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A influência do consumo de óleo de coco na próstata de Gerbilos de Mongólia durante o envelhecimento e seu impacto na hiperplasia prostática benigna

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conquistar novos mares, minha mãe Sueli.

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(BREWSTER, 1855, p 405)

RESUMO

A próstata é uma glândula altamente responsiva a alterações hormonais e consequentemente ao envelhecimento, uma vez que indivíduos idosos apresentam níveis diminuídos de andrógenos. Uma das respostas da próstata frente ao processo de envelhecimento é o desenvolvimento de hiperplasia prostática benigna (HPB). Essa patologia é altamente relacionada com aumento de inflamação e de estresse oxidativo e esses, por sua vez, aumentam durante o envelhecimento. O uso de terapias alternativas como tratamento para a HPB vem crescendo e tem demonstrado efeitos benéficos à próstata, sem os danosos efeitos colaterais das drogas sintéticas comumente utilizadas. Algumas propriedades do óleo de coco, como antioxidante e anti-inflamatória fazem dele um candidato para o tratamento alternativo de doenças prostáticas, e ainda esse óleo se mostrou capaz de interferir na HPB induzida por testosterona. Entretanto, até o momento, não havia nenhum estudo detalhado sobre o efeito do consumo do óleo de coco durante o envelhecimento e das respostas morfofisiológicas na próstata. Sendo assim, o presente trabalho avaliou os efeitos do consumo de óleo de coco durante o envelhecimento na morfologia prostática, bem como seus efeitos na HPB espontânea e sua relação com fatores intimamente associados a essa doença como o processo inflamatório e o estresse oxidativo. Para tal foram utilizados gerbilos machos adultos submetidos a três diferentes condições experimentais: animais que não receberam nenhum tratamento durante um ano (grupo IC), animais que receberam água (0,1ml), em dias alternados, durante um ano (grupo GC) e animais que receberam óleo de coco 0,1ml), em dias alternados, durante um ano (grupo CO) e as administrações foram via gavagem. Foram realizadas análises morfológicas, morfométricas, estereológicas, imuno-histoquímicas, sorológicas, da expressão de proteínas, citocinas e avaliação do estresse oxidativo na próstata ventral. Os resultados mostraram que a manipulação dos animais no procedimento de gavagem durante o envelhecimento agravaram as lesões prostáticas, aumentaram a expressão de receptores hormonais e aumentam a espessura e a proliferação de células do estroma muscular. Esse procedimento também interferiu na população de macrófagos e na expressão de citocinas pró-inflamatórias, metaloproteinases, além de aumentar o estresse oxidativo. Já o consumo de óleo de coco atenuou ou inibiu essas alterações relacionadas ao envelhecimento e intensificadas pelo procedimento de gavagem. Assim, o consumo de óleo de coco durante o envelhecimento resultou em efeitos favoráveis ao uso desse óleo como agente fitoterápico no tratamento de HPB.

Palavras-chave: Próstata. Envelhecimento. Hiperplasia. Óleo de coco. Gerbilos.

ABSTRACT

The prostate gland is highly responsive to hormonal changes and thus to aging since elderly individuals present lower androgen concentrations. One of the prostate responses to the aging process is the development of benign prostatic hyperplasia (BPH). This pathology is closely linked to increases in inflammation and oxidative stress, and these both increase during aging. The employment of alternative therapies as BPH treatment has been growing and has shown positive effects on the prostate, without the harmful secondary effects caused by the commonly available synthetic drugs. Some coconut oil properties, such as antioxidant and anti-inflammatory properties make it a candidate for alternative therapy to treat prostate diseases, and this oil has been shown to interfere with testosterone-induced BPH. However, until now, there was no detailed study about the effect of coconut oil consumption over aging and the morpho-physiological responses on the prostate. Thus, the present study assessed the effects of coconut oil consumption throughout aging on the prostatic morphology, as well as its effects on naturally occurring BPH and its interaction with factors closely associated with this disease, such as the inflammatory process and oxidative stress. For this purpose, adult male gerbils were submitted to three different experimental conditions: animals without any treatment for one year (IC group), animals which received water (0.1ml), every other day, for one year (CG group), and animals which received coconut oil 0.1ml), every other day, for one year (CO group). We performed a series of analyses of morphological, morphometric, stereological, immunohistochemical, serological, protein, and cytokine expression and evaluation of oxidative stress in the ventral prostate. The results showed that animal handling in the gavage procedure throughout aging aggravated the prostatic lesions, increased the hormone receptor expression, and increased the thickness and the proliferation of the muscular stroma. This procedure also influenced the macrophage population and the expression of pro-inflammatory cytokines, metalloproteinases, in addition to increasing oxidative stress. Coconut oil consumption, in contrast, attenuated or inhibited these age-related alterations intensified by the gavage procedure. Thus, coconut oil consumption over aging resulted in supportive data for the use of this oil as a phytotherapeutic agent in the HPB treatment.

Keywords: Prostate. Ageing. Hyperplasia. Coconut oil. Gerbil.

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LISTA DE ABREVIATURAS E SIGLAS

AR	Receptor de andrógeno
CML	Células musculares lisas
CP	Câncer de próstata
DHT	Di-hidrotestosterona
ERO	Espécie reativa de oxigênio
ERα	Receptor de estrógeno tipo alfa
ERβ	Receptor de estrógeno tipo beta
HPA	Hipotalâmico-pituitário-adrenal
HPB	Hiperplasia prostática benigna
HPG	Hipotalâmico-pituitário-gonadal
MEC	Matriz extracelular
MMP	Metaloproteinase
PAP	Fosfatase ácida prostática
PIN	Neoplasia intraepitelial
PSA	Antígeno prostático específico

SUMÁRIO

1 INTRODUÇÃO

1.1 A Próstata

1.1.1 Características gerais

Um dos resultados da evolução do sistema reprodutivo e da competição de espermatozoides como pressão seletiva foi o surgimento da próstata (RAMM; PARKER; STOCKLEY, 2005). A próstata é órgão do sistema genital masculino é do tipo glandular e sua secreção é fundamental para o sucesso reprodutivo (MARKER et al., 2003a), uma vez que garante aos espermatozoides condições ideais de sobrevivência e viabilidade durante e após a ejaculação (TABOGA; VILAMAIOR; GÓES, 2009). Nas últimas décadas diversos estudos têm revelado que essa glândula não é exclusiva do organismo masculino, sendo encontrada em fêmeas de diversos mamíferos, incluindo humanos (SANTOS; TABOGA, 2006; WHIPPLE, 2002). No homem e na maioria dos animais, este órgão é localizado próximo ao colo da bexiga e à uretra, possui um componente glandular e um muscular (Fig.1) que permite realizar sua função de produzir e liberar a maior fração do fluido seminal (ALUKAL; LEPOR, 2016; UNTERGASSER; MADERSBACHER; BERGER, 2005).

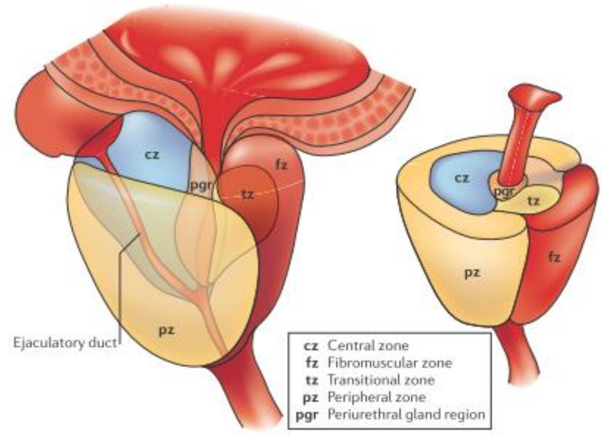

Figura 1. Posição anatômica da próstata, localizada abaixo da bexiga e circundando a uretra. Em humanos, a próstata é dividida em zona central (cz), transicional (tz) e periférica (pz), além das regiões fibromuscular (fz) e periuretral (pgr). Fonte: Modificado de (VERZE; CAI; LORENZETTI, 2016) .

A próstata apresenta morfologia variável entre as classes de mamíferos, em roedores, por exemplo, lobos distintos bilateralmente simétricos compõem o complexo prostático que circunda a uretra na base da bexiga, são designados como o lobos ventral, lateral, dorsal e ainda, associados às glândulas seminais, o lobo anterior ou glândula coaguladora (PRICE, 1963) (Fig. 2). O lobo ventral é o componente mais utilizado do complexo prostático em estudos para compreender a biologia da próstata (JESIK; HOLLAND; LEE, 1982). Essa preferência deve-se ao seu tamanho, à sua sensibilidade à ação de andrógenos, e maior incidência de hiperplasia e outras lesões nesta região da próstata (BANERJEE et al., 1998; SHAPPELL et al., 2004).

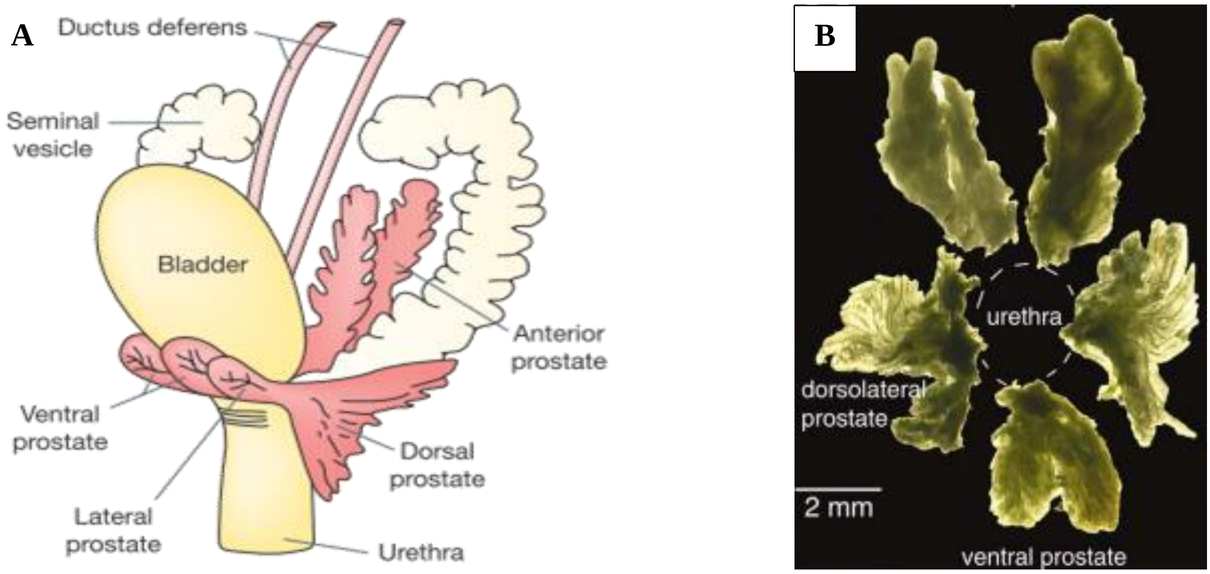

Figura 2. **A**: Desenho esquemático do complexo prostático de rato circundando a bexiga. **B**: Lobos prostáticos de rato isolados. Fonte: (AHMAD; SANSOM; LEUNG, 2008; MARKER et al., 2003b)

A próstata é classificada histologicamente como uma glândula túbulo-acinar composta, formada pelos compartimentos epitelial e estromal, que apresentam funções e populações celulares distintas, mas conservadas entre diferentes espécies como roedores, morcegos e humanos (ALBERNAZ et al., 2021; OLIVEIRA et al., 2016; VERZE; CAI; LORENZETTI, 2016). A atividade secretora da próstata ocorre principalmente na porção alveolar, entretanto os ductos também podem secretar alguns componentes para o conteúdo final da secreção prostática (REESE et al., 1986). A secreção é sintetizada em um epitélio, organizado em ácinos, composto de quatro tipos de células: basal, secretora, intermediária e neuroendócrina que são dependentes de hormônios esteroides e reagem diferentemente a cada um. As células secretoras são os tipos mais frequentes e a elas compete a síntese e secreção de proteínas como o antígeno prostático específico (PSA) e a fosfatase ácida prostática (PAP) (MARKER et al., 2003b; RISBRIDGER; TAYLOR, 2006). Na maioria dos mamíferos, as células basais se localizam entre o epitélio secretor e a membrana basal e são fonte progenitora das células secretoras, já as células neuroendócrinas são pouco frequentes e podem ser diferenciadas por técnicas específicas de coloração (MARKER et al., 2003a; REBELLO et al., 2021). Apesar das células secretoras, basais e as neuroendócrinas diferirem quanto à regulação hormonal, todas estão envolvidas na secreção de proteínas e substâncias de baixo peso molecular que também compõem o fluido prostático (BONKHOFF; REMBERGER, 1998). As células epiteliais são separadas do estroma por uma lâmina basal (CZYŻ; SZPAK; MADEJA, 2012) (Fig3).

Os ácinos secretores e ductos são envoltos por um estroma conjuntivo ricamente vascularizado (DE CARVALHO; TABOGA; VILAMAIOR, 1997). O estroma prostático consiste em um arranjo complexo de células musculares lisas (CML), que sofrem contração no processo de ejaculação (ROSS; PAWLINA, 1979), e fibroblastos imersos em uma matriz extracelular (MEC) (TUXHORN; AYALA; ROWLEY, 2001). Além de desempenhar um papel estrutural a CML e os fibroblastos são essenciais para a manutenção da homeostase da próstata, uma vez que produzem fatores autócrinos e parácrinos que regulam diversos processos fisiológicos no estroma (MARKER et al., 2003a; ROCHEL et al., 2007b). A atividade dessas células estromais mantém a estrutura básica da MEC sintetizando proteoglicanos (KOFOED et al., 1990), componentes de fibras colágenas e elásticas (DE CARVALHO; TABOGA; VILAMAIOR, 1997; VILAMAIOR et al., 2000) e metaloproteinases de MEC (MMPs) (KIANI et al., 2020) (Fig.4). Neste compartimento também se encontram células fagocitárias, como macrófagos e células endoteliais vasculares, além de telócitos, um tipo celular que faz intercomunicação entre as células estromais e participa do desenvolvimento e manutenção da próstata (SANCHES et al., 2017, 2021). O epitélio e o estroma compartilham características comuns em diferentes animais, independentemente de variações anatômicas da glândula, sendo assim, é possível fazer homologias e analogias morfo-funcionais entre animais de laboratório e o homem (PRICE, 1963).

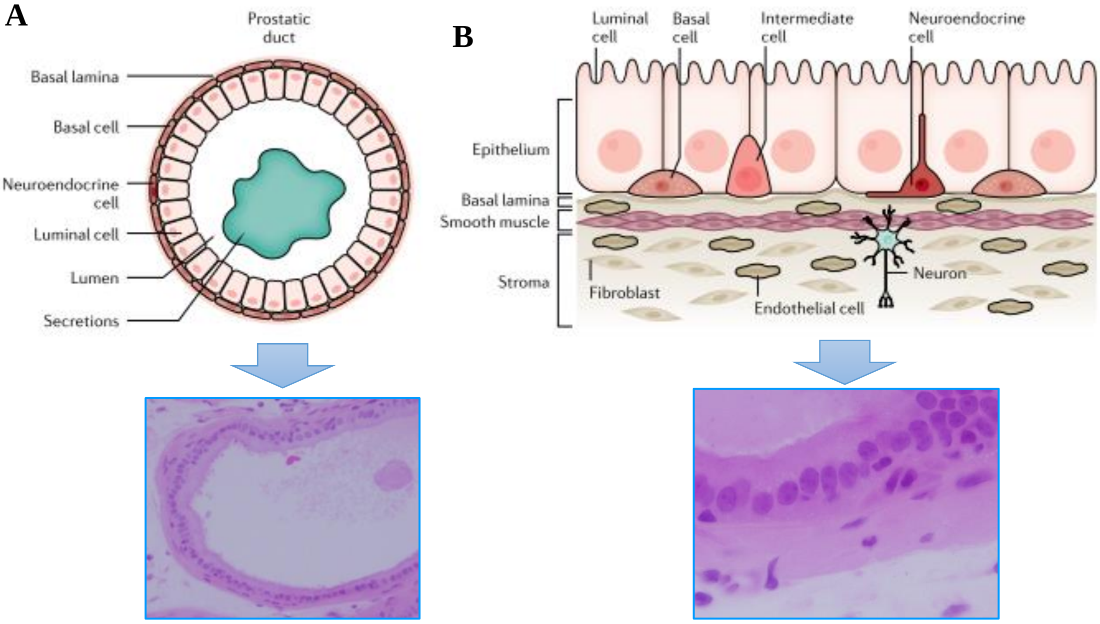

Figura 3. **A**: Na parte superior é mostrado o esquema ilustrativo do ácino prostático composto por células epiteliais (indicadas como células luminais), basais, e neuroendócrinas dispostas sobre uma lâmina basal, e a secreção é armazenada no lúmem; na parte inferior é mostrado um ácino em um corte histológico da próstata de Gerbilo da Mongólia corado com HE. **B**: Esquema do epitélio secretor composto por células epiteliais (células luminais), basais, intermediárias e neuroendócrinas. Abaixo do epitélio é mostrado a lâmina basal e o estroma contendo células musculares lisas, fibroblastos e células endoteliais. Abaixo é mostrado o epitélio e estroma em um corte histológico da próstata de Gerbilo da Mongólia corado com HE. Fonte:Arquivo pessoal e (REBELLO et al., 2021) modificado.

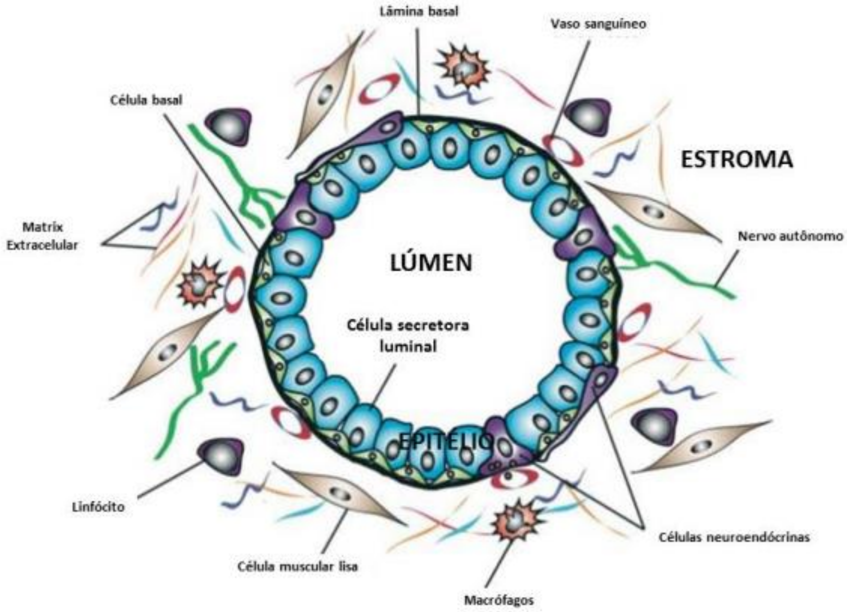

Figura 4. Esquema dos principais componentes do estroma e do epitélio. Fonte: Modificado de (BARRON; ROWLEY, 2012)

1.1.2 Controle hormonal

A próstata é regulada e dependente de andrógenos para o desenvolvimento, durante a embriogênese, e para sua manutenção ao longo da vida (MARKER et al., 2003a). A produção desses hormônios é regulada pelo eixo hipotálâmico-hipofisiário-gonadal (CUNHA et al., 2001; MARKER et al., 2003a) e eles agem em células alvo da próstata por meio da interação com os seus respectivos receptores hormonais específicos, como os receptores de andrógeno (AR) e os receptores de estrógeno (HSING; REICHARDT; STANCZYK, 2002). O principal hormônio andrógeno com interação prostática é a di-hidrotestosterona (DHT) que é resultante da conversão da testosterona pela enzima 5-α-redutase e, junto com a testosterona, ligam-se ao AR e ativam a maquinaria de transcrição de genes relacionados a atividades como crescimento celular, produção de PSA entre outros (TABOGA; VILAMAIOR; GÓES, 2009). Sendo assim, a expressão desse receptor, juntamente com os níveis de andrógenos, é responsável por controlar a razão entre proliferação e morte celular (TAN et al., 2015). Nesse cenário a atividade da 5-α-redutase tem papel fundamental no controle da proliferação celular prostática e consequentemente na modulação da hiperplasia dos compartimentos estromais e epiteliais (BECHIS et al., 2014).

Além dos andrógenos, os estrógenos também controlam a fisiologia da próstata, uma vez que expressa receptores de estrógenos do tipo alfa (ERα) e do tipo beta (ERβ) (MCPHERSON; ELLEM; RISBRIDGER, 2008). Além da expressão desses receptores, na próstata também ocorre a síntese de estrógeno por efeito da atividade da aromatase (CYP19) que converte testosterona em estradiol (GRINDSTAD et al., 2016). A participação dos estrógenos no controle da biologia prostática é apontada como um dos fatores potenciais de risco associados ao desenvolvimento de hiperplasia prostática benigna (HPB) e câncer de próstata (CP) (HENDERSON; FEIGELSON, 2000). Essa capacidade é diretamente relacionada com os níveis da expressão dos receptores ERα e ERβ e as diferentes respostas desencadeadas após a ligação dos hormônios com mesmos (CHOI et al., 2016). No tecido prostático, o aumento da expressão de ERα é associado à proliferação celular anormal e inflamação, já para o ERβ é atribuído um papel anti-proliferativo e anti-inflamatório (ELLEM; RISBRIDGER, 2009).

Durante o envelhecimento os níveis de testosterona diminuem (HARMAN et al., 2001). Uma das consequências dessa alteração hormonal é o comprometimento da dinâmica celular, ou seja, do equilíbrio entre proliferação e morte e acarretar no desenvolvimento de HPB que compromete a funcionalidade do sistema urinário, além de estar relacionada à iniciação do processo carcinogênico (BONKHOFF; REMBERGER, 1998). Em adição, alterações nos níveis androgênicos interfere em fatores de crescimento que podem estimular ou inibir, dependendo da situação, a divisão e a diferenciação celular (ROEHRBORN, 2008a) (Fig. 5). As consequências do envelhecimento na morfologia da próstata vão desde alterações dos componentes estromais, como alterações no arranjo de fibras colágenas e elásticas, alterações intracelulares, como aumento de acúmulo lipídico e de lipofusina, até o desenvolvimento de leões prostáticas como neoplasias intraepiteliais (PIN), atrofias e carcinomas microinvasivos (CAMPOS et al., 2008).

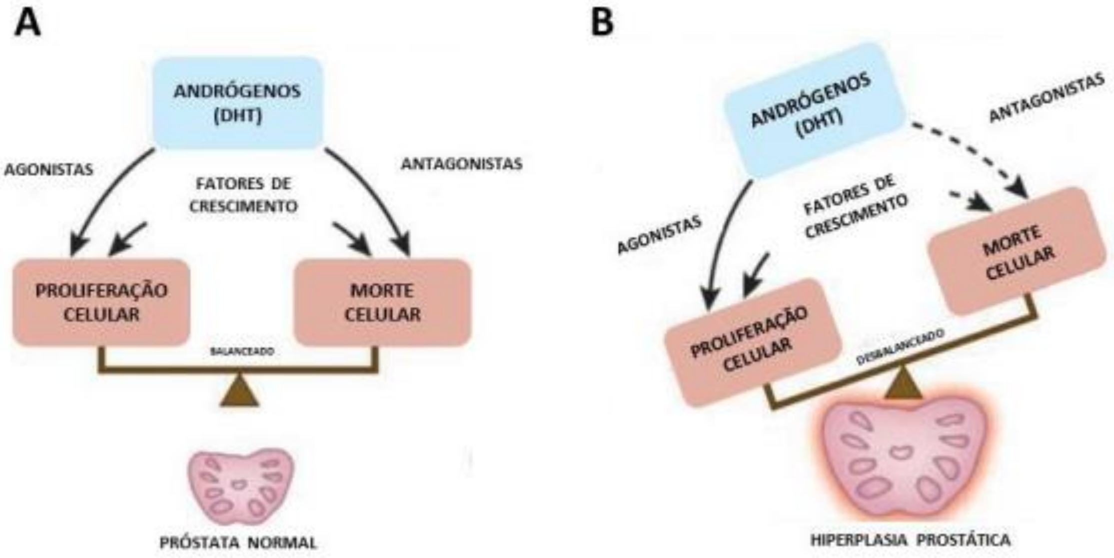

Figura 5. Esquema da ação de andrógenos e de fatores de crescimento na proliferação e na morte celular prostática em condições normais (A) e na HPB (B) Fonte:(ROEHRBORN, 2008).

1.2 Hiperplasia Prostática Benigna

A HPB é uma doença que afeta mais de cinquenta por cento dos homens com mais de cinquenta anos de idade (EGAN, 2016), sendo assim, dezenas de milhões de homens nessa faixa etária em todo o mundo possuem HPB e os dados epidemiológicos nos Estados Unidos, Europa e outras regiões sugerem que a incidência e prevalência dessa condição aumentará em um futuro próximo devido ao envelhecimento da população e o aumento da prevalência de síndrome metabólica e seus componentes (PATEL; PARSONS, 2014). Em geral, os dados da literatura sugerem que a patogênese da HPB é influenciada por diferentes mecanismos hormonais, em particular: os andrógenos e a atividade de seus receptores, a função dos fatores de crescimento e a importância do metabolismo do estrogênio, porem outros aspectos devem ser considerados, como inflamação crônica e os efeitos da síndrome metabólica (LA VIGNERA et al., 2016; NGAI et al., 2017).

1.2.1 Inflamação

A Inflamação e seus mediadores são relacionados diretamente com a HPB (DE NUNZIO; PRESICCE; TUBARO, 2016) e as células inflamatórias e seus produtos comumente associados com HPB são observadas no CP (KRUŠLIN et al., 2017). O aumento da população de células inflamatórias como linfócitos B e T na próstata, além da ativação de citocinas que aumentam a concentração de fatores de crescimento podem contribuir para o desenvolvimento da HPB (GANDAGLIA et al., 2017). Já os macrófagos desempenham um papel decisivo na imunidade inata e adaptativa e são cruciais no controle da resposta inflamatória e no processo de reparação e homeostase tecidual (VARIN; GORDON, 2009). Nesse cenário, alterações no ambiente prostático induzidas pela inflamação levam a várias mudanças na expressão gênica e subsequentemente resultam em inflamação crônica, isso contribui para mudanças na estrutura da próstata e para surgimento dos sintomas relacionados a HPB (TAOKA; KAKEHI, 2017). Estudos sugerem fortemente que a HPB é uma doença inflamatória imunológica (KRAMER; MARBERGER, 2006) e que a inflamação prostática deva ser considerada um novo campo na pesquisa básica e clínica em pacientes com HPB, assim como alvo para novas estratégias de tratamento (DE NUNZIO; PRESICCE; TUBARO, 2016).

1.2.2 Estresse Oxidativo

O aumento do estresse oxidativo, ou de espécies reativas de oxigênio (EROs), deve ser considerada no estudo da HPB, uma vez que desempenha um papel importante nessa patogênese (ZABAIOU et al., 2016). Em condições fisiológicas normais, a produção de EROs é uma condição normal e leva à maior fosforilação de I-kβ (Inibidor de Kappa Beta) e translocação do NF-kβ (Fator Nuclear Kappa Beta) para o núcleo, resultando na transcrição de enzimas antioxidantes e a manutenção do equilíbrio (TOMÁS-ZAPICO; COTO-MONTES, 2005). O estresse oxidativo é definido como uma interrupção do equilíbrio entre moléculas oxidantes e redutoras devido à produção excessiva de EROs, sendo que esse desequilíbrio leva ao dano oxidativo do DNA e fornece uma condição favorável para a etiopatogenia de diversas doenças incluindo prostáticas (KAYA et al., 2017). Nesse aspecto, sabe-se que o aumento do estresse oxidativo afeta todas as funções e processos celulares, incluindo replicação do DNA e divisão celular, e respostas inflamatórias, sendo considerado, portanto, um dos mecanismos que desencadeiam reações envolvidas no desenvolvimento e progressão da HPB (UDENSI; TCHOUNWOU, 2016).

1.2.3 Estresse Psicológico

Além de fatores fisiológicos relacionados a HPB já bem estabelecidos, como inflamação e EROs, outra temática ainda pouco elucidada tem chamado a atenção. O estresse psicológico é apontado como um agente causador de HPB por promover hiperatividade do sistema nervoso simpático (SNS) e como consequência interferir no controle de proliferação e morte celular (HUANG et al., 2009; ULLRICH; LUTGENDORF; KREDER, 2007). Além disso, há indícios que o estresse psicológico também interfira no eixo hipotalâmico-pituitário-gonadal (HPG) (ULLRICH et al., 2005). Dados recentes mostram que há uma relação entre o envelhecimento e o estresse psicológico, e essa relação influencia na progressão do PC por ação inflamatória (BELLINGER et al., 2021). Entretanto, estudos que induzem estresse em animais estão sujeitos a muitas variáveis expostas durante os experimentos, como a interferência do envelhecimento no eixo hipotalâmico-pituitário-adrenal (HPA), que interferem nos dados obtidos (GRAY; CHAOULOFF; HILL, 2015). Nesse cenário, a literatura que aborda os efeitos diretos do estresse psicológico na próstata é escassa, revelando um campo intrigante e pouco explorado.

1.3 Gerbilo da Mongólia

O Gerbilo da Mongólia (*Meriones unguiculatus*), uma espécie que pertence à família Muridae e subfamília Gerbillinae, é um roedor de pequeno porte com peso de aproximadamente 70 gramas em idade adulta. São animais de origem desértica e de simples manutenção em cativeiro, uma vez que apresenta comportamento dócil e se adaptam à diferentes condições (BATCHELDER et al., 2012; FISHER; LLEWELLYN, 1978). O emprego desse roedor como modelo experimental tem sido considerado em diversas áreas, como fisiologia (KHAKISAHNEH et al., 2019), genética (RAZZOLI et al., 2003) e comportamental (DENG; LIU; WANG, 2017). Outra área em que o gerbilo se mostra com um bom modelo experimental é a da reprodução, sendo utilizado em estudos de mama (LEONEL et al., 2020), testículo (PINTO-FOCHI et al., 2016) e próstata (GUERRA et al., 2019).

A próstata do gerbilo é parecida com as de outros roedores e é constituída pelos lobos ventral, anterior, dorsal e dorsolateral que estão associados à uretra (Fig.6) (ROCHEL et al., 2007a). Dentre os lobos da próstata de gerbilo, o lobo ventral é o mais estudado e é altamente responsivo aos níveis androgênicos, tanto em fêmeas quanto em machos (CORDEIRO et al., 2008). Considerando os principais aspectos envolvidos na HPB, o Gerbilo da Mongólia é um modelo muito interessante para o estudo dessa doença, posto que nesse animal há um aumento de ERO e também na suscetibilidade ao dano oxidativo durante o envelhecimento (SOHAL; AGARWAL; SOHAL, 1995). Além disso, esse animal apresenta HPB e PIN espontâneas quando idoso (CAMPOS et al., 2008; PEGORIN DE CAMPOS et al., 2006) e possui uma singularidade para estudos de metabolismo lipídico que é a relativa facilidade com que desenvolve hiperlipidemia em resposta à dieta, característica não compartilhada por outros roedores comumente utilizados (NICOLOSI et al., 1981).

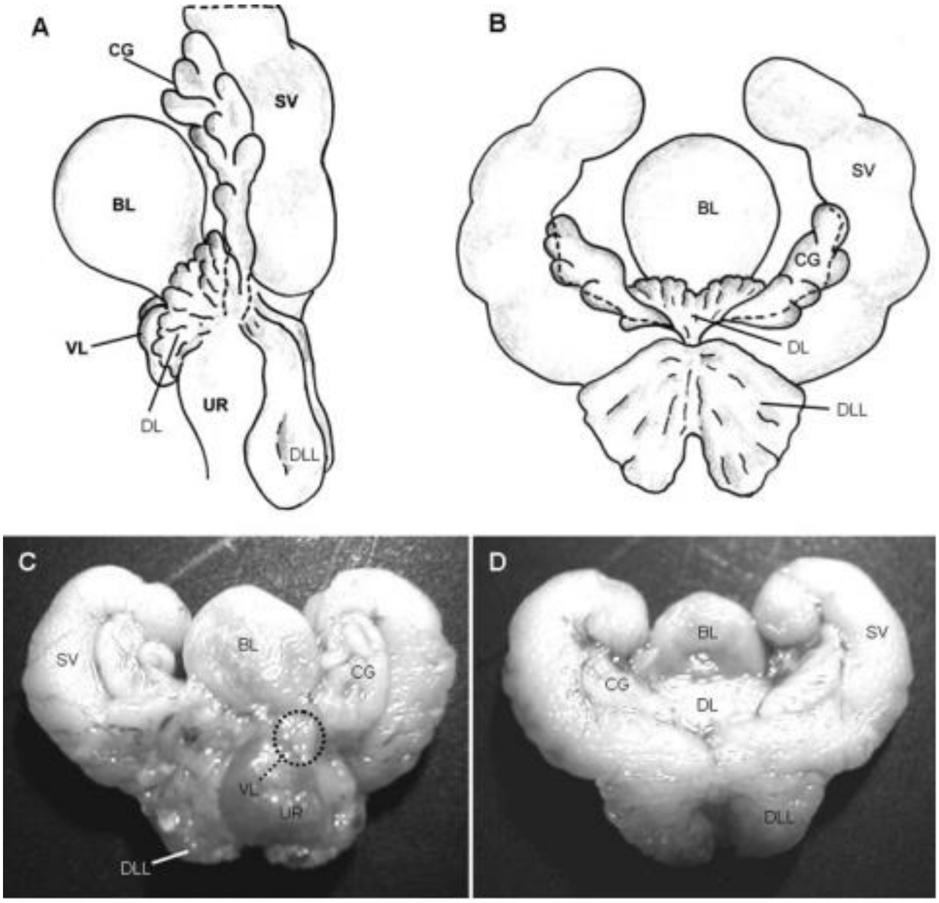

Figura 6. Complexo prostático do Gerbilo da Mongólia adulto em esquema de vista lateral (A) e dorsal (A) e do órgão fresco em vista ventral (C) e dorsal (D). Bexiga urinária (BL); glândula coaguladora ou lobo anterior (CG); lobo dorsal (DL); lobo dorsolateral (DLL); vesícula seminal (SV); uretra pélvica e músculo uretral (UR); lobo ventral (VL). Fonte: (ROCHEL et al., 2007b).

1.4 Fitoterapia

O crescente aumento de opções no mercado farmacêutico, torna a prescrição da terapia médica apropriada para pacientes com HBP uma tarefa cada vez mais complicada, uma vez que o equilíbrio final entre eficiência, efeitos adversos e custos é muitas vezes difícil de alcançar (SHUM; LAU; TEO, 2017). Nesse contexto, além das drogas sintéticas anti-androgênicas, comumente indicadas para o tratamento de HPB e complicações do trato urinário correlatas ao longo das últimas décadas (LAM; ROMAS; LOWE, 2003), medicamentos complementares e alternativos, como os fitoterápicos entraram em cena como uma opção de tratamento para BPH (KEEHN; TAYLOR; LOWE, 2016).

Cerca de trinta compostos fitoterapêuticos são utilizados tanto para “manter uma próstata saudável” quanto para o tratamento da HBP, como *Serenoa repens*, *Hypoxis rooperi*, *Secale cereale* (WILT et al., 2000), e seus mecanismos de ação variam desde anti-androgênicas até antioxidantes e anti-inflamatórias (ADARAMOYE et al., 2017; JEON et al., 2017; KEEHN; TAYLOR; LOWE, 2016). Dentre as possibilidades de fitoterápicos, um que chama a atenção é o óleo de coco, em razão de ser apontado como responsável por reduzir o aumento da glândula prostática em animais com HPB induzida após tratamento com testosterona (DE LOURDES ARRUZAZABALA et al., 2007).

1.4.1 Óleo de coco

O óleo de coco, obtido da espécie *Cocos nucifera,* é um alimento funcional emergente, comumente utilizado como medicina tradicional e um suplemento dietético no sul da Índia e muitos outros países tropicais. Esse óleo é relatado como responsável por reverter a condição de lipotoxicidade no fígado e melhorar a resistência à insulina (NARAYANANKUTTY et al., 2017). No que diz respeito a sua composição, este óleo é considerado saturado, pois apresenta cerca de 90% de ácidos graxos dessa natureza sendo o ácido láurico o lipídio mais abundante (AKPAN et al., 2006), seguido dos ácidos mirístico, palmítico, caprílico, cáprico e esteárico (SHAHIDI; AMBIGAIPALAN, 2015). Entretanto. há uma pequena fração de compostos insaturados (cerca de 9%), que compreendem os ácidos oleico e linoleico e estes são constituintes importantes das células, envolvidos na função celular, e podem interferir na fluidez da membrana e regular a sinalização celular, a expressão gênica e a inflamação (CALDER, 2008). Alguns desses componentes do óleo de coco ainda possuem atividade antioxidante (YEAP et al., 2015), e são capazes de alterar o perfil lipídico sérico (RESENDE et al., 2016).

2 JUSTIFICATIVA

Atualmente a literatura revela que diversos fatores influenciam no processo de desenvolvimento e progressão de HPB. Entre esses fatores, alguns são bem estabelecidos, como os níveis hormonais e a expressão de receptores androgênicos e estrogênicos (PRINS, 2008; ROEHRBORN, 2008b). Outras condições que modulam o desenvolvimento e a progressão dessa patologia são a inflamação e o estresse oxidativo (KRUŠLIN et al., 2017; ZHAO et al., 2021). Há ainda fatores pouco elucidados, como o estresse psicológico, capazes de interferir na HPB (ULLRICH et al., 2005). Considerando a suscetibilidade dessa doença a tantas variáveis fisiológicas, é considerável a diversidade de estudos sobre possíveis terapias para sanar esse problema que atinge mais de 50% dos homens com mais de 50 anos de idade. Nesse contexto, o estudo de possíveis agentes fitoterápico como tratamento para a HPB tem aumentado (KEEHN; TAYLOR; LOWE, 2016). O óleo de coco, que tem sido amplamente utilizado como suplementação alimentar, apresenta componentes que têm sido alvos de estudos por interferir em vias associadas à HBP e sua administração em animais com HPB mostrou-se capaz de reduzir o peso prostático absoluto (DE LOURDES ARRUZAZABALA et al., 2007). Entretanto, até o momento não há nenhum estudo sobre o resultado do consumo de óleo de coco durante o envelhecimento na HPB e em as condições fisiológicas que interferem nessa patologia, como inflamação e estresse oxidativo.

3 HIPÓTESE

Este trabalho teve como hipótese que o consumo de óleo de coco durante o envelhecimento influencie o desenvolvimento de HPB, bem como em condições associadas a esta patologia, como inflamação e estresse oxidativo.

4 OBJETIVO

4.1 Objetivo Geral

O presente trabalho tem como objetivo geral avaliar os efeitos do consumo de óleo de coco durante o envelhecimento na morfologia prostática de Gerbilos da Mongólia bem como seus efeitos na hiperplasia prostática benigna espontânea.

4.2 Objetivos específicos

- Avaliar a morfologia e expressão de receptores hormonais na próstata, além dos níveis hormonais séricos de animais que envelheceram consumindo óleo de coco e sob procedimentos de manipulação e gavagem.
- Verificar o status inflamatório da próstata dos animais submetidos aos procedimentos experimentais e tratados com óleo de coco.
- Analisar o efeito do consumo de óleo de coco e da manipulação experimental no perfil oxidativo e metabólico dos animais.

5 RESULTADOS

Os resultados do presente trabalho estão apresentados em três capítulos em forma de manuscritos, além disso é apresentado um capitulo extra, em anexo:

5.1 Capítulo 1 – Artigo 1.

Será submetido à revista “Brain, Behavior, and Immunity”

Coconut oil mitigates the effects of chronic stress on the prostate of gerbils.

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Abstract

Despite of benign prostatic hyperplasia (BPH) association with aging and inflammation, it is also influenced by chronic psychological stress which requires new strategies to minimize its impact on BPH. This work evaluated the coconut oil intake efecct on the prostate of aging gerbils under chronic stress conditions. For this purpose, three experimental groups were assigned: intact control, stress control and stress nourished with coconut oil. Gavage procedure was performed in alternate days, as chronic stress inducer, for one year. Cortisol, testosterone and estradiol serum levels were determined. Histopathological analysis, cell proliferation index (TUNEL and P-H-H3) and receptors of androgen and estrogen were evaluated on the prostate. The chronic stress induced by gavage aggravated prostatic lesions, increased androgen receptor (AR) and estrogen receptor alpha (ERα) expression. Coconut oil intake attenuated the stress-induced alterations in addition to increase apoptosis rate in the prostatic cells. In conclusion, chronic stress aggravated prostate lesions, such as BPH, whereas coconut oil treatment throughout aging mitigated the stress impacts on prostate health.

Keywords: Chronic stress; Ventral Prostate; Aging; Benign prostatic hyperplasia.

Introduction

The elderly population is increasing and it is expected that by 2050, sixteen percent of the global population will be over the age of 65 [1]. Aging is a complex process, highly variable among different species and strongly controlled by many genes, being susceptible to several factors [2]. Some lifestyle-related risk factors, such as smoking, alcohol consumption, and lack of physical activity, are pointed out as "aging accelerators” [3], including psychological chronic stress [4, 5]. Therefore, the search for improvements in the aging process has grown in recent decades.

Benign prostatic hyperplasia (BPH) is an aging-related disease and affects more than fifty percent of men over 50 years old [6], and, recent trends in the United States, Europe, and other regions suggest that its incidence and prevalence will increase due to increase in elderly population [7]. The BPH is influenced by several hormonal mechanisms, in particular, the androgens and their receptors activities, growth factors, estrogen metabolism, chronic inflammation and metabolic syndrome [8]. However, the association of stress and other psychological factors with BPH and lower urinary tract symptoms (LUTS) is not clear yet [9]. Chronic stress is pointed as a potential promoter of BPH in rats by overactivation of the sympathetic nervous system (SNS) and interference in epithelial proliferation [10]. In addition, social stress has also been reported as a plausible factor that affects prostate-specific antigen (PSA) levels [11].

The search for new strategies BPH treatment has increased and over the last decades, complementary and alternative medicines like phytotherapics agents have emerged [12]. Among them, coconut oil has been shown to reduce prostate gland enlargement in animals with testosterone-induced BPH, as observed in adult Sprague-Dawley rats [13], the cortisol levels in guinea pigs [14] and to interfere with depressive behavior in prenatally stressed rodents [15]. However, up to date, there are no evidence of its impact on psychological stress and BPH.

The use of animal models in stress research is complex since several conditions could interfere in the results, including animal handling and drugs administration [16]. Research concerning drug effects on stress-dependent BPH presents limitations in control groups, since the experimental condition of induced hyperplasia often interferes on the animal stress. An alternative solution to this limitation is the use of experimental models that develop BPH spontaneously. Mongolian gerbil (*Meriones unguiculatus*) represents an appropriate model to investigate the effects of different drugs as well as handling animals effects during the experimental procedures in BPH, since such rodents develop BPH, prostatic lesions and adenocarcinomas spontaneously along aging [17, 18]. In addition, gerbils increase cortisol levels in stressful conditions. Therefore, this work aimed to evaluate the animal handling-related stress and the coconut oil effect on gerbil prostate throughout aging.

Materials and Methods

Experimental design and sample collection

Animals were breed and raised at Institute of Biosciences, Letters and Exact Sciences /São Paulo State University (IBILCE/UNESP) with approval and in compliance with the institutional ethics committee guidelines (183/2018- CEUA- São José do Rio Preto). Animals were kept in ventilated mini-isolators (two animals per box) under controlled light and temperature conditions (12h light/12h dark - 24ºC) with water and chow *ad libitum*. For the experiment, 36 male Mongolian Gerbils were distributed equally into 3 groups: Intact control (IC): animals that did not undergo to any restrain or stress induction; Gavage control (GC): at the end of adulthood (100 days of age) animals were restrained for 30 seconds and received 0.1 ml of water by gavage; Coconut oil (CO): at the end of adulthood (100 days of age) animals were restrained for 30 seconds and received 0.1 ml of coconut oil (dose equivalent to 13ml for an adult man weighing 80kg [19]) by gavage. GC and CO groups received oral gavage on alternate day regimen for one year and were euthanized at 465 days of age. Gavage was performed by introduction of a reusable appropriate needle deep into the esophagus in non-anesthetized animals under restrainment condition. At the end of the experiment, animals were euthanized by deep anesthesia, weighed for biometric analysis, decapitated for blood collection and prostate harvested entirely. The blood was centrifuged, and serum stored at -80ºC. Prostates were weighed, and ventral lobes used for morphological and protein analysis. Tissues were either frozen immediately in liquid nitrogen (n=6) or fixed by immersion in 4% formaldehyde prepared in phosphate buffer, pH 7.2, for 24h (n=6). Following the formaldehyde fixing process, samples were washed with distilled water, dehydrated in an alcohol battery, immediately processed, and included in Histosec (Merck, Germany).

Hormonal analysis

Cortisol, testosterone, and 17-β-estradiol serum levels were quantified by enzyme-linked immunosorbent assay using high sensitivity specific commercial kits (IBL International; items number 52061, 52151, and 52041 respectively) according to the manufacturer's instructions and the readings determined in a microplate reader (TECAN - Infinite F50).

Morphological analysis

Tissues were sectioned at 4μm, and slides stained with hematoxylin-eosin (HE) for general histology. Stromal fibrillary components were accessed using the following staining methods: for collagen fiber analysis, slides were stained with Picrosirius Red; for reticular fiber, with Gömöri's reticulin; and, for the elastic fiber, with Weigert's resorcin-fuchsin method. Slides were analyzed with an Olympus BX60 light microscope (Olympus, Japan), and the images digitized with DP-BSW V3.1 (Olympus, Japan) and Image-ProPlus 6.1 software (Media Cybernetics, Silver Spring, MD). The relative frequency of prostatic compartments (epithelium, lumen, smooth muscle layer (SML), non-muscular stroma, and blood vessels) and stromal fibrillar components (collagen and elastic fibers) were obtained with M130 multipoint test system Weibel, 1978 [20] using 30 random fields per group. Height of the secretory epithelial cells (μm) and thickness of the smooth muscle layer (μm) were assessed in the histological sections using 200 measurements for each experimental group.

Incidence and multiplicity of lesions

Prostatic proliferative disorders incidence and multiplicity rates were obtained by histopathological analysis of 3 slides, from different histological depths, from each animal (n=18/group) and tissue sections examined by investigators blindly. Samples were stained by HE and digitized at ×400 magnification using the slide scanner system (Olympus VS120 - S5). Prostatic lesions classification was based on the well-established criteria of Bar Harbor [21], previously applied in the Mongolian gerbil [18, 22, 23]. The incidence was calculated as % = (number of animals with changes /number of animals per group), while the multiplicity as (number of changes in each animal / number of slides). The following prostatic lesions were considered: prostatic epithelial hyperplasia (PEH) - area of the epithelium with cell aggregation, stratification, and irregularly shaped cells; grade I intraepithelial neoplasia (PIN I) - areas with clustering of cells with enlarged nuclei of varying size, dense chromatin, and an occasional large and prominent nucleolus.; grade II intraepithelial neoplasia (PINII) - Similar to PIN I but with nuclei exhibiting strongly dense chromatin and frequently large nucleoli, basal cell discontinuity, and invasion of other prostatic compartments without muscular stroma disruption; cribriform intraepithelial neoplasia (PINC) - epithelium exhibiting PIN characteristics but displayed as micro-acini.; atrophy (ATR) - area with thinning of the epithelium and muscular stroma; microinvasive carcinoma (MC) - similar to PIN II but with microvessels and rupture of the adjacent muscular layer confirmed by immunohistochemistry staining for α-actin and reticular fibers disruption.

Cell proliferation index

Immunohistochemistry was performed for P-H-H3 as a marker of cell proliferation, according to the procedures described in the section "Immunohistochemistry" mentioned thereafter. To access the apoptosis rate, TUNEL assay was performed with ApopTag® commercial kit (Plus peroxidase in situ apoptosis detection kit- S7101- Merck, CA, USA) to detect DNA fragmentation following the manufacturer´s instructions. Apoptosis and proliferation frequencies were determined as the ratio of marked cells to the number of total cells. At least 6000 cells were counted in the epithelium and smooth muscular layer per group. The proliferation index was assessed by the frequency of proliferation to apoptosis ratio (P-H-H3/TUNEL).

Hormonal receptor expression analysis

Androgen receptor (AR) and estrogen receptors alpha and beta (ERα and ERβ) expression were assessed by immunohistochemistry and western blotting. Positive cells frequency was determined by the ratio immunolabeled cells to the total number of cells of the epithelium and smooth muscular layer. Protein quantification was determined by relative density to GAPDH using ImageJ 1.46r software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, MD, USA).

Immunohistochemistry

Anti-α-actin (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology, Dallas, TX, USA); anti- phospho-histone H3 (P-H-H3, rabbit polyclonal, H3, 1:75, Ser10, 9701, Cell Signaling, Danvers, USA); anti-AR (rabbit polyclonal IgG, N-20, 1:75, sc-816, Santa Cruz Biotechnology, Dallas, TX, USA); anti-ERα (polyclonal IgG rabbit, 1:50, PA5-34577, Invitrogen, Thermo Fisher Scientific, Rockford, IL, USA); and anti-ERβ (polyclonal IgG rabbit, 1:50, PA1-310B, Invitrogen, Thermo Fisher Scientific, Rockford, IL, USA) were used as primary antibodies. Antigen retrieval was performed by tissue sections embedding in 10mM citrate buffer (pH 6.0) for α-actin, PHH3, AR and ERβ; and TRIS-EDTA buffer (pH 9) for ERα. temperature and time for retrieval were 96°C for 40 minutes for PHH3, AR, and ERβ, 93°C for 20 minutes for α-actin, and a microwave sequence (2 x 7 min at low power and 1 x 7 min at medium-low power) for ERα. Endogen peroxidases were blocked by immersing the slides in 3% H_2O_2 at room temperature for 30 minutes. Then, non-specific proteins were blocked with 5% skimmed milk in TBS. Sections were incubated overnight at 4°C (PHH3, AR, ERα and ERβ) or for 1h at room temperature (α-actin) before secondary antibodies (Polymer kit Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) were applied for 1h at room temperature. Positive staining was detected using 3-30’-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstained with hematoxylin.

Western blot

Frozen samples (6 per group) were smashed and incubated for extraction (CelLytic MT cell lysis reagent, C3228, Sigma Aldrich) at 4 °C, for 60 minutes, under agitation. Protease activity was blocked by inhibitors (Protease Inhibitor Cocktail, P8340, Sigma Aldrich), followed by centrifugation for 20 min at 14,000 rpm, 4 °C. Protein quantification was determined by BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific) and the absorbance measured in a microplate reader (SPECTROstar Omega, BMG Labtech). The extracts were stored at −80 °C until analysis. For SDS-PAGE electrophoresis, 15ug of sample was loaded in each lane. The bands were transferred to a nitrocellulose membrane. Blots were blocked using skimmed milk 5% in TBS +0.1% Tween (TBSt), for one hour, at room temperature. Overnight incubation under agitation with anti-AR, ERα, ERβ (1:300, 1:1000, and 1:1000 dilutions, respectively), and GAPDH (mouse monoclonal MB374 Millipore, 1:10,000) primary antibodies, diluted in skimmed milk 1% in TBSt. Subsequently, the membranes were incubated at room temperature for one hour under agitation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 rabbit anti-mouse ab6728, Abcam, Cambridge, UK, or 1:10,000 goat anti-rabbit 115-035-144, Jackson Immunoresearch, Jannersville, PA, USA). Band visualization was performed with ECL system (32,109, Thermo Fischer) using ChemiDoc (ChemiDoc MP, BioRad) followed by quantification with ImageJ. Values were shown as mean of relative density to GAPDH (loading control)

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.00 software spreadsheets and graphs (GraphPad Software, San Diego, CA, USA). First, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was adopted. Differences were considered statistically significant when $p < 0.05$.

Results

GC animals showed lower body weight compared to IC, while CO unchanged (Table 1). Prostate weight decreased in CO compared to IC and GC (Table 1). Prostate relative weight was higher in GC compared to IC and CO (Table1). Serum hormone dosage in the GC group showed sharp decrease in cortisol levels, whereas in CO such reduction was milder (Table 1). Testosterone and estradiol levels unchanged under any condition (Table 1).

The examples of lesions evaluated are depicted in Figure 1. Secretory epithelium showed many regions with stratifications and folds that enter toward the lumen. All experimental groups also showed BPH and PIN, resulting in the irregular appearance of the alveoli (Figure 1). The smooth muscular layer (SML) also showed to be irregular in many regions, notably proximal to the lesions (Figure1). In GC group, the SML thickness and frequency increased compared to the other groups (Table 2). In the CO group these alterations were not observed, exhibiting epithelium length, SML thickness, and frequency of prostatic compartments similar to IC (Table2). Regarding the fibrillar components collagen and elastic fibers, stress induction and coconut oil treatment did not modify the occurrence of these components (Table 2, Figure2).

All experimental groups presented high incidence of lesions, showing 100% of prostatic epithelial hyperplasia (PEH), low-grade PIN (PIN I), and cribriform PIN (PINC) (Figure 3B). However, GC group showed the highest incidence of high-grade PIN (PIN II) with 66.66 % rate and the lowest incidence of atrophy (ATR) with 83.33 % (Figure 3B). On the other hand, the CO had 16.66 % of PIN II and 100 % of ATR similar to IC (Figure 3B). Incidence of microinvasive carcinoma (MC) for GC was 16.66% (Figure 3B). The multiplicity of the different lesions did not differ among the experimental groups (Figure 3A).

The staining patterns were shown in Figure 4 (A, C, E) for P-H-H3 and Figure 4 (B, D, F) for TUNEL. Apoptotic cells frequency in the epithelium and SML was higher in CO compared to IC; however, when each compartment was analyzed separately, apoptotic epithelial cells frequency in CO group increased compared to IC and GC (Figure 4B). SML P-H-H3 positive cells frequency in the GC group was higher than IC, but when combined to epithelium, there was no change in any experimental groups (Figure 4G). Considering SML only, the proliferation index increased in GC and CO (Figure 4H) but when combined to the epithelium or only epithelium itself, IC and GC had indexes close to each other and CO showed lower levels (Figure 4H).

Androgen receptor (AR) expression analysis were shown in Figure 5. GC group showed an increase in AR expression and CO was similar to IC (Figure 5E). AR expression changed particularly in the SML (Figure 5D). Regarding estrogen receptor beta (ERβ) positive cells and protein expression, as presented in figure 6, they did not change in GC and CO groups compared to IC (Figure 6 D and E) whereas estrogen receptor alpha (ERα) positive cells showed higher frequency in GC compared to IC particularly in the epithelial compartment (Figure 7D). CO group showed no change in the expression of these receptors (Figure 7).

Discussion

Prostate is highly susceptible to the aging and may respond differently to this process, leading to BPH, prostate enlargement, and prostate cancer progression [24]. An experimental model for studying these aging-related alterations is the Mongolian gerbil which exhibits many morphological changes in the prostate throughout the life, and in advanced age the presence of BPH and microinvasive carcinoma is often observed [18]. Despite of Mongolian gerbil has been considered an adaptable model for laboratory handling, and levels of stress markers normalize after a one-week adaptation period [25], this is the first study to report the long-term manipulation caused stress and impacted the aging process.

Laboratory routines may influence stress levels in animals [26]. Although the particularity of the gavage content may interfere with stress levels [27], the gavage itself is considered a stress inducer [28]. In addition to stress, long-term gavage can induce weight loss in mice [29], as we observed in GC group. Stress can be classified as acute or chronic based on the exposure duration to the stressor agent; the chronic stress-related conditions are time-dependent and can generate varied hypothalamic-pituitary-adrenal (HPA) axis responses [30].

Cortisol level is an endocrine stress marker, since the stress condition, acute or chronic, interferes in the HPA axis affecting its [16]. In addition, different stress conditions, can interfere in other neuroendocrine pathways and regulate glucocorticoid levels leading to different cortisol levels depending on the stressor [31]. Low cortisol level, presented by the GC group, corroborates with Fries et. al. who proposes hypocortisolism in rats because of chronic stress. Therefore, we consider that changes in cortisol levels and body weight of CG animals indicated that long-term handling promoted a state of chronic stress.

Given this scenario, it is interesting that coconut oil treatment during gavage attenuated the impact of stress on cortisol levels and animals' weight. Coconut oil has higher saturation status since around 90% of its composition is made of saturated fatty acids, being the lauric acid the most abundant, followed by myristic, palmitic, caprylic, capric, and stearic acids [32]. This oil has been reported to interfere in the HPA axis and attenuate neuroendocrine responses to stressors, such as increased or decreased cortisol levels [14, 33].

Coconut oil was shown to decrease the weight of hyperplasic prostate in rats [13], but our data show that regarding the relative weight, only the GC group showed a decrease in the weight of this gland. Although the decrease in prostate weight in CO can be due to anatomical variation, the effect of stress on relative prostate weight did not occur in the CO. The morphological characteristics of the Mongolian gerbil prostate are well established [18, 34, 35] and typically composed by alveoli, commonly referred as acini, formed by secretory epithelium that limits the luminal region where the secretion is stored. Surrounding the acinus there is the SML and between the acini, there is the stroma [34]. Prostate is susceptible to changes and gerbil prostate is highly responsive to variations in androgen and estrogen levels, reflecting in the gland secretory activity [36–38]. The gerbil prostate is also susceptible to interference from treatments using oils such as corn and mineral oil [39]. One of the morphological responses is the SML thickness increase and alteration in prostatic compartments frequency [36, 37, 40]. In the present study, we observed such effect in GC group which was attenuated by coconut oil treatment.

Alterations in cellular and extracellular matrix and their consequent increase are related with tumor progression [41]. Several studies showed that variations in SML were related with smooth muscle cells activity and a change in the extracellular matrix [42–44], and older animals have reactive stroma associated with malignant prostatic lesions [45]. Specially, the increased in collagen synthesis and remodeling provide a pro-tumoral environment [46, 47], which were not observed in GC and CO.

During aging, testosterone release is decreased and prostate responds with pronounced morphological changes [17] which is also observed for the Mongolian gerbil that exhibits prostatic lesions, including BPH and carcinoma, spontaneously at advanced age [18]. Our data corroborate the literature, since all old animals, presented lesions such as BPH and PIN at distinct grades. However, chronic stress seemed to intensify their gravity because high-grade PIN incidence raised, as well as the incidence of adenocarcinoma in GC. In breast tumors, chronic stress represents a risk factor for the development of more aggressive forms [48]. Psychological stress associated with aging modulates prostate cancer responses via changes in the inflammatory status [49]. Stress interferes in the autonomic nervous system (ANS), the HPA axis, and other neuroendocrine systems such as the reproductive axis, growth, and thyroid axis, as well as affecting the immune system [50]. Increased autonomous innervation of the prostate has been linked to prostate cancer progression [51]. These findings suggest that stress can induce BPH by interfering in regulatory systems, such as the HPA axis [9, 52]. Furthermore, inhibition of adrenergic receptors, responsible for receiving and transmitting signals of sympathetic and parasympathetic origin, led to higher rate of apoptosis in both benign and malignant conditions in the prostate [53].

Proliferative and apoptosis rate is directly related with prostatic lesions development and aggressiveness in gerbils and other animals [18]. Considering the potential action of stress as a lesion enhancer, GC group higher proliferation rate in the SML and thickness, taken together, it suggests that this prostate compartment is more susceptible to stress. Prostate stromal components, cells and extracellular matrix, are responsive to hormone levels variations and their receptors expression as well as growth factors [54–57], but the reasons for the greater responsiveness of this compartment to stress are still unknown. In contrast, coconut oil treatment indicated same proliferation rate compared with CG group but higher apoptosis rate when considered either the epithelium and SML together or the former alone, suggesting the coconut oil antiproliferative function. Its major component, lauric acid, has been reported as an anti-proliferative agent and apoptosis inducer in breast and endometrial cancer [58]. The stroma plays a critical role in mediating prostate carcinogenesis through AR and ERα, and the paracrine mechanisms of androgens and estrogens [41].

The prostate is an androgen-dependent gland since its development and differentiation as well as for its maintenance in adulthood, being mediated via AR. However, estrogens also influence prostate homeostasis, since it expresses both ERα and ERβ [59]. Chronic stress increased AR expression most remarkably in SML, while treatment with coconut oil maintained it similar to the intact control group. AR expression is directly related with the prostate cell proliferation [60]. In the Mongolian gerbil, under normal conditions, this receptor has a uniform immunolocalization between epithelium and stroma, but this location standard can be modified by genetic and epigenetic mechanisms and yet unknown factors [61]. Our data indicated that stress might be a candidate for one among them and coconut oil interferes on it.

Although ERβ expression remained unaltered, the ERα was shown to be responsive to stress. Estrogenic action within the prostate is complex, and both were reported to play different roles. ERα is related with the development and increase of proliferation, inflammation, and cancer, while ERβ has anti-proliferative, anti-inflammatory, and anticarcinogenic potential [62]. ERα expression herein consolidated our hypothesis that stress plays a pro-lesional role since GC group showed a higher frequency of ERα-positive cells. Interestingly, their immunolocalization was different from that of AR-positive cells. Studies regarding the localization of AR, ERα and ERβ expression in the gerbil ventral prostate suggest that the former is expressed in both the epithelium and SML, ERβ is more found in the epithelium, while ERα is in the SML [61, 63].

Conclusion

As expected, prostate from older gerbils showed BPH and PINs which are aging-related alterations. However, the long-term gavage procedure provided a situation of chronic stress that seemed to enhance their progression likely the microinvasive carcinoma development. On the other hand, coconut oil treatment throughout aging, minimized the chronic stress impacts. Although more studies are needed to elucidate the key mechanisms involved our results are provocative, especially because of changes in morphology and in the receptors expression, mainly in the stroma. Stromal alterations are common in older animals [45] and may drive malignant transformation in human prostatic epithelial cells [41]. Therefore, the effectiveness of a natural product, such as coconut oil, to mitigate the chronic stress impact on prostate along aging caught attention for further studies regarding this issue.

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References

1. United Nations Department of Economic and Social Affairs Population Division (2019) World Population Ageing 2019

2. Flatt T, Partridge L (2018) Horizons in the evolution of aging. BMC Biol 16:1–13. https://doi.org/10.1186/s12915-018-0562-z

3. Fiorito G, McCrory C, Robinson O, et al (2019) Socioeconomic position, lifestyle habits and biomarkers of epigenetic aging: a multi-cohort analysis. Aging (Albany NY) 11:2045–2070. https://doi.org/10.18632/aging.101900

4. Révész D, Verhoeven JE, Milaneschi Y, et al (2014) Dysregulated physiological stress systems and accelerated cellular aging. Neurobiol Aging 35:1422–1430. https://doi.org/10.1016/j.neurobiolaging.2013.12.027

5. Prather AA, Epel ES, Arenander J, et al (2015) Longevity factor klotho and chronic psychological stress. Transl Psychiatry 5: e585-6. https://doi.org/10.1038/tp.2015.81

6. Thomas D, Chughtai B, Kini M, Te A (2017) Emerging drugs for the treatment of benign prostatic hyperplasia. Expert Opin Emerg Drugs 22:201–212. https://doi.org/10.1080/14728214.2017.1369953

7. Patel ND, Parsons JK (2014) Epidemiology and etiology of benign prostatic hyperplasia and bladder outlet obstruction. Indian J Urol 30:170–176. https://doi.org/10.4103/0970-1591.126900

8. La Vignera S, Condorelli RA, Russo GI, et al (2016) Endocrine control of benign prostatic hyperplasia. Andrology 4:404–411. https://doi.org/10.1111/andr.12186

9. Ullrich PM, Lutgendorf SK, Kreder KJ (2007) Physiologic Reactivity to a Laboratory Stress Task Among Men with Benign Prostatic Hyperplasia. Urology 70:487–491. https://doi.org/10.1016/j.urology.2007.04.048

10. Huang S, Fang X, Meng Y, et al (2009) Sympathetic nervous system overactivity in the wistar rat with proliferative lesions of ventral prostate induced by chronic stress. Urol Int 83:230–235. https://doi.org/10.1159/000230030

11. Stone AA, Mezzacappa ES, Donatone BA, Gonder M (1999) Psychosocial stress and social support are associated with prostate-specific antigen levels in men: Results from a

community screening program. Heal Psychol 18:482–486. https://doi.org/10.1037//0278-6133.18.5.482

12. Keehn A, Taylor J, Lowe FC (2016) Phytotherapy for Benign Prostatic Hyperplasia. Curr Urol Rep 17:1–6. https://doi.org/10.1007/s11934-016-0609-z

13. de Lourdes Arruzazabala M, Molina V, Más R, et al (2007) Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. J Pharm Pharmacol 59:995–9. https://doi.org/10.1211/jpp.59.7.0012

14. Nemeth M, Schuster D, Millesi E, Wallner B (2021) Dietary fatty acids modulate cortisol concentrations and social dominance during social confrontations in adolescent male guinea pigs. Psychoneuroendocrinology 123:105045. https://doi.org/10.1016/j.psyneuen.2020.105045

15. Borsonelo EC, Suchecki D, Galduróz JCF (2011) Effect of fish oil and coconut fat supplementation on depressive-type behavior and corticosterone levels of prenatally stressed male rats. Brain Res 1385:144–150. https://doi.org/10.1016/j.brainres.2011.02.043

16. Gray JM, Chaouloff F, Hill MN (2015) To stress or not to stress: A question of models. Curr Protoc Neurosci 2015:8.33.1-8.33.22. https://doi.org/10.1002/0471142301.ns0833s70

17. Pegorin de Campos SG, Zanetoni C, Góes RM, Taboga SR (2006) Biological behavior of the gerbil ventral prostate in three phases of postnatal development. Anat Rec Part A, Discov Mol Cell Evol Biol 288:723–733. https://doi.org/10.1002/ar.a.20347

18. Campos SGP, Zanetoni C, Scarano WR, et al (2008) Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. Int J Exp Pathol 89:13–24. https://doi.org/10.1111/j.1365-2613.2007.00550.x

19. Reagan-Shaw S, Nihal M, Ahmad N (2007) Dose translation from animal to human studies revisited. FASEB J 22:659–661. https://doi.org/10.1096/fj.07-9574LSF

20. WEIBEL ER (1963) Principles and methods for the morphometric study of the lung and other organs. Lab Invest 12:131—155

21. Shappell SB, Thomas G V, Roberts RL, et al (2004) Prostate Pathology of Genetically Engineered Mice: Definitions and Classification. The Consensus Report from the Bar Harbor

Meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res 64:2270–2305. https://doi.org/10.1158/0008-5472.CAN-03-0946

22. Gonçalves BF, de Campos SGP, Zanetoni C, et al (2013) A new proposed rodent model of chemically induced prostate carcinogenesis: distinct time-course prostate cancer progression in the dorsolateral and ventral lobes. Prostate 73:1202–1213. https://doi.org/10.1002/pros.22669

23. Gonçalves BF, Zanetoni C, Scarano WR, et al (2010) Prostate carcinogenesis induced by N-methyl-N-nitrosourea (mnu) in gerbils: histopathological diagnosis and potential invasiveness mediated by extracellular matrix components. Exp Mol Pathol 88:96–106. https://doi.org/10.1016/j.yexmp.2009.09.017

24. Sampson N, Untergasser G, Plas E, Berger P (2007) The ageing male reproductive tract. J Pathol 211:206–218. https://doi.org/10.1002/path.2077

25. Liu X, Zheng X, Liu Y, et al (2019) Effects of adaptation to handling on the circadian rhythmicity of blood solutes in Mongolian gerbils. Anim Model Exp Med 127–131. https://doi.org/10.1002/ame2.12068

26. Balcombe JP, Barnard ND, Sandusky C (2004) Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 43:42–51

27. Brown AP, Dinger N, Levine BS (2000) Stress Produced by Gavage Administration in the Rat. Contemp Top Lab Anim Sci 39:17–21

28. Bonnichsen M, Dragsted N, Kornerup Hansen A (2005) The welfare impact of gavaging laboratory rats. Anim Welf 14:223–227

29. de Meijer VE, Le HD, Meisel JA, Puder M (2010) Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. Physiol Behav 100:387–393. https://doi.org/10.1016/j.physbeh.2010.04.001

30. Miller GE, Chen E, Zhou ES (2007) If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychol Bull 133:25–45. https://doi.org/10.1037/0033-2909.133.1.25

31. Gong S, Miao YL, Jiao GZ, et al (2015) Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. PLoS One 10:1–14. https://doi.org/10.1371/journal.pone.0117503

32. Moreau RA (2011) Corn Oil. In: Vegetable Oils in Food Technology. Wiley-Blackwell, Oxford, UK, pp 273–289

33. Nemeth M, Millesi E, Puehringer-Sturmayr V, et al (2016) Sex-specific effects of dietary fatty acids on saliva cortisol and social behavior in Guinea pigs under different social environmental conditions. Biol Sex Differ 7:1–15. https://doi.org/10.1186/s13293-016-0107-5

34. Rochel SS, Bruni-Cardoso A, Taboga SR, et al (2007) Lobe identity in the Mongolian gerbil prostatic complex: a new rodent model for prostate study. Anat Rec (Hoboken) 290:1233–1247. https://doi.org/10.1002/ar.20585

35. Dos Santos FCA, Carvalho HF, Góes RM, Taboga SR (2003) Structure, histochemistry and ultrastructure of the epithelium and stroma in the gerbil (Meriones unguiculatus) female prostate. Tissue Cell 35:447–457. https://doi.org/10.1016/S0040-8166(03)00071-5

36. Oliveira SM, Leite Vilamaior PS, Corradi LS, et al (2007) Cellular and extracellular behavior in the gerbil (Meriones unguiculatus) ventral prostate following different types of castration and the consequences of testosterone replacement. Cell Biol Int 31:235–245. https://doi.org/10.1016/j.cellbi.2006.10.006

37. Scarano WR, de Sousa DE, Campos SGP, et al (2008) Oestrogen supplementation following castration promotes stromal remodelling and histopathological alterations in the Mongolian gerbil ventral prostate. Int J Exp Pathol 89:25–37. https://doi.org/10.1111/j.1365-2613.2007.00559.x

38. Perez APS, Biancardi MF, Vilamaior PSL, et al (2012) Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. Microsc Res Tech 75:1084–1092. https://doi.org/10.1002/jemt.22034

39. Guerra LHA, Tamarindo GH, de Campos SGP, et al (2019) Do mineral and corn oil serve as potential endocrine disruptors in the gerbil prostate? Reprod Toxicol 90:141–149. https://doi.org/10.1016/j.reprotox.2019.09.004

40. Biancardi MF, Perez APS, Góes RM, et al (2012) Prenatal testosterone exposure as a model for the study of endocrine-disrupting chemicals on the gerbil prostate. Exp Biol Med 237:1298–1309. https://doi.org/10.1258/ebm.2012.012051

41. Cunha GR, Hayward SW, Wang YZ, Ricke WA (2003) Role of the stromal microenvironment in carcinogenesis of the prostate. Int J Cancer 107:1–10. https://doi.org/10.1002/ijc.11335

42. Horsfall DJ, Mayne K, Ricciardelli C, et al (1994) Age-related changes in guinea pig prostatic stroma. Lab Invest 70:753–763

43. Corradi LS, Góes RM, Carvalho HF, Taboga SR (2004) Inhibition of 5-alpha-reductase activity induces stromal remodeling and smooth muscle de-differentiation in adult gerbil ventral prostate. Differentiation 72:198–208. https://doi.org/10.1111/j.1432-0436.2004.07205004.x

44. Vilamaior PSL, Taboga SR, Carvalho HF (2005) Modulation of smooth muscle cell function: Morphological evidence for a contractile to synthetic transition in the rat ventral prostate after castration. Cell Biol Int 29:809–816. https://doi.org/10.1016/j.cellbi.2005.05.006

45. Montico F, Kido LA, San Martin R, et al (2015) Reactive stroma in the prostate during late life: The role of microvasculature and antiangiogenic therapy influences. Prostate 75:1643–1661. https://doi.org/10.1002/pros.23045

46. Provenzano PP, Eliceiri KW, Campbell JM, et al (2006) Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Med 4:1–15. https://doi.org/10.1186/1741-7015-4-38

47. Bauman TM, Nicholson TM, Abler LL, et al (2014) Characterization of fibrillar collagens and extracellular matrix of glandular benign prostatic hyperplasia nodules. PLoS One 9:1–9. https://doi.org/10.1371/journal.pone.0109102

48. Cormanique TF achi., Almeida LE liz. DF erret. de, Rech CA lb., et al (2015) Chronic psychological stress and its impact on the development of aggressive breast cancer. Einstein (Sao Paulo) 13:352–356. https://doi.org/10.1590/S1679-45082015AO3344

49. Bellinger DL, Dulcich MS, Molinaro C, et al (2021) Psychosocial Stress and Age Influence Depression and Anxiety-Related Behavior, Drive Tumor Inflammatory Cytokines and Accelerate Prostate Cancer Growth in Mice. Front Oncol 11:1–20. https://doi.org/10.3389/fonc.2021.703848

50. Chrousos GP, Gold PW (1992) The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis. JAMA J Am Med Assoc 267:1244–1252. https://doi.org/10.1001/jama.1992.03480090092034

51. Bauman J, McVary K (2013) Autonomic nerve development contributes to prostate cancer progression. Asian J Androl 15:713–714. https://doi.org/10.1038/aja.2013.113

52. Pond DA, Maratos J (1977) Psychosocial inter-relations of benign prostatic hypertrophy. J Psychosom Res 21:201–206. https://doi.org/10.1016/0022-3999(77)90091-5

53. Kyprianou N, Chon J, Benning CM (2000) Effects of alpha (1)-adrenoceptor (alpha(1)-AR) antagonists on cell proliferation and apoptosis in the prostate: therapeutic implications in prostatic disease. Prostate Suppl 9:42–46. https://doi.org/10.1002/1097-0045(2000)45:9+<42: aid-pros9>3.0.co;2-u

54. Tanner MJ, Welliver RC, Chen M, et al (2011) Effects of androgen receptor and androgen on gene expression in prostate stromal fibroblasts and paracrine signaling to prostate cancer cells. PLoS One 6: https://doi.org/10.1371/journal.pone.0016027

55. Antonioli E, Cardoso AB, Carvalho HF (2007) Effects of long-term castration on the smooth muscle cell phenotype of the rat ventral prostate. J Androl 28:777–783. https://doi.org/10.2164/jandrol.107.002873

56. Leach DA, Need EF, Toivanen R, et al (2015) Stromal androgen receptor regulates the composition of the microenvironment to influence prostate cancer outcome. Oncotarget 6:16135–16150. https://doi.org/10.18632/oncotarget.3873

57. Kajiwara S, Ishii K, Sasaki T, et al (2020) Castration-induced stromal remodeling disrupts the reconstituted prostate epithelial structure. Lab Investig 100:670–681. https://doi.org/10.1038/s41374-019-0352-4

58. Lappano R, Sebastiani A, Cirillo F, et al (2017) The lauric acid-activated signaling prompts apoptosis in cancer cells. 1–9. https://doi.org/10.1038/cddiscovery.2017.63

59. McPherson SJ, Ellem SJ, Risbridger GP (2008) Estrogen-regulated development and differentiation of the prostate. Differentiation 76:660–670. https://doi.org/10.1111/j.1432-0436.2008.00291.x

60. Culig Z, Santer FR (2014) Androgen receptor signaling in prostate cancer. Cancer Metastasis Rev 413–427. https://doi.org/10.1007/s10555-013-9474-0

61. Cordeiro RS, Scarano WR, Campos SGP, et al (2008) Androgen receptor in the Mongolian gerbil ventral prostate: Evaluation during different phases of postnatal development and following androgen blockage. Micron 39:1312–1324. https://doi.org/10.1016/j.micron.2008.02.008

62. Ellem SJ, Risbridger GP (2009) The dual, opposing roles of estrogen in the prostate. Ann N Y Acad Sci 1155:174–186. https://doi.org/10.1111/j.1749-6632.2009.04360.x

63. Rochel-Maia SS, Santos FCA, Alonso-Magdalena P, et al (2013) Estrogen Receptors Alpha and Beta in Male and Female Gerbil Prostates1. Biol Reprod 88:1–7. https://doi.org/10.1095/biolreprod.112.103614

Tables:

Table 1. Biometric and endocrine parameters of Mongolian gerbils in the intact control (IC), gavage control (GC) and coconut oil (CO) groups.

	Experimental groups		
	IC	GC	CO
Biometric data (g)			
Body weight	101.8 ± 13.85^{a}	91.98 ± 13.23^{b}	96.84 ± 14.69^{a,b}
Prostate weight	0.8966 ± 0.1232^{a}	0.8978 ± 0.1083^{a}	0.7947 ± 0.1103^{b}
Relative prostate weight x10^{-2}	0.8897 ± 0.1317^{a}	0.9803 ± 0.1141^{b}	0.8327 ± 0.1410^{a}
Hormonal data			
Cortisol (ng/mL)	136.5 ± 17.83^{a}	31.48 ± 22.10^{b}	89.70 ± 33.92^{c}
Testosterone (ng/mL)	1.85 ± 0.66	1.79 ± 0.42	1.8 ± 0.51
17β-estradiol (pg/mL)	35.67 ± 9.44	59.47 ± 33.96	40.20 ± 17.96

Values expressed as mean ± standard deviation. Superscript a and b represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

Table 2: Morphological parameters of Mongolian gerbil in the different experimental groups.

	Experimental groups		
	IC	GC	CO
Morphometric data (μm)			
Epithelium height	18.26 ± 1.65	20.58 ± 4.11	17.71 ± 1.57
SMLthickness	11.66 ± 1.01^{a}	14.01 ± 1.04^{b}	11.45 ± 1.16^{a}
Stereological data (%)			
Epithelium	22.31 ± 6.43	25.33 ± 10.50	18.49 ± 1.17
Lumen	48.10 ± 12.79	44.26 ± 11.76	53.54 ± 6.65
SML	$10.51 \pm 2.40^{a,b}$	15.90 ± 7.60^{b}	8.71 ± 2.07^{a}
Stroma	18.00 ± 6.65	14.92 ± 3.67	18.42 ± 6.49
Blood vessels	1.07 ± 0.61	2.15 ± 1.93	0.83 ± 0.58
Stromal fibers data (%)			
Collagen fibers	37.21 ± 9.14	42.52 ± 4.41	33.65 ± 6.49
Elastic fibers	0.28 ± 0.04	0.30 ± 0.02	0.30 ± 0.02

Values expressed as mean ± standard deviation. Superscript a and b represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

Figures:

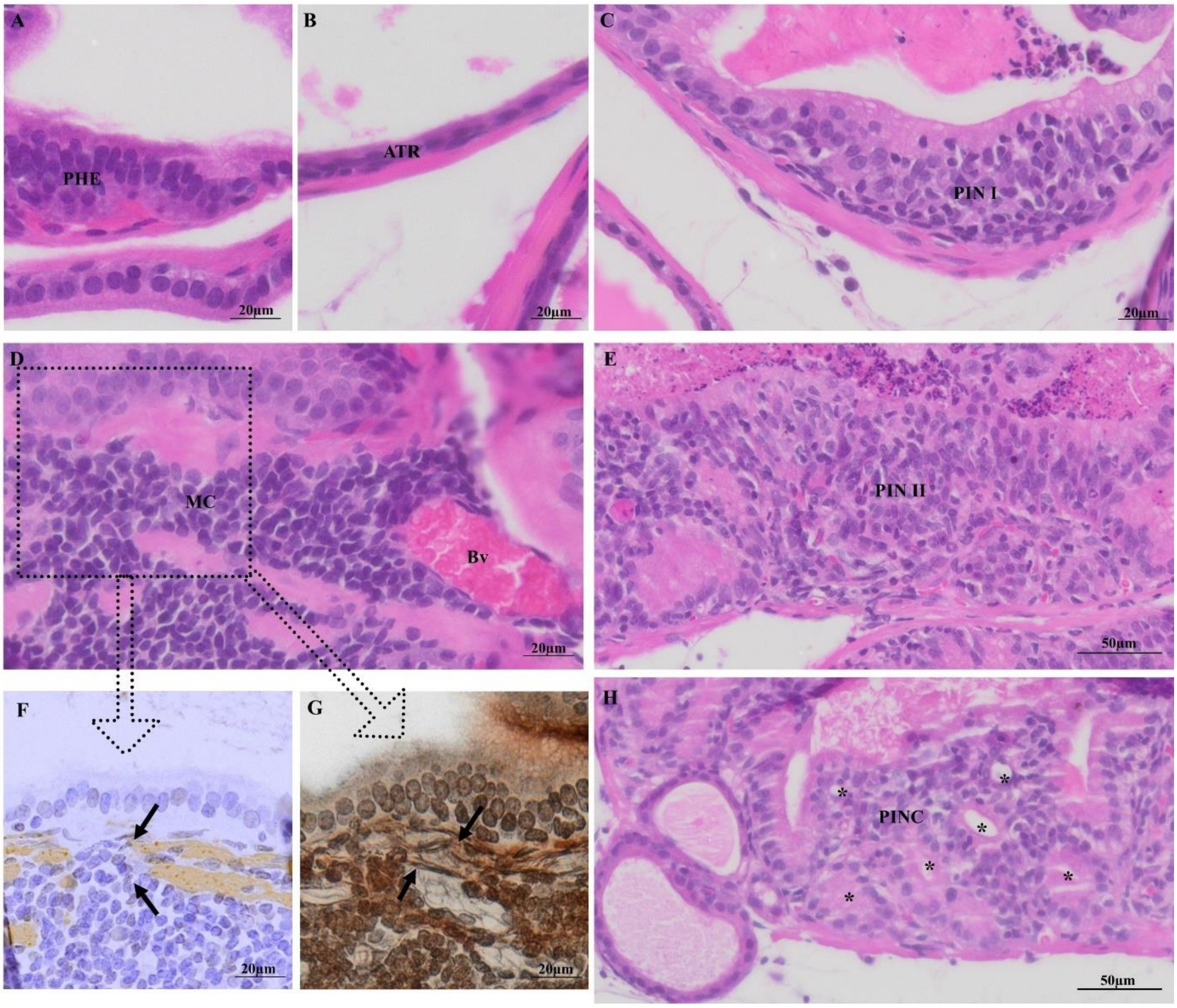

Figure 1: Lesions observed in the ventral prostate of old gerbils stained with HE (A, B, C, D, E and H), with Gomori's reticulin (G) and marking for a-actin (F). Prostatic epithelial hyperplasia - PEH (A); Epithelial atrophy - ATR (B); Grade I intraepithelial neoplasia - PIN I (C); Microinvasive carcinoma - CM (D); Grade II intraepithelial neoplasia - PIN II (E); Cribriform intraepithelial neoplasia - PINC (H) with micro- lumens (*). The region delimited by the dotted line in D is depicted in F and G. Arrows point to rupture of smooth muscle layer in F and to rupture of reticular fibers in G. Bv indicates blood vessel.

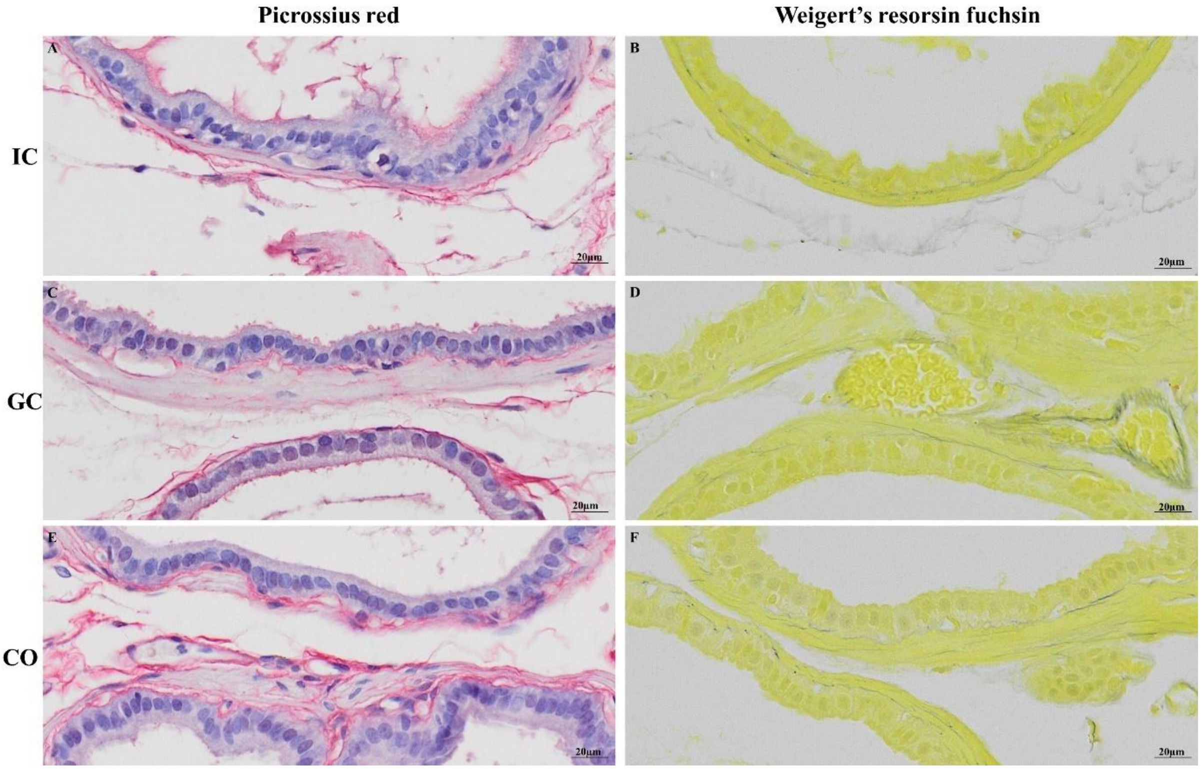

Figure 2: Fibrillar components to prostatic tissues from IC (A, B), GC (C, D) and CO (E, F) groups. Picrosirius staining identifies collagen in red shade in A, C and E. Resorcin-fuchsin staining identifies elastic fibers in blue in B, D, and F.

A

	Multiplicity		
	IC	**GC**	**CO**
Lesions			
PHE	18-76 (48.5)	4-61 (39.83)	30-69 (46.33)
PIN I	1-11 (6.33)	4-15 (10)	4-13 (8.67)
PIN II	0-2 (0.33)	0-37 (7.33)	0-1 (0.16)
PINC	6-73 (27.33)	4-60 (27.17)	15-52 (25.5)
ATR	2-33 (15.33)	0-23 (13)	2-44 (22)
CM	0 (0)	0-3 (0.66	0 (0)

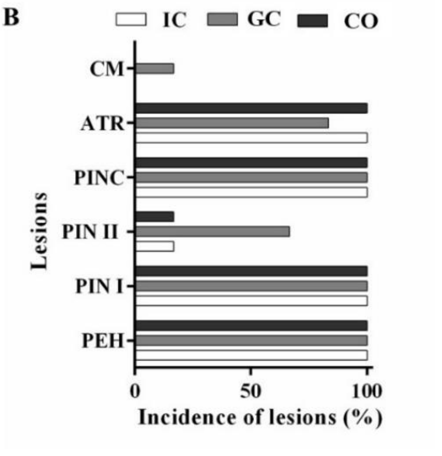

Figure 3: Analysis of incidence (B) and multiplicity (A) of lesions of the ventral prostate of the Mongolian Gerbil. Intact Control (IC), Gavage Control (GC) or Coconut Oil (CO) groups. Multiplicity data (A) correspond to the variation in the number of lesions per animal (n= 6 per group); the group mean is shown in parenthesis. Incidence data (B) indicate the percentage of animals affected. Prostatic epithelial hyperplasia (PEH); Grade I intraepithelial neoplasia (PIN I); Grade II intraepithelial neoplasia (PINII); Cribriform intraepithelial neoplasia (PINC); Epithelial atrophy (ATR) and Microinvasive carcinoma (CM).

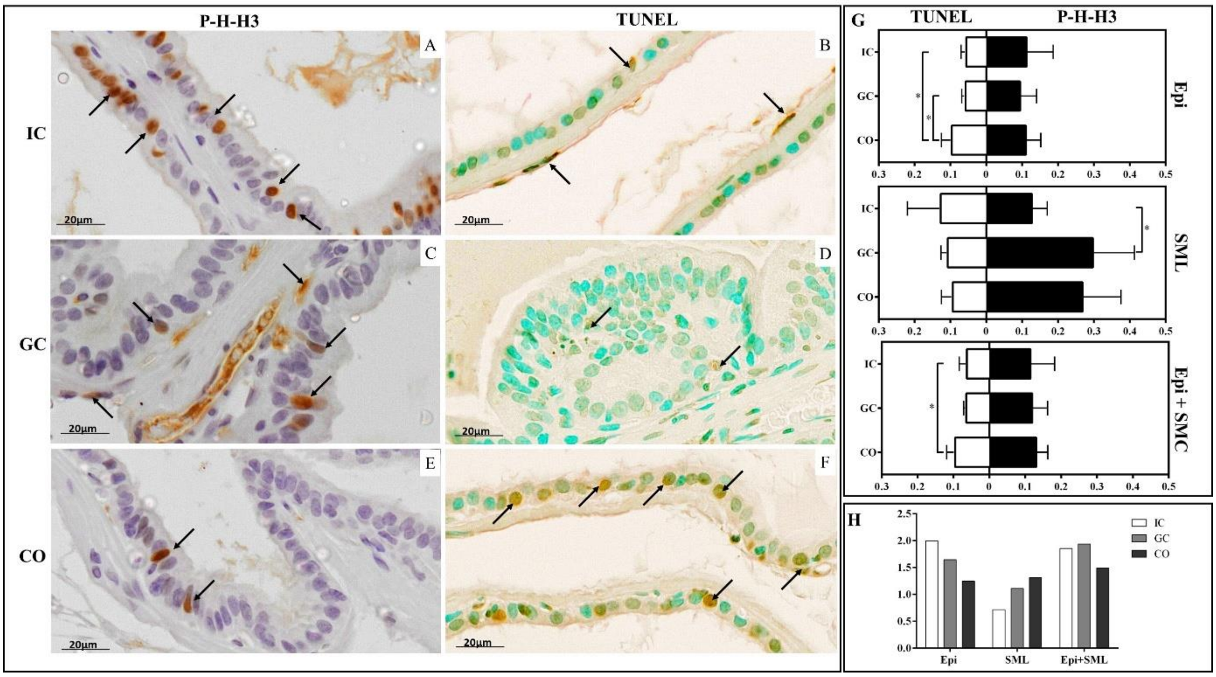

Figure 4: Immunohistochemistry for cell proliferation index data using P-H-H3 (A, C and E) for proliferating cells and TUNEL marking (B, D, and F) for apoptotic cells. In G are shown the frequency of cells positive for TUNEL (white columns) and P-H-H3 (black columns) in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML). * indicates statistical difference $p \leq 0.05$. In H is displayed the proliferation index (P-H-H3/TUNEL) of the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML). Arrows point to positive cells.

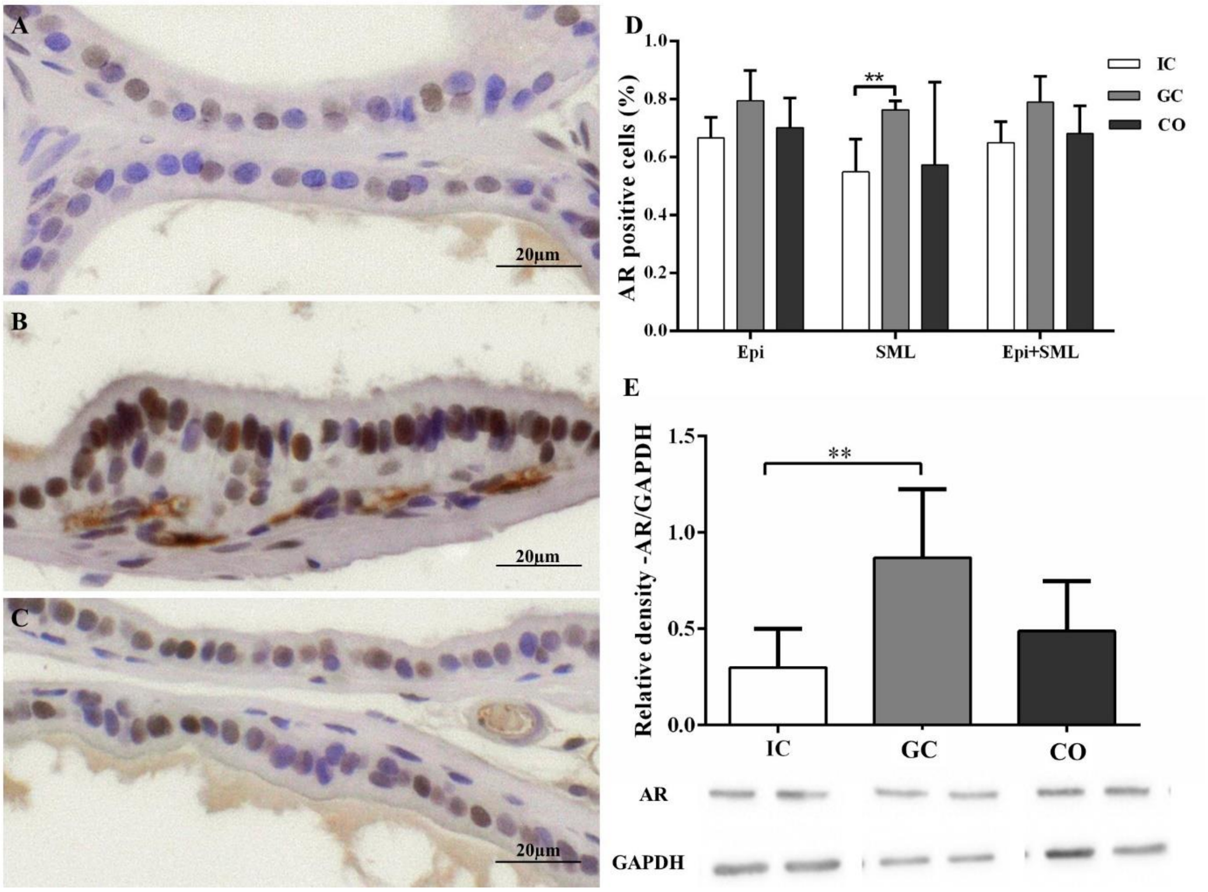

Figure 5: Immunohistochemistry for androgen receptor (AR) in the ventral prostate of Mongolian gerbils in intact control (A), gavage control (B) and coconut oil-treated (C) groups. Frequency of cells positive for AR in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) (D). Relative density of AR normalized to GAPDH, used here as a positive control (E). ** indicates statistical difference $p \leq 0.01$.

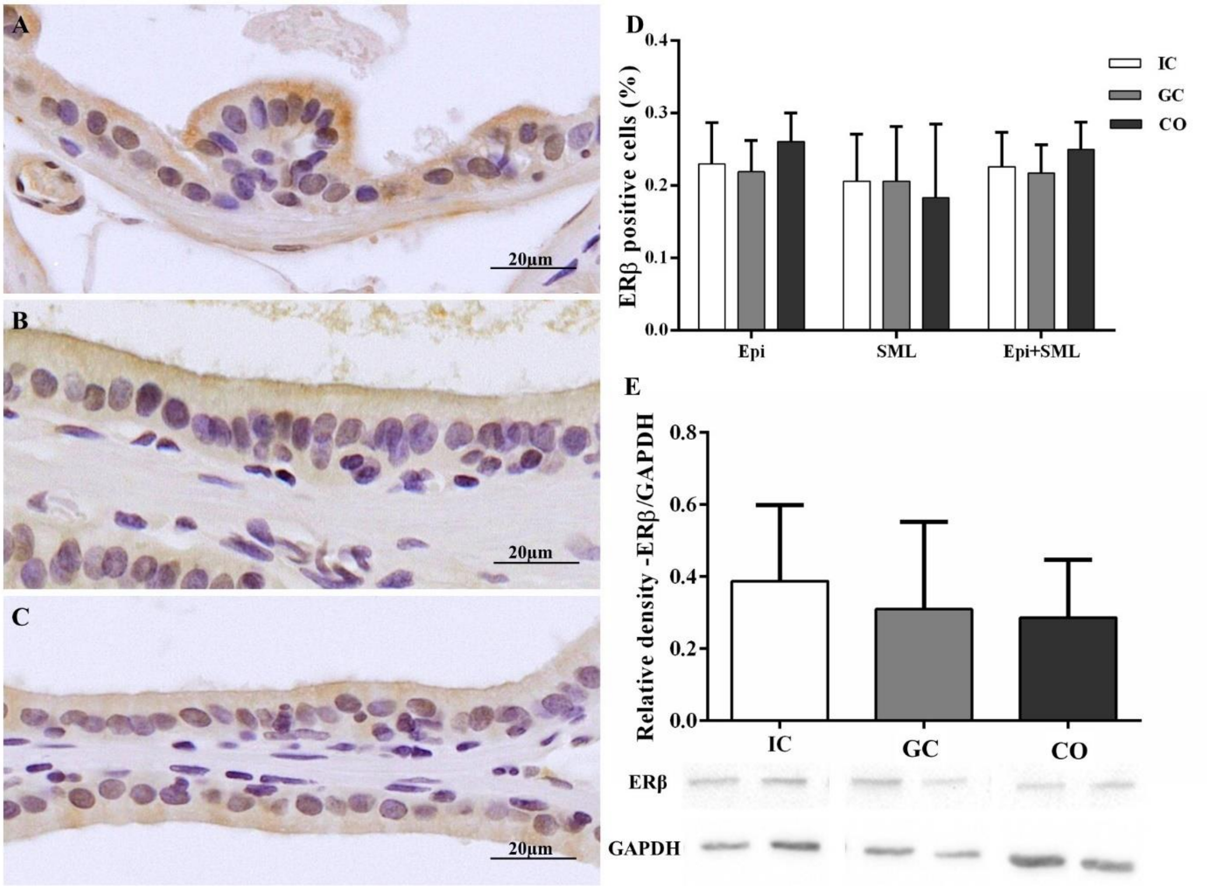

Figure 6: Immunohistochemistry for estrogen receptor beta (ERβ) in the ventral prostate of Mongolian gerbils in intact control (A), gavage control (B), and coconut oil-treated (C) groups. Frequency of cells positive for ERβ in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) (D). Relative density of ERβ normalized to GAPDH, used here as a positive control (E). There was no statistically significant difference between the groups.

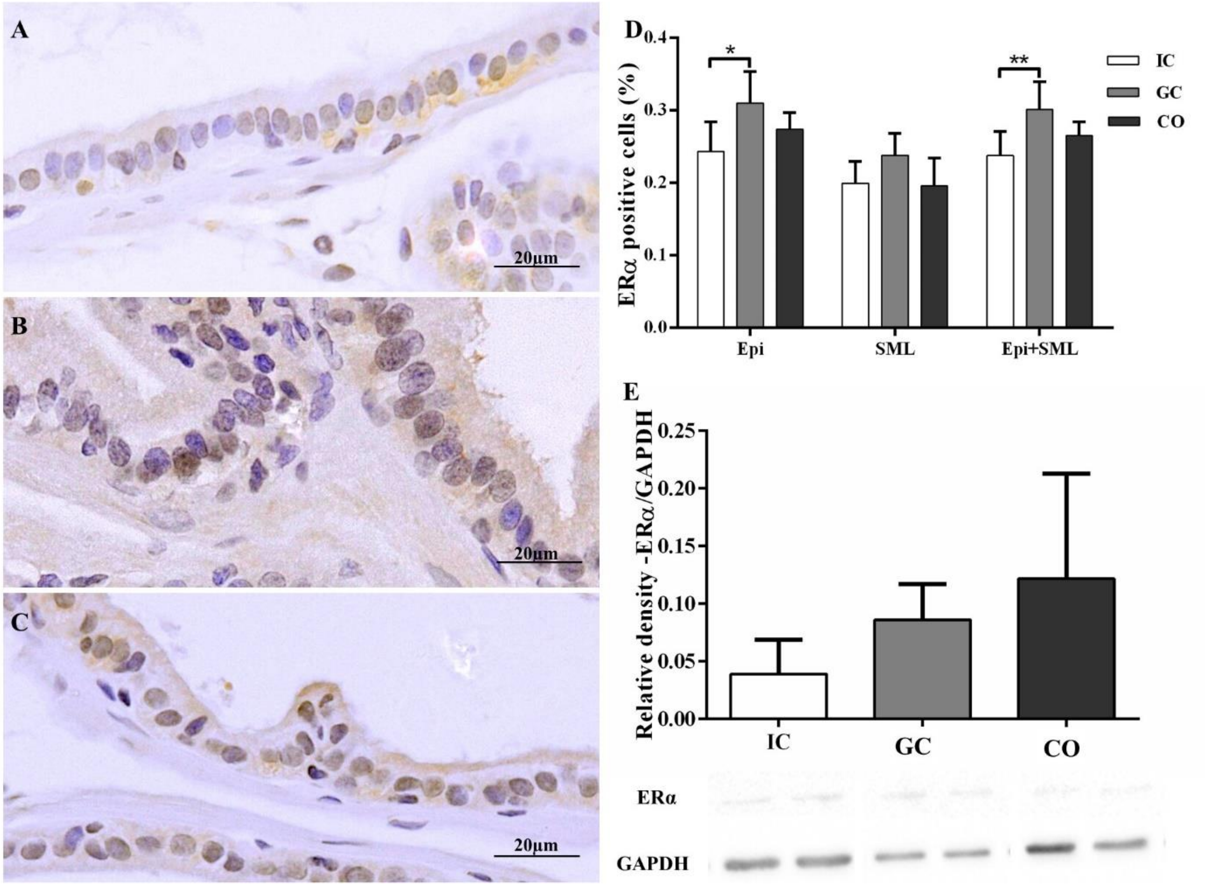

Figure 7: Immunohistochemistry for estrogen receptor alpha (ERα) in the ventral prostate of Mongolian gerbils in intact control (A), gavage control (B), and coconut oil-treated (C) groups. Frequency of cells positive for ERα in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) (D). Relative density of ERα normalized to GAPDH, used here as a positive control (E). There was no statistically significant difference between the groups. Statistical difference between the experimental groups is represented by * ($p \leq 0.05$) and ** ($p \leq 0.01$).

5.2 Capítulo 2 – Artigo 2.

Será submetido à revista “The Prostate”

Coconut oil attenuates the effects of the stress of animal handling on prostatic inflammation in old gerbils.

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Keywords: Phytotherapeutic, inflammatory cells, macrophage, vegetal oil, STAT3.

Abstract

Background

Inflammation is associated with age-related pathologies in the prostate, such as benign prostatic hyperplasia (BPH) and prostate cancer (PC), which are modulated by several components of the inflammatory process, like macrophages and cytokines. Coconut oil is indicated as a protective agent in age-related effects, including inflammation. In the present study, we have investigated the impact of coconut oil consumption and animal handling on the prostate throughout the aging process

Methods

Animals (Mongolian gerbil, 100 days old) were divided into three groups (n=11/group): Intact Control (IC group), Gavage Control (GC group) and Coconut Oil (CO group). The animals received via gavage 0.1ml of water (GC) or coconut oil (CO) on alternate days during one year. Inflammatory foci, mast cells, F4/80 and CD68 and CD163 macrophages, COX2, p-STAT3, IL-6, TNFα and metalloproteinases (MMPs) were analyzed.

Results

Inflammatory foci (IF) were present in the ventral prostate of all experimental groups. The occurrence of F4/80 and CD68 macrophages did not vary between the groups, whereas CD163 macrophages decreased in the CO epithelium. The presence of p-STAT3 decreased in the epithelium and overall prostate in the CO, but increased in the GC stroma. The presence of COX2, and the levels of IL-6 did not change. TNFα concentration increased in the GC, but did not change in the CO. The GC group also showed increased expression of MMP-2 and MMP-3.

Conclusions

Long-term animal handling changed the inflammatory profile in the prostate of the aged gerbils, while the administration of coconut oil throughout aging attenuated these effects.

Introduction

The prostate is a gland responsive to the aging process, and more than half of the population of men over fifty years old develops benign prostatic hyperplasia (BPH).[1] The aging process also changes the prostate inflammatory status, providing a microenvironment favorable to prostate cancer development and progression (PC).[2] Prostatic inflammation can cause infertility in young and adult men.[3] BPH is closely associated with inflammation, since several specific mediators of inflammatory pathways, such as interleukins and chemokines, modulate the condition of BPH.[4] In addition, the activity of inflammatory cells, such as mast cells and macrophages, with their different phenotypes, plays an important role in both BPH and PC.[4,5] These cells and the diverse inflammatory cytokines are found mainly in the stroma, which exhibits characteristics, acquired through aging, that contribute to the development of prostatic pathologies.[6] The prostate environment is thus sensitive to inflammation-related disorders that lead to hyper- or neoplastic processes.[7]

The use of natural compounds as phytotherapeutic agents, with mechanisms of action including inhibition of growth factors, anti-androgenic, anti-estrogenic, and anti-inflammatory activity is expanding.[8] In this scenario, studies have attributed antioxidant properties to coconut oil due to its protective role against injuries.[9–11] Coconut oil, obtained from the Cocos nucifera, is an emerging functional food commonly used as a traditional medicine and dietary supplement in South India and many other tropical countries, capable of diminishing liver lipotoxicity and insulin resistance. The potential to moderate metabolic and inflammatory disorders is also attributed to coconut oil; however, these properties vary in different studies [13–15]. This oil contains about 90% of saturated fatty acids, of which the most abundant is lauric acid, followed by myristic, palmitic, caprylic, capric, and stearic acids.[16,17]

Coconut oil decreased induced BPH in rats.[18] However, its effects on the prostate are poorly known and its consumption throughout aging has not been investigated yet Given that inflammation is closely associated with BPH, we investigated the effect of coconut oil consumption on the prostate throughout aging with regard to the inflammatory profile in the Mongolian gerbil, an experimental model that exhibits spontaneous BPH with advancing age.[19].

Materials and Methods

Animals and experimental design

Male gerbils (*M. unguiculatus*) (33 animals in total) were bred and kept in the Microscopy and Microanalysis Laboratory (Institute of Biosciences, Letters and Exact Sciences of São Paulo State University/UNESP), São José do Rio Preto, with approval and in compliance with the institutional ethics committee guidelines (183/2018- CEUA). At 100 days of age, animals were separated in ventilated polysulphone isolators (2 animals per box), kept under controlled temperature and light (25ºC, 12h light-dark cycle) and provided filtered water and food ad *libitum* during the experimental period.

Three experimental groups (n=11) were set up: the Intact Control group (IC) aged without any treatment; the Gavage Control group (GC) aged with administration of 0.1ml of filtered water by gavage; the Coconut Oil group (CO) aged with administration of 0.1ml of coconut oil (Nature Corp LTDA, Sorocaba, Brazil) by gavage. Oral gavage was performed on alternate day basis throughout the experiment, and the dosage provided corresponded to 13ml for an adult male (80kg), adjusted from Reagan-Shaw et al., 2007[20]. The aging period was 12 months, and at the end, all animals (15 months old) were anesthetized (3 mg/kg of Xylazine hydrochloride and 10 mg/kg of Ketamine hydrochloride) and euthanized by decapitation. The ventral prostates were removed and fixed in 4% buffered paraformaldehyde for 24 hours (n=5) or flash frozen in liquid nitrogen and stored at -80ºC (n=6).

Histological analysis

The fixed prostates were dehydrated, clarified in xylene and included in Histosec (Merck). The organs were sectioned at 3µm thickness and stained by Hematoxylin and Eosin for general morphological analysis and inflammatory foci (IF) identification, and 0.5% Toluidine Blue for mast cell (MC) analysis. The images were captured at ×400 magnification using the slide scanner system (Olympus VS120 - S5) and the analysis performed with OlyVIA 2.9 software (Olympus). Images at 1000x magnification were acquired using Olympus BX60 light microscope (Olympus, Japan) and digitalized with DP-BSW V3.1 software (Olympus).

The IF frequency was determined by the number of IF per area of the histological section. Three sections at different depths from the same prostate were analyzed for each animal. The IF was identified by morphological characteristics such as the concentration of immune system cells (mainly lymphocytes and neutrophils). The foci were analyzed in different prostatic compartments: the non-muscular stroma (stromal), associated to the muscular stroma (periductal), associated to the secretory epithelium (intraepithelial) and in the lumen (intraluminal). The frequency of IF per μm^2 were expressed as median and values for each animal were plotted.

Mast cell analysis was based on their morphological characteristics. Their frequency was determined by total number per area of each section and values for each animal plotted along with median of number of cells per μm^2.

Immunohistochemical analysis

The histological sections were deparaffinized, rehydrated and subjected to antigen retrieval in Tris EDTA buffer pH 9.0 for 60 min at 98°C. The endogenous peroxidase was blocked with 3% H_2O_2 and nonspecific protein blockage carried out with 5% skimmed milk in tris-buffered saline + 0.1% tween. Then, sections were incubated overnight with anti-F4/80 (# 70076, Cell Signaling, MA, USA), anti-CD163 (sc - 33560, Santa Cruz Biotechnology, CA, EUA), anti-CD68 (sc-58965/Santa Cruz Biotechnology), anti-Cox2 (♯ 12282, Cell Signaling, MA, USA) and anti-p-STAT3 (#9145; Cell Signaling, MA, USA) primary antibodies. sections were then washed and incubated with secondary antibodies labelled with peroxidase (CD163: Rabbit ABC Staining System SC - 2018; Santa Cruz Biotechnology) or Dako Polymer (F4/80, CD68, COX2, p-SATAT3: Novocastra Novolink RE7230-CE, Leica Biosystems) for 1h at room temperature. Subsequently, positive staining was detected using 3-30’-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems) and counterstained with hematoxylin.

Images were captured at ×400 magnification using the slide scanner system (Olympus VS120 - S5) and the analysis was performed with the OlyVIA 2.9 software (Olympus). Positive cells frequency was determined by the total number of DAB-labelled cells normalized to the area of the section. Values were expressed as mean ± SD of cells per μm^2. In addition to the frequency in the entire gland, the frequency of macrophages (F4/80, CD163, CD168), COX2 and p-STAT3 in each prostate compartment were also considered.

Western blot

Frozen samples were smashed and incubated for extraction (CelLytic MT cell lysis reagent, C3228, Sigma Aldrich) at 4 °C, for 60 minutes, under agitation. Protease activity was blocked by inhibitors (Protease Inhibitor Cocktail, P8340, Sigma Aldrich), followed by centrifugation for 20 min at 14,000 rpm, 4 °C. protein quantification was determined by BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific) and the absorbance measured in a microplate reader (SPECTROstar Omega, BMG Labtech). The extracts were stored at −80 °C until analysis. For SDS-PAGE electrophoresis, 15ug of sample was loaded in each lane. The bands were transferred to a nitrocellulose membrane. Blots were blocked using skimmed milk 5% in TBS +0.1% Tween (TBSt), for one hour, at room temperature. Overnight incubation under agitation with anti-MMP-2, MMP-3, MMP-9 (sc-13595/ Santa Cruz Biotechnology, 1:500; PA5-27936, Invitrogen, 1:500; sc-21733/Santa Cruz Biotechnology, 1:200, respectively), and GAPDH (MB374 Millipore, 1:10,000) diluted in skimmed milk 1% in TBSt was performed. Subsequently, the membranes were incubated at room temperature for one hour under agitation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 rabbit anti-mouse ab6728, Abcam, or 1:10,000 goat anti-rabbit 115-035-144, Jackson Immunoresearch). Band visualization was performed with ECL system (32,109, Thermo Fischer) using ChemiDoc (ChemiDoc MP, BioRad) followed by quantification with ImageJ. Values were shown as mean of relative density to GAPDH (loading control)

Analysis of inflammatory mediators

For prostate cytokine tests, 25 µL of sample (extracted tissue samples for Western blot) were analyzed with MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead panel (MCYTOMAG-70K-03; Millipore Corporation, USA), following the manufacturer’s instructions. IL-6 and TNFα cytokines were assessed using Luminex laser-based fluorescent analytical test instrumentation (MAGPIX, Austin, TX). Their concentrations were determined according to standard curves from each plate and expressed in picograms per milliliter (pg/ml).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.00 software spreadsheets and graphs (GraphPad Software, San Diego, CA, USA). First, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was adopted. Differences were considered statistically significant when $p < 0.05$.

Results

Histological analysis revealed that the gerbil prostate, taking into account the stroma, muscular stroma, epithelium and lumen compartments, is heterogeneous regarding IF. In all three groups, prostates showed regions with IF and regions lacking evidence of inflammation, with cells of the immune system spread throughout the tissue (Fig. 1). The presence of IF in at least one of the compartments was found in all three groups and is highlighted in Figure 1. In the stroma, the IF were commonly found near lymphatic vessels, whereas, in the periductal region, they were often associated with blood vessels. Acini containing intraluminal IF were formed by hyperplastic epithelium and inflammatory cell infiltration (Fig. 1). The presence of abundant lymphocytes and neutrophils was observed in all prostatic lesions, which showed inflammatory cells accumulated peripherally and between muscle and epithelial cells (Fig. 1). These large IF associated with prostatic lesions were more frequently observed in the IC and CG groups. The total number of IF showed a higher median in the CG concerning all analyzed compartments (Fig. 1). On the other hand, coconut oil treatment (CO) reduced it, and it was lower than in the control group (IC) in the stromal, periductal and intraepithelial compartments (Fig.1).

Mast cells were observed spaced throughout the stroma and were found either in the muscle stroma or occasionally between the epithelial cells (Fig. 1). Their number per area showed a high median in CG, and was lowest in CO (Fig. 1).

F4/80-positive macrophages in the ventral prostate were observed in all experimental groups, mostly in the stroma and occasionally in the epithelium (Fig. 2). Despite no difference being detected in their number between groups, we found an increase in the epithelium of IC, whereas in the stroma this effect was observed for CG (Fig. 3). This pattern of F4/80-positive macrophage distribution in prostatic compartments was also observed for CD68-positive macrophages (Fig. 2), but at a lower level (Fig. 3). Regarding CD163-positive macrophages, their frequency decreased in the epithelium of CO compared with IC (Fig. 3). However, considering the stromal compartments and the entire prostate, there was a tendency to increase in the IC and CG groups compared with CO (Fig. 3).

The cytoplasmic marker Cox2 was present in both epithelial and stromal compartments in all experimental groups (Fig. 2). In the present study, Cox2-positive cell frequency did not show significant differences between the groups, and the immunostaining patterns in the epithelium and stroma compartments were similar between the groups (Fig. 3). The IC and CO groups showed greater variation between the values per animal, while the CG showed less variation between these values and a lower median compared with CO (Fig. 3).

p-STAT3 immunolabeling in the epithelial and stromal compartments was observed in all experimental groups and, even though most of them were nuclear, some stromal cells showed cytoplasmic staining (Fig. 2). p-STAT3-positive cell quantification revealed that the CO group was smaller than the IC regarding the epithelial and epithelial-plus-stromal compartment (Fig. 3); However, the GC group was smaller in the epithelium but was higher in stroma.

High variation in IL-6 concentration was observed between GC and CO groups; however, there was no difference between the experimental groups (Fig. 4). Regarding TNFα, it was higher in the CG compared with IC, while in the CO it was unchanged (Fig. 4).

MMP-2 increased in the CG compared with CO (Fig. 5). Regarding MMP-3, CG showed higher relative density than IC, whereas MMP-9 did not change among any experimental groups (Fig. 5).

Discussion

Inflammatory processes in the prostate are common throughout aging and are associated with several age-related diseases.[21] In the present study, we evaluated the effect of coconut oil ingestion throughout aging on the prostate inflammatory profile. The effects of animal handling (over 1 year) were also considered. Prostate inflammatory foci, associated or not with lesions, is common in humans and older rodents,[2,6] and is mostly located in the stromal compartment associated with hyperplasic areas.[22] A consequence of aging is the recruitment of inflammatory cells into the prostate microenvironment, which may promote selective proliferation of transformed or non-transformed cells;[23] thus, our morphological data are in line with literature reports. The high IF medians and the association with prostatic lesions of the CG, being lower in the CO, drew our attention to the potential influence of animal handling on the inflammatory status of the gerbil prostate. Animal handling in experiments leads to a state of stress that may affect several physiological parameters, such as metabolism, influencing the immune cell infiltration pattern and increasing the risk of neuroinflammation.[24–26] A coconut oil diet is reported to attenuate metabolic and inflammatory alterations.[15,27] Concerning the inflammatory process, mast cells and macrophages play important roles, which affect prostate age-related pathologies, including BPH and PC.[28]

Mast cells may be triggered by different stimuli and release several mediators, such as cytokines and chemokines, as well as participating in tissue remodeling[29]. In the prostate, mast cells are usually clustered and associated with blood vessels, are abundant in the stroma,[7] and may favor BPH progression via the IL-6/STAT3/CyclinD1 pathway.[30] In contrast to the reports, in the old gerbil prostate, mast cells were found scattered throughout the stroma Fatty acid consumption due to coconut oil may change mast cell structure.[31,32] However, our results demonstrated that its consumption throughout aging did not affect the mast cell population in the prostate. It also had no effect on the F4/80-positive and CD68-positive macrophage cell frequency. Macrophages have roles in repair, immune responses to pathogens, and the maintenance of homeostasis.[33] These phagocytic cells can be associated with tumors (TAMs) and classified differently according to the activation pathway and polarization.[34,35] Although the present study did not aim at classifying their polarization into M1 or M2, we adopted three different markers to investigate whether coconut oil consumption may influence the subtypes.

F4/80 is a pan macrophage marker for mice,[36] with a high affinity for bone marrow-derived macrophages and correlated with epithelial proliferation and prostate regeneration.[37] Castro *et al* [38] reported that F4/80-positive macrophages increase in the prostate stroma of Mongolian gerbils with induced BPH and decrease when the animals are treated with β-caryophyllene. In the present study, pan macrophage distribution across the prostate tissue compartments was heterogeneous, with the highest concentration in the stroma. Our data are in agreement with that; however, regarding the epithelial compartment, the range of the number of F4/80 macrophages between the animals was lower in the CG and CO groups. This apparent lower variation was also noted for CD68 macrophages and confirmed for macrophages 163, indicating that animal handling, an experimental condition common to both GC and CO groups, may modify macrophage distribution between the stroma and epithelium.

CD68 macrophages are also considered pan macrophages,[39,40] and this cell type increases in both stroma and epithelium in conditions of BPH.[41,42] The literature shows that androgen deprivation alters the cell morphology of CD68 macrophages, as well as their distribution in the tissue, and also increases the percentage of these cells in the rat ventral prostate.[43] Our data suggest that, although the numbers of CD68 macrophages found halved compared with F4/80, these two pan macrophage marker types behave similarly in the gerbil prostate. CD68 and F4/80 macrophages in the gerbil prostate have already been quantified,[38,44] but ours is the first study to describe the possible effect of animal handling on the distribution pattern of macrophages in the prostate of older animals. Most of the available data on F4/80 and CD68 macrophages are based on in vitro studies or using established models such as rats and mice. Thus, further investigation is needed to ascertain whether the cell types themselves behave differently or whether this is a particularity of the model used.

CD163 has been employed as a marker for M2 macrophages in some studies;[45–48] however, its specificity when evaluated alone is controversial[49] and the association with other markers is required to confirm macrophage polarization.[50] Even though we did not analyze it, the decrease in CD163 cells on the prostate of animals treated with coconut oil was intriguing, especially since it happened only in the epithelial compartment. The distribution of CD163-positive macrophages throughout the compartments of the rodent prostate is controversial; in adult rats they were found only in the stroma,[43] while in aged castrated rats they were also found in the epithelium.[51] In Mongolian gerbils, CD163 macrophages occurred in both stroma and epithelium, and with distinct morphology according to their distribution.[44] In adult gerbils with induced BPH, the amount of these macrophages increased in the stroma and decreased when the animals were treated with β-caryophyllene.[38] According to these studies, the increase in CD163-positive macrophages was related to tissue remodeling due to hormonal changes, but the precise role of these cells in the rodent prostate is still unclear. The increased CD163 macrophage population is associated with tumor progression, worse prognosis, and even death as a consequence of PC.[46,47] Thus, we suggest that coconut oil consumption throughout aging has a positive effect on the CD163 macrophage population in the prostate. In addition to the integral cells of the immune system, we also investigated the performance of other inflammatory markers.

Cyclooxygenase is an enzyme involved in the conversion of arachidonic acid to prostaglandin and other eicosanoids, and the isoform 2 (COX2) is considered pro-inflammatory.[52] COX2 expression is elevated in advanced PC and associated with death from PC.[53,54] In the prostate of aged rats, COX-2 decreased apoptosis,[55] and its expression in the stroma increased with aging.[56] Increased COX-2 leads to activation of ER-β,[57] an essential receptor that plays a role in the proliferation and maintenance of the prostate and which reduces its expression in old gerbils.[58] Nevertheless, another study involving the adult gerbil prostate has shown that COX-2 expression is higher in the stroma than in the epithelium and that the induction of BPH led to increased frequency of COX-2-positive cells in both compartments.[38] Our data indicate that the distribution pattern between stroma and epithelium previously reported in adult animals is maintained in old animals. A high-fat diet, especially caprylic acid, promotes COX-2 expression,[59] and stearic and palmitic acids have also been reported to increase the expression of this enzyme.[60] Despite the fact that these fatty acids are present in coconut oil, we noticed that its consumption throughout aging did not interfere with COX2-positive cell frequency in the prostate. However, the influence of the oil intake on p-STAT3 expression was remarkable.

The STAT-3 canonical pathway is activated in the Janus-activated kinase/STAT signaling pathway, which, upon phosphorylation at a conserved tyrosine residue (Y705) and homodimerization, is transported into the nucleus and plays a role in activating transcription of a target gene.[61,62] Our data corroborated those reported for localization of the activated state of STAT3, since the majority of p-STAT3-positive cells showed nuclear marking, although some cells in the stroma showed cytoplasmic labeling. Leonel *et al*, 2021 reported the presence of pSTAT3-positive cells with cytoplasmic marking in the prostate stroma of Mongolian gerbils.[63] This transcription factor plays an important role in regulating the expression of genes involved in tumorigenesis, inhibition of apoptosis, and inflammatory mediators.[64] It is considered that p-STAT3 signaling contributes to the higher invasiveness and aggressiveness of PC.[65] Several compounds have been reported to be efficient in the treatment of cancer by interfering in pSTAT-3 action and consequently modulating different cell signaling pathways.[66–69] Although reports have shown that a high-fat diet accelerated PC growth in mice via IL-6/pSTAT3 signaling, our data indicate that treatment with coconut oil, a saturated fatty acid, decreases the amount of p-STAT3-positive cells. Again, we cannot ignore the possibility that animal handling interfered with the results, since the CG showed fewer p-STAT3-positive cells in the epithelium and higher numbers in the stroma. Alterations resulting from chronic psychological stress could interfere with the IL-6/STAT3 signaling pathway and promote prostate tumor growth.[71,72] Thus, even when prostate compartments were taken into consideration together, coconut oil treatment seemed to decrease the number of p-STAT3-positive cells. However, the potential interference of manipulating animals over a long experimental period should be further evaluated to confirm this finding.

One of the best-known STAT3 activators is IL-6. Although our data suggests a decrease in p-STAT3 expression due to coconut oil treatment, we cannot conclude that this interference involves IL-6, since the dosage of this interleukin, even though it showed higher concentrations in the CG and CO groups, did not result in a statistically significant difference between the experimental groups. Nevertheless, the fact that the two experimental groups that underwent handling over the experimental period showed higher concentrations of IL-6 is intriguing. The chronic stress condition may lead to increased levels of IL-6.[71,72] IL-6 is an inflammatory molecule that acts as a key mediator in tumorigenesis initiation, induction of aggressive PC phenotype, as well as PC progression to castration-resistant state and promotion of PC tumor metastasis.[73] The aging process results in a decrease in testosterone levels and this is linked to an increase in IL-6 levels;[74,75] however, this relation is not yet clear.[76] Given this, we suggest that long-term animal handling may potentiate the effects of aging on the prostate inflammatory profile, but more inflammation markers need to be taken into consideration.

TNFα is a cytokine commonly secreted by macrophages in the course of inflammation and is involved in a wide range of signaling pathways related to necrosis or apoptosis.[77] The inflammatory pathway involving TNFα is directly related to PC and it contributes to all stages of the malignant process.[78] In addition, the increase of this cytokine has a role in the aging of the rat prostate and in BPH.[79–81] Different inflammatory responses have been attributed to psychological stress situations, and the increase in levels of TNFα is a key indicator of this effect.[82–85] These studies indicate that the impact of stress on inflammation varies according to the experimental model used, as well as the wide variety of stress induction methods used. Therefore, we hypothesize that animal handling provoked an increase in prostatic TNFα levels. Coconut oil diminished the effect of handling since the TNFα levels in CO remained close to those of the control group. The protective effect of coconut oil on the inflammatory process in certain pathologies has been associated with the reduction of TNFα levels.[10,86] However, coconut oil combined with high-fat diets has been reported to increase TNFα levels.[27,87] Thus there is still much to be investigated about the effect of coconut oil treatment on the levels of this cytokine.

An important regulator of levels of TNFα and other cytokines is MMPs.[88] These proteins are part of a family of proteolytic enzymes, found mainly in the extracellular matrix, which act in tissue homeostasis, defense and repair, and modulate inflammation.[89] Increased levels of MMPs 2 and 9 are associated with the development and progression of prostate pathologies such as BPH and PC,[90–92] and also with prostatic inflammation.[93] MMP-3 is closely correlated with PC progression and is used as a biomarker in Gleason patterns.[94,95] The increase in MMPs 2 and 3 in the CG but not in CO supports our hypothesis that handling may enhance the effects of aging on the prostate and that coconut oil acts as a protector. Psychological stress elevates levels of MMP-2, 3, and 9 in the nervous system.[96] High levels of MMP-2 and MMP-9 have also been found in semen and attributed to psychological stress.[97] Little is known about the impact of coconut oil treatment on MMP levels. MMP-9 levels have been found to increase in NIH 3T3 cells and hamster liver treated with coconut oil,[98,99] but no changes were observed in our study.

Conclusions

Our data suggest that the one-year experimental procedure, in which the animals were manipulated every other day to administer coconut oil by gavage, interfered in the inflammatory parameters investigated here. The coconut oil treatment reduced the number of CD163 macrophages, p-STAT3-positive cells, TNF concentration, and the expression of MMP-2 and 3. We therefore believe that coconut oil exerts a protective and anti-inflammatory role in the prostate. Further studies to investigate the mechanisms of its action, as well as the impact on the prostate of long-term handling, may serve to elucidate these issues.

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References

1. Thomas D, Chughtai B, Kini M, Te A. Emerging drugs for the treatment of benign prostatic hyperplasia. *Expert Opin Emerg Drugs*. 2017;22(3):201-212. doi:10.1080/14728214.2017.1369953

2. Sfanos KS, Yegnasubramanian S, Nelson WG, De Marzo AM. The inflammatory microenvironment and microbiome in prostate cancer development. *Nat Rev Urol*. 2018;15(1):11-24. doi:10.1038/nrurol.2017.167

3. Verze P, Cai T, Lorenzetti S. The role of the prostate in male fertility, health and disease. *Nat Rev Urol*. 2016;13(7):379-386. doi:10.1038/nrurol.2016.89

4. Chughtai B, Forde JC, Thomas DDM, et al. Benign prostatic hyperplasia. *Nat Rev Dis Prim*. 2016;2:1-15. doi:10.1038/nrdp.2016.31

5. Hayashi T, Fujita K, Matsushita M, Nonomura N. Main Inflammatory Cells and Potentials of Anti-Inflammatory Agents in Prostate Cancer. Published online 2019.

6. Bianchi-Frias D, Vakar-Lopez F, Coleman IM, Plymate SR, Reed MJ, Nelson PS. The effects of aging on the molecular and cellular composition of the prostate microenvironment. *PLoS One*. 2010;5(9):1-16. doi:10.1371/journal.pone.0012501

7. Silva JAF, Calmasini F, Siqueira-Berti A, Moraes-Vieira PMM, Quintar A, Carvalho HF. Prostate immunology: A challenging puzzle. *J Reprod Immunol*. 2020;142(February):103190. doi:10.1016/j.jri.2020.103190

8. Fourcade RO, Théret N, Taïeb C. Profile and management of patients treated for the first time for lower urinary tract symptoms/benign prostatic hyperplasia in four European countries. *BJU Int*. 2008;101(9):1111-1118. doi:10.1111/j.1464-410X.2008.07498.x

9. Famurewa AC, Ekeleme-egedigwe CA, Nwali SC, et al. Dietary Supplementation with Virgin Coconut Oil Improves Lipid Profile and Hepatic Antioxidant Status and Has Potential Benefits on Cardiovascular Risk Indices in Normal Rats Dietary Supplementation with Virgin Coconut Oil Improves Lipid Profile and Hepat. *J Diet Suppl*. 2017;0(0):1-13. doi:10.1080/19390211.2017.1346031

10. Famurewa AC, Akunna GG, Nwafor J, Chukwu OC, Ekeleme-Egedigwe CA, Oluniran JN. Nephroprotective activity of virgin coconut oil on diclofenac-induced oxidative nephrotoxicity is associated with antioxidant and anti-inflammatory effects in rats. *Avicenna J phytomedicine*. 2020;10(3):316-324. doi:10.22038/ajp.2019.14263

11. Ogedengbe OO, Akang EN, Onanuga AIJIO, Peter UOAI. Adjuvant potential of virgin coconut oil extract on antiretroviral induced testicular toxicity : An ultrastructural study. 2017;(October):1-14. doi:10.1111/and.12930

12. Narayanankutty A, Palliyil M, Kuruvilla K, Raghavamenon AC. Virgin coconut oil reverses hepatic steatosis by restoring redox homeostasis and lipid metabolism in male Wistar rats. 2017;(September). doi:10.1002/jsfa.8650

13. Intahphuak S, Khonsung P, Panthong A. Anti-inflammatory, analgesic, and antipyretic activities of virgin coconut oil. *Pharm Biol*. 2010;48(2):151-157. doi:10.3109/13880200903062614

14. Harris M, Hutchins A, Fryda L. The impact of virgin coconut oil and high-oleic safflower oil on body composition, lipids, and inflammatory markers in postmenopausal women. *J Med Food*. 2017;20(4):345-351. doi:10.1089/jmf.2016.0114

15. Zicker MC, Silveira ALM, Lacerda DR, et al. *Virgin Coconut Oil Is Effective to Treat Metabolic and Inflammatory Dysfunction Induced by High Refined Carbohydrate-Containing Diet in Mice*. Vol 63. Elsevier Inc; 2019. doi:10.1016/j.jnutbio.2018.08.013

16. Moreau RA. Corn Oil. In: *Vegetable Oils in Food Technology*. Wiley-Blackwell; 2011:273-289. doi:10.1002/9781444339925.ch10

17. Akpan EJ, Etim OE, Akpan HD, Usoh IF. Fatty acid profile and oil yield in six different varieties of fresh and dry samples of coconuts (Cocos nucifera). *Pakistan J Nutr*. 2006;5(2):106-109. doi:10.3923/pjn.2006.106.109

18. de Lourdes Arruzazabala M, Molina V, Más R, et al. Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. *J Pharm Pharmacol*. 2007;59(7):995-999. doi:10.1211/jpp.59.7.0012

19. Campos SGP, Zanetoni C, Scarano WR, Vilamaior PSL, Taboga SR. Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. *Int J Exp Pathol*. 2008;89(1):13-24. doi:10.1111/j.1365-2613.2007.00550.x

20. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*. 2007;22(3):659-661. doi:10.1096/fj.07-9574LSF

21. Frungieri MB, Calandra RS, Bartke A, Matzkin ME. Ageing and inflammation in the male reproductive tract. *Andrologia*. 2018;50(11):1-9. doi:10.1111/and.13034

22. Kohnen PW, Drach GW. Patterns of inflammation in prostatic hyperplasia: a histologic and bacteriologic study. *J Urol*. 1979;121(6):755-760. doi:10.1016/s0022-5347(17)56980-3

23. McDowell KL, Begley LA, Mor-Vaknin N, Markovitz DM, Macoska JA. Leukocytic promotion of prostate cellular proliferation. *Prostate*. 2010;70(4):377-389. doi:10.1002/pros.21071

24. Ono M, Sasaki H, Nagasaki K, et al. Does the routine handling affect the phenotype of disease model mice? *Jpn J Vet Res*. 2016;64(4):265-271.

25. Gariépy JL, Rodriguiz RM, Jones BC. Handling, genetic and housing effects on the mouse stress system, dopamine function, and behavior. *Pharmacol Biochem Behav*. 2002;73(1):7-17. doi:10.1016/S0091-3057(02)00789-X

26. Swan MP, Hickman DL. Evaluation of the neutrophil-lymphocyte ratio as a measure of distress in rats. *Lab Anim (NY)*. 2014;43(8):276-282. doi:10.1038/laban.529

27. Ströher DJ, De Oliveira MF, Martinez-Oliveira P, et al. Virgin Coconut Oil Associated with High-Fat Diet Induces Metabolic Dysfunctions, Adipose Inflammation, and Hepatic Lipid Accumulation. *J Med Food*. 2020;23(7):689-698. doi:10.1089/jmf.2019.0172

28. Wu Z, Chen H, Luo W, Zhang H, Li G, Zeng F. The Landscape of Immune Cells In fi ltrating in Prostate Cancer. 2020;10(October):1-15. doi:10.3389/fonc.2020.517637

29. Amin K. The role of mast cells in allergic inflammation. *Respir Med*. 2012;106(1):9-14. doi:10.1016/j.rmed.2011.09.007

30. Ou Z, He Y, Qi L, Zu X, Wu L, Cao Z. Infiltrating mast cells enhance benign prostatic hyperplasia through IL-6 / STAT3 / Cyclin D1 signals. 2017;8(35):59156-59164.

31. van Haaster CMCJ, Engels W, Lemmens PJMR, Hornstra G, van der Vusse GJ. Formation of prostanoids and hydroxy fatty acids by stimulated peritoneal mast cells: Role of the dietary fat type in rat. *Biochim Biophys Acta (BBA)/Lipids Lipid Metab*. 1993;1167(2):147-154. doi:10.1016/0005-2760(93)90155-3

32. Engels W, Van Haaster CMCJ, Lemmens PJMR, Van Der Vusse GJ, Hornstra G. Dietary modulation of fatty acid composition of mast cell phospholipids does not affect histamine release induced by compound 48/80. *Inflamm Res*. 1997;46(5):185-190. doi:10.1007/s000110050170

33. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;496(7446):445-455. doi:10.1038/nature12034

34. Mantovani A, Allavena P. The interaction of anticancer therapies with tumor-associated macrophages. *J Exp Med*. 2015;212(4):435-445. doi:10.1084/jem.20150295

35. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686. doi:10.1016/j.it.2004.09.015

36. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol*. 1981;11(10):805-815. doi:10.1002/eji.1830111013

37. Ikehara A, Maeda H, Kimura T, Saito S, Ochiai A. Bone marrow-derived macrophages are associated with androgen modulated prostate regeneration. *Prostate*. 2012;72(1):1-11. doi:10.1002/pros.21399

38. Castro NF da C, Jubilato FC, Guerra LHA, Santos FCA dos, Taboga SR, Vilamaior PSL. Therapeutic effects of β-caryophyllene on proliferative disorders and inflammation of the gerbil prostate. *Prostate*. 2021;81(12):812-824. doi:10.1002/pros.24177

39. Sadasivan SM, Chen Y, Gupta NS, et al. The interplay of growth differentiation factor 15 (GDF15) expression and M2 macrophages during prostate carcinogenesis. *Carcinogenesis*. 2020;41(8):1074-1082. doi:10.1093/carcin/bgaa065

40. Gottfried E, Kunz-Schughart LA, Weber A, et al. Expression of CD68 in non-myeloid cell types. *Scand J Immunol*. 2008;67(5):453-463. doi:10.1111/j.1365-3083.2008.02091.x

41. Lu T, Lin WJ, Izumi K, et al. Targeting androgen receptor to suppress macrophage-induced EMT and benign prostatic hyperplasia (BPH) development. *Mol Endocrinol*. 2012;26(10):1707-1715. doi:10.1210/me.2012-1079

42. Lindholm PF, Lu Y, Adley BP, et al. Role of monocyte-lineage cells in prostate cancer cell invasion and tissue factor expression. *Prostate*. 2010;70(15):1672-1682. doi:10.1002/pros.21202

43. Silva JAF, Bruni-Cardoso A, Augusto TM, et al. Macrophage roles in the clearance of apoptotic cells and control of inflammation in the prostate gland after castration. *Prostate*. 2018;78(2):95-103. doi:10.1002/pros.23449

44. Castro NFC, Falleiros-Júnior LR, Zucão MI, et al. Ethinylestradiol and its effects on the macrophages in the prostate of adult and senile gerbils. *Cell Biol Int*. 2020;44(7):1467-1480. doi:10.1002/cbin.11342

45. Medrek C, Pontén F, Jirström K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC*

Cancer. 2012;12(1):1. doi:10.1186/1471-2407-12-306

46. Kim MG, Lim K, Lee YJ, et al. M2 macrophages predict worse long-term outcomes in human acute tubular necrosis. *Sci Rep*. 2020;10(1):1-7. doi:10.1038/s41598-020-58725-w

47. Erlandsson A, Carlsson J, Lundholm M, et al. M2 macrophages and regulatory T cells in lethal prostate cancer. *Prostate*. 2019;79(4):363-369. doi:10.1002/pros.23742

48. Topf MC, Tuluc M, Harshyne LA, Luginbuhl A. Macrophage type 2 differentiation in a patient with laryngeal squamous cell carcinoma and metastatic prostate adenocarcinoma to the cervical lymph nodes. *J Immunother Cancer*. 2017;5(1):2-6. doi:10.1186/s40425-017-0264-z

49. Barros MHM, Hauck F, Dreyer JH, Kempkes B, Niedobitek G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One*. 2013;8(11):e80908. doi:10.1371/journal.pone.0080908

50. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. 2019;19(6):369-382. doi:10.1038/s41577-019-0127-6

51. Werneck-Gomes H, Campolina-Silva GH, Maria BT, et al. Tumor-Associated Macrophages (TAM) are recruited to the aging prostate epithelial lesions and become intermingled with basal cells. *Andrology*. 2020;8(5):1375-1386. doi:10.1111/andr.12783

52. Hussain T, Gupta S, Mukhtar H. Cyclooxygenase-2 and prostate carcinogenesis. *Cancer Lett*. 2003;191(2):125-135. doi:10.1016/S0304-3835(02)00524-4

53. Edwards J, Mukherjee R, Munro AF, Wells AC, Almushatat A, Bartlett JMS. HER2 and COX2 expression in human prostate cancer. *Eur J Cancer*. 2004;40(1):50-55. doi:10.1016/j.ejca.2003.08.010

54. Richardsen E, Uglehus RD, Due J, Busch C, Busund L-T. COX-2 is overexpressed in primary prostate cancer with metastatic potential and may predict survival. A comparison study between COX-2, TGF-beta, IL-10 and Ki67. *Cancer Epidemiol*. 2010;34(3):316-322. doi:10.1016/j.canep.2010.03.019

55. Badawi AF, Liu Y, Eldeen MB, et al. Age-associated changes in the expression pattern

of cyclooxygenase-2 and related apoptotic markers in the cancer susceptible region of rat prostate. *Carcinogenesis*. 2004;25(9):1681-1688. doi:10.1093/carcin/bgh176

56. Pascal LE, Dhir R, Balasubramani GK, et al. E-cadherin expression is inversely correlated with aging and inflammation in the prostate. *Am J Clin Exp Urol*. 2021;9(1):140-149. http://www.ncbi.nlm.nih.gov/pubmed/33816702%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC8012829

57. Liu TT, Grubisha MJ, Frahm KA, et al. Opposing effects of cyclooxygenase-2 (COX-2) on estrogen receptor β (ERβ) response to 5α-reductase inhibition in prostate epithelial cells. *J Biol Chem*. 2016;291(28):14747-14760. doi:10.1074/jbc.M115.711515

58. Rochel-Maia SS, Santos FCA, Alonso-Magdalena P, et al. Estrogen receptors alpha and beta in male and female gerbil prostates. *Biol Reprod*. 2013;88(1):7. doi:10.1095/biolreprod.112.103614

59. Wang C, Wang J, Chen K, et al. Caprylic acid (C8:0) promotes bone metastasis of prostate cancer by dysregulated adipo-osteogenic balance in bone marrow. *Cancer Sci*. 2020;111(10):3600-3612. doi:10.1111/cas.14606

60. Liu J, Hu S, Cui Y, et al. Saturated fatty acids up-regulate COX-2 expression in prostate epithelial cells via toll-like receptor 4/NF-κB signaling. *Inflammation*. 2014;37(2):467-477. doi:10.1007/s10753-013-9760-6

61. Huang S. Regulation of metastases by signal transducer and activator of transcription 3 signaling pathway: Clinical implications. *Clin Cancer Res*. 2007;13(5):1362-1366. doi:10.1158/1078-0432.CCR-06-2313

62. Srivastava J, DiGiovanni J. Non-canonical Stat3 signaling in cancer. *Mol Carcinog*. 2016;55(12):1889-1898. doi:10.1002/mc.22438

63. Leonel ECR, Ruiz TFR, Bedolo CM, Campos SGP, Taboga SR. Inflammatory repercussions in female steroid responsive glands after perinatal exposure to bisphenol A and 17-β estradiol. *Cell Biol Int*. 2021;45(11):2264-2274. doi:10.1002/cbin.11665

64. Aggarwal BB, Sethi G, Kwang SA, et al. Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: Modern target but ancient

solution. *Ann N Y Acad Sci*. 2006;1091:151-169. doi:10.1196/annals.1378.063

65. Horinaga M, Okita H, Nakashima J, Kanao K, Sakamoto M, Murai M. Clinical and pathologic significance of activation of signal transducer and activator of transcription 3 in prostate cancer. *Urology*. 2005;66(3):671-675. doi:10.1016/j.urology.2005.03.066

66. Bosch-Barrera J, Menendez JA. Silibinin and STAT3: A natural way of targeting transcription factors for cancer therapy. *Cancer Treat Rev*. 2015;41(6):540-546. doi:10.1016/j.ctrv.2015.04.008

67. Eskandari E, Heidarian E, Amini S, Saffari-Chaleshtori J. Evaluating the effects of ellagic acid on pSTAT3, pAKT, and pERK1/2 signaling pathways in prostate cancer PC3 cells. *J Cancer Res Ther*. 2016;12(4):1266-1271. doi:10.4103/0973-1482.165873

68. Kim HJ, Jin BR, An HJ. Umbelliferone ameliorates benign prostatic hyperplasia by inhibiting cell proliferation and G1/s phase cell cycle progression through regulation of STAT3/E2F1 axis. *Int J Mol Sci*. 2021;22(16). doi:10.3390/ijms22169019

69. Xing WY, Zhang ZH, Xu S, et al. Calcitriol inhibits lipopolysaccharide-induced proliferation, migration and invasion of prostate cancer cells through suppressing STAT3 signal activation. *Int Immunopharmacol*. 2020;82(February):106346. doi:10.1016/j.intimp.2020.106346

70. Hayashi T, Fujita K, Nojima S, et al. High-fat diet-induced inflammation accelerates prostate cancer growth via IL6 signaling. *Clin Cancer Res*. 2018;24(17):4309-4318. doi:10.1158/1078-0432.CCR-18-0106

71. Cheng Y, Tang XY, Li YX, et al. Depression-induced neuropeptide y secretion promotes prostate cancer growth by recruiting myeloid cells. *Clin Cancer Res*. 2019;25(8):2621-2632. doi:10.1158/1078-0432.CCR-18-2912

72. Mohammadpour H, Bucsek MJ, Hylander BL, Repasky EA. Depression Stresses the Immune Response and Promotes Prostate Cancer Growth. *Clin cancer Res an Off J Am Assoc Cancer Res*. 2019;25(8):2363-2365. doi:10.1158/1078-0432.CCR-18-3980

73. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: The role of interleukin 6 (IL-6). *BJU Int*. 2014;113(6):986-992. doi:10.1111/bju.12452

74. Lamas CA, Kido LA, Hermes TA, et al. Brazilian berry extract (Myrciaria jaboticaba): A promising therapy to minimize prostatic inflammation and oxidative stress. *Prostate*.

2020;80(11):859-871. doi:10.1002/pros.24017

75. Maggio M, Basaria S, Ceda GP, et al. The relationship between testosterone and molecular markers of inflammation in older men. *J Endocrinol Invest*. 2005;28(11 Suppl Proceedings):116-119.

76. Mohamad NV, Wong SK, Wan Hasan WN, et al. The relationship between circulating testosterone and inflammatory cytokines in men. *Aging Male*. 2019;22(2):129-140. doi:10.1080/13685538.2018.1482487

77. Idriss HT, Naismith JH. TNFα and the TNF receptor superfamily: Structure-function relationship(s). *Microsc Res Tech*. 2000;50(3):184-195. doi:10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H

78. Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev*. 2006;25(3):409-416. doi:10.1007/s10555-006-9005-3

79. Rastrelli G, Vignozzi L, Corona G, Maggi M. Testosterone and Benign Prostatic Hyperplasia. *Sex Med Rev*. 2019;7(2):259-271. doi:10.1016/j.sxmr.2018.10.006

80. Lam YW, Tam NNC, Evans JE, Green KM, Zhang X, Ho S-M. Differential proteomics in the aging Noble rat ventral prostate. *Proteomics*. 2008;8(13):2750-2763. doi:10.1002/pmic.200700986

81. Hamid ARAH, Umbas R, Mochtar CA. Recent role of inflammation in prostate diseases: chemoprevention development opportunity. *Acta Med Indones*. 2011;43(1):59-65.

82. Zhao X, Cao F, Liu Q, et al. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-induced mouse models of depression. *Behav Brain Res*. 2019;364(May):494-502. doi:10.1016/j.bbr.2017.05.064

83. Song AQ, Gao B, Fan JJ, et al. NLRP1 inflammasome contributes to chronic stress-induced depressive-like behaviors in mice. *J Neuroinflammation*. 2020;17(1):1-13. doi:10.1186/s12974-020-01848-8

84. Marsland AL, Walsh C, Lockwood K, John-henderson NA. analysis. 2018;(412):208-219. doi:10.1016/j.bbi.2017.01.011.The

85. Himmerich H, Fischer J, Bauer K, Kirkby KC, Sack U, Krügel U. Stress-induced

cytokine changes in rats. *Eur Cytokine Netw*. 2013;24(2):97-103. doi:10.1684/ecn.2013.0338

86. Alghamdi BS. The Effect of Short-Term Feeding of a High-Coconut Oil or High-Fat Diet on Neuroinflammation and the Performance of an Object–Place Task in Rats. *Neurochem Res*. 2021;46(2):287-298. doi:10.1007/s11064-020-03163-3

87. García-Escobar E, Monastero R, García-Serrano S, et al. Dietary fatty acids modulate adipocyte TNFa production via regulation of its DNA promoter methylation levels. *J Nutr Biochem*. 2017;47:106-112. doi:10.1016/j.jnutbio.2017.05.006

88. Nissinen L, Kähäri VM. Matrix metalloproteinases in inflammation. *Biochim Biophys Acta - Gen Subj*. 2014;1840(8):2571-2580. doi:10.1016/j.bbagen.2014.03.007

89. Parks WC, Wilson CL, López-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol*. 2004;4(8):617-629. doi:10.1038/nri1418

90. Zhang L, Shi J, Feng J, Klocker H, Lee C, Zhang J. Type IV collagenase (matrix metalloproteinase-2 and -9) in prostate cancer. *Prostate Cancer Prostatic Dis*. 2004;7(4):327-332. doi:10.1038/sj.pcan.4500750

91. Mukherjee S, Siddiqui MA, Dayal S, Ayoub YZ, Malathi K. Epigallocatechin-3-gallate suppresses proinflammatory cytokines and chemokines induced by Toll-like receptor 9 agonists in prostate cancer cells. *J Inflamm Res*. 2014;7(1):89-101. doi:10.2147/JIR.S61365

92. König JE, Senge T, Allhoff EP, König W. Analysis of the Inflammatory Network in Benign Prostate Hyperplasia and Prostate Cancer. *Prostate*. 2004;58(2):121-129. doi:10.1002/pros.10317

93. Wilson MJ, Woodson M, Wiehr C, Reddy A, Sinha AA. Matrix metalloproteinases in the pathogenesis of estradiol-induced nonbacterial prostatitis in the lateral prostate lobe of the Wistar rat. *Exp Mol Pathol*. 2004;77(1):7-17. doi:10.1016/j.yexmp.2004.02.004

94. Srivastava P, Kapoor R, Mittal RD. Impact of MMP-3 and TIMP-3 gene polymorphisms on prostate cancer susceptibility in North Indian cohort. *Gene*. 2013;530(2):273-277. doi:10.1016/j.gene.2013.06.087

95. Serretta V, Abrate A, Siracusano S, et al. Clinical and biochemical markers of visceral

adipose tissue activity: Body mass index, visceral adiposity index, leptin, adiponectin, and matrix metalloproteinase-3. Correlation with Gleason patterns 4 and 5 at prostate biopsy. *Urol Ann*. 2018;10(3):280-286. doi:10.4103/UA.UA_188_17

96. Kucharczyk M, Kurek A, Detka J, et al. Chronic mild stress influences nerve growth factor through a matrix metalloproteinase-dependent mechanism. *Psychoneuroendocrinology*. 2016;66:11-21. doi:10.1016/j.psyneuen.2015.12.019

97. Mary AP, Nandeesha H, Papa D, Chitra T, Ganesh RN, Menon V. Matrix metalloproteinase-9 is elevated and related to interleukin-17 and psychological stress in male infertility: A cross-sectional study. *Int J Reprod Biomed*. 2021;19(4):333-338. doi:10.18502/ijrm.v19i4.9059

98. Cell NIH, Ika D, Meliala P, et al. The Role of Coconut Oil to Increase Expression of MMP-9, PDGF-. 2019;7(22):3733-3736.

99. Yang SF, Tseng JK, Chang YY, Chen YC. Flaxseed oil attenuates nonalcoholic fatty liver of hyperlipidemic hamsters. *J Agric Food Chem*. 2009;57(11):5078-5083. doi:10.1021/jf900499v

Figures:

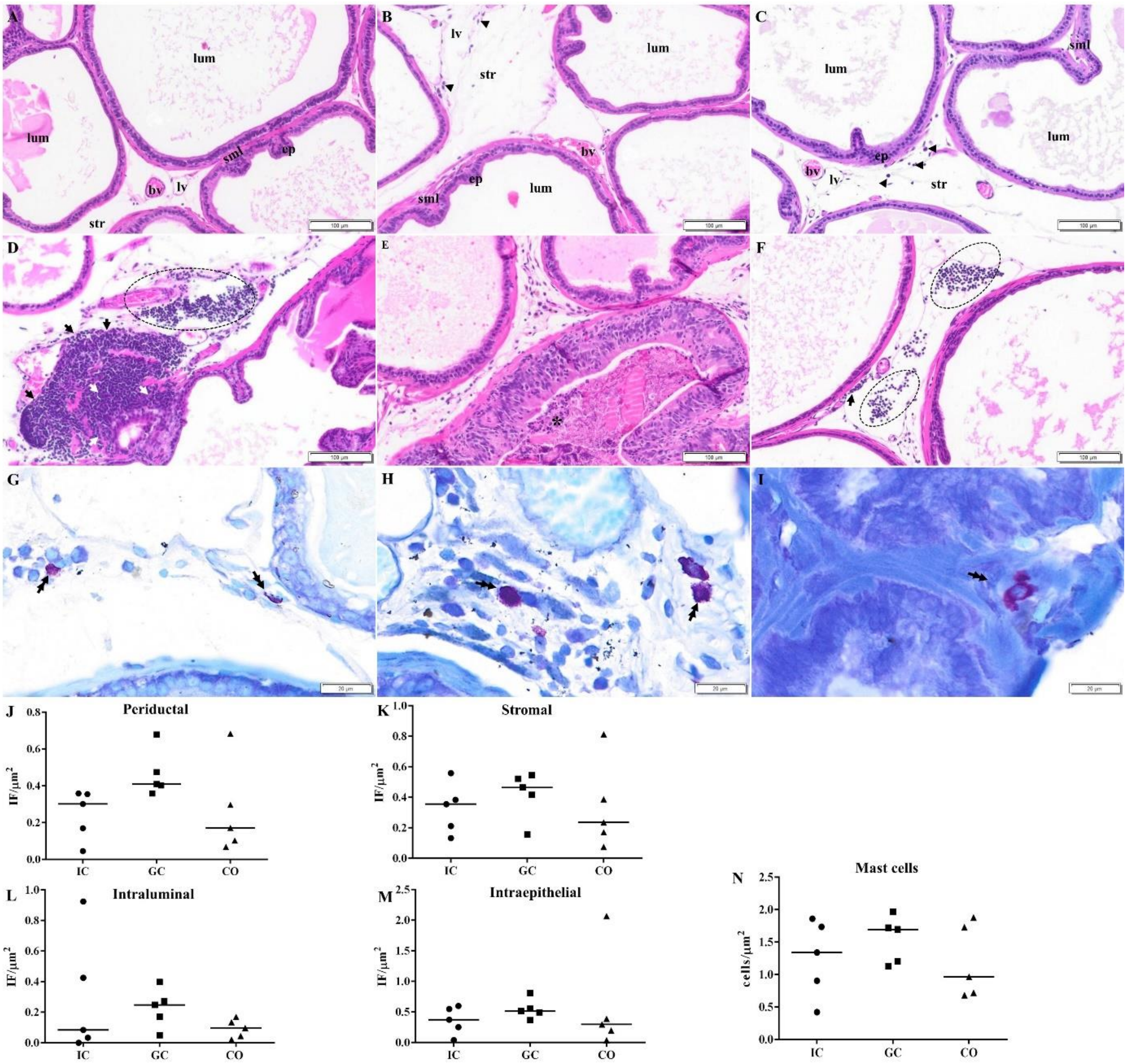

Figure 1: Histological sections of gerbil prostate stained with HE (A-F) at 100x magnification and stained with Toluidine Blue (H-I) at 400x magnification of groups IC (A, D, G), GC (B, E, H), and CO (C, F, I). Quantification of inflammatory foci (IF) in different compartments (J: periductal; K: stromal; L: intraluminal, and M: intraepithelial) and mast cells (N) per histological section area (µm^2). Regions without IF (A-C) showing lumen (lum), stroma (str), muscle stroma (sml), epithelium (ep), blood vessels (bv), and lymphatic vessels (lv); arrowheads point to cells of the immune system. IF in the different compartments (D-F), stromal (dotted line), periductal (black arrows), intraepithelial (white arrows), and intraluminal (asterisk). In G-I the double arrows point to mast cells highlighted in purple. In the graphics are plotted the values obtained of animals in IC (points), CG (square), and CO (triangle) groups and the median is shown.

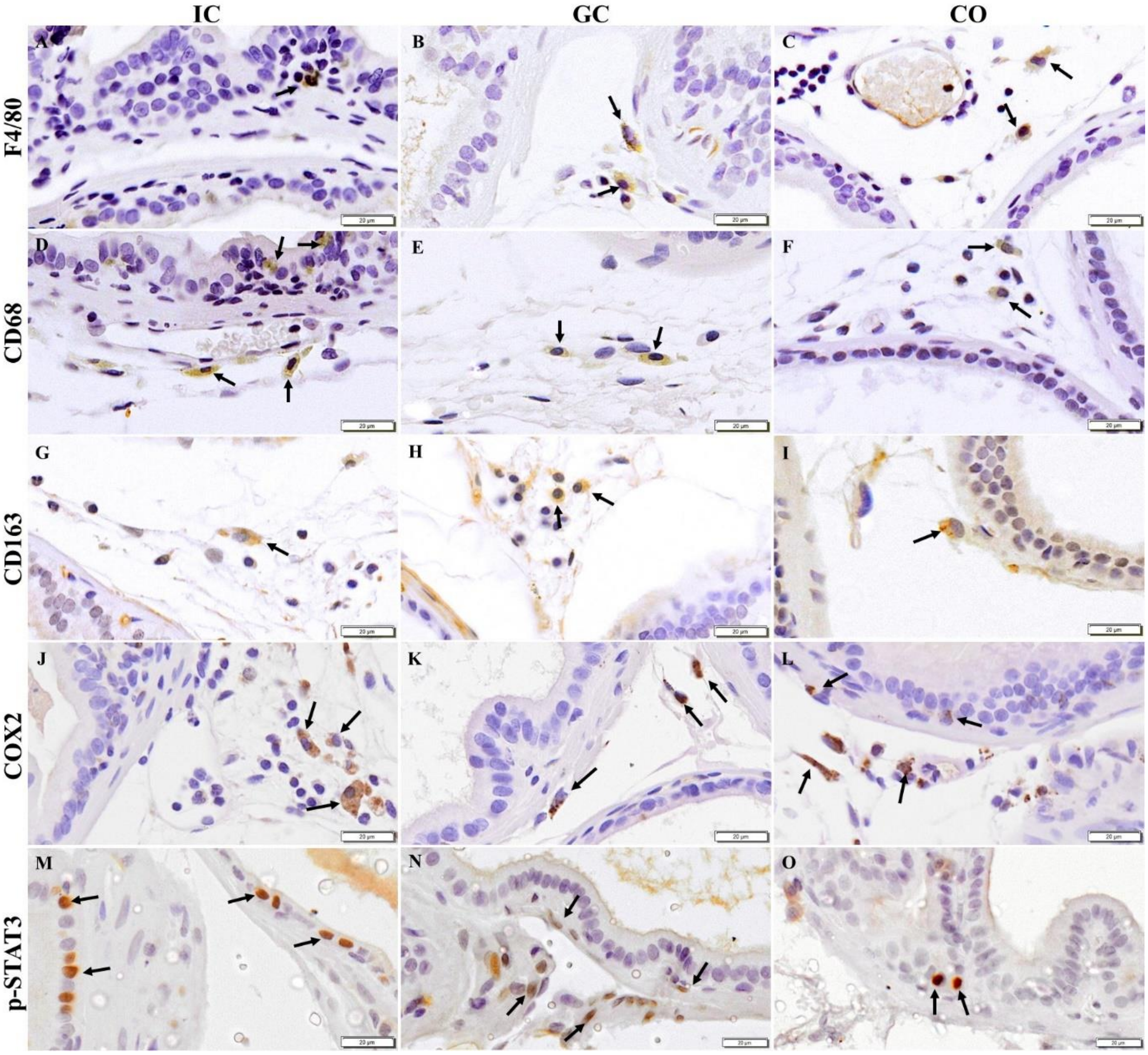

Figure 2: Immunohistochemistry for F4/80 (A-C), CD68 (D-F), CD163 (G-I), COX2 (J-L), p-STAT3 (M-O) of gerbil ventral prostate, at 400x magnification, of groups IC (A, D, G, J, M), GC (B, E, H, K, N) and CO (C, F, I, L, O). The arrows point staining positive for the respective antibodies.

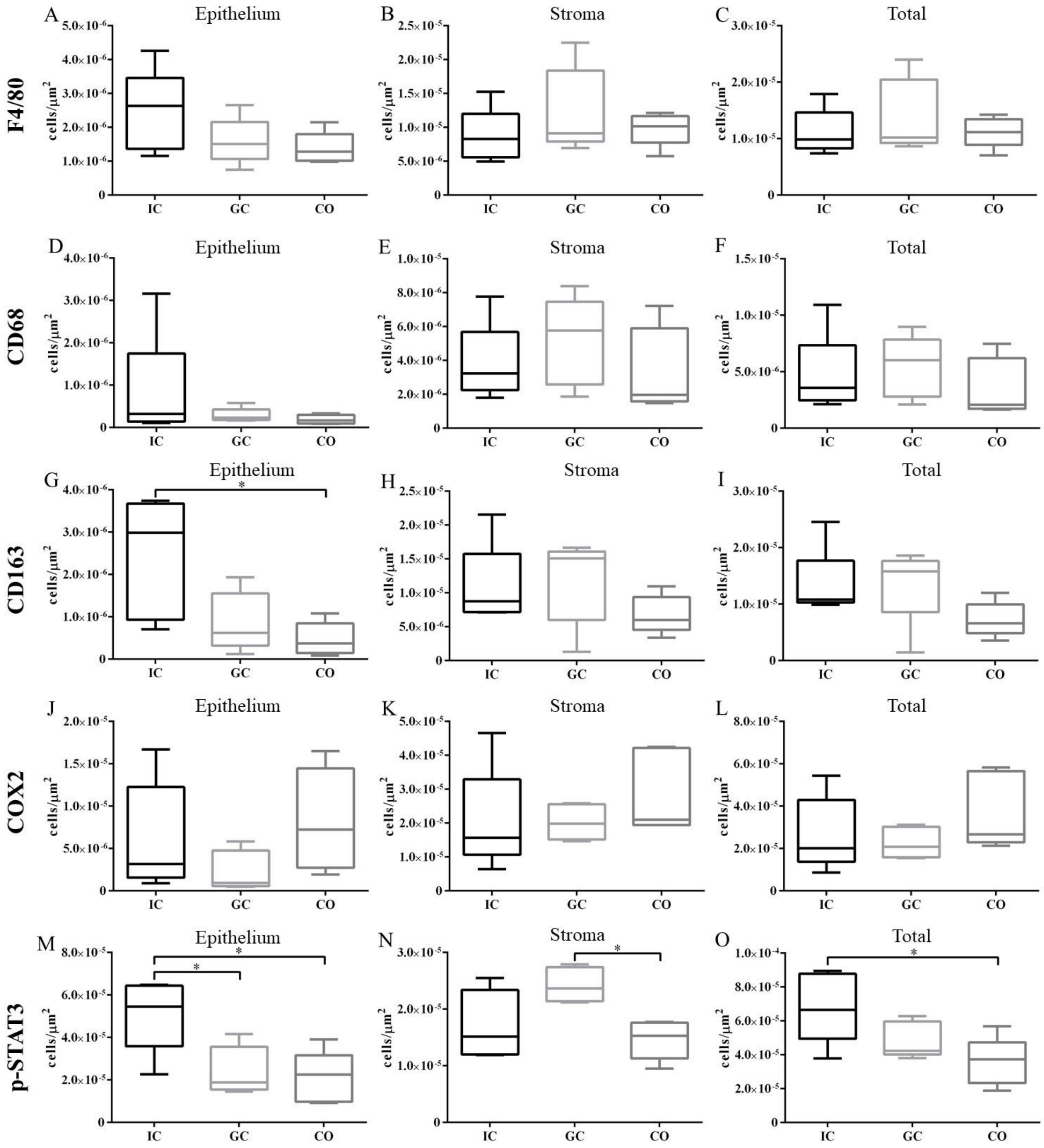

Figure 3: Quantification of positive cells for F4/80 (A-C), CD68 (D-F), CD163 (G-I), COX2 (J-L), p-STAT3 (M-O) per histological section area (µm^2). The data concerning the epithelium (A, D, G, J, M), stroma (B, E, H, K, N), and total prostate (C, F, I, L, O) are presented. Immunohistochemistry data were tested employing the Kruskal-Wallis test and are presented as box and wiskers and statistical difference between the experimental groups is represented by * ($p \leq 0.05$**)**.

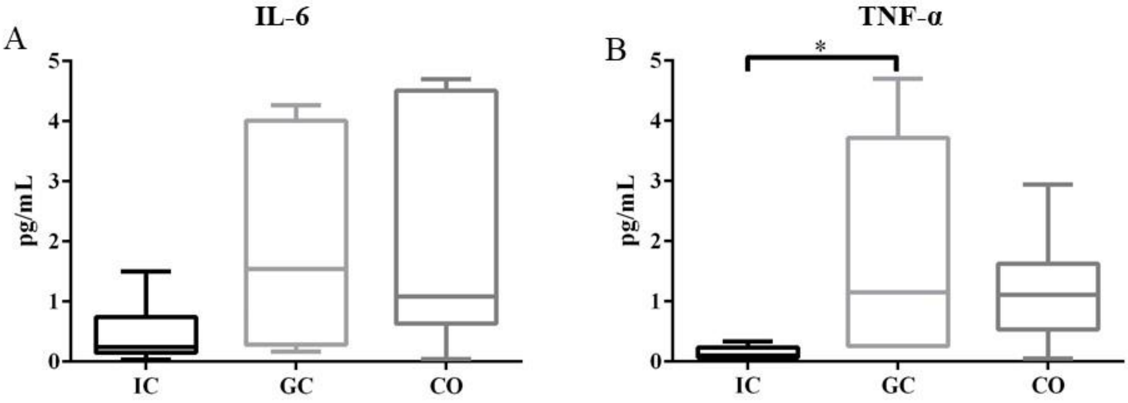

Figure 4: Concentrations (pg/mL) of IL-6 (A) and TNF-a (B) in the ventral prostate of gerbils in IC, GC, and CO groups. The data were tested employing the Kruskal-Wallis test and are presented as box and wiskers and statistical difference between the experimental groups is represented by * ($p \leq 0.05$**).**

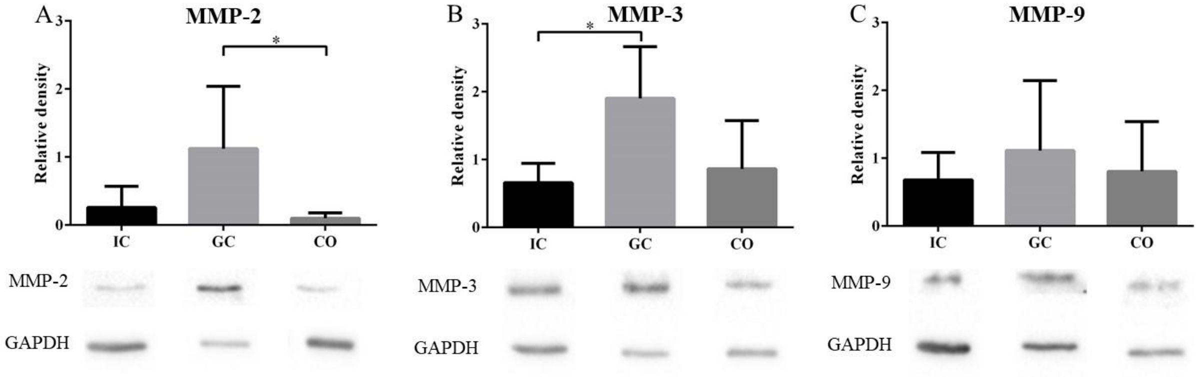

Figure 5: Relative density of MMP-2 (P), MMP-3 (Q), and MMP-9 (R) normalized to GAPDH, used here as a positive control. The data were tested employing one-way ANOVA and presented as mean and standard deviation and statistical difference between the experimental groups is represented by * ($p \leq 0.05$**)**

5.3 Capítulo 3 – Artigo 3.

Será submetido à revista “Phytotherapy Research”

Coconut oil intake throughout aging attenuates ROS increase arising from animal handling.

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Abstract

Aging increases oxidative stress in the prostate and leads to several pathologies such as benign prostatic hyperplasia (BPH) and prostate cancer (PCa). Coconut oil consumption may attenuate some physiological conditions associated with these diseases. In this work, we evaluated the effect of coconut oil intake throughout aging and the interference of animal handling along the experiment. Mongolian gerbils (*Meriones unguiculatus*) received coconut oil (extracted from *Cocos nucifera)* or water via gavage for one year and the prostates were analysed. Intracellular lipid accumulation, oxidative stress biomarkers, antioxidant enzymes activity, lipofuscin accumulation and lipid profile were analysed. Coconut oil consumption mitigated the increase of catalase and GST activity due to reactive oxygen species increase induced by animal handling, and decreased cholesterol and triglycerides serum levels. Animal handling decreased lipofuscin accumulation. In conclusion, animal handling of gavage procedure changed the prostate oxidative status and altered the lipid profile which was mitigated by coconut oil. Moreover, its intake attenuated the age-related metabolic changes, suggesting the coconut oil protective property in both conditions.

Keywords

Lipofuscin, metabolic aging, benign prostatic hyperplasia, vegetal oil.

Introduction

The prostate is highly responsive to aging process changing the endocrine balance, inflammatory status, and oxidative stress thereby providing a favorable microenvironment for benign prostatic hyperplasia (BPH) and prostate cancer (PCa) establishment (Udensi & Tchounwou, 2016). BPH rates increase with age up to 75% among men over 50 years (Egan, 2016). During the past decades, complementary and alternative medicines such as phytotherapic agents have emerged as a possible treatment for BPH and for " keeping a healthy prostate", such as *Serenoa repens*, *Secale cereale*, *Pygeum africanum*, and their mechanisms of action range from anti-androgenic to antioxidant and anti-inflammatory (Keehn, Taylor, & Lowe, 2016). Coconut oil, extracted from the *Cocos nucifera* plant is drawing interest as another possible phytotherapy due to its potential in reduce prostate weight in rats with induced BPH (de Lourdes Arruzazabala et al., 2007).

Dietary supplementation with coconut oil was reported to improve the lipid profile and regulate the antioxidant system by increasing superoxide dismutase (SOD) and catalase activity (CAT) while decreasing malondialdehyde (MDA) levels (Famurewa, Ekeleme-egedigwe, et al., 2017). However, the effects of coconut oil on lipid profile are controversial, given that its consumption worsened lipid parameters, such as triglyceride and LDL levels (Resende et al., 2016). The current data regarding coconut oil intake is based on short to moderate-term, and there are none about long-term. Therefore, this study aimed to investigate the effect of coconut oil consumption throughout aging on oxidative status and lipid profile in the prostate.

Materials and Methods

Animals and experimental design

The animals (male gerbils, 100 days old) were obtained and kept in the Institute of Biosciences, Letters and Exact Sciences of São Paulo State University/UNESP, São José do Rio Preto, with approval and in compliance with the institutional ethics committee guidelines (183/2018-CEUA). The animals were kept in ventilated polysulphone isolators (2 animals per box) under controlled temperature and light (25ºC, 12h light-dark cycle) with filtered water and feed *ad libitum*. The animals were equally distributed in three experimental groups: Intact Control group (IC), without any treatment; Gavage Control (GC), administered 0.1ml of filtered water by gavage every other day; Coconut Oil (CO) administered 0.1ml of coconut oil (Nature Corp

LTDA, Sorocaba, Brazil) by gavage every other day. The dosage provided corresponded to 13ml for an adult male (80kg), adjusted from Reagan-Shaw et al., 2007 (Reagan-Shaw, Nihal, & Ahmad, 2007). The length of the experiment was 12 months and at the end, all animals (15 months old) were anesthetized (3 mg/kg of Xylazine hydrochloride and 10 mg/kg of Ketamine hydrochloride) and euthanized by decapitation. The blood was collected and the serum stored (-80ºC). The ventral prostates were removed and fixed in 4% buffered paraformaldehyde (n=6/group) or flash-frozen in liquid nitrogen and stored at -80ºC (n=48).

Intracellular lipid accumulation analysis

The frozen prostates (n=6/group) were sectioned in a cryostat and the cryosections (25 µm thickness), placed on glass slides, fixed by formaldehyde 3.7% at 60ºC for 1h, washed in distilled water and then immersed in 60% isopropanol for 20 minutes. The slides were then incubated in a solution of 3% oil red O (ORO) in 60% isopropanol for 2h, washed in 60% isopropanol, and in water. Finally, the slides were covered with Fluoroshield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA; F6057). The sample was analyzed in a Zeiss LSM710 Confocal Microscope at 400x magnification and digitized with Zen 2010 software (Carl Zeiss, Thornwood, New York). The images (6 fields/animal) were analyzed in ImageJ software and the quantification of lipid accumulation was accessed by the ratio between total area stained by ORO and total area stained with DAPI. Based on our previous experience with Mongolian gerbil prostate cells, we assumed that nuclei size variations did not influence our analysis.

Oxidative status

Frozen prostate (n=8/group) were homogenized in 0.2 M phosphate buffer, 1 mM EDTA, using a homogenizer (OMNI). The homogenates were centrifuged (15.000g/ 10 min/ 4 °C) and the supernatants were used for analysis of nitric oxide (**NO**), superoxide dismutase (**SOD**), catalase (**CAT**) and glutathione S-transferase (GST) activities. The pellets were used for analyses of protein oxidation (carbonylated proteins - **CP**). Total protein was determined according to (LOWRY, ROSEBROUGH, FARR, & RANDALL, 1951) using bovine serum albumin (BSA) as a standard.

MDA content in prostate tissue (n=6) was determined using a Thiobarbituric Acid Reactive Substances (TBARS) assay kit (Cayman Chemical, Item Number 10009055) according to the manufacturer’s instructions. Upon boiling temperature, oxidized lipids produce MDA which can be measured calorimetrically at 530–540nm. The results were expressed in µM. **CP:** Based in the reaction of the carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH) The supernatant was measured in a microplate reader at 370 nm. The carbonylated proteins were expressed as nmole/mL based on the molar extinction coefficient of ε370 1⁄4 22 mmol/L × cm. **NO:** The samples were incubated with Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine, in 2.5% H_3PO_4) for 10 min at room temperature (Tsikas, 2007). The concentrations of NO were inferred from a standard curve using known concentrations of sodium nitrite. The absorbance was measured at 570 nm and the results expressed in µmol/L. **SOD:** Analysis based on the reduction of the $O^{2-\bullet}$ in H_2O_2, hence decreasing the auto-oxidation of pyrogallol (Dieterich, Bieligk, Beulich, Hasenfuss, & Prestle, 2000). Potassium phosphate buffer (5 mM, pH 8) was added to the samples and the reaction started by adding pyrogallol (100 mM). The reaction mixture was determined by absorbance reading at 570 nm. SOD activity was calculated as U/mg of protein, with one U of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. **CAT**: Catalase activity was measured by adapting the (Hadwan & Abed, 2016) .The samples were added to the substrate (65M H_2O_2 in 60 mM of potassium sodium phosphate buffer, pH 7.4) or in buffer (blank) and incubated (37ºC, 3 min), the reaction was stopped with the addition of molybdate of ammonia (32.4 mM), and the absorbance reading at 374 nm. The micromolar concentrations of H_2O_2 was calculated using a standard curve with a known concentration of H_2O_2. The catalase activity was expressed as U CAT/mg protein. **GST:** determined by the formation of glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB) (Habig, Pabst, & Jakoby, 1974). 1 mM of CDNB was added to the buffer containing 1 mM of GSH plus the sample, then the change monitored at 340nm for 90 sec. The molar extinction coefficient for CDNB was ε340 = 9.6 mM x cm. The results were expressed in µmole/min/g.

Lipofuscin histological analysis

The fixed prostates were dehydrated, clarified in xylene, and included in Histosec (Merck). The organs were sectioned at 3µm thickness and stained by the Gömöri - Halmi method following Behmer et al., 1976 (Behmer, Tolosa, & Freitas Neto, 1976). Images were captured at ×400 magnification using the slide scanner system (Olympus VS120 - S5). Images at 1000x magnification were acquired using Olympus BX60 light microscope (Olympus, Japan) and digitalized with DP-BSW V3.1 software (Olympus). The lipofuscin accumulation was measured with the OlyVIA 2.9 software (Olympus) by counting cells with high apical accumulation of lipofuscin per area of each section and values expressed as mean ± SD of cells per µm^2. We assumed that lipofuscin accumulation clusters of granules similar to the nucleus size of the surrounding cells.

Lipid profile

Lipid profile was evaluated in blood samples (n=6) using enzymatic tests to assess the total cholesterol and levels of HDL, LDL, and triglyceride (In vitro Diagnóstica Ltda, Itabira, MG, Brazil). All samples were evaluated in duplicate using Cary Eclipse Fluorescence Spectrophotometer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). First, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was adopted. Differences were considered statistically significant when $p < 0.05$.

Results

The accumulation of intracellular lipid, shown in figure 1 (A, C, E), was observed in all experimental groups. Lipids accumulated in isolated sites along the prostatic tissue. In these regions, the lipid accumulation was observed mainly at the base of the epithelial cells and as the accumulation increased inside the cells the droplets took over the cytoplasm. In the stromal cells close to these regions of lipid accumulation was also observed the presence of lipid accumulation, but lesser than in the epithelial cells. We did not observe the association between regions of lipid accumulation with prostatic lesions. The presence of regions with accumulation varied greatly among animals of each experimental group and its quantification showed a higher mean in the CO group despite of statistical analysis has shown no difference between the experimental groups (Fig 1G).

Regarding the antioxidant enzymes, CAT activity was increased in the GC when compared to the IC group, and the CO group showed no difference among the other groups. GST activity increased in the GC group compared with IC and CO, whereas in the CO group these levels increased compared with the IC whereas decreased compared to the GC group (Fig2). The levels of MDA, NO, CP and SOD activity remained unaltered among the experimental groups.

Lipofuscin granules were observed in the cytoplasm of epithelial cells, and clusters of these granules were found in the apical region of the cells, close the lumen. Cytoplasmic granules and lipofuscin accumulation were observed either in isolated or grouped cells within the prostatic acini, and no association with prostatic lesions was noted (Fig 1B, D, F). The quantification of cells bearing lipofuscin accumulation showed lower values in the GC group compared with the IC group, while the CO group showed no changes compared with the IC and GC groups (Fig 1H).

The experimental groups GC and CO showed a reduction in LDL and triglycerides serum levels compared with IC. The GC group showed higher HDL values while CO lower total cholesterol compared with the IC. The lipid profile data are shown in Table 1.

Discussion

Antioxidant capacity impairment is ranked among the leading causes of aging, being the prostate gland highly susceptible to oxidative damage (Udensi & Tchounwou, 2016). In the present study, we described the effect of long-term handling on prostate oxidative stress in the Mongolian gerbil and the protective role of coconut oil on the antioxidant system. Campos et al 2008 showed that this model is highly responsive to aging and with several alterations in the prostate, such as intracellular lipid accumulation in addition to develop BPH and PCa spontaneously. Our histological data corroborates the ultrastructural description of this work that showed intracellular lipid droplets accumulated, mainly at the base of epithelial cells, between a large number of mitochondria. The lipid droplets play important roles in the control of membrane lipid composition, mitochondrial function, and the protection of other organelles from lipotoxicity and oxidative stress (Jarc & Petan, 2019). However, our data indicated that animal manipulation and long-term coconut oil consumption did not change the intracellular lipid droplet amount or lipid peroxidation, since the MDA levels were also not changed.

Although SOD was unchanged, CAT activity increased in the GC group indicating that animal handling of the gavage procedure altered the activity of this enzyme. Studies employing animals as experimental models utilize gavage as the controlled route of administration, however, the gavage procedure is considered a stressor, and its effects are highly variable (Bonnichsen, Dragsted, & Kornerup Hansen, 2005). Psychological stress promotes the production of reactive oxygen species (ROS) and leads to DNA damage (Gidron, Russ, Tissarchondou, & Warner, 2006), and the increase in CAT activity was observed in rats under the induction of psychological stress, but no change in SOD levels (Duda et al., 2016). Due to this, we hypothesize that the animal handling increased CAT in response to ROS increase resulting from psychological stress, although it was not enough to cause damage to lipids and proteins, seen by the absence of changes in MDA and CP levels.

Our hypothesis regarding the animal handling to change ROS was bolstered by the GST increase in the GC group. Increased GST levels as a compensatory mechanism against increased oxidative stress have been related with aging (Vyskočilová et al., 2013). GST acts in the detoxification of electrophiles by glutathione conjugation and its activity modulates the development and progression of BPH and PCa (Udensi & Tchounwou, 2016). A putative result of the elevated antioxidant enzymes is the decreased lipofuscin accumulation. Lipofuscin originates from oxidative processes catalyzed by iron and due to its undegradability, it accumulates in post-mitotic cells, being considered an aging marker of aging (Brunk & Terman, 2002). Its accumulation in the prostate is remarkable in old mice, is responsive to hormonal alterations (Morrissey et al., 2002), and is reported as a prognostic in PCa (Mahmoodi, Zhang, Salim, Hou, & Garcia, 2006). Therefore, high levels of CAT and GST may be associated with increased ROS, however, these enzymes likely protected prostate cells against lipofuscin accumulation.

The coconut oil consumption through aging mitigated the effects of animal handling since in the CO group the levels of antioxidant enzymes and lipofuscin accumulation were similar to the IC group. Coconut oil plays an antioxidant and protective role against anti-cancer drugs (Famurewa, Aja, et al., 2017), and this potential is attributed of its components, such as ferulic acid and *p*-coumaric acid (Marina, Che Man, Nazimah, & Amin, 2009). In addition, coconut oil protects against alcohol-induced oxidative stress and maintains the homeostasis of serum lipid levels (Dosumu, Akinola, & Akang, 2012).

Along aging the total cholesterol and triglyceride levels increased and were related to several diseases (Kreisberg & Kasim, 1987). Low HDL levels and elevated triglycerides were reported to cause oxidative stress and prostate tissue remodeling (Vanella et al., 2014). Our data showed that decreased total cholesterol, increased HDL, and decreased LDL in the CO group were also noted in the GC group, suggesting animal handling as the inducer of these metabolic changes. However, we observed that coconut oil intake decreased total cholesterol and triglycerides levels. It is reported to normalize oxidative stress and lipid profile altered by psychological stress induction (Yeap et al., 2015). In conclusion, coconut oil may act as a protector against the increased ROS triggered by long-term handling of the animals, and also protect against metabolic changes resulting from aging.

Acknowledgements

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References

Behmer, O. A., Tolosa, E. M. C. de, & Freitas Neto, A. G. (1976). *Manual de Técnicas para Histologia Normal e Patológica* (H. Washington, ed.). São Paulo: EDART-São Paulo Livraria Editora Ltda.

Bonnichsen, M., Dragsted, N., & Kornerup Hansen, A. (2005). The welfare impact of gavaging laboratory rats. *Animal Welfare*, *14*, 223–227.

Brunk, U. T., & Terman, A. (2002). Lipofuscin: Mechanisms of age-related accumulation and influence on cell function. *Free Radical Biology and Medicine*, *33*(5), 611–619. https://doi.org/10.1016/S0891-5849(02)00959-0

Campos, S. G. P., Zanetoni, C., Scarano, W. R., Vilamaior, P. S. L., & Taboga, S. R. (2008). Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. *International Journal of Experimental Pathology*, *89*(1), 13–24. https://doi.org/10.1111/j.1365-2613.2007.00550.x

de Lourdes Arruzazabala, M., Molina, V., Más, R., Carbajal, D., Marrero, D., González, V., & Rodríguez, E. (2007). Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. *The Journal of Pharmacy and Pharmacology*, *59*(7), 995–999. https://doi.org/10.1211/jpp.59.7.0012

Dieterich, S., Bieligk, U., Beulich, K., Hasenfuss, G., & Prestle, J. (2000). Gene expression of antioxidative enzymes in the human heart: Increased expression of catalase in the end-stage failing heart. *Circulation*, *101*(1), 33–39. https://doi.org/10.1161/01.CIR.101.1.33

Dosumu, O. O., Akinola, O. B., & Akang, E. N. (2012). Alcohol-induced testicular oxidative stress and cholesterol homeostasis in rats - The therapeutic potential of virgin coconut oil. *Middle East Fertility Society Journal*, *17*(2), 122–128. https://doi.org/10.1016/j.mefs.2011.12.005

Duda, W., Curzytek, K., Kubera, M., Iciek, M., Kowalczyk-Pachel, D., Bilska-Wilkosz, A.,

… Antkiewicz-Michaluk, L. (2016). The Effect of Chronic Mild Stress and Imipramine on the Markers of Oxidative Stress and Antioxidant System in Rat Liver. *Neurotoxicity Research*, *30*(2), 173–184. https://doi.org/10.1007/s12640-016-9614-8

Egan, K. B. (2016). The Epidemiology of Benign Prostatic Hyperplasia Associated with Lower Urinary Tract Symptoms: Prevalence and Incident Rates. *Urologic Clinics of North America*, *43*(3), 289–297. https://doi.org/10.1016/j.ucl.2016.04.001

Famurewa, A. C., Aja, P. M., Maduagwuna, E. K., Ekeleme-Egedigwe, C. A., Ufebe, O. G., & Azubuike-Osu, S. O. (2017). Antioxidant and anti-inflammatory effects of virgin coconut oil supplementation abrogate acute chemotherapy oxidative nephrotoxicity induced by anticancer drug methotrexate in rats. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *96*, 905–911. https://doi.org/10.1016/j.biopha.2017.12.008

Famurewa, A. C., Ekeleme-egedigwe, C. A., Nwali, S. C., Agbo, N., Obi, J. N., Ezechukwu, G. C., … Nwali, S. C. (2017). Dietary Supplementation with Virgin Coconut Oil Improves Lipid Profile and Hepatic Antioxidant Status and Has Potential Benefits on Cardiovascular Risk Indices in Normal Rats Dietary Supplementation with Virgin Coconut Oil Improves Lipid Profile and Hepat. *Journal of Dietary Supplements*, *0*(0), 1–13. https://doi.org/10.1080/19390211.2017.1346031

Gidron, Y., Russ, K., Tissarchondou, H., & Warner, J. (2006). The relation between psychological factors and DNA-damage: A critical review. *Biological Psychology*, *72*(3), 291–304. https://doi.org/10.1016/j.biopsycho.2005.11.011

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferases: THE FIRST ENZYMATIC STEP IN MERCAPTURIC ACID FORMATION. *Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/https://doi.org/10.1016/S0021-9258(19)42083-8

Hadwan, M. H., & Abed, H. N. (2016). Data supporting the spectrophotometric method for the estimation of catalase activity. *Data in Brief*, *6*, 194–199. https://doi.org/https://doi.org/10.1016/j.dib.2015.12.012

Jarc, E., & Petan, T. (2019). Lipid Droplets and the Management of Cellular Stress. *The Yale Journal of Biology and Medicine*, *92*(3), 435–452.

Keehn, A., Taylor, J., & Lowe, F. C. (2016). Phytotherapy for Benign Prostatic Hyperplasia.

Current Urology Reports, *17*(7), 1–6. https://doi.org/10.1007/s11934-016-0609-z

Kreisberg, R. A., & Kasim, S. (1987). Cholesterol metabolism and aging. *The American Journal of Medicine*, *82*(1 SUPPL. 2), 54–60. https://doi.org/10.1016/0002-9343(87)90272-5

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Mahmoodi, M., Zhang, S., Salim, S., Hou, J. S., & Garcia, F. U. (2006). Lipofuscin pigment can be used as a prognostic marker in prostatic adenocarcinoma. *Annals of Diagnostic Pathology*, *10*(5), 257–262. https://doi.org/10.1016/j.anndiagpath.2005.12.007

Marina, A. M., Che Man, Y. B., Nazimah, S. A. H., & Amin, I. (2009). Antioxidant capacity and phenolic acids of virgin coconut oil. *International Journal of Food Sciences and Nutrition*, *60*(SUPPL. 2), 114–123. https://doi.org/10.1080/09637480802549127

Morrissey, C., Buser, A., Scolaro, J., O’Sullivan, J., Moquin, A., & Tenniswood, M. (2002). Changes in hormone sensitivity in the ventral prostate of aging Sprague-Dawley rats. *Journal of Andrology*, *23*(3), 341–351.

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2007). Dose translation from animal to human studies revisited. *The FASEB Journal*, *22*(3), 659–661. https://doi.org/10.1096/fj.07-9574LSF

Resende, N. M., Félix, H. R., Soré, M. R., M M Neto, A., Campos, K. E., & Volpato, G. T. (2016). The effects of coconut oil supplementation on the body composition and lipid profile of rats submitted to physical exercise. *Anais Da Academia Brasileira de Ciencias*, *88*(AHEAD), 933–940. https://doi.org/10.1590/0001-3765201620150302

Tsikas, D. (2007). Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *851*(1–2), 51–70. https://doi.org/10.1016/j.jchromb.2006.07.054

Udensi, U. K., & Tchounwou, P. B. (2016). Oxidative stress in prostate hyperplasia and carcinogenesis. *Journal of Experimental and Clinical Cancer Research*, *35*(1), 1–19. https://doi.org/10.1186/s13046-016-0418-8

Vanella, L., Russo, G. I., Cimino, S., Fragalà, E., Favilla, V., Li Volti, G., … Morgia, G. (2014). Correlation between lipid profile and heme oxygenase system in patients with benign prostatic hyperplasia. *Urology*, *83*(6), 1444.e7-13. https://doi.org/10.1016/j.urology.2014.03.007

Vyskočilová, E., Szotáková, B., Skálová, L., Bártíková, H., Hlaváčová, J., & Boušová, I. (2013). Age-related changes in hepatic activity and expression of detoxification enzymes in male rats. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/408573

Yeap, S. K., Beh, B. K., Ali, N. M., Yusof, H. M., Ho, W. Y., Koh, S. P., … Long, K. (2015). Antistress and antioxidant effects of virgin coconut oil in vivo. *Experimental and Therapeutic Medicine*, *9*(1), 39–42. https://doi.org/10.3892/etm.2014.2045

Tables

Table 1: Lipid profile in different experimental groups.

	IC	**GC**	**CO**
Lipid profile (mg/dL)			
Total cholesterol	87.78 ± 14.18^{a}	65.68 ± 14.98^{a,b}	53.38 ± 11.73^{b}
HDL	18.54 ± 5.49^{a}	33.90 ± 9.57^{b}	24.90 ± 6.12^{a,b}
LDL	25.64 ± 11.10^{a}	8.48 ± 8.13^{b}	7.78 ± 4.43^{b}
Triglycerides	198.0 ± 65.34^{a}	116.5 ± 35.66^{b}	66.0 ± 31.25^{b}

Values expressed as mean ± standard deviation. Superscript a and b represent statistically significant differences ($p \leq 0.05$) among the experimental groups.

Figures

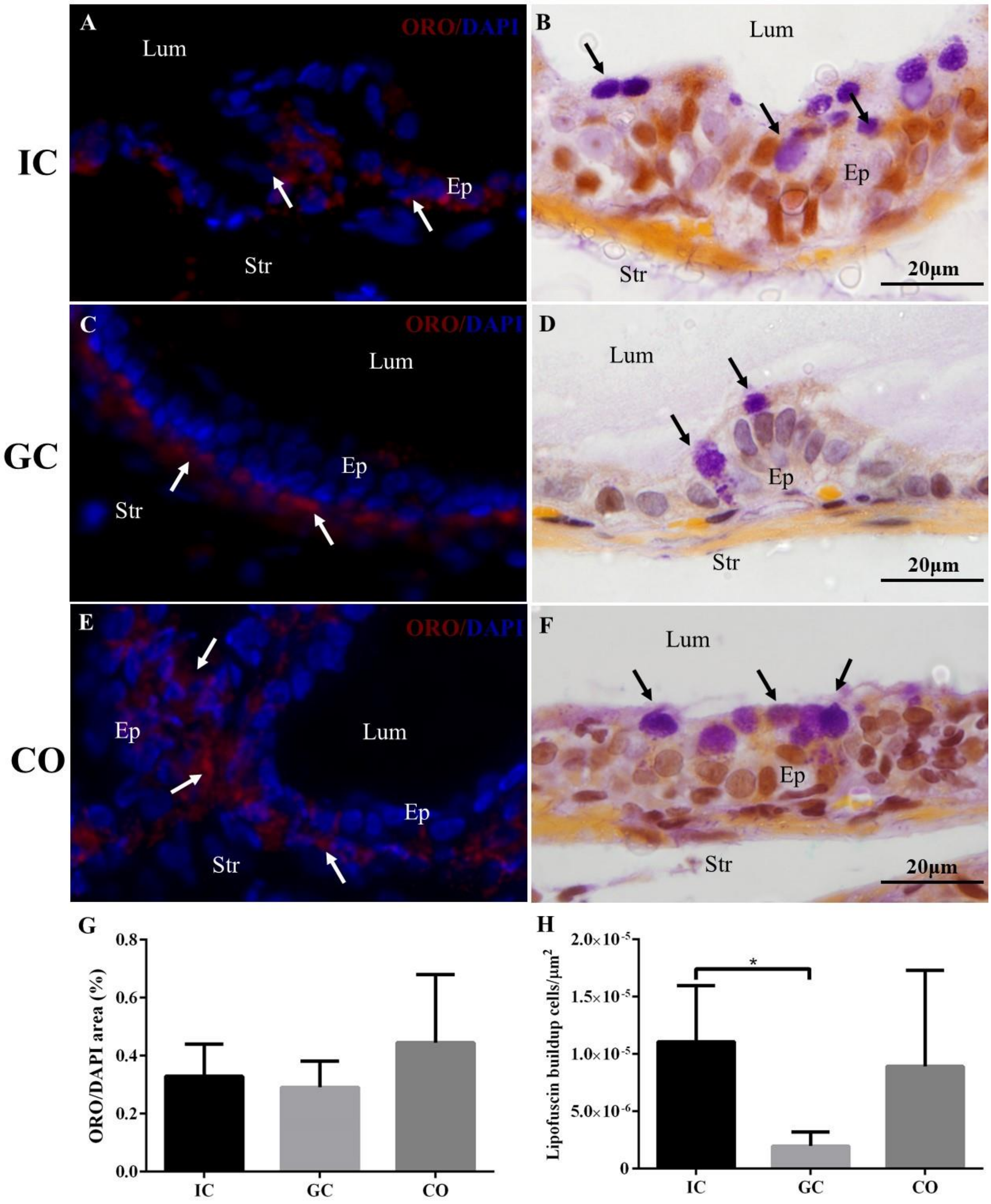

Figure 1: Histological sections of gerbil prostate stained with Oil Red O (ORO) and DAPI at 400x magnification (A, C, E) and Gömöri - Halmi at 1000x magnification (B,D,F) of groups IC (A,B), GC (C,D), and CO (E,F). White arrows point lipid droplets colored in red, black arrows point lipofuscin accumulation colored in purple. The regions of epithelium (Ep), lumen (Lum) and stroma (Str) are indicated. In G the percentages of ORO/DAPI area and in H the lipofuscin buildup cells/µm^2 are presented as mean + SD. The data of IC, GC and CO experimental groups were tested employing one-way ANOVA and statistical difference between the experimental groups is represented by * ($p < 0.05$).

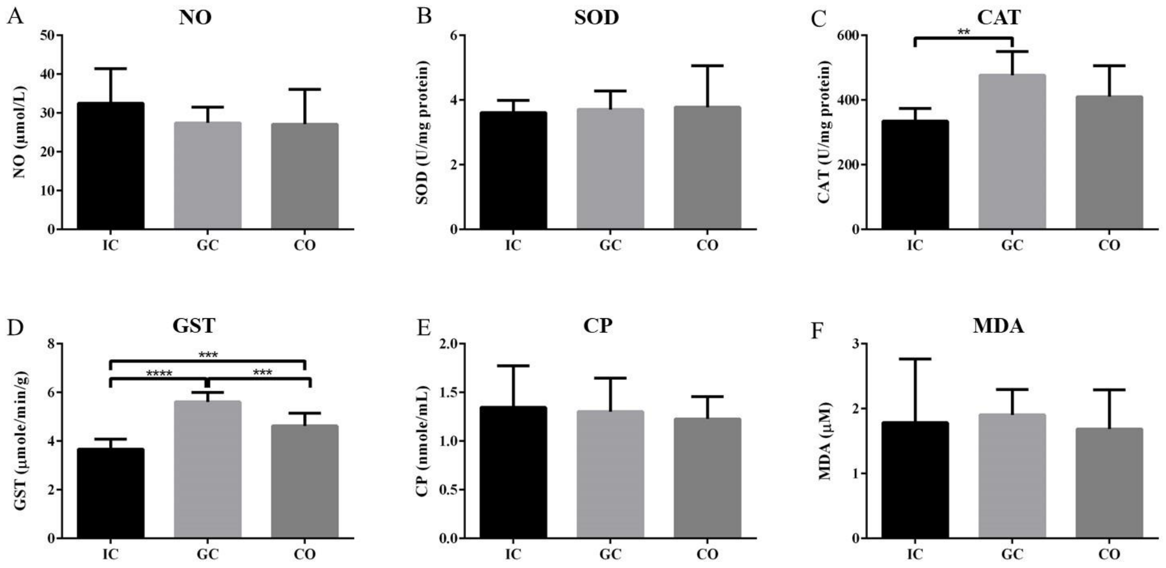

Figure 2: Antioxidant enzymes (A, E, F) and oxidative stress indexes (B, C, D): (A) Nitric Oxide – NO, (B) Superoxide dismutase – SOD, (C) Catalase – CAT, (D) Glutathione S-transferase – GST, (E) Carbonylated proteins – CP, (F) Malondialdehyde – MDA. The data of IC, GC and CO experimental groups were tested employing one-way ANOVA and presented as mean and standard deviation and statistical difference between the experimental groups is represented by ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

6 CONSIDERAÇÕES FINAIS E CONCLUSÃO

O desenvolvimento de lesões na próstata, como HPB e CP já é postulado como uma consequência do envelhecimento. A presente tese evidenciou que condições de estresse psicológico, geradas por procedimentos experimentais que incluíram contenção dos animais durante o envelhecimento, aceleram este processo. Nesse contexto estão envolvidas alterações na morfologia prostática, na expressão de receptores hormonais, índices de proliferação e de lesões. A inflamação modula a HPB por meio da ação de células, como macrófagos e mastócitos, citocinas e outros fatores. Nossos dados evidenciaram que a manipulação dos animais, especificamente o procedimento de gavagem, influencia na população de células inflamatórias, no nível de citocinas e expressão de metaloproteinases. Outros processos fisiológicos associados ao envelhecimento e HPB, como o aumento do estresse oxidativo e acúmulo de lipofuscina, também se mostraram susceptíveis a esse procedimento. Já o consumo de óleo de coco por animais sujeitos às mesmas condições experimentais, que levaram à uma aceleração dos efeitos relacionados ao envelhecimento, atenuou esses efeitos.

Essa tese conclui que, em condições de estresse psicológico que aceleraram os efeitos do envelhecimento na próstata, o consumo de óleo de coco durante o envelhecimento interfere no desenvolvimento de HPB, atenuando a gravidade das lesões prostáticas, diminuindo a inflamação e evitando o aumento do estresse oxidativo.

7 REFERÊNCIAS

ADARAMOYE, O. A. et al. Methyl jasmonate reduces testosterone-induced benign prostatic hyperplasia through regulation of inflammatory and apoptotic processes in rats. **Biomedicine and Pharmacotherapy**, [s. l.], v. 95, n. August, p. 1493–1503, 2017. Disponível em: <http://dx.doi.org/10.1016/j.biopha.2017.08.106>

AHMAD, I.; SANSOM, O. J.; LEUNG, H. Y. Advances in mouse models of prostate cancer. **Expert Reviews in Molecular Medicine**, [s. l.], v. 10, n. 16, p. 1–20, 2008.

AKPAN, E. J. et al. Fatty acid profile and oil yield in six different varieties of fresh and dry samples of coconuts (Cocos nucifera). **Pakistan Journal of Nutrition**, [s. l.], v. 5, n. 2, p. 106–109, 2006.

ALBERNAZ, E. S. S. et al. The prostate of the bat Artibeus lituratus: Seasonal variations, abiotic regulation, and hormonal control. **Journal of Morphology**, [s. l.], v. 282, n. 8, p. 1188–1207, 2021.

ALUKAL, J. P.; LEPOR, H. Testosterone Deficiency and the Prostate. [s. l.], v. 43, p. 203–208, 2016.

BANERJEE, P. P. et al. Age-dependent and lobe-specific spontaneous hyperplasia in the brown Norway rat prostate. **Biology of reproduction**, United States, v. 59, n. 5, p. 1163–1170, 1998.

BARRON, D. A.; ROWLEY, D. R. The reactive stroma microenvironment and prostate cancer progression. **Endocrine-related cancer**, [s. l.], v. 19, n. 6, p. R187-204, 2012.

BATCHELDER, M. et al. **Gerbils**. [s.l.] : Elsevier Inc., 2012. Disponível em: <http://dx.doi.org/10.1016/B978-0-12-380920-9.00052-3>

BECHIS, S. K. et al. Personalized medicine for the management of benign prostatic hyperplasia. **The Journal of urology**, [s. l.], v. 192, v. 2014/02/25, n. 1, p. 16–23, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24582540>

BELLINGER, D. L. et al. Psychosocial Stress and Age Influence Depression and Anxiety-Related Behavior, Drive Tumor Inflammatory Cytokines and Accelerate Prostate Cancer Growth in Mice. **Frontiers in Oncology**, [s. l.], v. 11, n. September, p. 1–20, 2021.

BONKHOFF, H.; REMBERGER, K. [Morphogenesis of benign prostatic hyperplasia and prostatic carcinoma]. **Der Pathologe**, Germany, v. 19, n. 1, p. 12–20, 1998.

BREWSTER, D. Memoirs of life, writings, and discovers of Sir Isaac Newton. **T. Constable and Company**, v. 2, p 1-557, 1855.

CALDER, P. C. Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. **Molecular Nutrition and Food Research**, [s. l.], v. 52, n. 8, p. 885–897, 2008.

CAMPOS, S. G. P. et al. Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. **International Journal of Experimental Pathology**, [s. l.], v. 89, n. 1, p. 13–24, 2008.

CHOI, H. M. et al. Cinnamomi Cortex (Cinnamomum verum) Suppresses Testosterone-induced Benign Prostatic Hyperplasia by Regulating 5α-reductase. **Scientific Reports**, [s. l.], v. 6, n. July, p. 1–12, 2016. Disponível em: <http://dx.doi.org/10.1038/srep31906>

CORDEIRO, R. S. et al. Androgen receptor in the Mongolian gerbil ventral prostate: Evaluation during different phases of postnatal development and following androgen blockage. **Micron**, [s. l.], v. 39, n. 8, p. 1312–1324, 2008.

CUNHA, G. R. et al. Estrogenic effects on prostatic differentiation and carcinogenesis. **Reproduction, fertility, and development**, Australia, v. 13, n. 4, p. 285–296, 2001.

CZYŻ, J.; SZPAK, K.; MADEJA, Z. The role of connexins in prostate cancer promotion and progression. **Nature reviews. Urology**, England, v. 9, n. 5, p. 274–282, 2012.

DE CARVALHO, H. F.; TABOGA, S. R.; VILAMAIOR, P. S. Collagen type VI is a component of the extracellular matrix microfibril network of the prostatic stroma. **Tissue & cell**, Scotland, v. 29, n. 2, p. 163–170, 1997.

DE LOURDES ARRUZAZABALA, M. et al. Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. **The Journal of pharmacy and pharmacology**, [s. l.], v. 59, n. 7, p. 995–9, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17637195>

DE NUNZIO, C.; PRESICCE, F.; TUBARO, A. Inflammatory mediators in the development and progression of benign prostatic hyperplasia. **Nature Reviews Urology**, [s. l.], v. 13, n. 10, p. 613–626, 2016. Disponível em: <http://dx.doi.org/10.1038/nrurol.2016.168>

DENG, K.; LIU, W.; WANG, D. Inter-group associations in Mongolian gerbils: Quantitative evidence from social network analysis. **Integrative Zoology**, [s. l.], v. 12, n. 6, p. 446–456, 2017.

EGAN, K. B. The Epidemiology of Benign Prostatic Hyperplasia Associated with Lower Urinary Tract Symptoms: Prevalence and Incident Rates. **Urologic Clinics of North America**, [s. l.], v. 43, n. 3, p. 289–297, 2016. Disponível em: <http://dx.doi.org/10.1016/j.ucl.2016.04.001>

ELLEM, S. J.; RISBRIDGER, G. P. The dual, opposing roles of estrogen in the prostate. **Annals of the New York Academy of Sciences**, [s. l.], v. 1155, p. 174–186, 2009.

FISHER, M. F.; LLEWELLYN, G. C. The Mongolian Gerbil: Natural History, Care, and Maintenance. **The American Biology Teacher**, [s. l.], v. 40, n. 9, p. 557–560, 1978. Disponível em: <https://doi.org/10.2307/4446413>

GANDAGLIA, G. et al. The role of prostatic inflammation in the development and progression of benign and malignant diseases. **Current Opinion in Urology**, [s. l.], v. 27, n. 2, p. 99–106, 2017.

GRAY, J. M.; CHAOULOFF, F.; HILL, M. N. To stress or not to stress: A question of models. **Current Protocols in Neuroscience**, [s. l.], v. 2015, n. January, p. 8.33.1-8.33.22, 2015.

GRINDSTAD, T. et al. Estrogen receptors α and β and aromatase as independent predictors for prostate cancer outcome. **Scientific Reports**, [s. l.], v. 6, n. August, p. 1–10, 2016. Disponível em: <http://dx.doi.org/10.1038/srep33114>

GUERRA, L. H. A. et al. Do mineral and corn oil serve as potential endocrine disruptors in the gerbil prostate? **Reproductive Toxicology**, [s. l.], v. 90, n. September, p. 141–149, 2019. Disponível em: <https://doi.org/10.1016/j.reprotox.2019.09.004>

HARMAN, S. M. et al. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. **The Journal of clinical endocrinology and metabolism**, United States, v. 86, n. 2, p. 724–731, 2001.

HENDERSON, B. E.; FEIGELSON, H. S. Hormonal carcinogenesis. **Carcinogenesis**, England, v. 21, n. 3, p. 427–433, 2000.

HSING, A W.; REICHARDT, J. K.; STANCZYK, F. Z. Hormones and prostate cancer: current perspectives and future directions. **Prostate**, [s. l.], v. 52, n. 3, p. 213–235, 2002. Disponível em: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation

&list_uids=12111697>

HUANG, S. et al. Sympathetic nervous system overactivity in the wistar rat with proliferative lesions of ventral prostate induced by chronic stress. **Urologia Internationalis**, [s. l.], v. 83, n. 2, p. 230–235, 2009.

JEON, W. Y. et al. Inhibitory effects of Ponciri Fructus on testosterone-induced benign prostatic hyperplasia in rats. **BMC Complementary and Alternative Medicine**, [s. l.], v. 17, n. 1, p. 1–10, 2017.

JESIK, C. J.; HOLLAND, J. M.; LEE, C. An anatomic and histologic study of the rat prostate. **The Prostate**, United States, v. 3, n. 1, p. 81–97, 1982.

KAYA, E. et al. Oxidative stress parameters in patients with prostate cancer, benign prostatic hyperplasia and asymptomatic inflammatory prostatitis: A prospective controlled study. **Advances in clinical and experimental medicine : official organ Wroclaw Medical University**, Poland, v. 26, n. 7, p. 1095–1099, 2017.

KEEHN, A.; TAYLOR, J.; LOWE, F. C. Phytotherapy for Benign Prostatic Hyperplasia. **Current Urology Reports**, [s. l.], v. 17, n. 7, p. 1–6, 2016. Disponível em: <http://dx.doi.org/10.1007/s11934-016-0609-z>

KHAKISAHNEH, S. et al. Thyroid hormones mediate metabolic rate and oxidative, anti-oxidative balance at different temperatures in Mongolian gerbils (Meriones unguiculatus). **Comparative biochemistry and physiology. Toxicology & pharmacology : CBP**, United States, v. 216, p. 101–109, 2019.

KIANI, A. et al. Activities and polymorphisms of MMP-2 and MMP-9, smoking, diabetes and risk of prostate cancer. **Molecular biology reports**, Netherlands, v. 47, n. 12, p. 9373–9383, 2020.

KOFOED, J. A. et al. Effects of castration and androgens upon prostatic proteoglycans in rats. **The Prostate**, United States, v. 16, n. 2, p. 93–102, 1990.

KRAMER, G.; MARBERGER, M. Could inflammation be a key component in the progression of benign prostatic hyperplasia? **Current opinion in urology**, [s. l.], v. 16, n. 1, p. 25–9, 2006. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16385197>

KRUŠLIN, B. et al. Inflammation in Prostatic Hyperplasia and Carcinoma—Basic Scientific Approach. **Frontiers in Oncology**, [s. l.], v. 7, n. April, p. 1–7, 2017. Disponível em:

<http://journal.frontiersin.org/article/10.3389/fonc.2017.00077/full>

LA VIGNERA, S. et al. Endocrine control of benign prostatic hyperplasia. **Andrology**, [s. l.], v. 4, n. 3, p. 404–411, 2016. Disponível em: <http://doi.wiley.com/10.1111/andr.12186>

LAM, J. S.; ROMAS, N. A.; LOWE, F. C. Long-term treatment with finasteride in men with symptomatic benign prostatic hyperplasia: 10-year follow-up. **Urology**, United States, v. 61, n. 2, p. 354–358, 2003.

LEONEL, E. C. R. et al. Impact of perinatal bisphenol A and 17β estradiol exposure: Comparing hormone receptor response. **Ecotoxicology and Environmental Safety**, [s. l.], v. 188, n. February 2019, p. 109918, 2020. Disponível em: <https://doi.org/10.1016/j.ecoenv.2019.109918>

MARKER, P. C. et al. Hormonal , cellular , and molecular control of prostatic development. **Developmental Biology**, [s. l.], v. 253, n. 2, p. 165–174, 2003. a.

MARKER, P. C. et al. Hormonal, cellular, and molecular control of prostatic development. **Developmental biology**, United States, v. 253, n. 2, p. 165–174, 2003. b.

MCPHERSON, S. J.; ELLEM, S. J.; RISBRIDGER, G. P. Estrogen-regulated development and differentiation of the prostate. **Differentiation**, [s. l.], v. 76, n. 6, p. 660–670, 2008.

NARAYANANKUTTY, A. et al. Virgin coconut oil reverses hepatic steatosis by restoring redox homeostasis and lipid metabolism in male Wistar rats. [s. l.], n. September, 2017.

NGAI, H.-Y. et al. Metabolic syndrome and benign prostatic hyperplasia: An update. **Asian journal of urology**, [s. l.], v. 4, n. 3, p. 164–173, 2017.

NICOLOSI, R. J. et al. Influence of dietary unsaturated and saturated fat on the plasma lipoproteins of Mongolian gerbils. **Atherosclerosis**, Ireland, v. 38, n. 3–4, p. 359–371, 1981.

OLIVEIRA, D. S. M. et al. The mouse prostate: a basic anatomical and histological guideline. **Bosnian journal of basic medical sciences**, [s. l.], v. 16, n. 1, p. 8–13, 2016.

PATEL, N. D.; PARSONS, J. K. Epidemiology and etiology of benign prostatic hyperplasia and bladder outlet obstruction. **Indian journal of urology : IJU : journal of the Urological Society of India**, [s. l.], v. 30, n. 2, p. 170–176, 2014.

PEGORIN DE CAMPOS, S. G. et al. Biological behavior of the gerbil ventral prostate in three phases of postnatal development. **Anatomical Record - Part A Discoveries in**

Molecular, Cellular, and Evolutionary Biology, [s. l.], v. 288, n. 7, p. 723–733, 2006.

PINTO-FOCHI, M. E. et al. Sexual maturation of the Mongolian gerbil (Meriones unguiculatus): A histological, hormonal and spermatic evaluation. **Reproduction, Fertility and Development**, [s. l.], v. 28, n. 6, p. 815–823, 2016.

PRICE, D. COMPARATIVE ASPECTS OF DEVELOPMENT AND STRUCTURE IN THE PROSTATE. **National Cancer Institute monograph**, United States, v. 12, p. 1–27, 1963.

PRINS, G. S. Endocrine disruptors and prostate cancer risk. **Endocrine Related Cancer**, [s. l.], v. 15, n. 3, p. 649–656, 2008. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2822396&tool=pmcentrez&rendertype=abstract>

RAMM, S. A.; PARKER, G. A.; STOCKLEY, P. Sperm competition and the evolution of male reproductive anatomy in rodents. **Proceedings of the Royal Society B: Biological Sciences**, [s. l.], v. 272, n. 1566, p. 949–955, 2005.

RAZZOLI, M. et al. AFLP to assess genetic variation in laboratory gerbils (Meriones unguiculatus). **The Journal of heredity**, United States, v. 94, n. 6, p. 507–511, 2003.

REBELLO, R. J. et al. Prostate cancer. **Nature Reviews Disease Primers**, [s. l.], v. 7, n. 1, 2021. Disponível em: <http://dx.doi.org/10.1038/s41572-020-00243-0>

REESE, J. H. et al. Differential distribution of pepsinogen II between the zones of the human prostate and the seminal vesicle. **The Journal of urology**, United States, v. 136, n. 5, p. 1148–1152, 1986.

RESENDE, N. M. et al. The effects of coconut oil supplementation on the body composition and lipid profile of rats submitted to physical exercise. **Anais da Academia Brasileira de Ciencias**, [s. l.], v. 88, n. AHEAD, p. 933–940, 2016. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0001-37652016005006106&lng=en&nrm=iso&tlng=en>

RISBRIDGER, G. P.; TAYLOR, R. A. Physiology of the male accessory sex structures: The prostate gland, seminal vesicles, and bulbourethral glands. **Knobil and Neill's Physiology of Reproduction**, [s. l.], p. 1149–1172, 2006.

ROCHEL, S. S. et al. Lobe identity in the Mongolian gerbil prostatic complex: A new rodent model for prostate study. **Anatomical Record**, [s. l.], v. 290, n. 10, p. 1233–1247, 2007. a.

ROCHEL, S. S. et al. Lobe identity in the Mongolian gerbil prostatic complex: a new rodent model for prostate study. **Anatomical record (Hoboken, N.J. : 2007)**, United States, v. 290, n. 10, p. 1233–1247, 2007. b.

ROEHRBORN, C. G. Pathology of benign prostatic hyperplasia. **International Journal of Impotence Research**, [s. l.], v. 20, n. SUPPL. 3, 2008. a.

ROEHRBORN, C. G. Pathology of benign prostatic hyperplasia. **International Journal of Impotence Research**, [s. l.], v. 20, n. SUPPL. 3, 2008. b.

ROSS, M. H.; PAWLINA, W. **Histology: A Text and Atlas, with Correlated Cell and Molecular Biology, 6th Edition**. [s.l.] : Lippincott, 1979. Disponível em: <https://books.google.com.br/books?id=P42YQgAACAAJ>

SANCHES, B. D. A. et al. Telocytes play a key role in prostate tissue organisation during the gland morphogenesis. **Journal of cellular and molecular medicine**, [s. l.], v. 21, n. 12, p. 3309–3321, 2017.

SANCHES, B. D. A. et al. Telocytes of the male urogenital system: Interrelationships, possible functions, and pathological implications. **Cell biology international**, England, v. 45, n. 8, p. 1613–1623, 2021.

SANTOS, F.; TABOGA, S. Female prostate: a review about the biological repercussions of this gland in humans and rodents. **Anim Reprod**, [s. l.], v. 3, n. 1, p. 3–18, 2006.

SHAHIDI, F.; AMBIGAIPALAN, P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. **Journal of Functional Foods**, [s. l.], v. 18, p. 820–897, 2015. Disponível em: <http://dx.doi.org/10.1016/j.jff.2015.06.018>

SHAPPELL, S. B. et al. Prostate Pathology of Genetically Engineered Mice: Definitions and Classification. The Consensus Report from the Bar Harbor Meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. **Cancer Research**, [s. l.], v. 64, n. 6, p. 2270–2305, 2004. Disponível em: <http://cancerres.aacrjournals.org/content/64/6/2270>

SHUM, C. F.; LAU, W.; TEO, C. P. C. Medical therapy for clinical benign prostatic hyperplasia: α1 Antagonists, 5α reductase inhibitors and their combination. **Asian journal of urology**, [s. l.], v. 4, n. 3, p. 185–190, 2017.

SOHAL, R. S.; AGARWAL, S.; SOHAL, B. H. Oxidative stress and aging in the Mongolian gerbil (Meriones unguiculatus). **Mechanisms of ageing and development**, Ireland, v. 81, n.

1, p. 15–25, 1995.

TABOGA, S. R.; VILAMAIOR, P. S.; GÓES, R. M. Androgenic and estrogenic modulation in the prostate: an approach in rodent experimental models with emphasis on structural biology | Modulação androgênica e estrogênica na próstata: uma abordagem em modelos experimentais de roedores com enfoque na biolog. **Arquivos brasileiros de endocrinologia e metabologia**, [s. l.], v. 53, n. 8, p. 946–955, 2009.

TAN, M. H. E. et al. Androgen receptor: structure, role in prostate cancer and drug discovery. **Acta pharmacologica Sinica**, [s. l.], v. 36, n. 1, p. 3–23, 2015.

TAOKA, R.; KAKEHI, Y. The influence of asymptomatic inflammatory prostatitis on the onset and progression of lower urinary tract symptoms in men with histologic benign prostatic hyperplasia. **Asian journal of urology**, [s. l.], v. 4, n. 3, p. 158–163, 2017.

TOMÁS-ZAPICO, C.; COTO-MONTES, A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. **Journal of pineal research**, England, v. 39, n. 2, p. 99–104, 2005.

TUXHORN, J. A.; AYALA, G. E.; ROWLEY, D. R. Reactive stroma in prostate cancer progression. **The Journal of urology**, United States, v. 166, n. 6, p. 2472–2483, 2001.

UDENSI, U. K.; TCHOUNWOU, P. B. Oxidative stress in prostate hyperplasia and carcinogenesis. **Journal of Experimental and Clinical Cancer Research**, [s. l.], v. 35, n. 1, p. 1–19, 2016. Disponível em: <http://dx.doi.org/10.1186/s13046-016-0418-8>

ULLRICH, P. M. et al. Stress, hostility, and disease parameters of benign prostatic hyperplasia. **Psychosomatic Medicine**, [s. l.], v. 67, n. 3, p. 476–482, 2005.

ULLRICH, P. M.; LUTGENDORF, S. K.; KREDER, K. J. Physiologic Reactivity to a Laboratory Stress Task Among Men with Benign Prostatic Hyperplasia. **Urology**, [s. l.], v. 70, n. 3, p. 487–491, 2007.

UNTERGASSER, G.; MADERSBACHER, S.; BERGER, P. Benign prostatic hyperplasia: Age-related tissue-remodeling. **Experimental Gerontology**, [s. l.], v. 40, n. 3, p. 121–128, 2005.

VARIN, A.; GORDON, S. Alternative activation of macrophages: Immune function and cellular biology. **Immunobiology**, [s. l.], v. 214, n. 7, p. 630–641, 2009. Disponível em: <http://dx.doi.org/10.1016/j.imbio.2008.11.009>

VERZE, P.; CAI, T.; LORENZETTI, S. The role of the prostate in male fertility, health and disease. **Nature Reviews Urology**, [s. l.], v. 13, n. 7, p. 379–386, 2016. Disponível em: <http://dx.doi.org/10.1038/nrurol.2016.89>

VILAMAIOR, P. S. et al. Collagen fiber reorganization in the rat ventral prostate following androgen deprivation: a possible role for smooth muscle cells. **The Prostate**, United States, v. 45, n. 3, p. 253–258, 2000.

WHIPPLE, B. Book Review: The Human Female Prostate: From Vestigial Skene’s Paraurethral Glands and Ducts to Woman’s Functional Prostate.By Milan Zaviacic. Slovak Academic Press, Bratislava, Slovakia, 1999, 171 pp., $49.00. **Archives of Sexual Behavior**, [s. l.], v. 31, n. 5, p. 457–458, 2002. Disponível em: <https://doi.org/10.1023/A:1019852411051>

WILT, T. J. et al. Phytotherapy for benign prostatic hyperplasia. **Public health nutrition**, England, v. 3, n. 4A, p. 459–472, 2000.

YEAP, S. K. et al. Antistress and antioxidant effects of virgin coconut oil in vivo. **Experimental and Therapeutic Medicine**, [s. l.], v. 9, n. 1, p. 39–42, 2015.

ZABAIOU, N. et al. Oxidative stress in benign prostate hyperplasia. **Andrologia**, [s. l.], v. 48, n. 1, p. 69–73, 2016.

ZHAO, M. J. et al. Oxidative Stress Links Aging-Associated Cardiovascular Diseases and Prostatic Diseases. **Oxidative Medicine and Cellular Longevity**, [s. l.], v. 2021, 2021.

8 ANEXO

Artigo Extra submetido à revista “Microscopy and microanalysis”

Prostatic morphological changes throughout life: Cytochemistry as a tool to reveal tissue ageing markers.

Running head: Cytochemical techniques to study prostate morphology.

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Abstract

The prostate undergoes normal or pathological morphological changes throughout life. An understanding of these changes is fundamental for the comprehension of ageing-related pathological processes such as benign prostatic hyperplasia (BPH) and cancer. In the present study, we show some of these morphological changes, as well as histochemical techniques like Weigert's resorcin-fuchsin method, Picrosirius Red, and Gömöri's reticulin for use as tools in the study of prostate tissue under light microscopy. For this purpose, prostates of the Mongolian Gerbil, an experimental model that develops BPH spontaneously, were analyzed at three life stages: young, adult, and old. The results showed that fibrillar components such as collagen, and reticular and elastic fibers, change throughout life. We also identified the accumulation of lipid droplets and lipofuscin granules evidenced by Oil red O and Gömöri - Halmi techniques respectively. The histochemical techniques presented here have been demonstrated to be useful and accessible tools in prostate studies.

Key words: Prostate, Light Microscopy, Histochemical, Ageing, Mongolian Gerbil, Lipofuscin.

Competing interests: The authors declare none competing interests.

Introduction

The search for a better understanding of the association between ageing and BPH and cancer respectively has motivated many physiological, cellular, and molecular studies (Bianchi-Frias et al., 2010; Corona et al., 2014; Crowell et al., 2020; De Magalhães, 2013). In addition to a knowledge of the particularities of the prostate gland of old mammals, the collation between young and adults is also important, as several conditions that occur during an animal's lifetime can influence prostatic health (Ren et al., 2020; Morrissey et al., 2002; Alexandre et al., 2020). In this context, histological analysis, in addition to the analysis of molecular and biochemical data, represents an indispensable and often sovereign tool in the diagnosis of age-related pathologies such as BPH and cancer.

Although the use of figures available in histology guides, books and even the use of virtual microscopy is a possibility in histological studies, the practice of microscopy is necessary for a reading of tissue samples and their anatomical variations (Pratt, 2009). In addition to mastery of the different methods, histological practice requires mastery of histochemistry, an investigative and diagnostic area (Wick, 2012). In view of this, although the practice of the pathology of benign prostatic hyperplasia (BPH) is well established (Roehrborn, 2008), there are few reports of methods suitable for light microscopy that can be applied in this practice.

Histological analysis is commonly linked to in vivo studies, although there are in vitro studies that require morphological evaluation. In the case of in vivo prostate studies, some animals, such as rats, dogs, and mice, are used to elucidate processes related to diseases that also affect humans (Silva et al., 2018; Angrimani et al., 2020; Mateus et al., 2019).

Some morphological changes, such as the presence of epithelial hyperplasia, prostatic intraepithelial neoplasia (PIN), and alterations in stromal fibrillary component disposition, are already established as histological markers for prostate of old animals (Untergasser et al., 2005; Campolina-Silva et al., 2020). These morphological disorders linked to ageing have already been described in both male and female Mongolian gerbils (Meriones unguiculatus), making it a valuable experimental model for the study of prostate ageing, since, in these animals, these changes occur spontaneously (Custodio et al., 2010; Campos et al., 2008).

Another detectable characteristic as a sign of cellular ageing is lipofuscin accumulation, which is directly related to oxidative stress and lipid degradation (Terman & Brunk, 2004). Furthermore, it is recognized that the accumulation of lipid droplets in the cytoplasm is closely associated with cell proliferation, growth, and cancer (Roman et al., 2020) and that lipofuscin accumulation is also considered a tracer for tumor prediction and aggressiveness (Mahmoodi et al., 2006). Nevertheless, the correct identification of this pigment is often difficult under light microscopy, as different stains may suggest dubious findings (Brennick et al., 1994).

In addition to morphological changes such as the appearance of lesions and changes in stromal components, ultrastructural analyses have shown that old Mongolian gerbils also have lipid droplets and osmiophilic granules similar to lipofuscin granules scattered throughout the cytoplasm of epithelial cells (Custodio et al., 2008; Campos et al., 2008). However, it is not yet clear how these indications of ageing develop throughout the lifetime of the animals. We therefore analyzed the ventral prostate of male gerbils in three development phases, young, adult, and old, in order to clarify how the accumulation of lipid droplets and lipofuscin that occurs throughout these phases may be employed as markers visible under light microscopy for the prostatic ageing study and BPH pathology

Materials and methods:

Animals, sample fixation

All the animals were born and raised in the Institute of Biosciences, Letters and Exact Sciences /São Paulo State University (IBILCE/UNESP) under the orientation and authorization of the Institutional Committee for Ethics in Animal Experimentation of the Institute (183/2018-CEUA - São José do Rio Preto). The animals were kept in polyethylene boxes under controlled light and temperature conditions (12h light/12h dark - 24ºC) with water and feed ad libitum until the time of removal of the prostate. In total, 27 male Mongolian Gerbils were distributed equally in 3 groups representing different stages of life. Young-group (YG) animals were killed at 1 month of age; adult-group (AG) animals at 3 months, and old-group (OG) animals at 15 months of age. For the removal of the prostate the animals were killed by inhalation of CO2 and, after dissection, the prostate was removed, washed in TBS and then submitted to the fixation process. For light microscopy analysis, the prostate was fixed by immersion in 4% formaldehyde prepared in phosphate buffer, pH 7.2, for 24h. For ultrastructural analysis the prostate was fixed in Millonig’s buffer, pH 7.3, containing 0.54% glucose for 24h. For lipid analysis, the prostate was frozen by submersion in liquid nitrogen immediately following removal.

General histological analysis

Following the paraformaldehyde fixing process, the samples were washed with distilled water and dehydrated in an alcohol battery. Immediately after dehydration the samples were processed and included in Histosec (Merck, Darmstadt, Germany). The tissues were sectioned at 4µm thick and the slides were stained with hematoxylin-eosin (HE) for general histology. The stromal fibrillary components were accessed using the following staining methods: for the collagen fiber analysis, the slides were stained with Picrosirius Red; for the reticular fiber analysis, the slides were stained with Gömöri's reticulin; and, for the elastic fiber analysis, with Weigert's resorcin-fuchsin method. The slides were analyzed with an Olympus BX60 light microscope (Olympus, Japan), and the resulting images were digitalized with DP-BSW V3.1 (Olympus) and Image-ProPlus 6.1 software (Media Cybernetics, Silver Spring, MD).

Lipofuscin histological analysis

Lipofuscin deposition was observed using the Gömöri - Halmi method following Behmer et al., 1976 (Behmer et al., 1976). This method is a variation of Gömöri's trichrome, which meant that a previous treatment of the tissue with potassium permanganate – sulphuric acid followed by metabisulphite washing was necessary. After fuchsin–paraldehyde staining the tissue was stained with iron hematoxylin, washed with 70% alcohol and 1% hydrochloric acid, washed in distilled water and then stained with Halmi stain. The sections were finally washed in 0.2% aqueous acetic acid solution and dehydrated, and the slides were mounted using Canadian balsam.

Lipid analysis

In order to assess the lipid content of the prostate cells, cryosections of prostate (25 µm thickness) were placed on glass depression slides and fixed by immersion in formaldehyde 3.7% at 60ºC for 1h. Once fixed, the cryosections were washed in distilled water and then immersed in 60% isopropanol for 20 minutes. The slides were then incubated in a filtered solution of 3% oil red O in 60% isopropanol for 2h, washed in 60% isopropanol, and washed in water. Finally, the glass slides were mounted with Fluoroshield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA; cat. no. F6057). The sample was analyzed in a Zeiss LSM710 Confocal Microscope with Zen 2010 software (Carl Zeiss, Thornwood, New York).

Ultrastructural analysis

The prostatic tissue ultrastructure was assessed by transmission electron microscopy (TEM). To do this after fixation, the prostatic fragments were washed in Milloning's buffer, postfixed with 1% osmium tetroxide for 2 h, washed in Milloning's buffer again, dehydrated in graded acetone series and then embedded in Araldite resin. Ultrathin sections were cut using a diamond knife and contrasted with 2% uranyl acetate for 30 min, followed by 2% lead citrate for 10 min. The samples were observed and evaluated with a LEO – Zeiss 906 transmission electron microscope (Zeiss, Germany).

Results

The general morphology of the prostate gland changes according to life stage

General morphological analysis determined that prostate morphology varies throughout the life of the gerbil. The acini that make up the prostate of young animals are composed of cuboidal epithelial cells with a basophilic cytoplasm and a voluminous, circular nucleus. Among the epithelial cells and near to the basal lamina smaller cells can be found, corresponding to basal cells. Surrounding this epithelium, it is possible to observe smooth muscle cells (SMC) organized in a thick layer. The SMC are bulky and have a voluminous nucleus that follows the format of the cell (Figure 1E).

In adult animals, the acini have a larger lumen and epithelial cells with forms varying between cuboidal and columnar and a more acidophilic cytoplasm where it is possible to observe regions that indicate aggregation of Golgi complex. The nucleus is located near the basal pole and smaller about the cytoplasm. In these animals, it was also possible to observe the presence of basal cells among the epithelial cells. In adulthood, the smooth muscle cells surrounding the epithelium are more compacted and have a more fusiform nucleus (Figure 2E).

Due to ageing, some alterations occur in the prostatic tissue. The alveoli show irregular lumens due to several epithelial folds and BPH that extend towards the lumen. The epithelium is often stratified and cells that exhibit different forms generally have a chromophobic region under the nucleus. Epithelial cells may exhibit different nuclear phenotypes, mainly in lesions like PIN. The SMC that surround the alveoli are most decompressed with each other, and often have large nuclei (Figure 3E).

The stromal fibrillary components change during life stages

The use of Picrossirius red dye highlighted collagen fibers in reddish shades; however not all the red collagen fibers (Figures 1I, 2I, 3I) were birefringent under the polarized light (Figures 1F, 2F, 3F). In general, collagen fibers accumulate just under the epithelium and are intermingled with the SMC, being abundant in the non-muscular stroma. It was also possible to highlight the collagen fibers with Gömöri's reticulin method. With this technique, the collagen fibers exhibited a shade variation from light brown to yellow, intermingled with reticular fibers ranging from brown to black. In all life phases, the reticular fibers intermeshed with the SMC intermingled with the collagen fibers (Figures 1G, 2G, 3G). The elastic fibers were highlighted by the Weigert's resorcin-fuchsin method and exhibit a blue shade (Figures 1D, 2D, 3D). The presence of these fibers is more conspicuous in the vessels present in the non-muscular stroma and not very marked in the muscular stroma, where they are more easily found at the base of the epithelium.

In young animals, collagen fibers appear continuously and in large amounts underneath the epithelium and between the SMC (Figure 1I). Under polarized light, these fibers exhibit various shades of yellow, red and green (Figure 1F). The reticular fibers were at the base of the epithelium and intermingled with the layer of muscle cells in a sinuous and often misaligned pattern (Figure 1G). The elastic fibers are discontinuous and vary widely in thickness. These fibers are discretely arranged between the SMC (Figure 1D).

In adult animals, the collagen fibers extend to a lesser amount in the sub-epithelial region and are very thin between the smooth muscle cells (Figure 2I). Under polarized light, the color shade of these fibers was less varied and less bright (Figure 2F). Concerning the reticular fibers, in adults, these fibers are interspersed with the muscle layer in a less intense and a more rectilinear way (Figure 2G). The elastic fibers are thinner and more delicate in a continuous and rectilinear way (Figure 2D).

In old animals, the collagen fibers exhibit a less linear arrangement, and accumulate between the smooth musculature (Figure 3I). Under polarized light, these fibers often form bright points and exhibit little variation between yellow and red shades (Figure 3F). Due to ageing, the reticular fibers become thicker, mainly at the base of the epithelium, and several regions exhibit a disarrangement and breakage of these fibers more pronouncedly around the lesions (Figure 3G). The elastic fibers exhibit a thicker shape and can display different directions of the fiber among the SMC.

Lipofuscin granules appear with ageing

Gomori - Halmi trichrome stained the nuclei in shades ranging from light purple to brown, muscle fibers in yellow and elastic fibers and lipofuscin in purple (Figures 1C, 2C, 3C). Taking into account the use of this staining to evaluate the presence of lipofuscin, the results presented here focused on the presence or absence of these granules.

The young animals showed a clear cytoplasm with no evidence of lipofuscin. In adult animals, on the other hand, although most epithelium secretory cells exhibited no lipofuscin-related content, some cells exhibited small purple spots dispersed in the cytoplasm (Figure 2C). In a few cells of adult animals, it was possible to observe the accumulation of these granules at the apical pole of these cells (Figure 2C). In the old animals, the epithelial cells have a lot of purple-stained spots scattered throughout the cytoplasm. In many cells, these granules accumulate in the apical region, forming large concentrated regions of lipofuscin (Figure 3C).

With increasing age, the secretory cells accumulate lipidic droplets.

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Morphological changes are also observed under transmission electron microscopy

In order to check the findings of the cytochemical techniques employed, the ultrastructural analysis was focused on structures previously observed under light, fluorescence and confocal microscopy. The cells present a clean cytoplasm in the epithelium, with no aggregation of organelles nor any sign of secretory vesicles. The cytoplasm reveals no lipid droplets or lipofuscin accumulation (Figure 1B). Below the epithelial cells there is an accumulation of collagen fibers that are also present among the smooth muscle cells and fibroblasts which are arranged under this collagen accumulation. The collagen fibers are short, vary in thickness, and are observed in different planes of section. The presence of elastin is sparse and presents as subtle electrodense speckles (Figure 1H).

The epithelial cells of the prostate of adult animals present a phenotype of synthetic and secretory activity, in which there are many organelles, such as a well-developed rough endoplasmic reticulum, mitochondria, and Golgi apparatus. The cytoplasm is teeming with secretion vesicles, mainly at the apical pole, while at the basal pole the accumulation of lipid droplets below the nucleus is remarkable. The presence of tiny osmiophilic bodies corresponding to small lipofuscin granules was also detected (Figure 2B). The collagen fibers beneath the epithelium exhibit a homogenous appearance; they are compacted and appear tightly compressed between the muscle cells. Elastin is deposited near the epithelium next to the collagen fibers (Figure 2H).

In old animals, the prostatic epithelium exhibits cells similar to those found in adult animals, albeit with many stratified regions and many lipid droplets dispersed throughout the cytoplasm. In the apical region, a deposit of lipofuscin granules can be found (Figure 3B). The collagen fibers are thick and homogeneous. These fibers accumulate in the subepithelial region and penetrate the epithelial folds. The muscle cells exhibit sinuous shapes and there is an accumulation of collagen and elastin between these cells (Figure 3H).

The main morphological aspects in the prostate at the three life periods, young, adult and old, are represented in figure 4

Discussion

Being familiar with the essential tools for histological practice, such as cytochemistry, enables researchers to identify and understand tissue composition. In histological analyses related to BPH and prostate health research, an ability to identify prostatic morphological changes that occur in the course of a lifetime is crucial. In the present study, some morphological changes that occur during the ageing of the prostate in an animal model are presented, as well as well-established techniques to observe such morphological changes, which can serve as tool for pathologists and researchers who need to examine prostate tissue.

Hematoxylin and Eosin stain is undoubtedly the most widely used stain in general histological analyses since it displays the cytoplasm presenting acidophilia (proteins stain pink or orange) and the nucleus presenting basophilia (stain dark blue). Although this method of staining may vary, when used appropriately the results are satisfactory for general histological analysis (Chan, 2014; Feldman & Wolfe, 2014; Wick, 2019). Histological sections stained with HE shows that the basic characteristics of prostate tissue vary over the lifetime. Since the gerbil attains puberty at 42 days of life and sexual maturity at 90 days of life (Pinto-Fochi et al., 2016), the young gerbil prostate (30 days of life) appears with no significant secretory activity. HE staining reveals this condition, displaying short epithelial cells and basophilic cytoplasm. This physiological status was confirmed by ultrastructural analysis. Besides this, the thick layer of voluminous SMC indicates weakly turgid alveoli, with no noteworthy luminous contents.

After attaining sexual maturity, there is increased prostatic activity controlled mainly by the action of sex hormones and their respective receptors such as the androgen receptor (AR) and the estrogen receptors α and β (McPherson et al., 2008). Evidence of this activity is provided by the increase in luminal space and in the height of the epithelium and secretory cells with acidophilic cytoplasm, which indicate high synthetic activity. The tension resulting from the increased luminal area makes the muscular layer more compact with more elongated SMC. It is known that with ageing, there is a decrease in the serum androgenic level, and the prostatic morphology of the gerbil reflects this hormonal condition (Corradi et al., 2009). Our data, such as the occurrence of epithelial folds, the development of HPB and prostate lesions, corroborate what has been previously described in the literature (Campos et al., 2008; Lee et al., 2011; Rastrelli et al., 2019; Verze et al., 2016; Taboga & De Campos Vidal, 2003).

The homeostasis of the prostate is maintained by an interaction between the epithelium and the stroma (Cunha et al., 1996) and, consequently, the morphological characteristics of the epithelium of each stage of life are accompanied by stromal changes. Analysis of the stromal fibrillary components revealed some of these alterations and also showed that these components are responsive to alterations in the prostate gland that is not purely structural and static (P S Vilamaior et al., 2000). Collagen is the most prevalent stromal component of extra cellular matrix, and its presence was evidenced by Picrossirius red staining. Picrossirius red is an usual stain for general identification of collagen fiber (Coleman, 2011) which can be analyzed under polarized light, making it possible to distinguish fibers in different arrangements and thicknesses (Lattouf et al., 2014).

Although this is not a specific method like immunohistochemistry, the different color shades resulting from polarization can be indicative of the presence of type I collagen, displaying shades from yellow to red, type III collagen, displaying greenish shades, and type II collagen, with different color shades (Montes & Junqueira, 1991; Junqueira et al., 1978). Subsequent studies have shown that the interference colors in different absorptiometry spectra refer to different degrees of aggregation (Taboga & Vidal, 2003). The reticular fibers were highlighted with Gömöri’s method (Gömöri, 1937). Although the specificity of this method has been questioned in some organs, such as the liver, spleen, and cardiac and skeletal muscle (Puchtler

& Waldrop, 1978), our research group demonstrated that it is a useful method in the analysis of the fibril components of the prostate indicating the length and thickness of the fibers, the possible formation of bridges between adjacent fibers and ruptures (Patrcia S L Vilamaior et al., 2000; Corradi et al., 2004; Campos et al., 2008). Histochemical analysis of the elastic fibers raised doubts in the reading of the slides, since there is no specificity for elastin (Ross, 1973). However, Weigert's resorcin-fuchsin staining has been successfully employed in the analysis of elastic fibers in rat and gerbil prostates, confirming the effectiveness of this method by ultrastructural analysis (De Carvalho et al., 1997; dos Santos et al., 2003)

The phenotypic characteristics of SMCs and the arrangement of the fibrillar components that make up the prostatic stroma are responsive to androgenic levels (Vilamaior et al., 2005). The presence of large amounts of collagen, combined with misaligned reticular fibers and a lack of elastic fibers intermingled with SMC, reveals the immature status of the prostate. The lack of secretory activity of the acini and the consequent small luminal volume results in the distended aspect of the stromal components (Vilamaior et al., 2006; P S Vilamaior et al., 2000). Sexually mature animals, whose prostate is involved in intense secretory activity, have extended collagen fibers, linear reticular fibers and very thin elastic fibers. It is known that prostate growth is directly related to the fibromuscular stroma, that it is androgen-dependent and that androgen influences collagen status (Müntzing, 1980). It is therefore not surprising that old animals exhibit distinct stromal characteristics since, with ageing, there is a decrease in serum androgen levels in gerbils (Pegorin de Campos et al., 2006).

In addition to a having thickening of reticular fibers at the base of the epithelium and thicker elastic fibers in different orientations, the stroma of the old animals showed non-linear collagen accumulation between the SMC, with redder and yellower shades under polarized light. Although the color shades revealed by Picrossirius red staining under polarized light are not specific for classifying collagen into type I, II, and III, different collagen fiber thicknesses result in different shades (Dayan et al., 1989). Furthermore, this staining was validated to qualify the collagen type in the uterine stroma and indicated that there is an increase in type I and III collagen in the uterus of old animals (Briley et al., 2017). Our results therefore enable us to report that the organization of the predominant collagen in young animals is different from that of old and adult animals, which suggests that the prostatic stroma of young animals is mainly composed of collagen type III, while the prostatic stroma of adult and old animals accumulate collagen types I and III, with collagen I predominating in the prostate of the old.

A morphological feature associated with ageing is the cellular accumulation of lipofuscin granules (Terman & Brunk, 1998). Lipofuscin accumulation occurs in the prostate of humans (Brennick et al., 1994) and rodent prostate (Jara et al., 2004) including the male and female Mongolian gerbil (Custodio et al., 2008; Campos et al., 2008). Although Sudan-Black-B stain has been used as a specific stain for lipofuscin (Georgakopoulou et al., 2013), the present study revealed for the first time the possibility of using the Gömöri - Halmi method to stain lipofuscin in a paraformaldehyde-fixed and paraffin-embedded prostate. This age-related pigment could be subtly detected in adulthood, dispersed in the cytoplasm, and markedly in old age, mainly in the apical region of the secretory cells. Age-related damage to biomolecules leads to catabolic impairment, generating lipofuscin, an intralysosomal material composed of proteins altered by oxidation and residues of lipid degradation (Terman & Brunk, 2006). Lipofuscin accumulation is also related to the oxidation of mitochondrial proteins and the consequent increase in oxidative stress (König et al., 2017).

Considering that lipid peroxidation is partially responsible for lipofuscin accumulation, we also analyzed the presence of lipid deposition using Oil red O staining. This stain makes it possible to identify endogenous and exogenous lipid deposits. It is used in cytopathology and surgical pathology (Wang et al., 2011) exhibiting fluorescence using the Texas red excitation filter (540-580 nm), which makes it possible to quantify lipid accumulation with the application of Oil red O (Koopman et al., 2001). The presence of lipid droplets in the prostate of old gerbils has already been observed under TEM (Pegorin De Campos et al., 2006); however, this is the first time that lipid accumulation over the lifetime of the Mongolian gerbil was shown under light microscopy. Lipid accumulation is observed in cytoplasmic droplets from the adult phase on, mainly in the region between the basal pole and the nucleus of the epithelial cell; however, with the considerable increase of these lipid droplets in old animals, they are dispersed throughout the cytoplasm at this stage of life. The occurrence of these droplets in adulthood and their accumulation may be related to androgen levels, since androgen is involved in the regulation of the genes involved in the importation, biosynthesis and degradation of lipids (Mah et al., 2020). In addition to its possible use as a biomarker of ageing, intracellular lipid accumulation is closely associated with the development and progression of prostate cancer (Mah et al., 2020; Mitra et al., 2017).

Conclusions

The prostate undergoes morphological changes throughout the life span. Recognizing these changes enables possible abnormalities to be diagnosed. Two histochemical techniques, Oil red O and Gömöri - Halmi, provide evidence for two ageing markers: the accumulation of lipid droplets and lipofuscin granules, respectively. The application of simple histochemical techniques can spotlight many of these morphological changes that occur throughout life, representing a useful and accessible tool in studies of ageing, in which the gerbil prostate is a suitable model for understanding the aspects involved in the ageing process.

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References

ALEXANDRE, S., SANTOS, A., CAROLINA, A., CAMARGO, L., CONSTANTINO, F. B., COLOMBELLI, K. T., MARCOS, L., PORTELA, F., FIORETTO, M. N., VIEIRA, C. S., PADILHA, P. M., OLIVEIRA, M. B. DE, LUIS, S., CARVALHO, R. F., JUSTULIN, L. A. & PAULO, S. (2020). Identification of potential molecular pathways involved in prostate carcinogenesis in offspring exposed to maternal malnutrition. 12, 1–25.

ANGRIMANI, D. S. R., BRITO, M. M., RUI, B. R., NICHI, M. & VANNUCCHI, C. I. (2020). Reproductive and endocrinological effects of Benign Prostatic Hyperplasia and finasteride therapy in dogs. Scientific Reports 10, 1–9. https://doi.org/10.1038/s41598-020-71691-7.

BEHMER, O. A., TOLOSA, E. M. C. DE & FREITAS NETO, A. G. (1976). Manual de Técnicas para Histologia Normal e Patológica. Washington, H. (Ed.). São Paulo: EDART-São Paulo Livraria Editora Ltda.

BIANCHI-FRIAS, D., VAKAR-LOPEZ, F., COLEMAN, I. M., PLYMATE, S. R., REED, M. J. & NELSON, P. S. (2010). The effects of ageing on the molecular and cellular composition of the prostate microenvironment. PLoS ONE 5, 1–16.

BRENNICK, J. B., O'CONNELL, J. X., DICKERSIN, G. R. & YOUNG, R. H. (1994). Lipofuscin Pigmentation (So-Called "Melanosis") of the Prostate. The American Journal of Surgical Pathology 18. https://journals.lww.com/ajsp/Fulltext/1994/05000/Lipofuscin_Pigmentation__So_Called__Melanosis___of.3.aspx.

BRILEY, S. M., JASTI, S., MCCRACKEN, J. M., HORNICK, J. E., PRITCHARD, M. T. & DUNCAN, F. E. (2017). mammalian ovary. 152, 245–260.

CAMPOLINA-SILVA, G. H., WERNECK-GOMES, H., MARIA, B. T., BARATA, M. C., TORRES, M. J., CONTRERAS, H. R., MAHECHA, G. A. B. & OLIVEIRA, C. A. (2020). Targeting Wistar rat as a model for studying benign, premalignant and malignant lesions of the prostate. Life Sciences 242, 117149. https://doi.org/10.1016/j.lfs.2019.117149.

CAMPOS, S. G. P., ZANETONI, C., SCARANO, W. R., VILAMAIOR, P. S. L. & TABOGA, S. R. (2008). Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. International Journal of Experimental Pathology 89, 13–24.

DE CARVALHO, H. F., VILAMAIOR, P. S. L. & TABOGA, S. R. (1997). Elastic system of the rat ventral prostate and its modifications following orchiectomy. Prostate 32, 27–34.

CHAN, J. K. C. (2014). The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. International Journal of Surgical Pathology 22, 12–32.

COLEMAN, R. (2011). Picrosirius red staining revisited. Acta Histochemica 113, 231–233.

CORONA, G., VIGNOZZI, L., RASTRELLI, G., LOTTI, F., CIPRIANI, S. & MAGGI, M. (2014). Benign prostatic hyperplasia: A new metabolic disease of the ageing male and its correlation with sexual dysfunctions. International Journal of Endocrinology 2014.

CORRADI, L. S., CAMPOS, S. G. P., SANTOS, F. C. A., VILAMAIOR, P. S. L., GÓES, R. M. & TABOGA, S. R. (2009). Long-term inhibition of 5-alpha reductase and aromatase changes the cellular and extracellular compartments in gerbil ventral prostate at different postnatal ages. International Journal of Experimental Pathology 90, 79–94.

CORRADI, L. S., GÒES, R. M., CARVALHO, H. F. & TABOGA, S. R. (2004). Inhibition of 5-α-reductase activity induces stromal remodeling and smooth muscle de-differentiation in adult gerbil ventral prostate. Differentiation 72, 198–208.

CROWELL, P. D., GIAFAGLIONE, J. M., HASHIMOTO, T. & GOLDSTEIN, A. S. (2020). Distinct cell-types in the prostate share an ageing signature suggestive of metabolic reprogramming. American journal of clinical and experimental urology 8, 140–151. http://www.ncbi.nlm.nih.gov/pubmed/32929410%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC7486537.

CUNHA, G. R., HAYWARD, S. W., DAHIYA, R. & FOSTER, B. A. (1996). Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. Acta anatomica 155, 63–72.

CUSTODIO, A. M. G., SANTOS, F. C. A., CAMPOS, S. G. P., VILAMAIOR, P. S. L., GÓES, R. M. & TABOGA, S. R. (2008). Ageing effects on the mongolian gerbil female prostate (Skene’s paraurethral glands): Structural, ultrastructural, quantitative, and hormonal evaluations. Anatomical Record 291, 463–474.

CUSTODIO, A. M. G., SANTOS, F. C. A., CAMPOS, S. G. P., VILAMAIOR, P. S. L., OLIVEIRA, S. M., GÓES, R. M. & TABOGA, S. R. (2010). Disorders related with ageing in the gerbil female prostate (Skene’s paraurethral glands). International Journal of Experimental Pathology 91, 132–143.

DAYAN, D., HISS, Y., HIRSHBERG, A., BUBIS, J. J. & WOLMAN, M. (1989). Are the polarization colors of Picrosirius red-stained collagen determined only by the diameter of the fibers? Histochemistry 93, 27–29.

FELDMAN, A. T. & WOLFE, D. (2014). Tissue processing and hematoxylin and eosin staining. Methods in molecular biology (Clifton, N.J.) 1180, 31–43.

GEORGAKOPOULOU, E. A., TSIMARATOU, K., EVANGELOU, K., FERNANDEZ, M. P. J., ZOUMPOURLIS, V., TROUGAKOS, I. P., KLETSAS, D., BARTEK, J., SERRANO, M. & GORGOULIS, V. G. (2013). Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A PORTA EA. 2002. Pigments in Ageing: An Overview. Ann N Y Acad Sci, 959(1):57–65 DOI: 10.1111/j.1749-6632.2002.tb02083.x.method applicable in cryo-prese. Ageing 5, 37–50. https://www.ageing-us.com/article/100527.

GÖMÖRI, G. (1937). Silver Impregnation of Reticulum in Paraffin Sections. The American journal of pathology 13, 993-1002.5. http://www.ncbi.nlm.nih.gov/pubmed/19970363%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1911151.

JARA, M., CARBALLADA, R. & ESPONDA, P. (2004). Age-induced apoptosis in the male genital tract of the mouse. Reproduction 127, 359–366.

JUNQUEIRA, L. C. U., COSSERMELLI, W. & BRENTANI, R. (1978). Differential Staining of Collagens Type I, II and III by Sirius Red and Polarization Microscopy. Archivum histologicum japonicum 41, 267–274.

KÖNIG, J., OTT, C., HUGO, M., JUNG, T., BULTEAU, A.-L., GRUNE, T. & HÖHN, A. (2017). Mitochondrial contribution to lipofuscin formation. Redox Biology 11, 673–681. https://www.sciencedirect.com/science/article/pii/S2213231717300423.

KOOPMAN, R., SCHAART, G. & HESSELINK, M. K. (2001). Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. Histochemistry and Cell Biology 116, 63–68.

LATTOUF, R., YOUNES, R., LUTOMSKI, D., NAAMAN, N., GODEAU, G., SENNI, K. & CHANGOTADE, S. (2014). Picrosirius Red Staining: A Useful Tool to Appraise Collagen Networks in Normal and Pathological Tissues. Journal of Histochemistry and Cytochemistry 62, 751–758.

LEE, C. H., AKIN-OLUGBADE, O. & KIRSCHENBAUM, A. (2011). Overview of Prostate Anatomy, Histology, and Pathology. Endocrinology and Metabolism Clinics of North America 40, 565–575.

DE MAGALHÃES, J. P. (2013). How ageing processes influence cancer. Nature Reviews Cancer 13, 357–365. http://dx.doi.org/10.1038/nrc3497.

MAH, C. Y., NASSAR, Z. D., SWINNEN, J. V. & BUTLER, L. M. (2020). Lipogenic effects of androgen signaling in normal and malignant prostate. Asian Journal of Urology 7, 258–270.

MAHMOODI, M., ZHANG, S., SALIM, S., HOU, J. S. & GARCIA, F. U. (2006). Lipofuscin pigment can be used as a prognostic marker in prostatic adenocarcinoma. Annals of Diagnostic Pathology 10, 257–262.

MATEUS, P. A. M., KIDO, L. A., SILVA, R. S., CAGNON, V. H. A. & MONTICO, F. (2019). Association of anti-inflammatory and antiangiogenic therapies negatively influences prostate cancer progression in TRAMP mice. Prostate 79, 515–535.

MCPHERSON, S. J., ELLEM, S. J. & RISBRIDGER, G. P. (2008). Estrogen-regulated development and differentiation of the prostate. Differentiation 76, 660–670.

MITRA, R., LE, T. T., GORJALA, P. & GOODMAN, O. B. (2017). Positive regulation of prostate cancer cell growth by lipid droplet forming and processing enzymes DGAT1 and ABHD5. BMC Cancer 17, 1–12.

MONTES, G. S. & JUNQUEIRA, L. C. (1991). The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Memorias do Instituto Oswaldo Cruz 86 Suppl 3, 1–11.

MORRISSEY, C., BUSER, A., SCOLARO, J., SULLIVAN, J. O., MOQUIN, A. M. Y. & TENNISWOOD, M. (2002). Ageing Sprague-Dawley Rats. 23.

MÜNTZING, J. (1980). Androgen and collagen as growth regulators of the rat ventral prostate. The Prostate 1, 71–78.

PEGORIN DE CAMPOS, S. G., ZANETONI, C., GÓES, R. M. & TABOGA, S. R. (2006). Biological behavior of the gerbil ventral prostate in three phases of postnatal development. The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology 288, 723–733.

PEGORIN DE CAMPOS, S. G., ZANETONI, C., GÓES, R. M. & TABOGA, S. R. (2006). Biological behavior of the gerbil ventral prostate in three phases of postnatal development. Anatomical Record - Part A Discoveries in Molecular, Cellular, and Evolutionary Biology 288, 723–733.

PINTO-FOCHI, M. E., NEGRIN, A. C., SCARANO, W. R., TABOGA, S. R. & GÓES, R. M. (2016). Sexual maturation of the Mongolian gerbil (Meriones unguiculatus): A histological, hormonal and spermatic evaluation. Reproduction, Fertility and Development 28, 815–823.

PRATT, R. L. (2009). Are we throwing histology out with the microscope? A look at histology from the physician's perspective. Anatomical Sciences Education 2, 205–209.

PUCHTLER, H. & WALDROP, F. S. (1978). Silver impregnation methods for reticulum fibers and reticulin: A re-investigation of their origins and specifity. Histochemistry 57, 177–187.

RASTRELLI, G., VIGNOZZI, L., CORONA, G. & MAGGI, M. (2019). Testosterone and Benign Prostatic Hyperplasia. Sexual Medicine Reviews 7, 259–271. https://doi.org/10.1016/j.sxmr.2018.10.006.

REN, X., ZHANG, T., CHEN, X., WEI, X., TIAN, Y., LI, G., ZHANG, X., ZHANG, W., YOU, Z., WANG, S. & QIN, C. (2020). Early-life exposure to bisphenol A and reproductive-related outcomes in rodent models: A systematic review and meta-analysis. Ageing 12, 18099–18126.

ROEHRBORN, C. G. (2008). Pathology of benign prostatic hyperplasia. International Journal of Impotence Research 20.

ROMAN, M., WROBEL, T. P., PANEK, A., PALUSZKIEWICZ, C. & KWIATEK, W. M. (2020). Lipid droplets in prostate cancer cells and effect of irradiation studied by Raman microspectroscopy. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids 1865, 158753. https://doi.org/10.1016/j.bbalip.2020.158753.

ROSS, R. (1973). The elastic fiber. A review. Journal of Histochemistry and Cytochemistry 21, 199–208.

DOS SANTOS, F. C. A., CARVALHO, H. F., GÓES, R. M. & TABOGA, S. R. (2003). Structure, histochemistry, and ultrastructure of the epithelium and stroma in the gerbil (Meriones unguiculatus) female prostate. Tissue & cell 35, 447–457.

SILVA, J. A. F., BRUNI-CARDOSO, A., AUGUSTO, T. M., DAMAS-SOUZA, D. M., BARBOSA, G. O., FELISBINO, S. L., STACH-MACHADO, D. R. & CARVALHO, H. F. (2018). Macrophage roles in the clearance of apoptotic cells and control of inflammation in the prostate gland after castration. Prostate 78, 95–103.

TABOGA, S. R. & DE CAMPOS VIDAL, B. (2003). Collagen fibers in human prostatic lesions: Histochemistry and anisotropies. Journal of Submicroscopic Cytology and Pathology 35, 11–16.

TABOGA, S. R. & VIDAL, B. DE C. (2003). Collagen fibers in human prostatic lesions: histochemistry and anisotropies. Journal of submicroscopic cytology and pathology 35, 11–16.

TERMAN, A. & BRUNK, U. (2006). Damage Is a Result of Normal Metabolism Accumulate Because Their. Ageing 8, 197–204.

TERMAN, A. & BRUNK, U. T. (1998). Lipofuscin: Mechanisms of formation and increase with age. Apmis 106, 265–276.

TERMAN, A. & BRUNK, U. T. (2004). Lipofuscin. The international journal of biochemistry & cell biology 36, 1400–1404.

UNTERGASSER, G., MADERSBACHER, S. & BERGER, P. (2005). Benign prostatic hyperplasia: Age-related tissue-remodeling. Experimental Gerontology 40, 121–128.

VERZE, P., CAI, T. & LORENZETTI, S. (2016). The role of the prostate in male fertility, health and disease. Nature Reviews Urology 13, 379–386. http://dx.doi.org/10.1038/nrurol.2016.89.

VILAMAIOR, P S, FELISBINO, S. L., TABOGA, S. R. & CARVALHO, H. F. (2000). Collagen fiber reorganization in the rat ventral prostate following androgen deprivation: a possible role for smooth muscle cells. The Prostate 45, 253–258.

VILAMAIOR, PATRCIA S L, FELISBINO, S. L., TABOGA, S. R. & CARVALHO, H. F. (2000). Collagen fiber reorganization in the rat ventral prostate following androgen deprivation: A possible role for smooth muscle cells. Prostate 45, 253–258.

VILAMAIOR, P. S. L., TABOGA, S. R. & CARVALHO, H. F. (2005). Modulation of smooth muscle cell function: Morphological evidence for a contractile to synthetic transition in the rat ventral prostate after castration. Cell Biology International 29, 809–816.

VILAMAIOR, P. S. L., TABOGA, S. R. & CARVALHO, H. F. (2006). Postnatal growth of the ventral prostate in Wistar rats: A stereological and morphometrical study. Anatomical Record - Part A Discoveries in Molecular, Cellular, and Evolutionary Biology 288, 885–892.

WANG, Y., GOULART, R. A. & PANTANOWITZ, L. (2011). Oil red O staining in cytopathology. Diagnostic cytopathology 39, 272–273.

WICK, M. R. (2012). Histochemistry as a tool in morphological analysis: A historical review. Annals of Diagnostic Pathology 16, 71–78.

WICK, M. R. (2019). The hematoxylin and eosin stain in anatomic pathology—An often-neglected focus of quality assurance in the laboratory. Seminars in Diagnostic Pathology 36, 303–311. https://www.sciencedirect.com/science/article/pii/S0740257019300619.

Figures

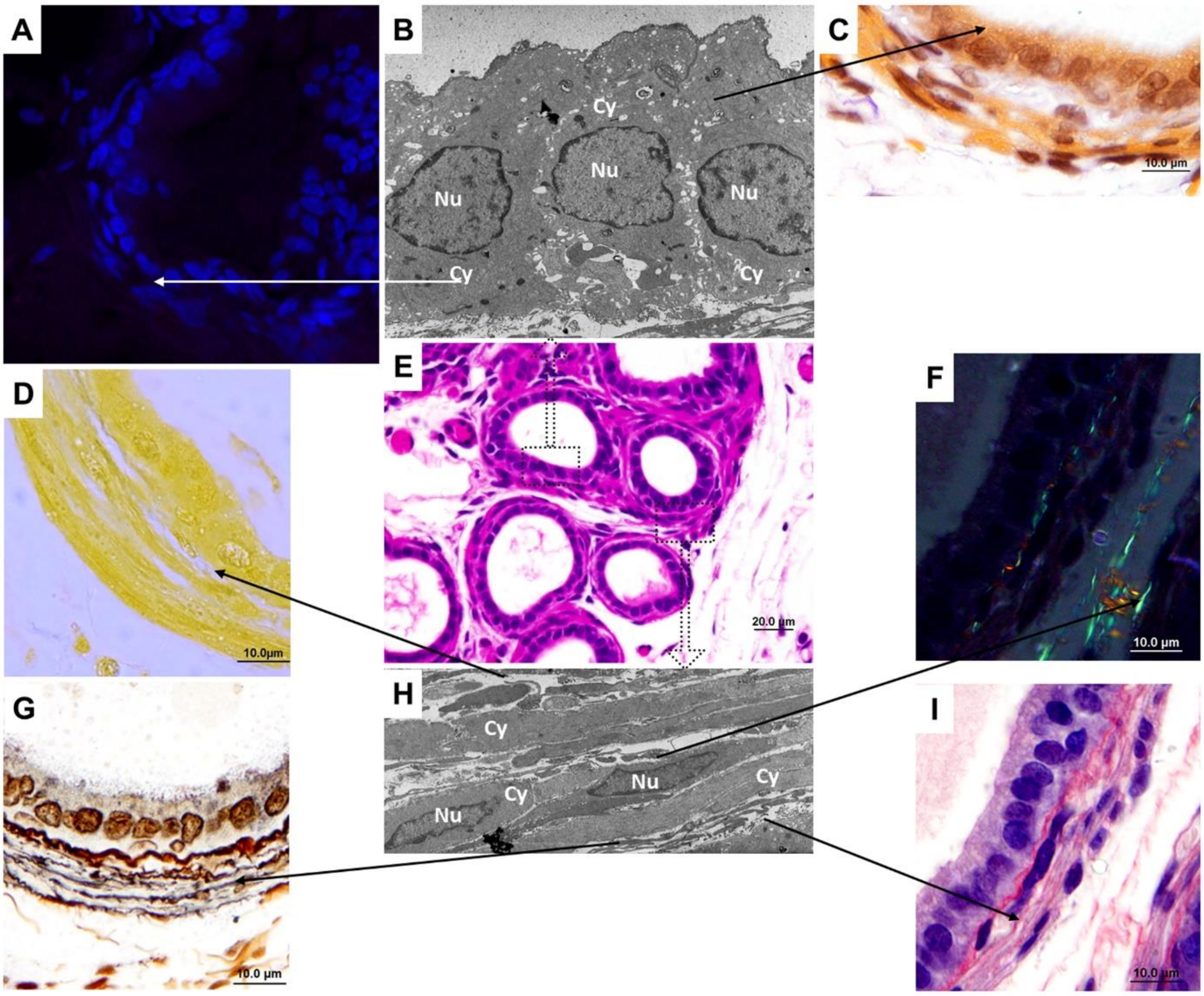


Figure 1: Prostate of young animals. A: Frozen prostate section stained with Oil Red O and DAPI; arrow points to basal pole of the epithelial cell. B: Ultrastructure of the epithelium; the white arrow starts from the basal pole and the black arrow starts from the apical pole; (Nu) nucleus, (Cy) cytoplasm; Magnification: 5019x. C: Histological sections stained with Gomori - Halmi trichrome; nucleus marked in brown; arrow points to apical pole of the epithelial cell. D: Histological sections stained with Weigert’s resorcin-fuchsin; arrow points to elastic fibers marked in blue. E: Histological sections stained with Hematoxylin-eosin; dotted area delimits region detailed in ultrastructure of the epithelium above and the muscular layer below. F: Histological sections stained with Picrossirius red under polarized light; arrow points to collagen fibers. G: Histological sections stained with Gömöri’s reticulin; arrow points to reticulin fibers marked in black. H: Ultrastructure of the muscular layer; the arrow starts from collagen, elastic, and reticular fibers; (Nu) nucleus, (Cy) cytoplasm; Magnification: 5019x. I: Histological sections stained with Picrossirius red; arrow points to collagen fibers marked in red.

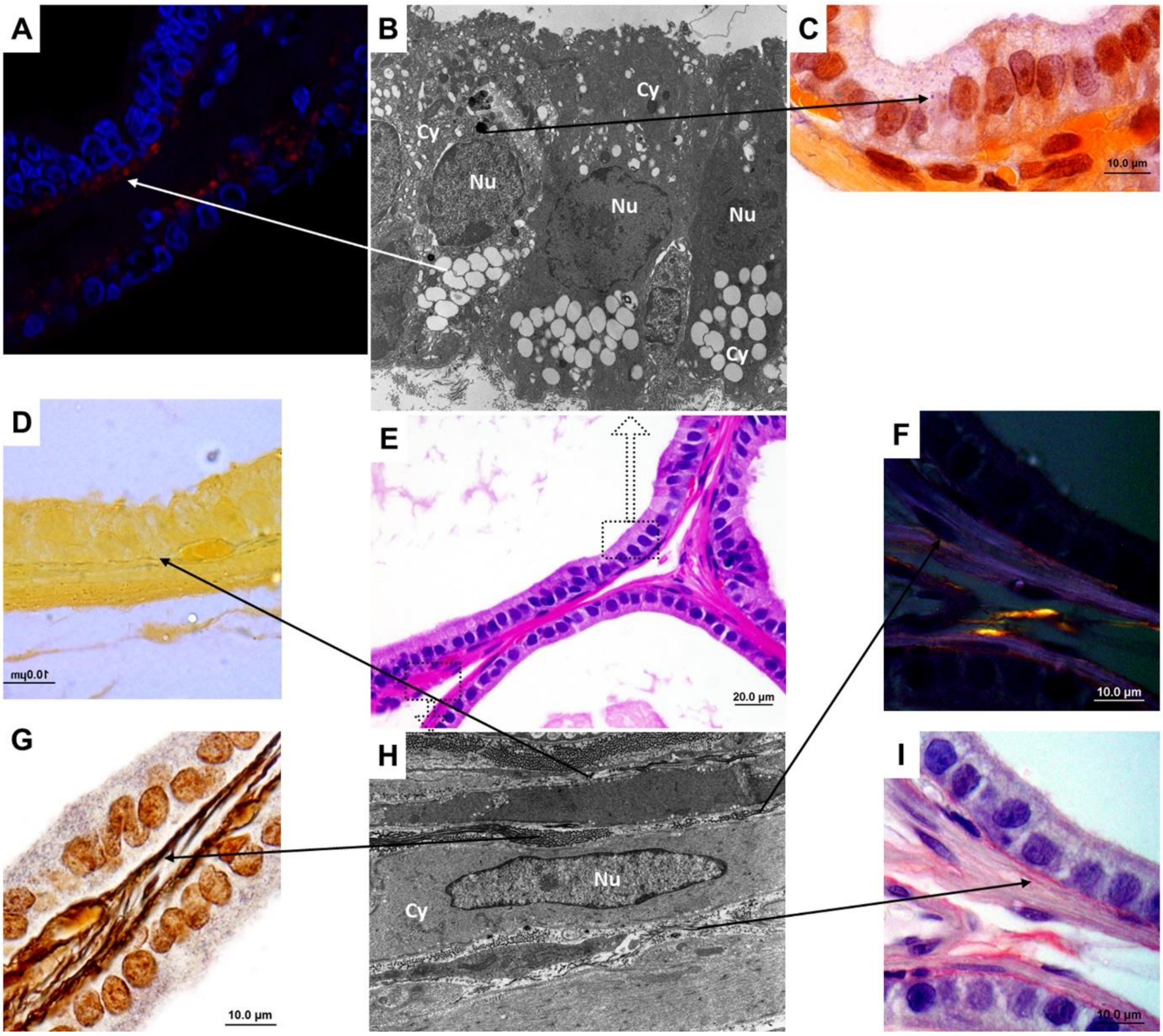


Figure 2: Prostate of adult animals. A: Frozen prostate section stained with Oil Red O and DAPI; arrow points to basal pole of the epithelial cell with lipidic droplets marked in red. B: Ultrastructure of the epithelium; white arrows start from lipid droplets and black arrows start from lipofuscin granules; (Nu) nucleus, (Cy) cytoplasm; Magnification: 4646x. C: Histological sections stained with Gomori - Halmi trichrome; nucleus marked in brown and lipofuscin granules marked in purple; arrow points to lipofuscin granules. D: Histological sections stained with Weigert's resorcin-fuchsin; arrow points to elastic fibers marked in blue. E: Histological sections stained with Hematoxylin-eosin; dotted area delimits region detailed in ultrastructure of the epithelium above and the muscular layer below. F: Histological sections stained with Picrossirius red under polarized light; arrow points to collagen fibers. G: Histological sections stained with Gömöri's reticulin; arrow points to reticulin fibers marked in black. H: Ultrastructure of the muscular layer; the arrow starts from collagen, elastic, and reticular fibers; (Nu) nucleus, (Cy) cytoplasm; Magnification: 4893x. I: Histological sections stained with Picrossirius red; arrow points to collagen fibers marked in red.

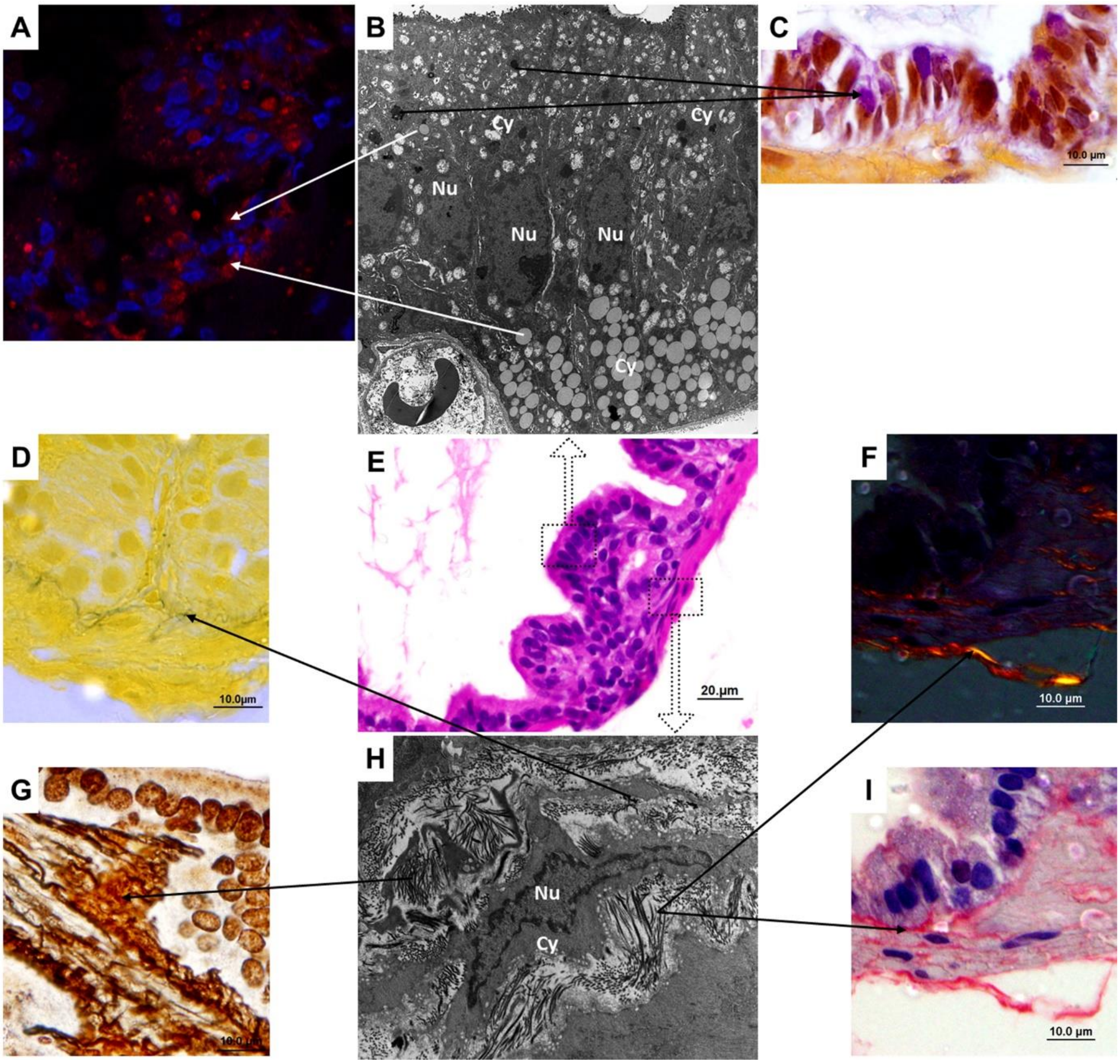
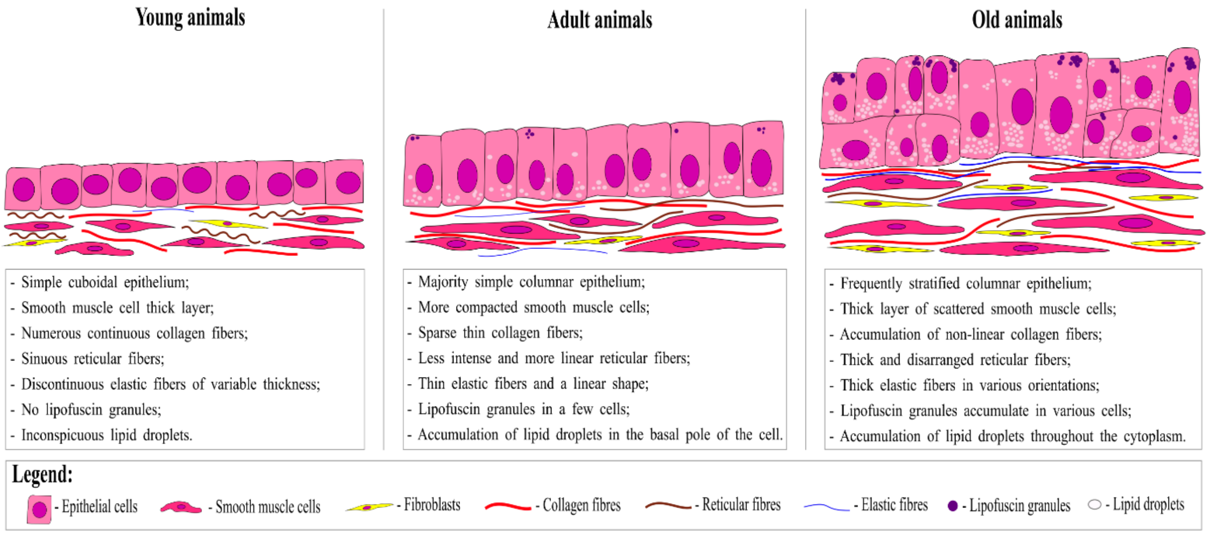


Figure 3: Prostate of old animals. A: Frozen prostate section stained with Oil Red O and DAPI; arrow points to basal pole of the epithelial cell with lipidic droplets marked in red. B: Ultrastructure of the epithelium; white arrows start from lipid droplets and black arrows start from lipofuscin granules; (Nu) nucleus, (Cy) cytoplasm; Magnification: 5294x. C: Histological sections stained with Gomori - Halmi trichrome; nucleus marked in brown and lipofuscin granules marked in purple; arrow points to lipofuscin granules. D: Histological sections stained with Weigert's resorcin-fuchsin; arrow points to elastic fibers marked in blue. E: Histological sections stained with Hematoxylin-eosin; dotted area delimits region detailed in ultrastructure of the epithelium above and the muscular layer below. F: Histological sections stained with Picrossirius red under polarized light; arrow points to collagen fibers. G: Histological sections stained with Gömöri's reticulin; arrow points to reticulin fibers marked in black. H: Ultrastructure of the muscular layer; the arrow starts from collagen, elastic, and reticular fibers;

(Nu) nucleus, (Cy) cytoplasm; Magnification: 7750x. I: Histological sections stained with Picrossirius red; arrow points to collagen fibers marked in red.



Young animals

- Simple cuboidal epithelium;
- Smooth muscle cell thick layer;
- Numerous continuous collagen fibers;
- Sinuous reticular fibers;
- Discontinuous elastic fibers of variable thickness;
- No lipofuscin granules;
- Inconspicuous lipid droplets.

Adult animals

- Majority simple columnar epithelium;
- More compacted smooth muscle cells;
- Sparse thin collagen fibers;
- Less intense and more linear reticular fibers;
- Thin elastic fibers and a linear shape;
- Lipofuscin granules in a few cells;
- Accumulation of lipid droplets in the basal pole of the cell.

Old animals

- Frequently stratified columnar epithelium;
- Thick layer of scattered smooth muscle cells;
- Accumulation of non-linear collagen fibers;
- Thick and disarranged reticular fibers;
- Thick elastic fibers in various orientations;
- Lipofuscin granules accumulate in various cells;
- Accumulation of lipid droplets throughout the cytoplasm.

Figure 4: The main morphological aspects in the prostate at the three life periods.