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Mônica Sousa Campos

Análise morfológica, imuno-histoquímica e ultraestrutural dos efeitos da exposição ao flavonóide crisina sobre a próstata de gerbilos adultos

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RESUMO

A crisina é um composto natural biologicamente ativo encontrado predominantemente em flores de maracujá azul, em mel, camomila, própolis e casca de frutas. Possui propriedades potentes como efeito anti-inflamatório, antioxidante e tem sido empregada para o tratamento de desordens reprodutivas. Essa flavona tem o potencial de aumentar os níveis de testosterona endógena, tanto por inibir a enzima aromatase, quanto por estimular a esteroidogênese testicular. Alterações no metabolismo hormonal mediadas por interferências exógenas podem interferir na fisiologia prostática resultando em modificações na atividade secretora da glândula e na regulação dos receptores hormonais. Assim, o objetivo deste estudo foi avaliar os efeitos da exposição à crisina sobre a próstata e gônadas de gerbilos adultos. Para avaliar o potencial androgênico da crisina, nós analisamos, comparativamente, os efeitos da testosterona sobre estes mesmos órgãos. Machos e fêmeas de gerbilos com 90 dias de idade foram tratados com crisina (50 mg/kg/dia) ou com cipionato de testosterona (1 mg/kg/semana) e divididos tem 3 subgrupos que foram eutanasiados após 3, 7 e 21 dias de tratamento. As próstatas foram coletadas para análises morfológicas, morfométrico-estereológicas, imuno-histoquímicas e ultraestruturais. Testículos e ovários foram submetidos a análises morfológicas e morfométrico-estereológicas. Os níveis séricos de testosterona e 17β-estradiol foram mensurados por ELISA. Machos e fêmeas tratados com crisina não apresentaram aumento dos níveis séricos de testosterona. No entanto, apenas as fêmeas tratadas com crisina durante 21 dias demonstraram aumento nos níveis séricos de estradiol. Foram observados tanto na próstata ventral masculina, quanto na próstata feminina em todos os tempos de tratamento com crisina, aumento da frequência de receptores androgênicos (AR), maior taxa de proliferação e hiperplasia glandular. Além disso, foi observada nas fêmeas em todos os tempos de exposição com crisina maior frequência imunomarcação para receptor estrogênico alfa (ERα). Ultraestruturalmente, machos e fêmeas tratados com crisina apresentaram intenso desenvolvimento das organelas da rota biossintética-secretora, acompanhada de intensa remodelação estromal, enquanto toxicidade celular foi observada apenas nas fêmeas. Nas gônadas masculinas o tratamento com crisina preservou a morfologia testicular. Em fêmeas, observou-se um maior número de folículos ovarianos em maturação. Comparativamente o tratamento com cipionato de testosterona foi capaz de causar lesões na próstata e gônadas, visto que focos de neoplasia intraepitelial prostática e degeneração gonadal foram observados em

ambos os sexos. Esses resultados demonstram que a exposição à crisina, preservou a morfologia testicular normal, aumentou o número de folículos ovarianos em crescimento, aumentou a atividade secretória e alterou a regulação de receptores hormonais na próstata, potencializando a resposta desta glândula aos efeitos biológicos dos esteróides endógenos. Assim nas condições experimentais desse estudo embora a crisina e a testosterona evocassem alterações morfológicas semelhantes na próstata gerbilos masculina e feminina, a suplementação com crisina foi mais favorável à saúde da próstata. Contudo, a administração de crisina como agente fitoterápico deve ser considerada com precaução, visto que esse flavonóide parece exercer efeitos diferenciais em machos e fêmeas.

Palavras chave: flavonoides, histopatologia, gerbilos, receptores hormonais, testosterona.

ABSTRACT

Chrysin is a naturally occurring biologically active compound found predominantly in blue passion flower, honey, chamomile, propolis and fruit peel. It has potent anti-inflammatory and antioxidant properties and has been used for the treatment of reproductive disorders. This flavone has the potential to increase endogenous testosterone levels both by inhibiting the aromatase enzyme and stimulating testicular steroidogenesis. Changes in hormone metabolism mediated by exogenous interferences may interfere with the prostatic physiology resulting in changes in the secretory activity of the gland and in the regulation of hormonal receptors. Thus, the objective of this study was to evaluate the effects of the exposure to chrysin on the prostate and gonads of adult gerbils. To evaluate the androgenic potential of chrysin, we comparatively analysed the effects of testosterone on these organs. Male and female gerbils aged 90 days were treated with either chrysin (50 mg/kg/day) or testosterone cypionate (1 mg/kg/week), subdivided into 3 subgroups and euthanized after 3, 7 and 21 days of treatment. The prostates and gonads were collected for morphological, morphometric-stereological, ultrastructural and immunehistochemical analyses. The testes and ovaries were subjected to morphological and morphometric-stereological analyses. Serum levels of testosterone and 17β-estradiol were determined using ELISA methods. Males and females treated with chrysin did not show increase in serum testosterone levels. However, only the females treated with chrysin for 21 days showed increase in the serum levels of estradiol. It was observed in both ventral prostate of males and in prostate of females in all the times of treatment with chrysin an increase in the frequency of androgen receptors (AR), a higher rate of proliferation and glandular hyperplasia. In addition, it was observed in all exposure times of females treated with chrysin a higher immunoreactivity frequency for alpha estrogen receptor (ERα). As to ultrastructure, males and females treated with chrysin showed intense development of the organelles of the biosynthetic-secretory route, accompanied by intense stromal remodeling. Cell toxicity was observed only in females. In the gonads of males the treatment with chrysin preserved testicular morphology. In females, we observed a larger number of ovarian follicles in maturation. Comparatively, the treatment with testosterone cypionate was able to cause lesions in the prostate and gonads, since the foci of prostatic intraepithelial neoplasia and gonadal degeneration were observed in both sexes. These results demonstrate that exposure to chrysin preserved normal testicular morphology, increased

the number of ovarian follicles in growth, higher activity of secretory and changed the regulation of hormone receptors in the prostate potentiating the response of this gland to the biological effects of endogenous steroids. Therefore, under the experimental conditions of this study, although the chrysin and testosterone evoked similar morphological changes to those in the prostate of both sexes, supplementation with chrysin appeared to be more favorable to the prostate health. However, the chrysin intake as a phytotherapic agent should be considered with caution given that this flavonoid seems to exert distinct effects in males and females.

Key words: flavonoids, histopathology, gerbils, hormonal receptors, testosterone.

SUMÁRIO

1 INTRODUÇÃO

1.1 PRÓSTATA MASCULINA

A próstata é uma glândula acessória do sistema genital masculino que, juntamente com a vesícula seminal, contribui com a produção de nutrientes para o fluido seminal e promove a manutenção do gradiente iônico e pH adequado desta secreção (UNTERGASSER et al., 2005). Adicionalmente, sua composição contempla substâncias antibacterianas, fatores de crescimento específicos, proteases como o antígeno específico prostático (PSA), e prostaglandinas que presumivelmente tornam o sistema imune feminino apto à fertilização (KELLY et al., 1991; SITARAM; NAGARAJ, 1995; GANN et al., 1999). Além disso, as células prostáticas possuem mecanismos enzimáticos capazes de ativar e coordenar a síntese de hormônios sexuais, responsáveis pela manutenção e crescimento glandular (HERMANN et al., 2000).

A próstata humana localiza-se no compartimento subperitoneal, entre o diafragma pélvico e a cavidade peritoneal, posterior à sínfise púbica, anterior ao reto e inferior à bexiga urinária, permitindo a palpação digital para exames investigativos (LEE et al., 2011). Apresenta estrutura compacta (Figura 1), sendo subdividida em três zonas: periférica, central e de transição (SELMAN, 2011). Estas zonas têm diferentes origens embrionárias e podem ser distinguidas através de análises histológicas, posição anatômica, funções biológicas e susceptibilidade a doenças prostáticas (LEE et al., 2011).

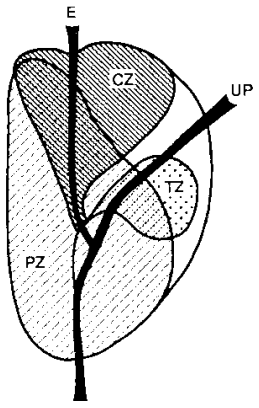

Figura 1. Divisão anatômica da próstata masculina. Representação esquemática da próstata humana adulta. Uretra proximal (UP), ducto ejaculatório (E) e as três maiores zonas glandulares descritas por McNeal. Zona central (ZC), zona periférica (ZP) e zona de transição (ZT). Adaptado de Timms (2008).

A próstata humana pesa aproximadamente um grama no momento do nascimento, aumenta para quatro gramas antes da puberdade e cresce até 20 gramas por volta dos vinte anos, sendo controlada e regulada por andrógenos sintetizados pelos testículos. Geralmente não ocorre mudança no peso prostático entre os vinte a trinta anos de idade (SWYER, 1944; BERRY et al., 1984).

Diferentemente da próstata humana, a próstata de roedores é uma glândula multilobada (RISBRIDGER; TAYLOR, 2006). Roedores do deserto do gênero *Meriones* estão amplamente distribuídos ao longo do norte da África e sudeste da Rússia e Ásia (ROBINSON, 1959). A espécie *Meriones unguiculatus* têm sido extensivamente adotada em experimentos biológicos e biomédicos em imunologia (NAWA et al., 1994), fisiologia (NOLAN et al., 1990) e morfologia (CUSTÓDIO et al., 2004; BIANCARDI et al., 2012; PEREZ et al., 2012). Além disso, esse modelo experimental tem sido utilizado em investigações da glândula prostática durante o envelhecimento (PEGORIM DE CAMPOS et al., 2006).

No gerbilo da Mongólia (*Meriones unguiculatus*) a próstata é uma glândula tubuloacinar dividida em quatro lobos (ventral, dorsolateral, dorsal e glândula coaguladora) (Figura 2), que em termos gerais, são muito semelhantes aos de outros roedores (ROCHEL et al., 2007). Cada lobo é um sistema complexo de ductos que partem da uretra e terminam em muitos ramos distalmente (JESIK et al., 1982; SUGIMURA et al., 1986).

A) **B)**

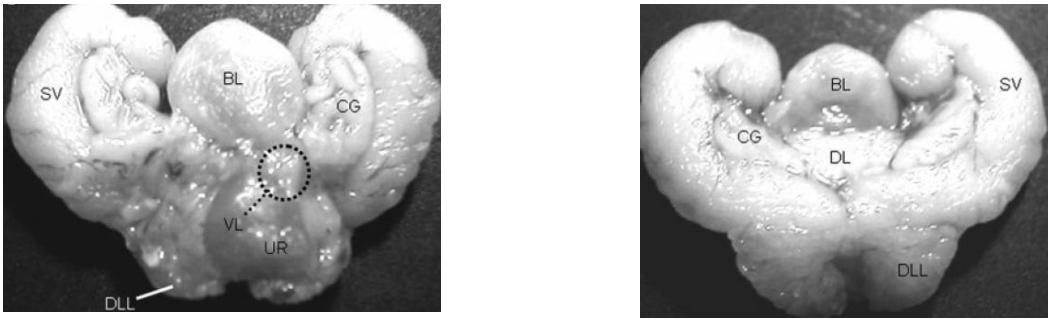

Figura 2. Representação do complexo prostático do gerbilo macho adulto. Vista ventral **(A)** e dorsal **(B)** com a bexiga (BL), glândula coaguladora (CG), lobo dorsal (DL), lobo dorsolateral (DLL), vesícula seminal (SV), lobo ventral (VL) e uretra (UR). Adaptado de Rochel et al., 2007.

Histologicamente, todos os lobos prostáticos do gerbilo, bem como de outros roedores, são compostos por um componente epitelial circundado por um estroma fibromuscular.

O compartimento epitelial é composto por células epiteliais colunares que são responsáveis por sintetizar e secretar para o compartimento luminal diversas proteínas, incluindo o antígeno específico prostático (PSA, do inglês *prostatic specific antigen*) e fosfatase ácida (PAP, do inglês, *prostatic acid phosphatase*), que irão fazer parte do ejaculado masculino (CHATTERJEE, 2003; ROCHEL et al., 2007). O epitélio prostático contém ainda células basais, menores e menos numerosas que as secretoras, geralmente restritas ao compartimento basal do epitélio, atuando como fonte progenitora das células secretoras prostáticas (LONG et al., 2005). Além disso, podem ser encontradas as células neuroendrócrinas, que são células intraepiteliais, que secretam numerosos peptídeos, neuropeptídios e hormônios, que estimulam o crescimento e a secreção prostática (ABRAHAMSSON; DI SANT'AGNESE, 1993).

Uma membrana basal altamente organizada e composta por glicoproteínas (laminina, fibronectina, entactina), glicosaminoglicanos e colágeno tipo IV separa fisicamente o compartimento epitelial do componente estromal (MORANI et al., 2008). O estroma prostático é formado por fibroblastos, células musculares lisas, células endoteliais, células nervosas e células imunológicas infiltradas, como mastócitos e linfócitos. As células estromais encontram-se imersas em uma matriz extracelular rica em proteínas estruturais como colágeno e elastina, proteoglicanos e glicoproteínas, além de enzimas específicas como as metaloproteinases de matriz (MMPs) (MATRISIAN, 1992; CARVALHO; LINE, 1996; VILAMAIOR et al., 2000; CHATTERJEE, 2003).

Vale ressaltar a importância do compartimento estromal na arquitetura glandular que tem papel fundamental nos processos de diferenciação epitelial, regulando as taxas de proliferação, diferenciação e apoptose (SUGIMURA et al., 1986; LEE, 1996). O estroma prostático é capaz de produzir pelo menos oito famílias de fatores de crescimento, destas, cinco são conhecidas por estarem envolvidas na regulação da proliferação e diferenciação do epitélio (WONG; WANG, 2000). Incluem TGF (fator de crescimento tumoral), FGF (fator de crescimento de fibroblastos), IGF (fator de crescimento semelhante à insulina), HGF (fator de crescimento do hepatócito) e famílias EGF (fator de crescimento epidérmico) (LONG et al., 2005). Assim, células musculares lisas e fibroblastos adicionalmente participam ativamente da síntese de fatores autócrinos e parácrinos importantíssimos para a comunicação entre os compartimentos e manutenção da homeostasia glandular (MUNTZING, 1981; IZUMIYA; NAKADA, 1997).

1.2 PROSTATA FEMININA

A próstata não é uma glândula exclusiva do sistema reprodutor masculino, sendo encontrada em diversas fêmeas de mamíferos, incluindo humanos e roedores (Figura 3) (BRAMBELL; DAVIS, 1940). O primeiro autor a descrever a existência da próstata feminina foi Reinier De Graaf em 1672. Dois séculos depois, Alexander Skene redescreveu esta glândula como sendo formada por dois ductos parauretrais principais que se abrem em ambos os orifícios da uretra, e desprovida de função secretória (SKENE, 1880). A partir de 1950, novas pesquisas foram realizadas em relação a essa temática, principalmente com referência ao papel biológico que esse órgão poderia desempenhar no organismo feminino (HUFFMAN, 1948; ZAVIACIC et al., 1993).

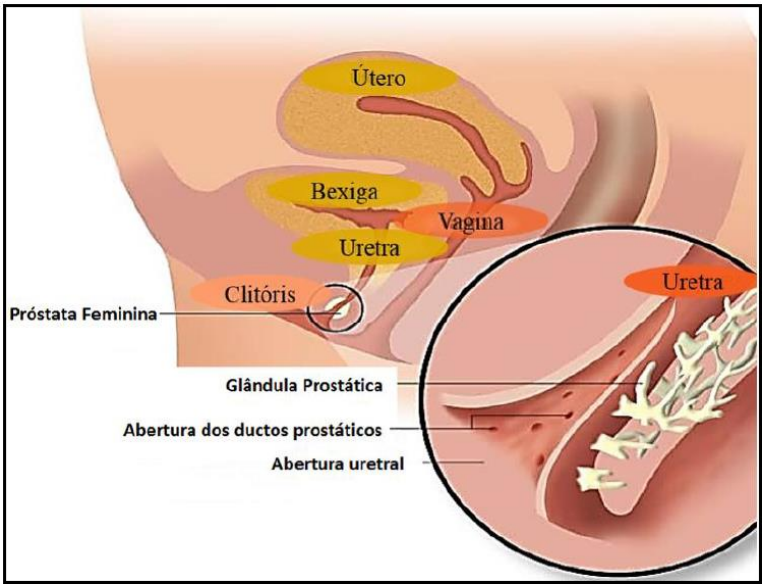

Figura 3. Representação esquemática da próstata feminina de humanos, mostrando a localização parauretral da glândula e a presença de ductos prostáticos que se abrem na luz uretral. Moalem (2009).

Paralelamente aos estudos em mulheres, diversas pesquisas têm demonstrado a ocorrência de próstata feminina em roedores como *Meriones unguiculatus* (SANTOS et al., 2003; CUSTÓDIO et al., 2004), *Lagostomus maximus maximus* (FLAMINI et al., 2002) e *Mastomys Erythroleucus* (BRAMBELL; DAVIS, 1940).

A próstata feminina de gerbilos (Figura 4) é composta por um conjunto de alvéolos, histologicamente semelhante ao lobo ventral do macho, que ficam concentrados em ambos os

lados da uretra, posição anatomicamente denominada de parauretral (SANTOS; TABOGA, 2006).

As similaridades fisiológicas existentes entre a próstata do gerbilo e a humana, tanto em machos quanto em fêmeas, sugerem que esse roedor é um excelente modelo experimental para investigar os aspectos funcionais do órgão normal e a instalação de desordens prostáticas.

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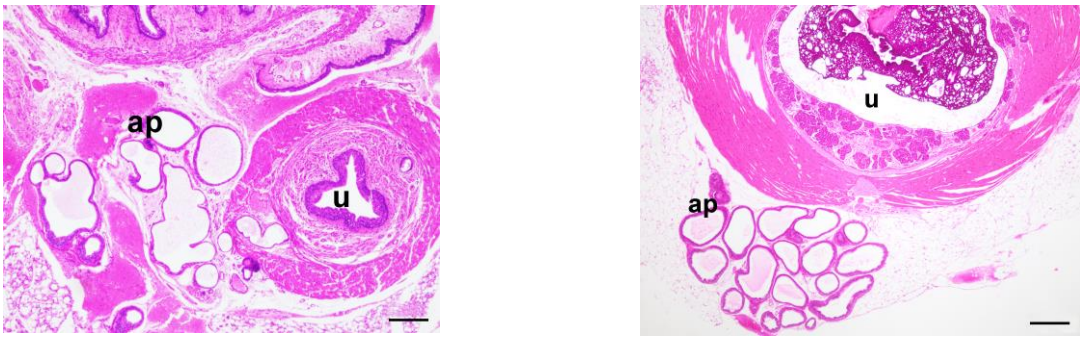

Figura 4. Fotomicrografia de luz, corada pela técnica em Hematoxilina e Eosina. **A)** próstata feminina do gerbilo da Mongólia, demonstrado localização parauretral. **B)** próstata ventral masculina do gerbilo da Mongólia. Alvéolos prostáticos (ap), uretra (u), barra de escala A e B: 200 µm. Fonte: próprio autor.

1.3 MODULAÇÃO HORMONAL PROSTÁTICA: EQUILÍBRIO ANDRÓGENO/ESTRÓGENO

A próstata é um órgão hormônio dependente, ao lado disso o seu crescimento seja normal ou neoplásico, sua manutenção e integridade funcional exigem um nível adequado e constante de andrógenos circundantes (ISAACS, 1984; WILSON, 2011). A testosterona (T) é o principal andrógeno no plasma masculino, sendo sintetizado pelas células de Leydig nos testículos fetal e adulto, interagindo com receptores específicos nos tecidos alvo por meio de difusão passiva (WILSON, 2011). Esse hormônio é crítico nos processos de masculinização dos ductos de Wolff, espermatogênese, funções sexuais, libido e produção de hormônios esteróides, atuando como um prohormônio (Figura 5), para a formação de metabólitos com maior afinidade pelos receptores específicos nos tecidos alvo (SORONEN et al., 2004; NICHOLSON; RICKE, 2011). Dentre esses metabólitos, está a diidrotestosterona (DHT), sintetizada a partir da conversão da T pela enzima 5α-redutase (RISBRIDGER et al., 2007). Durante o desenvolvimento fetal é a DHT que impulsiona a diferenciação do seio urogenital (UGS) na próstata, participa dos processos de virilização dos órgãos genitais externos e das características sexuais secundárias, além de ser responsável pela funcionalidade da próstata no indivíduo adulto

(SORONEN et al., 2004). Outros andrógenos importantes em homens são a dehidroepiandrosterona (DHEA), androstenediona e 5α-androstenediona, que podem ser convertidos em esteróides sexuais de maior afinidade, podendo agir na próstata em diferentes estágios, tanto no crescimento/funções normais quanto patológicas (BELANGER et al., 1994; ARNOLD, 2009).

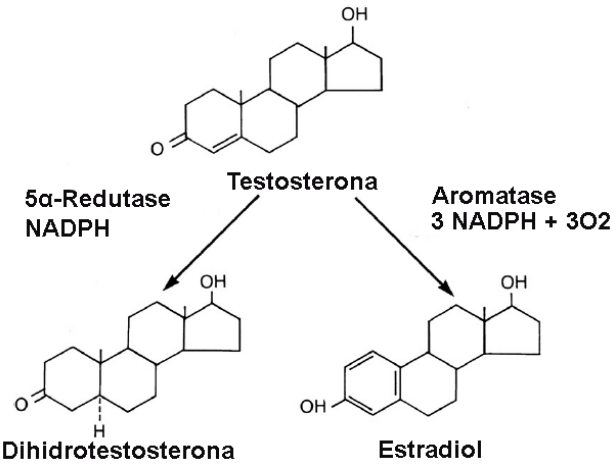

Figura 5. Conversão da testosterona em outros hormônios esteroides nas células de Leydig nos testículos. Adaptado de Wilson (2011).

Apesar de apresentarem funções distintas, tanto a T quanto a DHT exercem seus efeitos biológicos interagindo com o mesmo receptor androgênico (AR) (Figura 6), um membro da superfamília de receptores nucleares ativados por um ligante específico, que atua como fator de transcrição (MANGELSDORF; EVANS, 1995; LI; AZZAWI, 2009).

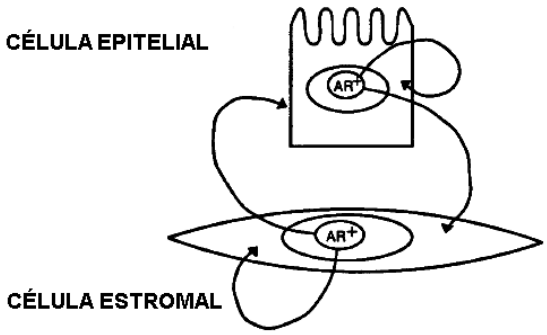

Figura 6. Representação esquemática do AR nas células epiteliais e estromais. As setas indicam direção dos processos induzidos por andrógenos. Adaptado de Trapman e Brinkmann (1996).

Após a interação do hormônio com o receptor, esse é translocado para o núcleo formando um complexo com as sequências específicas de DNA, denominadas de elementos andrógeno-responsivo (ARE, do inglês *androgen response elements*). Os ARE interagem com coativadores responsáveis por regular a expressão de mais de 100 genes prostáticos (MCKENNA et al., 1999; NELSON et al., 2002). Durante o desenvolvimento prostático, andrógenos estimulam fibroblastos e possivelmente os miócitos AR-positivos do compartimento mesenquimal a secretar fatores de crescimento, os quais atuam de modo parácrino sobre as células epiteliais vizinhas, induzindo o crescimento celular e o desenvolvimento glandular. Com referência a próstata adulta, os andrógenos atuam diretamente nas células epiteliais via AR para manter a viabilidade estrutural e funcional da glândula prostática. Assim, o AR tem fundamental importância nos diversos processos fisiológicos da próstata normal e neoplásica, e podem estar diretamente envolvidos no desenvolvimento e progressão de anormalidades andrógeno-dependentes (NELSON et al., 2002; CHATTERJEE, 2003).

Embora a próstata seja um órgão andrógeno dependente, os estrógenos têm participação direta sobre as funções normais prostáticas, especialmente o estradiol (E_2) (CHEN et al., 2008, MCPHERSON et al., 2008). O E_2 é sintetizado via aromatização de andrógenos, catalisada pela enzima aromatase, pertencente a família do citocromo P450 (O'DONNELL et al., 2001; KARKOLA; WAHALA, 2009; ELLEM; RISBRIDGER, 2010). A aromatase em humanos é codificada pelo gene CYP19 e sua expressão ocorre em vários tecidos tais como os ossos, ovários, testículos, cérebro, pele e tecido adiposo bem como na placenta através de um controle de promotores específicos (SIMPSON, 2003). A produção diária de estradiol em humanos do sexo masculino varia entre 40-50 µg, dos quais apenas 5-10 µg são derivados da secreção testicular direta, sendo considerado o estrógeno mais potente em homens, importantes nos processos de maturação óssea e no metabolismo de lipídios (EKMAN, 2000). Grande parte de estradiol no macho é derivado da conversão de testosterona via aromatase principalmente no tecido adiposo e músculo, no fígado e na glândula prostática (SCHWEIKERT et al., 1976; VERMEULEN et al., 2002).

A ação de estrógenos é tradicionalmente mediada pela interação com dois receptores nucleares (ERs), receptor de estrógeno alfa (ERα) e receptor de estrógeno beta (ERβ), pertencentes à superfamília do receptor nuclear (RN), ambos fatores de transcrição regulados pelos genes ESR1 e ESR2, respectivamente (CHEN et al., 2008; MORANI et al., 2008). O

receptor ERα é o responsável pela construção da glândula prostática antes da puberdade, enquanto o ERβ parece ter mais importância durante períodos mais tardios do desenvolvimento prostático, como a puberdade e a idade adulta, agindo juntamente com o AR na regulação da diferenciação e atividade funcional do tecido adulto (OMOTO et al., 2005; MCPHERSON et al., 2008).

Os mecanismos de ação do ERα, quando ativado, incluem proliferação aberrante do epitélio, inflamação e quando super ativados podem promover o desenvolvimento de lesões pré-malignas. Em contrapartida, efeitos benéficos dos estrógenos residem na ativação do ERβ, no qual atua na regulação dos efeitos antiproliferativos, anti-inflamatórios e potencialmente anticarcinogênicos (Figura 7). Assim, esses receptores em conjunto caracterizam efeitos agonistas e antagonistas na glândula prostática. Nesse sentido, tanto andrógenos quanto estrógenos desempenham papel importante na próstata e são críticos para crescimento e desenvolvimento normal da glândula, assim qualquer perturbação do ambiente hormonal endógeno pode causar alterações irreversíveis na morfofisiologia prostática (ELLEM; RISBRIDGER, 2009).

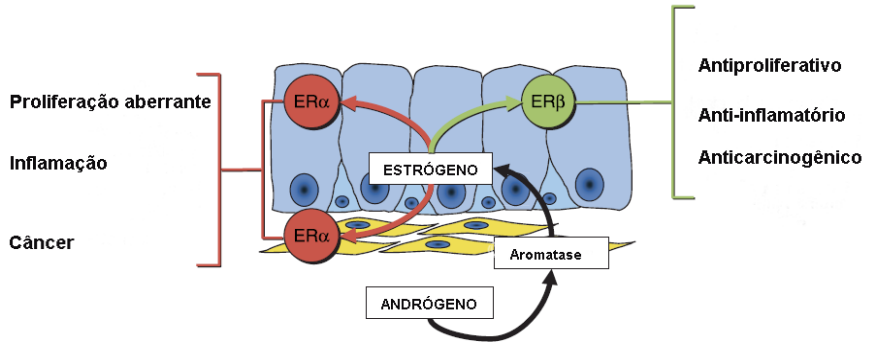

Figura 7. Esquema da sinalização local de estrógeno na próstata. A testosterona é metabolizada localmente em estrogéno pela enzima aromatase e atua via ERα ou ERβ. Os efeitos adversos via superativação do ERα no estroma e epitélio incluem proliferação aberrante, inflamação e eventualmente câncer. Em contraste, os estrógenos também são capazes de exercer efeitos benéficos via ERβ, causando respostas antiproliferativas, anti-inflamatórias e anticarcinogênicas. Adaptado de Risbridger et al. 2007.

1.4 COMPOSTOS FENÓLICOS

Compostos fenólicos ou polifenóis são importantes grupos de compostos que ocorrem naturalmente em plantas. Essas substâncias estão amplamente distribuídas, compreendendo no mínimo 8000 tipos de estruturas diferentes (BRAVO, 1998). Estes compostos são produzidos por

plantas como um metabólito secundário, sendo principalmente encontrados em grande concentração em frutas, vegetais, grande variedade de ervas e especiarias (SANDERSON et al., 2004).

Em geral, compostos fenólicos são divididos em grupos químicos dependendo de sua estrutura: fenol simples, ácidos fenólicos, flavonoides, cumarina, isocumarina, taninos e estilbenos (LIU, 2004). Essas substâncias são agentes redutores e juntamente com outros redutores dietéticos, como a vitamina C, E e carotenoides protegem os tecidos do corpo contra o estresse oxidativo, prevenindo o organismo de várias doenças como câncer, doenças cardiovasculares e inflamação (SCALBERT; WILLIAMSON, 2000; SUN et al., 2002).

Os flavonóides constituem a classe polifenólica mais importante, com mais de 5000 compostos já descritos. A estrutura básica dos flavonóides é composta por dois anéis benzênicos (Figura 8) (A e B) ligados através de um pirano heterocíclico ou anel de pirona com ligação dupla no meio, e podem ser divididos em várias classes de acordo com o grau de oxidação da molécula: flavonóis, flavonas, flavanonas, catequinas, antocianidinas, isoflavonas, diidroflavonois e chalconas (Figura 8) (COOK; SAMMAN, 1996; MIDDLETON et al., 2000).

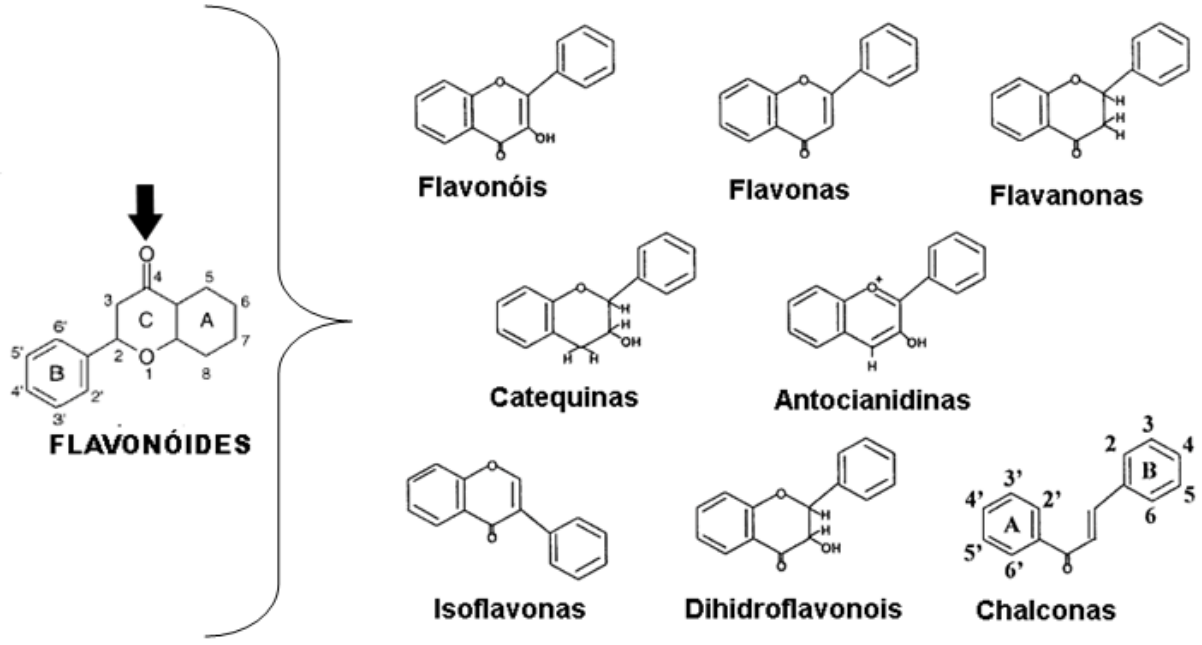

Figura 8. Estrutura das principais classes de flavonoides. Adaptado de Cook e Samman (1996).

A ingestão média de flavonoides nos Estados Unidos foi estimada em 1g/dia, embora tanto a fonte quanto a quantidade desses compostos variam sensivelmente em diferentes países (KUHNAU, 1976). Por exemplo, na dieta mediterrânea a quantidade consumida poderia ser consideravelmente maior, visto que a alimentação é rica em azeite, frutas cítricas e verduras e essas quantidades podem fornecer concentrações farmacologicamente significativas nos fluidos corporais e nos tecidos. Ademais, a ingestão dietética de flavonoides excede a da vitamina E, um antioxidade monofenólico, e a do β-caroteno quantificados em miligramas por dia (HERTOG et al., 1993). Ao lado disso, esses compostos fenólicos atuam como potentes quelantes de metais e eliminadores de radicais livres, atuando como antioxidantes naturais. Adicionalmente, exibem uma ampla gama de efeitos biológicos incluindo ações antibacteriana, anti-inflamatória, antialérgica, e ações vasodilatadoras, interferindo diretamente no nível fisiológico e bioquímico do organismo (COOK; SAMMAN, 1996).

Nesse contexto, a estrutura química desses compostos naturais e a expressão enzimática nos tecidos são determinantes para mensurar a taxa de absorção intestinal e a natureza dos metabólitos que circulam no plasma, assim como a farmacocinética e o destino metabólico de polifenóis ingeridos. Estudos realizados com vários compostos polifenólicos demonstraram que 75% a 99% dessas substâncias ingeridas não são encontrados na urina, isso implica que essas substâncias não foram absorvidas através da barreira intestinal, absorvida e excretada na bile ou metabolizada pela microflora do intestino (SCALBERT; WILLIAMSON, 2000). As concentrações de polifenóis intactos no plasma muitas vezes são baixas, por conseguinte reduzindo sua capacidade antioxidante. Em contrapartida, as formas ativas provavelmente serão metabólitos dessas substâncias que são muito mais abundantes no plasma, o que contribuem para aumentar a capacidade antioxidante no organismo (DONOVAN et al., 1999).

1.5 CRISINA

A crisina (5, 7-dihidroxiflavona) é um composto natural biologicamente ativo extraído de mel, plantas e própolis (Figura 9). Possui propriedades potentes tais como efeito anti-inflamatório, antioxidante, e promove morte celular perturbando a progressão do ciclo celular (SARAVANA; MANDAL, 2009). A crisina pode ser facilmente ingerida como suplemento dietético em capsulas contendo 500 miligramas e a dose sugerida pode chegar até seis capsulas por dia (WANG; MORRIS, 2007).

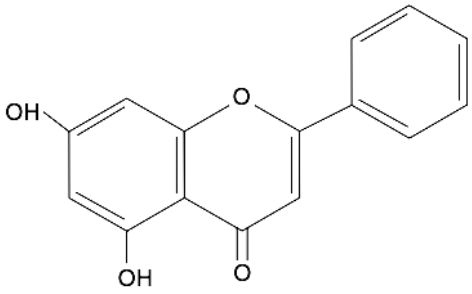

Figura 9. Estrutura química da crisina (5,7 dihidroxiflavona). Adaptado de Sathiavelu et al., 2009.

Nos processos inflamatórios o flavonoide crisina atua na supressão de enzimas tais como ciclooxigenase-2 (COX-2), prostaglandinas sintase e óxido nítrico sintase (NOS), enzimas envolvidas diretamente em diversos processos fisiológicos e patológicos (LIANG et al., 2001). Existem evidências que a ciclooxigenase-2 (COX-2) desempenha papel no desenvolvimento de tumores, assim como uma regulação inadequada dessa enzima prolonga a sobrevivência de células malignas, ocasionando alterações fenotípicas associadas ao potencial metastático (SURH et al., 2001). O óxido nítrico por sua vez está envolvido nos processos iniciais da carcinogênese, além de ser um promotor da tumorigênese e da transformação neoplásica (OHSHIMA; BARTSCH, 1994; ROBERTSON et al., 1996; KRONCKE et al., 2001).

Estudos demonstram que a crisina pode induzir apoptose em células malignas através da ativação de caspases, essenciais nos processos de apoptose e no bloqueio do ciclo do ciclo celular (WANG et al., 2004; WOO et al., 2004). Adicionalmente, estudos realizados com linhagem de células prostáticas humanas determinou os efeitos antiproliferativos e apoptóticos da crisina na progressão do câncer (SAMARGHANDIAN et al., 2011). Li e colaboradores (2010) abordaram o potencial anticancerígeno da crisina, avaliando a sensibilização dos efeitos desse flavonóide na morte celular apoptótica mediada pelo TNFα (fator de necrose tumoral), além de mecanismos moleculares envolvidos nesse processo. Além disso, esse composto possui várias outras atividades biológicas e tem demonstrado efeitos antioxidantes protetivos em vários órgãos como fígado, rim e colón, com destaque para o sucesso reprodutivo em roedores, ativando mecanismos protetivos na gônada masculina (CIFTCI et al., 2012; TAHIR; SUTANA, 2012).

Estudo realizado em roedores submetidos ao tratamento com esse composto demonstrou efeitos positivos nos testículos nos parâmetros espermáticos, na motilidade, concentração e

redução do estresse oxidativo nessa gônada (CIFTCI et al., 2012). Vale ressaltar que o desequilíbrio entre as espécies reativas de oxigênio e o mecanismo antioxidante nas células, resulta em produção excessiva de metabólitos de oxigênio, condição frequentemente denominada de “estresse oxidativo”, o que resulta em peroxidação lipídica da membrana, danos no DNA, assim como inativação de enzimas específicas (WISEMAN; HALLIWELL, 1996). Ao lado disso, os testículos são os principais alvos do estresse oxidativo, devido ao alto teor de lipídios poliinsaturados de membrana e é uma das principais condições de infertilidade e diminuição da função espermática (AITKEN; KRAUSZ, 2001). Nesse sentido, muitos estudos demonstram que a crisina tem o potencial de aumentar a atividade de enzimas antioxidantes tais como superóxido dismutase (SOD), catalase (CAT), e glutationa (GSH-Px), que em conjunto protegem o tecido contra o estresse oxidativo (SATHIAVELU et al., 2009; CIFTCI et al., 2012).

Além dessas propriedades farmacológicas, suplementos alimentares ricos em crisina estão relacionados em favorecer o aumento dos níveis séricos de testosterona. A crisina estimula a esteroidogênese testicular por aumentar a expressão do gene que codifica a proteína regulatória aguda da estereidogênese (StAR) (JANA et al., 2008). Essa proteína desempenha papel importante no processo de estereidogênese por facililitar o transporte de colesterol da membrana mitocondrial externa para a membrana mitocondrial interna, onde se encontra a enzima desmolase, pertencente ao complexo enzimático citocromo P450. Dessa forma ocorre a clivagem do colesterol em pregnenolona precursor da testosterona, aumentando a biodisponibilidade de testosterona sérica nas células de Leydig (PRIVALLE et al., 1983; PAYNE; YOUNGBLOOD, 1995). Além disso, a crisina aumenta a biodisponibilidade de testosterona sérica, por bloquear a atividade da enzima aromatase, que converte testosterona em estradiol (TA; WALLE, 2007).

Efeitos da crisina tem sido descrito no sistema genital de roedores, demonstrando que a exposição a essa flavona foi capaz de aumentar os níveis plasmáticos de testosterona em animais expostos durante 60 dias consecutivos a uma dosagem de 50 mg/kg/dia (CIFTCI et al., 2012). Similarmente verificou aumento das concentrações séricas de testosterona em aves expostos a essa flavona por um período de 12 dias consecutivos a uma dosagem de 75 mg/kg/dia (ALTAWASH et al., 2017).

Pesquisas demonstram que a crisina pode se ligar a ambas as formas de receptores estrogênicos (ERα e ERβ), desencadeando respostas estrogênicas ou antiestrogênicas (KUIPER et al., 1998; RESENDE et al., 2013). Além disso, estudos com outros tipos de flavonoides

(quercetina, apigenina, naringenina) apontam que estes compostos podem agir como antagonista do AR, via competição direta com os andrógenos endógenos (BOAM, 2015; WU et al., 2016). Na glândula prostática de gerbilos a exposição pré-puberal a essa flavona tem sido demonstrada por interferir na regulação dos receptores AR e ERα, potencializando a resposta glandular aos efeitos biológicos dos esteróides endógenos. Essas alterações resultaram em aumento da proliferação celular e maior atividade secretora da glândula (RIBEIRO et al., 2018). Assim os mecanismos de ação dessa flavona com receptores hormonais na glândula prostática ainda necessitam ser melhor elucidada.

Nesse contexto vale ressaltar que a próstata é um órgão extensivamente estudado por pesquisadores de todo o mundo, devido a sua alta propensão ao desenvolvimento de doenças. Por ser um órgão dependente e responsivo a hormônios, muitas desordens prostáticas têm sido atribuídas ao desequilíbrio ou desregulação hormonal sistêmica (SANTOS et al., 2006). Inúmeras pesquisas têm avaliado o potencial de substâncias naturais e/ou sintéticas em interferir com a homeostase desta glândula, investigando qual é a contribuição destes compostos para o surgimento de doenças (LIMA et al., 2015; COSTA et al., 2017). Estudos têm demonstrado que químicos ambientais sintéticos, como o bisfenol-A, parabenos e pesticidas apresentam atividade hormonal sobre a próstata, desencadeando respostas proliferativas (PRINS; PUTZ, 2008; RODRIGUEZ et al., 2015). Contudo, os efeitos de substâncias naturais hormonalmente ativas, como os flavonoides, ainda são pouco conhecidos. Assim torna se cada vez mais relevante avaliar os mecanismos e efeitos da crisina sobre os aspectos reprodutivos e o potencial dessa substancia em interferir com a produção e biodisponibilidade de hormônios esteroides no sistema reprodutor, visto que sensíveis modificações hormonais podem predispor a glândula ao desenvolvimento de lesões ao longo da vida.

2 OBJETIVOS

2.1 GERAL

- Avaliar os efeitos da exposição ao flavonoide crisina sobre a próstata e gônadas de machos e fêmeas de gerbilos adultos.

2.2 ESPECÍFICOS

- Avaliar se a exposição à crisina (3, 7 e 21 dias) pode causar alterações morfofisiológicas e morfométrico-estereológicas na próstata de gerbilos adultos.
- Investigar, por métodos ultraestruturais, o efeito dos tratamentos sobre a morfofisiologia das células prostáticas epiteliais e estromais.
- Avaliar se a crisina apresenta o mesmo potencial anabolizante dos andrógenos, por meio da comparação dos seus efeitos com os desencadeados pelo andrógeno sintético cipionato de testosterona.
- Analisar e quantificar por imuno-histoquímica os receptores nucleares AR e ERα nos compartimentos prostáticos de todos os grupos experimentais.
- Analisar o status proliferativo da glândula nos diferentes tratamentos, por meio da imuno-histoquímica para PCNA na glândula prostática de gerbilos de todos os grupos experimentais.
- Investigar os efeitos da crisina sobre os níveis hormonais séricos de testosterona e 17β estradiol.

3 METODOLOGIA

3.1 ANIMAIS

Neste experimento foram utilizados 140 gerbilos (*Meriones unguiculatus*), com 90 dias de idade, sendo 70 fêmeas e 70 machos. Estes animais foram mantidos em caixas de polietileno, com substrato de maravalha, sob condições controladas de luminosidade e temperatura média de 23ºC, sendo fornecidas água filtrada e ração "*ad libitum*". Todos os procedimentos e manipulações animais foram aprovadas pela Comissão de Ética no Uso de Animais da Universidade Federal de Goiás (CEUA/UFG, protocolo 111-17), seguindo a normativa do Conselho Nacional de Controle de Experimentação Animal (CONCEA).

3.2 OBTENÇÃO DA CRISINA

A síntese de crisina foi realizada em duas etapas, de acordo com protocolos descritos por Ramesh e co-autores, com modificações (RAMESH et al., 2015). Primeiramente foi preparado intermediário de chalcona usando a condensação de Claisen-Schmidt entre trihidroxiacetofenona e benzaldeído (PASSALACQUA et al., 2015). Esta reação foi realizada em hidróxido de sódio a 60% em etanol à temperatura ambiente durante 24 horas. A mistura reacional foi vertida em gelo triturado e acidificada com HCl a pH 3. O produto bruto estava sujeito a partição líquido-líquido com acetato de etilo. As fases orgânicas combinadas foram concentradas sob pressão reduzida e cromatografadas sobre gel de sílica, para fornecer 2', 4', 6'-trihidroxialcona com 44% de rendimento. Em seguida, a conversão de chalcona para flavona foi realizada por substituição nucleofílica intramolecular (ZERAIK et al., 2012). A solução de 2', 4', 6'-tri-hidroxialcona em glicerol e iodo foi submetida a refluxo durante 8 h. Após a conversão completa confirmada por análise de TLC, a mistura reacional foi extraída com acetato de etilo por partição líquido-líquido. O produto bruto foi purificado sobre gel de sílica, produzindo crisina (23%). A crisina foi obtida como um sólido amarelo pálido. 1H NMR (600 MHz, DMSO-d_6): 12.82 (*brs*, 1H), 8.06 (*d*,2H), 7.59 (*m*, 3H), 6.96 (*s*), 6.54 (*d*) e 6.23 (*d*). ^{13}C NMR (125 MHz, DMSO-*d6*): 182.3, 164.9, 163.7, 161.9, 157.9, 132.5, 131.3, 129.6, 126.8, 106.6, 105.0, 99.5 e 94.6.

3.3 DELINEAMENTO EXPERIMENTAL

Neste estudo foram formados os seguintes grupos experimentais:

Controle: 10 machos e 10 fêmeas com 90 dias de idade receberam por gavagem doses diárias do veículo de diluição da droga (óleo mineral – Nujol/Mantecorp - 0,1 ml/animal) durante vinte e um dias.

Crisina: 30 machos e 30 fêmeas com 90 dias de idade receberam por gavagem doses diárias de crisina (50mg/kg) diluída em 100 µl de óleo mineral (Nujol- Mantecorp). Estes animais foram divididos em três subgrupos que foram sacrificados após 3, 7 e 21 dias de tratamento.

Testosterona: 30 machos e 30 fêmeas com 90 dias de idade receberam doses subcutâneas de cipionato de testosterona (1mg/kg/semana) diluída em 100 µl de óleo mineral (Nujol-Mantecorp). Estes animais foram divididos em três subgrupos que foram sacrificados após 3, 7 e 21 dias de tratamento.

3.4 ANÁLISE BIOMÉTRICA

Os animais de todos os subgrupos experimentais foram anestesiados e eutanasiados com uma dose letal de anestésico (Cloridrato de cetamina, Cetamin®, Syntec) e relaxante muscular (Cloridrato de xilazina, Calmium®, Vetbrands), ou por meio de deslocamento cervical. Após a eutanasia, os animais foram submetidos a medidas de peso corporal e, em seguida, submetidos à coleta do complexo prostático (PrC) e gônadas. Após a dissecção, os PrCs, os ovários e os testículos foram pesados.

3.5 DOSAGEM HORMONAL SÉRICA

Os níveis séricos de testosterona e estradiol foram obtidos imediatamente após a eutanásia, por punção cardíaca dos gerbilos (n = 5 animais / grupo). Considerando o pequeno tamanho dos gerbilos, este procedimento permitiu obter um volume adequado de soro para realizar as dosagens. O soro foi separado por centrifugação (3000 rpm) e armazenado a -20 ° C para posterior análise hormonal. Os níveis séricos de testosterona e estradiol no soro foram determinados pelo imunoensaio enzimático competitivo (Monobind Inc., AccuBind, Lake Forest, EUA). A sensibilidade foi de 0,0576 ng / mL.

3.6 PROCESSAMENTO HISTOLÓGICO E ANÁLISE CITOQUÍMICA

O complexo prostático, testículos e ovários foram fixados por imersão em paraformaldeído a 4% (em tampão fosfato 0,1 M, pH 7,2; n = 3) por 24 horas, ou em metacarn (solução de metanol 60%, clorofórmio 30% e ácido acético 10%; n = 5) por três horas a 4ºC. Em seguida, os tecidos foram desidratados em bateria crescente de etanol, clarificados em xilol, embebidos em parafina (Histosec, Merck, Darmstadt, Alemanha) e seccionados a 5 μm em microtómio Leica (Leica RM2155, Nussloch, Alemanha).

Os cortes histológicos da próstata, testículos e ovários foram corados pela hematoxilina eosina (HE). As fibras colágenas e músculo foram identificados pelo método de coloração Tricrômico de Gömöri (BEHMER et al., 1976). Os espécimes foram analisados e digitalizados utilizando um microscópio de luz Zeiss Axioscope A1 (Zeiss, Alemanha).

3.7 ANÁLISE ESTEREOLÓGICA E MORFOMÉTRICA

As análises estereológicas e morfométricas foram realizadas a partir de lâminas coradas pela técnica da Hematoxilina Eosina e Tricrômico de Gomori respectivamente, e foi utilizado o Sistema Analisador de Imagens, com o programa Image- Pro Plus (Média Cybernetics).

As análises estereológicas foram realizadas para a obtenção da frequência relativa dos diferentes constituintes prostáticos (epitélio, lúmen, estroma muscular, estroma não muscular e conteúdo de colágeno) dos grupos experimentais em estudo. Para isso, foram capturados 30 campos aleatórios de cada subgrupo (6 campos fotomicrográficos/animal, n = 5) a partir de lâminas coradas pela Hematoxilina e Eosina ou Tricromico de Gomori. As medidas foram realizadas de acordo com o sistema de teste de multipontos M130 proposto por Weibel (1978) e aplicado à próstata por Huttunen et al. (1981). Assim, a partir dos dados obtidos para cada campo analisado, foi calculada a frequência relativa dos componentes em estudo.

Além disso, foi realizada a análise morfométrica para a determinação da altura (μm) do epitelio prostático dos diferentes grupos experimentais (300 medidas, 10 campos por animal, n = 3). As análises quantitativas foram realizadas usando o software Image-Pro Plus v 6.1 para Windows (Media Cybernetics Inc., Silver Spring, MD, EUA).

3.8 ANÁLISE ULTRAESTRUTURAL

Os fragmentos da glândula prostática dos diferentes grupos experimentais (n = 2) foram fixados com glutaraldeído 2,5% e ácido pícrico a 0,2% em tampão de cacodilato de sódio 0,1 M, pH 7,2, durante 24 horas a 4°C. Os fragmentos foram lavados duas vezes em tampão de cacodilato de sódio 0,1 M pH 7,2 e pós-fixados em tetróxido de ósmio a 1% preparado em tampão de cacodilato de sódio 0,1 M, pH 7,2, durante 2 h a 4°C. Após a pós-fixação, os fragmentos foram lavados duas vezes com água destilada durante 5 min cada, e sujeitos a desidratação com séries de acetona. Os materiais desidratados foram incorporados em resina Epon 812. Os materiais foram cortados em seções ultrafinas (50-70 nm) e contrastados com 3% de acetato de uranila e 3% de citrato de chumbo. As telas de cobre foram examinadas num microscópio electrónico de transmissão JEM-2100 (Jeol, Akishima, Japão) equipado com EDS (Thermo Scientific, Waltham, EUA) no Laboratório Multiusuário de Microscopia de Alta Resolução (LabMic / UFG).

3.9 ANÁLISE IMUNO-HISTOQUÍMICA

Para avaliar o efeito da exposição da crisina e testosterona sobre as próstatas de gerbilos foram verificadas as possíveis alterações na expressão de receptores hormonais em células epiteliais e estromais através das seguintes reações imuno-histoquímicas:

a) Receptor de andrógeno: AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA).
b) Receptor de estrógeno: Anti-ERα (rabbit polyclonal IgG, MC-20, Santa Cruz Biotechnology).
c) Receptor Anti-PCNA: Anti-PCNA (antígeno nuclear de proliferação celular).

Procedimentos gerais para imuno-histoquímica: os cortes histológicos desparafinizados e reidratados foram submetidos à recuperação antigênica em tampão citrato pH 6,0 a 100ºC por 45 minutos. Em seguida, os cortes foram lavados em PBS por 3 vezes de 5 minutos. O bloqueio de peroxidases endógenas foi realizado com H_2O_2 por 15 minutos e depois os cortes foram lavados em PBS por 3 vezes de 5 minutos. Os anticorpos primários para AR, ERα e PCNA foram incubados em diluições variadas (entre 1:50 a 1:500, de acordo com as instruções de cada anticorpo) a 4ºC overnight. Posteriormente, após serem lavados em PBS por 3 vezes de 5 minutos, e incubados com anticorpos secundários marcados com peroxidase (Rabbit ABC

Staining System SC-2018 – Santa Cruz Biotechnology, para AR, PCNA), os cortes foram revelados com a diaminobenzidizina (DAB). A contra coloração dos cortes foi feita com hematoxilina de Harris. Em seguida, as lâminas foram desidratadas em etanol, montadas em bálsamo do Canadá e avaliadas em microscopia de luz convencional.

3.10 QUANTIFICAÇÃO DE CÉLULAS AR, ER ALFA E PCNA POSITIVAS

Para a quantificação de células AR, ERα e PCNA positivas foram capturadas 30 imagens (n = 3 animais/grupo; aumento de 400x) para cada grupo experimental. Em cada campo, o número total de células positivas epiteliais e estromais foram obtidos como frequência relativa (%) em relação ao número total de células. Todas essas análises foram realizadas utilizando o sistema de análise de imagem previamente descrito.

3.11 ANÁLISE ESTATÍSTICA

A análise estatística dos dados morfométricos e estereológicos foram realizadas em planilhas e gráficos do software Statistic 6.0 (Copyright StatSoft, Inc. 1984-1996). Para a comprovação da significância dos resultados, foi utilizado o teste da ANOVA, seguido do desdobramento de Tukey, quando os dados apresentaram distribuição paramétrica, e o teste de Kruskal-Wallis, seguido do desdobramento de Dunn quando os dados tinham uma distribuição não-paramétrica. O nível de significância adotado para ambos os casos foi de 5% ($p \leq 0,05$).

4 RESULTADOS (CAPÍTULOS)

4.1 ARTIGOS 1: SHORT-TERM EXPOSURE TO CHRYSIN PROMOTES PROLIFERATIVE RESPONSES IN VENTRAL MALE PROSTATE AND FEMALE PROSTATE OF ADULT GERBILS

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Abstract

Chrysin (5,7-dihydroxyflavone) is a bioactive compound found in different fruits, vegetables, honey and propolis. This flavone has been indicated for the treatment of reproductive dysfunctions, mainly due to its antioxidant and hormonal properties. However, the effects of this polyphenol on the prostate are still poorly understood. The purpose of this study was to evaluate the effects of short-term chrysin exposure on the ventral male and female prostate of adult gerbils. To evaluate the androgenic potential of chrysin, gerbils were also exposed to testosterone. Male and female gerbils were exposed to chrysin (50 mg/kg/day) or testosterone cypionate (1 mg/kg/week) for 3, 7 and 21 days. Prostates were dissected-out for morphological, stereological and immunohistochemical analysis. Serum levels of testosterone and 17β-estradiol were measured by ELISA. Serum testosterone levels were not increased by chrysin supplementation in males or females. However, only females treated with chrysin for 21 days showed a raise in estradiol levels. Increased androgen receptor immunoreactivity, higher proliferation rates and glandular hyperplasia were observed in male and female prostates for all chrysin treatment times. Additionally, increased estrogen receptor alpha immunoreactivity was observed in all chrysin-treated females. Although chrysin and testosterone promoted similar morphological changes in the gerbil prostate, chrysin supplementation was more favorable to prostate health, since it resulted in lower incidences of hyperplasia and an absence of neoplastic foci.

Keywords: androgen receptor, estrogen receptor, flavonoids, immunohistochemistry, histopathology.

1. Introduction

Flavonoids are polyphenols found in many plants considered important components of the human diet (Pietta, 2000). Chrysin (5,7-dihydroxyflavone) is a naturally occurring compound of the flavone class found in passion blue flowers (*Passiflora coerulea*) (Wolfman et al., 1994), geranium leaf surface (*Pelargonium crispum*) (Williams et al. 1997), honey and propolis (Siess et al., 1996). This flavone shows a wide range of pharmacological effects, such as anti-inflammatory, anxiolytic, anticancer and antioxidant properties (Brown et al., 2007; Ali et al., 2014; Mani and Natesan, 2018).

Chrysin promotes testosterone-boosting effects, either by inhibiting aromatase catalytic activity (Kao et al., 1998; Jeong et al., 1999; Ta e Walle, 2007) or by stimulating testicular steroidogenesis via increased Star gene expression (Jana et al., 2008). Due to its hormonal properties, chrysin has been employed as a dietary supplement to obtain lean body mass (Wang and Morris, 2007), and for treatment of reproductive dysfunctions (Dhawan et al., 2002; Ly et al., 2015).

The effects of chrysin on reproduction are better understood in males. In rodents and birds, chrysin showed beneficial effects on spermatogenesis (Ciftci et al., 2012; Zhandi et al., 2017). In male rats, chrysin showed a protective role against testosterone-induced benign prostate hyperplasia (Shoieb et al., 2018). However, the effects of chrysin on the prostate gland remain poorly understood.

The prostate is an accessory gland of the reproductive system, whose function is to produce an alkaline secretion that promotes the nutrition and survival of spermatozoa (Biancardi et al., 2017). Several studies have reported the presence of prostate in women and female rodents (Zaviacic and Ablin, 2000; Flamini et al., 2002; Santos et al., 2008; Rodríguez-Castelán et al.,

2017). In gerbils, the female prostate presents structural homology with the ventral male prostate, making this a valuable model for comparative prostate studies (Santos and Taboga, 2006).

Prostate is a hormone-dependent gland that requires both androgens and estrogens for its growth, development and maintenance during adulthood (Ellem and Risbridger, 2009). In this context, we hypothesized that exposure to hormonally active phytochemicals may alter male and female prostate morphophysiology. Thus, the purpose of this study was to evaluate the effects of different periods of chrysin exposure (3, 7 and 21 days) on the ventral male prostate and the female prostate of adult gerbils. In addition, in order to evaluate the androgenic potency of chrysin, we compared, under the same experimental conditions, the effects of testosterone cypionate on the prostate of these animals.

2. Material and Methods

2.1 Chemical Procedures

Chrysin synthesis was performed in the Laboratory of Green and Medicinal Chemistry (IBILCE/UNESP) according to the method previously described in Ribeiro et al. (2018). Briefly, chalcone intermediate was obtained using Claisen-Schimidt condensation between trihydroxyacetophenone and benzaldehyde (Passalacqua et al., 2015). The conversion from chalcone to flavone was obtained by intramolecular nucleophilic substitution (Zeraik et al., 2012). After complete conversion was confirmed by TLC analysis, the reaction mixture was extracted with ethyl acetate by liquid-liquid partition. The crude product was purified over silica gel, yielding chrysin (23%). Chrysin was obtained as a pale yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6): 12.82 (*brs*, 1H), 8.06 (*d*, 2H), 7.59 (*m*, 3H), 6.96 (*s*), 6.54 (*d*) and 6.23 (*d*). ^{13}C NMR

(125 MHz, DMSO-*d6*): 182.3, 164.9, 163.7, 161.9, 157.9, 132.5, 131.3, 129.6, 126.8, 106.6, 105.0, 99.5 and 94.6.

2.2 Animals

All animals employed in this study were maintained in a temperature-controlled room (23ºC) on a 12 h light/dark cycle. Gerbils were kept on filtered water and standard rodent food *ad libitum* (Labina-Purina®; composition: 23% protein, 4% fat, 5% fiber and 12% minerals). Animal handling and experiments were performed according to the ethical guidelines (protocol no. 111/17 - CEUA/UFG), following the Guide for Care and Use of Laboratory Animals.

2.3 Experimental Design

One hundred twelve three month old gerbils (*Meriones unguiculatus*, Gerbillinae: Muridae) were employed in this study. Male and female gerbils were divided into fourteen groups (seven female groups and seven male groups, n = 8 animals/group): Control group (C): animals received daily oral doses of the dilution vehicle (mineral oil/Nujol-Mantecorp, 100 µL/animal) for 21 days. Chrysin group (Chr): animals received daily oral doses of chrysin (50 mg/kg) for 3, 7 and 21 days. Testosterone group (T): animals received subcutaneous injections of testosterone cypionate (1 mg/kg/week; Deposteron/EMS) for 3, 7 and 21 days. All gerbils were weighed and euthanized by cervical dislocation. The prostatic complexes (corresponding urethral segment, ventral, dorsolateral and dorsal prostate lobes in males, and vaginal segment, corresponding urethral segment and prostatic tissue in females) were dissected out and weighed.

2.4 Hormonal Serum Dosage

Blood samples of gerbils were obtained by cardiac puncture immediately after euthanasia (n = 5 animals/group). Serum was obtained by centrifugation (3000 rpm) and stored at -20°C for subsequent hormone analysis. Circulating serum testosterone and estradiol levels were determined by Competitive Enzyme immunoassay (Monobind Inc., AccuBind, Lake Forest, USA). The sensitivity was 0.0576 ng/mL.

2.5 Light Microscopy

The prostatic complexes were fixed by immersion in methacarn (60% methanol, 30% chloroform and 10% acetic acid; n = 5 animals/group) for four hours at 4°C, or in 4% paraformaldehyde (buffered in 0.1 M phosphate, pH 7.2; n = 3 animals/group) for 24 hours. Then, the tissues were dehydrated through a crescent ethanol series, clarified in xylol, embedded in paraplast (Histosec, Merck, Darmstadt, Germany) and sectioned at 5 μm on a Leica microtome (Leica RM2155, Nussloch, Germany). Sections were stained by hematoxylin-eosin (HE). Specimens were analyzed and digitized using a Zeiss Axioscope A1 light microscope (Zeiss, Germany).

2.6 Stereology

Stereological analyses were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines (Weibel, 1963). The relative frequency of each component of the prostatic tissue (epithelium, lumen, non-muscle stroma and muscle stroma) was determined. We chose 30 microscopic fields at random from each experimental group (six fields per animal; n = 5). We determined the relative frequency by counting the coincident points in the test grid and dividing them by the total number of points. Stereological analysis was performed using Image-Pro Plus software v6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

2.7 Histopathological Analysis

Histopathological analysis was performed using an Olympus light microscope (BX43, Olympus, Japan). Ventral male prostate and female prostate sections stained by HE (30 histological sections/group; n = 8 animals/group) were analyzed to determine the frequency (%) of prostatic disorders. For this, the total number of prostatic alveoli per section was determined, then the alveoli that presented foci of hyperplasia, neoplasia and luminal or perialveolar inflammation were discriminated. The percentage of altered alveoli was obtained in relation to the total alveoli number in each histological section.

2.8 Immunohistochemistry

Ventral male prostate and female prostate sections were subjected to immunohistochemistry analysis (n = 3 animals/group). Antibodies against androgen receptor (AR- rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), estrogen receptor alpha (ERα- rabbit polyclonal IgG, MC-20, sc-542, Santa Cruz Biotechnology) and proliferating cell nuclear antigen (PCNA- mouse monoclonal IgG2a, SC 56, Santa Cruz Biotechnology, CA, USA) were employed for immunostaining at a dilution of 1:100 overnight at 4°C. On the next day, Novocastra Post Primary and Polymer were used as secondary antibodies. The sections were stained with DAB Chromogen and DAB Substrate Buffer (in a proportion of 1:20), and finally counterstained with hematoxylin. The histological sections were analyzed using a Zeiss Axioscope A1 light microscope (Zeiss, Germany).

2.9 AR, ERα and PCNA Quantification

For AR, ERα, and PCNA quantification, 30 microscopic fields (n = 3 animals/group; magnification of 400x) were examined for each experimental group. In each field, the total number of positive epithelial and stromal cells was obtained as a relative frequency (%) in relation to the total number of cells. All these analyses were performed using the image analysis system previously described.

2.10 Statistical Analyses

The hypothesis tests employed to determine statistical significance were the Kruskal-Wallis test for non-parametric distributions and the ANOVA for parametric distributions. Further determination of the significant statistical differences between experimental groups was done using Dunn's test for non-parametric distributions and the Tukey's test for parametric distributions. The data were analyzed using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA). The level of significance was set at 5% ($p \leq 0.05$). Values are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Testosterone Plasma Levels

Chr-males showed a significant reduction of serum testosterone levels in 3D group, but these levels gradually recovered in 7D and 21D groups (Fig. 1a). Chr-females showed no significant changes in serum testosterone levels (Fig. 1c). T-males and T-females presented a significant increase in plasma testosterone levels only in 7D and 21D groups (Fig. 1b, d).

3.2 17β-estradiol Plasma Levels

Chr-males showed no significant changes in 17β-estradiol levels (Fig. 2a). T-males had a significant increase in estradiol levels only in the 7D group (Fig. 2b). Chr- and T-females presented higher 17β-estradiol levels only in the 21D group (Fig. 2c-d).

3.3 Body and Prostate Weight

Chr-males had body weight reduction in the 3D and 21D groups. In the T-males, a reduction in body weight was observed in the 3D group (Table 1). Chr- and T-treatments did not affect the prostatic complex weight of the male gerbils (Table 1). Chr- and T-females did not present alterations in body weight (Table 2). Only 7D Chr-females showed a significant increase in prostatic complex weight (Table 2).

3.4 Prostate Morphology, Stereology and Histopathology

Ventral prostate of the 3D and 7D Chr-males showed an increase in epithelium and muscle stroma frequency, and a reduction of the luminal compartment (Fig. 3a-c, Table 1). In 21D Chr-males, the ventral prostates presented both hypertrophic alveoli with large lumens (Fig. 3d) and hyperplastic alveoli (Fig. 3e). When compared to the C-group, the ventral prostates of 21D Chr-males had increased epithelial frequency and reduced luminal compartment (Table 1). In male gerbils, Chr-treatment caused a gradual increase in the hyperplastic alveoli frequency (C: 1.4 ± 0.4%, 3D: 14.7 ± 1.1%, 7D: 17.5 ± 1.0%, 21D: 26.8 ± 1.1%) (Table 3). T-male prostates (3D, 7D and 21D groups) presented glandular changes similar to those found in Chr-males, such as increased epithelium frequency, reduced lumen and increased frequency of alveolar hyperplasia (Fig. 3f-h, Tables 1 and 3). However, when compared to Chr-males, T-male prostates presented higher hyperplasia rates in all treatment phases (C: 1.4 ± 0.4%, 3D: 24.9 ± 1.3%, 7D: 24.1 ± 1.0%, 21D: 25.6 ± 1.4%, $p \leq 0.05$), in addition to isolated foci of neoplasia (Table 3).

Prostates of the 3D and 7D Chr-females presented increased frequency of epithelium and muscular stroma, and reduction of the luminal compartment (Fig. 3i-l, Table 2). Chr-females of the 21D group showed both hypertrophic and hyperplastic alveoli (Fig. 3l-m). When compared to the control group, 21D Chr-female prostates only presented increased epithelial frequency (Table 2). Chr-females of all experimental groups showed an increased frequency of hyperplastic alveoli and a reduction in the occurrence of inflammatory foci (Table 4). T-females showed increased epithelial frequency (especially in 3D and 7D groups) and reduced luminal compartment (only in 3D and 7D groups) (Fig. 3n-q, Table 2). In addition, in 7D T-females there was an increase in the muscular stroma frequency and a decrease in the non-muscular stroma frequency (Table 2). When compared to the control-group or to the Chr-group, T-females had a higher incidence of alveolar hyperplasia, inflammation and neoplasia (Table 4).

3.5 AR Immunostaining and Frequency

Males and females of the Chr- and T-groups showed a significant increase in AR-positive cells in the epithelial and stromal compartment (Fig. 4). However, in both treatments, no significant differences were observed between drug administration times (3D, 7D and 21D) (Fig. 4).

3.6 ERα Immunostaining and Frequency

In this study, we did not succeed with immunohistochemical reactions to ERα in males. However, Chr- and T-females showed a significant increase in stromal immunostaining for ERα at all treatment times (Fig. 5).

3.7 PCNA Immunostaining and Frequency

Males and females of the Chr- and T-groups showed a significant increase in PCNA-positive cells in the epithelium and stroma (Fig. 6, Table 5). In Chr- and T-males, the peak of epithelial and stromal proliferation was in the 3D group (Fig. 6b, e; Table 5). In Chr-females, the peak of epithelial proliferation was in the 7D group, and greater stromal proliferation was observed in the 3D and 7D groups (Fig. 6i-j, Table 5). On the other hand, in T-females peak of epithelial proliferation occurred in the 3D group, and there was a higher index of stromal proliferation in the 3D and 7D groups (Fig. 6l-m, Table 5).

4. Discussion

In this study, we evaluated the ventral male prostate and the female prostate after 3, 7 and 21 days of exposure to either chrysin or testosterone. Contrary to testosterone treatment, chrysin not increased testosterone plasma levels in male or female gerbils. However, chrysin presented similar potential to testosterone in increasing AR immunoreactivity and cellular proliferation in male and female prostates. In addition, Chr-females showed an increase in serum estradiol levels at 21 days of treatment and an increase in ERα-positive stromal cells at all treatment times.

Previous research has shown the testosterone-boosting properties of chrysin. Male rats that received 50 mg/kg/day of chrysin for 60 consecutive days showed a significant increase in the serum testosterone levels (Ciftci et al., 2012). Arthritic rats had serum testosterone levels restored after 21 days of chrysin supplementation (50 mg/kg/day) (Darwish et al., 2014). In contrast, in our study, males and females gerbils supplemented with 50 mg/kg/day of chrysin did not show a significant increase in testosterone levels in any of the analyzed times (3, 7 and 21 days). A recent study by Altawash and coworkers demonstrated that roosters treated for 12 consecutive weeks with chrysin at 25, 50 and 75 mg/kg/day showed an increase in serum testosterone levels only at 75 mg/kg/day (Altawash et al., 2017). Thus, we believe that the chrysin

dose employed in our study, as well as short exposure times, were not sufficient to promote testosterone-boosting responses in male and female gerbils.

Although plasma testosterone levels were not significantly increased, chrysin promoted considerable morphological changes in the male and female prostate. These morphological alterations were detected from the 3 days of treatment and were maintained until the end of the experiment (21 days). Chr-treated male and female prostates presented intense epithelial development, glandular hyperplasia, increased AR immunoreactivity and higher cell proliferation. These morphological changes were also observed, at least in part, in T-treated males and females, which showed an obvious increase in serum testosterone levels. These results suggest that chrysin may operate locally, causing increased intraprostatic testosterone bioavailability, or activating AR itself. Chrysin has been described as inhibiting aromatase, an enzyme that catalyzes the conversion of androgens to estrogens (Sanderson et al., 2004; Moon et al., 2006; Mani and Natesan, 2018). Since prostate cells express aromatase (Ellem and Risbridger, 2010; Morais-Santos et al., 2018), it is presumed that chrysin blocked androgen conversion into estrogens, increasing the intraprostatic bioavailability of androgens in male and female gerbils.

In this study, chrysin presented a similar potential to testosterone for increasing AR immunoreactivity in the male and female prostate. The effects of chrysin on AR regulation are poorly understood. I*n vitro* assays have shown that other flavonoids types, such as apigenin, fisetin and quercetin, have the potential to inhibit the AR signaling axis (Khan et al., 2008; Boam, 2015). On the other hand, a recent study published by our research group showed that prepubertal chrysin exposure increased AR immunoreactivity in the prostate of male and female adult gerbils (Ribeiro et al., 2018). These contradictory results indicate possible different mechanisms of action for chrysin using *in vitro* and *in vivo* models. However, AR upregulation in Chr-treated

males and females may be directly related to the increase in cell proliferation and prostatic hyperplasia observed in this study.

Chr-females, but not Chr-males, had increased 17β-estradiol serum levels at 21 days of treatment. This result was unexpected, since aromatase inhibitors tend to suppress estradiol serum levels (Risbridger et al., 2003; Corradi et al., 2009). Thus, the lower tissue estradiol bioavailability may have acted as a positive feedback on the hypothalamic-hypophyseal-gonadal axis, culminating in ovarian stimulation to restore estrogen levels. In addition, Chr-females showed increased ERα-reactivity in all treatment periods. These dissimilarities between males and females indicate that chrysin may present different mechanisms of action in both sexes, and demonstrate the estrogenic activity of this flavone in gerbil females. *In vitro* assays demonstrated that chrysin and other phytoestrogens were able to bind to ERα, triggering estrogenic agonist responses (Kuiper et al., 1998; Berthier et al., 2007; Leclercq and Jacquot, 2014). Since health and normal prostate morphology are maintained by the precise balance between androgens and estrogens (Ellem and Risbridger, 2009), chrysin dietary supplementation should be considered with caution, especially in females.

Although the results of this study demonstrated that chrysin and testosterone promoted similar effects on the prostate of both male and female gerbils, our histopathological examination indicated that testosterone was more deleterious than chrysin. T-males and T-females presented a prevalence of prostatic hyperplasia, whereas only T-females had a higher incidence of inflammatory and neoplastic foci. This evidence suggests that chrysin may be better than synthetic androgens in the treatment of reproductive dysfunctions, such as infertility and low libido.

5. Conclusion

Even in the short three day treatment, chrysin caused AR upregulation and increased cell proliferation rates in the ventral male and female prostate of adult gerbils. These precocious changes resulted in epithelial development and hyperplastic growth of the gland. In addition, chrysin treatment increased ERα immunoreactivity in the female prostate and raised serum estradiol levels in 21D females, indicating that chrysin exerted differential estrogenic effects in females. Although chrysin and testosterone evoked similar morphological changes in the male and female gerbil prostate, chrysin supplementation was more favorable to prostate health, since it resulted in a lower incidence of hyperplasia and absence of neoplastic foci.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Legends

Table 1. [1]Body and prostate complex weight in all experimental groups (n = 8/group). [2]Stereological data obtained for the ventral prostate in all experimental groups (n = 30 fields in 3 animals/group). Values are mean ± standard error of mean. Superscript letters (^{a,b,c}) represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Table 2. [1]Body and prostate complex weight in all experimental groups (n = 5/group). [2]Stereological data obtained for the female prostate in all experimental groups (n = 30 fields in 3 animals/group). Values are mean ± standard error of mean. Superscript letters (^{a,b,c}) represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Table 3. Alveolar disorders (%) in ventral male prostate of adult gerbils (n = 8 animals/group). Values are mean ± standard error of mean. Superscript letters $^{(a,b,c)}$ represent statistically significant differences between the control and time-related groups (3D, 7D, 21D; $p \leq 0.05$). Symbol (†) represents statistically differences between Chr- and T- treatments in each period of treatment.

Table 4. Alveolar disorders (%) in female prostate of the adult gerbils (n = 8 animals/group). Values are mean ± standard error of mean. Superscript letters $^{(a,b,c)}$ represent statistically significant differences between the control and time-related groups (3D, 7D, 21D; $p \leq 0.05$). Symbol (†) represents statistically differences between Chr- and T- treatments in each period of treatment.

Table 5. Frequency (%) of PCNA-positive cells in the ventral male and female prostate of all experimental groups. Values are mean ± standard error of mean. Superscript letters $^{(a,b,c,d)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 1. Testosterone plasma levels (ng/mL) in all experimental groups (n = 5 animals/group). Values are mean ± standard error of mean. Asterisks represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 2. 17β-Estradiol plasma levels (pg/mL) in all experimental groups (n = 5 animals/group). Values are mean ± standard error of mean. Asterisks represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 3. Histological sections of ventral male prostate and female prostate stained by HE method. C: control; Chr: chrysin; T: testosterone cypionate for 3 (3D), 7 (7D) and 21 (21D) days of treatment. Epithelium (Ep), lumen (L), stroma (S), hyperplasia (Hyp), prostatic intraepithelial neoplasia (PIN), inflammatory infiltrate (*). Scale bar: 50 μm; inserts (e, m, o): 20 μm.

Figure 4. AR immunostaining in ventral male prostate and female prostate. C: control; Chr: chrysin; T: testosterone cypionate. **(a-c)** Arrows indicate AR-positive cells in epithelium (Ep) and stroma (S) of the 7D male groups. Lumen (L). **(d-e)** Frequency (%) of AR-positive cells in the ventral male prostate of all experimental groups. **(f-h)** Arrows indicate AR-positive cells in epithelium and stroma of the 7D female groups. **(i-j)** Frequency (%) of AR-positive cells in the female prostate of all experimental groups. Values are mean ± standard error of mean. Superscript letters $^{(a,b,c)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 5. ERα immunostaining in female prostate. C: control; Chr: chrysin; T: testosterone cypionate. **(a-c)** Arrows indicate ERα-positive cells in stroma (S) of the 3D female groups. Epithelium (Ep), Lumen (L). **(d-e)** Frequency (%) of ERα-positive cells in the female prostate of all experimental groups. Values are mean ± standard error of mean. Superscript letters $^{(a,b,c)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 6. PCNA immunostaining in ventral male prostate and female prostate. C: control; Chr: chrysin; T: testosterone cypionate in 3 (3D), 7 (7D) and 21 (21D) days of treatment. Arrows

indicate PCNA-positive cells in epithelium (Ep) and stroma (S). Lumen (L).

Table 1

[1]Biometry (g)	Treatment	Groups			
		C	**3D**	**7D**	**21D**
Body weight	**Chr**	73.2 ± 2.2[a]	65.2 ± 1.2[b]	71.6 ± 1.7[a]	66.4 ± 1.5[b]
	T		62.8 ± 2.2[b]	64.8 ± 1.7[a]	69.6 ± 3.7[a]
Prostate complex weight	**Chr**	0.7 ± 0.05	0.7 ± 0.05	0.6 ± 0.09	0.6 ± 0.02
	T		0.7 ± 0.06	0.6 ± 0.07	0.9 ± 0.06
[2]Stereology (%)					
Epithelium	**Chr**	17.4 ± 1.2[a]	33.6 ± 1.1[b]	35.5 ± 1.3[b]	23.5 ± 1.2[c]
	T		31.1 ± 1.4[b]	28.3 ± 1.1[b. c]	25.8 ± 1.7[c]
Lumen	**Chr**	52.9 ± 1.9[a]	28.3 ± 1.9[b]	26.7 ± 1.5[b]	43.6 ± 2.7[c]
	T		35.6 ± 2.8[b]	37.1 ± 2.2[b]	46.1 ± 3.3[a]
Muscle stroma	**Chr**	9.8 ± 0.6[a]	12.4 ± 0.5[b]	15.3 ± 0.7[c]	10.1 ± 0.6[a]
	T		11.4 ± 0.7	12.3 ± 0.8	10.3 ± 0.8
Non-muscle stroma	**Chr**	19.9 ± 1.7	25.7 ± 2.1	22.6 ± 1.9	22.8 ± 2.3
	T		21.9 ± 2.4	22.2 ± 1.9	17.9 ± 2.2

Table 2

[1]Biometry (g)	Treatment	Groups			
		C	**3D**	**7D**	**21D**
Body weight	**Chr**	54.8 ± 3.0	65.2 ± 2.2	61.2 ± 1.9	58.4 ± 4.5
	T		58.0 ± 1.1	56.4 ± 2.6	59.2 ± 1.7
Prostate complex weight	**Chr**	0.13 ± 0.01[a]	0.16 ± 0.02[a]	0.23 ± 0.03[b]	0.16 ± 0.03[a]
	T		0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
[2]Stereology (%)					
Epithelium	**Chr**	16.1 ± 1.0[a]	31.3 ± 1.1[b]	32.3 ± 1.3[b]	24.7 ± 1.03[c]
	T		34.6 ± 1.4[b]	34.3 ± 1.7[b]	28.6 ± 1.2[c]
Lumen	**Chr**	34.6 ± 2.2[a]	19.0 ± 1.9[b]	14.4 ± 1.2[b]	27.8 ± 2.3[a]
	T		18.5 ± 1.6[b]	21.0 ± 1.7[b]	24.8 ± 2.1[b]
Muscle stroma	**Chr**	13.9 ± 0.9[a]	18.8 ± 1.3[b]	17.9 ± 1.0[b]	13.0 ± 0.9[a]
	T		16.0 ± 1.0[a]	19.2 ± 1.1[b]	17.4 ± 1.2[a]
Non-muscle stroma	**Chr**	35.7 ± 1.9[a]	31.0 ± 2.3	35.4 ± 1.3	34.5 ± 2.0
	T		30.9 ± 1.7[a]	25.5 ± 1.9[b]	29.3 ± 1.7[a]

Table 3

Alveoli histology (%)	Treatment	Groups			
		C	**3D**	**7D**	**21D**
Normal	**Chr**	98.4 ± 0.5[a]	84.0 ± 1.0[b]	80.1 ± 1.6[b]	72.1 ± 1.0[c]
	T		74.7 ± 1.2[b,†]	74.9 ± 1.1[b,†]	69.6 ± 2.3[b]
Hyperplasia	**Chr**	1.4 ± 0.4[a]	14.7 ± 1.1[b]	17.5 ± 1.0[b]	26.8 ± 1.1[c]
	T		24.9 ± 1.3[b,†]	24.1 ± 1.0[b,†]	25.6 ± 1.4[b]
Inflammation	**Chr**	0.2 ± 0.1	1.0 ± 0.3	2.5 ± 1.4	1.1 ± 0.5
	T		0.4 ± 0.2	0.4 ± 0.2	4.0 ± 1.4
Neoplasia	**Chr**	0	0	0	0
	T		0	0.6 ± 0.3	0.8 ± 0.3

Table 4

Alveoli histology (%)	Treatment	Groups			
		C	**3D**	**7D**	**21D**
Normal	**Chr**	90.4 ± 1.5^{a}	77.8 ± 2.1^{b}	71.3 ± 2.7^{b}	70.2 ± 2.8^{b}
	T		$43.5 \pm 2.7^{b,\dagger}$	$47.5 \pm 3.0^{b,\dagger}$	$52.3 \pm 2.7^{b,\dagger}$
Hyperplasia	**Chr**	8.2 ± 1.2^{a}	22.2 ± 2.1^{b}	27.8 ± 2.4^{b}	29.2 ± 2.8^{b}
	T		$48.4 \pm 2.6^{b,\dagger}$	$47.7 \pm 3.2^{b,\dagger}$	$42.4 \pm 2.9^{b,\dagger}$
Inflammation	**Chr**	2.0 ± 0.8^{a}	0^{b}	1.4 ± 0.7^{a}	0.2 ± 0.2^{a}
	T		$4.6 \pm 0.9^{\dagger}$	$3.0 \pm 0.7^{\dagger}$	$2.1 \pm 0.5^{\dagger}$
Neoplasia	**Chr**	0.4 ± 0.2^{a}	0	0	0
	T		$3.5 \pm 0.9^{b,\dagger}$	$1.8 \pm 0.6^{a,\dagger}$	$3.3 \pm 1.0^{b,\dagger}$

Table 5

		Groups			
	Treatment	**C**	**3D**	**7D**	**21D**
Male					
Epithelium	**Chr**	11.5 ± 0.6^{a}	23.6 ± 1.0^{b}	17.6 ± 0.8^{c}	16.1 ± 0.7^{c}
	T		23.03 ± 0.9^{b}	16.9 ± 0.5^{c}	16.5 ± 0.7^{c}
Stroma	**Chr**		42.7 ± 2.2^{b}	35.8 ± 1.2^{b}	36.8 ± 1.3^{b}
	T	14.6 ± 3.2^{a}	29.2 ± 1.1^{b}	24.4 ± 1.1^{b}	25.8 ± 1.0^{b}
Female					
Epithelium	**Chr**	13.8 ± 1.7^{a}	27.8 ± 1.2^{b}	32.5 ± 1.4^{c}	25.4 ± 1.6^{b}
	T		31.9 ± 1.2^{b}	25.1 ± 1.5^{c}	19.7 ± 0.9^{d}
Stroma	**Chr**	12.3 ± 0.7^{a}	52.0 ± 1.3^{b}	53.7 ± 1.6^{b}	32.3 ± 1.2^{c}
	T		32.8 ± 1.2^{b}	33.7 ± 1.2^{b}	26.8 ± 0.9^{c}

Figure 1

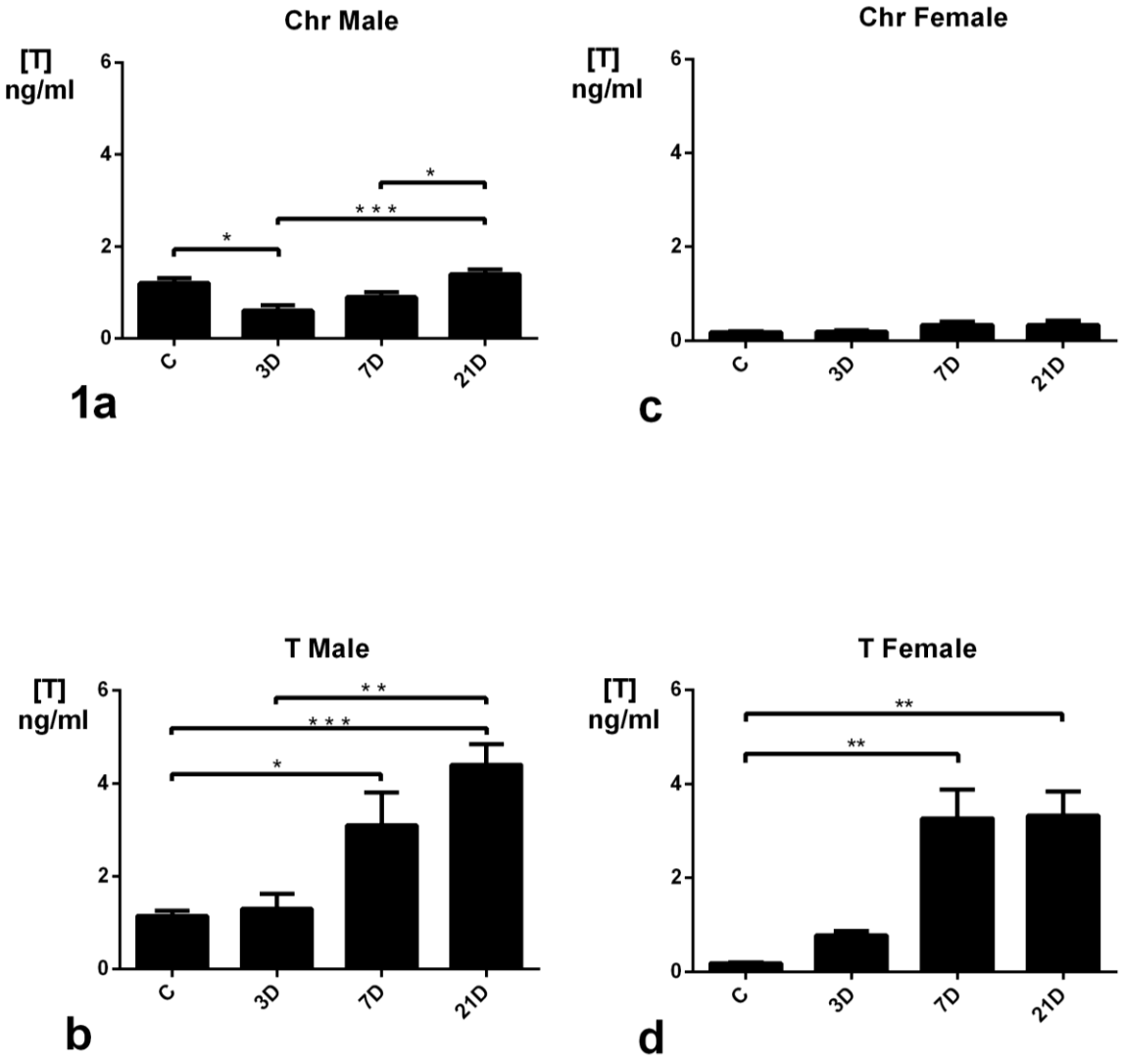
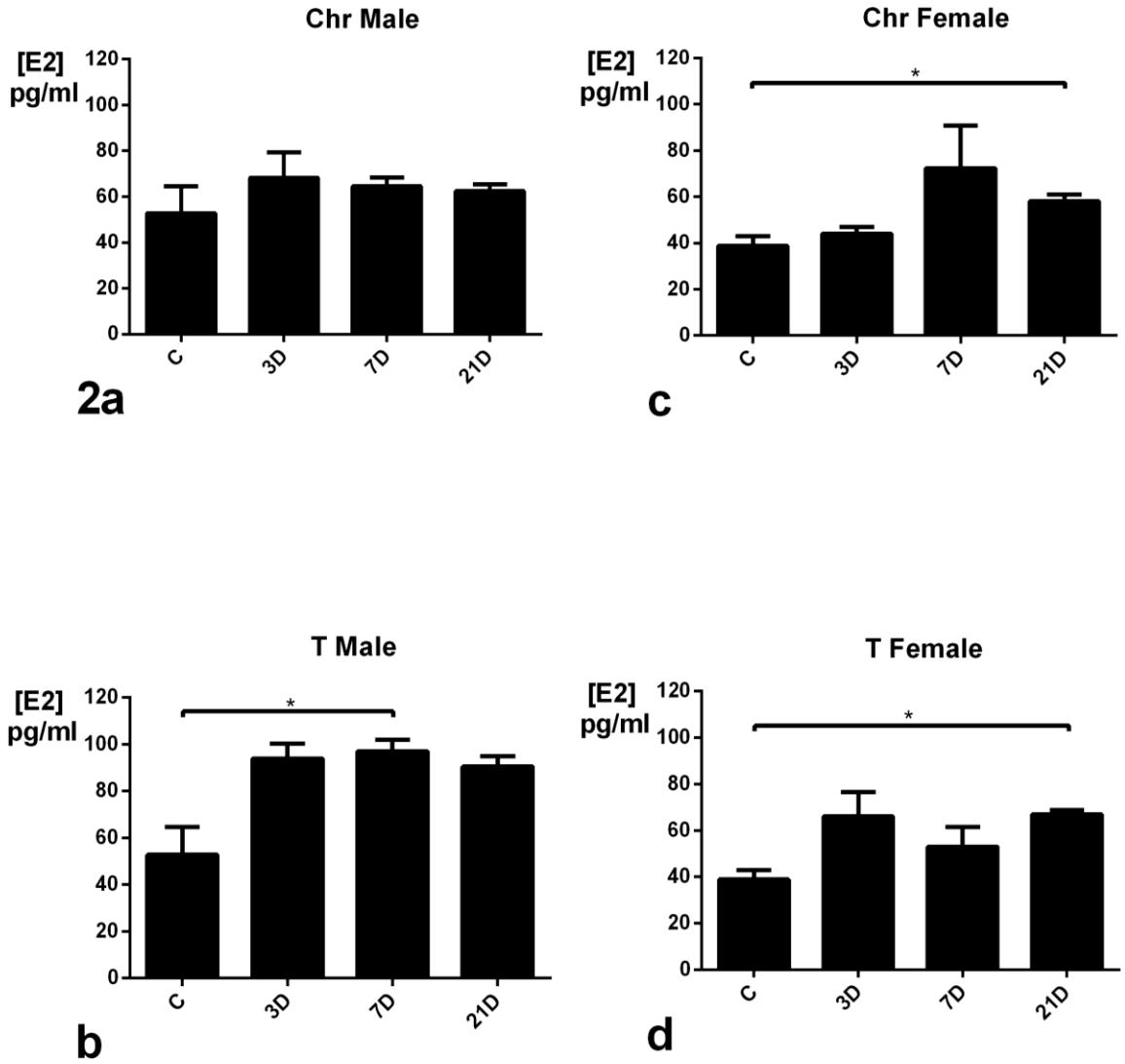

Chr Male
[T] ng/ml
6
4
2
0
C
3D
7D
21D
1a
Chr Female
c
T Male
b
T Female
d

Figure 2


Chr Male
[E2] pg/ml
120
100
80
60
40
20
0
C
3D
7D
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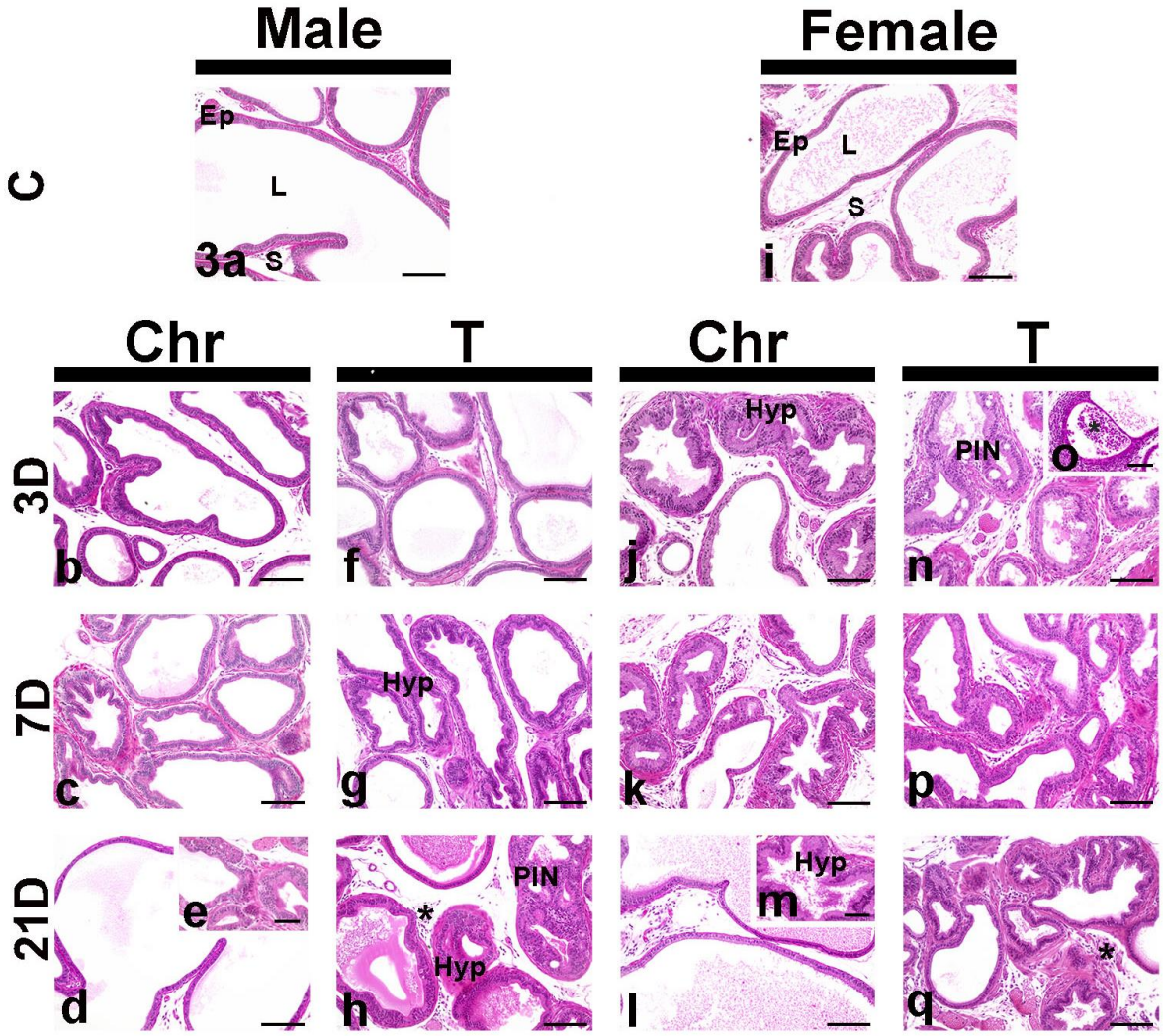
2a
Chr Female
*
c
T Male
b
T Female
d

Figure 3


Male
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C
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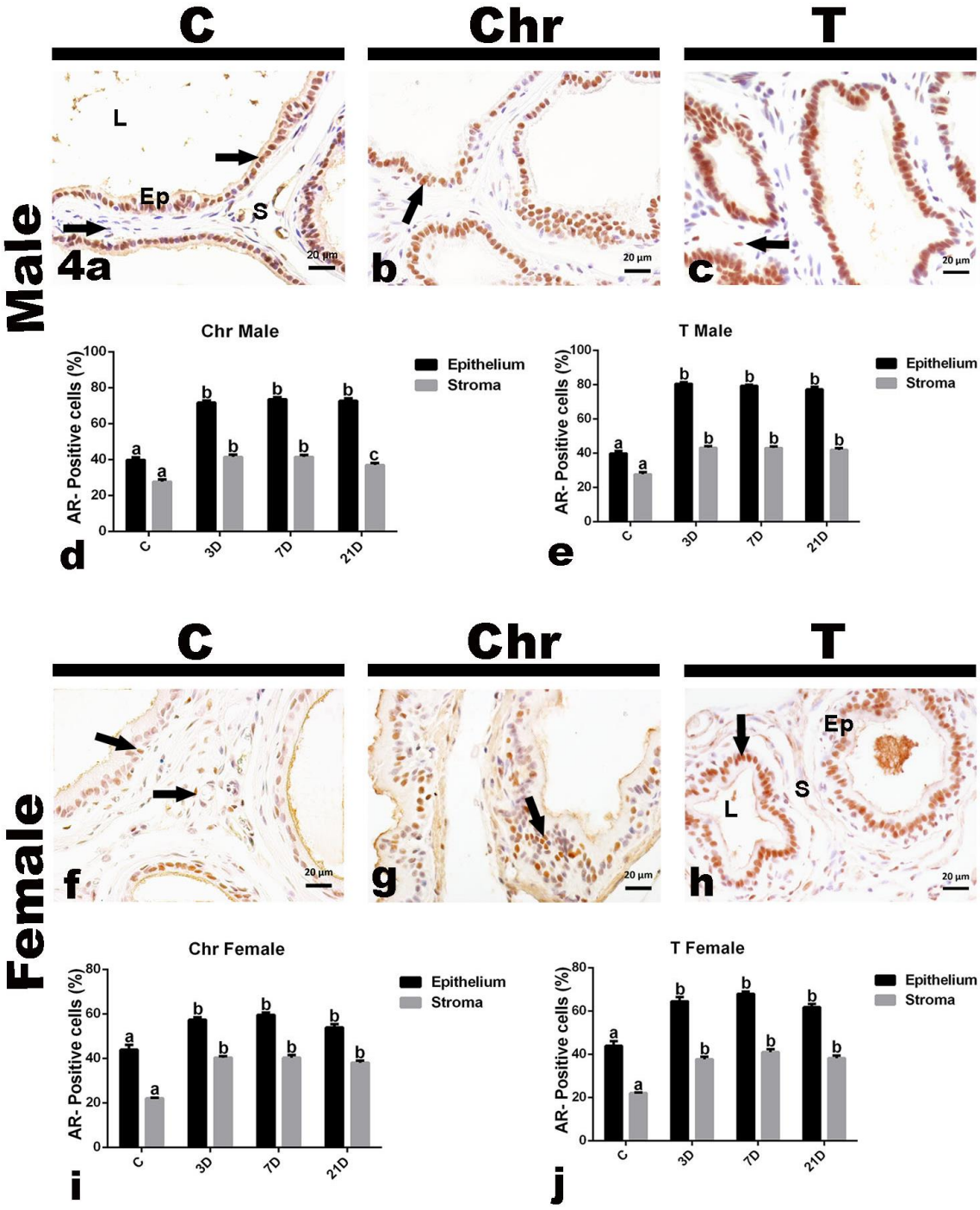
Figure 4


C
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Male
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20 µm
Chr Male
T Male
AR- Positive cells (%)
Epithelium
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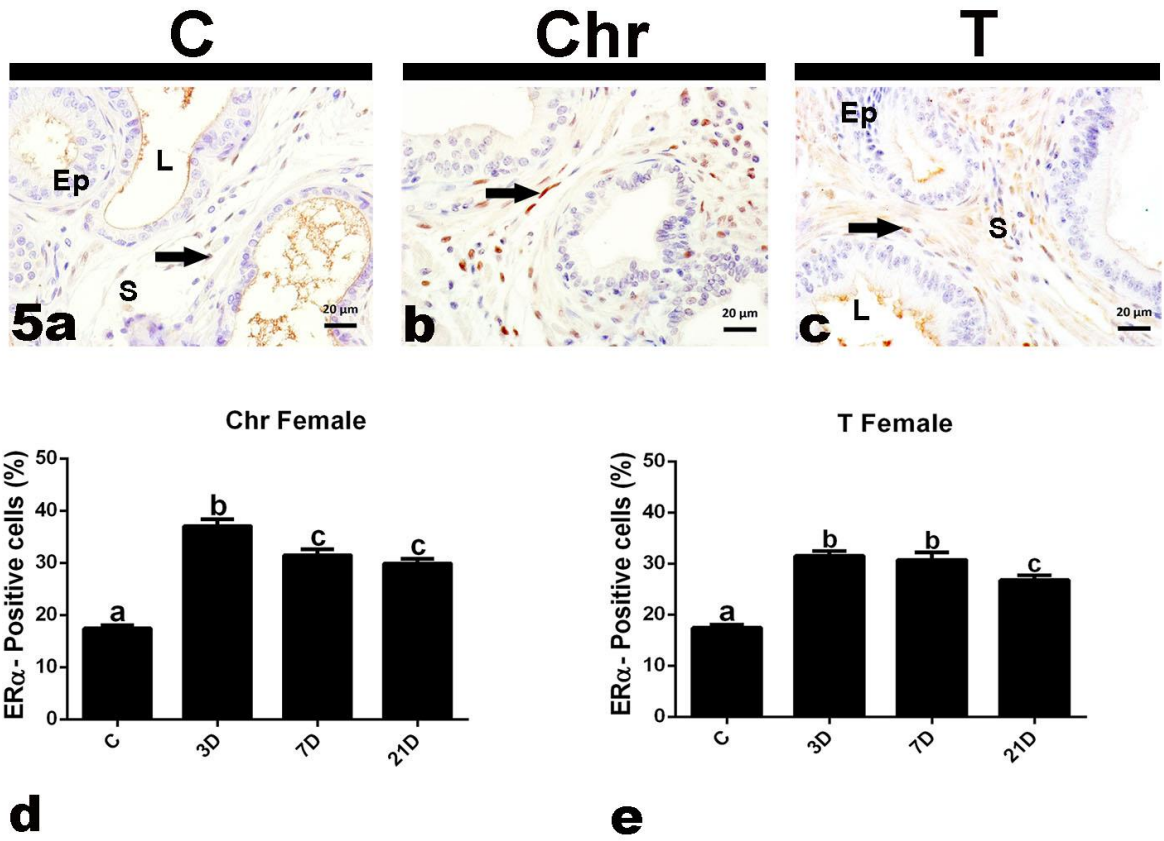
Figure 5


C
Chr
T
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L
S
5a
20 µm
b
c
Chr Female
T Female
ERα- Positive cells (%)
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10
20
30
40
50
a
b
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C
3D
7D
21D
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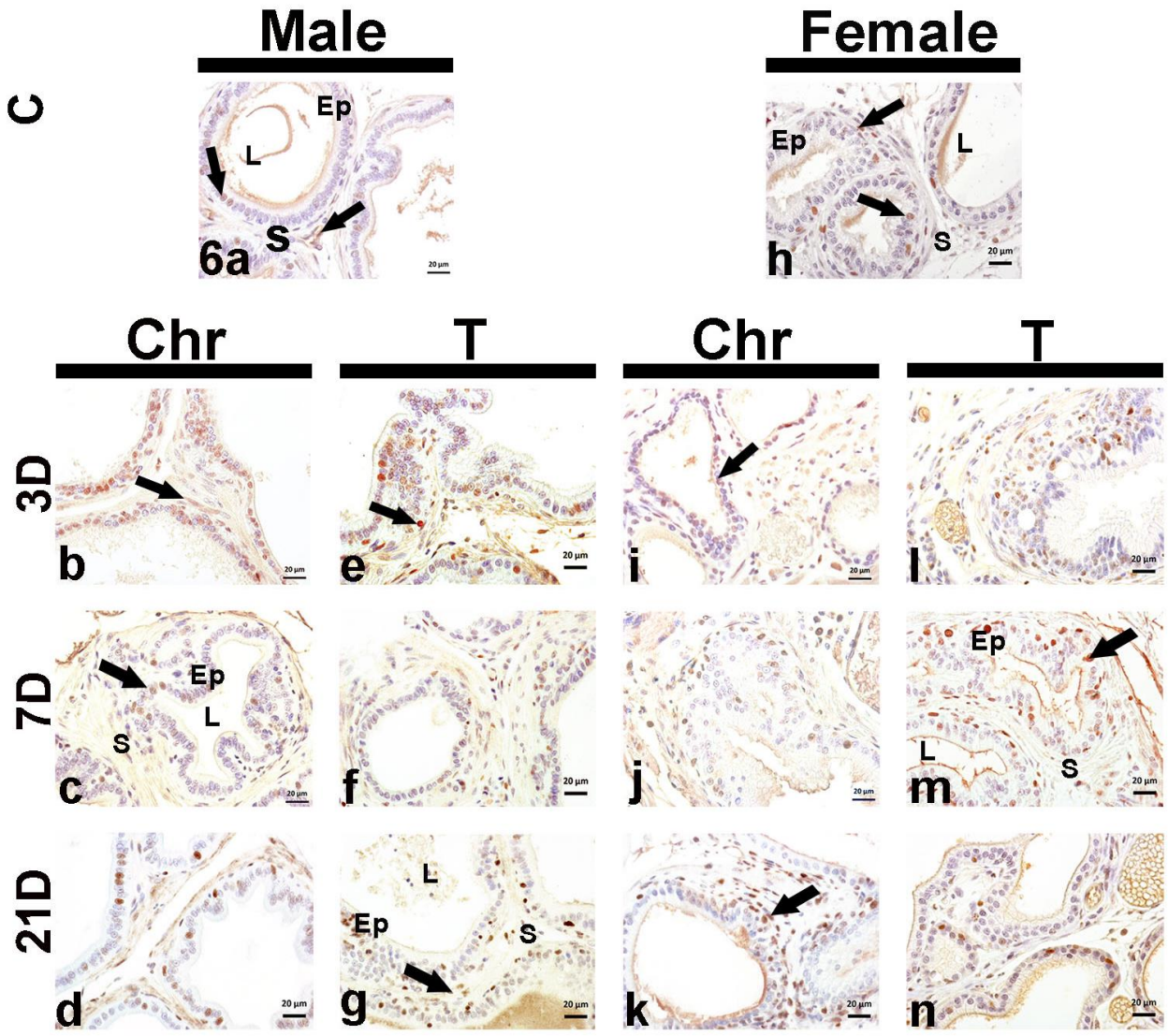
e

Figure 6



4.2 ARTIGO 2: ANABOLIC EFFECTS OF CHRYSIN ON THE VENTRAL MALE PROSTATE AND FEMALE PROSTATE OF ADULT GERBILS (*MERIONES UNGUICULATUS*)

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Abstract

Chrysin is a bioflavonoid found in fruits, flowers, tea, honey and wine, which has antioxidant, anti-inflammatory, anti-allergic and anticarcinogenic properties. This flavone has also been considered as beneficial for reproduction due its testosterone-boosting potential. Thus, the aim of this study was to evaluate the effects of chrysin on the prostate and gonads of male and female adult gerbils. In addition, a comparative analysis of the effects of testosterone on these same organs was conducted. Ninety-day-old male and female gerbils were treated with chrysin (50 mg/kg/day) or testosterone cypionate (1 mg/kg/week) for 21 days. The ventral male prostate and female prostate were dissected out for morphological, morphometric-stereological and ultrastructural assays. Testes and ovaries were submitted to morphological and morphometric-stereological analyses. Chrysin treatment caused epithelial hyperplasia and stromal remodeling of the ventral male and female prostate. Ultrastructurally, male and female prostatic epithelial cells in the chrysin group presented marked development of the organelles involved in the biosynthetic-secretory pathway, whereas cellular toxicity was observed only in female glands. Chrysin preserved the normal testicular morphology and increased the number of growing ovarian follicles. Comparatively, testosterone treatment was detrimental to the prostate and gonads, since foci of prostatic intraepithelial neoplasia and gonadal degeneration were observed in both sexes. Thus, under the experimental conditions of this study, chrysin was better tolerated than testosterone in the prostate and gonads.

Keywords: prostate gland, testosterone, fertility, morphology, ovary, testes

Introduction

The prostate is an accessory gland of the male reproductive system that secretes nutrients into the seminal fluid, promoting maintenance of the ionic gradient and pH of this secretion (Hayward et al., 1996; Untergasser et al., 2005). In addition, prostatic epithelial cells secrete antibacterial substances, specific growth factors, proteases and a variety of immunosuppressive agents, such as prostaglandins (Kelly et al., 1991; Gann et al., 1999).

The prostate is not an exclusive gland of the male reproductive system, being found in several female mammals, including humans and rodents (Zaviacic and Ablin, 2000; Biancardi et al., 2017). The growth, maturation and functionality of the prostate gland in both sexes are regulated by steroid hormones, especially by the balance between androgens and estrogens (Marker et al., 2003; Biancardi et al., 2017). Thus, bioactive compounds that have the ability to mimic endogenous steroids, such as flavonoids, can affect prostate physiology (Thelen et al., 2014; Boam, 2015; Ribeiro et al., 2018).

Flavonoids constitute a broad class of pigments (polyphenolic compounds) derived from secondary metabolism in plants and found in fruits, grains, barks, stems, flowers, tea and wine (Kumar and Pandey, 2013; Ly et al., 2015). Chrysin is a flavonoid of the flavone class with antioxidant, anti-inflammatory, anti-allergic, anticancer and anti-aging properties (Jana et al., 2008; Ali et al., 2014). One of the beneficial properties of chrysin is its ability to boost testosterone plasma levels (Jana et al., 2008; Ciftci et al., 2012). Studies have demonstrated that chrysin upregulates the synthesis of steroidogenic acute regulatory protein (StAR), a protein that facilitates cholesterol transfer in Leydig cells, resulting in increased testosterone biosynthesis (Jana et al., 2008). In addition, chrysin increases serum testosterone bioavailability by blocking

aromatase, an enzyme that converts testosterone into estradiol (Séraline and Moslemi, 2001; Ta and Walle, 2007).

Based on its hormonal properties, chrysin has been employed for the treatment of reproductive disorders, mainly to increase libido, and to improve fertility and steroid synthesis (Dhawan et al., 2002; Jana et al., 2008). Studies conducted in rodents have shown that chrysin has the potential to increase fertility rates in both males and females, particularly by improving morphophysiological aspects of gonads and ameliorating the gametogenesis process (Darwish et al., 2014; Ding et al., 2016). However, there is a lack of information about the action of this flavone on prostate morphology, especially *in vivo*. In this context, the objective of this study was to evaluate the effects of chrysin supplementation on the morphological aspects of the prostate and gonads of male and female adult gerbils. In addition, to evaluate the androgenic potency of chrysin, we compared the effects of testosterone on the prostate and gonads under the same experimental conditions.

Material and Methods

Chemical procedures

Chrysin synthesis was performed according to the protocol described in previous research published by Ribeiro and co-workers (2018). Briefly, chalcone intermediate was obtained using Claisen-Schimidt condensation between trihydroxyacetophenone and benzaldehyde (Passalacqua et al., 2015). The conversion from chalcone to flavone was obtained by intramolecular nucleophilic substitution (Zerack et al., 2012). After complete conversion confirmed by TLC analysis, the reaction mixture was extracted with ethyl acetate by liquid-liquid partition. The crude product was purified over silica gel, yielding chrysin (23%). Chrysin was obtained as a pale

yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6): 12.82 (*brs*, 1H), 8.06 (*d*,2H), 7.59 (*m*, 3H), 6.96 (*s*), 6.54 (*d*) and 6.23 (*d*). ^{13}C NMR (125 MHz, DMSO-*d6*): 182.3, 164.9, 163.7, 161.9, 157.9, 132.5, 131.3, 129.6, 126.8, 106.6, 105.0, 99.5 and 94.6.

Animals

All animals employed in this study were housed in new polyethylene cages and filtered water was provided from glass bottles. Males and females were housed separately (five animals per cage) in a temperature-controlled (23°C) room on a 12 h light/dark cycle. Gerbils were fed with rodent food *ad libitum* (Labina-Purina®; composition: 23% protein, 4% fat, 5% fiber and 12% minerals). Experiments were approved by the Ethical Committee of the Federal University of Goiás (license number 111/17 CEUA) and conducted following the Guide for Care and Use of Laboratory Animals.

Experimental design

Forty two adult gerbils (*Meriones unguiculatus*, Gerbillinae: Muridae), 21 males and 21 females, aged three months were employed in this study. The gerbils were assigned to one of three groups, with 5 animals per group - Control group (C): males and females received daily oral doses of the vehicle only (mineral oil/Nujol-Mantecorp; 100 µl/animal) for 21 days. Chrysin group (Chr): males and females received daily oral doses of chrysin (50 mg/kg) for 21 days. Testosterone group (T; positive control): males and females received subcutaneous injections of testosterone cypionate (1 mg/kg/week; Deposteron/EMS). After chrysin and testosterone exposure, all gerbils were euthanized with a lethal dosage of anesthesia (100 µl/100 g), which was prepared as a 1:1 (v/v) mixture of anesthetic (Cetamin, Syntec) and muscle relaxant (Xylazine, Vetbrands). Control females were euthanized in the proestrus phase. Ovaries, testes

and prostatic complex (urethral segment, ventral, dorsolateral, and dorsal prostate lobes in males, and urethral segment plus prostatic tissue in females) were dissected out.

Light microscopy

The prostatic complex (n = 5 glands/group), ovaries and testes were fixed by immersion in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) for four hours at 4°C. The organs were dehydrated through a crescent ethanol series, clarified in xylol, embedded in paraplast (Histosec, Merck, Darmstadt, Germany) and sectioned at 5 μm on a Leica microtome (Leica RM2155, Nussloch, Germany). The sections were stained by Gomori's trichrome or by hematoxylin-eosin (HE) (Behmer et al., 1976). The specimens were analyzed and digitized using a Zeiss Axioscope A1 light microscope (Zeiss, Germany).

Stereology and Morphometry

Stereological analyses of the prostate were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines (Weibel, 1963). The relative frequency of collagen and muscular stroma was determined. We chose 30 microscopic fields (at 400× magnification) at random from each experimental group (six fields per animal; n = 5 animals). We determined the relative frequency by counting the coincident points in the test grid and dividing them by the total number of points. Morphometric analysis of the prostate included the determination of epithelial cell height (μm) of the alveolar units (300 measurements; six fields per animal; n = 5 animals).

In the testicles, the diameter (μm) of the seminiferous tubules was determined (300 measurements; six fields per animal; n = 5 animals). Only cross-sections of the tubules were considered in this analysis. In the ovaries, the number of growing and atretic follicles per histological section was determined. The quantification of follicles was obtained after serial

sectioning of the whole ovary. Only longitudinal sections of the right and left ovaries, corresponding to the center of gonads, were used for counting (30 measurements; six fields per animal; n = 5 animals). It was considered as growing follicles the unilaminar primary follicles, multilaminar primary follicles, secondary or antral follicles, and mature or Graafian follicles. All quantitative analyses were performed using Image-Pro Plus software v6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical analyses

The tests employed to determine statistical significance were the Kruskal-Wallis test for non-parametric distributions and the ANOVA for parametric distributions. Further determination of the significant statistical differences between experimental groups was done using Dunn's test for non-parametric distributions and Tukey's test for parametric distributions. The data were analyzed using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA). The level of significance was set at 5% ($p \leq 0.05$). Values are presented as mean ± standard error of the mean (SEM).

Transmission electron microscopy

Prostate glands (n = 2 glands/group) were fixed with 2.5% glutaraldehyde plus 0.2% picric acid in 0.1 M sodium cacodylate buffer pH 7.2, for 24 hours at 4°C. The fragments were washed twice in 0.1 M sodium cacodylate buffer pH 7.2 and post-fixed in 1% osmium tetroxide prepared in 0.1 M sodium cacodylate buffer pH 7.2, for 2 h at 4°C. Following post-fixation, the fragments were washed twice with distilled water for 5 min each, and subjected to dehydration with crescent acetone series. The dehydrated materials were embedded in Epon 812 resin. The materials were cut into ultrathin sections (50-70 nm), and contrasted with 2% uranyl acetate and 0.2% lead citrate. Copper grids were examined in a JEM-2100 transmission electron microscopy

(Jeol, Akishima, Japan) equipped with EDS (Thermo Scientific, Waltham, EUA) in the Laboratório Multiusuário de Microscopia de Alta Resolução da Universidade Federal de Goiás (LabMic/UFG).

Results

Morphological and morphometric aspects of the prostate

Ventral prostates of Chr-males showed intense hyperplastic growth, whereas T-male prostates presented glandular hyperplasia and foci of intraepithelial neoplasia with a pseudocribiform pattern (Fig. 1b-c). This glandular growth was confirmed by the morphometric analysis, which showed a significant increase in epithelial height in the Chr and T groups (C = 14.5 ± 0.2 µm, Chr = 23.4 ± 0.4 µm, T = 23.0 ± 0.3 µm) (Fig. 2). Chr- and T-males showed intense stromal remodeling, with an increase in the relative frequency of collagen fibers located in the perialveolar stroma (Fig. 1b-c; Fig. 3a).

Prostates from Chr-treated females showed intense glandular development (Fig. 1d-e), with increased epithelial height (Fig. 2), stromal remodeling and increased relative frequency of collagen (Fig. 1e; 3b). T-treated females showed glandular alterations similar to those found in the Chr group, but with a higher degree of epithelial disorder (Fig. 1f; Fig. 2; Fig. 3b).

Ultrastructural analysis

Epithelial cells in prostates from male and female control animals showed similar ultrastructural characteristics (Fig. 4). They showed voluminous nuclei with decondensed chromatin, a basal cytoplasm rich in rough endoplasmic reticulum (RER) and mitochondria, and a supranuclear cytoplasm containing mitochondria, the Golgi complex (CG) and several secretory

vesicles (Fig. 4a, c). The perialveolar stroma contained mainly fibroblasts, smooth muscle cells, blood vessels and collagen fiber bundles. Smooth muscle cells formed concentric layers around the prostatic alveoli. These cells presented a fusiform shape and elongated nuclei (Fig. 4b, d).

In males and females, Chr treatment caused an increase in prostate secretory activity, characterized by the enlargement of all organelles involved in the biosynthetic-secretory pathway (Figs. 5 and 6). Prostatic epithelial cells became taller, with a highly developed RER in the basal cytoplasm, and with a voluminous apical cytoplasm filled with elongated mitochondria, secretory vesicles and Golgi complex cisterns. Epithelial cell nuclei became prominent, with evident nucleoli and thin chromatin (Fig. 5a-b; 6a-b). In both sexes, smooth muscle cells exhibited a spine-like shape and contractile appearance, with many caveolae closely associated with the plasma membrane. Thick bundles of collagen fibers were observed at the base of the epithelium and between smooth muscle cells (Fig. 5c-d; Fig 6e). Although Chr treatment caused similar anabolic responses in male and female prostates, only the females showed signs of toxicity, with degenerative-like epithelial cells observed in the prostate of all examined females (Fig. 6c-d). Degenerative cells presented large and electron-lucid vesicles, absent rough endoplasmic reticulum and Golgi cisterns, collapsed mitochondria and pyknotic nuclei (Fig. 6c-d).

The prostates of T-treated males and females also showed epithelial and stromal enlargement (Fig. 7). However, in these groups, there were numerous areas of epithelial stratification and nuclear polymorphism (Fig. 7a, c). In the stroma, smooth muscle cells had a voluminous cytoplasm rich in mitochondria with few membrane projections (Fig. 7b, d). Moreover, in females, signs of cellular toxicity were observed in the form of epithelial ceramide accumulation (Fig. 7c-d). The ceramide bodies were formed by concentric aggregates of membranes located between secretory cells (Fig. 7e).

Morphological alterations of testis and ovary

Morphological analysis of the testes and ovaries was performed in order to evaluate whether treatment with Chr or T could cause morphological changes in the gonads (Fig. 8). Chr-treated males showed morphologically normal seminiferous tubules with germ cells at all stages of differentiation (Fig. 8a-b). Chr-gerbils showed no variation in the diameter of the seminiferous tubules (Fig. 8d). Conversely, in the T-group, spermatogenic cells at different stages of differentiation, as well as several degenerating cells, were found in the lumen of the seminiferous tubules (Fig. 8c). In addition, only T-treated animals showed a significant increase in the diameter of the seminiferous tubules ($p \leq 0.05$; Fig. 8d).

Chr-females presented ovaries with a greater number of follicles in all stages of growth, such as unilaminar primary follicles, multilaminar primary follicles, secondary follicles and mature follicles (Fig. 8g-h). However, T-females had abnormal follicles, with increased granulosa cells and absence of an antral cavity (Fig. 8i, j). Many atretic follicles containing degenerating cells were observed throughout the ovarian cortex in T-group females. Ovarian stromal cells became prominent and vesiculated (Fig. 8i-j). Quantitative analysis demonstrated that Chr-females showed a significant increase in the number of growing follicles, whereas T-females showed a decrease in the number of growing follicles and an increase in the number of atretic follicles ($p \leq 0.05$; Fig. 8k).

Discussion

This study demonstrated that chrysin and testosterone had anabolic effects on the ventral male and female prostate of adult gerbils. Both treatments caused hyperplasia, stromal remodeling and stimulated the biosynthetic-secretory pathway in epithelial cells. However, in

contrast to testosterone, chrysin supplementation did not cause prostatic intraepithelial neoplasia in males and females, despite its toxic effect in the female prostate.

The effects of chrysin on the prostate gland *in vivo* are still poorly understood. The most studies have evaluated the effects of chrysin on different prostate cell lines, demonstrating its proapoptotic, antiproliferative and antioxidant properties *in vitro* (Khoo et al., 2010; Samarghandian et al., 2011; Ryu et al., 2017). A recent study showed that chrysin has antiproliferative effects in rats with testosterone-induced benign prostate hyperplasia (Shoieb et al., 2018). Moreover, a previous research developed by Ribeiro et al. (2018) demonstrated that prepubertal exposure to chrysin induces upregulation of androgen receptors in prostates of male and female gerbils, increasing the cell proliferation during adulthood. However, to date, no research has evaluated the effects of chrysin on the functional activity of prostate cells in adult animal models.

In the prostatic epithelium, Chr and T treatment caused epithelial hyperplasia and increase of cytoplasmic organelles related to the synthesis and secretion of prostatic glycoproteins, such as rough endoplasmic reticulum, Golgi complex, mitochondria and secretory vesicles. However, gerbils treated with chrysin did not show foci of prostatic intraepithelial neoplasia, which were more evident in animals treated with testosterone, as demonstrated in this study and in previous studies (Santos et al., 2006; Oliveira et al., 2007; Biancardi et al., 2012).

In the prostatic stroma, the Chr- and T-groups presented smooth muscle cells with spinous/contractile appearance, and an increased frequency of collagen fibers. The prostatic stroma is very sensitive to hormonal fluctuations, and its cellular and fibrillar components can undergo remodeling under androgen deprivation conditions (Vilamaior et al., 2000; Góes et al., 2007), as well as in hormonal stimulation assays (Corradi et al., 2004; Santos et al., 2007). In this study, the increased contractile activity of smooth muscle cells may have contributed to the

remodeling of bundles of collagen fibers, which became thicker and condensed close to the secretory alveoli.

In general, chrysin seems to have a more positive anabolic effect on the prostate than synthetic androgens, at least in male gerbils. In females, ultrastructural analysis demonstrated that both treatments had toxic effects, evidenced by changes in the prostate epithelial compartment. In Chr-treated females, we observed epithelial cells with degenerative-like features. T-treated females showed epithelial cells with ceramide multilayered bodies, which have previously been described as precursors of cell death by necrosis/apoptosis in different human prostate cancer cell lines (Wang et al., 2003). Although we observed heterogeneity regarding the morphological features of cellular toxicity in Chr- and T-females, the degenerative aspects observed in both groups suggest that the female prostate is more sensitive than the ventral male prostate to the action of compounds with androgenic potential. Similar results have been found in previous studies on gerbils, in which females exposed to androgens during prenatal development had a higher susceptibility to developing prostate disorders in comparison to males (Biancardi et al., 2012). Indeed, in the present study, we observed that chrysin preserved the gonadal morphology, which was demonstrated by the absence of signs of toxicity in the testes, and by the higher number of growing follicles in the ovaries. Conversely, T treatment caused the degeneration of germ cells in males, and increased follicular atresia in females. These effects of chrysin on gerbil gonads corroborates the results of previous studies that demonstrated the beneficial effects of chrysin and other flavonoids on gametogenesis in both rodents and birds (Ciftci et al., 2012; Ding et al., 2016; Jahan et al., 2016; Zhandi et al., 2017).

Chrysin is considered to be a beneficial phytotherapy with the potential to improve the health of individuals, as a consequence of its antioxidant, anti-allergic, anticarcinogenic, anti-inflammatory and anxiolytic properties (Jaganathan and Mandal, 2009; Khoo et al., 2010). Little

is known about the specific mechanisms of action of chrysin in the reproductive organs, although some of these benefits are thought to be a result of the antioxidant effects of flavonoids (Darwish et al., 2014). Flavonoids regulate antioxidant effects by suppressing the formation of reactive oxygen species (ROS), by regulating the antioxidant defenses in several organs and by promoting the production of ROS scavengers in tissues (Kumar and Pandey, 2013). Therefore, we conclude that supplementation with chrysin seems to be safer than treatment with synthetic androgens to improve reproductive parameters in male adult gerbils. This improvement in adult male gerbils may be related to either increased testosterone bioavailability or to the antioxidant activity of chrysin. However, chrysin had different effects in females, since the female prostate showed signs of cellular toxicity at a dose of 50 mg/kg/day. Additional studies will be necessary in order to generate more information regarding the mechanism of action of chrysin in the reproductive organs of male and female animal models.

Conclusion

Chrysin promoted an anabolic effect on the prostate of male and female gerbils, resulting in hyperplastic growth, stromal remodeling and stimulation of the biosynthetic-secretory pathway in prostate epithelial cells. Additionally, in females, chrysin caused cell toxicity in the secretory epithelium. In the gonads, chrysin preserved the normal morphology of the testis and stimulated follicular maturation in the ovary. Comparatively, testosterone treatment injured the prostate and gonads, since foci of prostatic intraepithelial neoplasia and gonadal degeneration were observed in both sexes. Thus, under the experimental conditions of this study, chrysin was better tolerated than testosterone in the prostate and gonads. However, the employment of chrysin as a phytotherapy agent should be considered with caution, since this flavonoid seems to present differential effects in males and females.

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Figure legends

Figure 1. Histological sections of the ventral male prostate and female prostate. Gomori's trichrome method. C: control; Chr: chrysin; T: testosterone cypionate. **(a)** Prostatic alveoli lined by cylindrical epithelium (Ep), and surrounded by scarce stroma (S) composed mainly by smooth muscle cells (SMC) and collagen fibers (arrow). Lumen (L). **(b)** Epithelial hyperplasia (Hyp) and stromal remodeling (S) induced by Chr treatment. Collagen fibers (arrow). **(c)** T treatment causes epithelial hyperplastic growth (Hyp), prostatic intraepithelial neoplasia (PIN) and stromal remodeling (S). Collagen fibers (arrow). **(d)** The female prostatic alveoli are lined by simple cuboidal epithelium (Ep). Smooth muscle cells (SMC) and collagen fibers (arrow) can be observed in the perialveolar stroma. Blood vessel (BV). **(e)** Chr-females presented epithelial hyperplasia (Hyp) and stromal remodeling (S). **(f)** In females, T treatment caused a greater degree of glandular hyperplasia than Chr treatment.

Figure 2. Prostatic epithelial height (μm) in males and females from all experimental groups (n = 300 measurements in 5 animals/group). Values are mean ± standard error of the mean. Superscript letters$^{(a,b)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 3. Relative frequency of collagen and smooth muscle cells (%) in all experimental groups (n = 30 fields in 5 animals/group). Values are mean ± standard error of the mean. Superscript letters$^{(a,b,c)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 4. (a) Ventral prostate epithelium in male adult gerbils. Basal cytoplasm of columnar epithelial cells showed abundant rough endoplasmic reticulum (RER). Secretory vesicles (V) and

Golgi complex (GC) were observed in the apical cytoplasm. Nucleus (N), nucleolus (arrow), blood vessel (BL), Lumen (L). **(b)** In control males, prostatic alveoli were surrounded by fusiform smooth muscle cells (SMC) and by thin bundles of collagen fibers (CO). Observe that smooth muscle cells presented a regular surface, devoid of projections (arrows), and showed many caveolae (arrowhead). Mitochondria (M). **(c)** Prostatic epithelium of the control female prostate. Secretory epithelial cells exhibit secretory vesicles (V), rough endoplasmic reticulum (RER) and Golgi complex (GC). Basal lamina (BL), lumen (L). **(d)** Stromal compartment in the control female prostate. Fusiform smooth muscle cells (SMC) were associated with thin collagen fiber bundles (arrow). Epithelium, (Ep), nuclei (N), blood vessel (BV).

Figure 5. Ventral prostate ultrastructure in chrysin-treated male gerbils. **(a)** Basal cytoplasm of the secretory epithelial cell showed a prominent rough endoplasmic reticulum (RER) and mitochondria (M). The large nuclei (N) showed decondensed chromatin and a prominent nucleolus (arrow). **(b)** Apical cytoplasm of a secretory epithelial cell exhibiting the Golgi complex (GC), elongated mitochondria (M), lysosomes (LY) and secretory vesicles (V). Lumen (L). **(c-d)** Spinous-like cytoplasmic projections (arrows) in smooth muscle cells (SMC). Numerous caveolae (arrowhead) were observed near the plasma membrane. Collagen fibers (CO) were observed between smooth muscle cells.

Figure 6. Prostate ultrastructure in chrysin-treated female gerbils. **(a)** Basal cytoplasm of the secretory epithelial cell showed a highly developed rough endoplasmic reticulum (RER) and mitochondria (M). Collagen fibers (CO) were observed at the base of the epithelium. Basal lamina (BL), nucleus (N). **(b)** Apical cytoplasm of a secretory cell exhibiting the Golgi complex (GC), mitochondria (M) and secretory vesicles (V). **(c-d)** Secretory epithelial cells showing a degenerative-like aspect: elongated and pyknotic nuclei (arrowheads), multivesiculated

cytoplasm (V) and several organelles with a degenerative appearance. Many degenerative mitochondria were observed close to the nucleus (arrow). Lumen (L), nuclei (N). **(e)** Smooth muscle cells with a spinous-like appearance (SMC). Caveolae (arrowhead) were observed near the plasma membrane of smooth muscle cells. Many thick collagen fiber bundles (CO) were observed between smooth muscle cells. Blood vessel (BV).

Figure 7. (a-b) Ventral prostate of T-treated male gerbils. **a:** Foci of epithelial stratification with polymorphic nuclei (N). Secretory cells exhibited many mitochondria (M) and secretory vesicles (V). **b:** Voluminous smooth muscle cells exhibiting mitochondria (M) and caveolae (arrowhead). Collagen (arrow). (**c-e)** Prostate gland of testosterone-treated female gerbils. **c-d:** Tall columnar cells showed abundant Golgi complex (CG), mitochondria (M) and large secretory vesicles (V). Ceramide concentric aggregation was observed between epithelial cells (arrows; insert). Scale bar figure (d): 600 nm. e: Perialveolar smooth muscle cells (SMC) were associated with thick collagen fiber bundles (arrow). Epithelium (Ep), basal lamina (BL), nuclei (N).

Figure 8. Histological/morphometrical features of the testis **(a-d)** and ovary **(e-k)** in adult gerbils in all experimental groups. HE staining. **(a)** Seminiferous tubules (ST) in control adult gerbils. Testiscular interstitium (i), lumen (L). (b) Chr-males showed seminiferous tubules (ST) with preserved germinative epithelium. Many spermatids were observed near to the lumen (arrow). **(c)** Germ cells and degenerative-like cells (arrows) inside the seminiferous tubules (ST). **(d)** Diameter of seminiferous tubules (μm) in all experimental groups. Values are mean ± standard error of the mean. Superscript letters$^{(a,b)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$). **(e)** Ovary morphology in control gerbils. Ovarian cortex showing antral follicle (a), and multilaminar primary follicle with granulosa cells (g) and oocyte (o). Ovarian stroma (S). **(f)** Detailed view of multilaminar primary follicle. Ovarian stroma (S),

multiple layers of granulosa cells (g). **(g)** Chr-treated gerbils showed many growing follicles. Antrum (a), granulosa cells (g), oocyte (arrow). **(h)** Normal aspects of multilaminar primary follicle and ovarian stroma (S) in Chr-females. **(i-j)** Testosterone-treated females showed increased granulosa layer (g), atretic follicles (arrowhead) and stromal cell vacuolization (arrow). **(k)** Quantification of growing and atretic follicles per longitudinal section, in all experimental groups. Values are mean ± standard error of the mean. Superscript letters$^{(a,b,c)}$ represent statistically significant differences between the experimental groups ($p \leq 0.05$).

Figure 1

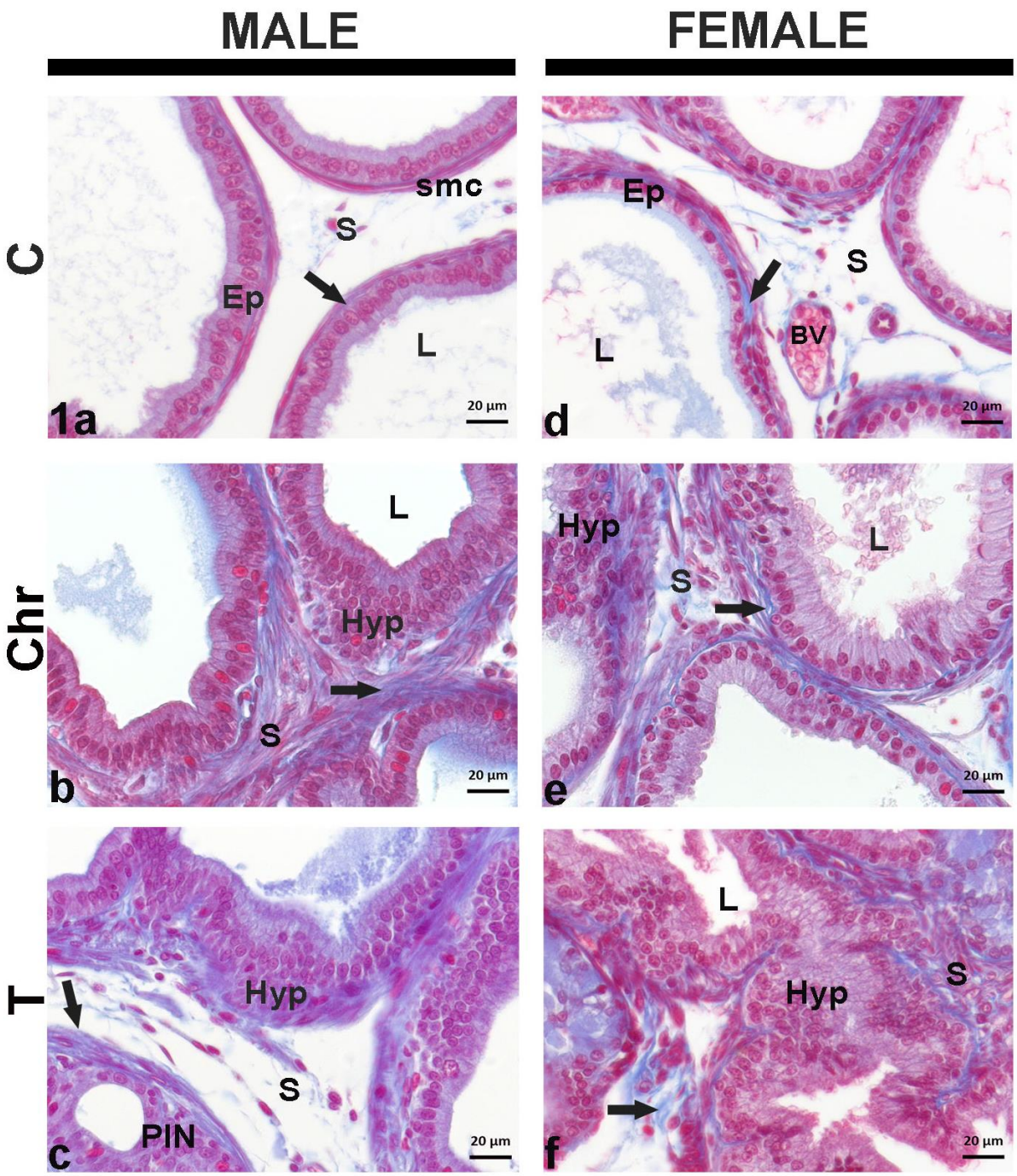


Figure 2

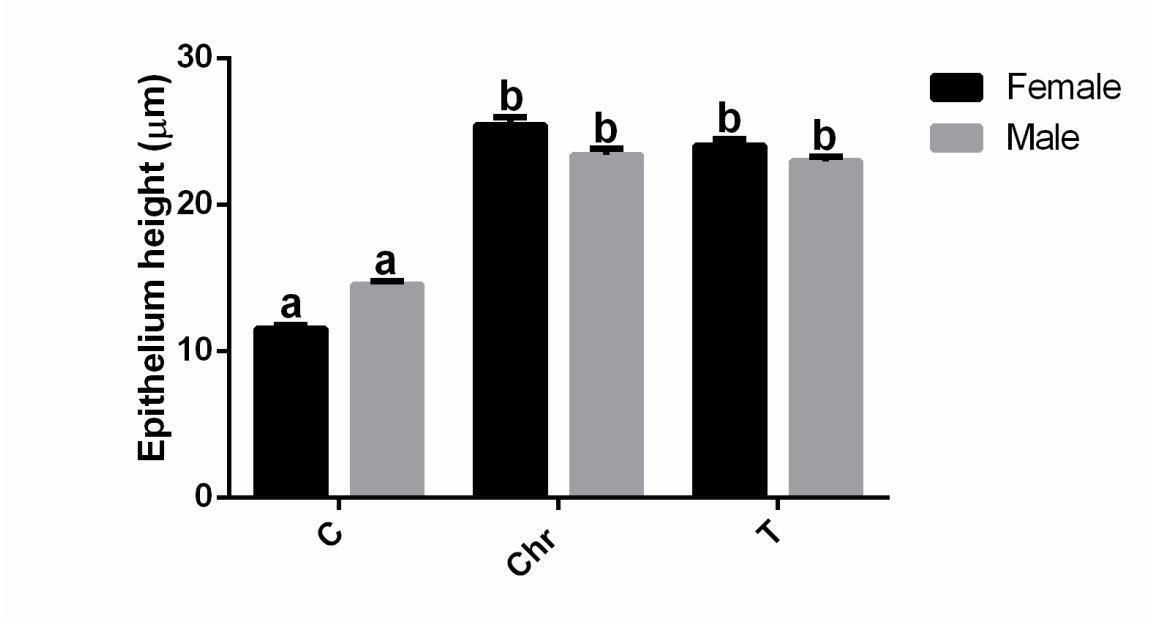
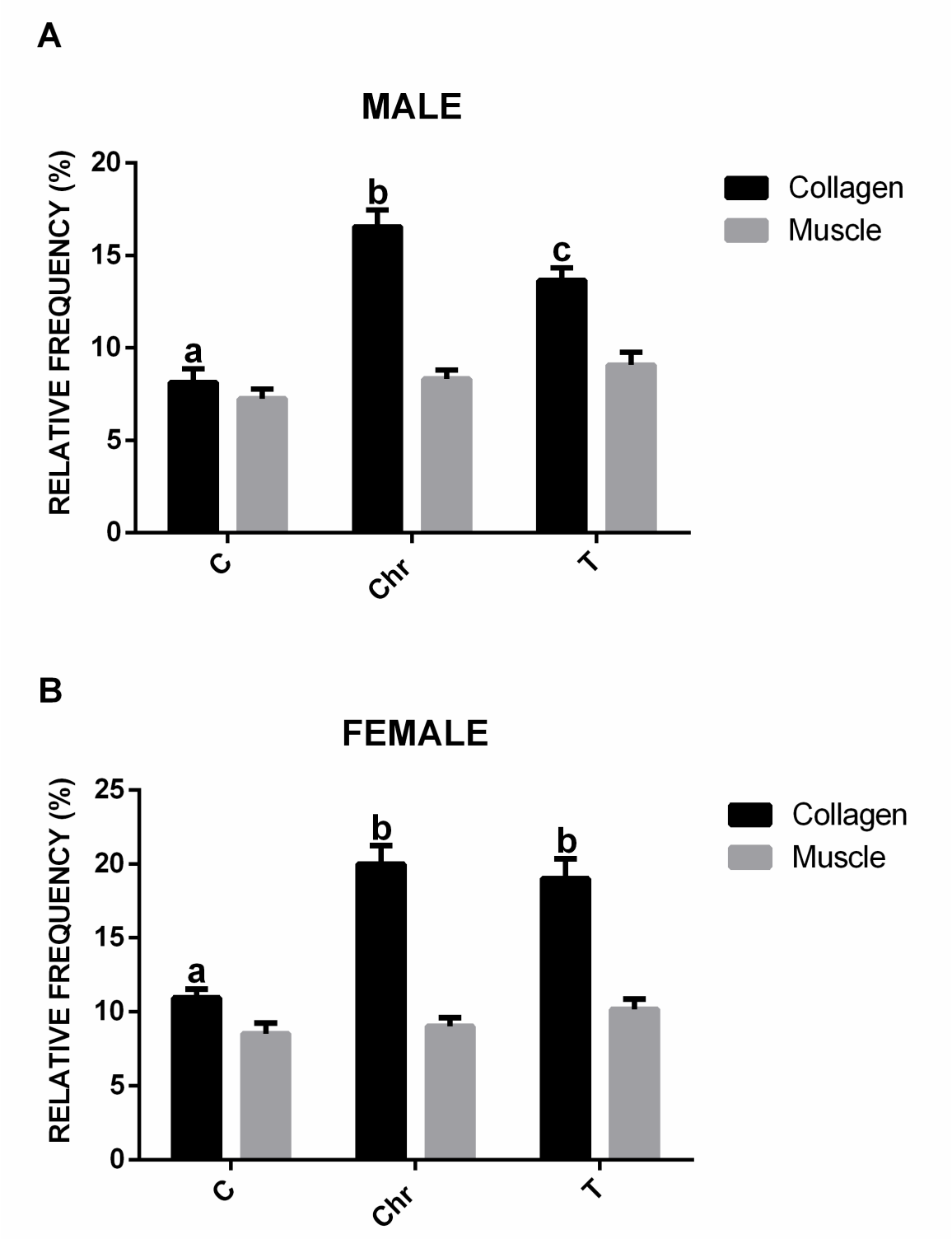


Figure 3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RELATIVE FREQUENCY (%)
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Collagen
Muscle

Figure 4

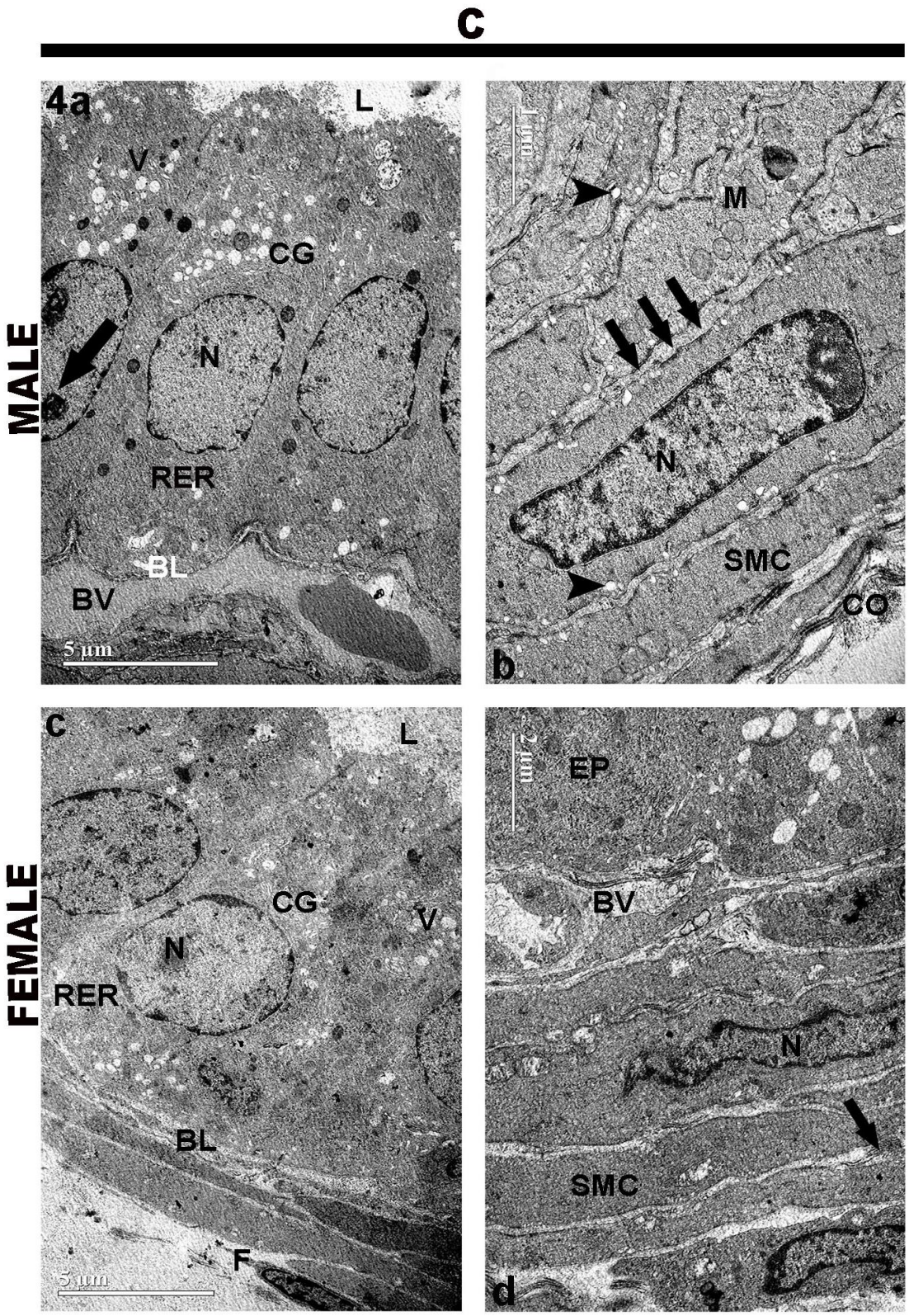
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Figure 5

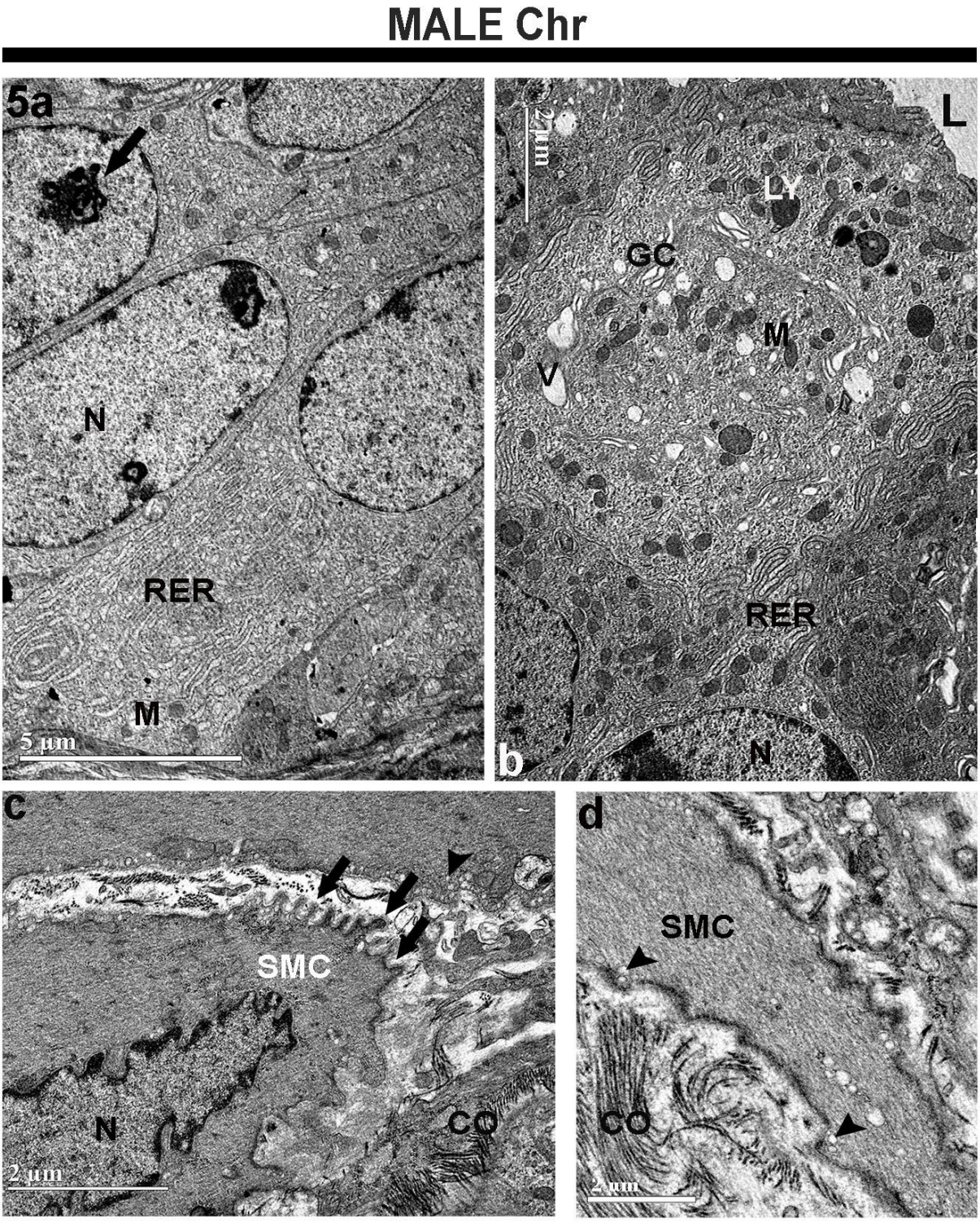
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Figure 6

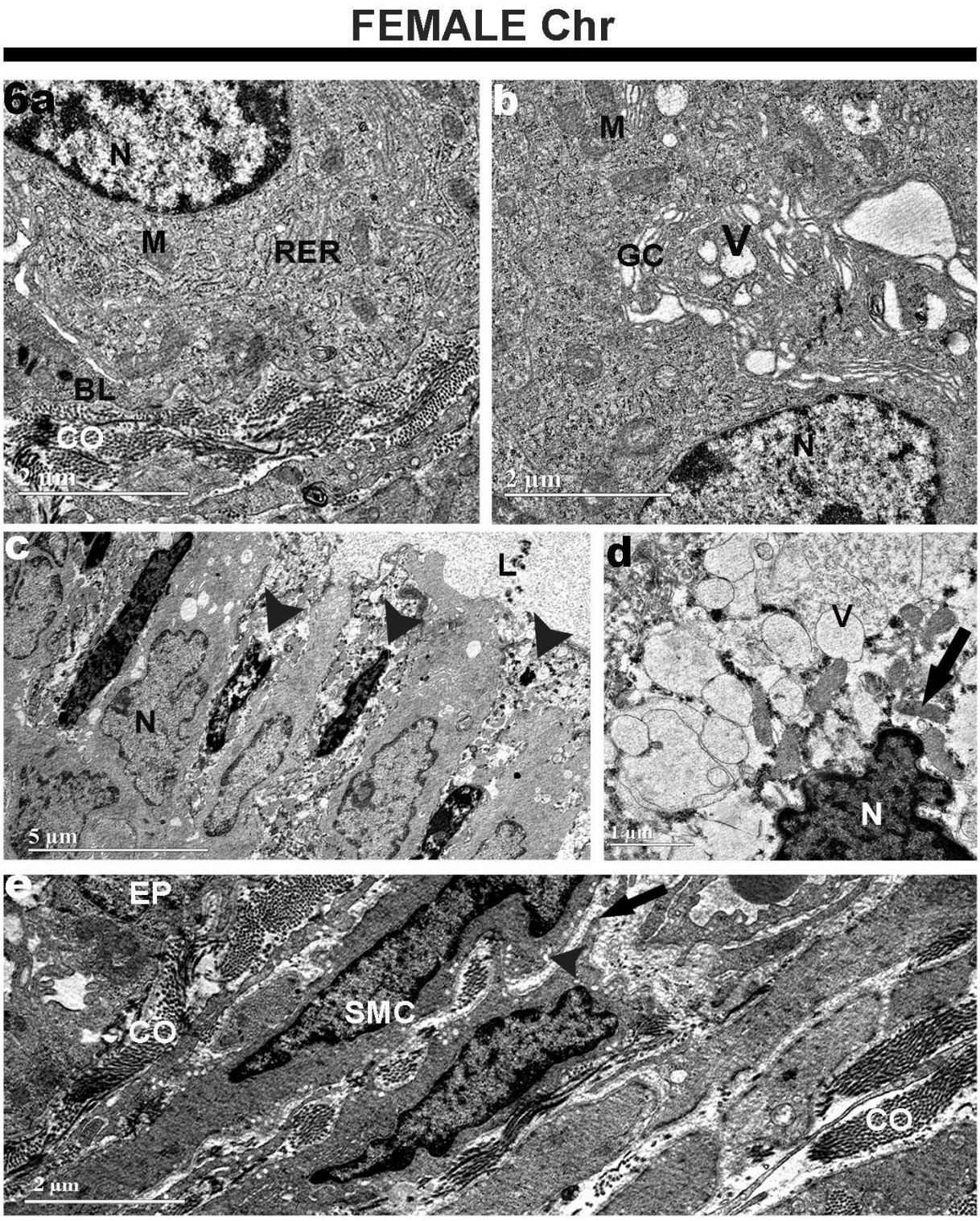
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Figure 7

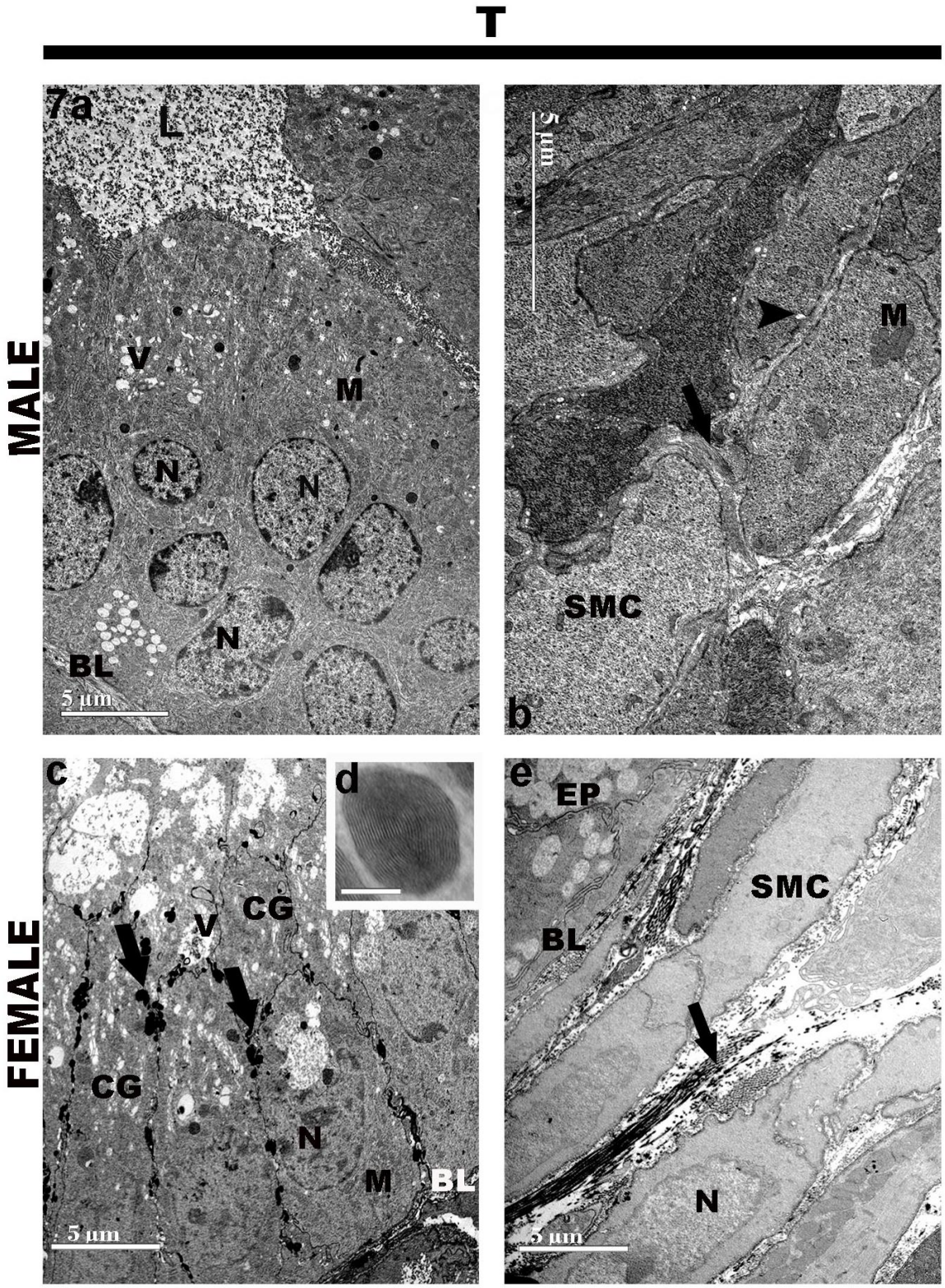
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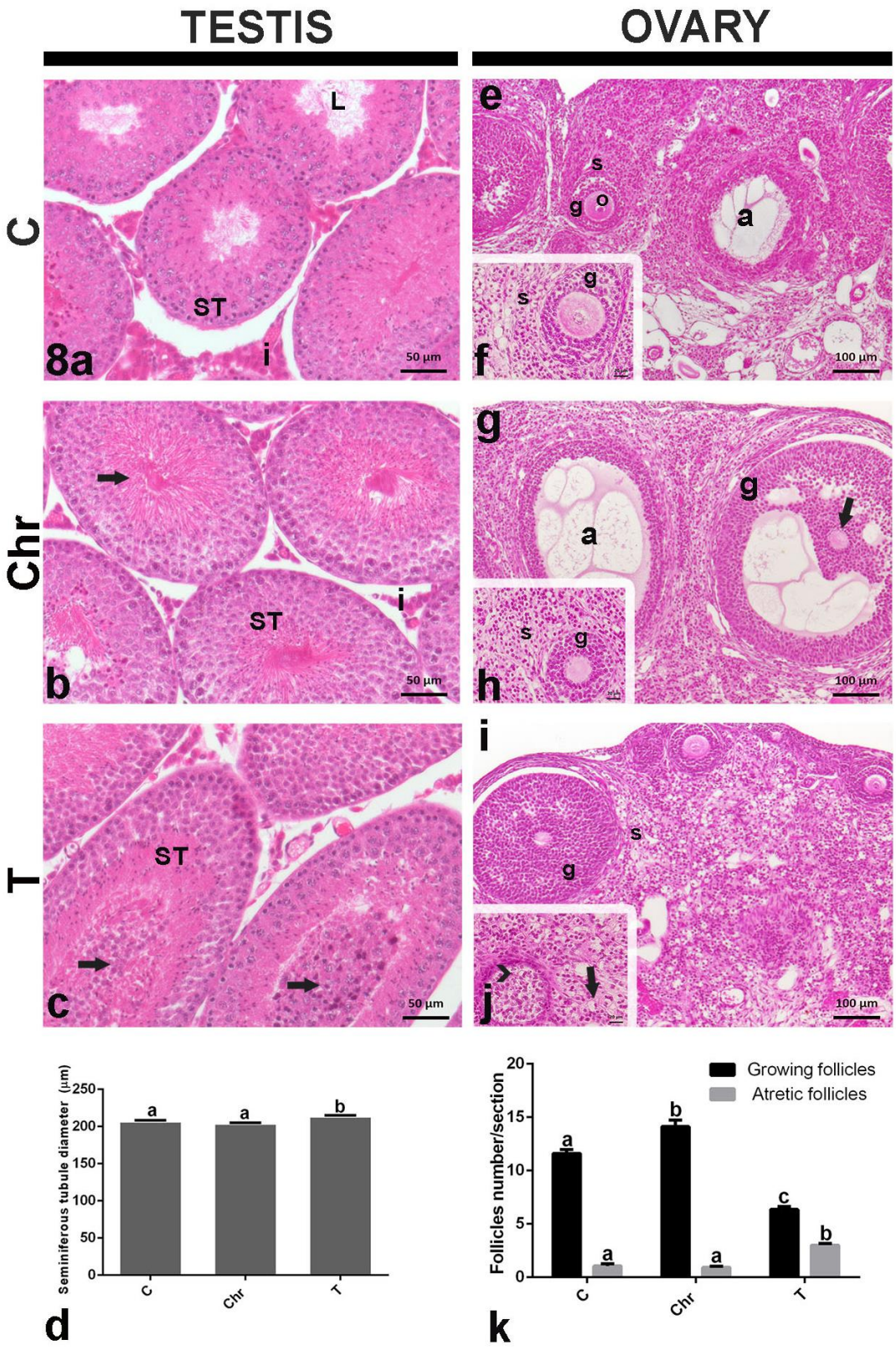
Figure 8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Seminiferous tubule diameter (µm)
250
200
150
100
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0
a
a
b
C
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k
Follicles number/section
20
15
10
5
0
Growing follicles
Atretic follicles
a
b
c
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5 CONCLUSÕES GERAIS

1. Machos e fêmeas de gerbilos tratados com crisina não apresentaram aumento dos níveis séricos de testosterona. No entanto, apenas as fêmeas tratadas com crisina durante 21D demonstraram aumento nos níveis séricos de estradiol.
2. Tanto na próstata ventral masculina, quanto na próstata feminina em todos os tempos de tratamento com crisina verificou-se o aumento da frequência de imunomarcação dos receptores androgênicos, aumento da taxa de proliferação celular resultante do intenso desenvolvimento epitelial e crescimento hiperplásico da glândula.
3. O tratamento com crisina favoreceu aumento das imunomarcações para ERα na próstata feminina e elevou os níveis séricos de estradiol em fêmeas de 21D, o que indica que a crisina exerceu efeitos estrogênicos diferenciais nas fêmeas.
4. Ultraestruturalmente, machos e fêmeas tratados com crisina apresentaram intenso desenvolvimento das organelas da via biossintética-secretora, acompanhada de intensa remodelação estromal, enquanto a presença de toxicidade celular foi observada apenas nas fêmeas.
5. Nas gônadas a crisina preservou a morfologia normal dos testículos e estimulou a maturação folicular nos ovários.
6. Comparativamente, o tratamento com cipionato de testosterona foi capaz de causar lesões na próstata e gônadas, visto que focos de neoplasia intraepitelial prostática e degeneração gonadal foram observados em ambos os sexos.
7. Assim, nas condições experimentais desse estudo, embora a crisina e a testosterona tenham promovido alterações morfológicas semelhantes na próstata de gerbilos masculina e feminina, a suplementação com crisina foi mais favorável à saúde da próstata, pois resultou em menor incidência de hiperplasia e ausência de focos neoplásicos.
8. Em conjunto, nossos resultados sugerem que a administração de crisina como agente fitoterápico deve ser considerada com precaução, visto que esse flavonóide parece exercer efeitos diferenciais em machos e fêmeas.

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