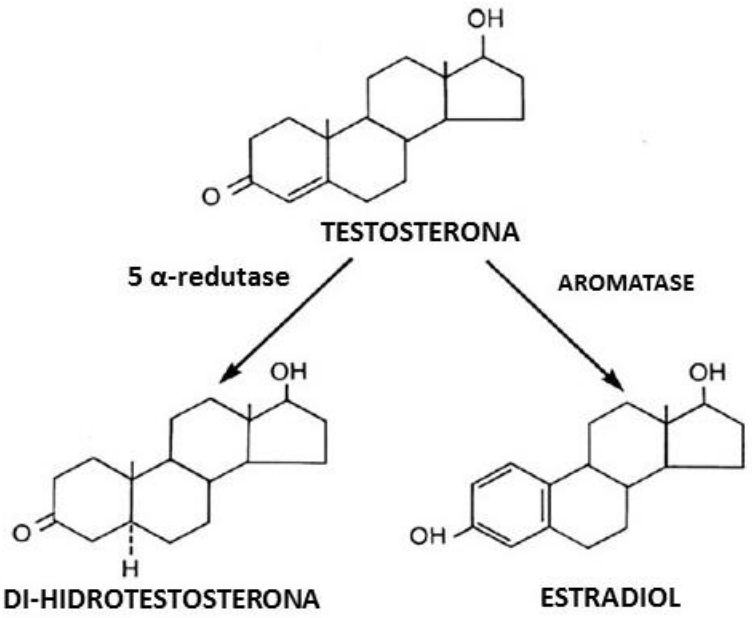
Os andrógenos prostáticos, oriundos de distintos locais do organismo, são encontrados na forma de di-hidrotestosterona (DHT) que é sintetizada a partir da T pela ação da enzima 5 α-redutase (5αR) (Figura 3) (ROBITAILLE; LANGLOIS, 2020; VERZE; CAI; LORENZETTI, 2016; WILSON, 2011). Existem três tipos de enzimas 5 α-redutases (5αR1, 5αR2 e 5αR3) e todas são expressas na próstata de mamíferos, porém a 5αR2 é a mais comum (ROBITAILLE; LANGLOIS, 2020; VICKMAN et al., 2020; ZHU; IMPERATO-MCGINLEY, 2009).

A T e a DHT ligam-se aos receptores de andrógenos (ARs) presentes no epitélio e no estroma prostático, entretanto a DHT tem mais afinidade com o AR (NIETO; RIDER; CRAMER, 2014; ROEHRBORN, 2008; THOMSON; CUNHA; MARKER, 2008). O AR é o mediador da ação androgênica em todos os tecidos que são andrógeno-dependentes (NIETO; RIDER; CRAMER, 2014). De acordo com o microambiente e os níveis hormonais, os ARs podem tanto estimular como inibir a proliferação celular (NIETO; RIDER; CRAMER, 2014). Além disso, o AR presente no estroma é necessário para a diferenciação e a proliferação das células epiteliais durante o desenvolvimento e a expressão de AR epitelial é essencial para que a glândula prostática exerça sua atividade secretória (CUNHA; CHUNG, 1981; DONJACOUR, 1993).

Os estrógenos podem ser convertidos, a partir da T, pela enzima aromatase, em estradiol (Figura 3), sendo o 17β-estradiol o mais comum nos homens (HO; HABIB, 2011; NICHOLSON; RICKE, 2011; WILSON, 2011). Eles também são hormônios importantes para a regulação do crescimento prostático durante todos os estágios da vida (ELLEM; RISBRIDGER, 2009). Na próstata, a ação dos estrógenos pode ser mediada pelos receptores de estrógeno do tipo α (ER-α) e do tipo β (ER-β) (COOKE et al., 2017). Nas diferentes espécies de mamíferos, esses receptores estão localizados em todos os compartimentos prostáticos, porém o ER-α pode ser mais

expresso no estroma e o ER-β no epitélio, o que indica que eles podem desempenhar efeitos distintos (HO; HABIB, 2011). O ER-α pode estar envolvido com o estímulo à proliferação e no estabelecimento da inflamação, enquanto que o ER-β pode exercer o papel anti-proliferativo, anti-inflamatório e apoptótico (HO; HABIB, 2011; RISBRIDGER; ELLEM; MCPHERSON, 2007; WARNER et al., 2020).

Figura 3. Conversão de testosterona em di-hidrotestosterona e estradiol.



FONTE: Extraído e adaptado de WILSON, 2011.

1.3. Hiperplasia prostática benigna e suplementação pela testosterona

A Hiperplasia Prostática Benigna (HPB) é uma doença prostática comum que afeta os homens, principalmente em idades avançadas (ELKAHWAJI, 2013; MADERSBACHER; SAMPSON; CULIG, 2019; VERZE; CAI; LORENZETTI, 2016). Entre as principais doenças urológicas, em 2017, a prevalência de HPB mundial foi três vezes maior que o câncer de próstata e sua incidência aumentou em 80% entre 1990 e 2017 (LAUNER et al., 2021). Histologicamente, a HPB é caracterizada pela proliferação das células epiteliais e estromais na zona de transição periuretral da próstata (MCNEAL, 1978; ROEHRBORN, 2008).

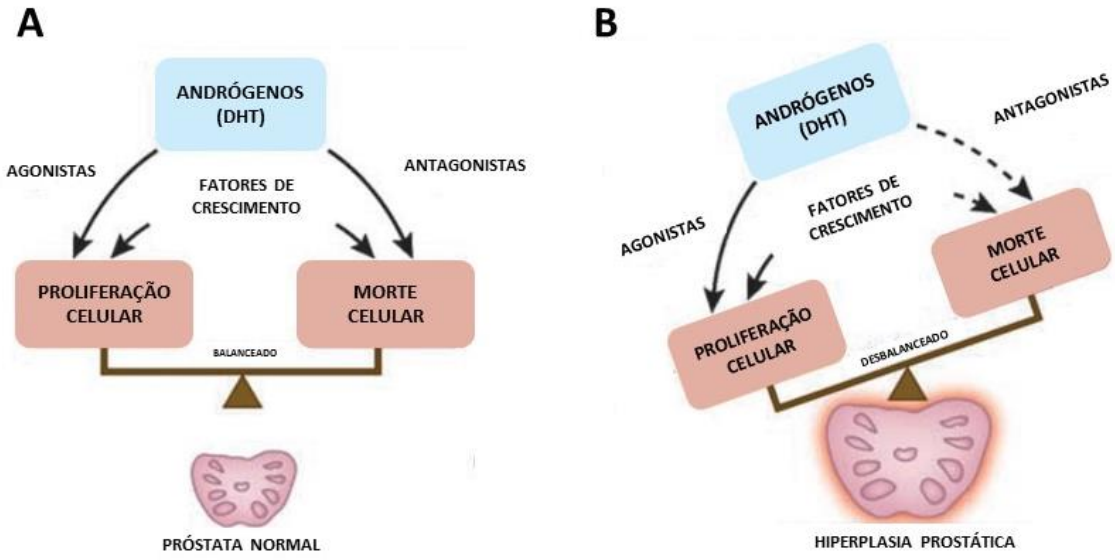
Na HPB, a proliferação celular pode resultar no aparecimento de lesões, como a neoplasia intraepitelial prostática (NIP). Essa lesão pode ser caracterizada pela hiperplasia das células epiteliais secretoras luminais, pelo aumento do volume dos

núcleos e nucléolos e pela redução das células basais (SHAPPELL et al., 2004; SHEN; ABATE-SHEN, 2010). Com a progressão da doença, pode ocorrer o rompimento da lâmina basal e a invasão de pequenos grupos de células, resultando no carcinoma microinvasivo (SHAPPELL et al., 2004).

Apesar da alta prevalência e do impacto socioeconômico, a patofisiologia da HPB ainda é pouco entendida (MADERSBACHER; SAMPSON; CULIG, 2019; NICHOLSON; RICKE, 2011; ROEHRBORN, 2008). Essa doença está associada à idade, inflamação, alterações metabólicas e hormonais (CHATTERJEE, 2003; CHUGHTAI et al., 2011; DE NUNZIO; PRESICCE; TUBARO, 2016; SHAH et al., 2021; UNTERGASSER; MADERSBACHER; BERGER, 2005). Em idosos, os sintomas do trato urinário inferior (LUTS) geralmente estão associados à HPB (LANGAN, 2019; SHAH et al., 2021; UNTERGASSER; MADERSBACHER; BERGER, 2005).

A próstata também é dependente de fatores de crescimento, que consistem em pequenas moléculas de peptídeos que podem estimular ou inibir, dependendo da situação, a divisão e a diferenciação celular, como exemplo o fator de crescimento de transformação β (TGF-β) (LEE, KEITH L; PEEHL, 2004). A interação entre esses fatores e os hormônios esteroides, como os andrógenos, pode alterar o balanço entre a proliferação e morte das células e, com isso, acarretar no surgimento da HPB (Figura 4) (LUCIA; LAMBERT, 2008; ROEHRBORN, 2008).

Figura 4. Representação esquemática da ação de andrógenos e de fatores de crescimento na proliferação e na morte celular prostática em condições normais (A) e na HPB (B).



FONTE: Extraído e adaptado de ROEHRBORN, 2008.

A sinalização hormonal da HPB está relacionada com a presença dos receptores de esteroides, como os ARs e os ERs (HO; HABIB, 2011; MADERSBACHER; SAMPSON; CULIG, 2019). As funções do ER-α e do ER-β na patogenia da HPB ainda não estão totalmente esclarecidas (NICHOLSON; RICKE, 2011).

A 5αR desempenha o papel importante na patogênese dessa doença, uma vez que inibidores dessa enzima têm sido amplamente utilizados no tratamento da HPB. Esses inibidores suprimem a conversão de T em DHT, diminuindo o volume da próstata e amenizando os sintomas da doença (ANDRIOLE et al., 2004; LOKESHWAR et al., 2019; MADERSBACHER; SAMPSON; CULIG, 2019; RITTMASTER, 2008; VICKMAN et al., 2020).

Os ARs exercem papel fundamental no desenvolvimento da HPB (IZUMI et al., 2013; VICKMAN et al., 2020). Estudos sugerem a associação entre a elevada expressão desses receptores com o aumento da proliferação celular no estroma e no epitélio prostático e inibição da apoptose, o que acarreta no crescimento da glândula prostática (BELLO et al., 1997; FICARRA et al., 2014; SILVA et al., 2001). Diante da função do AR na HPB, tratamentos que causam a redução desse receptor tem sido utilizados para aliviar os sintomas da doença e diminuir o tamanho da glândula (IZUMI et al., 2013).

A expressão de AR na próstata hiperplásica pode estar relacionada com o recrutamento de células inflamatórias (LU et al., 2012; WANG et al., 2012; WU et al., 2012). A inflamação também é um processo que está diretamente associado a HPB (DE NUNZIO; PRESICCE; TUBARO, 2016; FICARRA et al., 2014; GANDAGLIA et al., 2013). O aumento de infiltrados inflamatórios na próstata, como linfócitos B e T e macrófagos e a ativação de citocinas que aumentam a concentração de fatores de crescimento podem contribuir para o desenvolvimento da HPB (GANDAGLIA et al., 2013; LU et al., 2012; WANG et al., 2012; WU et al., 2012).

A HPB pode ser induzida pela suplementação hormonal, seja pela associação entre andrógenos e estrógenos ou somente pelo uso de hormônios androgênicos (MIAO et al., 2019; WANG et al., 2015). Os ésteres de T, como o propionato, o cipionato e o enantato são comumente usados em terapias de reposição hormonal em homens com baixos níveis ou ausência de T endógena (BI et al., 2018; MATSUMOTO; NAMIKI, 1994; REY; GRINSPON, 2020). Além disso, a utilização desses análogos de

andrógenos na indução de HPB é bem estabelecida em modelos experimentais (SAYED; SAAD; EL-SAHAR, 2016; TSAI et al., 2020; ZHANG et al., 2021).

A T é frequentemente utilizada em estudos que buscam esclarecer o funcionamento e a patogênese da hiperplasia (BOSLAND, 2014; CHEN et al., 2018; SHIRAI et al., 2000). Ademais, pesquisas realizadas com ratos, camundongos e cães, que recebem as aplicações subcutâneas dessas drogas androgênicas tem se demonstrado efetivas na indução da HPB para então realizar a investigação de novos tratamentos para essa doença (ABDEL-AZIZ et al., 2020; BASHA et al., 2019; HONG et al., 2020; KIRIYA et al., 2019; LI et al., 2018).

1.4. O gerbilo da Mongólia como um modelo para estudos da próstata

O gerbilo da Mongólia (*Meriones unguiculatus*) é um roedor pertencente à família Muridae, subfamília Gerbillinae (Figura 5 A). Eles são menores que ratos e maiores que camundongos e os machos, na fase adulta, pesam em torno de 70-100 g. Por serem animais de origem desértica, os gerbilos se adaptam à climas secos e quentes. Geralmente, em laboratório, eles apresentam comportamento dócil e são fáceis de manusear (BATCHELDER et al., 2012; FISHER; LLEWELLYN, 1978; RICH, 1968).

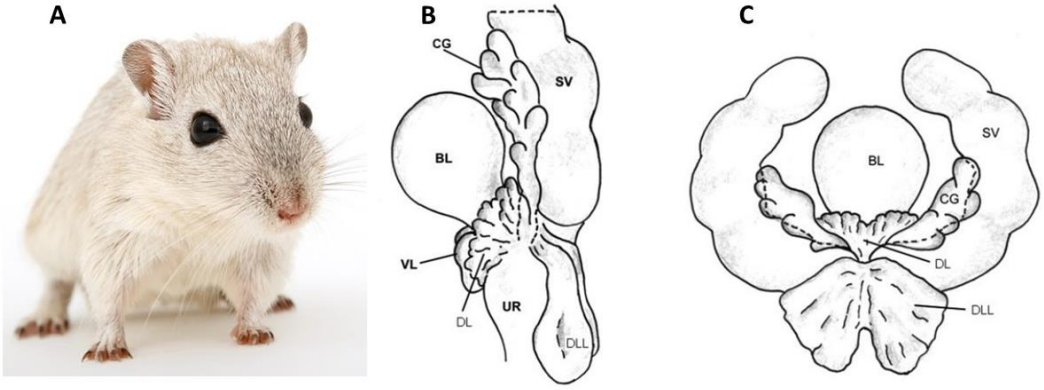
Esses roedores têm sido frequentemente utilizados como modelos experimentais em pesquisas científicas. Tais estudos abrangem diferentes áreas: genética (RAZZOLI et al., 2003); comportamental (DENG; LIU; WANG, 2017); imunologia (XU; WANG, 2011); fisiologia (KHAKISAHNEH et al., 2019; UMEZU; KURIBARA; TADOKORO, 1989); parasitologia (BELOSEVIC et al., 1983) e na reprodução, abrangendo as glândulas mamárias (LEONEL et al., 2017), a próstata (TABOGA; VILAMAIOR; GÓES, 2009) e os testículos (NEGRIN et al., 2018; PINTO-FOCHI et al., 2016).

Os estudos relacionados à próstata dessa espécie de roedor têm-se demostrado bem promissores na administração de hormônios (ANTONIASSI et al., 2017; BIANCARDI et al., 2015; FALLEIROS-JÚNIOR et al., 2016; PEREZ et al., 2016; SCARANO et al., 2008; ZANATELLI et al., 2021), no desenvolvimento de lesões associadas ao envelhecimento (CAMPOS et al., 2008; PEGORIN DE CAMPOS et al., 2006), nos efeitos de desreguladores endócrinos (COLLETA et al., 2015; DE JESUS et al., 2015; FACINA et al., 2018) e na indução de lesões proliferativas (GONÇALVES et al., 2010, 2013; QUINTAR et al., 2017). A próstata do gerbilo é semelhante à dos

demais roedores, sendo constituída pelos lobos ventral, anterior, dorsal e dorsolateral que estão associados à uretra (Figura 5 B,C) (ROCHEL et al., 2007).

O lobo ventral é o mais utilizado em estudos da próstata por ser altamente responsivo aos andrógenos (CORDEIRO et al., 2008). Em geral, a próstata dos gerbilos apresenta epitélio pseudoestratificado e o estroma conjuntivo rico em diversas células, entre elas as endoteliais, os fibroblastos e as células musculares lisas dispostas ao redor dos ácinos (PEGORIN DE CAMPOS et al., 2006; ROCHEL et al., 2007). A distribuição do estroma fibromuscular e dos componentes estromais na próstata de gerbilos é semelhante à encontrada na glândula prostática humana (PEGORIN DE CAMPOS et al., 2006).

Figura 5. Gerbilo da Mongólia (**A**). Complexo prostático do gerbilo adulto em vistas lateral (**B**) e dorsal (**C**). Bexiga urinária (BL); glândula coaguladora ou lobo anterior (CG); lobo dorsal (DL); lobo dorsolateral (DLL); vesícula seminal (SV); uretra pélvica e músculo uretral (UR); lobo ventral (VL).



FONTE: Extraído e adaptado de ROCHEL et al., 2007.

1.5. Efeitos do tratamento fitoterápico na próstata

O tratamento para a HPB pode ser realizado por medicamentos convencionais e por fitoterápicos (PAGANO et al., 2014). As drogas prescritas pela medicina convencional, como antagonistas α-1 dos receptores adrenérgicos ou inibidores da 5αR reduzem os sintomas da doença e o tamanho da próstata (ANDRIOLE et al., 2004; EDWARDS, 2008; RITTMASTER, 2008). Porém, esses remédios apresentam muitos efeitos colaterais como a disfunção erétil, a perda da libido, os problemas durante a ejaculação, dentre outros. Além disso, em alguns casos de HPB são

adotados a redução cirúrgica da glândula prostática (EDWARDS, 2008; PAGANO et al., 2014).

A fitoterapia é um tratamento alternativo para a HPB que vem sendo muito utilizado em países europeus, como a Áustria, por exemplo (PAGANO et al., 2014). Possivelmente, esse aumento no uso das plantas medicinais e compostos naturais deve-se a existência de poucos efeitos adversos durante o tratamento (PARK et al., 2019). Dentre esses compostos, é possível destacar o saw palmetto (*Seronoa repens*), a cereja africana (*Pygeum africanum*), a batata africana (*Hypoxis rooperi*), a abobrinha (*Cucurbita pepo*), o centeio (*Secale cereale*), a ortiga (*Urtica dioica*), o fitoesterol presente no abacate e em sementes oleaginosas (*β-Sitosterol*), o licopeno, o selênio e as isoflavonas de soja (KEEHN; TAYLOR; LOWE, 2016; PAGANO et al., 2014; PATERNITI et al., 2018).

1.6. β-Cariofileno

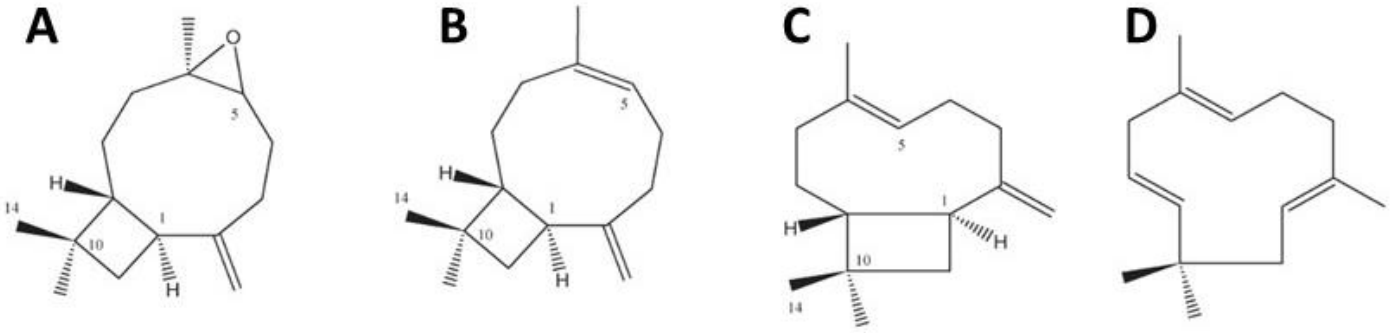
Os canabinoides (CBs) são uma família heterogênea de compostos que se ligam aos receptores de canabinoides. Esses compostos são divididos em três grupos, de acordo com a sua origem: os fitocanabinoides (compostos naturais presentes na *Cannabis sativa*), os endocanabinoides e os canabinoides sintéticos (análogos sintéticos de ambos os grupos, como o WIN-55,212-2 que é uma mistura de agonistas de CBs) (FRAGUAS-SÁNCHEZ; FERNÁNDEZ-CARBALLIDO; TORRES-SUÁREZ, 2016). Os receptores de CBs pertencem a família de receptores acoplados a proteína G e são divididos em dois subtipos: os receptores de canabinoides tipo 1 (RCB1) e os receptores de canabinoides tipo 2 (RCB2) (DÍAZ-LAVIADA, 2011; DOBOVIŜEK; HOJNIK; FERK, 2016).

Os RCB1 estão presentes principalmente no sistema nervoso central e também no sistema cardiovascular, respiratório, reprodutor e nos adipócitos. Os RCB2 são expressos preferencialmente em tecidos periféricos e no sistema imune, mas também são encontrados no trato gastrointestinal, no sistema nervoso central e periférico, nos ossos, no tecido adiposo e nos órgãos reprodutivos (DÍAZ-LAVIADA, 2011; DOBOVIŜEK; HOJNIK; FERK, 2016; WU, 2019). Ambos receptores estão presentes na próstata em condições normais e já foram encontrados em biopsias de pacientes com HPB (DÍAZ-LAVIADA, 2011).

O sesquiterpeno β-Cariofileno (BCP) é um fitocanabinoide agonista seletivo do RCB2. Na natureza, esse composto pode ser encontrado principalmente na forma de

trans-cariofileno ((E)-BCP), e também como (Z)- β-Cariofileno ou iso-cariofileno ((Z)-BCP), α-humuleno (α-cariofileno) e, após sofrer o processo de oxidação, como óxido de β-Cariofileno (BCPO) (Figura 6) (FIDYT et al., 2016; FRANCOMANO et al., 2019). O BCP está presente em diversos óleos essenciais e plantas alimentícias como na pimenta preta (*Piper nigrum L*.), no alecrim (*Rosmarinus officinalis*), na canela (*Cinnamomum spp.*), no orégano (*Origanum vulgare L.*), no manjericão (*Ocimum spp.*), no tomilho (*Thymus vulgaris*), na sálvia (*Salvia officinalis*), na hortelã (*Mentha piperita*), no gengibre (*Zingiber officinale*) e no cravo (*Syzygium aromaticum*). Além disso, ele também pode ser encontrado na citronela (*Cymbopogon*), no pinheiro (*Pinus*), na maconha (*Cannabis sativa*) e em plantas do gênero *Copaifera*, *Artemisia*, *Murraya*, *Cordia*, *Spiranthes*, *Ocimum*, entre outras (FRANCOMANO et al., 2019; MACHADO et al., 2018; OLIVEIRA et al., 2018; SHARMA et al., 2016).

Figura 6. Diferentes formas do BCP. Óxido de β-Cariofileno (A), (Z)- β-Cariofileno (B), trans-cariofileno (C), α-humuleno (D).



FONTE: Extraído e adaptado de FIDYT et al., 2016.

O uso do BCP é frequentemente utilizado como intensificador de sabor pela indústria alimentícia e na fabricação de cosméticos (GERTSCH et al., 2008; RASTOGI et al., 1998). Estudos indicam que o BCP possui propriedades benéficas em diferentes patologias, desempenhando o papel neuroprotetor (OJHA et al., 2016), anti-inflamatório (CHANG et al., 2013), antitumoral (PAVITHRA; MEHTA; VERMA, 2018), cardioprotetor (PODDIGHE et al., 2018), na redução do estresse oxidativo (AMES-SIBIN et al., 2018), entre outras funções.

Na próstata, as pesquisas existentes são restritas somente a linhagens celulares de câncer prostático e mostram o efeito antitumoral desse fitocanabinoide da forma de BCPO (KIM et al., 2014; PARK et al., 2011). Deste modo, por ser um

composto com potencial terapêutico e devido à escassez de estudos do BCP na próstata e em animais, são necessárias pesquisas para elucidar os efeitos desse composto na glândula prostática *in vivo* em diferentes condições experimentais e assim, validar o uso terapêutico em humanos.

2. JUSTIFICATIVA

O grande interesse em se compreender a biologia prostática deve-se tanto ao seu intrigante processo de desenvolvimento, quanto à alta incidência de doenças prostáticas, como adenocarcinoma e a HPB (MARKER et al.,2003; UNTERGASSER et al., 2005; RISBRIDGER et al., 2005). O desenvolvimento e a diferenciação prostática são regulados principalmente por andrógenos, e sensíveis alterações ou interferências nos níveis desses hormônios podem alterar o padrão normal de desenvolvimento dessa glândula (PRINS et al., 2008).

A utilização de compostos naturais para o tratamento de algumas doenças tem sido cada vez mais comum (PAGANO et al., 2014). Essas substâncias naturais desempenham algumas ações benéficas ao organismo e são importantes alvos terapêuticos no tratamento de doenças (PARK et al., 2019), como por exemplo o β-Cariofileno, composto que ainda tem sua ação pouco estudada na próstata (BEGNINI et al., 2014; FIDYT et al., 2016). Assim, é importante compreender os mecanismos de ação dessa substância sobre a próstata em condições de alterações proliferativas, como aquelas promovidas pela testosterona. Além disso, entender os mecanismos de atuação dos diferentes componentes celulares e macromoleculares frente a essa situação experimental, bem como suas inter-relações na próstata tornam-se instrumentos muito importantes para o entendimento do desenvolvimento, da estrutura e da fisiologia da glândula em processos proliferativos e inflamatórios.

3. HIPÓTESE

A hipótese deste trabalho é que a suplementação pela testosterona promova o estabelecimento da HPB e essa condição permaneça mesmo após o término da indução hormonal e, que o BCP possa amenizar ou reverter os efeitos provocados pela hiperplasia na próstata.

4. OBJETIVOS

4.1. *Objetivo geral*

O presente trabalho teve por objetivo avaliar os efeitos do β-Cariofileno sobre a próstata ventral de gerbilos adultos após a indução do ambiente hiperplásico pela suplementação com testosterona.

4.2. *Objetivos específicos*

- Analisar as alterações histopatológicas, hormonais e na frequência de receptores provocadas pela testosterona suplementada imediatamente e 30 dias após a indução da HPB;
- Determinar a incidência e multiplicidade das lesões prostáticas nas diferentes condições experimentais;
- Verificar a expressão dos receptores esteroides na próstata ventral;
- Avaliar a presença de diferentes tipos de células inflamatórias na próstata ventral dos gerbilos;
- Analisar a frequência de células em proliferação e em apoptose;
- Determinar os níveis de hormônios esteroides e o balanço entre eles nos diferentes grupos experimentais.

5. RESULTADOS

Os resultados do presente trabalho foram divididos em três artigos científicos. O artigo 1 e 3 estão em fase de elaboração e serão submetidos às revistas *Andrology* e *Biochemical Pharmacology"*, respectivamente. O artigo 2 apresenta os resultados já publicados na revista *The Prostate* (ISSN 1097-0045, 2021, 1-13. https://doi.org/10.1002/pros.24177).

5.1. Artigo 1.

Será submetido à revista *"Andrology".*

Testosterone supplementation alters the 5αR2, the proliferation and prostatic lesions even four weeks after the end of the hormonal administration in the ventral prostate of mature gerbils.

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Short title: Different periods after BPH-induced in gerbils.

Key words: androgens, prostate, testosterone, BPH, hormonal, disease.

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Abstract:

Background: Androgens are related to the development of Benign Prostatic Hyperplasia (BPH) and prostate cancer. Testosterone is frequently used as a potent prostate disease inductor. Studies with BPH-induced are very important and enable testing of new strategies of BPH treatment.

Objectives: The present study proposes to evaluate the effects of testosterone-induced BPH induction immediately and after four weeks to the end of hormonal administration in the ventral prostate of mature Mongolian gerbils, focusing on histopathologic alterations, distribution in the prostatic compartments, proliferation, inflammation, hormonal serum levels and in the expression of hormonal receptors.

Material and Methods: The animals were divided into control (C), that did not receive any treatment; and BPH-induced groups that received subcutaneous injections of cypionate testosterone (3mg/kg), in alternating days, for 30 days. Next, the animals were euthanized immediately (Testo) and 30 days (Testo+30) after the end of the hormonal administration. We performed biometric, hormonal, histopathologic, immunohistochemistry, and immunoblotting analyses.

Results: BPH-induced by testosterone increased the ventral prostate weight and the epithelium and muscular stroma proportion, which indicates that the hormonal administration was sufficient for the establishment of this hyperplasic condition even after four weeks after the end of the exogenous stimulus. The testosterone promoted the development of prostatic lesions, demonstrated the presence of microinvasive carcinoma only in Testo+30 and increased the multiplicity of subepithelial inflammation and PIN area in the Testo and Testo+30 groups. Regarding the expression of 5αR2 and AR-positive cells, there was an increase in both BPH-induced groups. The inflammatory and proliferative analysis showed an increase in the PHH3-positive cells in the Testo and Testo+30 groups, and it was higher even after 4 weeks after the hormonal administration; an increase in the incidence of COX2-positive cells and F4/80 macrophages in the BPH-induced groups. In addition, the hormonal administration also caused an increase in the frequency of metalloproteinase type 2 (MMP-2) and 9 (MMP-9).

Discussion: The androgens can stimulate cell proliferation and consequently the recruitment of cytokines and inflammatory cells that can promote the unbalance in the proportion of prostatic compartments. Testosterone altered the distribution of hormonal receptors, enzymes, macrophages, and proliferative cells. Moreover, this comparative

study contributes to a better understanding of testosterone in BPH development in distinct periods of analysis.

Conclusion: Testosterone administration caused the BPH establishment that remains after four weeks of the hormonal stimulation. The analysis of the ventral prostate in the Testo+30 group indicates that the testosterone continued to influence the prostate function, promoting an increase in the 5αR2 and consequent increase in the cell proliferation that can be related with the presence of microinvasive carcinoma. Therefore, the present study shows the testosterone effects in the prostate gland and demonstrates, for the first time, that the altered microenvironment remains even after 30 days of hormonal administration.

Introduction:

In males, the testosterone is the principal circulating androgen and is responsible for male sexual differentiation[1,2]. In target organs, such as the prostate, testosterone is converted into dihydrotestosterone (DHT) by the enzyme 5 α-reductase[1]. This hormone binds to the androgen receptor (AR), a principal sex steroid receptor in the prostate[3].

Although testosterone is essential for the development and maintenance of the male reproductive system[4–6], due to its proliferative effect, it has been implicated as a possible promoter of prostate diseases, such as cancer and benign prostatic hyperplasia (BPH)[2,5,7,8]. Studies indicate that testosterone administration in combination with *N*-nitroso-*N*-methyl urea (MNU), is frequently used as a potent inducer of carcinogenesis in animals prostate[7,9–11].

BPH is a common prostate disease and may be caused by aging[12,13], tissue remodeling[12], inflammation[14–16], imbalance of androgen/estrogen signaling[17,18], overexpression of stromal/epithelial growth factors[19], hypoxia[20], defects of stem cells[21] or epithelial-mesenchymal transitions[22,23]. Despite the high incidence of BPH, the knowledge about the pathophysiology of this disease is scarce[22–26].

The androgens are involved with the prostate proliferation process and the AR signaling can conduct the tumor progression and the BPH[6,24,27]. Furthermore, the inflammation may directly contribute to prostate proliferation and growth[16,28,29]. Studies suggest that macrophages responses initiate the inflammatory process, following the recruitment of T-lymphocytes[30,31]. Immediately, pro-inflammatory cytokines attract and maintain pro-inflammatory cells in the hyperplastic prostate[16,32].

BPH-induction in animals is frequently utilized to unveil the pathological mechanism[7] and testing novel therapeutic strategies[33–35]. Dogs, mice, and rats have been well-established experimental models for BPH induced by androgens[33,36,37]. In addition, the Mongolian gerbils (*Meriones unguiculatus*) are an alternative experimental model being a good promise for research involving induced neoplasia[9,11,38,39], spontaneous lesions[40,41], and hormonal treatment[42–48].

Usually, BPH is induced by subcutaneous injections of testosterone[35,49,50] and the treatment commonly lasts four weeks[24,34,51–53]. However, these studies are limited to investigating the BPH immediately after the hormonal induction. Thus, understanding the progression, maintenance or regression of BPH after the induction is necessary since this is precisely the period when treatments are tested in animal models[24,34,52,53]. Therefore, given the importance and the necessity of a greater understanding of this disease, the aim here was to evaluate the BPH induction by testosterone immediately and after 30 days of the end of the administration in the ventral prostate of mature gerbils.

Material and Methods:

Animals, ethics statement and experimental design

Thirty-six mature male Mongolian gerbils (3 months old) were used for this experiment. The procedures were conducted in accordance with the National Council for Animal Experimentation Control and were authorized by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP (protocol number 73/2017). The animals were maintained in polyethylene cages under controlled conditions of temperature (24 °C) and light (12 h dark/12 h light) and received filtered water and a rodent feed *ad libitum*.

The gerbils were divided into three groups (n=12/group). Control (C): untreated group; and BPH-induced groups that received subcutaneous injections of Testosterone Cypionate (*Deposteron, EMS sigma pharma*) diluted in corn oil (*Liza, Cargill, Brazil*) (3 mg/Kg, 0,1 ml/application)[54,55] during one month on alternate days and were euthanized shortly after this supplementation (testosterone – Testo group) and after 4 weeks of the end of supplementation (testosterone+30 – Testo+30 group). Testosterone is used as a BPH inductor.

The animals were anesthetized with ketamine (10 mg/kg) and xylazine (3 mg/kg) and euthanized by decapitation. After decapitation, the blood was collected,

centrifuged at 3000 rpm for 20 minutes, and the serum was frozen at -80°C. Dissection was performed, the prostatic complex was collected, and the ventral prostate was separated. For the western blot analysis, the ventral prostates were stored at -80°C. For the morphological and immunohistochemistry analysis, the fragments were fixed in 4% paraformaldehyde for 24 hours. Next, samples were processed for inclusion in paraffin (Histosec, Merck, Darmstadt, Germany) and sectioned at 3-5 µm thick to obtain the slides.

Biometrical analysis

The gerbils were weighed before decapitation. The weights of the adrenal, testis, prostatic complex (urethra and the ventral, dorsolateral and dorsal prostate), and ventral prostate were obtained after dissection. The relative weights of the prostatic complex and ventral prostate were calculated by dividing the organ weight by the body weight.

Hormonal data

The serum levels of testosterone and 17 β-estradiol were achieved by ELISA capture/sandwich. The tests were performed using a specific commercial kit, according to the manufacturer's instructions (IBL International, Hamburg, Germany – numbers 52151, sensitivity of 0.18 ng/ml and 52041, sensitivity of 10.60 pg/ml respectively). Readings were performed in a microplate reader (TECAN- Infinite F50).

Morphological and histopathological analysis

The histological sections were stained with Hematoxylin-Eosin (HE) and Picrosirius red for general morphological evaluation of the ventral prostate. Morphometric and stereological analyses were made on slides stained with Picrosirius red. Morphometry allowed us to determine the mean of epithelial cells height (µm) and smooth muscle cells thickness (µm). In these analyses, 210 measurements/per group were obtained (30 measurements/animal, n=7/group). The slides were randomly digitized at 1000x magnification in the slide scanner system (Olympus VS120-S5), and analyses were realized in Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA).

The stereological analyses were performed to obtain the relative frequency (%) of epithelium, muscular stroma, non-muscular stroma, and lumen. In this analysis, 42

ventral prostate fields (6 fields/animal, n=7/group) were randomly digitized at 200x magnification in a slide scanner system (Olympus VS120-S5), and analyses were realized in Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA). The relative frequency was estimated using the M130 multipoint test system proposed by Weibel, 1963[56] and applied to the prostate according to the procedure described in Huttunen et al. 1981.

The histopathological analysis was performed in thirty-five sections/group (n = 7/group) stained with HE and digitized at ×400 magnification using the slide scanner system (Olympus VS120-S5). The histopathological classification of prostate lesions and inflammation was performed according to well-established criteria [58], which has been previously applied in the Mongolian gerbil [9,11,59]. The incidence of prostate lesions and inflammations in the ventral prostate was obtained by the number of animals exhibiting each type of disorder divided by the number of animals/group and expressed as a percentage. The multiplicity was estimated by the number of foci of disorders present in each animal divided by the number of slides analyzed per animal. For the analysis of prostate lesions, we considered benign intraepithelial neoplasms (PIN), flat epithelial atypia (FEA), papillary epithelial atypia (PEA), and microinvasive carcinoma. For inflammatory analysis, we counted intraluminal, periductal, and subepithelial foci. These analyses provide support for identifying the establishment of BPH by biometric, morphometric, and stereological parameters.

Immunohistochemistry analysis

The slides were deparaffinized, rehydrated through graded alcohols and distilled water. The antigen retrieval was performed in 10 mM citrate buffer pH 6.0, at 92°C for 45 minutes (AR), 20 minutes (MMP-2, MMP-9), and in Tris EDTA buffer pH 9.0 for 60 min at 98°C (5αR2, PHH3, Cox2, and F4/80). The endogenous peroxidases blockage was performed in 3% hydrogen peroxide for 30 minutes and followed by nonspecific proteins blockage with 5% skimmed milk/tris buffered saline + tween 20 (TBSt). The sections were incubated overnight at 4°C with primary antibodies diluted in bovine serum albumin (BSA) 1% in TBSt: 5 α-reductase 2 (5α-R2) (rabbit polyclonal, sc-20659, Santa Cruz Biotechnology CA, EUA, 1:75), AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology CA, EUA, 1:75), phosphohistone H3 (PHH3) - for the cell proliferation analysis (rabbit polyclonal, Ser 10, ♯9701S, Cell Signaling, Danvers, MA, USA, 1:75), cyclooxygenase 2 (COX2) - for the inflammatory cell analysis (rabbit

monoclonal, IgG, D5H5, ♯12282, Cell Signaling, Danvers, MA, USA, 1:100), macrophages F4/80 (rabbit monoclonal, IgG, D2S9R, ♯70076, Cell Signaling, Danvers, MA, USA, 1:100), matrix metalloproteinase-2 (MMP-2) (mouse monoclonal, sc-13595, Santa Cruz Biotechnology, CA, EUA, 1:50) and matrix metalloproteinase-9 (MMP-9) (mouse monoclonal, sc-21733, Santa Cruz Biotechnology, CA, EUA, 1:50). After washing in TBSt, the sections were incubated with a Polymer kit (Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) for 40 minutes at room temperature. Positive staining was detected with diaminobenzidine tetrahydrochloride (DAB, Sigma) solution and the slides were counterstained with Mayer hematoxylin. For the negative control, the primary antibodies were replaced with BSA 1 %.

The sections were digitized in 400x magnification on a slide scanner system (Olympus VS120-S5) and the quantification were realized in Image-Pro-Plus software (Media Cybernetics). The frequency of the immunohistochemistry analysis was performed in seventy microscopic fields/group (n=7, 10 fields/animal) (5αR2, AR); fifty six microscopic fields/group (n=7, 8 fields/animal) (PHH3, COX2 and F4/80) and fifty microscopic fields/group (n=5, 10 fields/animal) (MMP-2 and MMP-9). The relative frequencies of 5αR2, MMP-2, and MMP-9 were evaluated by a multipoint system with 160 intersections (modified from Weibel, 1963[56,60]). To determine the relative frequencies of AR-positive cells in the ventral prostatic lobe and epithelial and stromal compartments were divided the number of positive nuclei by the total number of nuclei counted and the result was expressed as a percentage. The absolute frequency of PHH3, Cox2, and F4/80 were obtained by the counts of positive cells by histologic section.

Western blot analysis

The ventral prostates (n=5/group) were removed from -80ºC and homogenized in a lysis buffer according to the procedure described by Li et al., 2009[61]. The samples were centrifuged at 14,000 rpm for 20 min at 4°C. The Pierce BCA Protein Assay Kit (23227-Thermo Scientific, Rockford, IL, USA) was utilized for the protein quantification and a microplate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany) were used to read the absorbance. After, the protein extracts were stored at −80 °C until analysis.

The quantification of AR in ventral prostate samples was realized by western blot. For this, a total protein of 15 µg was pipetted into each well of the polyacrylamide

incorporated in detergent sodium dodecyl sulfate (SDS-page) gel and subjected to electrophoresis (105V for approximately 120 minutes). The transfer of the bands was realized during 60 minutes at 360mA in nitrocellulose membrane (Amersham Protram, 10,600.003, GE Healthcare, Darmstadt, Germany). Then, the membranes were washed in TBSt and the block of nonspecific binding was in 5% skimmed milk for 60 minutes. Subsequently, they were incubated at 4°C overnight under agitation with the respective primary antibodies, diluted in 1% milk: AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology CA, USA, 1:300) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (monoclonal rabbit, IgG, #2118, Cell Signaling, Danvers, MA, USA, 1:1000). After, the membranes were washed in TBSt and incubated, with the secondary peroxidase-conjugated anti-rabbit antibody (goat, IgG, #7074, Cell Signaling, Danvers, MA, USA) (1:2000) for 60 minutes, under agitation, at room temperature. Finally, the membranes were washed in TBSt and revealed with enhanced chemiluminescent (ECL) substrate to visualize the bands in the imaging system (ChemiDoc MP, BioRad, Hercules, CA, USA). The band densities were analyzed in densitometry software (Image J, version 1.52a, Wayne Rasband, NIH, USA) and normalized by GAPDH (positive control).

Statistical analysis

The quantitative results were submitted to statistical analysis using GraphPad Prism 6.00 software (GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov test was utilized to check the normality distribution of data. The parametric data were analyzed by one-way ANOVA followed by Tukey's test. The Kruskal-Wallis test followed by Dunn's test was adopted for non-parametric data. Differences were considered statistically significant when $p < 0.05$.

Results:

Testosterone administration alters the weight of the prostate, testis, and the serum levels of testosterone immediately and four weeks after the end of hormonal supplementation.

Testosterone administration did not interfere in the body and adrenal weight. However, the relative weight of the prostatic complex increased in the Testo group and the relative weight of ventral prostate increased in both BPH-induced groups that can

indicate the establishment of BPH even after the end of testosterone administration. In contrast, the weight of testis decreased in the Testo and Testo+30 groups (Table 1).

The hormonal parameters allow us to verify an increase in the serum levels of testosterone in the Testo group. We did not find differences in the levels of 17 β-estradiol in any evaluated group (Table 1).

Supplementation of testosterone affects the distribution of prostatic compartments and enhances histopathologic lesions.

The testosterone administration altered the distribution of prostatic compartments, increasing the epithelium and muscular stroma proportion, typical BPH phenotype (Figure 1B, H). The hormonal supplementation caused the appearance of tissue disorders (Figure 1 B-E, H-K). The morphometric parameter allows us to check an increase in the epithelium height and the muscle layer thickness in the Testo and Testo+30 groups (Table 2). In the stereological analyses, the frequency of epithelium increased and of lumen decreased in the groups that received testosterone. The muscular stroma frequency also increased in these groups and was higher in the Testo+30 group. Thus, the frequency of stroma in the Testo+30 was higher than the C group (Table 2).

In addition, we investigated the incidence and multiplicity of prostatic lesions of the ventral prostate in all experimental groups. The multiplicity of inflammation, the subepithelial area increased in the Testo and Testo+30 groups and the presence of intraluminal inflammatory cells was observed only in both BPH-induced groups. The administration of testosterone did not alter the multiplicity of flat and papillary epithelial atypia. However, in PIN multiplicity, we verified an increase in the Testo+30 compared to the C group. Only the Testo+30 group presented a microinvasive carcinoma that indicates that testosterone supplementation continues to influence the functioning of the gland even four weeks after the administration of the hormone has finished (Table 3).

Testosterone manipulation increases the content of 5αR2 and AR in the ventral prostate after testosterone manipulation.

We evaluated the expression of 5αR2 in all prostatic compartments by immunohistochemistry (IHC) (Figure 2 A-D) and we found an increase in the immunoreaction of this enzyme this analysis showed an increase in the frequency of

this marking in both BPH-induced groups and were higher in the Testo+30 group (Figure 2 I). We verified the distribution of AR-positive cells in all experimental groups by IHC (Figure 2 E-H). The frequency of these positive cells increased in the prostate of the Testo and Testo+30 groups (Figure 2 J). The AR-positive cells also increased in the epithelium of the Testo and Testo+30 groups (Figure 2 K). In the stroma, these cells increased only in the Testo and, in contrast, a reduction was verified in the Testo+30 group (Figure 2 K). Moreover, the analyses of the androgen receptor protein expression showed an increase of this protein in the Testo in comparison to the C group (Figure 2 L).

Testosterone administration increases the immunoexpression of PHH3, COX2, and F4/80 positive cells.

We also analyze the proliferative and inflammatory cells in the epithelial and stromal compartments (Figure 3 A-L). The immunohistochemistry analysis of PHH3-positive cells showed an increase in the Testo and Testo+30 groups in both compartments, and it was higher in the epithelium of the Testo+30 group (Figure 3 M). In addition, there was an increase in COX2-positive cells in the epithelium and stroma of the Testo and Testo+30 groups (Figure 3 N). The analysis of F4/80 positive cells revealed an increase only in the stroma of the Testo and Testo+30 groups (Figure 3 O).

Testosterone-induced BPH interferes in the distribution of MMP-2 and MMP-9 in the gerbil prostate.

Finally, we investigated the frequency of MMP-2 and 9 in the ventral prostate of experimental groups (Figure 4). Immunohistochemistry analyses showed that both MMPs were elevated in the Testo and Testo+30 groups, being higher in the Testo group (Figure 4 I, J).

Discussion

In this study, both BPH-induced groups presented an increase in the prostate weight and distribution of epithelium and muscular stroma, characteristics of the BPH condition. Testosterone supplementation caused the presence of intraluminal inflammation, of microinvasive carcinoma only in the Testo+30 group and the increase of subepithelial inflammation and PIN area in both BPH-induced groups. This shows

that testosterone administration beyond causing the development of BPH triggered the increase of some lesions and the presence of other proliferative foci in the prostate. The hormonal supplementation increased the expression of 5αR2, AR, and some inflammatory and proliferative cells in the Testo and Testo+30 groups. Our results show that the testosterone administration promoted the establishment of BPH and the hyperplastic condition is maintained even 30 days after the end of testosterone applications.

The relative ventral prostate weight was greater in the Testo and Testo+30 groups and this result is in agreement with others studies[50,52,62] where applications of exogenous testosterone directly influence the weight of the prostate. Testosterone supplementation affects the reproductive function, which may result in testicular atrophy in men[63,64] and rats[65], and cause oxidative stress in the testis of rats[66]. Here, the testis weight was reduced in animals with BPH. This result is directly related to androgen applications because the testis hypogonadism can occur during and after high use of testosterone by men[64,67]. Moreover, the decrease of testis weight is due the o hypothalamic-pituitary axis inhibition, reducing the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentration due to increased circulation of steroids, such as testosterone, as occurs in individuals who indiscriminately use these androgens for anabolic purposes[68].

The analyses of hormonal serum levels revealed a higher concentration of testosterone in the Testo group, as expected due to exogenous administration of testosterone[53,69–71]. A slight elevation of circulating testosterone can be related to prostate cancer in rat models[7,72]. Although the levels of this hormone reduced drastically in the Testo+30 group in comparison to the Testo group, the values were greater than the control group. This result can be occurring due to the end of the testosterone administration.

In contrast, the levels of 17 β-estradiol did not alter among the groups. However, studies related to BPH and estradiol are controversial, since they indicated that serum level of 17 β-estradiol is unchanged in old men with BPH[73] and also demonstrated an association between circulating estrogen and the risk of BPH[74]. Although estrogen serum levels did not change, the Testo group presented inversely proportional values for 17 b-estradiol and testosterone levels, indicating a greater aromatization and consequently elevated activity of 5aR.

BPH causes an enlargement of epithelial and stromal cells in the prostate[6,75]. Testo and Testo+30 groups presented similar results in morphometric and stereological analysis, as a hyperplasia of the epithelial and stromal compartments, characteristic of BPH[71,75]. This hyperplastic condition was greater in the muscular stroma of the Testo+30 group. In addition, the testosterone administration was sufficient to increase the frequency of subepithelial inflammation and PIN lesions. Prostatic inflammation can facilitate the cellular proliferation and consequent incidence of histopathological lesions in both benign and malignant conditions[75]. The association of prostatic lesions, inflammation and time after testosterone exposure may be related to the incidence of microinvasive carcinoma in the Testo+30 group. Moreover, the presence of prostatic lesions after the testosterone supplementation can be associated to alterations caused by the prolonged use of anabolic steroids[76,77].

Androgens are the main factor for prostate cell proliferation and is involved in BPH development[25,71]. 5αR2 is the most frequent isoenzyme in the prostate, has the greatest affinity for testosterone, and is overexpressed in BPH[6,78]. Our investigation showed that the frequency of 5αR2 is elevated in both testosterone treated groups. This data is consistent with the literature and indicate that 5αR2 is an important factor responsible for proliferative lesions and BPH establishment. The values were higher in the Testo+30 group, suggesting that the maintenance of BPH condition even after the hormonal manipulation.

AR is involved with the BPH development[79,80] that can explain the increase of AR-positive cells founded in the prostate and in the epithelial compartment in the Testo and Testo+30 groups. The stromal AR can alter various autocrine and/or paracrine growth factors and it may influence the initiation and progression of BPH[79,81,82]. These factors can justify the increase in AR-positive stromal cells founded in the Testo group. However, the AR-positive cells decreased in the stroma of the Testo+30 group, and the expression of AR protein in this group showed no difference. We believe that this reduction can be related to the end of the testosterone applications.

Cell proliferation is the main condition for the establishment of prostatic hyperplasia[15,26,27,83]. To evaluate the proliferative process, we examined the frequency of the positive cells for PHH3, a proliferation marker[84], in normal prostate and BPH. Our results demonstrated a rise in PHH3 frequency in the stroma and the epithelium of both BPH-induced groups, and it was higher in the epithelium of the Testo+30 group. We believe that these results are a reflection of the greater expression of 5αR2 in the

Testo+30 group and the AR in the epithelium of both BPH-induced groups since the 5αR2 and the AR activation are involved in the proliferation[6,78–80]. These observations suggest that testosterone administration caused the elevation of proliferative cells, contributing to the establishment of BPH, which persisted even after four weeks after the end of the hormonal supplementation.

In the BPH, stromal cells induce the production of pro-inflammatory cytokines and chemokines and these infiltrates can stimulate the proliferation of prostate stromal cells[26,29,85–88]. COX2 is an enzyme overexpressed during inflammation[89] and related to cell growth and progression of BPH[90,91]. This enzyme can be detected in prostatic inflammatory cells and it is enhanced in proliferative lesions associated with inflammation[52,86,92]. Here, we confirmed this, as the COX2 frequency increased in the epithelial and stromal of both BPH-induced groups.

Furthermore, macrophages are also associated with the establishment, progression, and inflammatory development of BPH[14,93,94]. A previous study with cell culture[85] showed that BPH prostate stromal cells can attract macrophage infiltration and these phagocytic cells could be related to the enhanced BPH prostate stromal cells proliferation. F4/80 is a glycoprotein expressed by murine macrophages [95] and was adopted as a marker for macrophages[96]. In the stroma of BPH-induced groups, we observed an increase of F4/80 positive cells. Studies in BPH cells related a raise in AR-positive cells that can promote the recruitment of macrophages[6,79,93]. These infiltrating cells play an important role in macrophage-induced prostatic stromal cell proliferation and can contribute to the establishment and the progression of BPH[85,93,97]. Although the Testo+30 group presented a decrease in AR-positive stromal cells, we believe that the AR, COX2, and F4/80 positive cells may be associated with enhanced proliferative cells of the gerbil prostate and may be a long-term effect of testosterone supplementation.

The exposure of gerbils to testosterone resulted in an increase of MMP-2 and MMP-9. These metalloproteinases are commonly associated with the degradation of basal membrane components, metastasis, and angiogenesis in prostate cancer[98–101] and are often used as markers of poor prognosis[102]. The tissue remodeling caused by the BPH may have altered the distribution of MMPs and these enzymes can be contributed to the establishment of the inflammatory and proliferative microenvironment.

In conclusion, the testosterone supplementation showed prostatic disorders characteristics of BPH in both groups. The hormonal administration caused the establishment of prostatic lesions and promoted the increase of the cellular proliferation occasioned by the higher expression of 5αR2 and AR. Furthermore, the hormonal supplementation increased the frequency of MMPs that can be associate with the inflammation observed in the prostate also playing a significant role in the BPH induction. Thus, the present study demonstrated that the dosage utilized of testosterone caused the BPH phenotype and the testosterone effects are persistent 30 days after the end of the exogenous stimulus, the period that researchers test new treatments for BPH, utilizing many conventional and herbal medicines[35,49,50,103].

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Conflict of interest:

The authors declare there are no conflicts of interest.

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Tables:

Table 1. Biometric and hormonal parameters of Mongolian gerbils in the groups control (C), testosterone (Testo) and testosterone euthanized after 30 days to the end of the applications (Testo+30).

	Experimental groups		
Parameters	C	Testo	Testo+30
Biometric data (%) n=7			
Body weight (g)	72.86 ± 5.001	73.14 ± 6.817	80.57 ± 5.968
Relative weight of prostatic complex (10^{-3})	8.185 ± 2.576[a]	16.74 ± 1.356[b]	14.50± 1.713[a,b]
Relative weight of ventral prostate (10^{-4})	2.504 ± 0.828[a]	4.139 ± 0.914[b]	4.527 ± 0.904[b]
Adrenal (g)	0.0420 ± 0.003	0.044 ± 0.007	0.034 ± 0.0081
Right testis (g)	0.490 ± 0.076[a]	0.409 ± 0.043[b]	0.366 ± 0.041[b]
Left testis (g)	0.501 ± 0.058[a]	0.407 ± 0.052[a,b]	0.327 ± 0.119[b]
Hormonal data (µm) n=7			
Testosterone (ng/ml)	1.099 ± 0.522[a]	33.43 ± 9.816[b]	9.025 ± 4.994[a]
17 β-estradiol (pg/ml)	17.55 ± 12.62	3.002 ± 3.475	12.41 ± 16.92

Values expressed as mean ± standard deviation.

[a,b] represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

One-way ANOVA was followed by Tukey and Kruskal-Wallis post-tests.

Table 2. Stereological and morphometric parameters of Mongolian gerbils in the groups control (C), testosterone (Testo) and testosterone euthanized after 30 days to the end of the applications (Testo+30).

	Experimental groups		
Parameters	C	Testo	Testo+30
Morphometric data (µm) n=7			
Epithelium height	13.79 ± 4.084[a]	24.43 ± 8.888[b]	22.65 ± 8.120[b]
SMC thickness	7.421 ± 2.460[a]	12.48 ± 4.649[b]	12.73 ± 5.052[b]
Stereological data (%) n=7			
Epithelium	16.52 ± 4.960[a]	25.07 ± 9.538[b]	23.08 ± 9.904[b]
Muscular stroma	7.802 ± 3.244[a]	9.725 ± 3.836[b]	11.72 ± 3.966[c]
Stroma	10.86 ± 7.114[a]	12.80 ± 10.28[a,b]	15.62 ± 6.958[b]
Lumen	57.00 ± 14.15[a]	36.52 ± 23.62[b]	43.11 ± 12.48[b]

Values expressed as mean ± standard deviation.

[a,b,c] represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

One-way ANOVA was followed by Tukey and Kruskal-Wallis post-tests.

Table 3. Incidence and multiplicity of prostatic lesions and inflammation in gerbils of the control (C), testosterone (Testo) and testosterone euthanized 30 days after the end of the applications (Testo+30) groups.

	Incidence (%)			**Multiplicity (mean ± SD)**		
	C	Testo	Testo+30	C	Testo	Testo+30
Inflammation						
Periductal	100	100	100	(0-5) 1.457	(0-19) 5.200	(0-20) 6.171
Subepithelial	100	100	100	(0-4) 1.714 [a]	(0-24) 5.600[b]	(0-18) 6.257[b]
Intraluminal	0	28,5	28,5	(0) 0	(0-3) 0.228	(0-15) 0.714
Flat epithelial atypia	100	100	100	(3-36) 18.57	(2-66) 23.77	(3-41) 19.54
Papillary epithelial atypia	100	100	100	(0-24) 12.71	(3-47) 17.09	(4-57) 20.34
PIN	100	100	100	(0-7) 2.343[a]	(0-65) 10.57[a,b]	(0-78) 16.34[b]
Microinvasive carcinoma	0	0	28,5	(0) 0	(0) 0	(0-1) 0.058

[a,b] represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

One-way ANOVA was followed by Tukey and Kruskal-Wallis post-tests.

Figures:

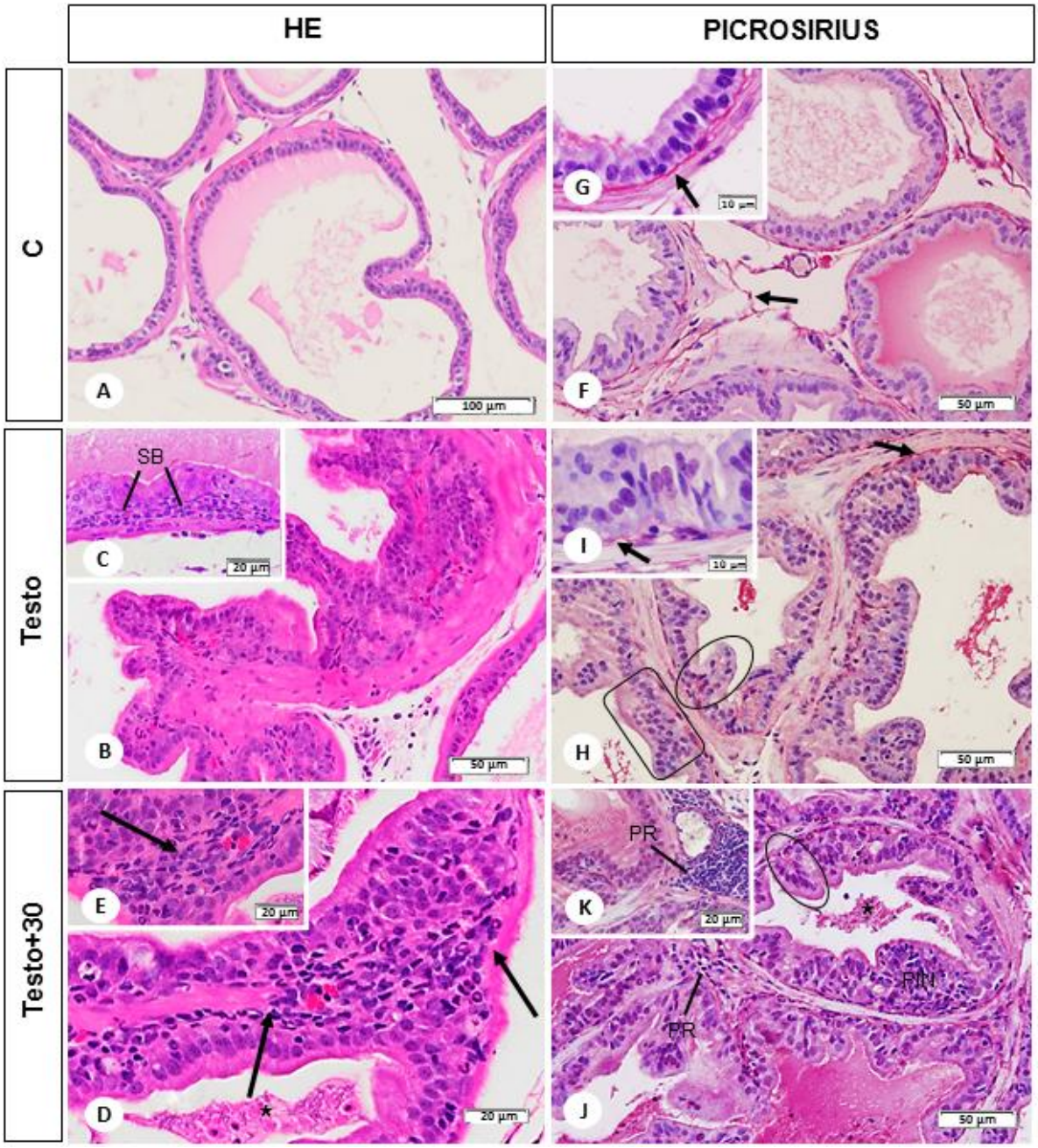

Figure 1. Testosterone administration alters the distribution of ventral prostate compartments. Sections of all experimental groups stained with HE (A- E) and picrosirius red (F- K). Groups: C (A, F, G), Testo (B, C, H, I) and Testo+30 (D, E, J, K). Normal aspect in A, F, G and hyperplastic tissue in B-E, H-J. Short arrows indicate the collagen (F-I). The flat epithelial atypia (square), papillary epithelial atypia (circles), periductal (PR) and subepithelial (SB) inflammation and prostatic intraepithelial hyperplasia (PIN) were verified in all groups. The intraluminal inflammation (asterisks) was observed in both testosterone groups and the microinvasive carcinoma (long arrows) only in the Testo+30.

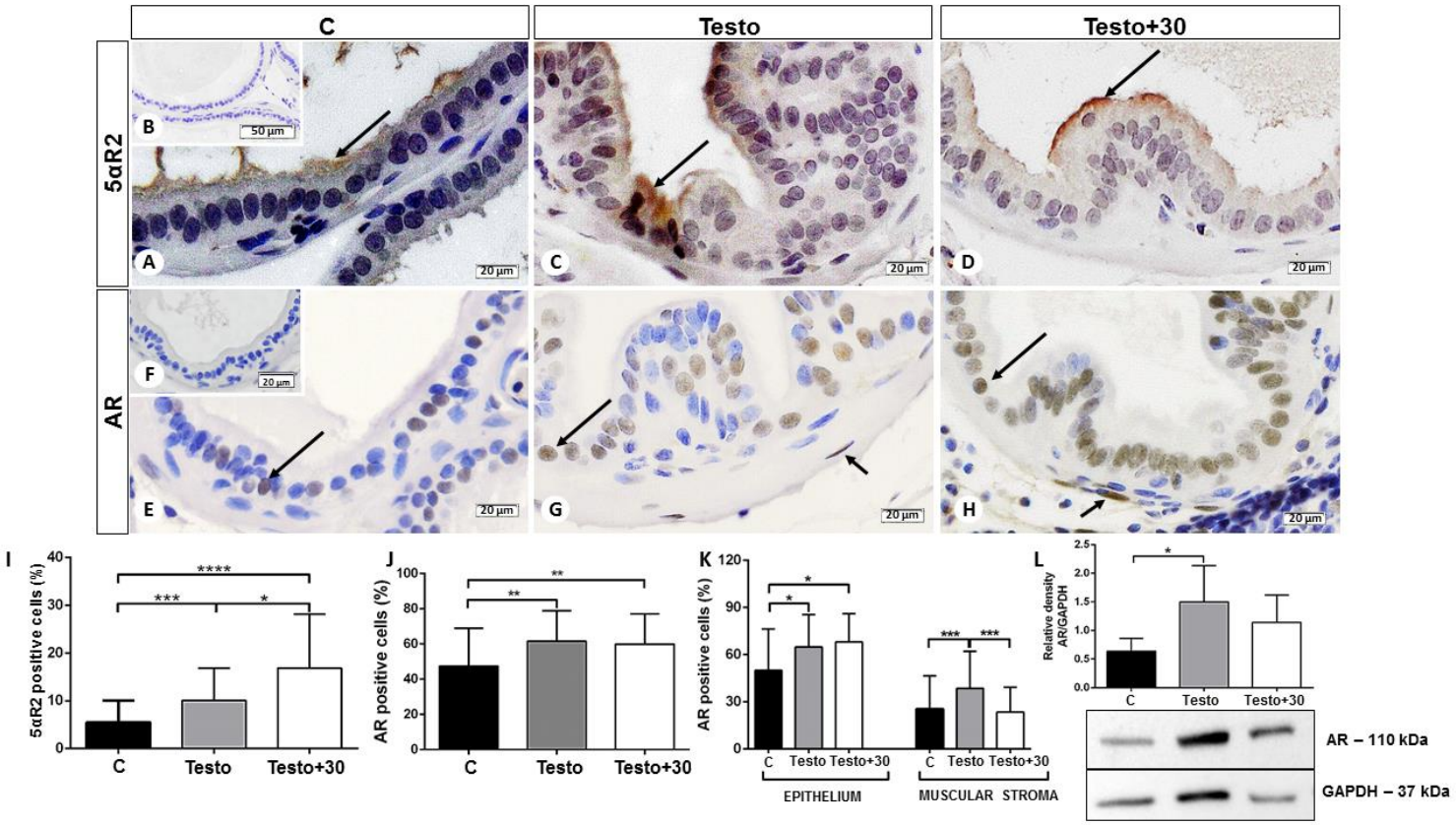

Figure 2. The hormonal supplementation influenced in the immunoexpression of 5 αR2 and AR-positive cells and in the AR protein expression of the ventral prostate. Immunohistochemical staining of 5 αR2 (A-D), AR (E-H) and the respective negative controls of reaction (B, F) in the ventral lobe of Mongolian gerbils. Determination (%) of 5 αR2 stained (I) and AR-positive cells (J) in the gerbil prostate, and of AR-positive cells in the epithelium and stroma (K). Relative density of AR (L) normalized to GAPDH, here utilized as positive control. The error bars indicated the standard deviation. * , **, *** and **** indicate statistically significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$ respectively), according to the Kruskal-Wallis test followed by the post hoc Dunn test. Positive stainings are present in the epithelium (long arrows) and in the stroma (short arrows).

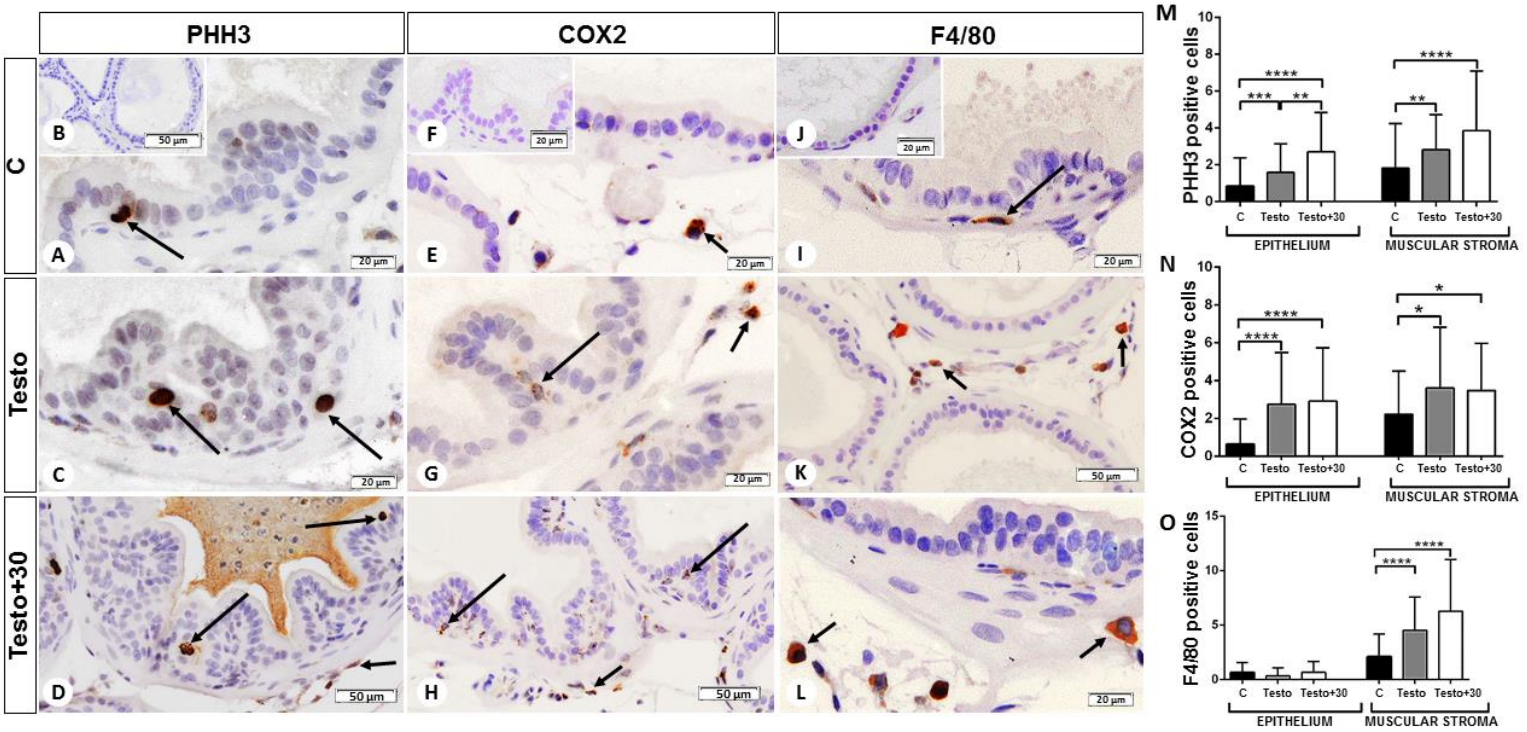

Figure 3. BPH causes an increase of proliferative and inflammatory cells in Mongolian gerbil prostate. Immunohistochemistry for PHH3 (A-D), COX2 (E-H) and F4/80 (I-L) positive cells in the ventral prostate. In B, F, J the sections did not incubate with primary antibodies (Negative controls of reaction). The dates represent averages of the absolute frequency (%) of PHH3 (M), COX2 (N) and F4/80 (O) positive cells in the epithelial (long arrows) and stromal compartments (short arrows). The error bars indicated the standard deviation. * , **, *** and **** indicate statistically significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$ respectively), according to the Kruskal-Wallis test followed by the post hoc Dunn test.

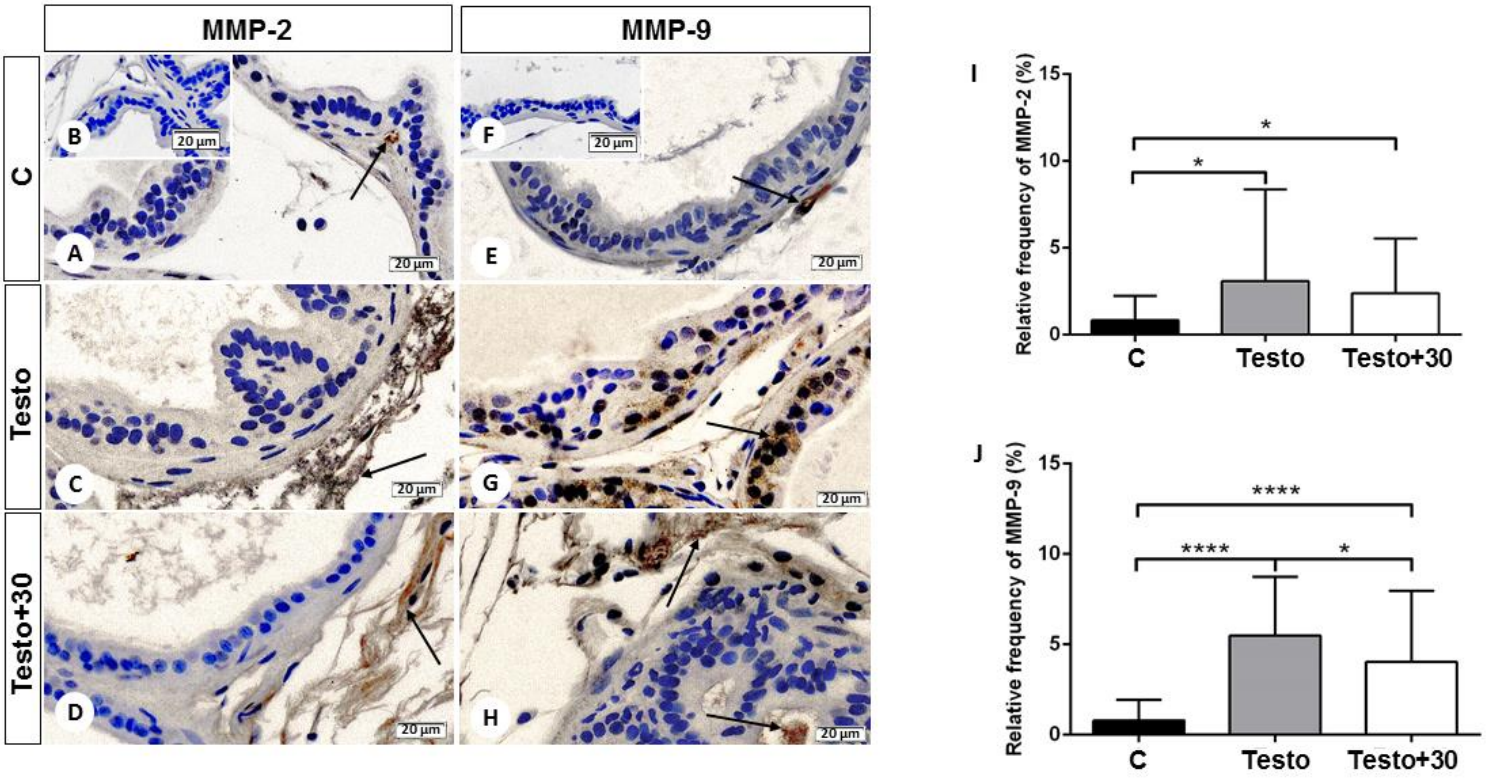

Figure 4. Frequency of MMP-2 and 9 increase in ventral prostate of Mongolian gerbils of BPH –induced groups. A-D and E-H represents the immunoreaction of MMP-2 and 9, respectively. Negative controls of reaction are indicate in B, F. Positive staining are indicated by arrows. The dates represent averages of the relative frequency (%) of MMP-2 (I) and MMP-9 (J). The error bars indicated the standard deviation. * and **** indicate statistically significant differences ($p < 0.05$ and $p < 0.0001$ respectively), according to the Kruskal-Wallis test followed by the post hoc Dunn test.

[1] **5.2. Artigo 2.**

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ORIGINAL ARTICLE

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Therapeutic effects of β-caryophyllene on proliferative disorders and inflammation of the gerbil prostate

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Abstract

Background: The prostate is susceptible to changes in androgen levels, which can play an important role in the development of Benign Prostatic Hyperplasia (BPH). Natural compounds have beneficial properties for organisms and can be an important therapeutic strategy in the treatment of diseases. β-Caryophyllene (BCP) is a phytocannabinoid present in several medicinal and food plants species and has shown beneficial effects in different organs. However, little is known about its effects on the prostate. The present study seeks to evaluate the effects of exposure to BCP on the morphophysiology of the ventral prostate of adult gerbils supplemented with testosterone.

Methods: Animals were distributed into four groups (n = 8/group): Intact control (C); β-Caryophyllene (BCP): β-Caryophyllene (50 mg/kg/day); Testosterone (T): animals received subcutaneous injections of Testosterone Cypionate (3 mg/Kg), on alternate days, for one month and were euthanized 30 days supplementation ended; Testosterone and β-Caryophyllene (TBCP): animals were exposed to testosterone cypionate (3 mg/Kg) to induce hyperplastic alterations followed by daily BCP (50 mg/kg). Morphological, biometric, immunohistochemical, and serological analyses were performed.

Results: Proliferative disorders and inflammatory foci were present in the ventral prostate of all experimental groups. An increase in the multiplicity of benign intraepithelial neoplasm (PIN) and subepithelial inflammatory foci was observed in T group. The incidence of intraluminal inflammatory foci and microinvasive carcinoma was verified only in the T group. Cellular rearrangement and tissue remodeling occurred in the prostate of groups exposed to phytocannabinoids. A reduction was observed in the frequency of PHH3 and Cox2 markers in the prostatic epithelium of TBCP in comparison with T. A decrease in F4/80 and CD163 positive macrophages were also observed in the prostatic stroma of the TBCP group in comparison with T. The results suggest that BCP had favorable effects on BPH, reducing the proliferation and frequency of some inflammatory cells.

Conclusions: BCP impacts the tissue remodeling process in the premalignant prostate environment and that the use of this phytocannabinoid can have a promising effect in the handling of BPH.

Keywords: BPH, inflammatory cells, macrophages, phytocannabinoid, tissue remodeling.

Introduction

The prostate can be characterized as a hormone-dependent and immunocompetent tissue organization that encompasses several cell types, such as lymphocytes, granulocytes, and macrophages.[1,2] This gland is part of the reproductive system and is susceptible to hormonal, biochemical, metabolic, genetic and micronutrient influences.[3] This factors may be related to the development and progression of prostatic diseases as Benign prostatic hyperplasia (BPH) and cancer.[1] BPH is defined by the growth of the gland due to interference in the balance between growth factors that affect stroma and epithelium relationships, which increases the volume of the prostate.[3] This comorbidity is directly associated with an increased risk of developing prostate cancer.[4] Pathologically, it is associated with an increase in proliferating cells and the establishment of inflammation in the prostate tissue.[5,6] The inflammatory environment and uncontrolled cell proliferation leads to tumorigenesis.[5] The experimental models are important in the study of this disorder that can be induced by androgen analogs.[7–11]

Androgens are essential steroid hormones for prostate development and homeostasis.[12–14] These hormones are already established as risk factors for the pathogenesis of BPH and are associated with elevated inflammatory cell levels.[5,6,9,15–17] In BPH, inflammation is characterized by increased production of pro-inflammatory chemokines and cytokines and by the association of stromal cells and infiltrated immune cells, such as macrophages.[18–21] This inflammatory process is also associated with oxidative stress, which is mediated by nitric oxide synthase (NOS) and cyclooxygenase (Cox), among others.[21–23]

The current adopted treatments for BPH includes the use of conventional drugs and medicinal plants.[24] Pharmacology has made great advances related to the treatment of this disease, but many medications have impairments in terms of selectivity and prolonged use, including sexual dysfunction.[25] Phytotherapeutic agents have shown great benefits in prostatic hyperplasia treatment,[24] but the understanding of the mechanisms performed by these plants is not well understood yet.[26] Thus, there is a need for further studies with new drugs and compounds for BPH.[25]

Beta-Caryophyllene (BCP) is a compound extracted from medicinal and food plants, such as black pepper (*Piper nigrum L.*), rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum spp.*), oregano (*Origanum vulgare L.*), basil (*Ocimum spp.*), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), mint (*Mentha piperita*), ginger

(*Zingiber officinale*), cloves (*Syzygium aromaticum*), citronella, (*Cymbopogon*), pine tree (*Pinus*), *Cannabis sativa*, and plants of the Copaifera genus, among others.[27–29] Its use has been approved by the Food and Drug Administration (FDA)[29,30] and the European Food Safety Authority (EFSA)[29], and it is used in the manufacture of cosmetics[31] and as a flavor enhancer.[27,29]

BCP is a sesquiterpenoid; it belongs to the cannabinoid family and is a selective agonist of type 2 cannabinoid receptors (CB2-R)[29,31]. CB2-Rs are metabotropic receptors found in peripheral tissues and cells of the immune system (mast cells, macrophages, B-lymphocytes). CB2-Rs are involved in pathological processes such as inflammation, neurodegenerative pathologies (Parkinson's disease, Alzheimer's disease, multiple sclerosis) and some types of cancer.[29] They are therefore important targets for the study of agonist molecules and in the search for treatment for pathological conditions.[29,32]

This sesquiterpenoid plays important pharmacological functions related to anti-inflammatory properties,[30,33–35] hepatoprotection,[36] neuroprotection,[27,37–39] cardioprotection,[40] apoptosis,[26] in anticancer therapies,[26,41,42] and in improving rheumatoid arthritis.[32,34] Furthermore, BCP inhibits the proliferation of a variety of tumor cells present in the lung, liver, breast and prostate.[26,41–43]

Despite relevant results, studies of BCP in the prostate are still limited to the action of this compound in prostate cancer cell lines.[26,42,44] There is therefore a need to assess the effects of BCP on the prostate *in vivo,* aiming to evaluate a physiological pharmacokinetic environment prone to metabolic alterations. A relevant experimental model for studies related to the prostate is the Mongolian gerbil (*Meriones Unguiculatus*). These rodents have tissue composition similar to the human prostate and have shown to be promising in the scientific studies.[45–48] The present study sought to analyze, for the first time in vivo environment, the effects of BCP on the ventral prostate of Mongolian gerbils under normal and BPH conditions, focusing on the proliferation and inflammation aspects of this gland.

Materials and Methods

Animals

For this experiment, thirty two adult Mongolian gerbils (*Meriones unguiculatus*) (3 months old) obtained from the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State

University (UNESP) were used. The animals were divided into four groups (n = 8/per group), maintained in polyethylene cages with wood shavings substrate, under controlled light (12h light/dark cycle) and temperature conditions (24 ± 2°C). They received feed and filtered water *ad libitum*. The experiments were carried out following the ethical principles recommended by the National Council for Animal Experimentation Control (CONCEA) and the procedures involved were approved by the Ethics Committee on the Use of Animals at IBILCE/UNESP (Proc. No. 173/2017).

Experimental design

The experimental design is described in Figure 1. The animals were distributed in the following experimental groups: Intact control (C): the animals received no treatment during the experiment; β-Caryophyllene (BCP): animals received 50 mg/kg/day[37] of β-Caryophyllene (*W225207, Sigma-Aldrich, St. Louis, MO, USA,*) diluted in corn oil (*Cargil, Brazil*) via gavage during 30 consecutive days; Testosterone (T): the animals received, on alternate days and for a month, subcutaneous injections of Testosterone Cypionate (*Deposteron, EMS*) diluted in corn oil (3 mg/Kg)[49,50] and were euthanized 30 days after the end of supplementation and Testosterone and β-Caryophyllene (TBCP): the animals received, on alternate days and for a month, subcutaneous injections of Testosterone Cypionate diluted in corn oil (3 mg/Kg)[49,50] and then, via gavage, followed by 50 mg/kg/day[37] of β-Caryophyllene diluted in corn oil via gavage for the 30 consecutive days.

Biometric analysis

The animals from all experimental groups were anaesthetized with Xylazine hydrochloride (3 mg/kg) and Ketamine hydrochloride (10 mg/kg) and euthanized by decapitation. The body blood was collected and stored at -80°C. The prostatic complex and the ventral prostate were weighed and fixed. The relative weight of the prostatic complex and of the ventral prostate was calculated as a proportion, dividing the organ weight by the body weight.

Hormonal analysis

The serum levels of testosterone were quantified by ELISA using a high sensitivity specific commercial kit (*IBL International, Hamburg, Germany - item number 52151).* The readings were performed in a microplate reader (*TECAN- Infinite F50*).

Histological processing and histochemical analysis

The collected organs were fixed in 4% buffered paraformaldehyde, dehydrated in ethanol, clarified in xylol, and then included in Histosec (*Merck*). The organs were sectioned at 3-5µm and stained with the following techniques: Hematoxylin-Eosin (HE) and Picrossirius for general analysis of the morphological aspects of the gland and Gömori Reticulin staining, for reticular and collagen fibers analysis.

Incidence and multiplicity of lesions

The analysis of proliferative disorders was performed in 5 slides per animal (n = 8/group) stained by HE and digitized at 400x magnification using the slide scanner system (*Olympus VS120-S5*). The histopathological classification of prostate lesions was accomplished according a well-established criteria[51], which has been previously applied in the Mongolian gerbil.[52–54] The incidence of prostatic alterations was calculated by dividing the number of animals with changes by the number of animals per group, expressed in %. Also, the multiplicity was evaluated, in which the number of changes present in each animal was divided by the number of slides analyzed per animal.

The presence of benign intraepithelial neoplasms (PINs), intraluminal, periductal and subepithelial inflammatory foci was evaluated. The presence of flat epithelial atypia (FEA) and papillary epithelial atypia (PEA) was also quantified. Briefly, FEA consists in a cell grouping by the acini that does not project into the lumen, resulting in an epithelial stratification and PEA corresponds to the grouping of cells in the acini that are projected into the lumen.[48]

Immunohistochemical analysis

The absolute frequency of phosphohistone H3 (PHH3) positive cells (for analysis of cell proliferation), cyclooxygenase 2 (Cox2) immunostaining (for inflammatory signaling cells) and macrophages F4/80 and CD163 marking positives were calculated and the distribution of smooth muscle cells with the use of an α-actin marker.

The slides were deparaffinized, and rehydrated histological sections were subjected to antigen retrieval in Tris EDTA buffer pH 9.0 for 60 minutes at 98° C (PHH3, Cox2, F4/80 and CD163) or for 20 minutes at 98°C in 10mM citrate buffer pH 6.0 (α-actin). The blocking of endogenous peroxidases was performed with 3% H2O2, followed by nonspecific protein blockage with 5% skimmed milk in TBS + 0.1% tween (TBSt) for 30 minutes. The sections were then incubated with primary antibodies

overnight (Table 1), before being washed and incubated with secondary antibodies labeled with peroxidase (CD163: *Rabbit ABC Staining System SC-2018 -Santa Cruz Biotechnology CA, USA*) or Dako Polymer (PHH3, Cox2, F4/80 and α-actin: *kit Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA*). The sections were stained with diaminobenzidine (*DAB, Sigma, St. Louis, MO, USA*) and counterstained with Mayer's Haematoxylin.

The images were captured at 400x magnification using the slide scanner system (*Olympus VS120-S5*). The counts of positively labeled cells were performed in 70 microscopic fields per group (n = 7/group) (as an adaptation of the previously described protocol[55]) using Image-Pro-Plus software (*Media Cybernetics*).

Statistical analysis

Statistical analysis was performed on GraphPad Prism 6.00 software spreadsheets and graphs. The quantitative analysis was based on two-way ANOVA followed by Bonferroni post-tests for multiple proofs. The level of significance adopted was 5% ($p < 0.05$).

Results

Biometric Analysis

Biometric data shows that there was an increase in body weight in the T group compared to the other groups (Table 2). Regarding the weight of the prostatic complex, there was an increase in the T and TBCP groups in comparison to C and BCP groups (Table 2).

For the absolute and relative weight of the ventral prostate, an increase was observed in the groups supplemented by testosterone (T and TBCP) compared to animals in groups C and BCP (Table 2).

Serum hormonal levels

The hormonal dosage showed an increase in serum testosterone levels in the T and TBCP groups compared to C and BCP (Table 2).

Histological analyses

As expected, the Morphological analysis revealed that the ventral prostate is composed of several acini and ducts surrounded by a layer of muscle, blood, reticular and collagen cells that make up the fibromuscular stroma. The epithelium is usually

simple and can vary from cubic to prismatic, and this gland presents the luminal compartment, where seminal fluid is stored (Figure 2 A, H, I, M, N, Q).

Histological analysis showed incidence of proliferative disorders in the epithelial and stromal compartment from T group compared to the others. Benign intraepithelial neoplasia (PIN) (Figure 2 D, F, G, J, K, P, R), inflammatory cell foci in the subepithelial region (Figure 2 D, E) and the luminal compartment (Figure 2 D, J, O), in addition to a large concentration of inflammatory cells in the stroma (Figure 2 D, J, O, S, U) and breakdown in the pattern of organization of collagen and reticular fibers (Figure 2 M-P) were present. The presence of planar (Figure 2 C, L), papillary (Figure 2 A, B, C, D, F, J, O, P) and microinvasive carcinoma in the T group (Figure 2 S, T) was also noted.

The multiplicity of these alterations in the different experimental groups made it possible to verify differences in subepithelial inflammations, which were higher in the T group in comparison to C, BCP, and TBCP and in the PINs, in which an increase was observed in the T group when compared to C and BCP (Figure 3 A). The presence of microinvasive carcinoma was observed only in T (Figure 3 B). The other alterations were noted in all groups (Figure 3).

Immunohistochemical analysis

1. Proliferative index (PHH3): The anti-PHH3 antibody, showed the presence of proliferative cells in the epithelium and stroma of the ventral prostate in all experimental groups (Figure 4 A-F). It was possible to verify an increase in the absolute frequency of these cells in the epithelium of animals in the T group when compared to the other groups (Figure 4 L). This increase was also observed in the stromal compartment of groups BCP, T, and TBCP in comparison to C (Figure 4 L).

2. Inflammatory maker (Cox2): The cytoplasmic marker Cox2 was present in the stromal and epithelial compartments (Figure 4 G-K). The immunostaining analyses showed an increase in Cox2 positive cells in the T group epithelium when compared to C, BCP, and TBCP (Figure 4 M). In the stroma, there was also an increase in Cox2 positive cells in the T and TBCP groups in comparison to the C and BCP groups (Figure 4 M).

3. Macrophage infiltration (F4/80, CD163): F4/80 immunostaining revealed the presence of macrophages in the ventral prostate of all groups (Figure 5 A-G). The quantification of positive F4/80 cells showed an increase of this marker in the intraepithelial region of TBCP in comparison with C, BCP, and T (Figure 5 N). Incidence

of F4/80 positive cells was higher in the stroma of the TBCP group in comparison to C, in BCP when compared to C and in the stromal compartment of the T in comparison to the other groups (Figure 5 N). The quantification of CD163 positive cells in the stromal and intraepitheial compartments (Figure 5 H-M) showed a decrease in the frequency of these cells in the stroma of the TBCP group in comparison to T (Figure 5 O).

Discussion

In the present study, we analyzed the influence of BCP on the ventral prostate of gerbils under normal conditions and with BPH. This phytocannabinoid showed positive effects on inflammatory and proliferative disorders, in which a decrease in the frequency of PHH3 and Cox2 positive cells in the prostatic epithelium of the TBCP group in comparison to T. In addition, in the prostate stroma of these same experimental groups, there was a decrease in macrophages after treatment with BCP in animals with BPH. Also, it was observed that the testosterone dosage used was sufficient to induce the hyperplastic environment.

The gerbil is an alternative experimental model for prostate cancer studies[52,56]. In chemical induction, they develop prostatic lesions in a reduced period of latency, with a satisfactory incidence in comparison to others rodents [52,57,58]. This observation is important for establishing the gerbil as a suitable model for evaluating prostate carcinogenesis[52]. Our research group has interesting results with gerbils chemically induced tumors [52,53,56], development of spontaneous neoplasm in senile age [59,60] and hormonal treatment [61–65] as well as observed in the present study.

The prostate is sensitive to changes in the levels of androgens.[66] This sensitivity explains the increase in the prostatic complex weight and the relative weight of the ventral prostate in groups supplemented with testosterone. This increase in prostate weight is consistent with previously published research which demonstrated that treatment with this hormone induces prostate growth.[7,16,50]

Since BPH is a multifactorial disease characterized by the proliferation of cells in the prostatic compartments,[66,67] it was possible to observe proliferative disorders, such as PINs, in greater numbers in the prostate ventral of animals in the T group compared to C and BCP. Although there were no statistical differences between the groups regarding other lesions, the presence of inflammation was frequent in animals supplemented with testosterone and in similar frequency between C and BCP. Thus,

from the histological analysis of the ventral prostate, it is possible to infer that the dose of testosterone used was sufficient to induce all the BPH typic alterations. This statement can be corroborated by the increase in serum levels of this androgen in the groups that received supplementation.[9,11,14,68]

The increased incidence of PHH3 positive cells in the epithelium of the T group shows the presence of proliferating cells, as seen in BPH. This marker is specific and consistent for determining proliferation and has been widely applied in studies related to neoplasms,[69] such as prostate cancer.[70] Thus, given the specificity of PHH3 and based on results observed, it is possible to infer that exposure to the phytocannabinoid BCP reduces the proliferation of cells in the hyperplastic ventral prostate epithelium of Mongolian gerbils, also producing an anti-proliferative effect.

Proliferative changes promote an increase in frequency of inflammatory cells, which lead to lesions in the epithelial and stromal compartments.[71,72] Such changes lead to the prostatic tissue remodeling process, which can alter the pattern of organization of the collagen and reticular fibers, as seen in the testosterone supplemented groups. These fibers are structural components of the extracellular matrix[52,61,62,73–76] and become thicker and denser in regions with lesions in response to supplementation by testosterone in gerbils[62] and human BPH.[75] Besides, these changes can promote structural changes associated with benign and malignant disorders and the constant recruitment of more inflammatory cells.[13]

Prostatic inflammation occurs as a response to the pathological condition and is associated with BPH and prostate cancer.[21,77–79]It is divided into acute inflammation (characterized by the action of neutrophils) and chronic inflammation (characterized by the predominantly presence of lymphocytes and macrophages)[18,80]. These cells are involved in the secretion of different cytokines and growth factors that are released in greater quantity due to the increase in the number of immune cells, and this can stimulates cell proliferation and the recruitment of more inflammatory cells in the tissue.[2,77,79,80] Different cell types have been implicated in the pathogenesis of prostate diseases, such as cancer.[5,17,19,77,80–83] For example, tumor-associated macrophages (TAMs)[84], T cell subtypes[81] and B cells[85], and mast cells have been present in the prostate tumor microenvironment and might contribute to disease progression. [80,86,87]

One of the enzymes expressed during the inflammatory process is Cox2.[23,88] Coxs are located in the luminal region of the endoplasmic reticulum and the nuclear membrane of cells. This isoform play a crucial role in tumor angiogenesis, cell

proliferation, and inhibition of apoptosis[44] and is overexpressed in prostate cancer and BPH,[44] which explain the increased frequency of Cox2 positive cells in the T group.[44] This implies that BCP also has an anti-inflammatory action on the prostate since treatment with this phytocannabinoid decreased the frequency of Cox2 in BPH, corroborating studies that show the decrease of this enzyme in microglial cells and rheumatoid arthritis of rats after treatment with BCP.[33,34]

BPH is related to the presence of pro-inflammatory cytokines, ILs, increase of B and T lymphocytes and macrophages in the stromal, intraepithelial and luminal regions of prostatic tissue. [3,18,80] Macrophages are capable of performing phagocytosis and constitute several cell classifications with a wide variety of phenotypes, biological activities and functions in homeostatic and pathological conditions.[89] During prostatic inflammation, macrophages are the most recruited among different types of activated immune cells.[5] These phagocytes are involved in changes in the stromal microenvironment and can alter the interactions between the stroma and the epithelium in the ventral prostate of Mongolian gerbils.[90]

The F4/80 is a murine-specific pan macrophage marker and expressed on TAMs.[91,92] Our study allowed us to observe an increase in the frequency of these cell types in the prostate stroma of animals with BPH. This result may have been caused by the fact that macrophages are related to the development and progression of BPH.[55,77,93] The proliferation and inflammation present in the hyperplastic prostate microenvironment stimulate the macrophages infiltration and these can promote the proliferation of stromal cells.[55] This may also explain the increased frequency of proliferative and inflammatory cells in the stroma of the BPH group.

In addition, an increase was observed in the frequency of F4/80 positive cells in the epithelium of the TBCP group. We believe that this increase was not enough to favor the progression of tissue inflammation, as was verified by a decrease in PHH3 and Cox2 in the epithelial compartment of this group. It was also observed that the amount of these macrophages increased in the stroma of the group exposed only to phytocannabinoids. Thus, exposure to BCP can contribute to the recruitment of these phagocytes in an attempt to favor prostatic tissue homeostasis.

A reduction was also verified in the frequency of positive markings for F4/80 in the stroma prostate of the TBCP group. We believe that this variation in the frequency of pan macrophages in groups exposed to BCP can result in positive effects for the

ventral prostate, since macrophages can perform several functions, according to the environment in which they are recruited.[89]

The frequency of positive CD163 cells was also analyzed. CD163 is used in association with other markers to identify M2 type macrophages,[89,94–96] and this specificity may explain the decreased variation in the frequency of these cells compared to the frequency obtained for positive F4/80 macrophages. In excessive frequency and uncontrolled activity, macrophages can contribute to the spread and development of different diseases, such as cancer.[5,89,97,98] This may have favored the increase in CD163+ markings in the prostatic stroma of animals with BPH, in response to the proliferative stimulus and increased inflammation triggered by testosterone supplementation. In addition, there was a significant reduction in CD163 positive cells in the stroma of animals with BPH shortly after exposure to BCP.

We believe that these analyses indicate the possible anti-inflammatory effect of BCP on the prostate. The dose utilized is well established for neuroprotective effects[38,39] and as a modulator of the inflammatory response[99]. In addition, have been described others actions in lower doses as anti-inflammatory[33], antioxidant[100], hepatoprotective[36], and sedative[101], muscle relaxant[102] and a reduction of oxidative stress[34] in a concentration greater than 50 mg/kg/day[29]. Thus, is important and necessary other studies with this phytocannabinoid to understand more deeply the effects of BCP on the prostate.

Conclusion

The present research presents innovative results not described, so far, on the therapeutic effects of BCP on BPH *in vivo*. It was possible to observe the protective effect of the compound on prostatic proliferation and inflammation in animals that received testosterone. Our preliminary results show that the use of BCP can be promising in the treatment of proliferative prostatic disorders and BPH.

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Tables:

Table 1. Descriptions of antibodies applied in the immunohistochemistry.

Antibodies	Description	Dilution
PHH3	Rabbit Polyclonal (Ser 10, #9701S, Cell Signaling, Danvers, MA, USA)	1:75
Cox2	Rabbit Monoclonal (IgG, D5H5, #12282, Cell Signaling, Danvers, MA, USA)	1:100
F4/80	Rabbit Monoclonal (IgG, D2S9R, #70076, Cell Signaling, Danvers, MA, USA)	1:100
CD163	Rabbit Polyclonal (sc-33560, Santa Cruz Biotechnology CA, EUA)	1:50
α- actin	Mouse monoclonal (sc-32251, Santa Cruz Biotechnology CA, EUA	1:100

Table 2. Biometric and hormonal parameters of Mongolian gerbils in the control (C), β-Caryophyllene (BCP), Testosterone (T), and Testosterone and β-Caryophyllene (TBCP) groups.

	Experimental groups			
Parameters	**C**	**BCP**	**T**	**TBCP**
Biometric data (n=8/group)				
Body weight (g)	72.80 ± 8.715[a]	73.90 ± 6.537[a]	81.00 ± 8.547[b]	74.42 ± 5.357[a]
Prostatic complex weight (mg)	662.3 ± 154.5[a]	658.0 ± 163.6[a]	1163 ± 127.8[b]	1169 ± 118.4[b]
Prostatic complex relative weight (x 10^{-3})	9.323 ± 1.379[a]	8.845 ± 1.920[a]	14.48 ± 1.968[b]	14.97 ± 1.395[b]
Ventral lobe weight (mg)	19.95 ± 6.370[a]	19.06 ± 7.540[a]	38.40 ± 9.081[b]	36.70 ± 8.266[b]
Ventral lobe relative weight (x 10^{-4})	2.735 ± 0.769[a]	2.550 ± 0.878[a]	4.799 ± 1.298[b]	4.954± 0.992[b]
Hormonal data (n=7/group)				
Testosterone (ng/ml)	2.187 ± 0.924[a]	1.765 ± 1.019[a]	8.686 ± 4.722[b]	6.082 ± 1.387[b]

Values expressed as mean ± standard deviation.

[a,b] represent statistically significant differences ($p \leq 0.05$) between the experimental groups.

Two-way ANOVA was followed by Bonferroni post-tests.

Figures:

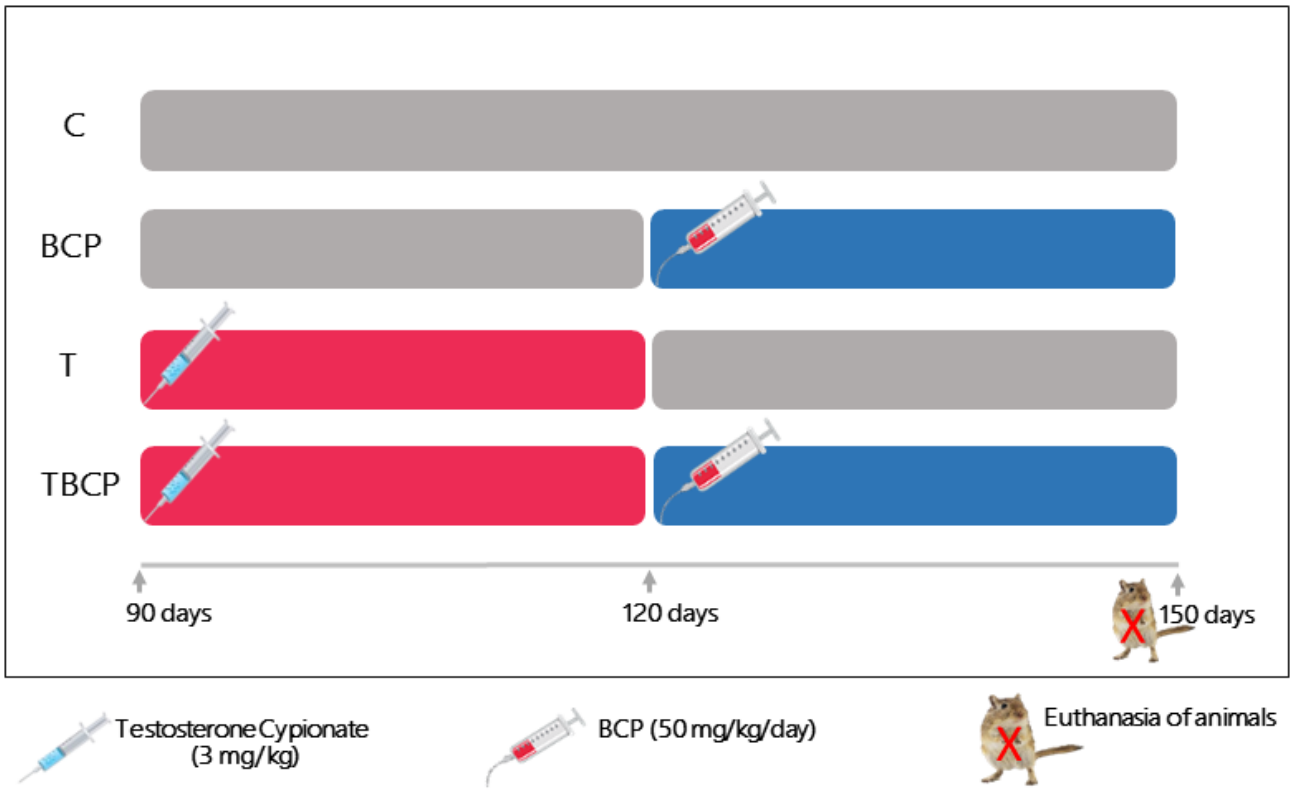

Figure 1. Schematic representation of treatments and experimental groups.

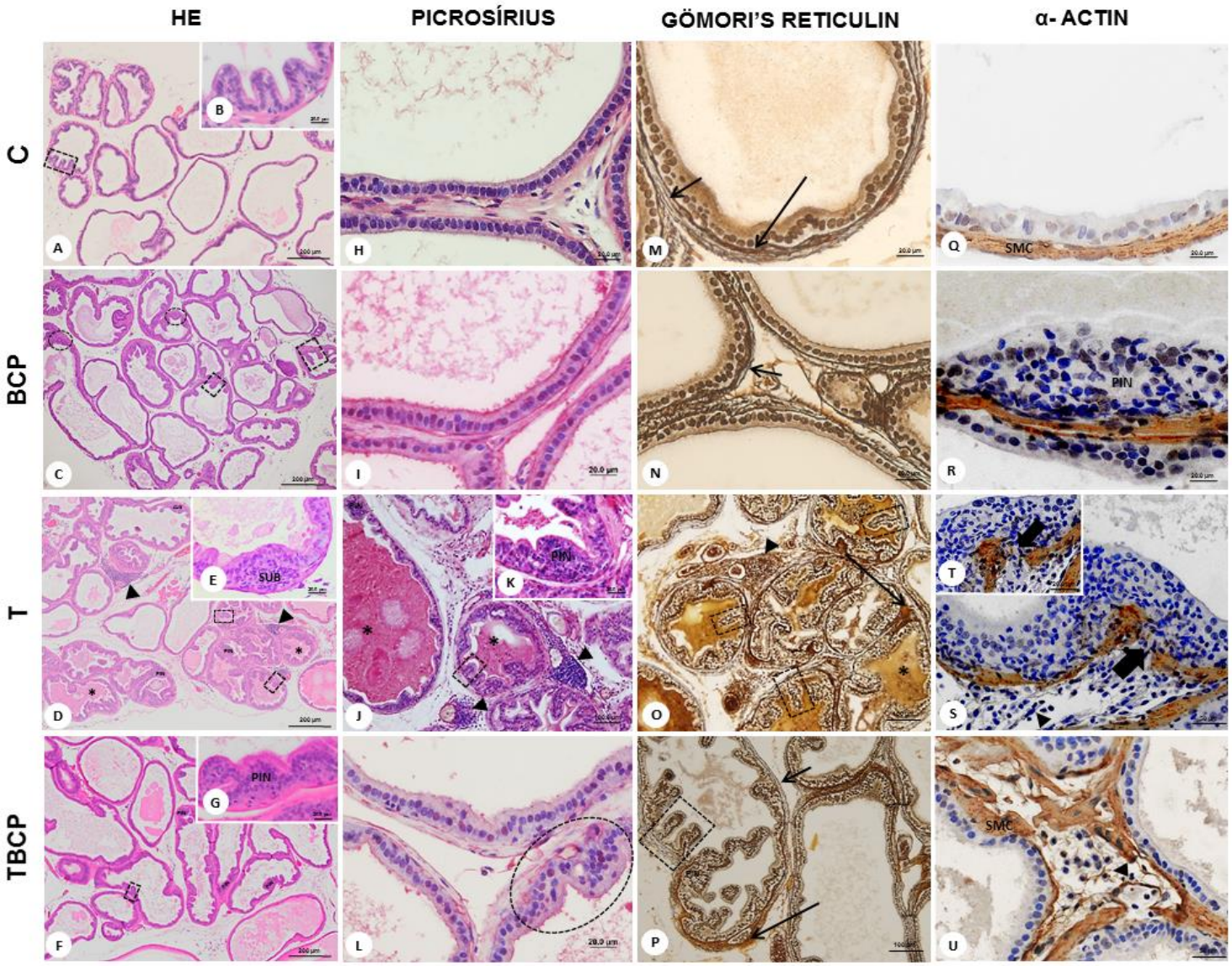

Figure 2. Histological sections of the ventral prostate of Mongolian gerbils from different experimental groups. Normal aspect (A, H, I, M, N, Q), Prostatic intraepithelial neoplasia (PIN) (D, F, G, J, K, P, R), intraluminal inflammatory foci (*) (D, J, O), periductal foci (arrowhead) (D, J, O, S, U), subepithelial inflammatory foci (SUB) (D, E), flat epithelial atypia (circles) (C, L), papillary epithelial atypia (squares) (A, B , C, D, F, J, O, P), reticular fibers (short arrows) (M, N, P), collagen fibers (long arrows) (M, O, P), microinvasive carcinoma (thicker arrows) (S, T) and smooth muscle cells (SMC) (Q, U).

A

MULTIPLICITY				
	C	BCP	T	TBCP
Flat epithelial atypia	3-49 (18.93)	3-41 (14.28)	2-43 (18.53)	4-69 (23.50)
Papillary epithelial atypia	0-29 (13.03)	0-44 (14.83)	0-75 (18.38)	0-69 (18.15)
Periductal Inflammation	0-6 (1.275)	0-7 (1.725)	0-18 (5.875)	0-21 (4.925)
Intraluminal Inflammation	0 (0)	0 (0)	0-12 (0.625)	0 (0)
Subepithelial Inflammation	0-5 (1.575)[a]	0-11 (1.400)[a]	0-20 (5.525)[b]	0 (0)[a]
PIN	0-8 (2.175)[a]	0-18 (4.475)[a]	0-84 (14.58)[b]	0-29 (7.375)[a,b]
Microinvasive carcinoma	0 (0)	0 (0)	0-1 (0.050)	0 (0)

B

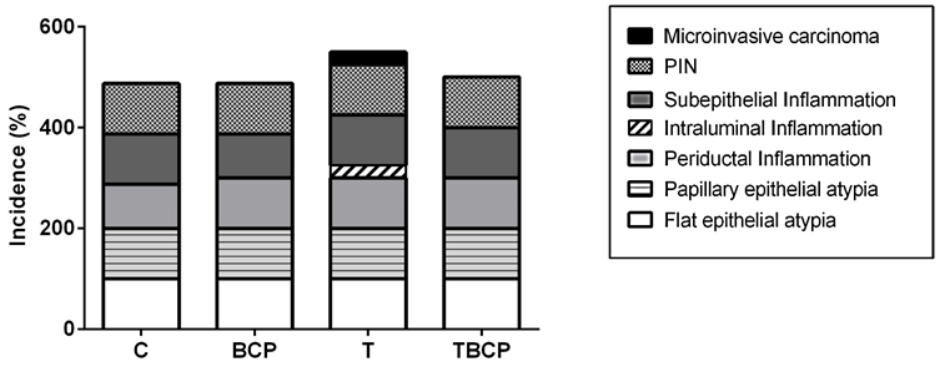

Figure 3. Analyses of multiplicity (A) and incidence (B) of prostatic lesions in ventral lobes of Mongolian gerbils. Control (C), β-Caryophyllene (BCP), testosterone (T), and Testosterone and β-Caryophyllene (TBCP) groups. Superscript letters a and b indicate significant differences between the groups. Two-way ANOVA was followed by Bonferroni post-tests (A). Incidence analysis was performed in percentage (B). Values expressed as mean ± standard deviation.

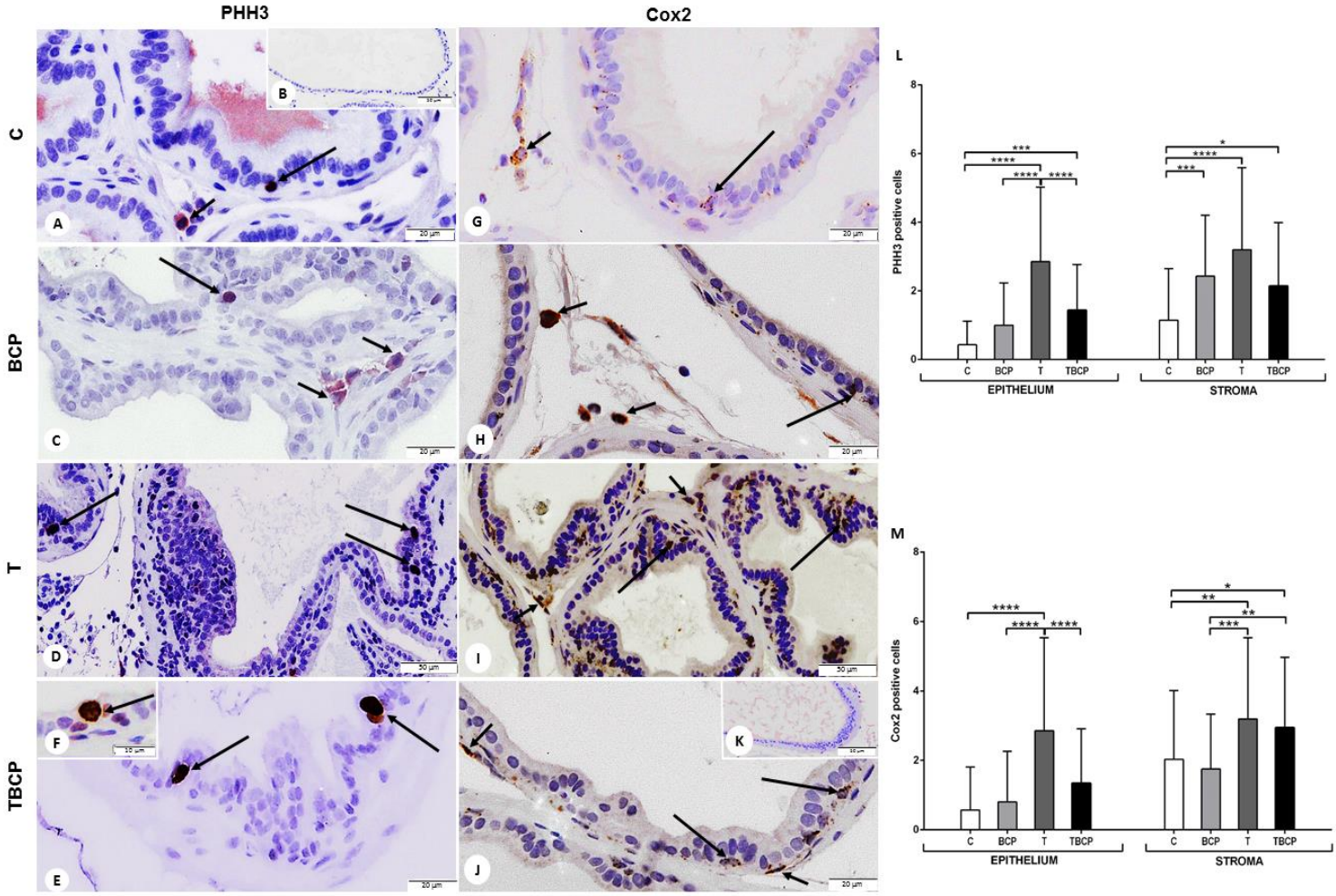

Figure 4. Histological sections of the gerbil prostate subjected to immunostaining for proliferating cells (PHH3) (A-F) and for inflammatory cells (Cox2) (G-K). Control Group (A, B, G), BCP (C, H), T (D, I) and TBCP (E, F, J, K). Negative controls (B, K). Positive staining was present in the epithelium (long arrows) and stroma (short arrows). Absolute frequency of PHH3 (L) and Cox2 (M) positive cells present in the epithelium and stroma of the ventral prostate of groups C, BCP, T, TBCP. The statistical analyses were based on the two-way ANOVA test followed by the Bonferroni test (*post hoc*) (I). * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

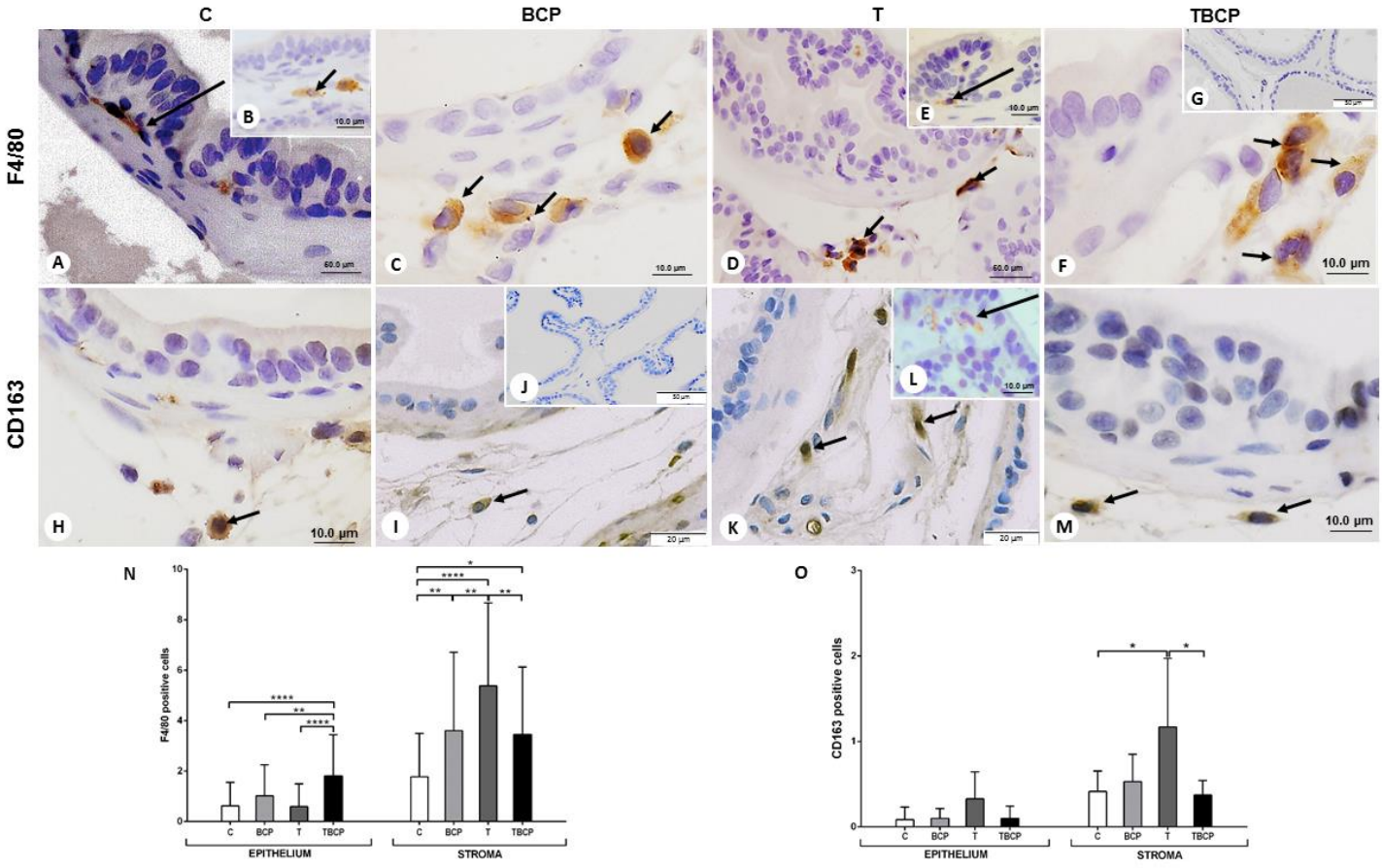

Figure 5. Immunohistochemical identification of macrophages through reaction with the anti-F4/80 (A-G) and CD163 (H-M) antibody. Control Group (A, B, H), BCP (C, I, J), T (D, E, K, L) and TBCP (F, G, M). Negative controls (G, J). Positive markings for F4/80 and CD163 in the intraepithelial region (long arrows) and stroma (short arrows). Absolute frequency of positive F4/80 (N) and CD163 (O) cells present in the intraepithelial region and stroma of the ventral prostate of groups C, BCP, T, TBCP. The statistical analyses were based on the two-way ANOVA test followed by the Bonferroni test (*post hoc*) (H). * p <0.05; ** p <0.01; **** p <0.0001.

5.2. Artigo 3.

Será submetido à revista *"Biochemical Pharmacology".*

β-caryophyllene treatment regulates the expression of hormonal receptors and apoptotic activity in testosterone-induced benign prostatic hyperplasia.

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Abstract

Benign prostatic hyperplasia (BPH) consists of an excessive growth of prostate epithelium and stroma and is associated with inflammation and an increase in proliferative cells. In recent years, studies of phytochemicals have been conducted and have shown positive results in the treatment of prostatic diseases. β-caryophyllene (BCP) is a phytocannabinoid with anti-inflammatory effects, which has been little studied in prostatic pathologies. In the present study, we evaluated the effects of BCP on both normal and BPH conditions of the Mongolian gerbil prostate, focusing on tissue morphology, the expression of hormone receptors, and apoptosis. Adult male gerbils were divided into four groups (n = 12) and exposed (T and TBCP) or not (Control and BCP) to testosterone cypionate (3 mg/kg), as a hyperplastic inductor. The TBCP and BCP groups were then treated with BCP (50 mg/kg/day). Testosterone exposure increased the prostatic volume, the expression of 5 α-reductase type 2 (5αR2), epithelial androgen receptor (AR) and estrogen receptor α (ER-α) in the T group. BCP induced a reduction in areas of the epithelium and stroma, and AR-positive cells in hyperplastic tissue of the TBCP group. Western blot only showed variations in ER- β expression, which was higher after the BCP administration in animals with BPH. Moreover, the serum levels of 17-β estradiol were hardly detected in the BCP and TBCP groups. The number of apoptotic cells increased in the BCP and TBCP groups. The administration of BCP in animals with BPH regulates the expression of hormonal receptors and induces apoptosis. These results suggest that BCP is beneficial and can reverse the effects of BPH.

Keywords: prostatic hyperplasia, cannabinoid, hormonal receptors, apoptosis.

Introduction

Benign prostatic hyperplasia (BPH) is a common prostatic disease that constitutes a serious public health concern [1]. BPH is characterized by abnormal growth of the prostate resulting from deregulation of the proliferation of epithelial and stromal cells and an increase in extracellular matrix components such as glycoproteins, proteoglycans and collagens [2,3].

Androgens and estrogens mediate the hormonal regulation of the prostatic cells [4,5]. However, the difference between the expression levels of androgen and estrogen receptors (AR and ERs) in BPH and the normal prostate has been a matter for debate, since levels may increase or decrease in BPH tissues [6–10]. In addition, the activity

of 5 α-reductase plays an important role in the pathogenesis of this disorder since inhibitors of this enzyme have been widely used in BPH treatment [11,12].

Even though many studies have been made of the molecular and endocrine processes [13–16], the etiology of BPH is still unclear [17,18]. To elucidate the pathological mechanisms and develop therapeutic strategies, the use of animals as experimental models has been extensive [17]. Thus, various animal species have been utilized, which develop BPH either spontaneously (associated with age) [19] or after induction [20–22].

The Mongolian gerbil (*Meriones unguiculatus*) is an experimental model commonly utilized in prostate gland studies [23–26]. They are easily handled in the laboratory and are thus increasingly used for biomedical research [27,28]. The prostate in these animals is similar to the human prostate both in morphological terms and, differ from other rodents, with regard to lobe compaction [29,30].

New treatment alternatives for prostate diseases, such as BPH, are being sought and tested by different researchers [17,31–33]. In this context, one promising treatment previously proposed for prostatic disorders is the use of cannabinoid receptor (CB) agonists [34,35]. These receptors are divided into cannabinoid receptors type 1 (CB1) and type 2 (CB2) and they present different distributions in the organism [34,36,37].

CB1 is mostly localized in the central nervous system and CB2 is frequently found in peripheral and reproductive tissues and immune cells [34,36,38,39]. CBs modulate several signaling pathways involved in the modulation of proliferation and cell death [35,40]. Recent studies in colon, breast, and prostate cancerous cell lines showed overexpression of CB2, suggesting that this receptor is involved in the regulation of tumor cell metastasis [35].

β-caryophyllene (BCP) is a sesquiterpene classified as a phytocannabinoid ligand of CB2 [39,41,42]. It is found in *Cannabis sativa* and several other plant species such as black pepper, clove, hops, copaiba, and oregano [36,37,39,43,44]. The pharmacological proprieties of BCP include antioxidant, anti-inflammatory, antimicrobial, cardioprotective, neuroprotective, and apoptotic effects [39,43,45–48].

Although BCP has demonstrated beneficial effects in many disorders [44,48], studies in the prostate are still restricted to β-caryophyllene oxide (BCPO), a derivate of BCP [36,49,50] and little is known about BCP action in prostatic disorders, such as BPH. Recently, we investigated the effects of BCP in the ventral prostate under normal

and BPH conditions. Proliferation and inflammation markers were reduced in the BCP treated groups, suggesting a beneficial effect of this phytocannabinoid on BPH [51]. Thus, to complement the analysis of the effects of BCP in the prostate, our proposal in the present study has been to evaluate the morphology, the expression of sex hormonal receptors, and the apoptosis rates in the BPH and normal prostate tissue compartments, since these parameters have not been analyzed in the prostate after the administration of BCP.

Material and Methods

Experimental design

Forty-eight male gerbils (90 days old) were utilized in this experiment. The animals were provided by the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP). They were maintained in ventilated polyethylene cages under controlled conditions of light (12 h dark/12 h light) and temperature (24 °C) and received a rodent feed and filtrated water *ad libitum*.

The animals were randomly divided into four groups (n=12 per group). The intact control (C) did not receive any treatment. The β-Caryophyllene (BCP) group received 0.1 ml/day of β-Caryophyllene (W225207; Sigma-Aldrich, 50 mg/kg/day) [52] diluted in corn oil (Cargil, Brazil) for 30 days (during the fourth month old), via gavage. The BPH induced groups: testosterone (T) - animals received 0.1 ml of testosterone cypionate (Deposteron, EMS) [53,54] diluted in corn oil (3 mg/Kg), on alternate days, during 30 days via subcutaneous injections and they were euthanized one month after the end of the hormonal application; and testosterone + β-Caryophyllene (TBCP) - animals received subcutaneous injections of 0.1 ml of testosterone cypionate diluted in corn oil (3 mg/Kg), on alternate days, during 30 days and then, the BCP treatment (50 mg/kg/day) diluted in corn oil in 0.1 ml/day for 4 weeks, via gavage. At the end of the experiment, all animals, with 150 days old, were anesthetized with xylazine (3 mg/kg) and ketamine (10 mg/kg) and euthanized by decapitation. Immediately after decapitation, the blood was collected, centrifuged at 3000 rpm for 20 minutes and serum was frozen at -80°C. The dissection was performed, the prostatic complex was collected, and the ventral prostate was separated. For the western blots analysis, the ventral prostates were storage at - 80°C. For morphological and immunohistochemistry analysis, these prostatic tissue were fixed in 4% paraformaldehyde for 24 hours. Next,

samples were processed for inclusion in paraffin (Histosec, Merck, Darmstadt, Germany).

The experiments were conducted according to the ethical principles recommended by the National Council for Animal Experimentation Control and the procedures involved were approved by the Ethics Committee on the Use of Animals at IBILCE/UNESP (protocol number 73/2017).

Morphological analyses

Histological sections were stained with Picrosirius red for the general morphological evaluation and stereological and morphometric analyses. The stereological analyses were performed to obtain the relative frequency (%) of tissue compartments (epithelium, muscular stroma, non-muscular stroma, lumen, collagen fibers, and blood vessels). For that, 48 ventral prostate fields/group (6 fields/animal, n=8/group) were randomly digitized at 200x magnification in a slide scanner system (Olympus VS120-S5), and analysis were made using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA). The relative frequency was estimated using the M130 multipoint test system [55] applied to the prostate according to the procedure described in Huttunen et al. 1981.

Morphometry was also performed to determine the mean height (μm) of epithelial cells, and thickness (μm) of smooth muscle cells (SMC) layer. For each analysis, 240 measurements/experimental group were obtained (30 measurements/animal, n=8/group). Histological sections were randomly digitized at 1000x magnification in the slide scanner system (Olympus VS120-S5), and analysis were made using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA).

Immunohistochemistry analyses

The primary antibodies used for immunohistochemistry (IHC) were: enzyme 5 α-reductase 2 (5αR2) (rabbit polyclonal, sc-20659, Santa Cruz Biotechnology CA, EUA, dilution 1:75), androgen receptor (AR) (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology CA, EUA, dilution 1:75), estrogen receptor alpha (ER-α) (rabbit polyclonal, IgG, MC-20, sc-542, Santa Cruz Biotechnology, CA, USA, dilution 1:50) and estrogen receptor beta (ER-β) (mouse polyclonal, IgG2a, B-1, sc-390243, Santa Cruz Biotechnology, CA, USA, dilution 1:50). For immunoreactions, sections were deparaffinized, rehydrated and antigen retrieval was performed in 10 mM citrate

buffer pH 6.0, at 92°C for 45 minutes (AR), at 100°C for 15 minutes (ER-β) and a microwave sequence (2 x 7 min at low power and 1 x 7 min at medium low power) (ER-α). The blockage of endogenous peroxidases was performed in 3% hydrogen peroxide for 30 minutes, and followed by nonspecific proteins blockage with 5% skimmed milk/tris buffered saline + tween 20 (TBSt). The sections were incubated overnight at 4°C with primary antibodies diluted in bovine serum albumin (BSA) diluted to 1% in TBSt (BSA 1%). Some sections did not incubate with primary antibodies, only with BSA 1%, to provide the negative control of reaction. After washing in TBSt, the sections were incubated with a Polymer kit (Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) for 40 minutes at room temperature. Positive staining was detected using 3-30′-diaminobenzidine tetrahydrochloride (DAB, Sigma) solution and sections were counterstained with Mayer hematoxylin.

Histological sections were digitalized on slide scanner system (Olympus VS120-S5) in 400x magnification. Eighty-four microscopic fields per group (n=7/12 measurements/animal) were analyzed to determine the relative frequency of AR, ER-α, and ER-β positive cells in the ventral prostate and in the epithelial and stromal compartments separately. These frequencies were calculated dividing the number of positive nuclei by the total number of nuclei counted and expressing the result as a percentage. The relative frequency of 5αR2 were evaluated by a multipoint system with 160 intersections (modified from Weibel, 1963) [55,57], in seventy microscopic fields per group (n=7/10 measurements/animal).

Western blot

For protein extraction, after storage at -80°C, the ventral prostates (n=5/group), were homogenized in a lysis buffer according to the protocol described by Li et al., 2009 [58]. The homogenate was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant, stored at -80°C. The protein quantification of the extracts was performed with the Pierce BCA Protein Assay Kit (23227-Thermo Scientific, Rockford, IL, USA) and for read the absorbance was used a microplate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany). After, the extracts were stored at −80 °C until analysis.

To perform Western blots, 15 µg of total proteins were pipetted into each well of the 12% polyacrylamide incorporated in detergent sodium dodecyl sulfate (SDS – page) gel and subjected to electrophoresis (105V for approximately 2 hours). The

bands were transferred (360mA during 60 minutes) to nitrocellulose membrane (Amersham Protram, 10,600.003, GE Healthcare, Darmstadt, Germany). Subsequently, the membranes were washed in TBSt and nonspecific binding was blocked in 5% milk for 60 minutes. Then, they were incubated overnight at 4°C under agitation with the following primary antibodies, diluted in 1% milk: AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology CA, USA, 1:300), ER-α (polyclonal IgG rabbit, PA5-34577, Invitrogen, Thermo Fisher Scientific, USA, 1:1000), ER-β (polyclonal IgG rabbit, PA1-310B, Invitrogen, Thermo Fisher Scientific, USA, 1: 1: 1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (monoclonal rabbit, IgG, #2118, Cell Signalling, Danvers, MA, USA, 1:1000). Then, the membranes were washed in TBSt and incubated, for 60 minutes at room temperature, under agitation with the secondary peroxidase conjugated anti-rabbit antibody (goat, IgG, #7074, Cell Signalling, Danvers, MA, USA) (1:2000). After this period, the membranes were washed in buffer and revealed with enhanced chemiluminescent (ECL) substrate to visualize the bands in the imaging system (ChemiDoc MP, BioRad, Hercules, CA, USA). Then, the band densities were analyzed in densitometry software (Image J, version 1.52a, Wayne Rasband, NIH, USA), and normalized by positive control GAPDH.

Detection of DNA Fragmentation in Situ (TUNEL)

Apoptotic cells in the epithelium and stroma were detected using the DNA fragmentation assay, based on the TUNEL reaction, (ApopTag Plus in situ, Apoptosis Detection Kit, Millipore, S7101, CA, USA). Histological sections were deparaffinized, washed in phosphate buffered saline (PBS) for 5 minutes, and incubated with proteinase K for 7.5 minutes. After, they were washed 2 X 2 minutes in distilled water and subjected to inactivation of endogenous peroxidase (3% H202 in PBS, for 10 minutes). Subsequently, they were washed 2 X 5 minutes in PBS, kept in the Equilibration buffer for 20 seconds, and sequentially incubated with the enzyme terminal deoxynucleotidyl transferase (TdT) for 60 minutes at 37°C. Finally, the sections were washed in a stop/wash buffer for 10 minutes, incubated with anti-digoxigenin peroxidase for 30 minutes, revealed by diaminobenzidine (DAB), and counterstained with methyl green.

Seventy microscopic fields per group (n=7/10 measurements/animal) were analyzed to determine the relative frequency of apoptotic cells in the epithelium and

stroma of the ventral prostate. Histological sections were digitalized on slide scanner system (Olympus VS120-S5) in 400x magnification. The frequencies were calculated dividing the number of positive nuclei by the total number of nuclei counted and expressing the results as percentages.

Analysis of serum hormone levels

Serum level of 17-β estradiol were quantified by capture/sandwich ELISA using commercial kit, according to the manufacturer's instructions (IBL International, Hamburg, Germany - numbers 52041, sensitivity of 10.60 pg/ml). Readings were performed using a microplate reader (TECAN- Infinite F50).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.00 software spreadsheets and graphs. To prove the significance of the results between groups C, BCP, T, and TBCP, two-way ANOVA tests were performed, followed by Tukey for multiple proofs. The results were considered statistically significant at $p \leq 0.05$ and are presented as mean ± standard deviation.

Results

Stereological and morphometric analyses showed a decrease in the frequency of tissue compartments in TBCP animals and in cell height/thickness.

We analyzed the morphology of the prostate in all experimental groups and detected differences in the distribution of the epithelial and muscular stroma compartments, especially in the T group (Figure 1E, F). The evaluation of stereological parameters showed an increase in the relative frequency of the epithelium and muscular stroma in the T group. The TBCP group showed a reduction in the relative frequency of the epithelium, muscular and non-muscular stroma when compared to the T group. Regarding the lumen, we verified a decrease in the frequency of this compartment in the T group. Furthermore, there were no significant differences in the frequency of collagen and blood vessels (Table 1). Morphometric analysis showed that T treatment caused an increase in epithelial height and thickness and BCP administration was able to ameliorate this effect of T on ventral prostate (Table 1).

BCP and T administration modify the immunohistochemistry parameters of 5αR2, AR, ER-α, and ER-β

We utilized immunohistochemistry to detect the expression of the enzyme 5αR2 in the ventral prostate of all groups (Figure 2 A-E). This enzyme increased in all groups in comparison to C, especially in T group. The TBCP group showed lower values than the T group, although it was not significant (Figure 2 L).

The immunoreactivity of AR in the nucleus exhibited positive epithelial and stromal cells in all experimental conditions (Figure 2 F-K). The AR-positive cells increased in the T group in comparison to all other groups, and there was a decrease in BCP in comparison to the C group (Figure 2 M). We evaluated the prostate compartments separately and observed the same result in the epithelium. There were no differences between the groups regarding stromal expression of AR (Figure 2 N).

Subsequently, the analysis of ER-α showed positive cells in the epithelial and stromal compartments in all experimental groups (Figure 3 A-E). The relative frequency of these positive cells in the ventral prostate increased in all groups in comparison to control; nonetheless, the percentage of positive cells was higher in the TBCP group when compared to the BCP group (Figure 3 K). We also verified the frequency of ER-α-positive cells in the epithelium and stroma. These cells increased in all groups when compared to C in both prostatic compartments (Figure 3 L), with the exception, of the stroma, of the T group when compared to the BCP and TBCP groups (Figure 3 L).

We also investigated the distribution of ER-β-positive cells in the tissue compartments of the ventral prostate (Figure 3 F-J). ER-β-positive cells decreased in the T group in comparison to all other groups, and increased after the BCP administration in hyperplasic prostate when compared to T group (Figure 3 M). In the epithelium, we noted the same result (Figure 3 N); however, in the stroma, only T animals presented an increase of positive cells when compared to those in the BCP group (Figure 3 N).

The whole tissue expression of the ER-β receptor increased in the TBCP group

We analyzed the expression of androgen and estrogen receptors by western blot in all experimental groups (Figure 4). For AR, although there were no statistical differences, there was a tendency towards a similar pattern to that observed in the immunohistochemistry quantification of the prostate and in the epithelial compartment

(Figure 4 A). We observed no significant differences in the expression of ER-α among the groups (Figure 4 B). The BCP administration in animals with BPH increase the ER-β expression when compared to the T group (Figure 4 C).

BCP increased apoptosis in the epithelial and stromal compartments of the ventral prostate.

We also evaluated the frequency of apoptotic cells in the tissue compartments of the ventral prostate (Figure 5). There was an increase in the BCP and TBCP groups in comparison to the C group; the frequency in the TBCP group was higher than that in the T group as well. In the stroma, the T group apoptotic rate was lower than that in the C and BCP groups (Figure 5 F).

The serum levels of 17 β-estradiol decreased in the BCP and TBCP groups.

The quantification of serum 17 β-estradiol in the gerbils from the different experimental groups is shown in Figure 6. This analysis showed a drastic reduction in the groups that received the phytocannabinoid, which were hardly detected by the sensitivity of the kit (10.60 pg/ml). Moreover, only one animal of the TBCP group presented sensitivity for the quantification, which justifies the absence of the quantification in this analysis.

Discussion

Due to the lack of BCP studies in prostatic research, and based on previous findings describing this compound's therapeutic effects on proliferation and inflammation [51], we proposed an analysis of the impact of BCP on the morphology, apoptosis, and expression of sexual hormone receptors in the normal and hyperplasic prostate. The present study demonstrated, for the first time, that this cannabinoid influences the functioning of the prostate and alters some parameters analyzed.

The analysis of morphometric and stereological parameters allowed us to verify an increase in the frequency and height/thickness of the epithelium and stroma in the T group, with a consequent reduction of the lumen. These findings may have been caused by the administration of testosterone, complementing the observations previously described [51], confirming that the supplementation of this hormone was sufficient to cause BPH. This hyperplasic condition causes an overgrowth of epithelial and stromal tissues [33,58,59].

In contrast, in the TBCP group, the morphometric and stereological analysis showed a reduction in the epithelium and stroma and an increase of lumen in comparison to the T group. These data indicate that BCP has beneficial effects on the balance of tissue compartments in BPH conditions. In addition, we are unaware of the existence of any studies of BCP in relation to these morphological parameters in the hyperplasic prostate. Due to the positive effects of the cannabinoid [36,37,44], we therefore suggest that it has the potential to be used in the treatment of BPH.

BPH results in an abnormal prostate growth [60] that can be related to changes in the balance of hormonal receptor expression (AR and ER) [1,7,61]. AR activation triggers proliferation [6,7,64] and here, the BCP administration altered the number of AR-positive cells, decreasing the expression of this androgenic receptor. Our results suggest that BCP can be beneficial for the gland, ameliorating the effects of BPH. Cannabinoids, such as BCP, have already had their anti-proliferative effects demonstrated [44,47,50]. Indeed, our previous study showed that BCP reduces the number of proliferative cells in BPH [51]. This anti-proliferative action explains the protective functions of some cannabinoids in androgen-dependent prostate cancer cells [65–67].

The prostate is a highly androgen-dependent organ [62]. Testosterone is necessary for normal development and is an essential component of the pathophysiology of BPH [6]. Although some studies have found similar levels of AR in the normal and BPH prostate [10], many studies have shown an increase of this receptor in hyperplastic tissue [60,68–70]. Our findings corroborate these studies: AR percentage increased in the ventral prostate and in the epithelium compartment of the T group. Although not to any significant extent, we found a similar pattern of AR expression in immunohistochemistry and Western blot analyses.

5αR2 is mandatory in the conversion of androgenic hormones into dihydrotestosterone (DHT) and, consequently, in binding to its respective receptor (AR). It is thus the most common type of enzyme found in the prostate [6,62,63], since DHT is directly related to prostatic functionality. Thus, the 5αR2 increase observed, especially in the T group, was probably caused by the T supplementation, assuming that, in these animals, more testosterone had been converted into DHT.

Moreover, estrogens are also important in the triggering and establishment of prostate disorders such as BPH [71]. We therefore investigated the expression and distribution of ERs α and β in the ventral prostate. The percentage of ER-α increased

in all treated groups, being similar to the C group only in the stroma of the T group. ER-α is involved in cell proliferation and BPH establishment [72–74], which explains the results obtained by IHC. This receptor is predominantly detected in the stroma [71], and studies have described a reduction in ER-α in the stromal compartment in the BPH condition [9,60]. Although immunohistochemical analysis of ER-α expression showed higher values in the BCP groups, according to the findings of the other parameters evaluated here, this compound presents positive effects in both normal and hyperplasic conditions.

ER-β has demonstrated anti-proliferative functions [71,75], which may be related to the development of BPH, decreasing the expression in the T group. We found an increase of ER- β by IHC and western blot in the TBCP group showing a possible activity of the phytocannabinoid against the proliferative effects of BPH. This indicates that treatment with BCP had a protective effect and can mitigate the effects of the T supplementation in the prostate.

Estrogens may have a direct influence on cannabinoid receptors like CB1 and CB2 [65]. In ovariectomized female and intact male rats, the administration of a non-selective CB agonist decreases serum levels of luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH), resulting in a decrease of circulating estrogen levels [65,76]. BCP is a cannabinoid receptor agonist [36–38,77]; however there are no studies of its effect on the levels of sex hormones and on the prostate. Considering this information, we believe that the drastic reduction of 17 β-estradiol levels in Mongolian gerbils of BCP groups is related to the administration of BCP.

Furthermore, although BCP performed an anti-apoptotic function in some previously described situations [37,38,83], this cannabinoid is highly associated with the induction of apoptosis [43–45,77,84] and suppression of proliferation, as an anti-cancer and anti-inflammatory property [36]. Our findings demonstrated there was an increase in cells in apoptosis after the administration of BCP under normal and BPH conditions. We suggest that BCP may be associated with these results since, in the prostate cancer cell, β-caryophyllene oxide increased apoptotic rates [36,50,85].

In conclusion, the parameters verified here complement our understanding of the beneficial effects of BCP in the normal and BPH prostate. This gland expresses components of the endocannabinoid pathway, and synthetic cannabinoids and phytocannabinoids described in the literature may be applied to achieve a protective effect against proliferative disorders. Thus, the results presented here contribute to an

understanding of the action of BCP in the prostate, which contributes to mitigating the proliferative disorders in the prostate. Further studies are needed to test and clarify how the administration of BCP may be beneficial in BPH disorders.

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Conflict of interest

The authors declare there are no conflicts of interest.

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Table:

Table 1. Stereological and Morphometric parameters of Mongolian gerbils in the control (C), β-Caryophyllene (BCP), Testosterone (T), and Testosterone and β-Caryophyllene (TBCP) groups.

	Experimental groups			
Parameters	**C**	**BCP**	**T**	**TBCP**
Stereological data (%) n=8				
Epithelium	16.22 ± 5.005[a]	15.02 ± 3.744[a]	22.24 ± 9.589[b]	13.88 ± 5.016[a]
Muscular stroma	10.34 ± 3.358[a]	11.49 ± 4.034[a]	15.21 ± 4.716[b]	10.69 ± 3.139[a]
Non muscle stroma	14.98 ± 9.166[a,b]	15.67 ± 9.845[a,b]	19.58 ± 9.010[b]	13.43 ± 10.17[a]
Lumen	57.16 ± 14.03[a]	56.36 ± 13.00[a]	41.31 ± 12.91[b]	60.61 ± 11.82[a]
Blood vessels	0.886 ± 1.087	0.906 ± 1.041	0.837 ± 1.134	0.423 ± 0.576
Collagen	0.461 ± 0.290	0.534 ± 0.292	0.432 ± 0.180	0.495 ± 0.252
Morphometric data (μm) n=8				
Epithelium height	13.11 ± 4.426[a]	13.38 ± 4.322[a]	22.72 ± 8.039[b]	15.60 ± 4.241[c]
SMC thickness	7.105 ± 2.547[a]	8.410 ± 2.544[b]	12.46 ± 4.779[c]	9.347 ± 3.207[d]

Values expressed as mean ± standard deviation.

[a,b,c,d] represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

Two-way ANOVA was followed by Tukey post-tests.

Figures:

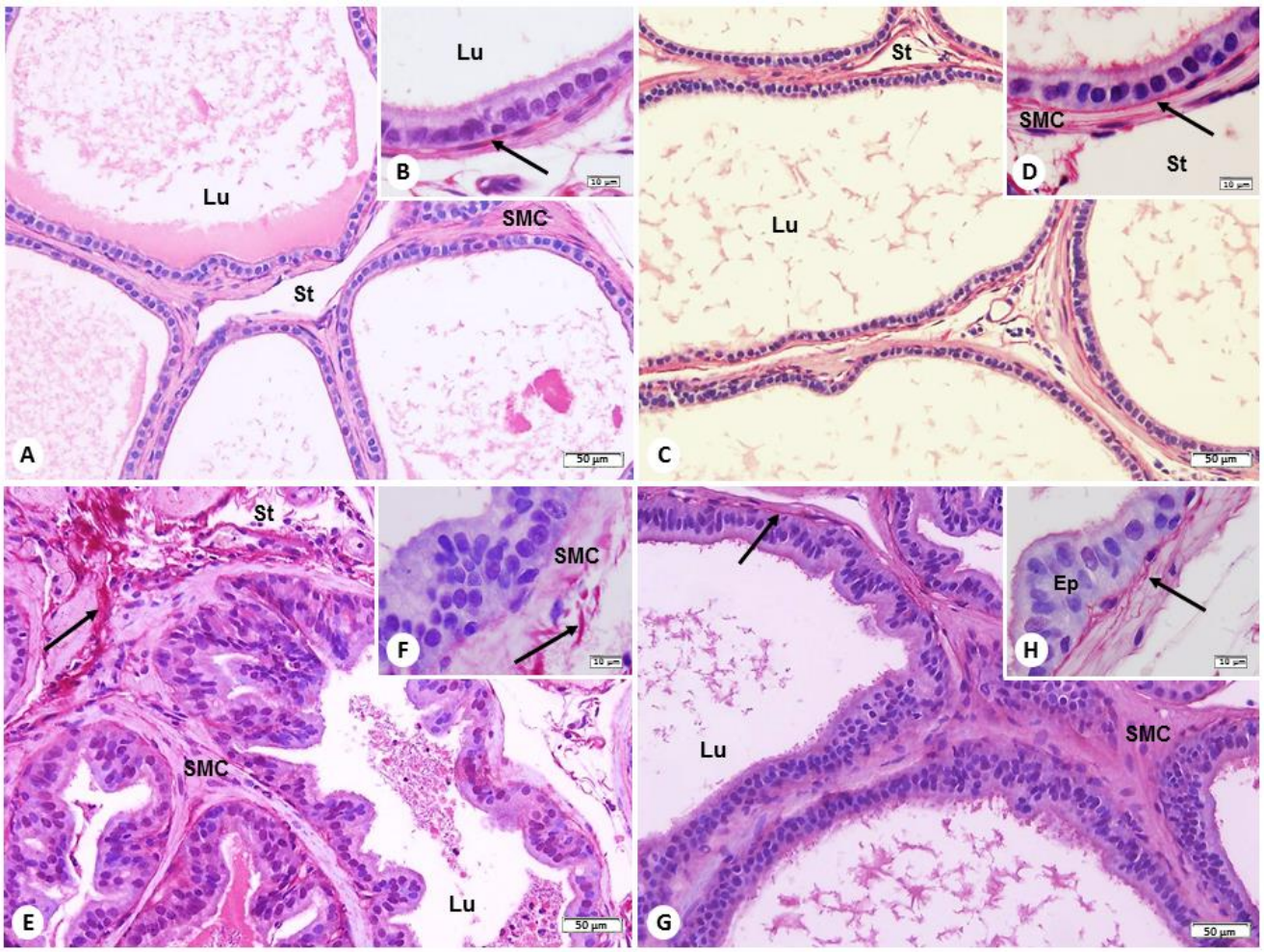

Figure 1. Morphologic aspects of the gerbil ventral prostate stained by picrosirius red. Groups: C (A, B); BCP (C, D); T (E, F) and TBCP (G, H). The prostate from C and BCP groups show a normal morphologic pattern (A-D). Testosterone administration promotes alterations in the distribution of the prostatic compartments (E, F). TBCP group also exhibited histological alterations but are less intense after the BCP treatment (G, H). Lu (lumen), St (stroma), SMC (smooth muscle cells), Ep (epithelium), and arrows (collagen).

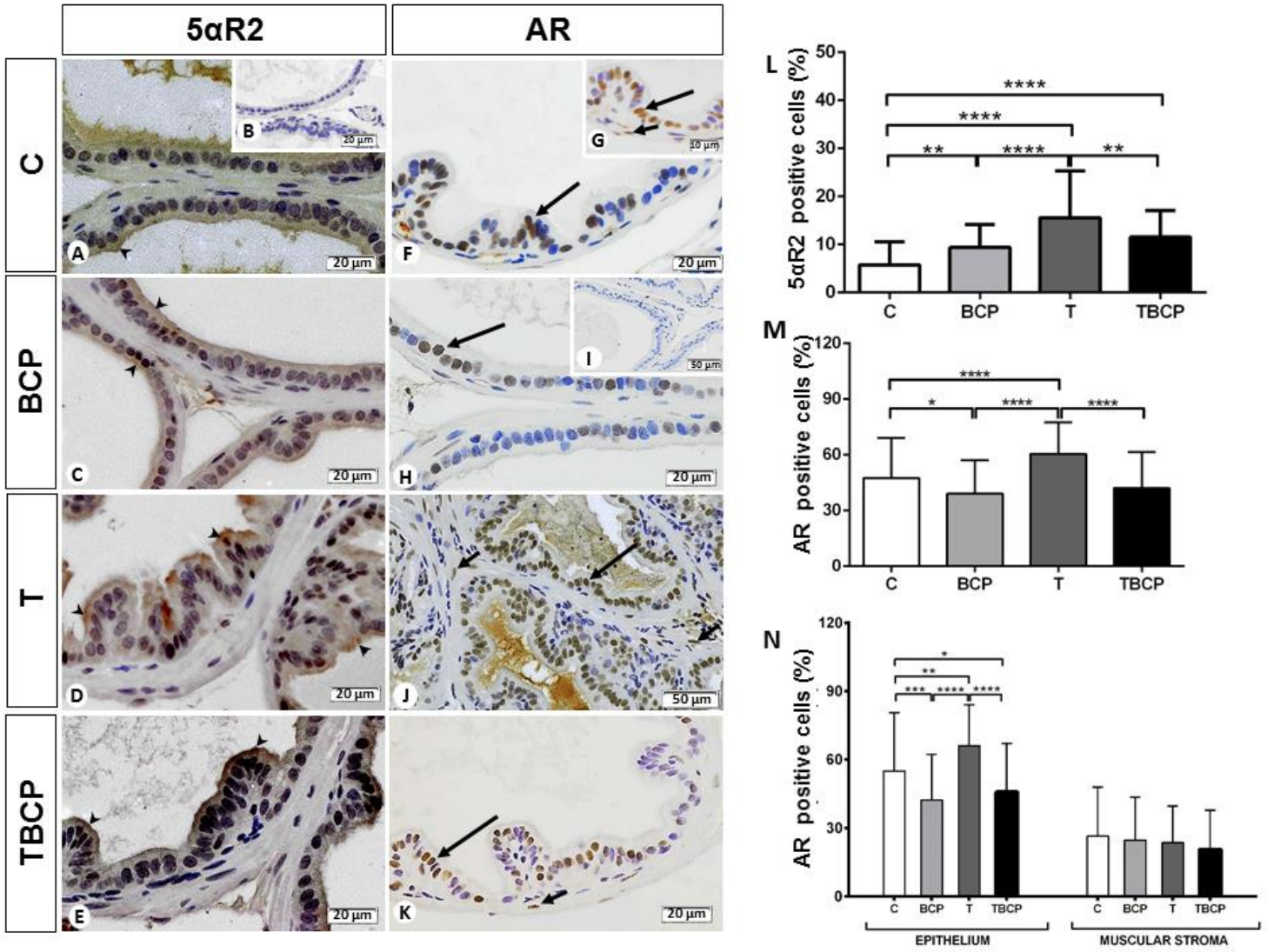

Figure 2. 5αR2 and androgen receptor (AR) immunostainings in the different experimental groups. C (A, B, F, G), BCP (C, H, I), T (D, J), and TBCP (E, K). The negative controls were indicated in B and I. 5αR2 immunostainings are indicated in the nuclei and cytoplasm by arrowheads and AR-positive cells are appointed in the nuclei of epithelium (long arrows) and stroma (short arrows). Data represent averages of the relative frequency (%) of 5αR2 (L) and AR (M, N) positive cells in the prostate and the bars indicate the standard deviation. *, **, ***, and **** indicate statistically significant differences ($p<0.05$; $p<0.01$; $p<0.001$ and $p<0.0001$, respectively), according to the two-way ANOVA test followed by the Tukey test.

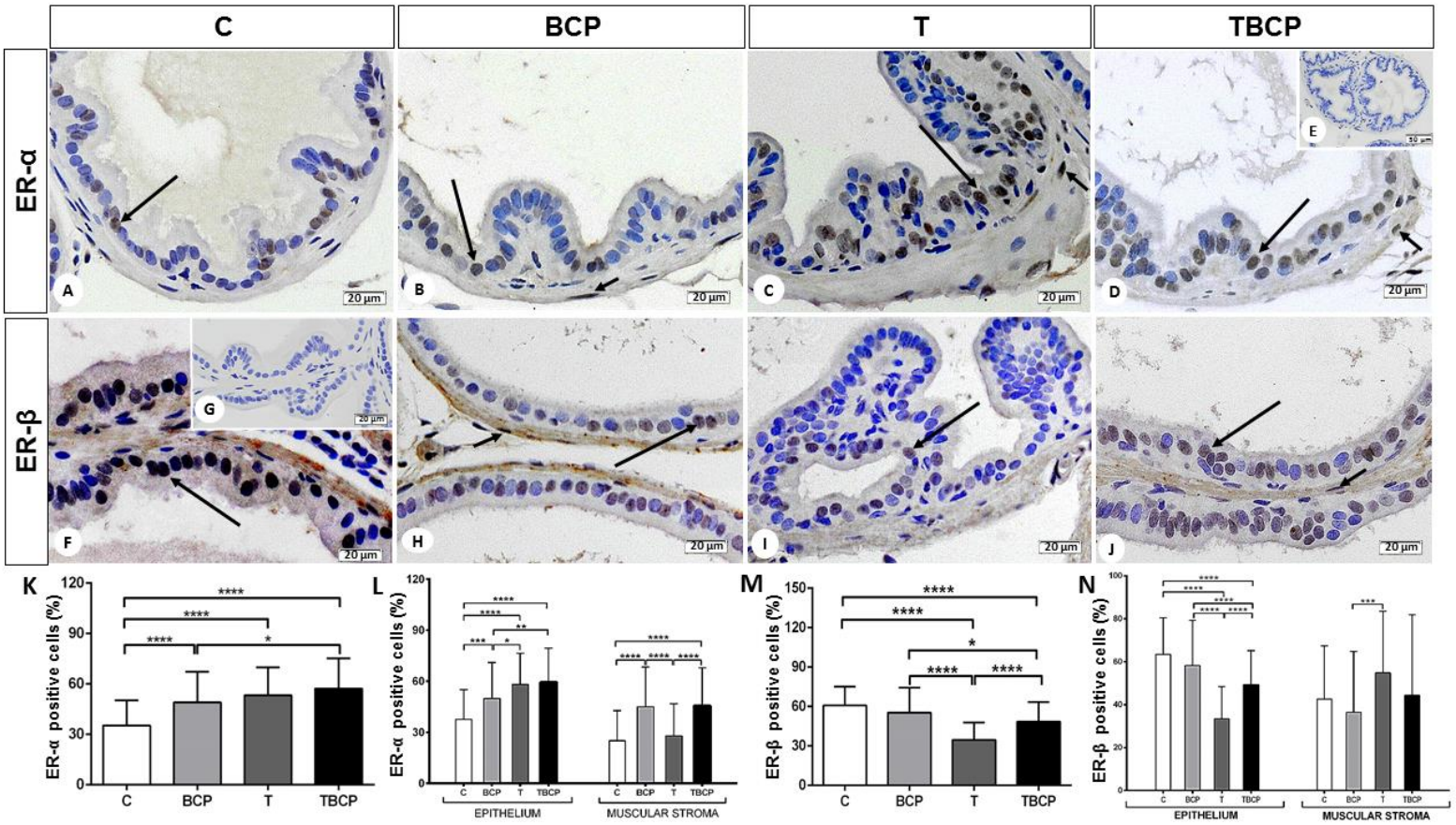

Figure 3. ER-α and ER-β positive cells in the ventral prostate of Mongolian gerbils. The negative controls of immunoreactions are indicated in E and G. Long arrows (epithelium) and short arrows (stroma) indicate immunostainings in the nuclei of positive cells. Data represent averages of the relative frequency (%) of ER-α (K, L) and ER-β (M, N) positive cells and the bars indicate the standard deviation. *, **, ***, and **** indicate statistically significant differences ($p<0.05$; $p<0.01$; $p<0.001$ and $p<0.0001$, respectively), according to the two-way ANOVA test followed by the Tukey test.

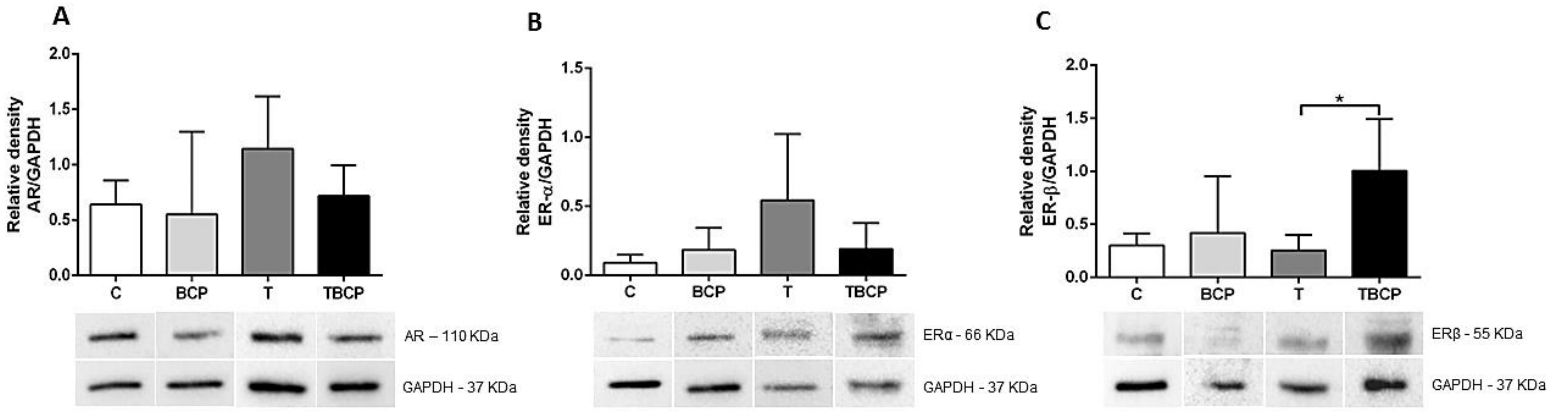

Figure 4. Relative density of AR, ER-α, and ER-β normalized by GAPDH, applied as positive control. Data represent averages of the relative density of AR (A), ER-α (B), and ER-β (C) expression and the bars indicate the standard deviation. * indicate statistically significant difference ($p<0.05$), according to the two-way ANOVA test followed by the Tukey test.

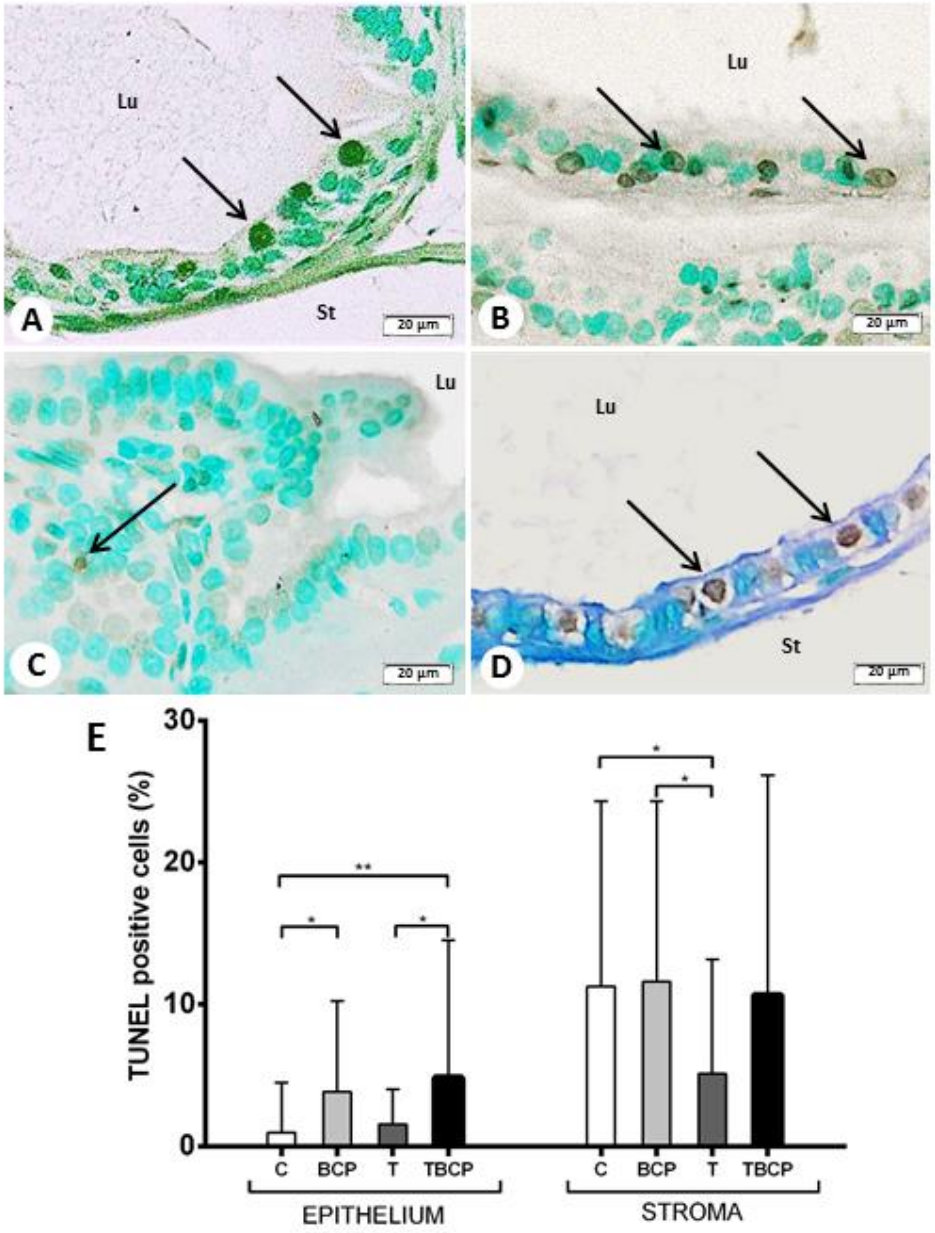

Figure 5. Apoptotic cells in the histological sections of ventral prostate subjected to TUNEL reaction. C (A), BCP (B), T (C) and TBCP (D). Data represents averages of the relative frequency of cells in apoptosis (E) and the bars indicate the standard deviation. * and ** indicate statistically significant differences ($p<0.05$ and $p<0.01$respectively), according to the two-way ANOVA test followed by the Tukey test. Lumen (lu), stroma (St), apoptotic cells (arrows).

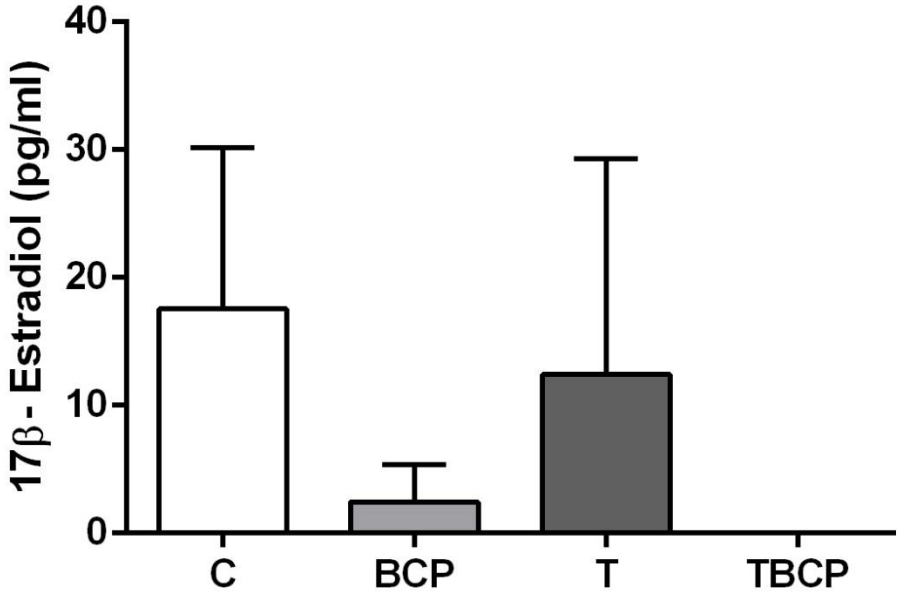

Figure 6. Serum hormonal profile: levels of 17 β-estradiol in Mongolian gerbils from the experimental groups. Data represent averages of the hormonal serum levels and the bars indicate the standard deviation, according to the two-way ANOVA test followed by the Tukey test.

6. CONSIDERAÇÕES FINAIS

- A suplementação pela testosterona foi suficiente para estimular o aparecimento de desordens proliferativas e propiciar o microambiente característico da HBP;

- A comparação entre os grupos com HPB mostrou que essa condição hiperplásica foi mantida mesmo após 30 dias do término das aplicações hormonais, período em que muitos compostos com potencial para o tratamento dessa patologia são testados;

- O BCP apresentou resultados positivos na proliferação e na inflamação prostática dos gerbilos com a HPB. A administração desse fitocanabinoide aumentou a taxa de células em apoptose e alterou a frequência dos receptores hormonais na próstata ventral nas condições normal e hiperplásica. Essas informações sugerem que o BCP apresentou efeitos protetivos e pode ser promissor no tratamento da HPB.

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ANEXO A. Certificado de Experimentação Animal

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que a proposta intitulada "Efeitos da exposição ao β-Cariofileno na próstata ventral de gerbilos adultos após a suplementação pela Testosterona", registrada com o nº. 173/2017 - CEUA, sob a responsabilidade da Professora Doutora Patrícia Simone Leite Vilamaior, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em reunião de 12 de setembro de 2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	01/10/2017 a 31/03/2021
Espécie/linhagem/Raça	Gerbil
Nº de animais	100 (cem)
Peso/Idade	70g
Sexo	Machos
Origem	Biotério de criação e manutenção de roedores do Laboratório de Microscopia e microanálises do Instituto de Biociências, Letras e Ciências Exatas de São José do Rio Preto.

São José do Rio Preto, 12 de setembro de 2017.

Prof. Dr. Luiz Henrique Florindo
Presidente da CEUA

Instituto de Biociências, Letras e Ciências Exatas – Comissão de Ética no Uso de Animais
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UNIVERSIDADE ESTADUAL PAULISTA
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Campus de São José do Rio Preto

TERMO DE REPRODUÇÃO XEROGRÁFICA

Autorizo a reprodução xerográfica do presente Trabalho de Conclusão, na íntegra ou em partes, para fins de pesquisa.

São José do Rio Preto, 30 / 09 / 2021

Mayara F. C. Casho

Assinatura do autor