UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

JULIANA DOS SANTOS MALDARINE

CARCINOGÊNESE PROSTÁTICA INDUZIDA POR ENU, TESTOSTERONA E ESTRADIOL EM FÊMEAS DO GERBILO DA MONGÓLIA (*MERIONES UNGUICULATUS*)

PROSTATIC CARCINOGENESIS INDUCED BY ENU, TESTOSTERONE AND ESTRADIOL IN FEMALE MONGOLIAN GERBILS (*MERIONES UNGUICULATUS*)

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2023

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Molecular e Morfofuncional, na área de Biologia Celular.

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Molecular and Morphofunctional Biology in the area of Cell Biology.

Orientador: Prof. Dr. Sebastião Roberto Taboga

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA JULIANA DOS SANTOS MALDARINE E ORIENTADA PELO PROF. DR. SEBASTIÃO ROBERTO TABOGA

CAMPINAS

2023

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

Maldarine, Juliana dos Santos, 1992-
M292c Carcinogênese prostática induzida por ENU, testosterona e estradiol em fêmeas do gerbilo da Mongólia (*Meriones unguiculatus*) / Juliana dos Santos Maldarine. – Campinas, SP : [s.n.], 2023.

Orientador: Sebastião Roberto Taboga.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Neoplasias da próstata. 2. Próstata feminina. 3. Adenocarcinoma. 4. Gerbilo da Mongólia. I. Taboga, Sebastião Roberto. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações Complementares

Título em outro idioma: Prostatic carcinogenesis induced by ENU, testosterone and estradiol in female Mongolian gerbils (*Meriones unguiculatus*)
Palavras-chave em inglês:
Prostatic neoplasm
Female prostate
Adenocarcinoma
Mongolian gerbil
Área de concentração: Biologia Celular
Titulação: Doutora em Biologia Molecular e Morfofuncional
Banca examinadora:
Sebastião Roberto Taboga [Orientador]
Sabrina Thalita dos Reis Faria
Ana Cláudia Polli Lopes
Taize Machado Augusto
Luciano de Figueiredo Borges
Data de defesa: 28-09-2023
Programa de Pós-Graduação: Biologia Molecular e Morfofuncional

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-0604-1703
- Currículo Lattes do autor: http://lattes.cnpq.br/8103244199029134

Campinas, 28 de setembro de 2023.

COMISSÃO EXAMINADORA

Prof. Dr. Sebastião Roberto Taboga

Profa. Dra. Sabrina Thalita dos Reis Faria

Profa. Dra. Ana Cláudia Polli Lopes

Profa. Dra. Taize Machado Augusto

Prof. Dr. Luciano de Figueiredo Borges

Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Biologia Molecular e Morfofuncional do Instituto de Biologia.

AGRADECIMENTOS

Agradeço primeiramente ao meu orientador, Prof. Dr. Sebastião Roberto Taboga, por todos os anos de orientação, desde o Estágio Básico até chegar à etapa final do Doutorado, sempre muito prestativo e paciente comigo. Foram períodos muito ricos de aprendizado, não somente no âmbito profissional, como no pessoal também. Com toda certeza, é uma inspiração para mim!

Ao meu coorientador, Dr. Bruno Domingos Azevedo Sanches, que esteve comigo em todos os momentos da vida acadêmica, me incentivando e auxiliando a vencer todos os obstáculos que percorreram este caminho, e no qual eu pude estabelecer uma grande amizade. Tenho imensa estima e admiração por você.

Aos colegas de laboratório, em especial minha coorientada Vitória Alário, com a disposição de sempre e conseguindo tornar tudo mais leve, mas também Carol Bedolo, Gustavo Amaro, Tati Scarpelli, Alana Della Torre, Vitor Grigio, Luiz Guerra, Guilherme Tamarindo, Simone Colleta, Thallez Ruiz, Stella Bicalho, a todos os nossos ICs, e claro, ao nosso técnico Luiz Roberto Falleiros, que desviava um pouco a seriedade do ambiente, proporcionando muitos momentos de diversão no LMM. Cada um deles me ajudou de alguma forma, e sou muito grata pelo convívio.

Um agradecimento particular às professoras Dr. Patrícia Vilamaior e Dr. Rejane Goés, pela disponibilidade de utilizar seus laboratórios e diversos itens sem os quais não seria possível avançar em vários experimentos, além da gigantesca troca de experiências enquanto aluna de vocês. As admiro muito!

À Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (nº 2019/14201-4), pela concessão da bolsa de Doutorado e Reserva Técnica no período de

01/03/2020 a 31/07/2023, viabilizando a execução deste projeto. Ainda, ao Conselho Nacional de Desenvolvimento Científico Tecnológico – CNPQ (nº 42490/2019-9) pela concessão inicial da bolsa de Doutorado no período de 01/08/2019 a 01/03/2020. O presente trabalho também foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Às melhores amigas que alguém poderia ter, Natália Troiano, Beatriz Lopes e Andréia Moraes, que nos momentos difíceis sempre me deram força, assim como estiveram comigo para comemorar todas as vitórias. Gostaria de agradecer também aos meus amigos de longa data, Maxwell Ponte, Ana Mattos, Carolina Valério e Guilherme Silva, por todo o apoio que me deram.

Por fim, os maiores agradecimentos são para minha mãe, Eunice de Almeida Santos, e irmãs, Priscila dos Santos Maldarine e Vanessa Maldarine Barbosa; vocês são meu alicerce e este trabalho eu dedico a vocês. Ao meu pai, Paulo, minha sobrinha, Júlia, minhas tias, Juciene, Eliana, Márcia, Célia, meu tio Waner e minha avó Alvarina, e todos os membros da minha família que me motivaram durante essa jornada.

Resumo

A próstata não é uma glândula exclusiva dos machos, estando presente em fêmeas de diversas espécies, inclusive em uma parcela das mulheres. O gerbilo da Mongólia (*Meriones unguiculatus*) é uma espécie de roedor na qual a próstata é encontrada na maioria das fêmeas e há evidências, tanto nessa espécie quanto na humana, de que ela pode sofrer das mesmas doenças que acometem a próstata dos machos, como prostatites e adenocarcinomas. Tendo em vista que a maioria dos modelos experimentais de indução de carcinogênese são descritos para a próstata dos machos, torna-se relevante a criação de um método que contemple também a glândula das fêmeas, bem como um possível alvo terapêutico, no contexto de carcinogênese induzida. Além disso, a complexidade da glândula aumenta quando levamos em consideração que ela também possui telócitos em seu estroma, células intersticiais que possuem papel na organização da próstata. Porém, ainda faltam estudos acerca destas células em tumores prostáticos. Desse modo, o presente trabalho teve por objetivo desenvolver um método de indução de câncer de próstata nas fêmeas por meio da exposição ao N-ethyl-N-nitrosurea (ENU), associado a testosterona (T) e ao estradiol (E2). Ainda, objetivou-se avaliar as alterações geradas pela formação do microambiente tumoral, bem como investigar os telócitos neste contexto, além de um tratamento subsequente com o Tamoxifeno (TAM), uma droga antiestrogênica. Nossos dados evidenciaram que o tratamento com ENU+T+E2 levou ao desenvolvimento de focos de adenocarcinoma em mais da metade das fêmeas, bem como a descontinuidade da musculatura lisa perialveolar, além de aumento no número de macrófagos e vasos sanguíneos. Os telócitos estão ausentes em regiões adjacentes as lesões epiteliais, como hiperplasia e PINs, demonstrando uma possível relação destes com a manutenção da arquitetura prostática, assim como podem ter um papel favorável à inflamação e angiogênese, uma vez que foram encontrados próximos a células imunes e vasos. Já o tratamento com TAM evidenciou uma diminuição na proliferação celular e na inflamação, bem como levou ao aumento de apoptose, demonstrando ser um potencial alvo terapêutico. Em conclusão, o presente trabalho gerou novos conhecimentos sobre a histopatologia da próstata de fêmeas, ampliou as informações sobre os telócitos em contextos normais e patológicos, além de trazer novas possibilidades de terapia e manipulação experimental com o modelo de carcinogênese induzida.

Abstract

The prostate is not a gland exclusively of males, being also present in females of several species, including in a portion of the women. The Mongolian gerbil (*Meriones unguiculatus*) is a rodent species in which the prostate is found in most females and there are indications, both in this species and in humans, that it can suffer from the same diseases that affect the prostate of males, such as prostatitis and adenocarcinomas. Considering that most experimental models of carcinogenesis induction are described for the male prostate, it becomes relevant to create a method that also includes the female gland in this context. In addition, the complexity of the gland increases when we consider that it also has telocytes in its stroma, which are interstitial cells that are present in several organs and are known to play a role in the prostate organization. However, there is still a lack of studies about these cells in prostatic tumors. In view of this, the present work aimed to develop a method of inducing prostate cancer in females through exposure to N-ethyl-N-nitrosurea (ENU), associated with testosterone (T) and estradiol (E2). Still, we aimed to evaluate the changes generated by the formation of the tumor microenvironment, in addition to investigating the telocytes in this context, besides a subsequent treatment with Tamoxifen (TAM), an antiestrogenic drug. Our data showed that the treatment with ENU+T+E2 led to the development of adenocarcinoma foci in more than half of the treated females, as well as the discontinuity of the perialveolar smooth muscle, in addition to an increase in the number of macrophages and blood vessels. Telocytes are absent in regions adjacent to epithelial lesions, such as hyperplasia and PINs, demonstrating a possible relationship between these cells and the maintenance of prostatic architecture, as well as they may have a role in inflammation and angiogenesis, since they were found close to immune cells and blood vessels. TAM treatment evidenced decreases in cell proliferation and inflammation, besides leading to an increase in apoptosis index, demonstrating to be a potential therapeutic target. In conclusion, the present work generated new knowledge about the histopathology of the prostate of females, expanded the information about the telocytes in normal and pathological conditions, as well as brought new possibilities of therapy and experimental manipulation with the model of induced carcinogenesis.

LISTA DE ABREVIAÇÕES

α-SMA (Alpha-Smooth Muscle Actin)

Ad (Adenocarcinoma)

AR (Androgen Receptor)

BM (Basement Membrane)

BPA (Bisphenol-A)

BSA (Bovine Serum Albumin)

BV (Blood Vessel)

CAFs (Cancer-Associated Fibroblasts)

CD163 (Cluster of Differentiation 163)

CD34 (Cluster of Differentiation 34)

CD31 (Cluster of Differentiation 31)

CD68 (Cluster of Differentiation 68)

CRPC (Castration Resistant Prostate Cancer)

DAB (3'-diaminobenzidine)

DAPI (4',6-diamidino-2-phenylindole)

ENU (N-ethyl-N-nitrosourea)

Ep (Epithelium)

ERα (Estrogen Receptor α)

E (Eosinophil)

E2 (17β-Estradiol)

FAP (Fibroblast Activation Protein)

FITC (Fluorescein Isothiocyanate)

GPER (G Protein-coupled Estrogen Receptor)

H (Hyperplasia)

H&E (Hematoxylin-Eosin)

ICC (Intersticial Cajal Cells)

L (Leukocytes)

Lc (Lymphocyte)

M (Macrophage)

Mi (Mitochondria)

MNU (N-methyl-N-nitrosourea)

N (Nucleus)

Nu (Nucleolus)

PA (Prostatic Alveoli)

PBS (Phosphate-buffered Saline)

PCNA (Proliferating Cell Nuclear Antigen)

PH.H3 (Phospho-Histone H3)

PIN (Prostatic Intraepithelial Neoplasia)

PFA (Paraformaldehyde)

SERM (Selective Estrogen Receptor Modulator)

Smc (Smooth Muscle Cell)

St (Stroma)

T (Testosterone cypionate)

TAM (Tamoxifen)

Tc (Telocyte)

TGFβ1 (Transforming Growth Factor β1)

TNFR1 (Tumor Necrosis Factor Receptor 1)

Tp (Telopode)

SUMÁRIO

I. INTRODUÇÃO

I.1. A próstata das fêmeas

Diferentemente do que se pensava, a próstata não é uma glândula presente apenas em machos, sendo encontrada em fêmeas de diversas espécies (Biancardi *et al.,* 2010; Aguiar *et al.*, 2013; Santos *et al.*, 2022), inclusive em mulheres, na qual também recebe a denominação de glândula parauretral de Skene. Apesar de inicialmente ter sido considerada apenas um órgão vestigial (Skene, 1880), muitos estudos conduzidos principalmente pelo professor patologista Milan Zaviacic e colaboradores (1999, 2000) já demonstraram que a próstata feminina é funcional e possui uma secreção alcalina similar à dos machos, tendo também um papel acessório na reprodução, de modo a diminuir a acidez no trato reprodutor feminino e consequentemente auxiliar na sobrevivência dos espermatozoides, além de lubrificar o canal vaginal, facilitando a passagem dos mesmos (Zaviacic & Ablin, 2005). Dietrich e outros pesquisadores (2011) relataram que a próstata está presente em aproximadamente metade das mulheres, formando um aglomerado de estruturas glandulares ao redor da porção mediana da uretra. Ainda, há evidências de que a mesma pode desenvolver quadros patológicos que são frequentes na próstata de homens, como prostatites e adenocarcinomas (Gittes, 2002; Pongtippan *et al*., 2004; Massari *et al.*, 2014; Muto *et al.,* 2017; Tregnago & Epstein, 2018; Gao *et al.*, 2022).

Contudo, a maioria dos estudos sobre a próstata de mulheres são realizados *post mortem*, o que dificulta ainda mais a obtenção desse material (Costa *et al.,* 2016). Assim, modelos animais tem sido uma alternativa viável, especialmente roedores. Nosso grupo de pesquisa tem utilizado o gerbilo da Mongólia (*Meriones unguiculatus*) por vários anos (**Figura 1**), sendo uma espécie de roedor que possui diversas vantagens, tais como micção infrequente, por ser de origem semiárida (Vincent *et al.*, 1980; Leonel *et al.*, 2021), facilidade de ser manuseado, pois não é tão grande como os ratos nem tão pequeno como os camundongos, e principalmente por aproximadamente 90% das fêmeas possuir uma próstata funcional, diferentemente de fêmeas de outras espécies, além da semelhança histológica com a próstata das mulheres (Santos & Taboga, 2006; Biancardi *et al.*, 2017). Tal fato possibilita análises comparativas entre a glândula de machos e de fêmeas, que são homólogas e similares histologicamente (Rochel *et al.*, 2007; Sanches *et al.*, 2016), e, como trabalhos anteriores já demonstraram, são animais que apresentam, com o envelhecimento, elevada probabilidade de

desenvolver espontaneamente algum tipo de lesão pré-maligna e maligna (Santos & Taboga, 2006; Campos *et al.*, 2008, Custodio *et al.*, 2008, 2010; Biancardi *et al.*, 2017; Leonel *et al.*, 2021).

Figura 1. Gerbilo da Mongólia (*Meriones unguiculatus*), modelo experimental utilizado em nosso laboratório. Sob licença Creative Commons.

I.2. Regulação hormonal prostática

Diversos estudos já demonstraram que o estímulo para induzir o desenvolvimento inicial dos brotamentos e ramificações epiteliais se dá por ação dos andrógenos, tendo a testosterona um papel central neste processo, atuando por meio da interação com os receptores de andrógenos (AR) (Thomson & Marker, 2006; Prins & Putz, 2008). Entretanto, sabe-se que os estrógenos também têm papel na regulação da próstata, auxiliando tanto no crescimento quanto na diferenciação da mesma por meio de receptores específicos de estrógenos (ERs) (Santos & Taboga, 2006; McPherson *et al.*, 2008). Trabalhos anteriores evidenciaram que a expressão aumentada do receptor de estrógeno α (ERα) está correlacionada com a proliferação de células do estroma prostático (Ellem & Risbridger, 2009; Takizawa *et al.*, 2015), que consequentemente agiriam sobre o epitélio por meio da liberação de fatores de crescimento e citocinas, induzindo a proliferação de células epiteliais. Ainda, foi visto que o ERα está

associado com o desenvolvimento e progressão do câncer de próstata (Bonkhoff, 2017), de tal forma que os estrógenos, preferencialmente, poderiam favorecer o crescimento desses tumores.

Nas fêmeas adultas do gerbilo, a concentração de testosterona circulante é relativamente maior do que em fêmeas de outras espécies, como ratos; contudo, este número ainda é inferior com relação aos machos da mesma espécie (Albert *et al.*, 1990; Santos *el al.*, 2006). Fochi e outros pesquisadores (2013) mostraram que os níveis séricos de testosterona em machos são cerca de 5 vezes mais elevados do que nas fêmeas. Porém, as concentrações séricas de estradiol em fêmeas adultas é o dobro do que o observado em machos (Zanatelli *et al*., 2014). Dessa forma, pode-se especular que apesar da testosterona ser essencial para o início do desenvolvimento prostático, nas fêmeas a glândula se desenvolve em um meio mais estrogênico e menos androgênico do que nos machos, sendo assim, os estrógenos poderiam ter um papel fundamental para o surgimento de próstata nas mesmas (Custodio *et al.*, 2008).

Silva e colaboradores (2013) demonstraram que a restrição hormonal causada pela retirada de ovários (ovariectomia) consequentemente levou a uma diminuição dos níveis de estradiol (E2) e afetou a histoarquitetura prostática, causando regressão da mesma e diminuição da atividade secretora dos alvéolos. Tal observação mostra que esse hormônio é importante para a manutenção da homeostase da glândula das fêmeas, da mesma maneira que a testosterona o é para machos, pois alterações semelhantes foram observadas em experimentos de privação androgênica, com a retirada dos testículos em gerbilos e ratos (Oliveira *et al.*, 2007; Felix-Patricio *et al.*¸2017). Ainda, análises comparativas entre machos e fêmeas de gerbilo expostos ao E2 durante o período intrauterino e analisados logo após o nascimento evidenciaram aumento no número de brotamentos e proliferação epitelial na próstata das fêmeas, ao passo que o tratamento levou a um aspecto hipomórfico na glândula dos machos (Sanches *et al.* 2017a). Quando ambos os sexos foram expostos durante o desenvolvimento intrauterino e analisados na vida adulta, dosagens baixas de E2 já foram suficientes para gerar alterações na estrutura das ramificações prostáticas nas fêmeas, que diminuem em número, porém apenas dosagens mais altas foram capazes de levar a alterações semelhantes em machos (Sanches *et al.* 2017b). Nosso grupo de pesquisa também conduziu vários trabalhos relativos à exposição de hormônios exógenos e desreguladores endócrinos, como o bisphenol-A (BPA), que mimetiza a ação do estradiol e que também levou a uma redução da proliferação na próstata de fêmeas expostas a dosagens baixas, enquanto somente dosagens mais altas acarretaram mudanças significativas em machos (Rodríguez *et al.*, 2016). Em conjunto, esses estudos

evidenciam que tanto o desenvolvimento quanto a fisiologia da próstata das fêmeas apresentam maior sensibilidade a variações nos níveis de E2 do que a dos machos, o que também pode ocorrer com a exposição a diferentes compostos (Sanches *et al.*, 2019).

I.3. Indução de carcinogênese em fêmeas

Apesar de a próstata das fêmeas ser semelhante à dos machos em termos de susceptibilidade à quadros patológicos, como câncer, e mesmo sendo importante se investigar a ocorrência de câncer na glândula feminina em estudos de manipulação hormonal e possíveis tratamentos, ainda não se constava, na literatura, um modelo de indução de carcinogênese para a próstata das fêmeas. Este modelo seria importante tendo em vista a dificuldade de se obter material humano, ainda mais de próstata de mulheres, e das limitações acerca da realização de estudos de manipulação experimental para esta glândula. O método de indução tumoral utilizado via exposição a N-methyl-N-nitrosurea (MNU) e testosterona se mostrou útil para machos de gerbilo da Mongólia, rato e camundongo (Gonçalves *et al.*, 2010; Sharmila *et al.*, 2014; Galheigo *et al.*, 2016), levando os primeiros a desenvolverem adenocarcinoma em mais da metade dos animais em todos os lobos da próstata (Gonçalves *et al*., 2010, 2013); entretanto, tal metodologia não obteve sucesso na indução de tumores malignos na próstata de fêmeas do gerbilo (dados não publicados). Desse modo, pensando em se desenvolver um método de indução de carcinogênese na próstata que também contemple as fêmeas, um agente carcinogênico ainda mais potente, N-ethyl-N-nistrosurea (ENU), foi escolhido para substituir o MNU. O ENU promove mutações aleatórias ao transferir o grupo etil para os pares de bases, geralmente transições AT-GC, como é o caso do MNU, mas também transversões AT-TA (Cordes, 2005). Outros estudos mostraram que o ENU é o composto mutagênico mais potente já descoberto em camundongos, principalmente em células-tronco espermatogoniais, sendo que uma dosagem de 250 mg/kg foi capaz de gerar uma taxa de mutação 5 vezes maior do que a gerada pela dose mais efetiva de raios-X (Russell *et al*., 1979; Probst & Justice, 2010). Doses elevadas de ENU já desenvolveram neoplasias em vários órgãos, tais como próstata, pâncreas, rins, tireoide, intestino e pulmões. Uma única aplicação intraperitoneal de 180 mg/kg de ENU desenvolveu tumores mamários em 90% das fêmeas de rato analisadas (Stoica, Koestner, Capen, 1983). Por fim, em comparação ao MNU, o ENU foi considerado menos tóxico, o que

é benéfico tanto para a sua manipulação quanto para seu uso nos animais (Wechsler, 1971; Druckrey, Ivankovic, Gimmy, 1973), podendo ser administrado em dosagens mais elevadas.

Por fim, levando em consideração a maior responsividade que a próstata das fêmeas tem ao E2, atrelou-se o tratamento do ENU e da T com a exposição ao E2. Dados relativos à criação desta nova metodologia foram compilados no **artigo 1**, no qual foram utilizadas técnicas histoquímicas, análises ultraestruturais e ensaios de imunofluorescência e imuno-histoquímica para fatores relacionados ao microambiente tumoral, bem como análises sorológicas hormonais.

I.4. A crescente complexidade do estroma e os telócitos

O estroma já foi visto como um ambiente estático que daria apenas suporte aos epitélios, possuindo tipos celulares variados, como células musculares lisas, células nervosas e fibroblastos. Conforme o conhecimento sobre as células epiteliais aumentava, especialmente pela alta incidência de tumores de origens epiteliais (Pentheroudakis, Golfinopoulos, Pavlidis, 2007; Mullangi & Lekkala, 2022), o próprio estroma era visto como coadjuvante. No entanto, esse cenário começou a se modificar com a realização de estudos do tecido cicatricial, onde detectou-se fibroblastos que portariam algumas características de células musculares lisas, os quais foram chamados de miofibroblastos por Gabbiani e colaboradores (1971). Ao longo dos anos, estes fibroblastos modificados foram encontrados no estroma de tumores de diversas origens e evidências se acumularam de que o estroma teria um papel muito importante na progressão da carcinogênese epitelial (Iozzo, 1995; Hanahan & Weinberg, 2011). Foi cunhado o termo “estroma reativo” para designar o estroma que envolve os tumores epiteliais e que é afetado por eles, de modo a se tornar um vetor do próprio avanço da carcinogênese (Tuxhorn, Ayala, Rowley, 2001). Desse modo, os fibroblastos e as células musculares lisas, antes inofensivos, dariam origem a variantes bastante agressivas: os CAFs (*Cancer-associated fibroblasts*) e os miofibroblastos. Estes novos tipos de células seriam a base da formação de um estroma modificado, capaz de reagir aos sinais do epitélio cancerígeno e facilitar a migração de células deste epitélio e a blindagem das mesmas perante o sistema imune, além do aumento da angiogênese e alterações fisiológicas capazes de criar um ambiente ideal para a progressão do câncer (Tuxhorn *et al*., 2002).

A complexidade estromal aumenta quando se considera a presença de células intersticiais de função *pacemaker* semelhantes às células intersticiais de Cajal (ICCs), que antes eram consideradas exclusivas do trato gastrointestinal, sendo, posteriormente, detectadas em outros órgãos, como ureteres, vesícula biliar e próstata (Streutker *et al*., 2007; Pasternak *et al*., 2013; Corradi *et al.*, 2013), bem como com a classificação de células semelhantes às ICCs CD34-positivas como um novo tipo celular devido às suas diferenças morfofuncionais, denominado telócito (Popescu & Faussone-Pellegrini, 2010). As principais características que diferenciam os telócitos de outras células estromais são os telopódios, extensões do citoplasma, que por sua vez são divididos em podômos, regiões mais dilatadas, e podômeros, regiões mais finas, levando a uma estrutura semelhante a um colar de contas (**Figura 2**). Entretanto, por possuirem dimensões extremamente pequenas, tais estruturas somente são observáveis por meio da ultraestrutura (Popescu & Faussone-Pellegrini, 2010; Cretoiu *et al.*, 2020; Sanches *et al*., 2020). Foi hipotetizado que os telócitos seriam capazes de se diferenciar em células musculares lisas (Bei *et al.*, 2015) ou mesmo em fibroblastos ou miofibroblastos (Díaz-Flores *et al*., 2016), porém sua origem permanece incerta.

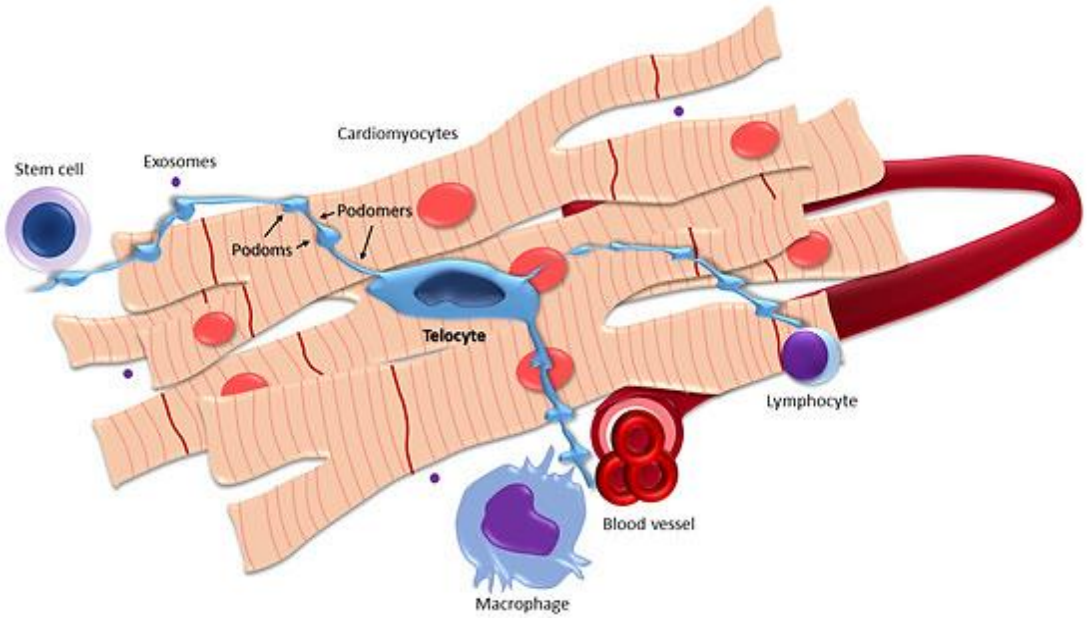

Figura 2. Telócitos encontrados em tecido muscular estriado cardíaco exibindo seus característicos telopódios, com a alternância entre podômos e podômeros, próximos de cardiomiócitos, células do sistema imune, células-tronco e vasos sanguíneos. **Nota:** Reimpresso de "Cardiac telocytes 16 years on — What have we learned so far and how close are we to routine application of the knowledge in cardiovascular regenerative medicine?", por Klein *et al.*, 2021. Sob licença *Creative Commons*.

Acumulam-se evidências de que os telócitos diferem dos fibroblastos e células musculares lisas em termos de proteoma, morfologia e expressão gênica (Zheng *et al*., 2014a; Kang *et al*., 2015; Xiao & Bei, 2016), além disso, tais células divergem também dos pericitos (Bei *et al*., 2015) e das células-tronco mesenquimais (Zheng *et al*., 2013). Os telócitos possuem características de células progenitoras, tal como foi proposto para um amplo *subset* de células que apresentam a marcação para o CD34 (Sidney *et al*., 2014), e, como supracitado, foi suposto que estas células poderiam dar origem a fibroblastos e miofibroblastos de modo a participar ativamente no processo de reparo tecidual na pele (Díaz-Flores *et al*., 2016) ou mesmo originar cardiomiócitos no tecido muscular estriado cardíaco (Bei *et al.,* 2015). Além disso, os telócitos apresentam intensa síntese de fatores parácrinos em suas vesículas, além da expressão de proteínas associadas a homeostase e reparo estromais (Zheng *et al*., 2014b), e acredita-se que também possuiriam um papel na organização do próprio estroma, como foi verificado no tecido muscular estriado cardíaco (Bani *et al*., 2010) e na próstata (Sanches *et al*., 2017c), atuando de modo a compartimentalizar, por meio de sua ampla rede de telopódios, microambientes estromais, por exemplo, separando a musculatura lisa do estroma adjacente e envolvendo o interstício. No entanto, faltam estudos acerca dos telócitos em quadros patológicos, particularmente na carcinogênese prostática.

Assim, no **artigo 2** foram utilizadas técnicas histoquímicas e de imunofluorescência, além de análises ultraestruturais para a análise dos telócitos na próstata de fêmeas do gerbilo no cenário prostático normal e tumoral induzido pelo tratamento com ENU, T e E2.

I.5. Terapias antiestrogênicas no combate ao câncer de próstata

Segundo a literatura, cerca de 20 casos de adenocarcinoma prostático já foram reportados em mulheres até o momento (Gao *et al.*, 2022). Histologicamente, a maioria deles demonstrou glândulas malformadas com padrão cribiforme, neoplasia epitelial, citoplasma com aspecto vacuolizado, e marcação positiva para o antígeno específico da próstata (PSA) (Tregnago & Epstein, 2018; Kyriazis *et al.*, 2020; Lenz *et al.,* 2021; Kaufman *et al.,* 2021). Apesar de ser considerada rara, essa enfermidade é, muitas vezes, confundida com outras desordens do trato urogenital, uma vez que parte dos cânceres de uretra tem origem prostática (Reis *et al*., 2011; Lenz *et al.,* 2021; Kunc & Biernat, 2021). Sendo assim, o número de casos

pode ser bem maior do que o reportado, o que levanta preocupações em termos de saúde pública.

Assim, em face à necessidade de se buscar um alvo terapêutico para adenocarcinomas da próstata feminina e levando em conta a susceptibilidade que a próstata da fêmea do gerbilo tem aos estrógenos, o presente trabalho propôs a utilização do tamoxifeno (TAM), um composto antiestrogênico amplamente utilizado no combate ao câncer de mama hormônio-dependente (Visvanathan *et al*., 2019; Emons, Mustea, Tempfer, 2020). TAM age como um antagonista do receptor de estrógeno (ER) na mama, ao passo que em outras regiões, como endométrio e osso, possui ação agonista (Li *et al*., 2017; Emons, Mustea, Tempfer, 2020; Lafront *et al*., 2020). Sua forma ativa, 4-hydroxytamoxifen (4HT), é convertida por meio do citocromo P450 e tem alta afinidade pelo ER, de forma a competir com o estradiol endógeno pela ligação com o receptor. Na mama, sabe-se que, ao se ligar ao ERα, o TAM promove respostas antagonistas ao bloquear a sinalização estrogênica, inibindo a proliferação celular (Hasegawa *et al*., 2018; Pepe *et al*., 2021). Por fim, o tratamento com TAM também pode se mostrar uma alternativa em cânceres de próstata resistente a castração (CRPC), uma vez que esses tumores, geralmente ER-positivos, já não respondem as terapias antiandrogênicas convencionais (Semenas *et al*., 2021).

Os resultados deste trabalho foram compilados no **artigo 3**, de modo que foram utilizadas técnicas histoquímicas, ensaios de imunofluorescência e imuno-histoquímica para fatores relacionados ao microambiente tumoral e ensaios para detecção de apoptose por meio da fragmentação do DNA, bem como análises sorológicas hormonais.

II. OBJETIVOS

O presente trabalho teve como objetivo a criação de um modelo de indução de câncer na próstata de fêmeas do gerbilo da Mongólia submetidas ao tratamento com ENU, T e E2. Além disso, objetivou-se identificar, quantificar e caracterizar as lesões encontradas e alterações de elementos estromais associados à formação do microambiente tumoral, destacando-se os telócitos. Por fim, objetivou-se propor um possível tratamento com um antiestrogênico, TAM, para a carcinogênese prostática induzida por ENU+T+E2.

III. PRODUÇÕES CIENTÍFICAS

III.1. Artigo 1

A model of carcinogenesis induction in the female prostate of Mongolian gerbil (*Meriones unguiculatus*)

Juliana S Maldarine[a], Bruno D A Sanches[a], Vitória A Santos[b], Gustavo M Amaro[b], Rejane M Góes[b], Patricia S L Vilamaior[b], Sebastião R Taboga[a,b]

[a]Department of Structural and Functional Biology, Institute of Biology, State University of Campinas (UNICAMP), Bertrand Russel Av., Campinas, São Paulo, Brazil.

[b]Department of Biological Sciences, Laboratory of Microscopy and Microanalysis, Sao Paulo State University (UNESP) Cristóvão Colombo St., 2265, São José do Rio Preto, São Paulo, Brazil.

Correspondence to: Dr. Sebastião R. Taboga, Department of Biology, Laboratory of Microscopy and Microanalyses, 2265 Cristóvão Colombo Street, São José do Rio Preto, São Paulo, e-mail: sebastiao.taboga@unesp.br

This dissertation text is adapted from the manuscript submitted to the Scientific Journal *Experimental and Molecular Pathology*

III.1.1. Abstract

The prostate is an important accessory gland of the mammalian reproductive system. Differently from what was initially thought, the gland is not exclusive to males, having been found in females of several species, such as dogs, rats, and gerbils; it is also present in part of the women population, being also called Skene's gland. There is evidence that the female prostate undergoes the same pathological conditions which affect the male prostate, such as prostatitis and even adenocarcinoma. Given the difficulties faced when investigating carcinogenesis in female prostate, Mongolian gerbils can be a useful model since 90% of the female individuals develop a functional gland. In the present study, we treated adult females with a single dose of N-ethyl-N-nitrosourea (ENU), a potent carcinogen, along with alternating weekly exposure to testosterone and estradiol for 24 weeks. The treatment was successful to inducing malignant lesions in 67% of females, which were classified as well-differentiated adenocarcinoma. Also, the data showed alterations regarding the stroma, such as discontinuity of smooth muscle surrounding the alveoli, besides increased vascularization and inflammatory response. In conclusion, our study developed for the first time a methodology for carcinogenesis induction in female prostate, which is relevant for better understanding this condition in females and for the search of new therapeutic targets.

Keywords: Prostate cancer, reactive stroma, angiogenesis, inflammation, adenocarcinoma, female prostate

Abbreviation List

ENU (N-ethyl-N-nitrosourea), PIN (Prostatic Intraepithelial Neoplasia), HE (Haematoxylin-Eosin), E2 (17β-estradiol), T (Testosterone cypionate), AR (Androgen Receptor), ERα (Estrogen Receptor α), PCNA (Proliferating Cell Nuclear Antigen), PH.H3 (Phospho-Histone H3), α-SMA (α-Smooth Muscle Actin), CD163 (Cluster of Differentiation 163), CD68 (Cluster of Differentiation 68), PFA (Paraformaldehyde), PBS (Phosphate-buffered Saline), DAPI (4′,6-Diamidino-2-Phenylindole), FITC (Fluorescein Isothiocyanate), BSA (Bovine Serum Albumin)

III.1.2. Introduction

The prostate is an accessory gland which constitutes the mammalian reproductive system; its main function is to produce a secretion that will be incorporated in the seminal liquid (Verze *et al.*, 2016). Although it was previously thought to be exclusive to males, this gland was also reported in females hundreds of years ago (De Graaf, 1672). As a matter of fact, back in 1880, it had already been named by the gynecologist Alexander Skene as "Skene's paraurethral gland". However, little was known about this gland, and it was considered a vestigial organ. This initial designation is still frequently used, although since it is very similar to the male prostate in histological and morphological terms, it should, according to Zaviacic and colleagues (1999), receive the same nomenclature. Since then, researchers have been focusing on identifying and expanding knowledge on the female prostate in several species, such as dogs (Aguiar *et al.*, 2013), rodents (Biancardi *et al.*, 2010; Santos *et al.*, 2022), and humans, in which it is present in approximately 50% of the women (Dietrich *et al.*, 2011). It has already been reported that this gland can be affected by the same disorders found in men, such as prostatitis and even adenocarcinoma (Gittes, 2002; Pongtippan *et al.*, 2004; Massari *et al.*, 2014; Muto *et al.*, 2017; Tregnago & Epstein, 2018; Bondili *et al*., 2021; Slopnick *et al*., 2022), which can raise concerns in terms of public health. However, given the difficulty in investigating this gland in women, since it is mostly obtained from cadavers (Costa *et al.*, 2016), animal models have been a viable alternative.

Our research group used the Mongolian gerbil (*Meriones unguiculatus*) in experiments, for this rodent is easy to handle and maintain in laboratory. The female gerbil has been very useful to understanding the female prostate development, physiology, and susceptibility to disorders, since a functional gland is found in approximately 90% of female individuals, besides being homologous to the ventral lobe of males and to the human female prostate (Santos & Taboga, 2006; Biancardi *et al.*, 2017). Although it is not completely elucidated, a function attributed to the female gland is the production of a secretion which could benefit reproduction. The secretion goes from the urethra into the entrance of the vagina and helps to reduce the acidity in that environment (for it is alkaline, similarly to that of males), and enables sperm capacitation (Zaviacic & Ablin, 2005). It is also known that both female and male gerbil prostates can develop spontaneous lesions with aging (usually after two years of life), such as neoplasia (Leonel *et al*., 2021), which might be correlated to alterations in

hormone levels typical of aged animals (Campos *et al.*, 2008; Custódio *et al.*, 2008). In that sense, this species can be an interesting model for cancer research (Leonel *et al*., 2021).

Despite the morphophysiological similarities, the male and female prostates of the gerbil also have several differences. The female gland is approximately four times smaller than that of males and has only one ventrolateral lobe (Sanches *et al.*, 2016), while the male prostate is divided into ventral, dorsal, dorsolateral, and anterior lobes (Rochel *et al.*, 2007). Nevertheless, one of the main differences is hormone-related, since the female gerbil has higher levels of circulating estradiol than the male, while the testosterone concentration is higher in males (Fochi *et al.*, 2013; Zanatelli *et al.*, 2014). For this reason, any minimal hormonal imbalance in estrogen levels can cause greater impacts on the female prostate, if compared to males. Hormonal manipulation experiments have shown that intrauterine exposure to low doses of 17β-estradiol (E2) led to a decrease in the number of female prostatic branching in the initial development, which was only achieved for the male with higher doses (Sanches *et al.*, 2017a). Also, the administration of bisphenol-A (BPA), an endocrine-disruptor that mimics estrogen action, in gerbil neonates led to a reduction in female prostate proliferation index even at lower doses, while only higher doses had a similar effect on males (Rodríguez *et al.*, 2016). Other studies have reported that estradiol is necessary for prostate homeostasis, and that a prolonged administration of this hormone can cause alterations in the prostate architecture; the same happens for supplementation with testosterone, which could compromise the gland functionality leading to the development of pre-malignant and malignant lesions (Silva *et al.*, 2013; Zanatelli *et al.*, 2014). Hence, it can be assumed that estrogens are also important when prostate tumors are concerned, including cancer.

The number of studies related to cancer, especially prostate cancer, exponentially increases every year, since it is the second leading cause of death in men (Bray *et al.*, 2018). Several methodologies have already been described for tumor induction in different species, such as dogs, mice, rats, and gerbils (Park *et al.*, 2010; Keller *et al.*, 2013; Gonçalves *et al.*, 2013; Bosland *et al.*, 2022). Previous studies conducted with MNU carcinogen and additional testosterone supplementation could induce carcinogenesis in the prostates of male gerbils (Gonçalves *et al.*, 2010; Quitar *et al.*, 2017). However, the same method was not effective for the female prostate, in which only pre-malignant lesions were found along with other components that evidenced the formation of the reactive stroma (Gonçalves *et al.*, unpublished results) which represents stromal alterations that can contribute to the onset of a tumor

microenvironment (Tuxhorn, Ayala, Rowley, 2001). Considering the similarities with the male gland, but also the female prostate particularities, such as the high impact estrogens have on its physiology, a new induction model is proposed herein, adapted from the previous study conducted in our laboratory. Therefore, an even more potent carcinogen, N-ethyl-N-nitrosourea (ENU), known to generate widespread mutation in mouse genome (Russell *et al.*, 1979; Probst & Justice, 2010), along with weekly exposure to testosterone and estradiol, were used. Hence, the aim of this study is to propose a new methodology to carcinogenesis induction in the female prostate.

III.1.3. Material and Methods

Animals and carcinogenesis induction

Forty adult female (6-months-old) Mongolian gerbils (*Meriones unguiculatus*) were housed in polyethylene cages in 25°C room temperature, with unlimited access to filtered water and food. The experiments followed the ethical guidelines of São Paulo State University (UNESP) (CEUA-209/2019). The animals were weighted and then euthanized by subcutaneous injection of ketamine (100 mg/kg), and xylazine (11 mg/kg). Blood samples were collected after the decapitation for posterior serological analysis and stored at -80ºC.

In order to induce carcinogenesis and the formation of the reactive stroma in the female prostate, a methodology adapted from Gonçalves and collaborators (2013) for tumor induction in the male prostate was used. In this study, the females (N=20) were submitted to a high dose of testosterone cypionate (T) (20 mg/kg) subcutaneously and, after three days, a single dose of the carcinogen N-ethyl-N-nitrosourea (ENU; Chem Service, West Chester, PA-USA) (100 mg/kg) was injected intraperitoneally, followed by alternating subcutaneous exposure to 17-β-estradiol (E2) (2 mg/kg) and testosterone cypionate (2 mg/kg) once a week for 24 weeks. The control group (N=20) was established by replacing the carcinogenic treatment with corn oil (100 µl/dose) in all applications.

Histological processing

The prostates were fixed in 4% PFA (buffered in 0.1 M phosphate, pH 7.4) for 24 h, washed in H_2O, dehydrated, clarified, and then embedded in paraffin. The glands were sectioned at 5 μm in a microtome (RM2155, Leica) and mounted on histological slides. Some of these slides were stained with Haematoxylin-Eosin (HE) for general morphological purpose, and with Gömöri Reticulin for the detection of reticular fibers.

Classification and quantification of prostate lesions

The entire prostates of the females were serially sectioned, and HE histological sections were randomly chosen (5 sections per animal) along the gland. The histopathological classification of lesions was accomplished according to a methodology described by Shappell and colleagues (2004); the lesions were categorized in 5 types: hyperplasia, atrophy, low-grade PIN (Prostatic Intraepithelial Neoplasia), high-grade PIN, and adenocarcinoma. The grading scale described for the TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) mice by Berman-Booty and colleagues (2012) was used for the adenocarcinoma classification. Incidence of prostate lesions was quantified by identifying the different lesions in relation to the total sample number, while the multiplicity was calculated by the frequency of each lesion found in the histological section relative to the total number of examined animals in both groups.

Transmission electron microscopy (TEM)

The ultrastructural data was obtained following Sanches and coworkers' protocol (2020), in which fragments of female prostates from the control and ENU/T/E2-treated groups were cut into small pieces and fixed in 3% glutaraldehyde and 0.25% tannic acid solution in Millonig buffer (pH 7.3) together with 0.54% glucose for one day. The samples were then fixed with 1% osmium tetroxide for 1 h, then dehydrated, and embedded in Araldite resin. Ultrathin sections (50–75 nm) were prepared with a diamond knife and stained with 2% alcoholic uranyl acetate for 30 min, and then with 2% lead citrate in a 1 M sodium hydroxide solution for 10 min. The samples were analyzed in a Hitachi HT 7800 at 80 kV electron microscopy.

Immunofluorescence assays

After the tissues were deparaffinized and rehydrated, the exposure of the antigens was carried out by heat treatment for 20 min at 95°C with the slides immersed in citrate buffer. Blocking of non-specific bindings was performed for 30 min using the UltraCruz® Blocking Reagent (sc-516214, Santa Cruz Biotechnology) and the slides were washed three times for 5 min each in PBS with 0.3% Triton-X between each step. The female prostate sections from the control and the ENU/T/E2-treated groups were subjected to immunofluorescence assays for α-SMA (monoclonal mouse, IgG1κ, B4, sc-53142, Santa Cruz Biotechnology), in order to assess how smooth muscle layers were arranged in the prostate. To observe activated macrophages, immunofluorescence assays were performed for CD68 (monoclonal rabbit, 97778S, Cell Signaling Technology) and for CD163 (monoclonal rabbit, 93498S, Cell Signaling Technology). These antibodies were incubated overnight at a dilution of 1µg/100µl of BSA (Bovine serum albumin) 1% in PBS. In the next morning, the slides were subjected to a secondary antibody incubation with FITC-labelled (Fluorescein Isothiocyanate) goat anti-mouse (F0257; Sigma-Aldrich), 1:100 dilution in 1% bovine serum albumin (BSA) for 2 h at room temperature, washed in PBS, and finally stained with DAPI (4′,6-Diamidino-2-Phenylindole) (F36924; Life Technology). The histological slides were analyzed with a ZeissImager M2 fluorescence microscope coupled with AxioVision (Zeiss) software.

Immunohistochemical assays

The gerbil female prostate sections were subjected to immunohistochemistry assays according to the methodology performed by Sanches and colleagues (2017a). The antibodies were used at a dilution rate of 1:100 for detecting androgen receptor (AR) (rabbit polyclonal IgG, s-816; Santa Cruz Biotechnology), estrogen receptor α (ERα) (rabbit polyclonal IgG, PA5-34577; Invitrogen, ThermoFisher Cientific), proliferating cell nuclear antigen (PCNA) (mouse monoclonal IgG2a, sc-56; Santa Cruz Biotechnology), and phosphor-histone H3 (PH.H3) (polyclonal rabbit, 9701, Cell Signaling Technology). The secondary antibodies were rabbit anti-mouse IgG (Post Primary, Novolink; Leica Biosystems) and anti-rabbit Poly-HRP-IgG (Polymer, Novolink; Leica Biosystems). The incubation time for both was 45 min at 37ºC. The revelation was conducted with 3’-diaminobenzidine (DAB), and the sections were

counterstained with Harris hematoxylin. Negative controls were obtained by omitting the incubation of the primary antibody. Histological sections were analyzed with the Olympus BX60 light microscope.

Quantitative analysis

Thirty random prostate fields (stained with HE) at a magnification of 400X of each group were analyzed using ImageJ software (National Institute of Mental Health, Bethesda, Maryland). The stereological analysis was carried out using Weibel's method (1979) graticulated system of 120 points and 60 lines in order to compare the relative proportion (% of volume) of each prostatic component (epithelium, stroma, and blood vessels) between the treated and control groups. The relative values were determined by counting the points that coincided and dividing them by the total number of points. For the morphometric analysis, twenty random prostate fields at a magnification of 200X were stained by Gömöri Reticulin to measure the area covered by reticular fibers. Also, twenty random prostate fields at a magnification of 200X from immunofluorescence assays for α-SMA (α-Smooth Muscle Actin) of each group were analyzed to measure the area of the smooth muscle layer that surrounds the alveoli.

Finally, thirty to fifty random prostate fields at a magnification of 400X were used to count the total number of cells of each group for CD68 (Cluster of Differentiation 68) and CD163 (Cluster of Differentiation 163) immunofluorescence assays, and for AR (Androgen Receptor), ERα (Estrogen Receptor α), PCNA (Proliferating Cell Nuclear Antigen) and PH.H3 (Phospho-Histone H3) immunohistochemical assays, in order to separate the positive and negative stained cells, in accordance with the methodology described by Maldarine and colleagues (2020). A minimum of 1,000 cells were counted per group, and the percentage of positive cells was determined in relation to the total number of cells.

Serological analysis

The blood of female gerbils from the experimental groups was collected after decapitation. The serum was separated by centrifugation (3,000 rpm for 20 min) and stored at -80ºC until analysis. Hormonal dosages of circulating estradiol and testosterone were given in

duplicate by ELISA high sensitivity kits (17β-Estradiol EIA Kit, RE52041, Tecan, IBL-International; Testosterone EIA Kit, RE52151, Tecan, IBL-International) following the manufacturer's instructions. The readings were performed using the SpectraMax Plus 384 reader, at 405 nm.

Statistical analysis

T-test was used for two-sample comparison, and for multiple samples we used ANOVA coupled with Tukey post-hoc test. Significance was set at $p<0.05$ and results were expressed as mean ± standard deviation. All statistical tests were performed using Statistica 7.0 (StatSoft).

III.1.4. Results

Histopathology of the gland

The histological sections stained with HE from animals in the control group showed a typical architecture of the prostate, in which the epithelium with cubic or columnar cells is surrounded by a fibromuscular stroma (Fig. 1A, B), although a small focus of low-grade PIN can be observed (Fig. 1C). The prostate samples from the individuals in the ENU/T/E2 group indicated an atrophy process of the epithelium, in which cells became flat (Fig. 1D), as well as hyperplasia and PIN foci, in which the epithelium became stratified. Hyperplasia foci are verified when the number of epithelial cells increase and can extend to other alveoli, yet the cellular nuclei commonly maintain their characteristic pattern (Fig. 1E); Low-grade PIN foci in turn are characterized by proliferative sites of epithelial cells that form invaginations into the lumen, although they are usually confined to the same alveolus and the nuclei vary in shape and size. The high-grade PIN phenotype is more severe in comparison to the low-grade PIN one, with greater nuclear changes and prominent nucleoli, in addition to the formation of microacini in the epithelial compartment (Fig. 1F). Adenocarcinoma foci were found in 67% of the female prostates of the treated group. The stromal reaction, a process in which there is a replacement of smooth muscle cells for other stromal cells, such as myofibroblasts or activated fibroblasts, is verified around well-differentiated adenocarcinoma (Fig. 1G, H). This type of lesion is

classified when the cells invade the peripheral stroma after the basement membrane rupture (Fig. 1I). The increased proliferation of the epithelium was quantitatively verified by the significant increase in the epithelial compartment volume of the ENU/T/E2 group if compared to the control group (29.6 ± 11.2 and 15.3 ± 5.2 %, respectively) (Fig. 1J), although no significant differences were found in the stromal compartment volume between the control and treated groups (21.3 ± 7.2 and 19 ± 6.7 %, respectively) (Fig. 1K). There was also a significant increase in the volume of blood vessels in the treated group in comparison to the control (3.6 ± 1.9 and 1 ± 0.5 %, respectively) (Fig. 1L).

Quantification of prostate lesions and prostate weight

At least one focus of hyperplasia and atrophic alveoli was verified in all the prostate samples from the two experimental groups. Interestingly, all control group samples had at least one focus of low-grade PIN, whereas in the group that received the carcinogen plus testosterone and estradiol, that lesion was found in 83% of the animals. Sixty-seven percent of female prostate samples from the control group had at least one focus of high-grade PIN, while 83% of animals from the treated group presented that lesion. Only 17% of females from the control group presented adenocarcinoma foci; however, the lesion was found in most animals from the group treated with ENU/T/E2 (67%). The multiplicity of alveoli atrophy doubled in the treated group compared to the control, while for the adenocarcinoma it was 7 times higher (Table 1). There were no significant differences in the weight of the Mongolian female gerbils, nor in the prostate absolute and relative weight between both experimental groups (Table 2).

Ultrastructural analysis

The ultrastructural data of gerbil female prostates from the control group showed a normal gland, in which secretory cells present a typical nuclear phenotype, along with few secretory vesicles in the apical side in the cytoplasm and an intact basement membrane adjacent to the epithelium (Fig. 2A, B). In the group treated with ENU/T/E2, it is possible to observe the disorganization in the prostate architecture. In hyperplasia foci, secretory vesicles were dispersed in the cytoplasm, giving the cells a multilocular or vacuolized aspect, and some cells' nuclei became irregular (Fig. 2C, D). A proliferative aggregate of epithelial cells towards the

lumen is seen in low-grade PIN focus (Fig. 2E). Atrophic alveoli with squamous cells are also evident, as well as immune cells that are often found in the interalveolar stroma (Fig. 2F).

Reticular fibers analysis

The reticular fiber network can be seen in a typical and organized orientation circulating the prostate alveoli in the control group (Fig. 3A, B), whilst in the tumorigenic-induced group some of the fibers either lost their orientation or became fragmented in the stroma (Fig. 3C, D). In addition, in the latter, the area of the subepithelial layer of reticular fibers increased, which was statistically confirmed (5 ± 2.4 and 2.8 ± 1.35 %, respectively) (Fig. 3E).

Smooth muscle analysis

The arrangement of the smooth muscle layer is well-defined around the alveoli in the control group (Fig. 4A, B), whereas in the treated group the perialveolar smooth muscle had a discontinuous appearance, i.e., did not completely involve the alveoli (Fig. 4C, D). The smooth muscle area presented a significant decrease in the group treated with ENU/T/E2 compared to the control group (1.8 ± 0.8 and 2.7 ± 0.7 %, respectively) (Fig. 4E).

Macrophages analysis

In the control group, few cells were immunoreactive to CD68 and CD163 in the stromal compartment (Fig. 5A, B, E, F). Immunostaining of both factors was increased in the interalveolar region for the group that received the tumorigenic treatment (Fig. 5C, D, G, H). Statistical data showed that the number of CD68-positive cells was 3 times higher for the treated group than for the control (10.6 ± 3.3 and 3 ± 1.5 %, respectively) (Fig. 5I), and the same was observed for the number of CD163-positive cells (10.3 ± 2.9 % in the treated group and 3.6 ± 1.8 % in the control group) (Fig. 5J).

Steroidal receptors analysis

Regarding the androgen receptor (AR) immunostaining, the epithelial and stromal compartments of the female prostate had few positive cells for the control group (Fig. 6A, B). For the ENU/T/E2 group, glands that presented histopathologic disorders, such as hyperplasia and PIN foci, had an increase in the number of AR-positive cells in both glandular compartments in comparison to the control group (Fig. 6C, D), which was confirmed by the statistical analysis (59.8 ± 16.6 and 22.6 ± 11.5 % in the epithelium and 33.4 ± 4.5 and 12.6 ± 5.2 % in the stroma, in the respective groups) (Fig. 6I). A similar pattern was observed for the estrogen receptor (ERα) staining. The normal epithelium and stroma of the female prostates for the control group had few cells immunoreactive to ERα (Fig. 6E, F), whilst for the tumorigenic-induced group the secretory epithelial (especially those with lesions) and stromal compartments showed a remarkable and significant increase in ERα-positive cells compared to the control (71.6 ± 9.9 and 29.3 ± 15.2 % in the epithelium and 56.1 ± 16.2 and 24.4 ± 10.6 % in the stroma, in the respective groups) (Fig. 6G, H, J).

Proliferation index

The immunohistochemical assays for the proliferating cell nuclear antigen (PCNA) revealed that in the control group, few of the epithelial and stromal cells were marked (Fig. 7A, B). The number of PCNA-positive cells had a significant increase in the epithelium of prostates from the ENU/T/E2 in comparison to the control group, which was corroborated by the quantitative analysis (32.9 ± 16.5 and 20 ± 10.1 % respectively), even though significant alterations were not found in the stroma (20.8 ± 10.2 and 15.4 ± 6.3 %, respectively) (Fig. 7C, D, I). Immunostaining of phospho-histone H3 (PH.H3) is seen in both the epithelial and stromal compartments of the prostates for the control group (Fig. 7E, F). As for the treated group, there is an increase in the number of PH.H3-positive cells in the two compartments in relation to the control, which was statistically significant (60 ± 8.9 and 47.7 ± 7.5 % in the epithelium, and 57.7 ± 6.6 and 47 ± 4.9 % in the stroma, in the respective groups) (Fig. 7G, H, J).

Hormonal serum levels

The female Mongolian gerbil presented a marked increase in the serum levels of 17β-estradiol, which was 6.5 times higher in the tumor-induced group if compared to the control (293.2 ± 79.5 and 45.5 ± 33.8 pg/mL, respectively) (Fig. 8A). The circulating testosterone levels showed a slight, yet not significant, increase in the treated group in relation to the control (646.5 ± 253.7 and 635.5 ± 178.3 pg/mL, respectively) (Fig. 8B). The ratio between testosterone and 17β-estradiol was almost 10 times lower in the treated group in comparison to the control group (2.1 ± 0.4 and 20.5 ± 11.1 pg/mL, respectively) (Fig. 8C).

III.1.5. Discussion

Most gerbil female prostates examined in the carcinogenic-induced group presented adenocarcinoma foci similar to those reported for models created for male individuals (Gonçalves *et al.,* 2013). Since gerbils are phylogenetically close to other rodents, such as mice (Chevret & Dobigny, 2005; Zorio *et al.,* 2019; Leonel *et al.,* 2021), the lesions found in this study would be equivalent to grade 5 of the grading scale used for the transgenic adenocarcinoma of the mouse prostate (TRAMP) (Berman-Booty *et al.,* 2012). At this stage, adenocarcinomas are considered well-differentiated, when cells retain characteristics from the original organ, and invasive, when these cells can migrate and invade other tissues (Gordetsky & Epstein, 2016; Kanan *et al.,* 2019). In our experiment, it was possible to observe the rupture of the basement membrane by epithelial cells that invaded the stroma yet maintaining typical characteristics, as well as a stromal reaction which resulted in the loss of smooth muscle layer involving the alveoli and its replacement for other stromal cells commonly associated with the tumor microenvironment, such as myofibroblasts and activated fibroblasts (Tuxhorn *et al.,* 2002; Andersen *et al.,* 2018). Taboga and coworkers (2008) have already shown that the smooth muscle cells became atrophic and lost interaction with each other in prostate tumors, which contributed to basement membrane disruption as well as epithelial cell invasion. Disorganization and increase in other elements of the stroma, such as reticular fibers, had already been reported in previous studies (Costa *et al.,* 2004; Gonçalves *et al.,* 2010) and are connected to stromal remodelling, which contributes to tumor initiation and cell invasion (Malik *et al.,* 2015; Luthold *et al.,* 2022). It is widely known that the maintenance of a regular

crosstalk between the epithelium and the stroma is essential to the prostate physiology (Gonçalves *et al.*, 2015; Güney *et al.*, 2020; Gonzáles *et al.*, 2022). Therefore, structural alterations in the stroma are associated with tumor microenvironment onset and progression (Jurj *et al.*, 2022), consisting of the so-called "reactive stroma", which was firstly described by Tuxhorn, Ayala and Rowley (2001).

Angiogenesis, essential for any tumor cell nutrition and growth, is a process that generates neovascularization, which is very common in cancer (Hanahan & Weinberg, 2011; Aguilar-Cazares *et al.*, 2019). In the present study, the blood vessels volume was almost four times higher in the treated group compared to the control. Previous studies on the induction of carcinogenesis in male gerbil also reported how increased vascularization and a higher number of prostate lesions were correlated (Gonçalves *et al.*, 2010). Inflammation, also commonly associated with cancer (Hanahan & Weinberg, 2011), is evidenced by the high number of macrophages in the interalveolar region of the carcinogenic-induced group. Macrophages are very heterogeneous cells which can be activated by different pathways; hence, different subpopulations can be found in tissues (Siefert *et al.*, 2021). Recent studies showed that CD68, a general macrophage marker, is associated with pro-inflammatory responses and implicated in various types of tumors, such as glioblastoma, thyroid carcinoma, and liver hepatocellular carcinoma (Shapouri-Moghaddam *et al.*, 2018; Zhang *et al.*, 2022). CD163 is a specific marker of M2 subtype macrophages, usually present in anti-inflammatory processes, since these cells secrete more cytokines and growth factors, also stimulating angiogenesis (Hu *et al.*, 2017), as well as functioning as biomarkers in colorectal (Krijgsman *et al.*, 2020) and prostate (Erlandsson *et al.*, 2019) cancers.

Two factors related to proliferation — proliferating cell nuclear antigen (PCNA) and phospho-histone H3 (PHH3) — were highly expressed in the carcinogenic-induced group. PCNA, a nuclear protein associated with DNA replication and repair, is considered a biomarker of cancer since it is overexpressed in all kinds of tumor cells, regardless of what tissue they originated from (Dillehay *et al.*, 2014). High levels of PHH3, a protein present in the nucleosome, have also been positively correlated with several types of cancer, such as melanoma, breast cancer (Hao *et al.*, 2018), and prostate cancer (Nowak *et al.*, 2014).

Besides malignant lesions, benign and pre-malignant lesions were also found in the experimental groups. The treatment with ENU/T/E2 led to a considerable increase in the incidence and multiplicity of alveoli atrophy foci as well as secretory epithelial involution,

which has also been previously reported in aged female gerbils (Oliveira *et al.*, 2010). Although none of the groups differed regarding the occurrence of hyperplasia or high-grade PIN foci, the control group had higher incidence of low-grade PIN. A plausible explanation is that, according to previous studies, both female and male gerbils can develop spontaneous lesions in their prostate as it ages, in particular hyperplasia and PIN, possibly due to hormonal imbalance typical of aging, since, in the case of females, ovaries no longer produce steroids (Campos *et al*., 2008; Custódio *et al.*, 2008; Biancardi *et al.*, 2017).

Our findings indicate remarkably increased circulating 17β-estradiol (E2) levels in the treated group, which can be correlated to the high number of cells expressing estrogen receptor alpha (ERα) in the gland. ERα is a nuclear factor implicated in the transcription regulation (Pagano *et al.*, 2020), in which its expressions increased in aggressive prostate cancer in mouse models (Takizawa *et al.*, 2015), besides being implicated in tumor progression (Bonkhoff, 2018). It is known that the gerbil female prostate is very sensitive to estrogens and that high levels of ERα can induce epithelial proliferation, while in males the androgen receptor (AR) would be the main responsible for this process (Rochel-Maia *et al.*, 2013). Several studies have demonstrated that the exposure to a more estrogenic environment can lead to different alterations in the physiology of both male and female prostates throughout life. Intrauterine exposure to E2 led to cellular proliferation and budding growth in the neonate females' prostates, while the proliferation index reduced, and the gland had a hypomorphic aspect in males (Sanches *et al.*, 2017a). Adult females exposed to E2 during fetal life also suffered alterations in prostate ramification even at lower dosages; for males, only higher dosages could change the branching dynamic as well as cell proliferation (Sanches *et al.*, 2017b). Bisphenol-A, an endocrine disruptor that mimics the estradiol action, also generates gender differences in gerbils, since female prostates exposed to lower dosages of this compound early in the development showed a more proliferative profile and increased expression of ERα in adult life, while in males only higher dosages could result in significant alterations in the gland (Rodríguez *et al.*, 2016). Accordingly, estradiol has shown to be essential for the integrity and regulation of the female prostate; its supplementation combined with progesterone can also stimulate tissue recovery, reversing the glandular regression and the decreased secretory activity caused by ovariectomy (Zanetelli *et al.*, 2014). In this sense, it is possible to deduce that a hyperestrogenic environment may lead to several alterations that could be even more aggressive to the female prostate than to that of males (Sanches *et al.*, 2019).

Previous studies conducted by our research group have used MNU plus testosterone as a method of carcinogenesis induction in male gerbil prostates (Gonçalves *et al.*, 2010), which was not successful in the female, since it caused only pre-malignant lesions (Gonçalves *et al.*, unpublished results). In the present study, we used N-ethyl-N-nitrosourea (ENU), one of the most potent mutagens for mice (Russell *et al.,* 1979, Probst & Justice, 2010). Reports have shown that this carcinogenic agent induced tumors in various organs, such as lungs, testis, and mammary glands, because it activates DNA damage pathways through random mutations by the transference of the ethyl group into base-pairs (Stoica, Koestner, Capen, 1983; Cordes, 2005; Bodakuntla *et al.,* 2014; Dai *et al.,* 2019). Thus, the association of a highly mutagenic agent with exposure to E2, given the huge impact it has on the female gland as discussed previously, combined with exposure to testosterone, which is already known to have proliferative effects on the gerbil female prostate (Santos *et al.,* 2006) and to be even more harmful for the female than for the male prostate (Biancardi *et al.,* 2012), has been efficient to produce a novel model of malignant tumor induction in the female prostate.

Therefore, new studies are necessary to better understand the female prostate under pathological conditions since there is evidence that it can respond differently and be even more sensitive to some compounds than that of males (Sanches *et al.,* 2019). This could have implications in public health since the gland is also found in approximately half of the women (Dietrich *et al.* 2011), and reports have shown it can develop the same disorders found in the male prostate, such as prostatitis and adenocarcinoma (Gittes, 2002; Pongtippan *et al.,* 2004; Massari *et al.,* 2014; Muto *et al.,* 2017; Tregnago & Epstein, 2018; Bondili *et al*., 2021; Slopnick *et al*., 2022). Novel models, such as the present one, are important because they may help elucidate how aggressive tumors can arise and progress from the female prostate to other organs, such as the urethra. These tumors are often mistaken for syndromes of the urogenital tract, neglecting the importance of this gland in women, since they may be subjected to higher concentrations of estradiol, as well as other xenoestrogens and endocrine disruptors, which can be harmful to the female prostate, whereas not to the male's (Biancardi *et al.,* 2017; Sanches *et al.,* 2019).

Finally, our study showed for the first time a method for inducing carcinogenesis in the female prostate, which paves the way for a new set of experimental investigations on prostate cancer histopathology in females and how it could differ from that in males. Moreover, the impact of estrogen on the carcinogenesis shown in this model indicates that anti-estrogenic

therapies could be a promising approach for treating prostate cancer in females, even though further studies are needed to test it.

Declaration of interest: none.

Funding

This work was supported by FAPESP (São Paulo Research Foundation); Contract number: 2018/08945-8 (to BDAS); 2018/23383-6 (to SRT) and 2019/14201-4 (to JSM) and CNPq (National Council for Scientific and Technological Development); Contract number 302938/2020-6 (to SRT).

Acknowledgements

The authors are grateful to FAPESP and CNPq for the fundings. Also, to Luiz Roberto Falleiros Jr. for technical support, as well as all coworkers at the Laboratory of Microscopy and Microanalysis (LMM).

III.1.6. References

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III.1.7. Figures, tables and legends

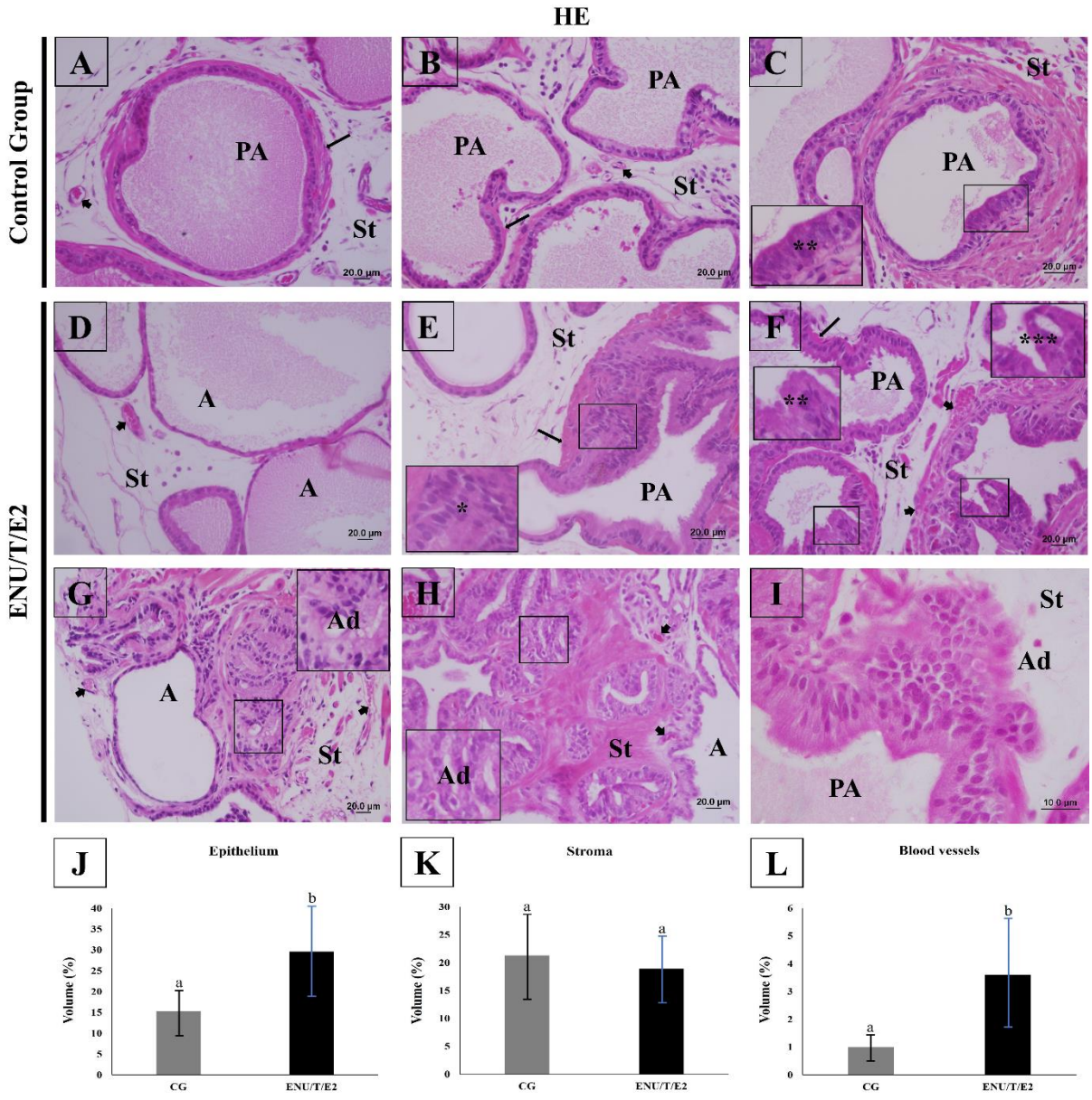

Fig. 1. Histological sections stained with HE and volume of prostate compartments of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) General view of alveoli of the control group, in which the epithelium layer with a normal phenotype can be seen surrounded by a fibromuscular stroma. (C) A small focus of low-grade PIN is also verified in this experimental group. (D) In the treated group, atrophic alveoli with flat cells can be observed. (E) General aspect of gland with hyperplasia and a stratified secretory epithelium. (F) Alveolus with a focus of low-grade PIN and high-grade PIN containing intraepithelial arcs

around a well-vascularized stroma can be verified. (G, H) Well-differentiated adenocarcinoma consisting of a cluster of epithelial cells and microacini around an evident stromal reaction. (I) Invasive adenocarcinoma with neoplastic cell proliferation invading the adjacent stroma through rupture of the basement membrane. (J) The quantification showed a significant increase in the epithelial volume in the treated group compared to the control group (K) Volume quantification of the stromal compartment showed no significant differences between both groups. (L) The volume of blood vessels increased significantly in the group treated with ENU/T/E2 in comparison to the control group. PA (Prostate alveoli), St (Stroma), Ad (Adenocarcinoma foci), Arrow (Smooth muscle), Arrowhead (Blood vessels), A (Atrophic alveoli), * (Hyperplasia foci), ** (Low-grade PIN foci), *** (High-grade PIN foci). Different letters (a, b) indicate significant statistical differences between groups.

Table 1 – Incidence and multiplicity of lesions of Mongolian gerbil female prostate

	Histopathological lesions				
	Hyperplasia	Atrophy	Low-grade PIN	High-grade PIN	Adenocarcinoma
Incidence (%)					
CG	100	100	100	67	17
ENU+T+E2	100	100	83	83	67
Multiplicity (Mean ± S.D.)					
CG	4.7 ± 1.1[a]	2.6 ± 1.6[a]	1.3 ± 0.4[a]	0.5 ± 0.5[a]	0.0 ± 0.0[a]
ENU+T+E2	3.8 ± 1.8[a]	5.5 ± 2.5[b]	1.2 ± 0.7[a]	1.1 ± 0.9[a]	0.7 ± 0.5[b]

Values represent mean ± standard deviation (S.D.) and different superscript letters (a, b) represent significant statistical differences between groups at $p \leq 0.05$. Statistical analysis based on the T test.

Table 2 – Body weight, absolute and relative prostate weight of the female Mongolian gerbil

	Groups	
	Control Group	ENU+T+E2
Female gerbils		
Body weight (g)	71.5 ± 5.3[a]	74.4 ± 6.2[a]
Prostate weight (g)	0,97 ± 0,03[a]	0,103 ± 0,03[a]
Prostate relative weight (%)	0,0013 ± 0,0004[a]	0,0014 ± 0,0005[a]

Values represent mean ± standard deviation (S.D.) and different superscript letters (a, b) represent significant statistical differences between groups at $p \leq 0.05$. Statistical analysis based on the T test.

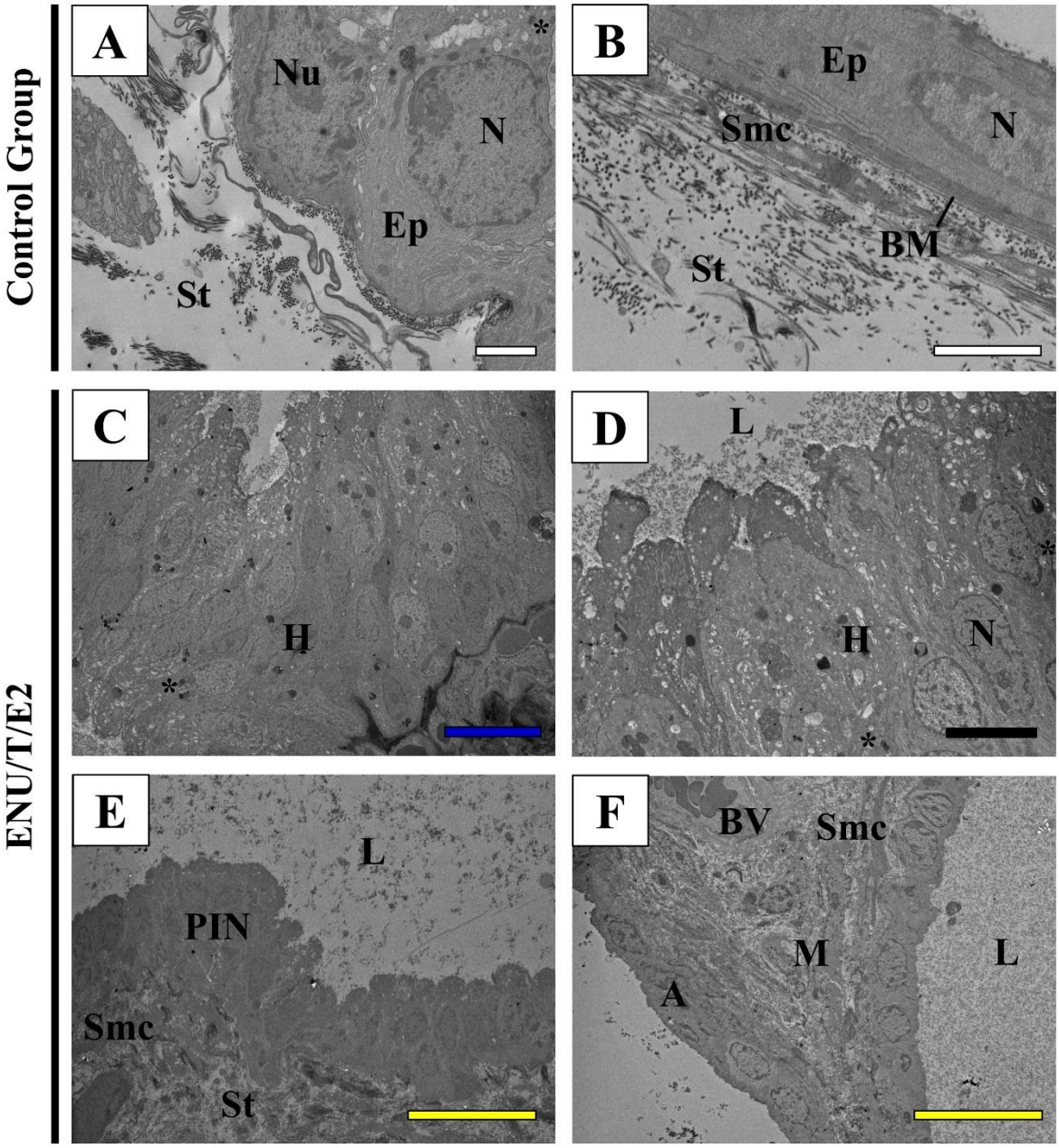

Fig. 2. Ultrastructural aspects of the prostate of female Mongolian gerbils from control and ENU/T/E2 treated groups. (A, B) In the control group, the secretory cells have regular nuclei (N), normally small and mostly round with a small nucleolus and finely granular chromatin. In the cytoplasm, the secretory vesicles are seen in the typical apical orientation. (C, D) In the focus of hyperplasia in the treated group, a non-polarized dispersion of secretory vesicles can be verified in the cytoplasm, which gives these cells a multilocular appearance. Irregular nuclei can also be observed, with a coarse chromatin distribution. (E) Low-grade PIN with atypical epithelial cells forming proliferative aggregates that invade the lumen. (F) Epithelial atrophy

process, in which the prostatic epithelium loses the pseudostratified characteristic organization and becomes squamous. Also, macrophages are seen in the interalveolar region in the stroma. Ep (Epithelium), N (Nuclei), Nu (Nucleolus), L (Lumen), St (Stroma), Smc (Smooth muscle cells), BV (Blood vessel), BM (Basement membrane), M (Macrophage), PIN (Prostatic intraepithelial neoplasia), A (Atrophic aveoli), H (Hyperplasia foci), * (Secretory vesicles), White bar (2 µm), Black bar (5 µm), Blue bar (10 µm), Yellow bar (20 µm).

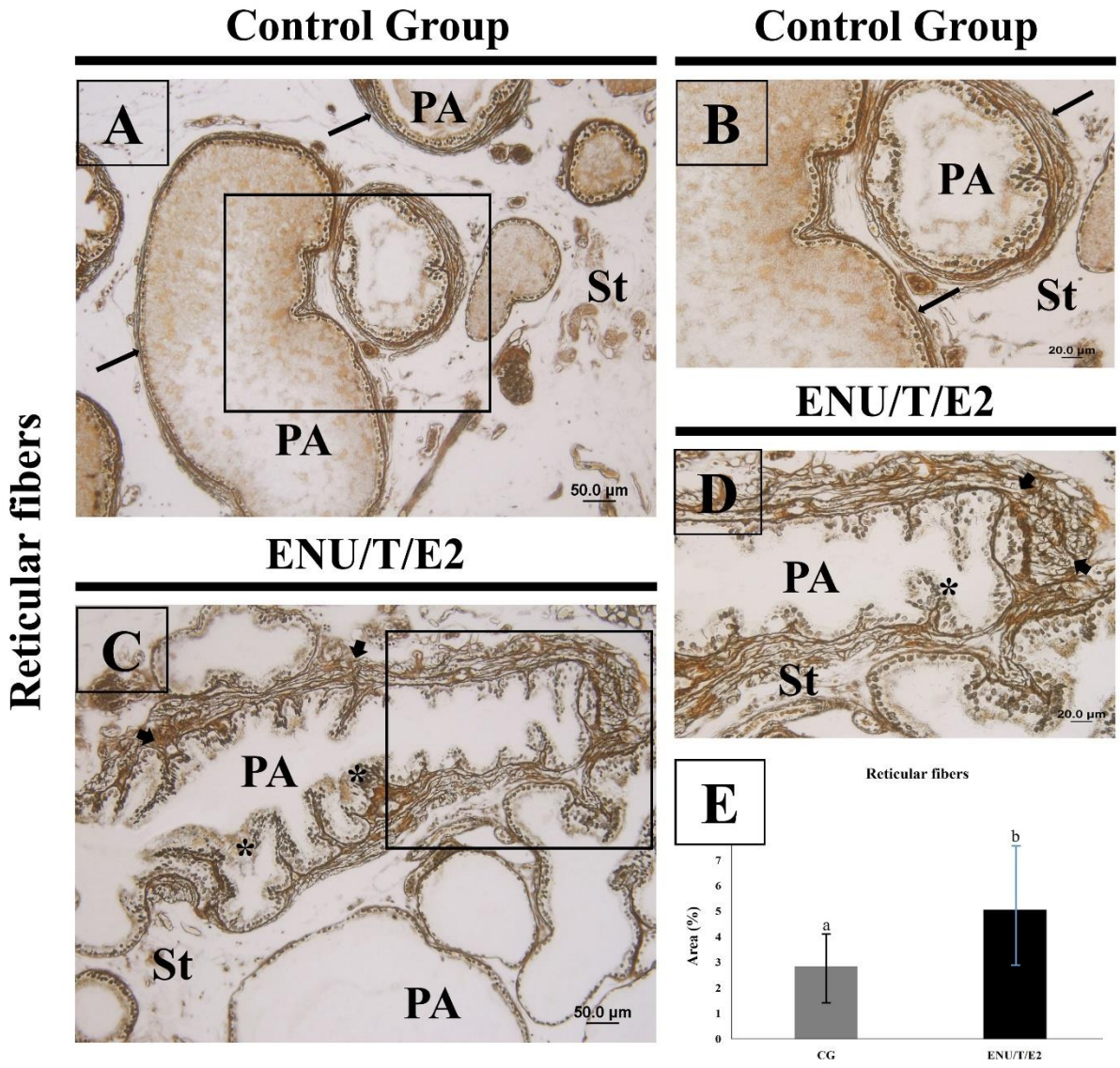

Fig. 3. Gömöri stain performed on histological sections of the prostate of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) In the control group, the normal phenotype of the reticular fiber network is organized in parallel around the alveoli in the stroma, (C, D) whereas in the treated group the fibrillar components are disorganized, which is associated with proliferative focus. Some of the fibers lost their orientation, becoming perpendicular to the alveoli and fragmented in several areas. (E) The quantification data showed a significant increase in the reticular fibers area in the treated group. PA (Prostate alveoli), St (Stroma), Arrow (Reticular fibers), Arrowhead (Fragmentation of the reticular fibers), * (Hyperplasia foci). Different letters (a, b) indicate significant statistical differences between groups.

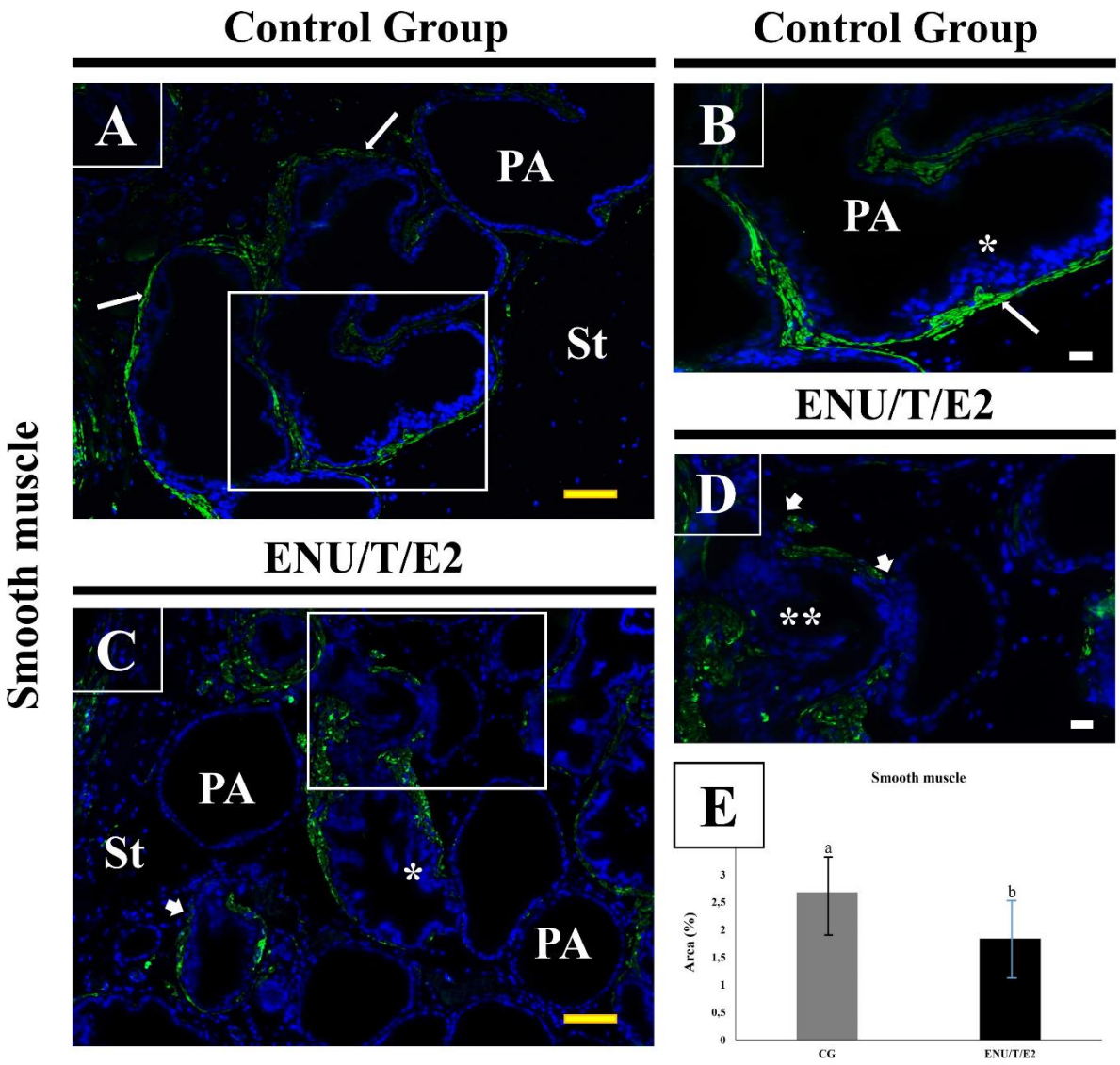

Fig. 4. Immunofluorescence assays for α-Actin performed on histological sections of the prostate of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) A well-defined smooth muscle layer surrounds the prostatic alveoli in the control group. (C, D) In the ENU/T/E2 group, there are areas of discontinuity in the perialveolar smooth muscle. (E) The quantification data revealed a significant decrease in smooth muscle area in the treated group compared to the control group. PA (Prostate alveoli), St (Stroma), Arrow (Well-defined smooth muscle layer), Arrowhead (Discontinuity of the smooth muscle layer), * (Hyperplasia foci), ** (Low-grade PIN foci), Yellow bar (50 μm), White bar (20 μm). Different letters (a, b) indicate significant statistical differences between groups.

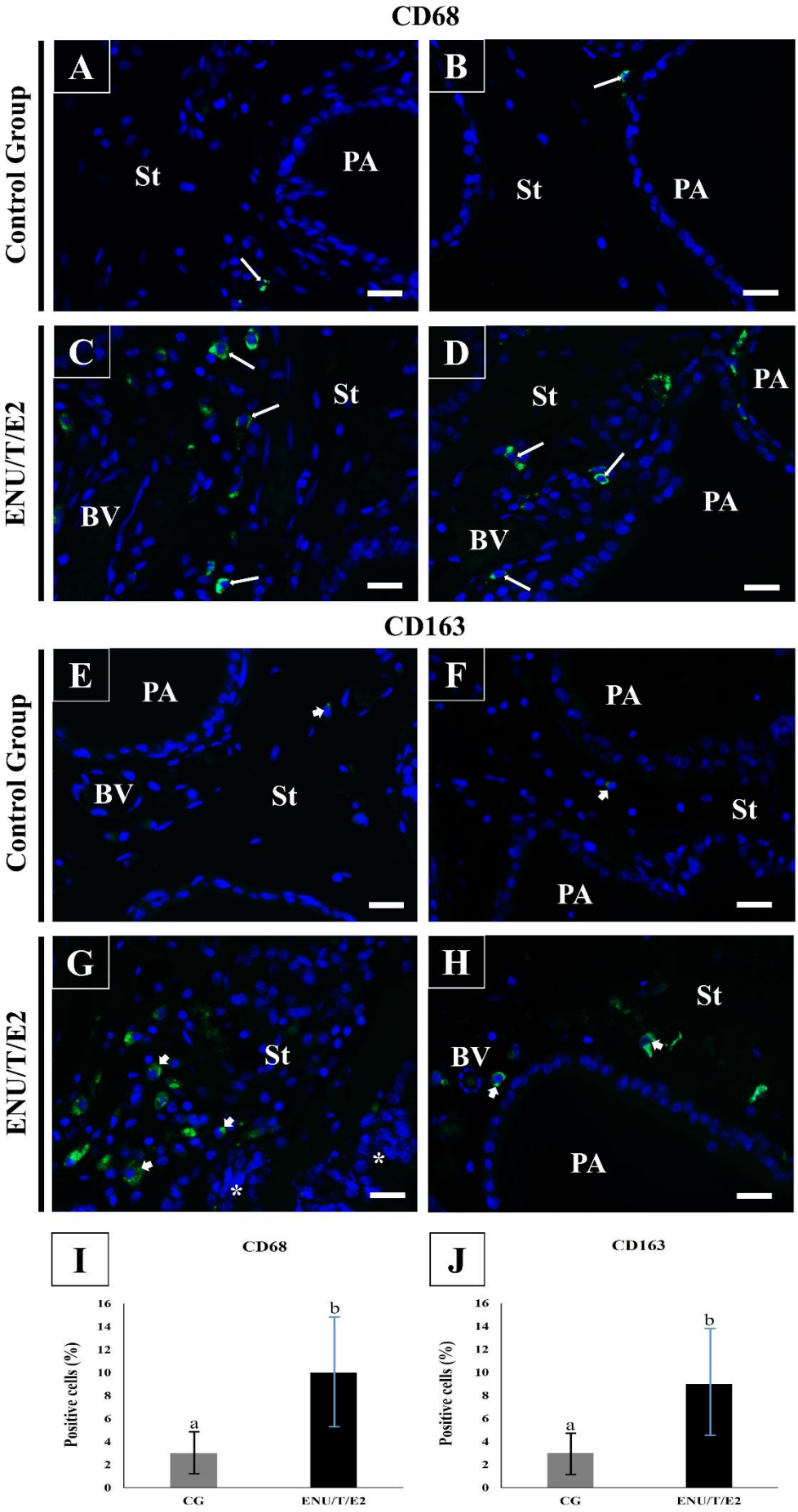
CD68
Control Group
A
St
PA
B
St
PA
ENU/T/E2
C
St
BV
D
St
PA
PA
BV
CD163
Control Group
E
PA
BV
St
F
PA
St
PA
ENU/T/E2
G
St
*
*
H
St
BV
PA
I
CD68
Positive cells (%)
16
14
12
10
8
6
4
2
0
a
b
CG
ENU/T/E2
J
CD163
Positive cells (%)
16
14
12
10
8
6
4
2
0
a
b
CG
ENU/T/E2

Fig. 5. Immunofluorescence assays for CD68 and CD163 performed on histological sections of the prostate of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) In the stroma of the control group, a few CD68-positive stromal cells can be verified between the alveoli. (C, D) In the treated group, the CD68 immunostaining is increased in the interalveolar region. (E, F) The same pattern is observed for the CD163 marking, in which some dispersed cells were positive in the control group, whereas many cells were positive for this factor in the group treated with ENU/T/E2 in the interalveolar region (G, H). (I) There was a significant increase in the number of CD68-positive cells in the treated group compared to the control group, and the same pattern can be seen for the CD163-positive cells (J). PA (Prostate alveoli), St (Stroma), BV (Blood vessels), Arrow (CD68-positive cells), Arrowhead (CD163-positive cells), * (Hyperplasia foci), White bar (20 µm). Different letters (a, b) indicate significant statistical differences between groups.

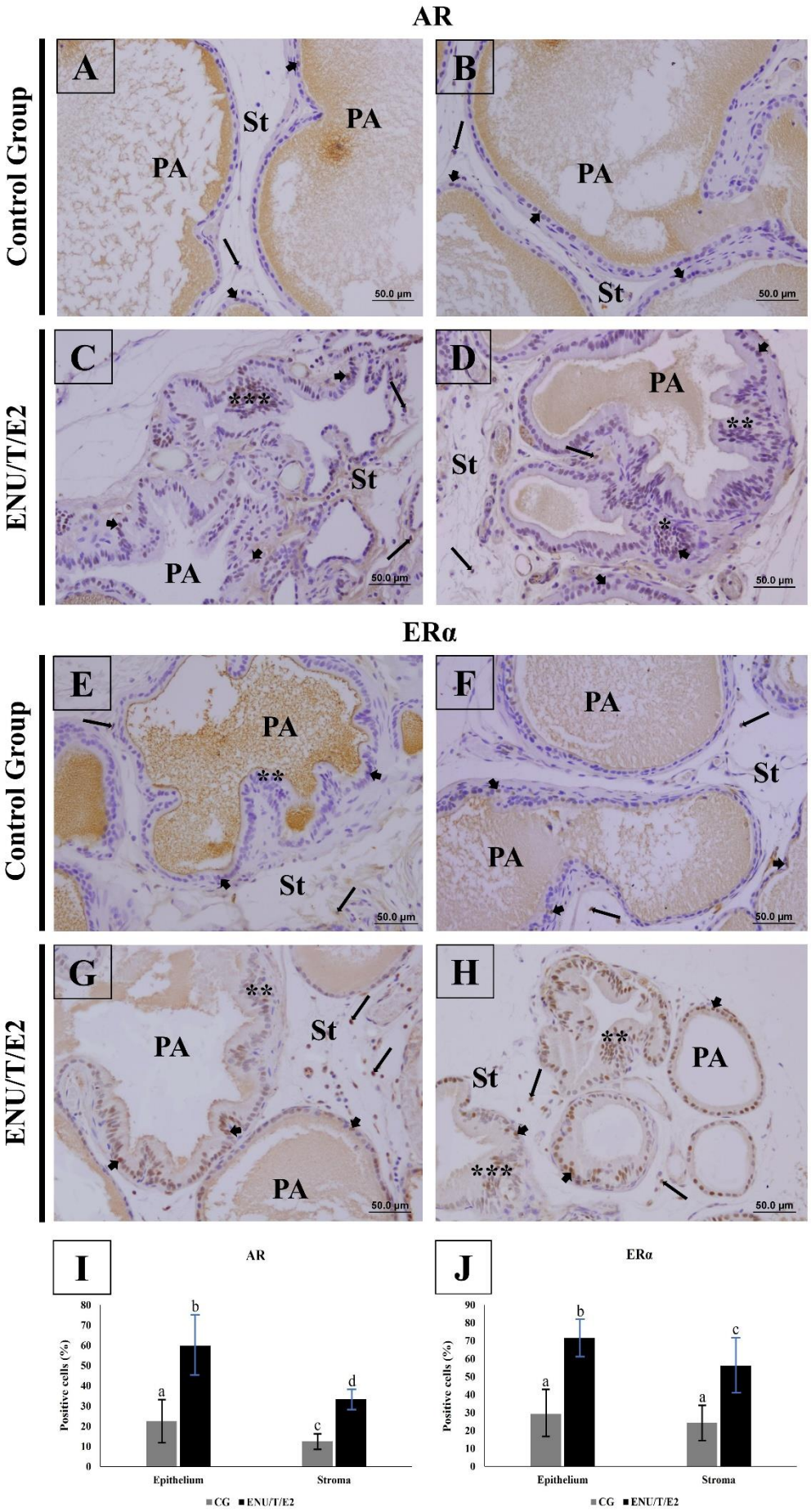
AR
Control Group
ENU/T/E2
A
B
C
D
St
PA
50.0 µm
ERα
E
F
G
H
I
J
Positive cells (%)
Epithelium
Stroma
CG
ENU/T/E2

Fig. 6. Immunohistochemical assays for AR and ERα performed on histological sections of the prostate of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) AR-positive cells are found in the epithelium and stroma of the gland in the control group. (C, D) In the treated group, there is an increase in the presence of the AR-positive cells both in the epithelium and stroma, especially in areas with proliferative aggregates. (E, F) In the control group, ERα-positive cells can be seen in the prostate epithelium and stroma. (G, H) An increase in this factor is observed in proliferative and non-proliferative areas of the epithelium as well as in the interalveolar stroma in the treated group. (I) The quantification data showed a significant increase in the percentage of AR-positive cells in both epithelial and stromal prostate compartments in the group treated with ENU/T/E2 compared to the control group. (J) The same can be observed for the ERα immunostaining. PA (Prostate alveoli), St (Stroma), Arrow (Positive-stromal cells for each factor), Arrowhead (Positive-epithelial cells for each factor), * (Hyperplasia foci), ** (Low-grade PIN foci), *** (High-grade PIN foci). Different letters (a, b, c, d) indicate significant statistical differences between groups.

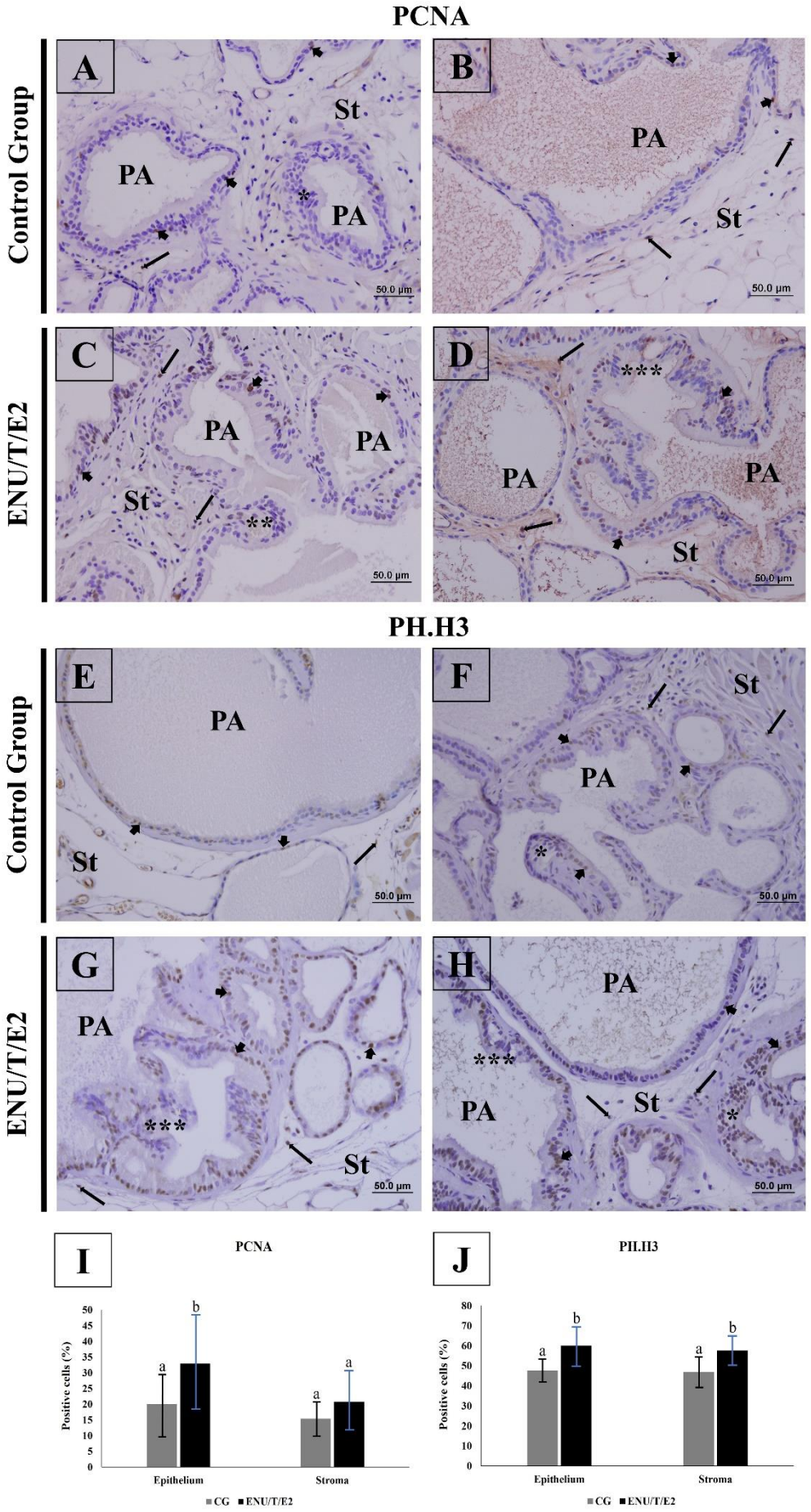
PCNA
Control Group
ENU/T/E2
A
B
C
D
St
PA
50.0 µm
PH.H3
E
F
G
H
I
PCNA
J
PII.II3
Positive cells (%)
Epithelium
Stroma
CG
ENU/T/E2

Fig. 7. Immunohistochemical assays for PCNA and PH.H3 performed on histological sections of the prostate of female Mongolian gerbils from control and ENU/T/E2-treated groups. (A, B) In the control group, PCNA-positive cells can be observed in the prostate epithelium and interalveolar region. (C, D) An increase in PCNA-positive cells is observed in the epithelial compartment, yet not in the interalveolar region in the treated group. (E, F) PH.H3-positive cells can be found in both prostate compartments in the control group. (G, H) In the group treated with ENU/T/E2, there is an increase in the presence of these factors both in the epithelium and stroma. (I) The quantification data showed a significant increase in the percentage of PCNA-positive cells in the epithelium, even though the stroma did not present significant differences in the treated group compared to the control group. (J) There was a significant increase in the number of PH.H3-positive cells both in the epithelium and stroma. PA (Prostate alveoli), St (Stroma), Arrow (Positive-stromal cells for each factor), Arrowhead (Positive-epithelial cells for each factor), * (Hyperplasia foci), ** (Low-grade PIN foci), *** (High-grade PIN foci). Different letters (a, b) indicate significant statistical differences between groups.

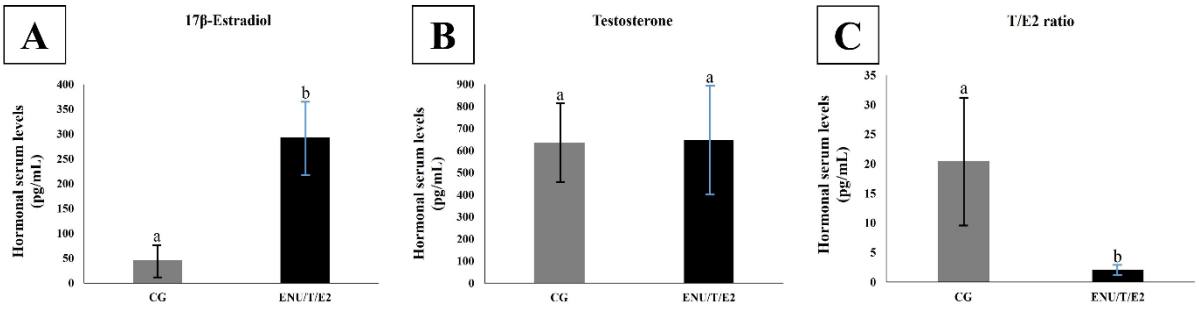

Fig. 8. Serological data of 17β-Estradiol and Testosterone serum levels of female Mongolian gerbil from control and ENU/T/E2-treated groups. (A) There was a significant increase in the hormonal serum levels of 17β-Estradiol in the group treated with ENU/T/E2 compared to the control. (B) As for the Testosterone hormonal serum levels, there were no significant statistical differences between the groups. (C) The T/E2 ratio showed a significant statistical decrease in the treated group in comparison to the control. Different letters (a, b) indicate significant statistical differences between groups.

III.2. Artigo 2

The complex role of telocytes in female prostate tumorigenesis in a rodent model

Juliana S Maldarine[a], Bruno D A Sanches[a], Vitória A Santos[b], Rejane M Góes[b], Patricia S L Vilamaior[b], Hernandes F Carvalho[a], Sebastião R Taboga[a,b]

[a]Department of Structural and Functional Biology, Institute of Biology, State University of Campinas (UNICAMP), Bertrand Russel Av., Campinas, São Paulo, Brazil.

[b]Universidade Estadual Paulista – UNESP, Department of Biological Sciences, Laboratory of Microscopy and Microanalysis, Cristóvão Colombo St., 2265, São José do Rio Preto, São Paulo, Brazil

Correspondence to: Dr. Sebastião R. Taboga, Department of Biology, Laboratory of Microscopy and Microanalyses, 2265 Cristóvão Colombo Street, São José do Rio Preto, São Paulo, e-mail: sebastiao.taboga@unesp.br

Conflict of Interest Statement: The authors declare that there are no conflicts of interest.

Ethics approval statement: The experiments were carried out following the ethical principles recommended by the National Council for Animal Experimentation Control (CONCEA) and the procedures involved were approved by the Ethics Committee on the Use of Animals at IBILCE/UNESP (Proc. No. CEUA-209/2019).

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

This dissertation text is adapted from the manuscript published in the Scientific Journal *Cell Biology International*

III.2.1. Abstract

The prostate is not an organ exclusive to the male. It is also found in females of several species, including humans, which is homologous to the male prostate. Evidence is accumulating that stromal alterations are central to tumorigenesis. Equally, telocytes, a recently discovered type of interstitial cell, are necessary for the maintenance of stromal organization. However, it is still uncertain whether there are telocytes in the female prostate and if they play a role in a tumor microenvironment. The present study used ultrastructural and immunofluorescence techniques to investigate the telocytes in the Mongolian gerbil female prostate, a rodent model that often has a functional prostate in females, in a model of induced-tumorigenesis with N-ethyl-N-nitrosurea (ENU), testosterone and estradiol. The results point to the presence of telocytes in the female prostate in the perialveolar and interalveolar regions, and reveal that these cells are absent in regions adjacent to benign and premalignant lesions, in which the perialveolar smooth muscle is altered. Additionally, telocytes are also closely associated with infiltrated immune cells in the stroma. Our data suggest that telocytes are important for both the maintenance of smooth muscle and prostatic epithelium integrity, which indicates a protective role against the advancement of tumorigenesis. However, telocytes are also associated with immune cells and a pro-inflammatory/pro-angiogenic role for these cells cannot be ruled out, implying that telocytes have a complex role in prostatic tumorigenesis in females.

Keywords: Reactive stroma, angiogenesis, inflammation, stromal remodelling, smooth muscle cell, ENU, PIN, hyperplasia, estradiol, testosterone.

Abbreviation List

ENU (N-ethyl-N-nitrosourea), RSH (Reactive Stroma Hypothesis), PIN (Prostatic Intraepithelial Neoplasia), CD34 (Cluster of Differentiation 34), HE (Haematoxylin-Eosin), E2 (17-β-estradiol), α-SMA (α-Smooth Muscle Actin), CAFs (Cancer-associated fibroblasts), IgG1 (Immunoglobulin G1), CD163 (Cluster of Differentiation 163) TNFR1 (Tumor Necrosis Factor Receptor 1) PBS (Phosphate-buffered Saline), DAPI (4′,6-Diamidino-2-Phenylindole), FITC (Fluorescein Isothiocyanate), PFA (Paraformaldehyde), BSA (Bovine Serum Albumin),

T (Testosterone), FAP (Fibroblast Activation Protein), pMSCs (Perivascular-resident Mesenchymal Stem Cells), TGFβ1 (Transforming Growth Factor β1)

III.2.2. Introduction

Interrelationships between the stroma and the epithelium of the prostate are not only important during development, but also in the maintenance of the adult gland and in the installation of pathological conditions, including cancer (Cunha, 1984; Marker *et al*., 2003; Cunha *et al*., 2018; Le *et al*., 2020; Tyekucheva *et al*., 2017; Bahmad *et al*., 2021). Tumorigenesis in the prostate has been shown to be progressively associated with many changes in stromal cells, with the appearance of altered fibroblasts, CAFs (Cirri & Chiarugi, 2012; Bonollo *et al*., 2020; Gunaydin, 2021) and myofibroblasts or α-SMA + CAFs (Krystyna *et al*., 2019; Liu *et al*., 2019). The extracellular matrix has also been characterized in these situations (Stewart *et al.*, 2004; Nallanthighal *et al*., 2019). This altered stroma affects the prostatic epithelium, because it stimulates and supports the progression of tumorigenesis. This concept was synthesized in the Reactive Stroma Hypothesis (RSH) (Tuxhorn, Ayala, Rowley, 2001). Though the majority of studies related to the RSH concerned the male prostate, there is a growing number of studies indicating a functional prostate is found in females of different species (Gross & Didio, 1987; Flamini *et al*., 2002; Santos & Taboga, 2006; Aguiar *et al*., 2013). In women (Zaviacic *et al*., 2000; Biancardi *et al*., 2017), the gland is often known as Skene gland, and is homologous to the prostate in men (Wernert *et al*., 1992; Dietrich *et al*., 2011). Despite being smaller and located in the middle part of the urethra (Costa *et al*., 2016), there are indications that the prostate in women can also develop pathological conditions, such as inflammation (Gittes & Nakamura, 1996) and cancer (Pongtippan *et al*., 2004; Thum *et al*., 2017). Some of the tumours detected in the urethra have a prostatic origin in women (Reis *et al*., 2011; Muto *et al*., 2017).

Regarding the study of female prostates, the Mongolian gerbil has been a good option as it is a rodent model with a high frequency of functional prostate in females (Santos & Taboga, 2006). Additional work has shown that aging (Custodio *et al*., 2010) and exposure to endocrine disruptors during development (Perez *et al*., 2011, 2016; Campos *et al*., 2015) promote changes in the female prostate resembling those typically found in the male prostate,

such as the presence of benign/pre-malignant lesions, foci of hyperplasia and PIN, concomitant with inflammatory infiltrates. However, a model of exposure of the female prostate to a carcinogenic agent, such as ENU, which is successful in inducing tumours in the mammary glands, lungs and testicles, among other organs, has not yet been evaluated (Stoica, Koestner, Capen, 1983; Cordes, 2005; Bodakuntla *et al*., 2014; Dai *et al*., 2019). In this respect it is also possible that this agent may accelerate the formation of a reactive stroma in the female prostate and constitute a useful tool for the study of prostate tumorigenesis in females. It is noteworthy that, despite the similarities, the prostate in the female gerbil has some marked differences to that of the male, such as increased dependency on progesterone (Fochi *et al*., 2013) and estradiol (Sanches *et al*., 2016a, 2017a). The female prostate is also more susceptible to endocrine disruptors, so that concentrations of some compounds that affect the female prostate would not affect the male prostate (Maldarine *et al*., 2019, 2020; Sanches *et al.*, 2019).

During the last decade, the detection of telocytes revealed that the prostatic stroma is more complex than was previously thought (Corradi *et al*., 2013). Telocytes are interstitial cells that were first described by Popescu and Faussone-Pellegrini (2010), comprising a set of CD34-positive interstitial cells detected in different tissues that were very similar to the interstitial Cajal cells. However, they have particular morphological differences, such as the presence of thin moniliform cytoplasmic extensions (telopodes), in addition to caveolae. These cells have been detected in the stroma of several organs such as the heart (Gherghiceanu & Popescu, 2012; Kostin, 2016), liver (Xiao *et al*., 2013; Liu *et al*., 2016), kidneys (Zheng *et al.*, 2012), mammary glands (Petre *et al*., 2016; Sanches *et al*., 2020a), and testes (Marini *et al*., 2018a; Liu *et al*., 2019), among others. Although there is no single function for these cells, their role in the organization of the stroma is especially notable (Sanches *et al*., 2021a, 2021b). As in other organs, prostatic telocytes spread their telopodes throughout the stroma (Corradi *et al*., 2013). These extensions connect the telocytes to each other and to other cell types, such as smooth muscle cells, fibroblasts, endothelial cells, nervous cells, and immune cells, among others (Sanches *et al*., 2021b). In the prostate, as in the myocardium (Bani *et al.*, 2010), digestive tract (Liang *et al*., 2019), uterus (Roatesi *et al*., 2015; Kagami *et al*., 2020) and other organs (Marini *et al*., 2018b), telocytes exist in close association with muscle cells. Particularly in the prostate, telocytes involve the smooth muscles and seem to be essential for the maintenance of these layers, preserving their integrity as the prostatic alveoli dilate (Sanches *et al.*, 2020b). Nevertheless, castration experiments, which generate a hypoandrogenic

environment, have shown that loss of the telopode network of telocytes occurred together with the disorganization of the smooth muscle around the alveoli. Accordingly, recovery of this telopode network via testosterone supplementation occurred concomitantly with the reorganization of the smooth muscle layers (Felisbino *et al*., 2019).

Although knowledge about telocytes in the prostate has advanced, the possible role of these cells in tumorigenesis is still uncertain. Given that telocytes are important for the organization of the stroma, it can be speculated that their telopode network must be altered in the reactive stroma onset, but data in this regard are still lacking in the literature. In other organs, such as the testicles, it is known that the absence of telocytes would lead to the complete loss of tissue organization in seminoma, a type of testis cancer (Marini *et al*., 2019). So, it is reasonable to assume that the telocytes would exert a counterforce to the advance of stromal alterations that are concomitant with tumorigenesis. However, it is also important to take into account the fact that telocytes also have a considerable immune role and may act as either a pro-inflammatory (Chi *et al*., 2015; Jiang *et al*., 2018) or an anti-inflammatory agent (Ibba-Manneschi *et al.*, 2016; Wang *et al*., 2020). In the prostate, evidence suggest that these cells contribute to angiogenesis and are sensitive to pro-inflammatory factors (Hussein & Mokhta, 2018; Soliman *et al.*, 2021; Sanches *et al.*, 2020b), which was also verified for lungs and other organs (Manole *et al*., 2011; Mitrofanova *et al*., 2020). Regarding reactive stroma, it is interesting to note that there is an increase in the amount of CD34-positive cells at the onset of stromal alterations (San Martin *et al*., 2014) and that these cells may be bone marrow-derived mesenchymal progenitor cells or even telocytes (Sanches *et al*., 2021b).

As telocytes also exist at the interface between prostate epithelium and stroma, it is tempting to speculate that these cells could be the first to undergo changes in the initial phase of tumorigenesis, because they secrete paracrine factors (Sanches *et al*., 2016b). Therefore, the present study made use of a model of induced tumorigenesis in prostates of Mongolian gerbil females to evaluate possible alterations that telocytes may undergo and a role for these cells in the reactive stroma initiation.

III.2.3. Material and Methods

Animals and tumorigenic induction

Gerbil adult females of six-months old were housed in polyethylene cages in 25°C room temperature, with unlimited access to filtered water and food. The experiments followed the ethical guidelines of São Paulo State University (UNESP) (CEUA-209/2019). The animals were euthanized by subcutaneous injection of ketamine (100 mg/kg), and xylazine (11 mg/kg), and then decapitated.

To induce tumorigenesis and the formation of reactive stroma in the female prostate, something that has not yet been reported in the literature, we adopted a methodology adapted from Gonçalves and collaborators (2013) of tumorigenesis induction in the male prostate. In our protocol, we used a high dose of testosterone cypionate (20 mg/kg), followed after three days by exposure to a single dose of the carcinogen N-ethyl-N-nitrosourea (ENU; Chem Service, West Chester, PA-USA) (100 mg/kg), followed by alternating weekly exposure to 17 β-estradiol (2 mg/kg) and testosterone cypionate (2 mg/kg) over 24 weeks. The control group was established with the same methodology, but with the use of corn oil (100 µl/dose) in all applications (Fig. 1).

Histological processing

The prostates were fixed in 4% PFA, washed in H_2O, dehydrated, clarified and then embedded in paraffin. The glands were sectioned at 5 µm and mounted on histological slides. Some of these slides were submitted to the Haematoxylin-Eosin (HE) for general histological analysis and to Picrosirius Red staining for the detection of collagen fibres (Junqueira *et al*., 1979).

Collagen fibers quantification

Some female prostate sections were stained with Picrosirius Red without Haematoxylin to measure the area covered by collagen fibers at a magnification of 200X using ImageJ with a protocol adapted from Felix-Patricio and coworkers (2017). The fibers were also observed under polarized light at the same magnification to better observe its organization, in

which the natural birefringence of collagen fibers is contrasted to a dark field (Coelho *et al*., 2018).

Transmission electron microscopy (TEM)

The ultrastructural data was obtained following Sanches and coworkers' protocol (2020b), in which fragments of female prostates from the control and ENU/T/E2-treated groups were cut and fixed in 3% glutaraldehyde and 0.25% tannic acid solution in Millonig buffer (pH 7.3) together with 0.54% glucose for one day. The samples were then fixed with 1% osmium tetroxide for 1 h, then dehydrated, and embedded in Araldite resin. Ultrathin sections (50–75 nm) were prepared with a diamond knife and stained with 2% alcoholic uranyl acetate for 30 min, and then with 2% lead citrate in a 1 M sodium hydroxide solution for 10 min. The samples were analyzed in a Hitachi HT 7800 at 80 kV electron microscopy.

Immunofluorescence of paraffin-embedded tissue sections

The prostate samples were fixed in 4% PFA (pH 7.4) for 24 hrs. After fixation, the tissues were washed, dehydrated, embedded in paraffin, and then sectioned at 5 µm using a microtome. The antigens were exposed by heat treatment for 20 min at 95°C in citrate buffer. Blocking of non-specific bindings was performed in half an hour, and the slides were washed three times for 5 min each in PBS with 0.3% Triton- X between each step. Immunofluorescence assays were performed for α-SMA (monoclonal mouse, IgG1κ, B4, sc-53142, Santa Cruz Biotechnology), to observe possible alterations in smooth muscle organization in the prostate. To detect myofibroblasts or α-SMA+ CAFs, double immunofluorescence assays were performed for α-SMA/vimentin (monoclonal mouse, IgG1κ, B4, sc-53142, Santa Cruz Biotechnology/ polyclonal rabbit, 5741S, Cell Signalling Technology). To verify telocytes in the adult female prostate from the control and the ENU/T/E2-treated groups, double immunofluorescence assays for CD34/CD31 (monoclonal mouse, IgG1κ, B-6, sc-74499 FITC; monoclonal mouse, IgG1κ, H-3, sc-376764 AF594, Santa Cruz Biotechnology) were carried out.

Finally, in order to observe elements of the reactive stroma, immunofluorescence assays were performed for the CAF marker FAP (monoclonal mouse, IgG1, F11-24, sc-65398,

Santa Cruz Biotechnology, Dallas, TX, USA), the activated macrophage marker CD163 (monoclonal mouse, IgG1κ, GHI/61, sc-20066, Santa Cruz Biotechnology), and the inflammation-associated receptor TNFR1 (polyclonal rabbit, IgG, H-271, sc-7895; Santa Cruz Biotechnology). The antibodies were incubated overnight at a dilution of 1:100. In the next morning, the slides incubated with fluorophore-conjugated antibodies were washed three times with PBS and were stained with DAPI (F36924; Life Technology). For the antibodies that were not fluorophore-conjugated, the slides were subjected to secondary antibody incubation, in which they were incubated with FITC-labelled goat anti-mouse (sc-2011; Santa Cruz Biotechnology) and goat anti-rabbit Texas Red-labelled (sc-2780; Santa Cruz Biotechnology) at 1:100 dilution in 1% bovine serum albumin (BSA) for 2 hr at room temperature, washed in PBS and finally stained with DAPI (F36924; Life Technology). The analysis was made with a ZeissImager M2 fluorescence microscope.

Statistical analysis

Data were initially evaluated using two-sample t-Test. Significance was set at $P<0.05$. All statistical tests were performed using Statistica 7.0 (StatSoft).

III.2.4. Results

Morphological analysis

In the control group, normal gland epithelium and stroma could be observed, in which the prostatic alveoli were surrounded by a well-defined layer of smooth muscle and the prostatic epithelium varied from cubic to pseudostratified (Fig. 2A, B). In the group treated with ENU/T/E2, alterations in the epithelium and stroma of the prostate became more frequent, especially with regard to the occurrence of foci of hyperplasia and PIN, in which the prostatic epithelium became stratified and the smooth muscle layers lost their characteristic organization and extended between the alveoli (Fig. 2C, D). Collagen fibers are organized around the alveoli in the control group (Fig. 2E). These fibers are red under polarized light which indicates a greater density (Fig. 2F). In the treated group, picrosirius staining showed the expansion of collagen fibers deposition in the interalveolar stroma (Fig. 2G). Under polarized light, it was

possible to verify both regions of thickening and discontinuity of the collagen matrix in the periphery of alveoli (Fig. 2H). However, although there was a visible increase in the collagen fibers area in the treated group compared to the control group, it was not quantitatively significant (1,2 ± 0,45 % and 0,51 ± 0,12 %, respectively) (Fig. 2I).

Ultrastructure

The ultrastructure data revealed telopode's aspect, which were divided into dilated sections, the podoms, and filament-like sections, the podomers (Fig. 3A, B). It was possible to verify the soma of a spindle-shaped telocyte. Telocytes were found to exist both in the periepithelial region and in the periphery of the smooth muscle cells, enveloping them (Fig. 3C). In addition, telocytes were also found in association with blood vessels in the interalveolar region (Fig. 3D). In the group treated with ENU/T/E2, in regions of unaltered epithelium, the perialveolar muscle appeared discontinuous, but it was still possible to observe the occurrence of telocytes (Fig 3E). However, in the periepithelial stroma adjacent to the hyperplasia foci, there were no telocytes (Fig. 3F). Nor were telocytes found in the stroma adjacent to PIN foci (Fig. 3G, H). Interestingly, it was possible to verify that a telocyte is closely associated with the prostatic epithelium in a region of discontinuity of the perialveolar smooth muscle (Fig. 3I, J).

Ultrastructural data from the female prostate of Mongolian gerbils from the control group and the ENU/T/E2-treated group also showed the relationship between telocytes and immune cells. In normal tissue, telocytes can be seen in the stroma in the vicinity of eosinophils (Fig. 4A, B) and macrophages (Fig. 4C). In tissue treated with ENU/T/E2 there were infiltrated leukocytes occupying part of the stroma between the alveoli (Fig. 4D). Telocytes maintained contact with macrophages in the perialveolar region (Fig. 4E, F), and with lymphocytes in the periphery of blood vessels (Fig. 4G, H). Telopode networks were in contact with macrophages in the periphery of the prostatic epithelium in regions where the smooth muscle layer was discontinuous (Fig. 4I).

Immunofluorescence assays

In the control group, there was a thin layer of smooth muscle surrounding the alveoli (Fig. 5A, B). In the group treated with ENU/T/E2, the perialveolar smooth muscle was altered,

with either discontinuities or thickenings, in hyperplasia foci (Fig. 5C), and such changes were also observed in PIN foci (Fig. 5D). In the control group there was no overlap of α-SMA and vimentin in the perialveolar smooth muscle region. However, overlapping was seen in the interalveolar region, which characterizes the typical pattern of myofibroblast (Fig. 5E). Fibroblasts were also seen in the stroma, indicated by vimentin-positive cells (Fig. 5F). In the ENU/T/E2-treated group, both in regions in which the smooth muscle surrounding the alveoli becomes thickened, and in regions of discontinuity around the PIN foci, there were also vimentin-positive cells, which is suggestive of fibroblasts (Fig. 5G). The colocalization of α-SMA and vimentin could be seen in the interalveolar region, indicating the presence of myofibroblasts. Furthermore, regions of discontinuity could be seen in the smooth muscle layers that surrounded the alveoli (Fig. 5H).

In the control group, fibroblast-like CD34-positive cells that did not co-localize with CD31 were found both in the perialveolar and interalveolar region, which is an immunophenotype indicating the presence of telocytes. In addition, cells showing the colocalization of CD34 and CD31, which are probably bone marrow-derived mesenchymal progenitor cells, could also be seen (Fig. 6A-C). In the group treated with ENU/T/E2, CD34-positive fibroblast-like cells were found near the alveoli, as well as cells showing colocalization between CD34 and CD31 (Fig. 6D). Co-localization of both factors was also confirmed between the alveoli (Fig. 6E). CD34-positive fibroblast-like cells lacking colocalization with CD31 were also found in the interalveolar region, indicating the presence of telocytes. Moreover, cells showing colocalization between CD34 and CD31 could also be seen near these cells (Fig. 6F).

Fibroblast-like FAP-positive cells were found in the stroma between the alveoli in the control group, indicating the presence of CAFs (Fig. 7A, B). These cells were also found in the stroma of the group treated with ENU/T/E2 (Fig. 7C, D). CD163-positive cells were found in the interalveolar stroma of the control group, indicating the presence of activated macrophages (Fig. 7E). These cells were also present in the interalveolar stroma of the treated group (Fig. 7F). TNFR1-positive cells were found both in the stroma of the control (Fig. 7G) and that of the treated group (Fig. 7H), which indicates the occurrence of a pro-inflammatory stimulus in the female prostate in both scenarios. The differences observed between reactive and normal stroma, including the telocytes between stromal cell types, are summarized in a schematic drawing (Fig. 8).

III.2.5. Discussion

Our data showed that adult females submitted to treatment with ENU/T/E2 developed benign lesions, such as hyperplasia foci, and premalignant ones, such as PIN foci. Therefore, the methodology proved to be successful for studying the formation of the reactive stroma and the process of prostatic tumorigenesis in females (Tuxhorn, Ayala, Rowley, 2001; San Martin *et al*., 2014). In addition to changes in the prostatic epithelium, we also observed a thickening or discontinuity in the perialveolar smooth muscle layers around PIN and hyperplasia foci. The collagen matrix was also altered in the treated group showing thickening in the interalveolar region as well as expansion and discontinuities in the perialveolar region. Similar changes were reported in previous studies of the male prostate, in which the collagen matrix was likewise altered in the carcinogen-treated gerbils and mice (Gonçalves *et al*., 2010; Galheigo *et al*., 2016). Together, the morphological data indicate for the first time in the literature that exposure to a carcinogenic agent implies the early process of tumorigenesis in the female prostate and the onset of the reactive stroma.

Ultrastructural analysis evidenced the telocytes in the female prostate for the first time. These cells generally have the same location as in the male prostate, being present in the periepithelial region, involving smooth muscle layers, as well as in the periphery of blood vessels and in the interstitium. In view of this set of evidence, it can be hypothesized that the role of telocytes in the normal female prostate is to give support to stromal tissue organization, which has been proposed previously for prostatic telocytes in males (Sanches *et al*., 2017b). As for the treated group, telocytes are absent from the stroma adjacent to benign lesion foci, such as hyperplasia, and premalignant lesions, such as in PIN. In these cases, the perialveolar smooth muscle becomes either discontinuous or thickened, which indicates that the telocytes, as previously proposed for the male prostate, are important for maintaining prostate smooth muscle layers (Felisbino *et al*., 2019; Sanches *et al*., 2020b).

In general, prostatic telocytes can be considered an important and hitherto unknown agent for the integrity of both the prostate smooth muscle and epithelium, and the loss of these cells in the perialveolar region can be taken as another factor involved in the progression of so-called reactive stroma. This is in line with studies carried out on tumorigenesis in the testes, in which the loss of telocytes is seen along with tumour progression as the testis stroma loses its typical organization (Marini *et al*., 2019). However, in addition to the protective role that

telocytes may play in the perialveolar smooth muscle in the prostatic epithelium, it was found that prostatic telocytes existed in close association with immune cells in both the control and the treated group. This association has been verified in several other organs (Chi *et al*., 2015; Ibba-Manneschi *et al*., 2016; Jiang *et al*., 2018; Wang *et al*., 2020), including the prostate (Sanches *et al*., 2021b), in which telocytes produce pro-angiogenic factors, such as VEGF, and become sensitive to pro-inflammatory factors, since they express TNFR1, with aging (Sanches *et al*., 2020b). In the treated group, telocytes are also in the periphery of leukocyte infiltrates that occupy part of the interalveolar region.

Moreover, telocytes also maintain contact with immune cells, such as lymphocytes, eosinophils and macrophages, in the perialveolar region and in the periphery of blood vessels. In other organs, it has been found that telocytes are capable of activating macrophages (Chi *et al*., 2015; Jiang *et al*., 2018) and can produce pro-inflammatory cytokines (Albulescu *et al*., 2015; Yang, 2016), acting in a pro-inflammatory manner, although they can act in an anti-inflammatory way in cases of fibrosis and scarring (Ibba-Manneschi *et al*., 2016). In view of the pro-inflammatory potential of telocytes, a promoter role of telocytes to the reactive stroma formation/progression cannot be ruled out, since inflammation is one of the main changes verified in prostatic tumorigenesis (De Marzo *et al*., 2007; Sfanos & De Marzo, 2012; De Bono *et al*., 2020). In addition, our data indicate that there is a close association between telocytes and epithelial cells, it is possible to speculate that vesicle exchange between these cells may consist of a mechanism whereby telocytes act maintaining prostate epithelium architecture. The study of these vesicles produced by telocytes and their role in intercellular communication has gained increasing attention in other organs (Fertig *et al*., 2014; Cismasiu & Popescu, 2015; Cretoiu *et al*., 2016), but the precise role of these vesicles is not clear.

Our immunofluorescence data corroborated the presence of telocytes in the perialveolar and interalveolar region, as well as indicating the presence of myofibroblasts between the alveoli, but not in the perialveolar region, suggesting that, adjacent to the hyperplasia and PIN foci, the cells found were in fact smooth muscle cells and not myofibroblasts. In addition, CAFs and macrophages were found in the interalveolar stroma. Together, these data indicate the occurrence of a reactive stroma in the prostate of treated females, despite the fact that activated fibroblasts and macrophages were also found in the control group.

An interesting finding in the female prostate was the presence of CD34-positive and CD31-negative cells that do not present the typical morphology of telocytes and occupy part of the interalveolar stroma. These infiltrated cells are probably bone marrow-derived mesenchymal progenitor cells, such as fibrocytes or other progenitor CD34-positive cells (Barth & Westhoff, 2007; Keeley *et al*., 2009; Sidney *et al*., 2014). In this sense, the evidence that there is an increase in stromal cells that are positive do CD34 in during the progression of tumorigenesis (San Martin *et al*., 2014; Montico *et al*., 2015) does not necessarily indicate an increase in the presence of telocytes, but possibly bone-marrow-derived mesenchymal progenitor cells or even perivascular-resident mesenchymal stem cells (pMSCs). The precise origin of these CD34-positive cells remains unclear, and, in the Microvascular Hypothesis of San Martin and colleagues (2014), their accumulation in the vicinity of perialveolar blood vessels was presumed to be one of the first alterations in the formation of reactive stroma. Furthermore, telocytes were already assumed to be potential sources of myofibroblasts (Díaz-Flores *et al*., 2016). Although this assumption cannot be completely dismissed, our data showed that the telocyte networks remain intact in the interalveolar stroma, where the myofibroblasts were found, which indicates that telocytes are not progenitors of myofibroblasts in the female prostate. However, since telocytes produce TGFβ1 (Sanches *et al*., 2016b, 2017b), they may contribute to the differentiation of bone-marrow-derived mesenchymal progenitor cells into myofibroblasts, which is yet another pathway by which telocytes could contribute to the progression of reactive stroma and prostatic tumorigenesis. Nonetheless, further studies are needed to confirm this.

III.2.6. Conclusion

The present study has demonstrated that the prostate of Mongolian gerbil females is sensitive to the carcinogen ENU and that it undergoes the same changes observed in the male prostate, such as hyperplasia and pre-malignant lesions. These changes occur concomitantly with other typical reactive stroma-associated alterations, such as thickened/interrupted smooth muscle layers, presence of infiltrated leukocytes, CAFs and myofibroblasts. In addition, our research is pioneering in demonstrating the presence of telocytes in the female prostate and showing that these cells have a dual role in relation to reactive stroma onset and the early prostatic tumorigenesis. Firstly, the absence of telocytes in the perialveolar region is verified in

hyperplasia and PIN foci, which indicates that telocytes are important for the integrity of both the smooth muscle layers and the prostatic epithelium. Secondly, telocytes are closely associated with infiltrating immune cells and blood vessels, and it cannot be ruled out that they may have a pro-inflammatory/angiogenic role, especially those present in the interalveolar stroma. Thus, prostatic telocytes may act against the tumorigenesis, through their role in maintaining the integrity of smooth muscle and the epithelium, but they may possibly act in the opposite direction, stimulating inflammation/angiogenesis in the stroma through interactions with infiltrating immune cells.

Declaration of interest: none.

Funding

FAPESP (São Paulo Research Foundation); Contract number: 2018/08945-8 (to BDAS); 2018/23383-6 (to SRT) and 2019/14201-4 (to JSM) and CNPq (National Council for Scientific and Technological Development); Contract number 302938/2020-6 (to SRT).

Acknowledgements

The authors are grateful to FAPESP and CNPq for the fundings. Also, to Luiz Roberto Falleiros Jr. for technical support, as well as all coworkers at the Laboratory of Microscopy and Microanalysis (LMM).

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III.2.8. Figures and legends

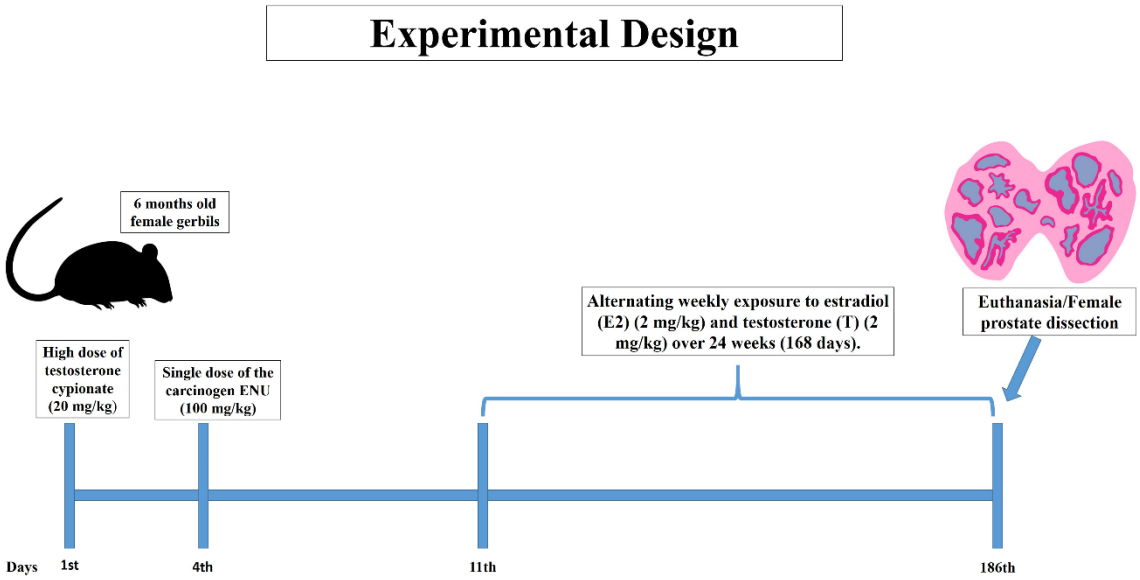

Fig. 1. Experimental design. Six-months-old female gerbils (*n* = 6) were exposed to a high dose of testosterone cypionate (20 mg/kg), followed after three days by exposure to a single dose of the carcinogen N-ethyl-N-nitrosourea (100 mg/kg), followed by alternating weekly exposure to 17-β-estradiol (2 mg/kg) and testosterone cypionate (2 mg/kg) over 24 weeks. The same methodology was established in the control group (*n* = 6), but with the use of corn oil (100 µl/dose) in all applications.

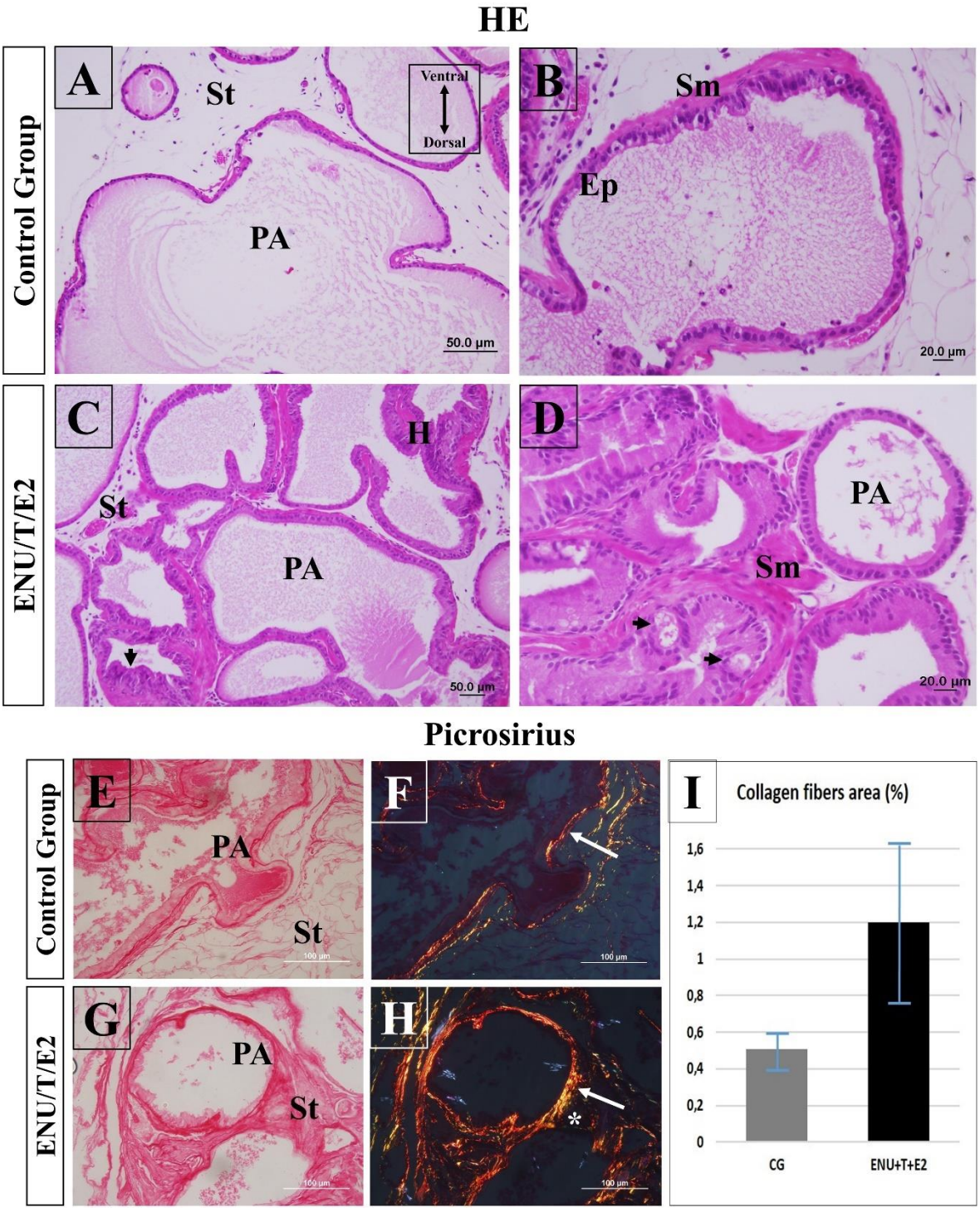

Fig. 2. Histological sections stained with HE and with Picrosirius of Mongolian gerbil female prostates from the control and the ENU/T/E2-treated group. (A, B) In the control group the normal prostatic epithelium, which varies from cubic to pseudo-stratified, can be observed, and a well-defined smooth muscle layer surrounds the prostatic alveoli in the stroma. (C, D)

Alterations in the epithelium and stroma become more frequent in the group treated with ENU/T/E2, especially with regard to the occurrence of hyperplasia and PIN foci, in which the prostatic epithelium becomes stratified, and the smooth muscles layers lose their characteristic organization and extend between the alveoli. (E) In the control group, Picrosirius staining shows the organization of the collagen fibers of the normal gland, with the occurrence of organized fibers surrounding the prostatic epithelium. (F) The same picrosirius image is observed under polarized light, in which a network of collagen fibers is verified in the perialveolar region, these are red under polarized light which indicates greater density of collagen in this region. (G) On the other hand, in the treated group, picrosirius staining shows the expansion of collagen fibers deposition in the interalveolar stroma. (H) The same picrosirius image is observed under polarized light, in which it is possible to verify both regions of thickening and discontinuity of the collagen matrix in the periphery of alveoli. (I) The quantification of the area covered by collagen fibers showed no statistically significant difference between the groups despite the increase verified in the ENU/T/E2 treated group. PA (Prostate alveoli), St (Stroma), Ep (Epithelium), Sm (Smooth muscle), H (Hyperplasia foci), Arrow (Collagen fibers in the perialveolar region), Arrowhead (PIN foci), Asterisk (Collagen matrix discontinuity in the perialvelar region).

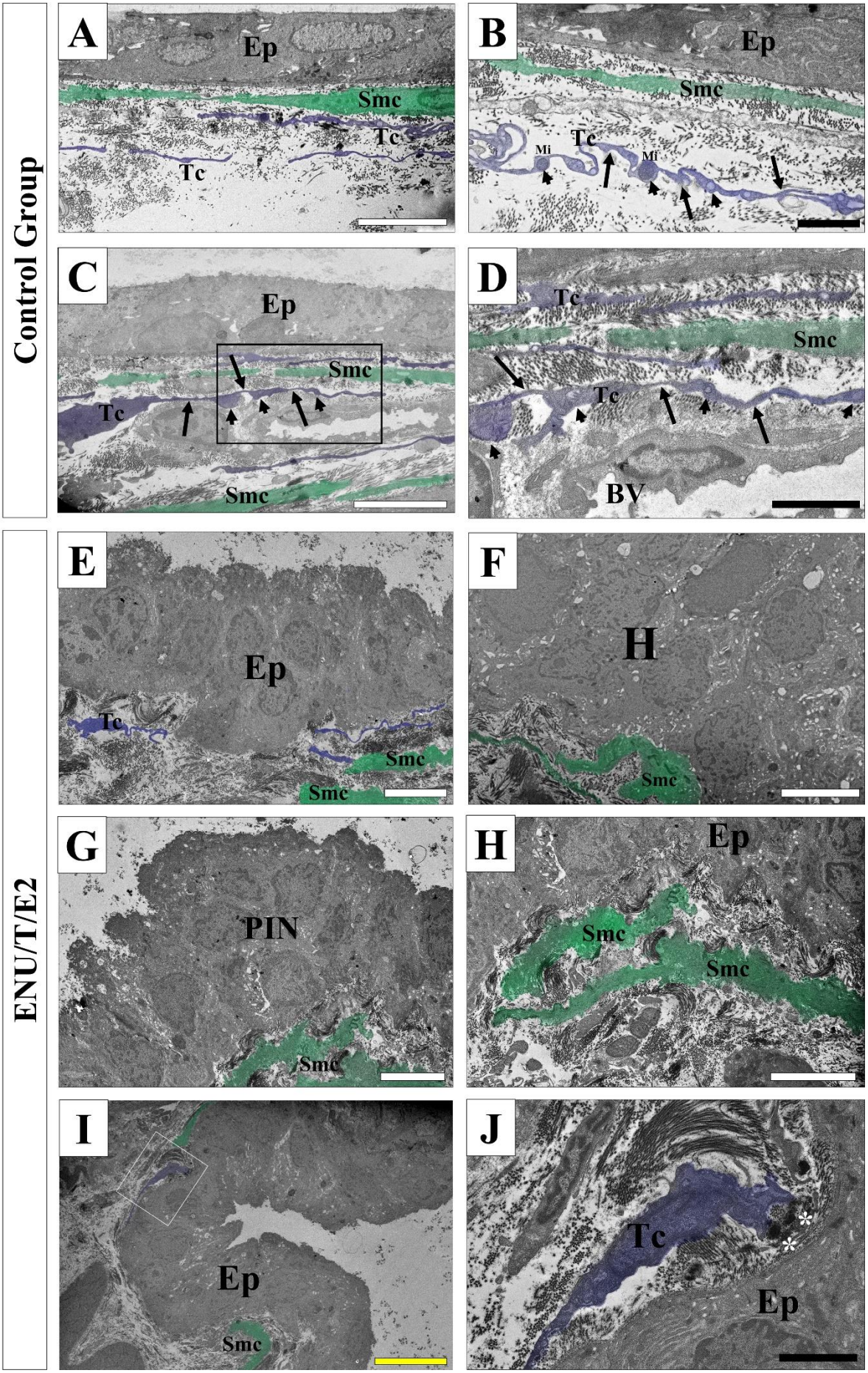
Control Group
A
Ep
Smc
Tc
Tc
B
Ep
Smc
Tc
Mi
Mi
C
Ep
Smc
Tc
Smc
D
Tc
Smc
Tc
BV
ENU/T/E2
E
Ep
Tc
Smc
Smc
F
H
Smc
G
PIN
Smc
H
Ep
Smc
Smc
I
Ep
Smc
J
Tc
Ep

Fig. 3. Ultrastructural data of the prostate of female Mongolian gerbils from the control and the ENU/T/E2 treated group. (A, B) Telocytes are seen next to perialveolar smooth muscle in the control group. It is possible to observe the telopodes, which are divided into podoms, the dilated sections, and podomers, the fibrillar-like sections. (C) The fusiform-shaped soma of a telocyte can also be seen. In addition, it is possible to verify that the telocytes exist both in the periepithelial regions and in the periphery of the smooth muscle cells, enveloping them. (D) Telocytes also exist in association with blood vessels in the interalveolar stroma. (E) In the group treated with ENU/T/E2, in regions where the epithelium is unaltered, the perialveolar musculature appears to be discontinuous, but telocytes can be observed. (F) However, there are no telocytes in the periepithelial stroma adjacent to hyperplasia foci. (G, H) Telocytes were also absent from PIN foci adjacent stroma. (I, J) Telocytes are seen close to prostatic epithelium in a region of perialveolar smooth muscle discontinuity. Ep (Epithelium), Smc (Smooth muscle cells), Tc (Telocyte), Mi (Mitochondria), BV (Blood vessel), Arrow (Podomers), Arrowhead (Podoms), PIN (Prostatic intraepithelial neoplasia), H (Hyperplasia foci), Asterisk (extracellular vesicles), White bar (5 µm), Black bar (2 µm), Yellow bar (10 µm).

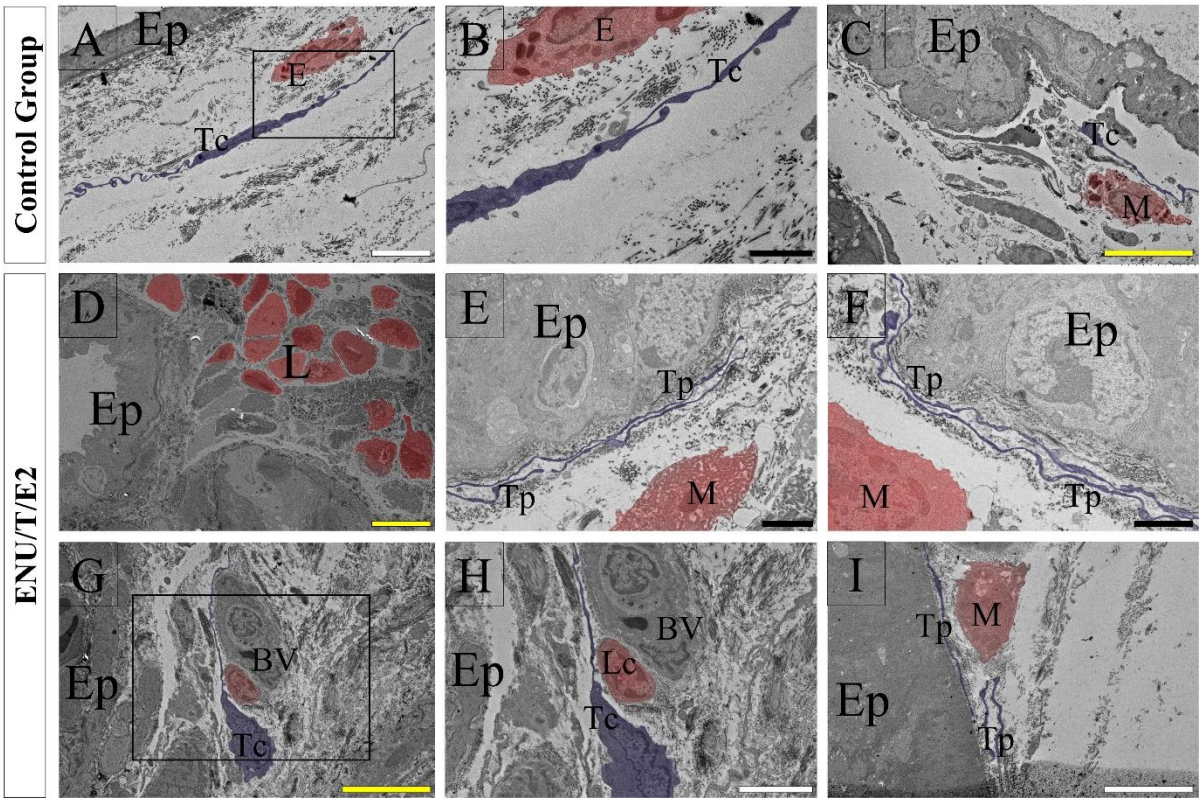

Fig. 4. Ultrastructural data from the prostate of female Mongolian gerbils from the control group and the group treated with ENU/T/E2, showing the association of telocytes with immune cells. (A, B) In normal tissue, telocytes can be seen in the stroma in the vicinity of eosinophils, (C) as well as of macrophages. (D) In the group treated with ENU/T/E2, infiltrated leukocytes occupy part of the interalveolar stroma. (E, F) In the perialveolar region, telocytes maintain contact with macrophages. (G, H) Telocytes are also close to lymphocytes in the periphery of blood vessels. (I) Telocyte telopode networks also make contact with macrophages in the periphery of the prostatic epithelium in regions where the smooth muscle layer is discontinuous. Ep (Epithelium), E (Eosinophil), Tc (Telocyte), M (Macrophage), L (Infiltrated Leukocytes), Tp (Telopode), BV (Blood vessel), Lc (Lymphocyte), White bar (5 µm), Black bar (2 µm), Yellow bar (10 µm).

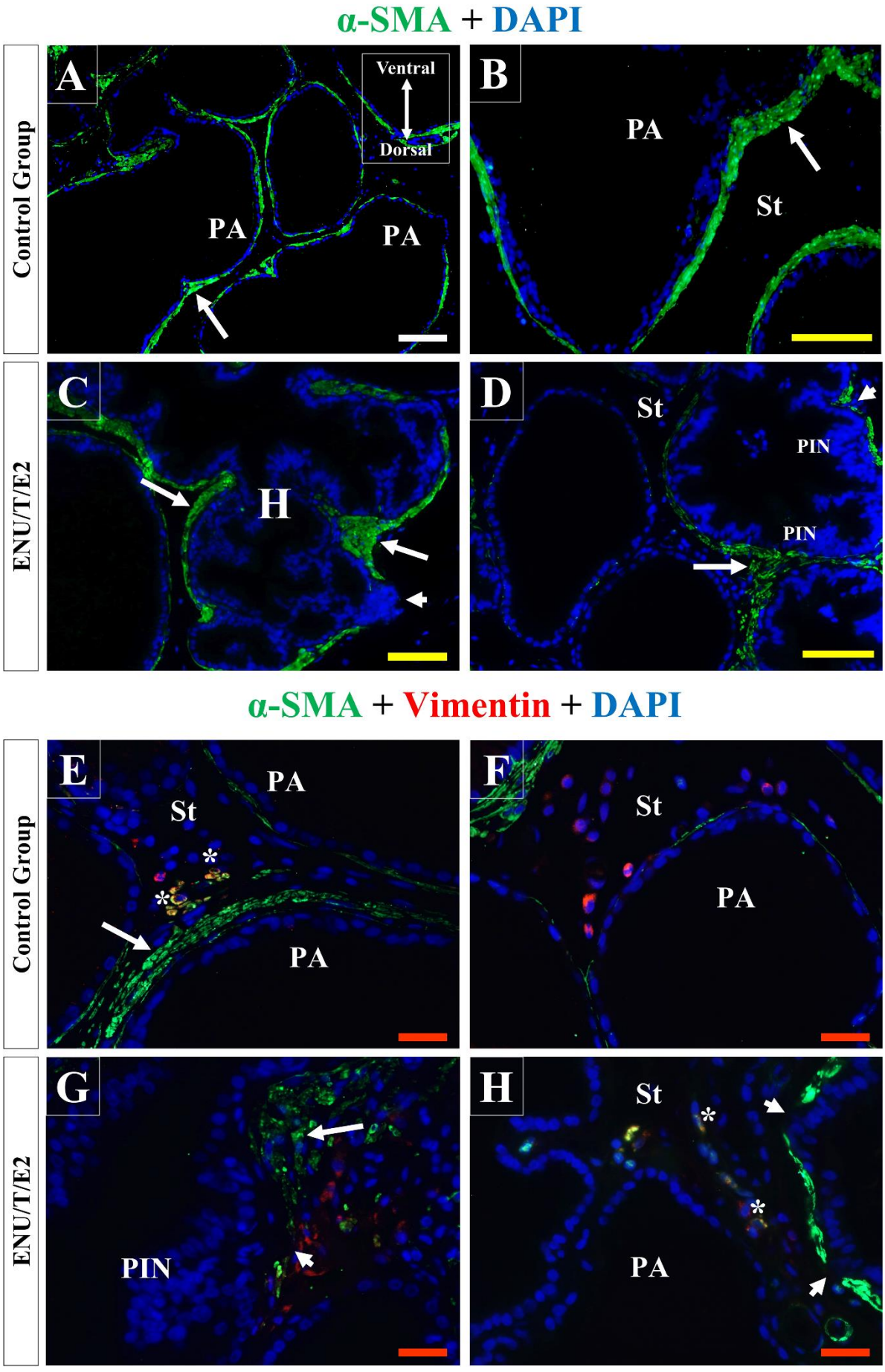
α-SMA + DAPI
Control Group
ENU/T/E2
A
Ventral
Dorsal
PA
PA
B
PA
St
C
H
D
St
PIN
PIN
α-SMA + Vimentin + DAPI
Control Group
ENU/T/E2
E
PA
St
PA
F
St
PA
G
PIN
H
St
PA

Fig. 5. Immunofluorescence assays for α-SMA and double immunofluorescence assays for α-SMA and vimentin performed on histological sections of the prostate of female Mongolian gerbils from the control and the ENU/T/E2-treated group. (A-B) In the control group, a thin layer of perialveolar smooth muscle surrounds the alveoli. (C) In the group treated with ENU/T/E2, alterations in the prostatic epithelium can be seen, such as in the hyperplasia foci, where the perialveolar smooth muscle is also altered, either with regions of discontinuity or with regions of thickening. (D) A similar pattern of changes in the smooth muscle surrounding the alveoli is also seen in the PIN foci. (E, F) In the control group there is no colocalization of α-SMA and vimentin in the perialveolar smooth muscle region. The overlap is seen in the interalveolar region, the typical pattern of the myofibroblast immunophenotype. (F) Vimentin-positive cells are also found in the stroma, indicating the presence of fibroblasts. (G) In the group treated with ENU/T/E2, it is possible to discern regions in which the perialveolar smooth muscle becomes thickened and others where it is discontinuous around the PIN foci. There are only immunostained cells for vimentin, which are possibly fibroblasts. (H) The colocalization of α-SMA and vimentin can be verified in the interalveolar stroma, possibly indicating myofibroblasts. Regions of smooth muscle discontinuity surrounding the alveoli can also be seen. PA (Prostatic Alveoli), St (Stroma), Arrow (α-SMA immunolabeling in the perialveolar region), Arrowhead (Discontinuation of α-SMA immunolabeling in the peralveolar region), Asterisk (Colocalization of α-SMA and vimentin), White bar (100 μm), Yellow bar (50 μm), Orange bar (20 μm).

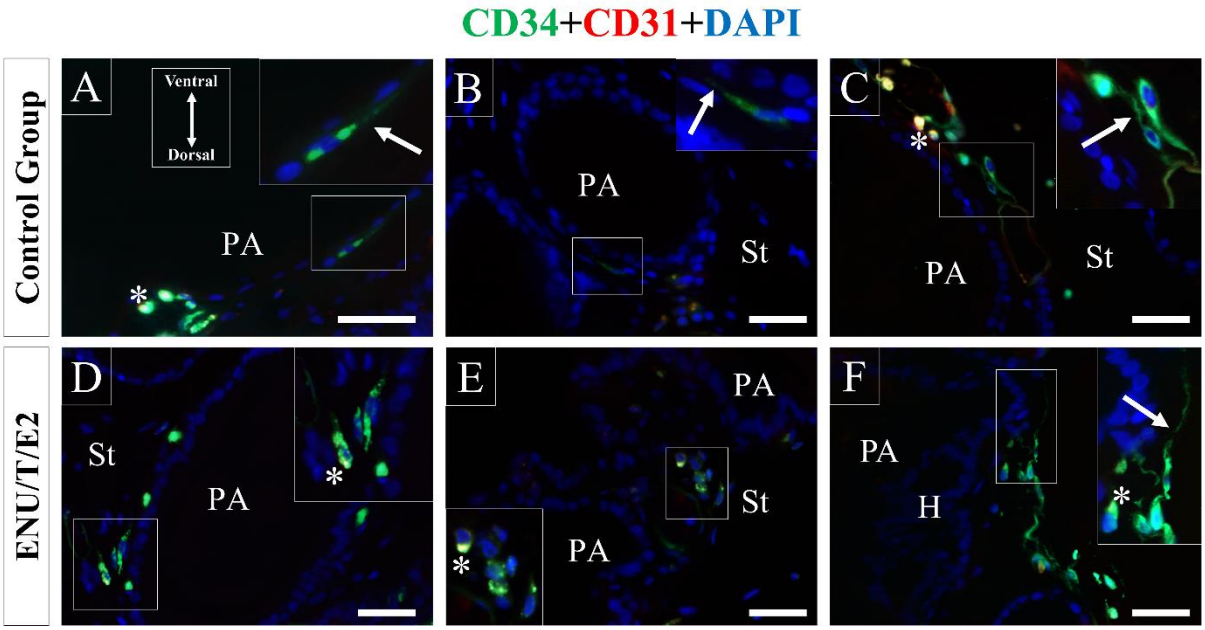

Fig. 6. Immunofluorescence assays for CD34/CD31 in histological sections of the prostate of female Mongolian gerbils from the control and ENU/T/E2-treated groups. (A-C) In female prostates of the control group, fibroblast-like CD34-positive cells that do not show colocalization with CD31 are found in the perialveolar and interalveolar region, indicating the presence of telocytes, but there are also cells where CD34 colocalizes with CD31, which are possibly bone-marrow-derived mesenchymal progenitor cells. (D) In the group treated with ENU/T/E2, CD34-positive fibroblast-like cells are also found near the alveoli, in addition to cells showing colocalization of CD34 and CD31 in this region. (E) Colocalization of CD34 and CD31 is also verified in the interalveolar region. (F) Fibroblast-like CD34-positive cells showing no colocalization with CD31 are also found in the interalveolar region, indicating the presence of telocytes. Close to these cells, there are cells showing colocalization of CD34 and CD31. PA (Prostate alveoli), St (Stroma), H (Hyperplasia foci), Arrow (Exclusively CD34-positive cells), Asterisk (Cell showing colocalization of CD34 and CD31), Bar (20 µm).

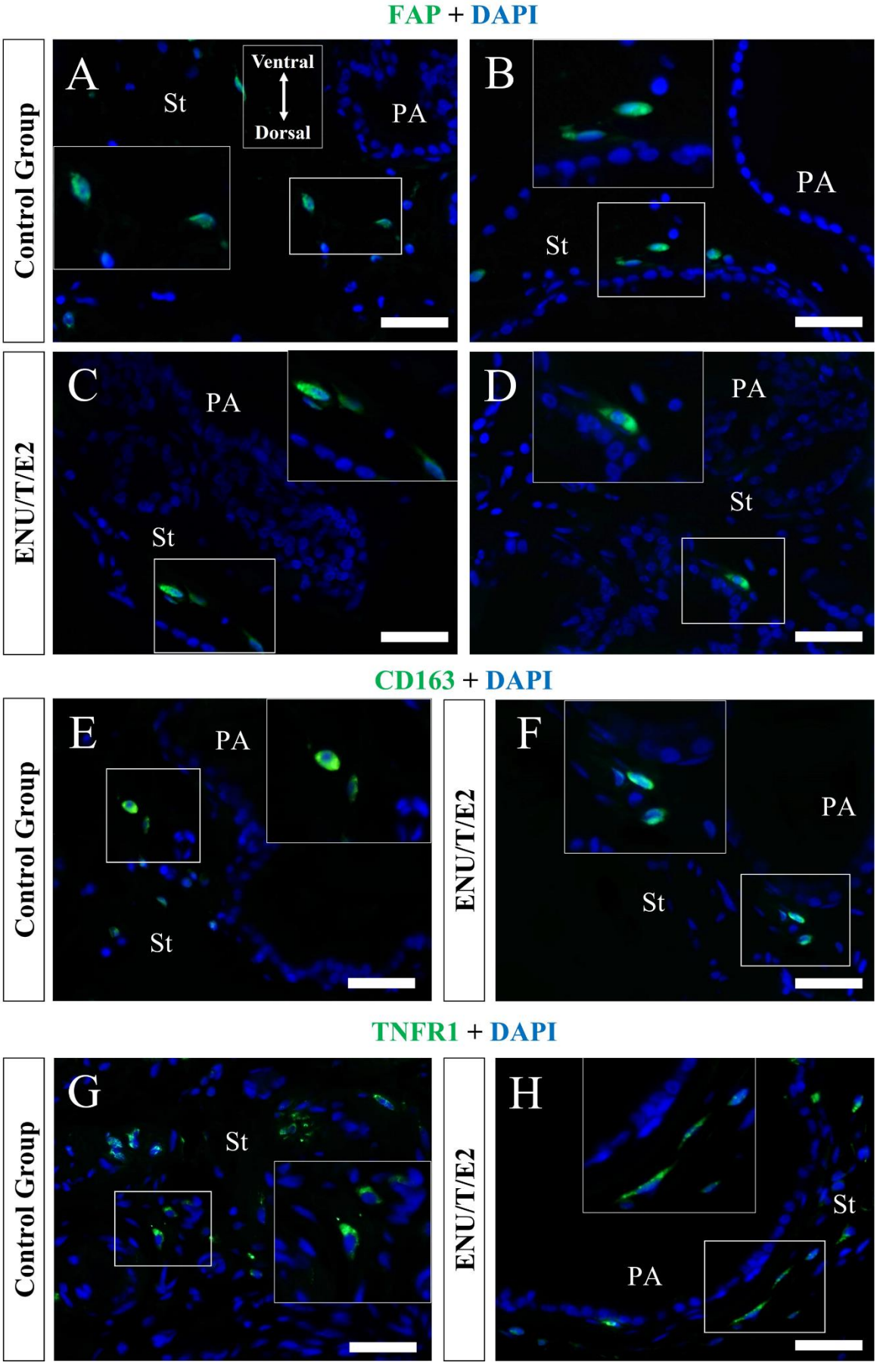
FAP + DAPI
Control Group
ENU/T/E2
A
St
Ventral
Dorsal
PA
B
PA
St
C
PA
St
D
PA
St
CD163 + DAPI
Control Group
E
PA
St
ENU/T/E2
F
PA
St
TNFR1 + DAPI
Control Group
G
St
ENU/T/E2
H
St
PA

Fig. 7. Immunofluorescence assays for FAP, CD163 and TNFR1 in histological sections of the prostate of female Mongolian gerbils from the control and the ENU/T/E2-treated groups. (A, B) FAP-positive fibroblast-like cells are seen in the interalveolar stroma in the control group, indicating the presence of CAFs. (C, D) These cells are also verified in the stroma of the group treated with ENU/T/E2. (E) CD163-positive cells are found in the interalveolar stroma of the control group, indicating the presence of activated macrophages. (F) These cells are also present in the interalveolar stroma of the treated group. (G) TNFR1-positive cells are observed both in the stroma of the control group and that of the treated group (H). This finding indicates the occurrence of a pro-inflammatory stimulus in the female prostate in both scenarios. PA (Prostate alveoli), St (Stroma), Bar (20 µm).

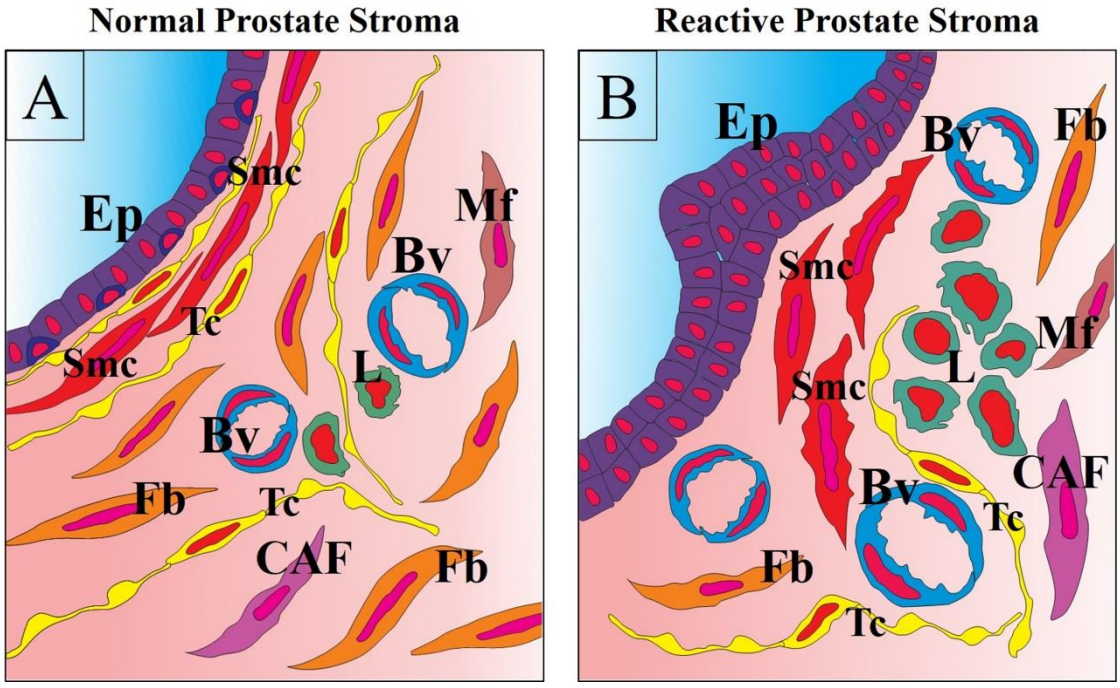

Fig. 8. Representation of the normal stroma of the prostate and the alterations verified with reactive stroma onset. (A) In the normal stroma of the prostate gland, the perialveolar/periductal smooth muscle layer is continuous and interspersed between the telocytes in the periepithelial region and those at the interface between the smooth muscle cells and the remainder of the prostatic stroma. Other telocytes occupy the interalveolar stroma in contact with blood vessels and leukocytes. In addition to fibroblasts, the presence of CAFs and myofibroblasts can also be observed among other stromal cells. (B) With the reactive stroma formation, the layer of muscle cells becomes discontinuous or thickens in regions surrounding epithelial lesions, such as in cases of PIN and hyperplasia. Interestingly, telocytes are not present in the stroma adjacent to these lesion foci, suggesting that these cells may help to maintain the integrity not only of the smooth muscle layers, but also of the prostatic epithelium itself. Finally, the tissue becomes more vascularized and there is often the presence of leukocyte infiltrates; cell types such as CAFs and myofibroblasts are also found in reactive stroma. Ep (Epithelium), Tc (Telocyte), L (Leukocytes), BV (Blood vessel), Fb (Fibroblast), Smc (Smooth muscle cell), CAF (Cancer-associated fibroblast), Mf (Myofibroblast).

III.3. Artigo 3

Tamoxifen treatment decreases proliferation and inflammation associated with induced-cancer of the female prostate in gerbil

Juliana S Maldarine[a], Bruno D A Sanches[a], Luiz Henrique Alves Guerra[b], Gustavo M Amaro[b], Hernandes F Carvalho[a], Rejane M Góes[b], Patricia S L Vilamaior[b], Sebastião R Taboga[a,b]

[a]Department of Structural and Functional Biology, Institute of Biology, State University of Campinas (UNICAMP), Bertrand Russel Av., Campinas, São Paulo, Brazil.
[b]Department of Biological Sciences, Laboratory of Microscopy and Microanalysis, Sao Paulo State University (UNESP) Cristóvão Colombo St., 2265, São José do Rio Preto, São Paulo, Brazil.

Corresponding author: Dr. Sebastião R. Taboga, Department of Biology, Laboratory of Microscopy and Microanalyses, 2265 Cristóvão Colombo Street, São José do Rio Preto, São Paulo, e-mail: sebastiao.taboga@unesp.br

Ethics approval statement: The experiments were carried out following the ethical principles recommended by the National Council for Animal Experimentation Control (CONCEA) and the procedures involved were approved by the Ethics Committee on the Use of Animals at IBILCE/UNESP (Proc. No. CEUA-209/2019).

This dissertation text is adapted from the manuscript that will be submitted to the Scientific Journal *The Prostate*

III.3.1. Abstract

Previous studies have already demonstrated that the female prostate, also known as Skene's gland, can undergo several pathologies similar to those of males, including cancer. The presence of a functional prostate is ubiquitous in females of Mongolian gerbil, so this species is promising for research on female prostate. In this study we aimed to evaluate the impact of Tamoxifen (TAM) in female gerbils that were previous submitted to a carcinogenic-induction model, in which the animals were exposed to a single dose of N-ethyl-N-nitrosourea (ENU), followed by an alternating exposure of testosterone and estradiol for 24 weeks. Later, female gerbils were treated with TAM for 2 weeks every other day. Our results showed that TAM decreases proliferation and inflammation, as well as promotes apoptosis in the female prostate. TAM exposure also implicated in hormonal variations since the circulating testosterone levels diminished together with the expression of androgen and estrogen receptors. Finally, this study evidenced that TAM is a promising therapeutic strategy for female prostate cancer.

Keywords: ENU, tamoxifen, antiestrogen therapy, inflammation, female prostate cancer, apoptosis

Abbreviation List

ENU (N-ethyl-N-nitrosourea), PIN (Prostatic Intraepithelial Neoplasia), HE (Haematoxylin-Eosin), E2 (17β-estradiol), TAM (Tamoxifen), T (Testosterone cypionate), AR (Androgen Receptor), ERα (Estrogen Receptor α), PCNA (Proliferating Cell Nuclear Antigen), PH.H3 (Phospho-Histone H3), CD163 (Cluster of Differentiation 163), CD68 (Cluster of Differentiation 68), CRPC (Castration Resistant Prostate Cancer), PBS (Phosphate-buffered Saline), DAPI (4′,6-Diamidino-2-Phenylindole), FITC (Fluorescein Isothiocyanate), BSA (Bovine Serum Albumin), PFA (Paraformaldehyde)

III.3.2. Introduction

The existence of a female prostate has been debated over the decades and, despite resistance from part of the scientific community, several studies have evidenced that the glandular tissue surrounding the female urethra has morphological and immunohistochemical similarity to the prostate of men (Zaviacic *et al.*, 2000; Tomalty *et al*., 2022, 2023). In this sense, it was confirmed the presence of a functional prostate in about half of women (Dietrich *et al*., 2011), besides being found in females of other species (Biancardi *et al.*, 2010; Aguiar *et al.*, 2013; Santos *et al.,* 2022). It was suggested that the female gland plays a role in reproduction, so that its secretion participates in the nutrition and survival of spermatozoa as they pass through the vaginal tract (Santos & Taboga, 2006; Biancardi *et al*., 2017). Still, clinical cases that demonstrate the emergence of various pathological conditions in women prostate, such as adenocarcinoma, are increasing (Heller, 2015; Thum *et al*., 2017; Tregnago & Epstein, 2018; Kyriazis *et al*., 2020; Bondili *et al*., 2021; Gao *et al*., 2022), similar to that of men prostate (Kaufman *et al.,* 2020). A literature review showed that 20 cases of prostate adenocarcinoma have already been reported in women until now (Gao *et al.,* 2022). Although being considered rare, this disease is often confused with other disorders of the urogenital tract, and it was found that part of urethral cancers arises from the prostate (Reis *et al*., 2011; Kunc & Biernat, 2021). Therefore, the number of cases may be higher than reported, which raises concerns in terms of public health. Nevertheless, there are limitations with regard to obtaining material to deepen the knowledge about women's prostates under normal and pathological conditions, and most studies are carried out *post-mortem* (Santos & Taboga, 2006; Costa *et al*., 2016).

In this sense, the Mongolian gerbil (*Meriones unguiculatus*) has become an interesting experimental model, once the prostate is found in most females (Santos & Taboga, 2006; Biancardi *et al.,* 2017). As in males, it is known that the morphology and physiology of the female gland are regulated by steroid hormones, specifically testosterone (T) and estradiol (E2) (Custodio *et al.*, 2010; Biancardi *et al.*, 2015, 2017). Additionally, it has been discovered that the female prostate has different responsibility to these hormones, showing to be more sensitive to E2 than that of males, and that alterations on hormone levels can lead to the emergence of several types of lesions (Custodio *et al*., 2008; Sanches *et al.*, 2019). It is known that estrogens, particularly E2, are implicated in prostate cancer progression (Bonkhoff, 2018).

Moreover, studies with rodents and humans showed the relation between high estrogen levels and tumor aggressiveness (Dobbs *et al*., 2019; Lafront *et al*., 2020). Other works demonstrated a positive correlation between estrogen receptor α (ERα) and tumor progression, since this receptor has a role in cell proliferation and growth (Bonkhoff, 2018; Liu, Ma, Yao, 2020). Hence, the development of therapies that focus on the estrogen signaling pathway may become useful for prostate cancer treatment, as seen for breast cancer (Chaput & Sumar, 2022).

Our research group developed a method of inducing carcinogenesis using a potent carcinogen, N-ethyl-N-nitrosurea (ENU), which promotes random mutations in the mouse genome (Russell *et al.*, 1979; Probst & Justice, 2010), in association with testosterone and estradiol (unpublished data). In this model, most females developed malignant lesions that would be equivalent to grade 5 on the scale used for the transgenic adenocarcinoma of the mouse prostate (TRAMP) (Berman-Booty *et al.*, 2012), in which adenocarcinomas are classified into well-differentiated, when cells still retain their organs typical characteristics, and invasive, once they have the potential to migrate and invade other regions (Gordetsky & Epstein, 2016; Kanan *et al.*, 2019).

So, given the female gland's susceptibility to estrogens and the need to find therapeutic targets for adenocarcinomas of the female prostate, the present study proposes the use of tamoxifen (TAM), an antiestrogenic drug widely used in the treatment of hormone-dependent breast cancer (Visvanathan *et al*., 2019; Emons, Mustea, Tempfer, 2020). TAM acts as an antagonist of estrogen receptor (ER) in the mammary gland, whereas in other regions, such as endometrium and bone, it has an agonist action (Li *et al*., 2017; Patel & Bihani, 2018; Emons, Mustea, Tempfer, 2020). Its active form, 4-hydroxytamoxifen (4HT), is converted by cytochrome P450 and has high affinity for the ER, consequently competing with estrogens for binding to the receptor. In the breast, TAM promotes antagonist responses when binds to ERα, blocking estrogen signaling and inhibiting cell proliferation (Hasegawa *et al*., 2018; Pepe *et al*., 2021). Finally, TAM administration is found to be an alternative to castration-resistant prostate cancer (CRPC), since these tumors, which are commonly ER-positive, no longer respond to the conventional antiandrogenic therapies (Semenas *et al*., 2021).

III.3.3. Material and Methods

Animals, treatments, and sample preparation

Forty adult female (6-months-old) Mongolian gerbils (*Meriones unguiculatus*) were housed in polyethylene cages in 25°C room temperature, with unlimited access to filtered water and food. The experiments followed the ethical guidelines of São Paulo State University (UNESP) (CEUA-209/2019). The animals were weighted and then euthanized by subcutaneous injection of ketamine (100 mg/kg), and xylazine (11 mg/kg). Blood samples were collected for posterior serological analysis at -80ºC. The female prostates were collected and immersed in 4% PFA (pH 7.4), washed in H_2O, dehydrated, clarified, and then embedded in paraffin. The glands were sectioned at 5 μm and mounted on histological slides. Some of these slides were submitted to the Haematoxylin-Eosin (HE) for general histological analysis.

In the experimental groups, the carcinogenesis was induced in the female prostate following the same methodology from Maldarine and collaborators (unpublished data). In the group treated with TAM, the females were submitted to a high dose of testosterone cypionate (T) (20 mg/kg), followed after three days by exposure to a single dose of the carcinogen N-ethyl-N-nitrosourea (ENU; Chem Service, West Chester, PA-USA) (100 mg/kg) and then by alternating exposure to 17-β-estradiol (E2) (2 mg/kg) and testosterone cypionate (2 mg/kg) once a week over 24 weeks. Additionally, in the next two weeks, they were exposed to TAM (5 mg/kg) every other day. The control group was established with the same protocol but replacing TAM by its diluent. After dissection, the prostate glands were collected and fixed in 4% buffered paraformaldehyde (buffered in 0.1 M phosphate, pH 7.4) for 24 h, washed in water, dehydrated in ethanol, clarified in xylol, and then embedded in paraffin (Histosec; Merck, Darmstadt, Germany). The organs were then sectioned at 5 μm using a microtome (RM2155, Leica, Nussloch, Germany) and mounted on histological glass slides. Some of the slides were stained with Haematoxylin-Eosin (HE) histochemical technique for general morphological purpose.

Classification and quantification of prostate lesions

The entire prostates of the females were serially sectioned, and HE histological sections were randomly chosen (5 sections per animal) along the gland. The histopathological classification of lesions was accomplished according to the methodology described by Shappell and colleagues (2004); the lesions were categorized in 5 types: hyperplasia, atrophy, low-grade PIN (Prostatic Intraepithelial Neoplasia), high-grade PIN, and adenocarcinoma. The grading scheme described for the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice by Berman-Booty and colleagues (2012) was used for the adenocarcinoma classification. Incidence of prostate lesions was quantified by identifying the different lesions in relation to the total sample number, while the multiplicity was calculated by the frequency of each lesion found in the histological section relative to the total number of examined animals in both groups.

Detection of steroid receptors and cell proliferation

The histological sections of gerbil female prostates were used in immunohistochemistry assays for the detection of AR, ERα, PCNA and PH.H3 according to the methodology performed by Sanches and colleagues (2017). The antibodies were used (in a dilution of 1:100) for the detection of androgen receptor (AR) (rabbit polyclonal IgG, s-816; Santa Cruz Biotechnology), estrogen receptor α (ERα) (rabbit polyclonal IgG, PA5-34577; Invitrogen, ThermoFisher Cientific), proliferating cell nuclear antigen (PCNA) (mouse monoclonal IgG2a, sc-56; Santa Cruz Biotechnology) and phospho-histone H3 (PH.H3) (polyclonal rabbit, 9701, Cell Signaling Technology). The secondary antibodies were rabbit anti-mouse IgG (Post Primary, Novolink; Leica Biosystems) and anti-rabbit Poly-HRP-IgG (Polymer, Novolink; Leica Biosystems). The incubation time for both was 45 min at 37ºC. The revelation was made with 3’-diaminobenzidine (DAB), and the sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the incubation with the primary antibody. Histological sections were analysed with the Olympus BX60 light microscope.

Detection of macrophages

After the tissues were deparaffinized and rehydrated, the exposure of the antigens was carried out by heat treatment for 20 min at 95°C with the slides immersed in citrate buffer. Blocking of non-specific bindings was performed for 30 min using the UltraCruz® Blocking Reagent (sc-516214, Santa Cruz Biotechnology) and the slides were washed three times for 5 min each in PBS with 0.3% Triton-X between each step. The female prostate sections from the control and the TAM-treated groups were subjected to immunofluorescence assays for CD68 (monoclonal rabbit, 97778S, Cell Signaling Technology) and for CD163 (monoclonal rabbit, 93498S, Cell Signaling Technology), in order to verify the presence of activated macrophages. These antibodies were incubated overnight at a dilution of 1µg/100µl of BSA (Bovine Serum Albumin) 1% in PBS. In the next morning, the slides were subjected to a secondary antibody incubation with FITC-labelled (Fluorescein Isothiocyanate) goat anti-mouse (F0257; Sigma-Aldrich), 1:100 dilution in 1% BSA for 2 h at room temperature, washed in PBS, and finally stained with DAPI (4′,6-Diamidino-2-Phenylindole) (F36924; Life Technology). The histological slides were analyzed with a ZeissImager M2 fluorescence microscope coupled with AxioVision (Zeiss) software.

Detection of apoptosis

The TUNEL reaction was performed following the manufacturer's instructions (ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101, EMD Millipore Corporation, California, USA) and using the same methodology from Campos *et al*. (2008). The tissue sections were deparaffinized, rehydrated and Tris base buffered saline (TBS, pH 7.6), and then they were digested by Proteinase K (1.5 µL in 300 µL Tris pH 8.0) for 15 minutes at room temperature. The revelation was made with 3'-diaminobenzidine (DAB), and the sections were counterstained with Harris hematoxylin. Histological sections were analysed with the Olympus BX60 light microscope.

Quantitative analysis

Thirty random prostate fields (stained with HE) at a magnification of 400X of each group were analyzed using ImageJ software (National Institute of Mental Health, Bethesda, Maryland). The stereological analysis was carried out using Weibel's method (1979) graticulated system of 120 points and 60 lines in order to compare the relative proportion (% of volume) of each prostatic component (epithelium, stroma, and blood vessels) between the treated and control groups. The relative values were determined by counting the points that coincided and dividing them by the total number of points.

Also, thirty to fifty random prostate fields were used to count the total number of cells of each group for CD68 (Cluster of Differentiation 68) and CD163 (Cluster of Differentiation 163) immunofluorescence assays, as well as for AR (Androgen Receptor), ERα (Estrogen Receptor α), PCNA (Proliferating Cell Nuclear Antigen) and PH.H3 (Phospho-Histone H3) immunohistochemical assays and for the TUNEL reaction, in order to separate the positive and negative stained cells, in accordance with the methodology described by Maldarine and colleagues (2020). A minimum of 1,000 cells were counted per group, and the percentage of positive cells was determined in relation to the total number of cells.

Serological analysis

The blood of female gerbils from the experimental groups was collected after decapitation. The serum was separated by centrifugation (3,000 rpm for 20 min) and stored at -80ºC until analysis. Hormonal dosages of circulating serum estradiol and testosterone were given in duplicate by ELISA high sensitivity kits (17β-Estradiol EIA Kit, RE52041, Tecan, IBL-International; Testosterone EIA Kit, RE52151, Tecan, IBL-International) following the manufacturer's instructions. The readings were performed using the SpectraMax Plus 384 reader, at 405 gm (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

T-test was used for two-sample comparison, and for multiple samples we used ANOVA coupled with Tukey post-hoc test. Significance was set at $p<0.05$ and results were expressed as mean ± standard deviation. All statistical tests were performed using Statistica 7.0 (StatSoft).

III.3.4. Results

Histopathology of the Mongolian gerbil female gland

Histological sections stained with HE evidenced the female prostates architecture. Both groups presented several lesions, ranging from the benign ones, such as hyperplasia and atrophy, to the premalignant and malignant phenotype, such as low-grade PIN, high-grade PIN and adenocarcinoma. The atrophic alveoli present flattened epithelial cells, and basal cells are rarely visualized (Fig. 1A, D). In the hyperplasia foci, cell proliferation leads to stratification of the secretory epithelium, which loses the pseudostratified characteristic, although no changes are verified in the cellular nuclei (Fig. 1 A, E). Low-grade PIN phenotype is classified when epithelial cells extend into the lumen, forming invaginations (Fig. 1B, D). This type of lesion differs from the high-grade PIN phenotype in terms of severity, in which the latter present greater alterations relative to the nucleus, which becomes more irregular, as well as the formation of microacini within the epithelial layer itself (Fig. 1B, E). Adenocarcinoma was the most severe lesion that were observed in both groups. Most adenocarcinoma foci are classified as well-differentiated, when the epithelial cells invade the adjacent stroma, through the rupture of the basement membrane, however, these cells still retain their original organ typical characteristics (Fig. 1C, F). There were no significant differences in the epithelial compartment volume between the control group and the TAM-treated group (19.04 ± 6.9 % and 17.6 ± 4.7 %, respectively) (Fig. 1G). There were also no significant changes in the volume of the stromal compartment (17.9 ± 6.6 % in the control group and 15.4 ± 4.8 % in the treated group), nor in the volume of blood vessels between both groups (2.5 ± 1.5 % in the control group and 2.8 ± 1.7 % in the group treated with TAM) (Fig. 1H, I).

Quantification of lesions and prostate weight

At least one focus of alveoli atrophy, hyperplasia and low-grade PIN was found in all the examined prostates from the two experimental groups. High-grade PIN and adenocarcinoma foci were present in 67% of animals from both groups. Regarding the multiplicity of the lesions, no significant differences were verified between groups (Table 1). There were also no significant differences in the weight of the Mongolian female gerbils, nor in the prostate absolute and relative weight between the control group and the group treated with TAM (Table 2).

Steroidal receptors

The immunohistochemical assays for the AR evidenced several positive cells both in the epithelium and stroma of the prostate in the control group (Fig. 2A, B). In the treated group, both compartments of the female gland presented fewer positive cells compared to the control (Fig. 2C, D), which was corroborated by the quantitative analysis (41.1 ± 9.8 and 58.9 ± 8.6 % in the epithelium and 28.5 ± 7.6 and 43.5 ± 7.5 % in the stroma, in the respective groups) (Fig. 2I). As for the ERα, a similar pattern was verified (Fig. 2E-H). The group treated with TAM presented a significant decrease in the ERα staining both in the epithelial and stromal compartments in comparison to the control group (35.4 ± 7.5 and 59.7 ± 7.4 % in the epithelium and 31.4 ± 11.5 and 57.4 ± 12.1 % in the stroma, in the respective groups) (Fig. 2J).

Proliferation index

The immunostaining for the PCNA revealed several positive cells in the control group, in both the epithelial and stromal compartments (Fig. 3A, B). However, the number of PCNA-positive cells had a significant decrease in the epithelium and stroma in the prostates from the TAM-treated group in relation to the control (Fig. 3C, D), which was statistically significant (29.4 ± 6.7 and 49.9 ± 7.9 % in the epithelium and 31.9 ± 12 and 50.6 ± 11.4 % in the stroma, respectively) (Fig. 3I). Regarding the PH.H3 immunostaining, several cells are positive for this factor in the two compartments in the control group (Fig. 3E, F). Similar to the PCNA marking, there was also a decrease in the number of positive cells in both the epithelium

and in the interalveolar region in the group treated with TAM compared to the control group (Fig. 3G, H), which was statistically confirmed (37.3 ± 6 and 43.2 ± 8.2 % in the epithelium, and 32.5 ± 8.5 and 43.3 ± 8.8 % in the stroma, in the respective groups) (Fig. 3J).

Apoptosis

The TUNEL reaction evidenced few positive cells in the epithelium and the interalveolar region of the female gland in the control group (Fig. 4A, B). On the other hand, in the group treated with TAM there was an increase in the apoptosis in both compartments (Fig. 4C, D), which was statistically significant (11 ± 5.3 and 6.1 ± 1.6 % in the epithelium and 14.4 ± 4.8 and 8.1 ± 3.1 % in the stroma, in the respective groups) (Fig. 4E).

Macrophages

The immunofluorescence assays for CD68 showed several positive cells between the prostate alveoli in the control group (Fig. 5A, B). In contrast, few positive cells for this factor were verified in the stroma of the TAM-treated group (Fig. 5C, D). Immunostaining for CD163 showed the positive cells in the interalveolar region in the control group (Fig. 5E, F). In the treated group, few cells were immunoreactive to this factor in the stromal compartment (Fig. 5G, H). Statistical data confirmed the decrease in the number of CD68-positive cells in the TAM-treated group compared to the control (9.8 ± 2.4 and 12.5 ± 3.4 %, respectively) (Fig. 5I), and the same was observed for the number of CD163-positive cells (9.7 ± 2.8 % in the treated group and 12.2 ± 3.8 % in the control) (Fig. 5J).

Hormonal serum levels

The serological data showed no alterations in 17β-estradiol levels between both groups (216.8 ± 114.1 pg/mL in the control group and 131.6 ± 45.2 pg/mL in the group treated with TAM) (Fig. 6A). However, there was a significant decrease in the circulating testosterone levels in the treated group compared to the control (300.5 ± 135.2 and 523.5 ± 194 pg/mL, respectively) (Fig. 6B). The ratio between testosterone and 17β-estradiol showed no significant

differences in the treated group in comparison to the control group (2.2 ± 0.4 and 2.2 ± 1.1 pg/mL, respectively) (Fig. 6C).

III.3.5. Discussion

Following the experimental model previously developed by our research group of chemically-induced cancer on Mongolian gerbil female prostates (unpublished data), in this study the animals were treated with N-ethyl-N-nitrosurea (ENU), testosterone and estradiol, and, considering the impact estrogens have on the female gland, they were later exposed to TAM, an antiestrogenic compound widely used in breast cancer treatment (Visvanathan *et al*., 2019; Chaput & Sumar, 2022). Although we did not observe a reduction of cancerous lesions, our findings revealed that TAM has pro-apoptotic and anti-proliferative effects, as well as anti-inflammatory, in the induced-carcinogenesis scenario, demonstrating to be a potential therapeutic target for pathologies that can occur in the female prostate.

TAM is a non-steroidal drug that competes with estrogen for ER, inhibiting or regulating estrogenic signaling (Patel & Bihani, 2018; Ibrahim *et al.*, 2019; Dehghan *et al*., 2021). Considered a selective estrogen receptor modulator (SERM), the complexity of its mechanism of action is related to the type of interaction with the ER and, depending on the tissue, it can generate antagonistic responses, as in the mammary glands, or agonistic responses, as in the endometrium and bone (Li *et al*., 2017; Emons, Mustea, Tempfer, 2020; Patel & Bihani, 2018), besides having other pharmacological activities non-ER related (Nasu *et al*., 2008; Pepe *et al*., 2021; Sfogliarini *et al*., 2022). The effectiveness of treatment with TAM is well established mainly in women at high risk of developing breast cancer (Nazarali & Narod, 2014; Chaput & Sumar, 2022), since it prevents the proliferative action of estrogens and blocks the growth of tumor cells in breast tissue (Li *et al*., 2017). As for the gerbil prostate, it was observed that TAM has an agonist action (Santos *et al*., 2008), as well as it diminishes the survival of human prostate cancer cells and in mouse models (Shim *et al*., 2009, Semenas *et al*., 2021).

Several studies have already demonstrated the correlation between the overexpression of ERα, a transcription factor implicated in the regulation of growth promoting genes, and metastatic tumors and CRPC (Ricke *et al*., 2008; Semenas *et al*., 2021). When activated, this receptor regulates the Notch1 signaling pathway, related to epithelial-

mesenchymal transitions, promoting the formation and invasion of tumor cells (Shen *et al*., 2019). Our data showed that E2 serum levels were not altered, similarly to what was verified in another study that used treatment with TAM in gerbil females (Santos *et al*., 2008); however, we observed a reduction in the expression of ERα in the prostatic tissue. It can be hypothesized that TAM, acting as an agonist in the female prostate, can initially potentiate the estrogenic effect, saturating the receptors, which would lead to a subsequent drop.

Additionally, there was a decrease in testosterone serum levels as well as in AR expression, a nuclear factor considered a biological marker in several types of cancers, such as melanoma, glioblastoma, and breast and prostate cancers (Wang *et al*., 2017; Zalcman *et al*., 2018; You *et al*., 2022; Westaby *et al*., 2022). Previous works have shown that circulating testosterone diminished in patients with breast cancer that were treated with TAM (Lonning *et al*., 1995; Hadji *et al*., 2012; Baumgart *et al*., 2014), which corroborates our findings. Regarding the AR, studies have shown that its overexpression altered TAM mechanism of action, which lead to resistance to treatment with this compound in breast cancer cells (Amicis *et al*., 2010; Ciupek *et al*., 2015). Therefore, AR can act as a modulator of TAM, so that the higher its expression is, the lower the effect of TAM treatment will be. In our research, AR expression is decreased in the prostate, hence it can be deduced that TAM may have a greater action on the prostate, competing against E2.

Our results also revealed a reduction in cell proliferation, in addition to an increase in cell apoptosis after TAM use. The observed drop in ERα expression can be positively correlated with the decrease in proliferative activity, since it is known that this receptor activates cell proliferating and growth pathways (Liu, Ma, Yao, 2020; Dehghan *et al*., 2021). In parallel, TAM may also act on ER-independent signaling pathways, as observed in studies with skin cancer cells (Hasegawa *et al*., 2018). Such data related the antiproliferative effects of the drug to a rise in intracellular calcium concentration and activation of protein kinase C (PKC). Moreover, Dehghan and collaborators (2021) showed that the treatment with TAM inhibited growth of thyroid cancer cells, blocked proliferation, and induced apoptosis, which was also verified in isolated breast cancer cell lineages. One of the suggested mechanisms involves the mitochondrial pathway and caspase-3 activation. TAM negatively regulates Bcl-2 expression, an anti-apoptotic protein, and stimulates the expression of Bax, a pro-apoptotic protein, and caspase-3 (Li *et al*., 2017; Rouhimoghadam *et al*., 2018; Dehghan *et al*., 2021). Still, other works showed that TAM decreases the expression of apoptosis suppressor genes, such as

NEAT1, which is active in several tumors, including of ovary, lung and liver (Ding *et al*., 2017; Yu *et al*., 2019; Kou *et al*., 2020). Xenograft tumors in mice corroborate the observations that treatment with TAM induces a high level of apoptosis and cell arrest, as well as inhibited metastasis in CRPC that are ER-positive, emerging as an alternative for cancers that no longer respond to the usual anti-androgenic therapies (Semenas *et al*., 2021).

The immunofluorescence assays evidenced a reduction of activated macrophages in our experiment. Although there are several classifications regarding their immunophenotypes currently, this cell type is conventionally divided into two: M1 e M2 (Sfogliarini *et al*., 2022). M1 macrophages, the classic and general phenotype, have pro-inflammatory properties and are related to the normal pathway of reaction against pathogens and infections, being usually evidenced by expression of CD68 (Shapouri-Moghaddam *et al*., 2018; Zhang *et al.*, 2022). On the other hand, M2 macrophages have anti-inflammatory properties and are involved in tissue healing, revascularization, and suppression of inflammation, as well as they are detected by expression of CD163 (Mantovani & Locati, 2013; Hu *et al*., 2017; Sfogliarini *et al.*, 2022). Both types can be present in processes associated with tumor formation and progression (Liu *et al*., 2021), and, as verified in our previous carcinogenesis induction model, the expression of the two macrophage markers increased significantly (unpublished data). Conversely, in the present study it was found that both CD68 and CD163 expression decreased in prostate stroma, so that we can suggest that TAM has anti-inflammatory effects in chemically induced carcinogenesis. In line with this idea, many studies demonstrated TAM anti-inflammatory activity in malignant tumors, as in pancreatic cancer (Cortes *et al*., 2018; Pein & Oskarsson, 2019) and in aggressive prostate cancer (Semenas *et al*., 2021; Tong, 2022). In mice, Han and other researchers (2010) showed that this drug prevented macrophages from producing nitric oxide (NO), which is a molecule related to inflammation and carcinogenesis process, and whose inhibition favors the treatment of cancer. Other studies have shown that TAM, besides directly affecting macrophages through G protein-coupled estrogen receptor (GPER), affecting the migration of these cells, can also lead to changes in extracellular matrix (ECM) composition by reducing its stiffness, which limits macrophage infiltration and cell invasion (Hattar *et al*., 2009; Pein & Oskarsson, 2019). Hence, the immunomodulator effects of this compound in tissues may or may not be related to ER signaling pathway (Sfogliarini *et al*., 2022).

Still, it is pertinent to point out that, despite being considered a safe medication with few adverse effects, there is evidence that, in certain cases, prolonged treatment with TAM may

be harmful. The side effects reported in the literature involve development of gynecological diseases, such as endometrial cancer, which occur especially in postmenopausal women with a history of uterine pathology (Nasu *et al*., 2008; Emons, Mustea, Tempfer, 2020). These effects demonstrate TAM complex mechanism of action, which is organ-specific and can trigger different responses through different pathways, as well as may affect the physiology of various hormone-dependent organs, such as the prostate (Santos *et al*., 2008).

Finally, this investigation brings for the first time a potential therapeutic target to ameliorate the tumor microenvironment generated in cancer-induced female prostates. Our findings are potentially relevant in terms of public health as approximately half of women have a functional prostate similar to that of men (Dietrich *et al*., 2011; Biancardi *et al*., 2017) and there is a growing number of studies associating the female prostate, also known as Skene's gland, with the development of various pathologies, including adenocarcinoma (Heller, 2015; Thum *et al.*, 2017; Tregnago & Epstein, 2018; Kyriazis *et al*., 2020; Bondili *et al*., 2021; Gao *et al*. 2022). These female prostate diseases are often confused with those derived from urogenital tract and there is evidence that some diagnosed urethral carcinoma in women have a prostatic origin (Reis *et al*., 2011; Kunc & Biernat, 2021). Thus, the real number of female prostate cancer cases may be underestimated, and further studies are important to better understand the physiology and the pathological conditions that affect female prostate.

Declaration of interest: none.

Funding

This work was supported by FAPESP (São Paulo Research Foundation); Contract number: 2018/23383-6 (to SRT), 2019/14201-4 (to JSM) and 2021/02303-7 (to HFC) and CNPq (National Council for Scientific and Technological Development); Contract number: 302938/2020-6 (to SRT), 465699/2014-6 (to HCF) and 104276/2023-1 (to BDAS).

Acknowledgements

The authors are grateful to FAPESP and CNPq for the fundings. Also, to Luiz Roberto Falleiros Jr. for technical support, as well as all coworkers at the Laboratory of Microscopy and Microanalysis (LMM).

III.3.6. References

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III.3.7. Figures, tables and legends

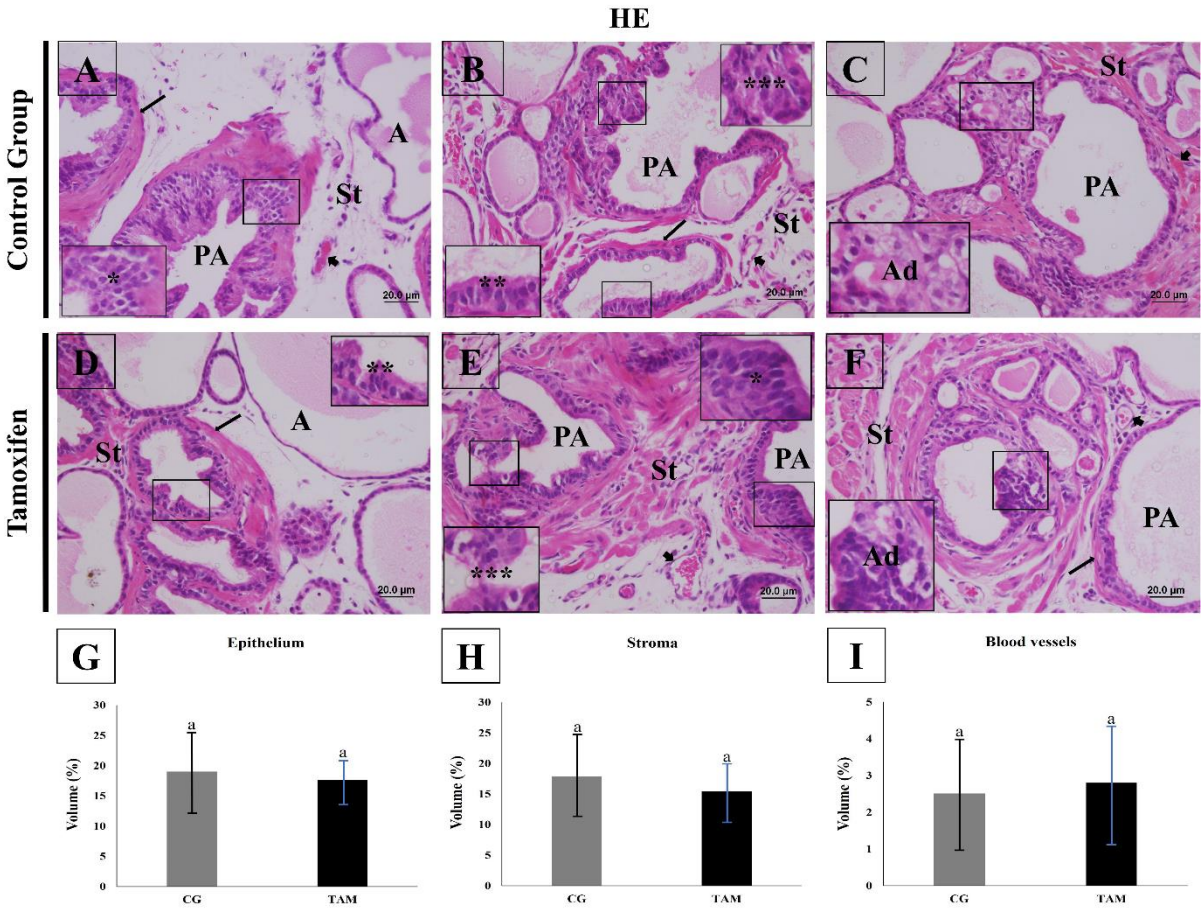

Fig. 1. Histological sections stained with HE and volume of prostate compartments of female Mongolian gerbils from control and TAM-treated groups. (A, B) Atrophic alveoli with flat cells and hyperplasia foci are observed in the control group, together with low-grade PIN and high-grade PIN foci containing intraepithelial arcs. (C) Well-differentiated adenocarcinoma foci are often seen in this group. (D-F) All of those lesions are also verified in the group treated with TAM. (G-I) Volume quantification of the epithelial, stromal and blood vessels compartments showed no significant differences between both groups. PA (Prostate alveoli), St (Stroma), Ad (Adenocarcinoma foci), Arrow (Smooth muscle), Arrowhead (Blood vessels), A (Atrophic alveoli), * (Hyperplasia foci), ** (Low-grade PIN foci), *** (High-grade PIN foci). Different letters (a, b) indicate significant statistical differences between groups.

Table 1 – Incidence and multiplicity of lesions of Mongolian gerbil female prostate

	Histopathological lesions				
	Hyperplasia	Atrophy	Low-grade PIN	High-grade PIN	Adenocarcinoma
Incidence (%)					
CG	100	100	100	67	67
TAM	100	100	100	67	67
Multiplicity (Mean ± S.D.)					
CG	7.3 ± 2.5[a]	7.4 ± 2.1[a]	0.8 ± 0.2[a]	0.7 ± 0.6[a]	0.3 ± 0.3[a]
TAM	6.6 ± 2.5[a]	11 ± 5.5[a]	1.1 ± 0.7[a]	0.7 ± 0.7[a]	0.4 ± 0.3[a]

Values represent mean ± standard deviation (S.D.) and different superscript letters (a, b) represent significant statistical differences between groups at $p \leq 0.05$. Statistical analysis based on the T test.

Table 2 – Body weight, absolute and relative prostate weight of the female Mongolian gerbil

	Groups	
	CG	TAM
Female gerbils		
Body weight (g)	70.6 ± 2.8[a]	70.7 ± 3.9[a]
Prostate weight (g)	0,124 ± 0,05[a]	0,110 ± 0,02[a]
Relative prostate weight (%)	0,0018 ± 0,0007[a]	0,0016 ± 0,0003[a]

Values represent mean ± standard deviation (S.D.) and different superscript letters (a, b) represent significant statistical differences between groups at $p \leq 0.05$. Statistical analysis based on the T test.

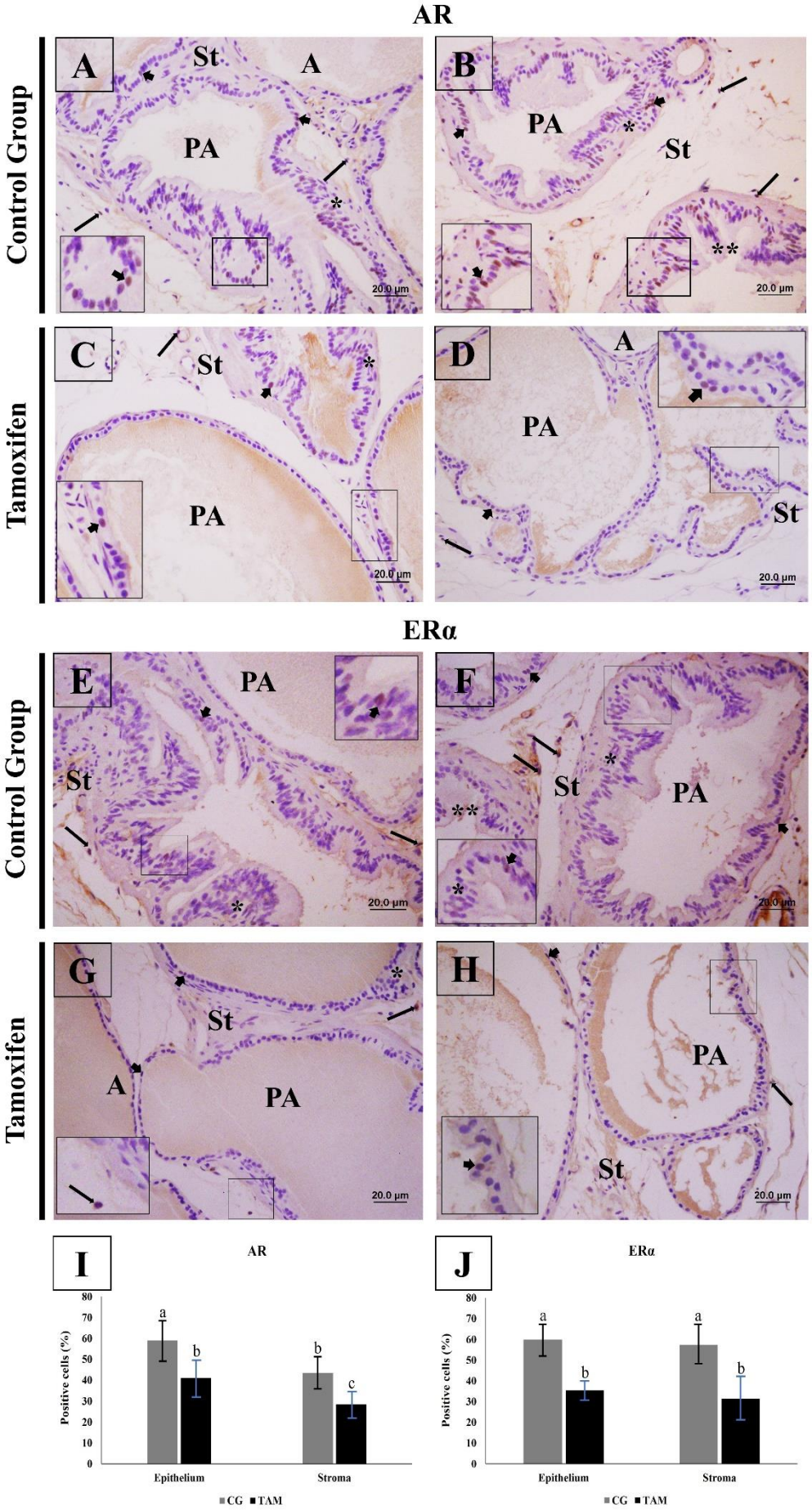
AR
Control Group
Tamoxifen
A
B
C
D
St
A
PA
*
**
20.0 µm
ERα
Control Group
Tamoxifen
E
F
G
H
I
J
Positive cells (%)
Epithelium
Stroma
CG
TAM
a
b
c

Fig. 2. Immunohistochemical assays for AR and ERα performed on histological sections of the prostate of female Mongolian gerbils from control and TAM-treated groups. (A, B) AR-positive cells are often seen in the epithelial and stromal compartments of the gland in the control group. (C, D) The presence of AR-positive cells decreases in both the epithelium and the stroma in the treated group. (E, F) In the control group, ERα-positive cells can be observed in the epithelium as well as in the interalveolar stroma. (G, H) The staining for this factor is decreased in the prostate epithelium and stroma in the group treated with TAM. (I) The quantification data showed a significant decrease in the percentage of AR-positive cells in both epithelial and stromal prostate compartments in the group treated with TAM compared to the control group. (J) There was also a significant decrease in the number of ERα-positive cells both in the epithelium and stroma in the treated group. PA (Prostate alveoli), St (Stroma), Arrow (Positive-stromal cells for each factor), Arrowhead (Positive-epithelial cells for each factor), A (Atrophic alveoli), * (Hyperplasia foci), ** (Low-grade PIN foci). Different letters (a, b, c) indicate significant statistical differences between groups.

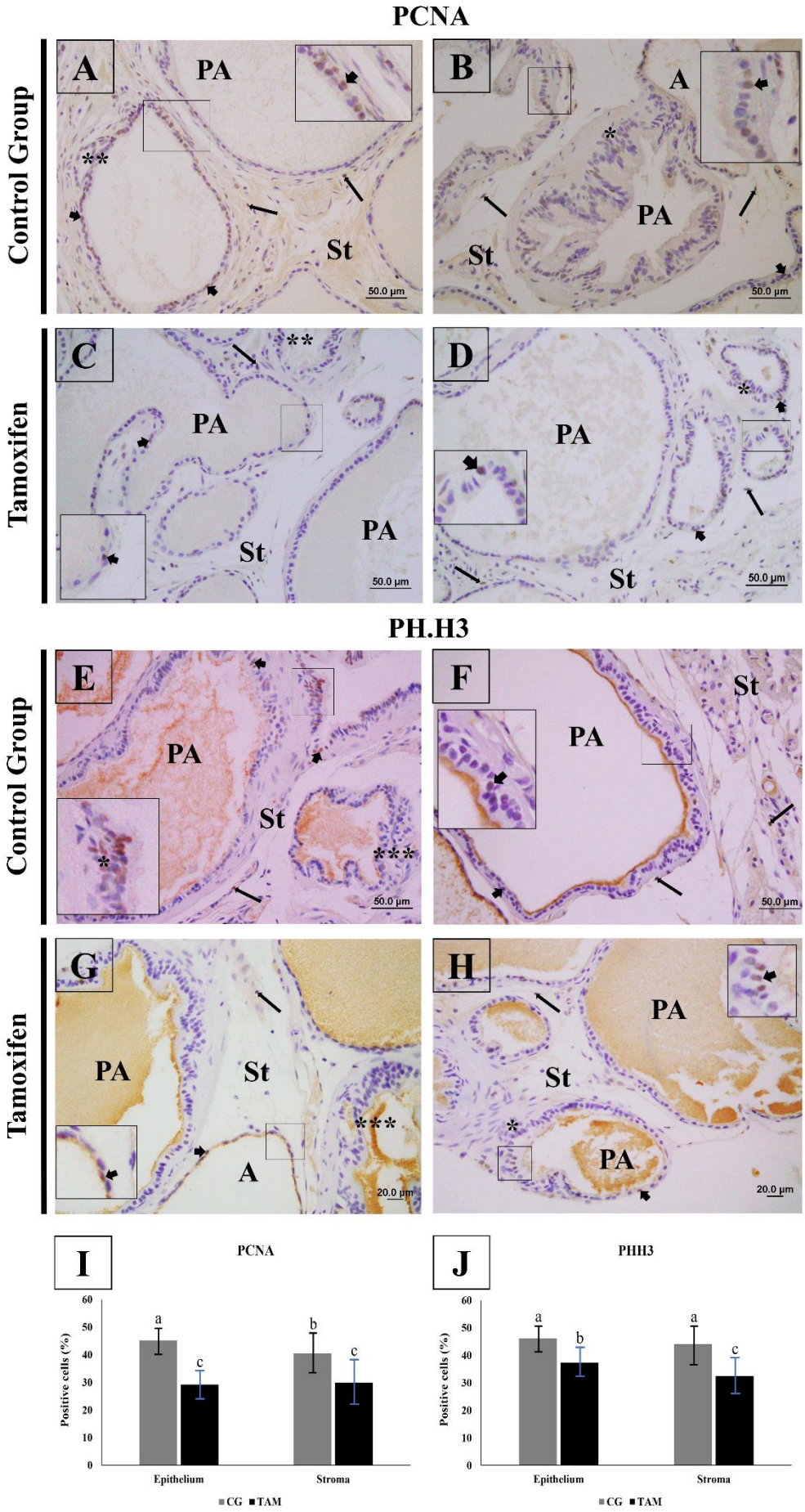
PCNA
Control Group
Tamoxifen
A
B
C
D
PA
St
A
50.0 µm
PH.H3
E
F
G
H
20.0 µm
I
PCNA
J
PHH3
Positive cells (%)
Epithelium
Stroma
CG
TAM
a
b
c

Fig. 3. Immunohistochemical assays for PCNA and PH.H3 performed on histological sections of the prostate of female Mongolian gerbils from control and TAM-treated groups. (A, B) PCNA-positive cells can be verified in both prostate compartments in the control group. (C, D) In the group treated with TAM, there is a decrease in the presence of this factor both in the epithelium and stroma of the gland. (E, F) In the control group, PH.H3-positive cells can be seen in the prostate epithelium and interalveolar region. (G, H) As for the TAM-treated group, a decrease in PH.H3-positive cells can be observed in both prostate compartments. (I) The quantification data showed a significant decrease in the percentage of PCNA-positive cells in the epithelium in the TAM-treated group compared to the control group. (J) The same is observed for the PH.H3 immunostaining. PA (Prostate alveoli), St (Stroma), Arrow (Positive-stromal cells for each factor), Arrowhead (Positive-epithelial cells for each factor), A (Atrophic alveoli), * (Hyperplasia foci), ** (Low-grade PIN foci), *** (High-grade PIN foci). Different letters (a, b, c) indicate significant statistical differences between groups.

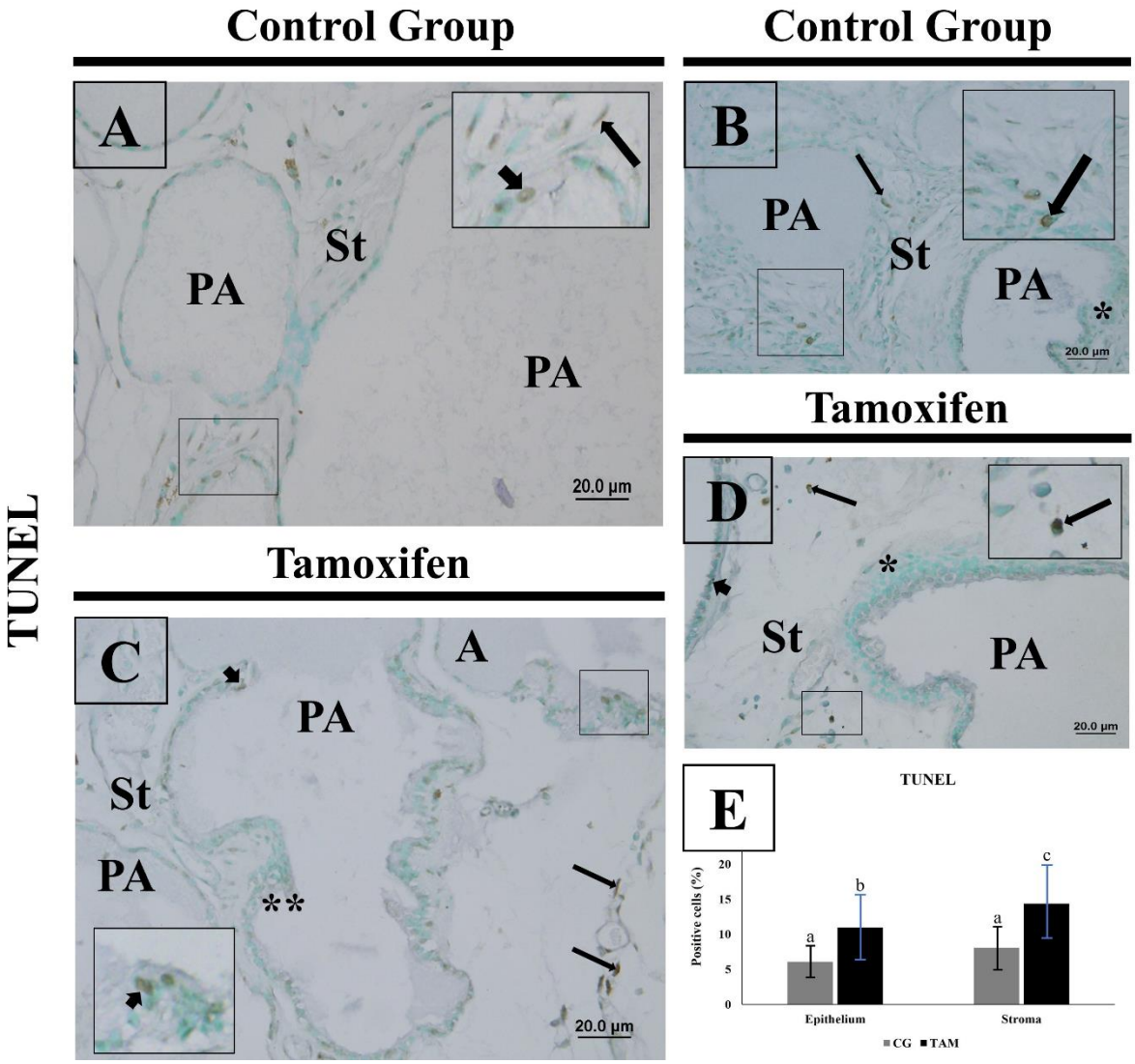

Fig. 4. TUNEL reaction was performed on histological sections of the prostate of female Mongolian gerbils from the control and the TAM-treated group. (A, B) In the control group, a few positive cells can be verified in the epithelial and stromal compartments. (C, D) The staining is increased in both prostate compartments in the group treated with TAM. (E) There was a significant increase in the number of positive cells in both prostate compartments in the TAM-treated group compared to the control group. PA (Prostate alveoli), St (Stroma), Arrow (Positive epithelial cells), Arrowhead (Positive stromal cells), A (Atrophic alveoli), * (Hyperplasia foci), ** (Low-grade PIN foci). Different letters (a, b, c) indicate significant statistical differences between groups.

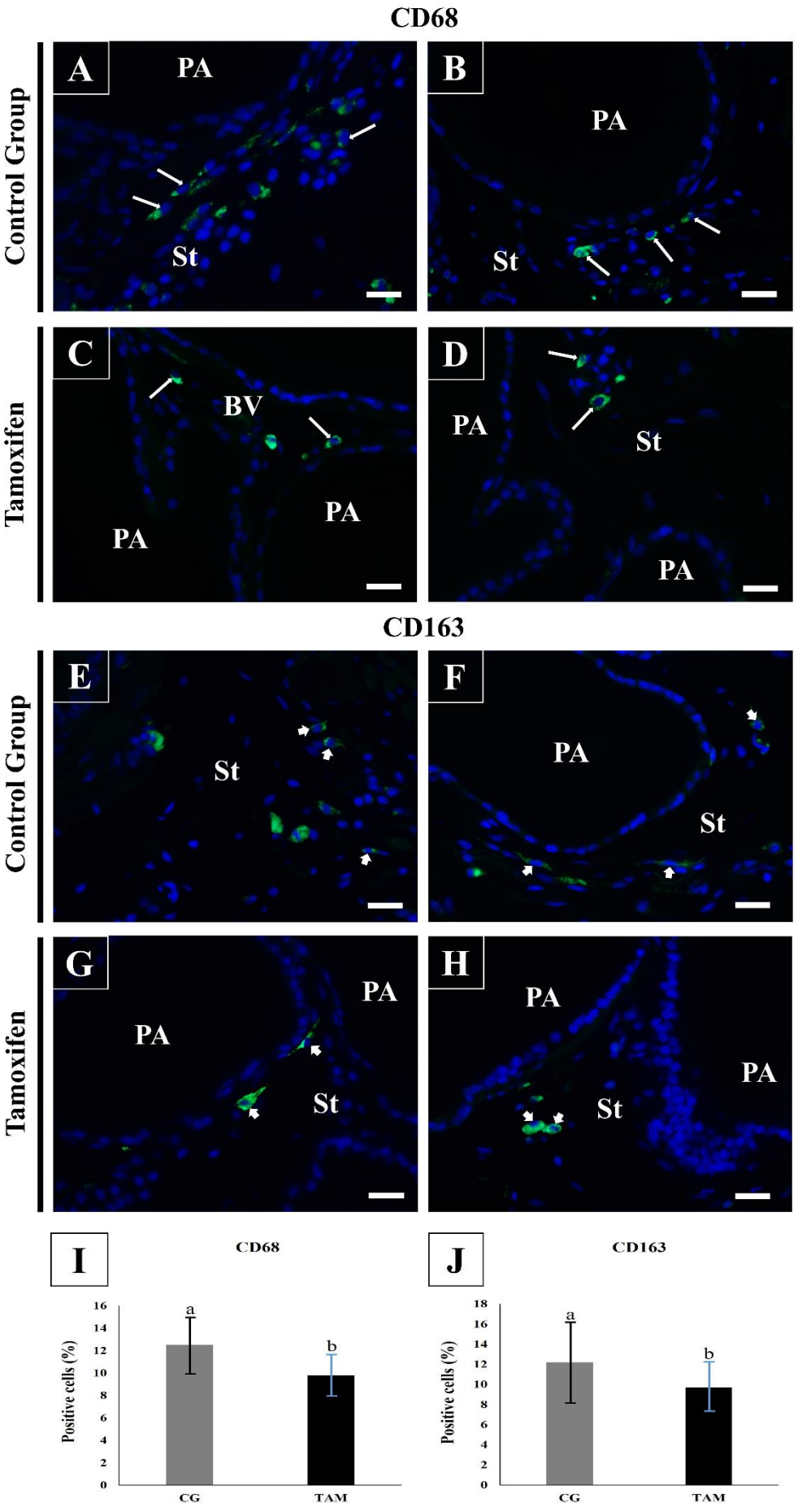
CD68
Control Group
A
PA
St
B
PA
St
Tamoxifen
C
BV
PA
PA
D
PA
St
PA
CD163
Control Group
E
St
F
PA
St
Tamoxifen
G
PA
PA
St
H
PA
PA
St
I
CD68
Positive cells (%)
16
14
12
10
8
6
4
2
0
a
b
CG
TAM
J
CD163
Positive cells (%)
18
16
14
12
10
8
6
4
2
0
a
b
CG
TAM

Fig. 5. Immunofluorescence assays for CD68 and CD163 performed on histological sections of the prostate of female Mongolian gerbils from control and TAM-treated groups. (A, B) In the control group, several CD68-positive stromal cells can be observed between the alveoli. (C, D) The CD68 immunostaining decreased in the interalveolar region in the group treated with TAM. (E, F) The same pattern is observed for the CD163 marking, in which many cells were positive for this factor between the alveoli in the control group, whereas some dispersed cells were positive in the interalveolar region in the TAM-treated group (G, H). (I) There was a significant decrease in the number of CD68-positive cells in the treated group compared to the control group, and the same pattern can be seen for the CD163-positive cells (J). PA (Prostate alveoli), St (Stroma), Arrow (CD68-positive cells), Arrowhead (CD163-positive cells), White bar (20 µm). Different letters (a, b) indicate significant statistical differences between groups.

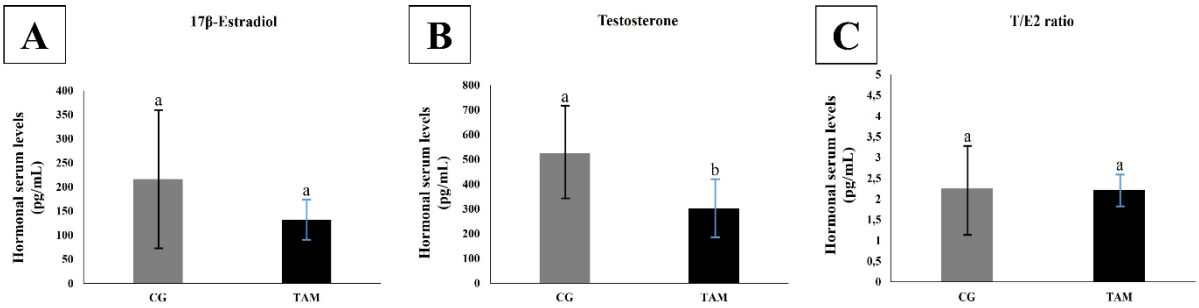

Fig. 6. Serological data of 17β-Estradiol and Testosterone serum levels of female Mongolian gerbil from control and TAM-treated groups. (A) There were no significant statistical differences between the groups in the hormonal serum levels of 17β-Estradiol. (B) As for the Testosterone hormonal serum levels, there was a significant decrease in the group treated with TAM compared to the control group. (C) The T/E2 ratio showed no significant statistical differences in the treated group in comparison to the control. Different letters (a, b) indicate significant statistical differences between groups.

IV. DISCUSSÃO

Nossos dados compilados no **artigo 1** demonstram que o tratamento com ENU, T e E2 proposto para a indução de câncer na próstata de fêmeas do gerbilo foi bem-sucedido, de modo que a maioria das fêmeas desenvolveu focos de adenocarcinoma. Essas lesões foram semelhantes às encontradas na próstata de machos da mesma espécie no modelo de indução tumoral utilizando o MNU em associação com a testosterona, criado por Gonçalves e colaboradores (2010, 2013); a mesma metodologia não havia sido eficiente para o gerar tumores malignos na glândula das fêmeas (dados não publicados). Sendo assim, nosso trabalho traz pela primeira vez um método capaz de induzir carcinogênese na próstata de fêmeas, o que se torna relevante levando-se em consideração a presença de próstata em pelo menos metade das mulheres (Dietrich *et al.*, 2011), além de diversos casos clínicos já terem evidenciado que tais podem ser acometidas por lesões malignas, frequentemente encontradas na próstata masculina, como adenocarcinoma (Massari *et al.*, 2014; Muto *et al.* 2017, Tregnago & Epstein 2018; Kunc & Biernat, 2021; Gao *et al.*, 2022). A escolha do ENU, agente químico mais mutagênico que existe perante os camundongos, gerando mutações pontuais que se espalham por todo o genoma (Russell *et al.*, 1979; Probst & Justice, 2010), juntamente com a exposição ao E2, tendo em vista o impacto que os estrógenos possuem sobre a glândula das fêmeas, além da T, foram o diferencial nesse novo modelo experimental de indução tumoral.

Ao analisarmos diferentes regiões da próstata das fêmeas, observamos que o tratamento aumentou tanto a incidência quanto a multiplicidade de focos adenocarcinoma. Em termos patológicos, o conceito de agressividade do tumor indica que, quanto maior o grau, maior a probabilidade desses tumores migrarem para outros tecidos e gerarem metástases, sendo chamados de tumores invasivos; se tiverem graus menores, os tumores podem ser estruturais, e ficar restritos ao local de origem durante anos, recebendo a denominação de tumores *in situ* (Gordetsky & Epstein, 2016; Kanan *et al.*, 2019). Dessa forma, pensando-se na classificação desses tumores, e como não há uma escala descrita para fêmeas ou mesmo para machos do gerbilo, foi utilizado como base o modelo de classificação de grau de lesões descrito para o TRAMP (Berman-Booty *et al.*, 2012), já que se sabe que os camundongos são filogeneticamente próximos ao gerbilo (Chevret & Dobigny, 2005, Zorio *et al.*, 2019; Leonel *et al.*, 2021). Assim, a maioria dos focos de adenocarcinoma encontrados seriam equivalentes ao grau 5, seguindo este modelo. Nesse estágio, os tumores são classificados como invasivos e

bem diferenciados, ou seja, as células epiteliais ainda não perderam suas características e continuam formando uma estrutura semelhante a uma glândula normal, com aspecto geralmente cribiforme; ainda, ocorre a ruptura da membrana basal, assim como uma reação estromal que leva a perda da musculatura lisa ao redor dos alvéolos, que em conjunto possibilitam a invasão das células epiteliais no estroma (Berman-Booty *et al.*, 2012; Silva *et al.*, 2015).

Sabe-se que a grande maioria dos tumores da próstata tem origem epitelial, e, desse modo, a maior parte dos trabalhos na literatura tem se centrado em análises e classificações do epitélio (Pentheroudakis, Golfinopoulos, Pavlidis, 2007; Mullangi & Lekkala, 2022), enquanto era atribuído um papel secundário para o estroma. Entretanto, evidências foram se acumulando ao longo dos anos e mostraram que o estroma também sofria uma série de alterações que poderiam auxiliar no desenvolvimento e progressão dos tumores, demonstrando que é um processo multifatorial complexo (Tuxhorn, Ayala, Rowley, 2001). Os elementos estromais, assim como as células epiteliais, sofrem modificações, de modo a ocorrer intenso remodelamento e proliferação no estroma. Células musculares lisas e fibroblastos passam a ser substituídos por miofibroblastos e fibroblastos associados ao câncer (CAFs) levando a liberação de fatores de crescimento e citocinas, neovascularização, e recrutamento de células imunes, como os macrófagos. Este processo pode estar relacionado com a hipótese do "estroma reativo", termo criado por Tuxhorn e outros pesquisadores (2001, 2002), de modo que o estroma passa a ter um papel preponderante para a indução inicial do microambiente tumoral e manutenção deste, sendo distinto de um estroma considerado normal. Tais modificações estromais já foram descritas em vários tipos de tumor, como os de cólon, próstata e mama (Tuxhorn, Ayala, Rowley, 2001; Tuxhorn *et al.*, 2002; Tomas & Kruslin, 2004).

Dentre os elementos estromais que poderiam sofrer alterações em um contexto de estroma reativo e formação de um microambiente tumoral, estão os telócitos. Este tipo celular foi descrito a relativamente pouco tempo (Popescu & Faussone-Pellegrini, 2010), e, apesar do interesse crescente sobre estas células, até o presente momento são poucos os estudos sobre o comportamento dos telócitos tanto em condições normais quanto patológicas na próstata. Sendo assim, nosso grupo de pesquisa passou a aprofundar os conhecimentos sobre essa célula na próstata de machos (Corradi *et al.*, 2013, Sanches *et al*., 2020), além de outros órgãos (Zani *et al.*, 2018), assim como foi pertinente verificar como elas poderiam reagir em um cenário de indução tumoral, como foi o caso desta pesquisa, que foi a primeira a demonstrar telócitos na próstata das fêmeas. Assim, de modo complementar ao primeiro trabalho, o **artigo 2** foi

produzido, no qual a ênfase não esteve no método de indução e ocorrência de lesões, mas sim em uma célula em particular.

Levando-se em consideração que os telócitos são células difíceis de serem distinguíveis em microscopia óptica comum, a realização deste trabalho se baseou principalmente nas análises de microscopia eletrônica de transmissão (TEM), onde as características particulares deste tipo celular são vistas com mais detalhes, tais como os prolongamentos citoplasmáticos, denominados telopódios, e a alternância entre regiões dilatadas e finas, os podômos e podômeros respectivamente (Corradi *et al.*, 2013; Sanches *et al.*, 2020). Na ultraestrutura, foram encontradas regiões de lesões benignas e pré-malignas, como hiperplasia e neoplasia intraepitelial prostática (PIN), respectivamente; entretanto, não foram observadas regiões de adenocarcinoma. Os miofibroblastos e fibroblastos ativados, característicos do estroma reativo, também foram evidenciados no estroma. Foi visto que os telócitos estão ausentes em regiões adjacentes aos focos de lesões, assim como a musculatura lisa ao redor dos alvéolos, nestas mesmas regiões, sofreu alterações, se tornando descontinua ou espessa. Tal fato indica que estas células seriam necessárias para a integridade do epitélio e a manutenção da camada muscular perialveolar, algo que já havia sido sugerido para os telócitos na próstata dos machos (Felisbino *et al.*, 2019; Sanches *et al.*, 2020). Ainda, estas células foram vistas próximas a vasos sanguíneos e células imunes, sugerindo que eles podem ter um papel pró-angiogênico/pró-inflamatório, como já foi visto em outros órgãos (Chi *et al.*, 2015; Ibba-Manneschi *et al.*, 2016; Jiang *et al.*, 2018).

Dando sequência aos resultados obtidos no **artigo 1**, além dos focos de adenocarcinoma, foi evidenciado alta incidência e multiplicidade de atrofia alveolar, o que demonstra uma desorganização da arquitetura prostática, algo que também é verificado em quadros de hipoandrogênese e castração (Goés *et al.*, 2013; Zanatelli *et al.*, 2021). Os dados de ultraestrutura mostraram as alterações epiteliais relacionadas ao processo de atrofia com mais detalhes, de modo que o compartimento epitelial, antes cúbico ou cilíndrico, passa a ser escamoso, assim como ocorre achatamento do núcleo. O presente trabalho também mostrou a ocorrência de outras lesões benignas e pré-malignas, tanto no grupo tratado quanto no controle, tais como focos de hiperplasia, PIN de baixo e de alto grau, que foram classificadas de acordo com Shappell e colaboradores (2004). A presença desses tipos de lesões no grupo controle corrobora estudos anteriores que demonstraram que o gerbilo é uma espécie que naturalmente desenvolve lesões benignas e pré-malignas na próstata com o envelhecimento, geralmente

associadas com alterações no equilíbrio hormonal (Campos *et al*., 2008; Custodio *et al.,* 2008; Biancardi *et al.,* 2017). Partindo desse princípio, análises sorológicas foram feitas para T e E2, dois hormônios intrinsicamente relacionados ao desenvolvimento e manutenção da fisiologia prostática (McPherson *et al.*, 2008; Rochel-Maia *et al.,* 2013). Nossos dados evidenciaram um expressivo aumento no nível de E2 circulante, ao passo em que não houve diferença nos níveis séricos de T. A razão T/E2 diminuiu, indicando um meio mais estrogênico, o que também foi verificado em outros trabalhos, no qual foi hipotetizado que um ambiente hiperestrogênico poderia ter relação com a susceptibilidade a quadros patológicos, como o câncer (Prins *et al.*, 2007; Usoro *et al.*, 2015).

Muitos estudos do nosso grupo de pesquisa se centraram na associação entre desenvolvimento e câncer, visto que vários genes e fatores de crescimento presentes durante o desenvolvimento inicial da próstata também são expressos no câncer (Isali *et al.,* 2019; Teishima *et al*., 2019; Wang *et al.,* 2020). Assim, iniciaram-se os trabalhos de exposição intrauterina a xenoestrógenos, bem como desreguladores endócrinos, conhecidos por causarem desequilíbrio hormonal, sobre os fetos de gerbilo (Silva *et al.,* 2013; Zanatelli *et al.,* 2014; Rodríguez *et al.,* 2016; Sanches *et al.*, 2017a, 2017b), o que levou ao entendimento de que animais expostos a estes compostos, especialmente os que possuem ação estrogênica, ainda durante a vida fetal, teriam uma predisposição maior a desenvolver alterações no desenvolvimento pós-natal, bem como tumores prostáticos malignos, conforme envelhecem (Biancardi *et al.,* 2017; Sanches *et al*., 2019). Dessa forma, pode-se supor que há no câncer de próstata dos gerbilos, seja dos machos ou das fêmeas, a atuação de um componente hormonal, já que, por ser uma glândula sexual acessória, os hormônios endócrinos são necessários para a homeostase desta (McPherson *et al.,* 2008; Rochel-Maia *et al.* 2013). Por fim, no caso das fêmeas, como os níveis séricos de T são geralmente muito menores do que os encontrados em machos, e o contrário é verificado para os estrógenos, como o E2. Assim, entende-se que estes últimos exercem um papel essencial na próstata (Fochi *et al*., 2013; Rochel-Maia *et al.,* 2013; Zanatelli *et al.,* 2014). O desenvolvimento inicial da próstata das fêmeas expostas ao E2, mesmo em dosagens baixas, teve impactos maiores nas glândulas das fêmeas adultas em comparação aos machos (Sanches *et al*., 2017a, 2017b), o que mais uma vez nos demonstra que mudanças mínimas nos níveis deste hormônio são suficientes para gerar alterações mais agressivas para as fêmeas do que o seriam para machos (Sanches *et al.,* 2019).

Considerando-se a maior sensibilidade que as fêmeas possuem ao E2, posteriormente ao tratamento com ENU+T+E2, elas foram expostas ao tamoxifeno (TAM), uma droga amplamente utilizada contra o câncer de mama (Visvanathan *et al*., 2019; Chaput & Sumar, 2022), e os resultados foram compilados no **artigo 3**. Apesar de não observarmos redução quanto às lesões encontradas, nossos resultados revelaram que o TAM possui efeito pró-apoptótico e antiproliferativo, além de anti-inflamatório, no quadro de carcinogênese quimicamente induzida, demonstrando ser um potencial alvo terapêutico para patologias que podem ocorrer na próstata das fêmeas.

TAM compete com os estrógenos por seus receptores (ER), atuando como um modelador seletivo dos receptores de estrógeno (SERM) (Patel & Bihani, 2017; Dehghan *et al*., 2021). Dependendo do órgão e do tipo de interação que ele tem com o receptor estrogênico, este composto pode gerar respostas antagonistas, como no caso da mama, ou agonistas, como no endométrio e osso (Li *et al*., 2017; Emons, Mustea, Tempfer, 2020; Lafront *et al*., 2020), além de possuir outras atividades farmacológicas não relacionadas ao ER (Nasu *et al*., 2008; Pepe *et al*., 2021; Sfoglianari *et al*., 2022). Sabe-se que o tratamento com TAM é bem-sucedido em mulheres com alto risco de desenvolver câncer de mama (Nazarali & Narod, 2014; Chaput & Sumar, 2022), já que ele inibe a proliferação celular, antes ocasionada pelos estrógenos, assim como diminui o crescimento das células tumorais nas glândulas mamárias (Li *et al*., 2017). Na próstata do gerbilo, já foi verificado que o TAM possui ação agonista quanto ao ERα (Santos *et al*., 2008), bem como diminui a sobrevivência de células do câncer de próstata tanto em humanos quanto em camundongos (Shim *et al*., 2009, Semenas *et al*., 2021).

A relação entre uma superexpressão do receptor de estrógeno α (ERα) com tumores metastáticos e câncer de próstata resistente a castração (CPRC) é bem documentada (Ricke *et al*., 2008; Semenas *et al*., 2021). ERα é um fator de transcrição que, ao ser ativado, regula vias de sinalização, tais como NOTCH1, relacionada a transições epitélio-mesenquimais, e promove o crescimento e invasão das células tumorais (Shen *et al.*, 2019). Observamos redução na expressão deste receptor em nosso experimento, entretanto, os níveis séricos de E2 não se alteraram, o que já foi visto em outro estudo que utilizou o tratamento com TAM em fêmeas do gerbilo (Santos *et al.*, 2008). Dessa forma, hipotetizamos que o TAM, ao agir de forma agonista na próstata das fêmeas, potencializa o efeito estrogênico, inicialmente saturando os receptores, o que implicaria em queda a posteriori.

Adicionalmente, verificou-se uma queda nos níveis séricos de testosterona, assim como uma diminuição na expressão do receptor de andrógeno (AR), um fator nuclear considerado marcador biológico em diversos tipos de cânceres, como melanomas, glioblastomas, câncer de mama e de próstata (Wang *et al*., 2017; Zalcman *et al*., 2018; You *et al*., 2022; Westaby *et al*., 2022). Trabalhos anteriores demonstraram que a testosterona circulante diminui em pacientes com câncer de mama tratadas com TAM (Lonning *et al*., 1995; Hadji *et al*., 2012; Baumgart *et al*., 2014), corroborando nossos achados. Tal fato pode ser explicado por um possível aumento na atividade da aromatase, enzima responsável pela conversão periférica de testosterona em estradiol (Ma *et al.,* 2005; Lakshman *et al.,* 2010), em uma tentativa do próprio organismo para compensar o bloqueio estrogênico gerado pelo TAM na glândula mamária. Com relação ao AR, estudos evidenciaram que uma superexpressão do mesmo alterou o mecanismo de ação do TAM, induzindo a uma resistência ao tratamento com o fármaco em células de câncer de mama (Amicis *et al*., 2010; Ciupek *et al*., 2015). Assim, o AR pode agir como um modulador do TAM, de modo que quanto maior a sua expressão, menor vai ser o efeito do tratamento com o fármaco. Nossa pesquisa mostrou que a expressão do AR diminui na próstata, e, dessa forma, pode-se deduzir que o TAM pode ter uma maior ação na próstata, competindo contra o E2.

Nossos resultados também demonstram redução na proliferação celular, assim como aumento de apoptose após o tratamento com TAM. A queda observada na expressão de ERα pode ser positivamente correlacionada à diminuição da atividade proliferativa, uma vez que este receptor ativa vias de proliferação e crescimento celular (Liu, Ma, Yao 2020; Dehghan *et al*., 2021). Paralelamente, TAM também pode atuar em vias de sinalização independente de ER, como foi visto em estudos com células de câncer de pele (Hasegawa *et al*., 2018). Tais dados relacionaram os efeitos antiproliferativos do tratamento com o fármaco a um aumento na concentração de cálcio intracelular e ativação da proteína kinase C (PKC). Em adição, Dehghan e colaboradores (2021) mostraram que a exposição ao TAM inibiu o crescimento de células do câncer de tireoide ao bloquear a proliferação e induzir a apoptose, algo que também foi verificado em linhagens celulares isoladas de câncer de mama. Um dos mecanismos sugeridos envolve a via mitocondrial e ativação da Caspase-3. TAM regula negativamente a expressão de Bcl-2, proteína anti-apoptótica, e estimula a expressão de Bax, proteína pró-apoptótica, e de Caspase-3 (Li *et al*., 2017; Rouhimoghadam *et al*., 2018; Dehghan *et al*., 2021). Ainda, sabe-se que o TAM diminui a expressão de genes supressores da apoptose celular, como NEAT1,

ativo em diversos tipos de tumores, como de ovário, fígado e pulmão (Ding *et al*., 2017; Kou *et al*., 2020; Yu *et al*., 2019). Tumores xenográficos em camundongos corroboram as observações de que o tratamento com TAM induz a um nível elevado de apoptose e a inibição do crescimento celular, além de inibir metástase em CRPC positivos para o ER, surgindo como uma alternativa para tipos de câncer que não respondem mais a terapias antiandrogênicas (Semenas *et al*., 2021).

Ainda, os dados evidenciaram redução de macrófagos ativados. Apesar de atualmente existirem várias classificações quanto ao imuno-fenótipo deste tipo celular, eles são convencionalmente divididos em dois: M1 e M2 (Sfogliarini *et al*., 2022). Macrófagos do tipo M1, o fenótipo mais clássico e geral, possuem propriedade pró-inflamatória, ou seja, estimulam a inflamação nos tecidos, estando relacionados com a via normal de reação contra patógenos e infecções, e são geralmente evidenciados pela marcação para o CD68 (Shapouri-Moghaddam *et al*., 2018; Zhang *et al.*, 2022). Já os do tipo M2 possuem características anti-inflamatórias, e estão envolvidos na cicatrização do tecido, revascularização e supressão da inflamação (Hesketh *et al*., 2017), bem como são detectados pela marcação para o CD163 (Mantovani & Locati, 2013; Hu *et al*., 2017; Sfogliarini 2022). Ambos os tipos podem estar presentes em processos associados a formação de tumores (Pan *et al*., 2020), e, como verificado em nosso modelo prévio de indução de carcinogênese, a expressão para os dois marcadores de macrófagos aumentou significativamente (dados não publicados). Inversamente, no presente trabalho foi observado que tanto a expressão de CD68 quanto a de CD163 diminuíram no estroma prostático, de modo que podemos sugerir que o TAM possui efeitos anti-inflamatórios na carcinogênese quimicamente induzida. Em consonância com essa ideia, vários trabalhos evidenciaram a atividade anti-inflamatória do TAM em diferentes tipos de tumores, como no câncer de pâncreas (Cortes *et al*., 2019; Pein & Oskarsson, 2019) e no câncer de próstata agressivo (Semenas *et al*., 2021; Tong, 2022). Em camundongos, Han e outros pesquisadores (2010) mostraram que o TAM impediu a produção de óxido nítrico (NO) pelos macrófagos, molécula relacionada ao processo de inflamação e carcinogênese, cuja inibição auxilia no tratamento do câncer. Outros estudos demonstraram que o fármaco, além de afetar os macrófagos diretamente por meio do receptor de estrógeno acoplado à proteína G (GPER), diminuindo a migração destas células, também pode levar a alterações na composição da matriz extracelular (ECM) de forma a reduzir a rigidez da mesma, o que limitaria a infiltração e ancoragem destas células, bem como a invasão celular (Hattar *et al*., 2009; Pein & Oskarsson,

2019). Assim, os efeitos imunomoduladores do TAM nos tecidos podem ou não estar relacionados a via de sinalização do ER (Sfogliarini *et al*., 2022).

Adicionalmente, é pertinente ressaltar que, apesar de ser considerada uma medicação segura, justamente pela baixa toxicidade e por ter poucos efeitos adversos, o tratamento prologando com TAM pode levar ao surgimento de doenças ginecológicas, como câncer de endométrio, que ocorrem especialmente em mulheres pós-menopausa com histórico de patologias no útero (Nasu *et al*., 2008; Emons, Mustea, Tempfer, 2020). Tais efeitos demonstram o complexo mecanismo de ação do TAM, que, dependendo do tecido, pode atuar como agonista ou antagonista, podendo afetar a fisiologia de diversos órgãos hormônio-dependentes, como a próstata (Santos *et al*., 2008).

Por fim, estre trabalho trouxe pela primeira vez um potencial alvo terapêutico para atenuar o microambiente tumoral gerado pela indução química de carcinogênese na próstata das fêmeas. Abordagens como esta são relevantes tendo em vista que estudos sugerem que até metade das mulheres pode ter uma próstata funcional similar à masculina (Dietrich *et al*., 2011; Biancardi *et al*., 2017). Ademais, é crescente o número de trabalhos associando a próstata feminina, também conhecida como glândula de Skene, com o desenvolvimento de adenocarcinoma (Heller, 2015; Thum *et al*., 2017; Tregnago & Epstein 2018; Kyriazis *et al*., 2020; Bondili *et al*., 2021; Gao *et al*., 2022). Muitas vezes, essas enfermidades são confundidas com doenças relacionadas ao trato urogenital, uma vez que existem evidências de que alguns carcinomas uretrais em mulheres tem origem prostática (Reis *et al*., 2011; Lenz *et al*., 2021; Kunc & Biernat, 2021). Sendo assim, o número real de casos pode estar sendo subestimado, o que gera preocupação a nível de saúde pública.

V. CONCLUSÕES

O presente trabalho mostrou pela primeira vez uma metodologia para a indução de câncer na próstata de fêmeas, algo que antes só era visto para machos. O tratamento com ENU, T e E2 promoveu o aparecimento de tumores malignos na próstata de mais da metade das fêmeas do gerbilo, além de lesões benignas, como hiperplasia, e pré-malignas, como PIN de baixo e alto grau. O microambiente tumoral foi evidenciado pelas diversas alterações na estrutura estromal, como a perda de musculatura lisa ao redor dos alvéolos, que possivelmente

foi substituída por outros tipos celulares comumente encontrados no contexto de “estroma reativo”, como miofibroblastos e CAFs. Ainda, houve aumento de vasos sanguíneos, o que indica a ocorrência do processo de angiogênese, bem como um influxo de macrófagos, gerando aumento na resposta inflamatória. Adicionalmente, tanto os níveis circulantes de estradiol quanto os receptores estrogênicos no epitélio e estroma aumentaram drasticamente, enquanto a razão T/E2 diminuiu consideravelmente, o que demonstra que os estrógenos, em especial o estradiol, tem um grande impacto na manutenção da histofisiologia prostática e na promoção de quadros patológicos, como o câncer.

As diferenças intersexuais, em termos de sensibilidade hormonal e maior susceptibilidade ao desenvolvimento de lesões, se tornam preocupantes já que concentrações de estradiol que não são nocivas para o macho podem o ser para a fêmea. Visto que aproximadamente metade das mulheres possuem próstata, e, assim como a próstata de homens, pode desenvolver quadros patológicos, como prostatites ou até mesmo adenocarcinoma, a glândula das mulheres pode ser um órgão negligenciado em termos de saúde pública, já que elas também podem estar sujeitas a concentrações maiores de determinados compostos com ação estrogênica que não teriam efeitos danosos para o homem.

Ainda, por meio do tratamento proposto, foi possível investigar o comportamento dos telócitos em um contexto de formação tumoral. Estas células estão ausentes em regiões adjacentes a lesões, bem como a camada de musculatura lisa se mostra alterada próxima a essas regiões, sofrendo descontinuidade. Além disso, observou-se que os telócitos estão em contato com vasos sanguíneos e várias células imunes no estroma. Desse modo, sugere-se que os telócitos seriam necessários tanto para a integridade do epitélio e da camada de musculatura lisa perialveolar, assim como para a organização do próprio estroma, ao mesmo tempo em que poderiam atuar a favor da inflamação e angiogênese.

Por fim, o modelo de terapia baseado no TAM, um antiestrogênico comumente utilizado em terapias contra o câncer de mama, mostrou ser efetivo para atenuar o microambiente tumoral. A atividade proliferativa e resposta inflamatória diminuíram, bem como houve aumento de morte celular programada. Novos estudos serão necessários para aprofundar o conhecimento sobre a histologia da próstata feminina tanto em condições normais quanto patológicas, bem como as implicações que o uso de possíveis terapias, como o tratamento com TAM, podem causar a curto e longo prazo em órgãos hormônio-dependentes,

como a próstata, uma vez que já foi visto que a utilização prolongada desse composto pode levar a enfermidades ginecológicas.

VI. REFERÊNCIAS

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VII. ANEXOS

VII.1. Termo de aprovação da pesquisa pela Comissão de Bioética e/ou Biossegurança pertinente

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

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

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação do papel dos telócitos na formação do estroma reativo em um modelo de carcinogênese induzida na próstata de fêmeas do gerbilo (*Meriones unguiculatus*)", registrada com o nº. 209/2019 - CEUA, sob a responsabilidade do Professor Doutor Sebastião Roberto Taboga e da Doutoranda Juliana dos Santos Maldarine, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em reunião de 22 de outubro de 2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	22/10/2019 a 15/06/2023
Espécie/linhagem/Raça	*Meriones unguiculatus*
Nº de animais	120(cento e vinte)
Peso/Idade	0,7 gramas/06 meses
Sexo	Fêmea
Origem	Biotério do Instituto de Biociências, Letras e Ciências Exatas da UNESP, campus de São José do Rio Preto/SP.

São José do Rio Preto, 28 de janeiro de 2020.

Profa. Dra. Eliane Gonçalves de Freitas
Presidente da CEUA

Observações:

1) O Relatório Final deverá ser encaminhado em Formulário próprio à CEUA no prazo de até 30 (trinta) dias após o término da pesquisa;
2) Qualquer alteração na pesquisa deverá ser encaminhada à CEUA para apreciação.

Instituto de Biociências, Letras e Ciências Exatas – Comissão de Ética no Uso de Animais
Rua Cristovão Colombo, 2265 - Jardim Nazareth - CEP 15054-000 São José do Rio Preto – SP - Brasil
Tel: (17) 3221-2480 / 3221-2563

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"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

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

ATESTADO

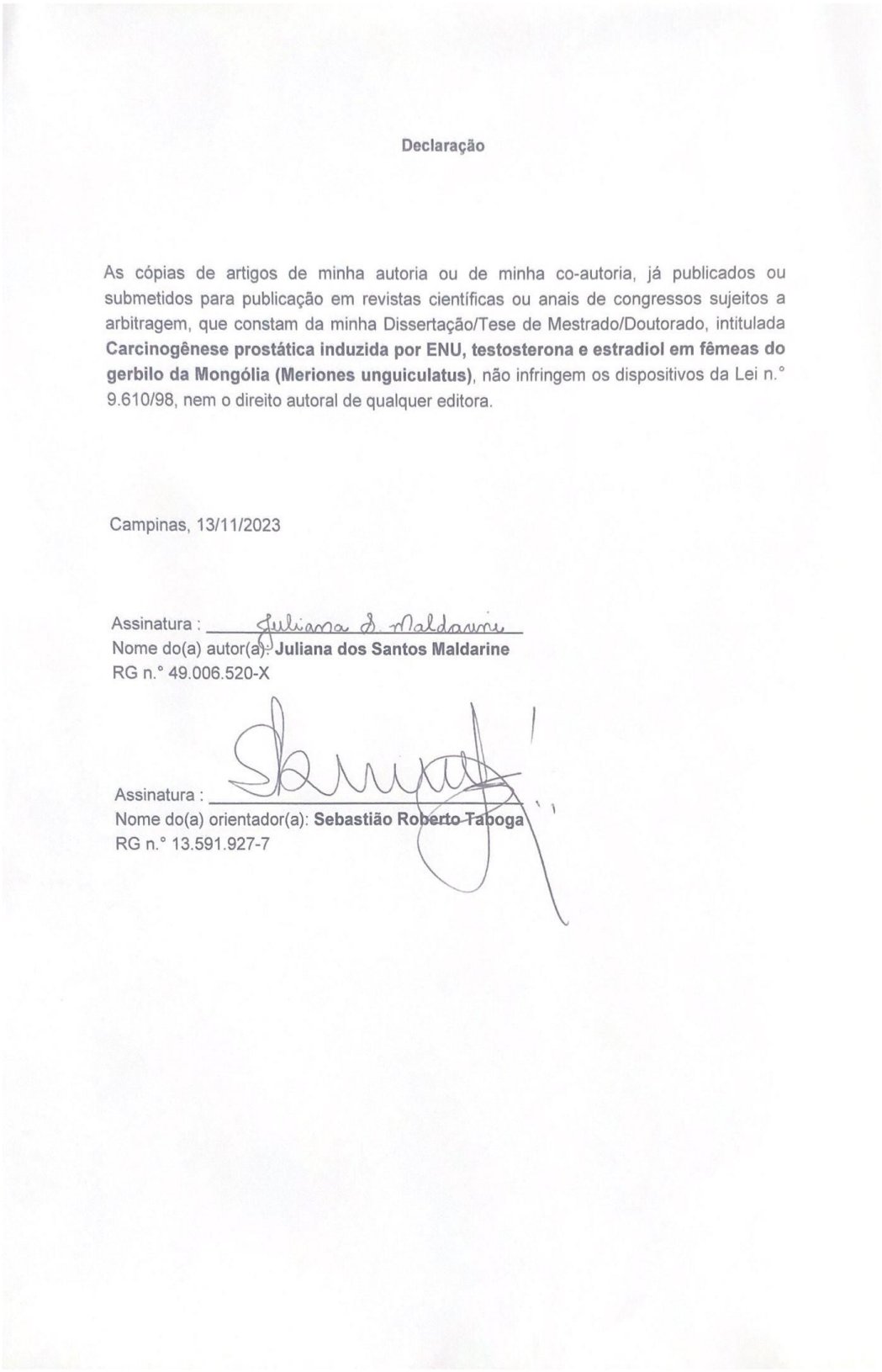
Atestamos que a proposta intitulada "Avaliação do papel dos telócitos na formação do estroma reativo em um modelo de carcinogênese induzida na próstata de fêmeas do gerbilo (*Meriones unguiculatus*)", registrada com o nº. 209/2019 - CEUA, sob a responsabilidade do Professor Doutor Sebastião Roberto Taboga e da Doutoranda Juliana dos Santos Maldarine, aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em 22 de outubro de 2019, teve seu título alterado, a pedido dos responsáveis pela pesquisa e aprovado ad referendum da CEUA em 04 de agosto de 2023, para *"CARCINOGÊNESE PROSTÁTICA INDUZIDA POR ENU, TESTOSTERONA E ESTRADIOL EM FÊMEAS DO GERBILO DA MONGÓLIA (MERIONES UNGUICULATUS)"*.

São José do Rio Preto, 08 de agosto de 2023.

Profa. Dra. Patricia Simone Leite Vilamaior
Presidente da CEUA

VII.2. Direitos autorais

1. Declaração



Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Carcinogênese prostática induzida por ENU, testosterona e estradiol em fêmeas do gerbilo da Mongólia (Meriones unguiculatus)**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 13/11/2023

Assinatura : Juliana S. Maldarine
Nome do(a) autor(a): **Juliana dos Santos Maldarine**
RG n.° 49.006.520-X

Assinatura : _____
Nome do(a) orientador(a): **Sebastião Roberto Taboga**
RG n.° 13.591.927-7

2. Autorização da editora para utilização do artigo "*The complex role of telocytes in female prostate tumorigenesis in a rodent model*"