UNIVERSIDADE ESTADUAL DE CAMPINAS

Heitor Verardi

EFEITO DA PENICILAMINA SOBRE A REGRESSÃO PROSTÁTICA SEGUIDA À CASTRAÇÃO

Tese apresentada ao Instituto de Biologia da UNICAMP para obtenção do título de Mestre em Biologia Celular e Estrutural, na área de Biologia Celular

Orientador: Prof. Dr. Hernandes F. Carvalho

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Titulares:

Prof. Dr. HERNANDES FAUSTINO DE CARVALHO

Profa. Dra.VALÉRIA HELENA ALVES CAGNON QUITETE

Prof. Dr. SEBASTIÃO ROBERTO TABOGA

Suplente:

IARA MARIA SILVA DE LUCCA

Sumário

Dedicatória

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Resumo

A regressão prostática que se segue à castração representa um sistema bastante dinâmico de interações células estromais-células epiteliais-matriz extracelular. A definição de alguns parâmetros associados à matriz extracelular tem sido alvo de pesquisas em nosso laboratório e, neste trabalho, procuramos determinar o efeito da penicilamina, uma dimetilcisteína, com capacidade de quelar cátions divalentes e de interagir com o piridoxol, cofatores da enzima lisil oxidase, sobre a regressão prostática em ratos. Foi demonstrado que a aplicação de dosagem subcrônica de penicilamina reduz a perda de peso da próstata ventral após castração e que este efeito é revertido pela administração simultânea de sulfato de cobre e vitamina B6. Histologicamente, a penicilamina causa o colapso das estruturas epiteliais que apresentam alta densidade celular, quando comparadas com animais castrados não tratados. Este efeito é caracterizado por um atraso na morte celular típica das células epiteliais que se segue à castração. As células musculares lisas apresentam comportamento semelhante na presenção ou ausência da penicilamina, perdendo os contatos com outras células musculares lisas e adquirindo um aspecto espinhoso. A análise de cortes histológicos corados pelo tricrômico de Masson sugere uma menor deposição de colágeno, que apresenta-se disperso no estroma ao invés de organizados em fibras onduladas e intimamente associado com as células musculares lisas. Estes aspectos foram confirmados à microscopia eletrônica de transmissão que mostrou também existirem células epiteliais que se projetam em direção ao estroma, perdendo o contato com as demais células epiteliais, mas mantendo-se separadas do estroma pela membrana basal. O sulfato de cobre em conjunto com a vitamina B6 revertem parcialmente os efeito causados pela penicilamina.

Introdução

O aparelho reproduto masculino é formado pelos testículos, ductos genitais, glândulas acessórias (vesículas seminais, próstata e glândulas bulbo-uretrais) e pênis. A próstata é a maior das glândulas acessórias e apresenta grande expressão funcional (Netter, 1965). No homem, ela é um órgão glandular e fibro-muscular, do tamanho aproximado de uma noz, circundando a parte prostática da uretra (Moore, 1990).

No homem, a próstata é constituída por um conjunto de glândulas túbulo-alveolares ramificadas cujos ductos desembocam na uretra prostática e que estão organizadas em quatro lobos distintos: os lobos anterior e posterior, os lobos laterais e um lobo mediano. Destes, os laterais são os mais desenvolvidos (Netter, 1965). As glândulas são revestidas internamente por um epitélio cilíndrico simples secretor que se assenta sobre uma membrana basal que o separa de um estroma vascularizado rico em células musculares lisas. Nas células epiteliais aparece grande quantidade de lisossomos com intensa atividade de fosfatase ácida. Uma delgada cápsula fibro-elástica com muitas células musculares lisas envolve a próstata, enviando trabéculas que penetram na glândula (Cormack, 1991; Junqueira e Carneiro, 1995).

A próstata normal, ainda que estruturalmente presente já por volta da 12ª semana de vida intra-uterina, permanece rudimentar através da infância e se desenvolve somente após a puberdade sob a influência dos níveis aumentados de andrógenos circulantes. Testosterona é o hormônio mais importante nesse processo, sendo sintetizado pelas células de Leydig do testículo, sob a influência do hormônio luteinizante (LH) de origem hipofisária que , por sua vez, é dependente de um estímulo hipotalâmico através do hormônio de liberação do hormônio luteinizante (LHRH). Os níveis de testosterona são mantidos dentro dos limites normais (10 – 35 μg/L) por um mecanismo de retroalimentação negativa. Embora muito menos expressiva, o cortex da adrenal é uma outra fonte de andrógenos. Sob a influência do hormônio adrenocorticotrófico (ACTH), andrógenos são liberados para a circulação a partir das adrenais. Quando a função testicular está intacta,

Abstract

The prostatic involution that follows castration is a dinamic system in which interactions amongst eptihelial cells-stromal cell-extracellular matrix take place. We are interested in defining some characteristics of the extracellular matrix in this model and so, in this work, we decided to investigate the effects of a sub-chronic dosage of penicillamine, a dimethyl-cysteine, able to chelate divalent cations and to interact with pyridoxol, co-factors of the enzyme lysyl-oxidase, upon the prostatic regression in rats. It was shown that penicillamine reduces the weight loss after castration and that this effect is reverted by the simultaneous administration of copper sulfate and vitamin B6. Histologically, penicillamine causes a collapse of the epithelial structures, which are highly cellular as compared to those in non-treated castrated rats. This effect was shown to result from a delay in the apoptotic epithelial cell death. Smooth muscle were not affected by penicillamine, showing the same behavior as in non-treated rats, loosing contacts to other smooth muscle cells and acquiring a spinous aspect. Analysis of Masson's trichromic stained sections suggested a reduced accumulation of collagen, which was dispersed in the stroma, instead of being organized in undulated fibers in close association with the smooth muscle cells. These aspects were confirmed by electron microscopy, which also demonstrated the existence of some epithelial cells projecting from the epithelium into the adjacent stroma after penicillamine treatment. These cells loose the contacts to other epithelial cells but are still envelopped by the basal membrane. Copper sulfate in association with vitamin B6 partially reverts the effects promoted by penicillamine.

esta menor atividade androgênica adicional tem pouco impacto; entretanto, após a castração seus efeitos residuais androgênicos podem ter discreta ação sobre células prostáticas que são dependentes de andrógenos.

O crescimento normal, a diferenciação e a manutenção da integridade estrutural e funcional da próstata, vesículas seminais e glândulas bulbouretrais é dependente de níveis constantes de andrógenos circulantes e ocorrem através de interações entre o mesênquima e o epitélio durante o desenvolvimento (Price, 1963, Aumüller e Seitz 1990; Rosai et al 1996; Hayward et al, 1997; Thomson et al, 1997) e o estroma e o epitélio, no animal adulto.

A supressão de andrógenos (por castração ou pela administração de anti-andrógenos) causa a atrofia dos órgãos dependente de andrógeno (Cormack, 1991). Este processo de redução do tamanho e de perda da função é chamado de involução ou regressão.

No rato, a próstata é uma estrutura complexa formada por um par de lobos ventrais no istmo da bexiga e dois lobos dorsolaterais com seus ductos. O conjunto dorsolateral circunda a uretra na base da bexiga (Price, 1963, Sugimura et al, 1986). Os lobos diferem entre si com relação às suas secreções (Aumüller e Seitz, 1990), topografia e organização estrutural (Jezik et al, 1982).

Na próstata ventral, o epitélio que reveste as unidades secretoras e seus ductos é constituído por células cilíndricas altas, sendo possível diferenciá-las em luminais e basais através da utilização de anticorpos monoclonais contra citoqueratinas específicas (Prins et al, 1991). As unidades secretoras apresentam um extenso pregueamento do epitélio secretor e podem, por isso armazenar a secreção produzida (Cormack, 1991). Além disto, o sistema de ductos pode ser dividido em três regiões morfológica e funcionalmente diferentes em relação à células epiteliais que o revestem (Lee, 1990). Na extremidade distal das estruturas secretoras existem células cilíndricas altas com núcleo apical (Lee et al, 1994), a região intermediária é revestida por células secretoras de proteínas prostáticas específicas, enquanto que as células epiteliais da região proximal são cúbicas baixas, sofrendo muitas delas, morte celular programada (Nemeth e Lee, 1996).

A membrana basal localizada entre epitélio e o tecido conjuntivo da próstata tem grande importância no controle das atividades celulares e, principalmente, na fisiologia das células epiteliais. Os principais componentes das membranas basais (dentre eles o colágeno tipo IV e a laminina) foram detectados na próstata humana normal (Knox et al, 1994), e em carcinomas com diferentes graus de diferenciação tumoral, à exceção do colágeno VII que está ausente nos ácinos neoplásicos. Além dos componentes da membrana basal, Knox e colaboradores (1994) puderam também investigar duas integrinas (receptores celulares para componentes da matriz extracelular), que estão intimamente associadas com a adesão de células epiteliais à membrana basal, encontrando que a integrina $\alpha 6\beta 4$, associada à formação de hemidesmossomos, também estava ausente nos ácinos neoplásicos. Estas observações a respeito da membrana basal do epitélio prostático não foram, entretanto, estendidos ao modelo representado pela próstata ventral de ratos, embora existam referências a um aspecto ondulado da membrana basal das estruturara epiteliais em animais castrados (Holterhus et al, 1993). Por outro lado, Carvalho e Line (1996) caracterizaram as modificações associadas a membrana basal das células epiteliais e das células musculares lisas que se seguem à privação de andrógenos, mostrando que eventos apoptóticos seguem a perda de adesão da célula à membrana basal e que existe um retardo nas absorção das membranas basais residuais que se tornam extremamente pregueadas mas que permanecem apresentando moléculas intactas de laminina.

O estroma da próstata apresenta diversos tipos celulares cada qual desempenhando papel importante na manutenção da forma e função tecidual. Os fibroblastos são células fusiformes, basófilas em colorações de rotina, sendo responsáveis pela produção da matriz extracelular. As células musculares lisas são fusiformes com um único núcleo central e são responsáveis pelos mecanismos de contração. Macrófagos, ricos em lisossomos, são responsáveis pelos mecanismos iniciais de defesa. As células endoteliais são achatadas e revestem internamente os vasos. Associados aos vasos encontram-se os pericitos, células estas que possuem natureza mesenquimal indiferenciada.

O termo estroma serve para designar, no adulto, o componente não epitelial de um órgão secretor. O estroma no adulto é equivalente ao mesênquima do estágio embrionário (Cunha et al, 1976; 1985). O trabalho de Nemeth e Lee (1996) comprovou diferentes distribuições de musculatura lisa e tecido fibroso ao longo dos ductos prostáticos.

A musculatura lisa é mais abundante nas regiões intermediária e proximal, sendo mais espessa que o tecido fibroso na região distal.

O efeito da castração foi bem determinado em hamsters por Ortiz (1953). A influência de hormônios androgênicos na proliferação celular e arquitetura tecidual da próstata humana foi descrita por Voogt e colaboradores (1987).

A grande redução do tamanho e peso do órgão é atribuída inicialmente a uma parada na síntese e uma acelerada liberação da secreção luminal, seguida pela diminuição do tamanho das células epiteliais e por processos de morte e degeneração celular, resultando em lóbulos reduzidos e células epiteliais baixas (Ortiz, 1953; Brandes, 1966). É observado ainda um declínio na síntese de DNA e de proteínas, no conteúdo e na complexidade do RNA e uma diminuição de receptores de andrógenos (Aumüller e Seltz, 1990).

Os efeitos da testosterona sobre os componentes nucleares das células epiteliais glandulares foram estudados. Ocorre um decréscimo de volume nuclear (66%) e nucleolar, deformação dos núcleos por grandes invaginações do envoltório nuclear, abundância de cromatina condensada, pequena quantidade de fibrilas pericromatinicas e diminuição da fração do volume nuclear ocupado pelas fibrilas ribonucleoproteícas não nucleolares (Echeverria et al, 1991). As modificações ocorridas nas células epitelias da porção glandular já foram intensamente investigadas. Há, ainda, necessidade de se conhecer melhor as modificações morfológicas e bioquímicas que ocorrem no estroma prostático para um perfeito entendimento das relações epitélio–estroma.

Durante a regressão prostática, existe um aparente aumento na área seccional ocupada por matriz extracelular e por células estromais (Kerr e Searle, 1973). Dentre estas últimas, as mais proeminentes são as células musculares lisas. Além de um aparente aumento das células musculares lisas ao redor das estruturas epiteliais (lóbulos e ductos), elas assumem um fenótipo mais sintético, com uma fração miofibrilar reduzida (Zhao et al, 1992).

Embora tenha sido sugerido que o aumento do estroma ocorre sem a síntese de componentes colagênicos, conforme sugerido pela quantificação de hidroxiprolina (Kerr e Searle, 1973) e que estas alterações possam mesmo ocorrer sem a síntese *de novo* de

colágeno, a síntese de outros componentes e a reorganização do estroma prostático devem ser destacadas como função das células do estroma. Da mesma forma, a reativação da glândula pela aplicação de testosterona deve envolver remoção ou reconstituição de componentes da matriz extracelular.

Vollmer e colaboradores (1994) investigaram a presença de tenascina na próstata normal e as modificações que ocorrem frente à remoção do estímulo androgênico. Os autores identificaram um aumento na quantidade de tenascina, estimada através do produto da reação imunocitoquímica e correlacionaram este aumento a um estado menos diferenciado do órgão, o que foi também detectado em outros órgão dependentes da estimulação hormonal.

Nakada e colaboradores (1994) detectaram um aumento no conteúdo de elastina, colágeno e glicoproteínas estruturais decorrente da castração, mas a distribuição destes componentes no estroma e as interações com outros elementos não foram identificadas. Já Carvalho e colaboradores (1997a) demonstraram a existência de um sistema de microfibrilas da matriz extracelular composta por colágeno do tipo VI e por microfibrilas compostas por fibrilina, enquanto Carvalho e colaboradores (1997b) demonstraram modificações estruturais associadas ao sistema elástico, seguindo as modificações mais gerais associadas à próstata em regressão. Em suas observações os autores constataram figuras associadas à elastogênese e sugeriram que a remodelação prostática envolve também a síntese de alguns componentes da matriz extracelular.

É evidente que durante a involução prostática devido a privação de hormônios androgênicos obtidos por castração ou tratamentos químicos, a perda da função secretora está associada à morte de células epiteliais e a um intenso processo de modificações que, ao nosso entender, resultam não somente da degradação dos componentes da matriz extracelular, mas também da reorganização de componentes pré-existentes e mesmo da síntese de novos componentes da matriz extracelular. Este nítido processo de remodelação resulta do redirecionamento das funções das células estromais, entre elas as próprias células musculares lisas.

Considerados alguns dos aspectos histológicos e ultra estruturais do estroma prostático frente à castração, tentaremos caracterizar o efeito da penicilamina sobre os

componentes fibrilares colagênicos e elásticos, bem como alguns aspectos dos proteoglicanos presente na próstata ventral de ratos, durante a involução promovida pela castração. Esperamos poder contribuir para melhor compreensão dos fenômenos associados à regressão prostática, em especial da interrelação entre diferentes componentes fibrilares da matriz extracelular, ao interferir com a enzima lisil oxidase que tem importante papel na formação de ligações cruzadas presentes ente moléculas de colágeno e entre as moléculas de pró-elastina.

A penicilamina foi isolada em 1953 da urina de pacientes com doença hepática que estavam recebendo penicilina. A descoberta de suas propriedades quelantes permitem o seu uso em pacientes com doença de Wilson (degeneração hepatolenticular devido a um excesso de cobre) e intoxicações com metal pesado. Quimicamente a penicilamina é dimetilcisteina. O D isomero é usado clinicamente; o L isomero forma complexos quelantes. Penicilamina é um efetivo quelante do cobre, mercúrio e zinco e promove a excreção destes metais na urina. É bem absorvida (40 – 70%) no trato gastrintestinal e, por isso, tem vantagem sobre outros agentes quelantes. Alimentação, antiácidos e ferro reduzem sua absorção. Picos de concentração no sangue são obtidos entre uma e três horas após a administração (Gilman et al, 1990).

A penicilamina também teve ampla aplicação clínica no tratamento de doenças como a esclerose sistêmica (Rodnam 1981) e foi utilizada neste trabalho por interferir na formação de fibrilas de colágeno e de fibras elásticas, inibindo a formação de ligações cruzados

Objetivos

O presente estudo procurou averiguar o efeito da penicilamina, um agente anti-fibrosante, sobre a regressão prostática de ratos, com ênfase nos eventos celulares e da matriz extracelular e a modulação destes efeitos pela suplementação de vitamina B6 e de íons cobre, em níveis estrutural e ultra-estrutural.

Effects of penicillamine on the rat ventral prostate regression after castration

H. Verardi[1,2] and H. F. Carvalho[1]

[1]Dept. Cell Biology - UNICAMP, Campinas SP and

[2]Dept. Morphology, Passo Fundo University, Passo Fundo RS, Brazil

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Correspondence and reprints addressed to:

Hernandes F. Carvalho
Dept. Cell Biology - UNICAMP
CP6109
13083-970 Campinas SP, Brazil
Tel./Fax: 0055 19 788 7821
E-mail: hern@unicamp.br

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Abstract: The effect of penicillamine on the prostatic regression after castration was examined by histochemistry and TEM. Penicillamine impaired the accumulation of collagen in the rat ventral prostate (VP) and it apparently resulted in the colapse of the regressing epithelial structures. Pencilamine also caused a delay in apoptotic loss of epithelial cells, resulting in highly cellular epithelial structures, even after 21d after castration. At this point, some epithelial cells, though still lined by the basal membrane, projected into the adjacent stroma. The phenotypical modifications of the smooth muscle cells were not affected penicillamine. Vitamin B6 and Cu^{2+} supplementation partially restored the normal aspects of the prostatic regression, in respect to both the epithelial elimination and the reorganization of the collagen fibrils into convoluted fibers.

Introduction

Prostatic regression after castration involves extensive remodelling of the stromal compartment, besides the loss of epithelial cells. Early events in the prostatic reaction to androgen deprivation involve endothelial cell death and blood flow reduction (Shabsigh et al., 1998, 1999), which preceed the epithelial cell loss by apoptosis (Kerr & Searle, 1973; Hu et al., 1998). Late events correspond to an extensive stromal reorganization in both cellular and extracellular matrix compartments. A rapid reduction of the epithelial compartment occurs in the first days after castration due to the elimination of the luminal secretion products, a reduction in the number of epithelial cells, and also a reduction in the synthetic machinery of the remaining cells (Brandes, 1966, Carvalho et al., 1997a). After the first week, there is still a progressive but slower weight loss, likely associated with a continuous loss of epithelial cells.

Given this dynamic of the prostate gland after androgen withdrawal, we may expect a concerted action of stromal cells, in adapting the adjacent stroma to the reducing area occupied by the epithelial structures, as it occurs in the developing and growing prostate (Cunha, 1976; Cunha et al., 1985). However, these modifications are not completely understood but seems to involve the active reorganization of the pre-existing fibrillar and non-fibrillar components and also the *de novo* synthesis of extracellular matrix macromolecules by the fibroblasts and smooth muscle cells (SMC).

We have previously reported modifications associated with the basement membrane (Carvalho & Line, 1996) and with the elastic system components (Carvalho et al., 1997a) in the regressing ventral prostate (VP) of the rat. We have also demonstrated that type VI collagen is present and corresponds to a major component of the prostatic stroma (Carvalho et al., 1997b) Collagen accumulation in the regressing prostate was also described in the VP of rats treated with estradiol (Nakada et al., 1994) and in the regressing prostate of castrated rats (Vilamaior, 1998). An increased expression of tenascin was also observed in the regressing prostate (Vollmer et al., 1994). Furthermore, the blood flow reduction reported to play an important role in the prostatic response to castration (Shabsigh et al., 1998, 1999) may also involve modifications of the extracellular matrix.

Collagen is thought to perform an important role in prostatic physiology. Müntzing (1980) has even suggested a growth regulation role for collagen in the VP. Although there is evidence that collagen content is hormonally controlled in the VP (Nakada et al., 1994; Suzuki & Nakada, 1996), structural aspects of its remodelling after castration is still lacking. Furthermore, it is not yet defined which cells are responsible for the collagen associated modifications, but an active role of smooth muscle cells on the stromal remodelling is suggested by the conversion from a contractile to a more synthetic phenotype (Zhao et al., 1992).

In this work we used penicillamine, an anti-fibrotic drug, to interfere with collagen metabolism and investigate its influence on the prostatic regression after castration. Histology and transmission electron microscopy were used to follow the behavior of epithelial cells, smooth muscle cells, collagen and elastic system components. We could show that penicillamine reduces the accumulation of collagen fibers in the rat VP after castration, causes a collapse of the epithelial structure and delays the loss of epithelial cells by apoptosis. Simultaneous administration of vitamin B6 and Cu^{2+} partially reverts the observed effects of penicillamine.

Material and methods

Animals: Three-month-old Wistar rats were used. They were kept in individual cages and had food and water *ad libitum*. Castration was achieved surgically by scrotal incision.

Drug administration: A subchronic dosage of penicillamine was administered to castrated and non-castrated rats. DL-Penicillamine (DL-2-Amino-3-Mercapto-3-methylbutanoic acid (91.25mg/kg of body weight) in water was given orally in a daily basis. To another group, vitamin B6 (VitB6)(4.56mg/kg of body weight) and copper sulfate (45.7mg/kg of body weight) was administered in addition to penicillamine.

Histological procedures: The ventral prostate (VP) was dissected out 72h, 96h and 120h, and 7, 14 and 21 days after castration. Fragments of the organ were fixed by immersion in 4% formaldehyde in phosphate buffered saline (PBS) for 12h, washed in PBS, dehydrated in an ascending ethanol series, cleared in Cedar wood oil and embedded in Paraplast Plus

medium. Six µm thick sections were obtained and stained with hematoxylin and eosin, Masson's trichromic or subjected to the Feulgen's reaction.

Transmission electron microscopy: Tissue fragments were fixed by immersion in 3% glutaraldehyde and 0.25% tannic acid in Millonig's buffer (Cotta-Pereira et al. 1976) for at least 4h. After washing, the material was post-fixed in 1% osmium tetroxide in the same buffer, washed again, dehydrated in acetone and embedded in Epon 812. Silver sections were obtained with a diamond knife and contrasted with uranyl acetate and lead citrate. Material was examined and micrographed in a Leo 906 transmission electron microscope.

Apoptosis index: The number of apoptotic epithelial cells was determined by counting morphologicallly identified apoptotic cells (Hu et al., 1998) in preparations subjected to the Feulgen's reaction. Eigthteen animals were castrated and divided into two groups which received or not penicillamine as above. Three animals from each group killed at 72, 96 and 120h after castration. Six random microscopical fields per experimental point were taken at random, photomicrographed and the percentual of apoptotic cells determined.

Results

The reduction in VP weight under the different experimental procedures used in this work is observed in Fig. 1. Penicilamine delays the weight reduction in castrated rats, while in untreated, castrated rats and castrated rats administered with penicillamine plus VitB6 and Cu^{2+} showed a similar weight reduction pattern.

Figure 2 shows the aspects of the epithelium and associated stroma in the control animals treated with penicillamine for 14d. Similar aspects were seen at days 7 and 21.

The histological aspects of the VP of castrated animals treated with penicillamine 7 and 14d after castration are shown in Figs. 3 and 4, respectively. The epithelial structures are large, cells are short but present in numerous. The cell nucleus is compact, and the cytoplasm is scarce.

Fig. 5 is a Masson's trichromic stained section of the VP of non-castrated rat treated with penicillamine for 14d. Collagen fibers are scarce and dispersed in the stroma. SMC form a continuous layer below the epithelium. At the seventh day after castration, under treatment with penicillamine (Fig. 6) the epithelial structures are collapsed and folded outwards. Fourteen days after castration and penicillamine administration resulted in

collapsed epithelial structures, some of which are expanded when not associated with smooth muscle cells (SMC) (Fig. 7). Figs. 8 and 9 are details of the VP prostate 14d after castration under penicillamine administration. SMC are typically spinous, but collagen accumulation is low. Collagen fibers are diffuse and not undulated, as seen in non-treated, castrated rats at the same experimental point. After twenty-one days, under penicillamine administration (Fig. 10), the VP of castrated rats showed large epithelial structures which are still rich in epithelial cells. The cells were tall and showed large and homogeneous nuclei. A second cell layer between the epithelium and the SMC was observed. Collagen accumulation was low and the fibers were dispersed in the stroma.

Simultaneous administration of vitB6 and Cu^{2+} apparently reduced the effects of penicillamine on the non-castrated animals (Fig. 11). Epithelial structures, SMC, fibroblasts are apparently normal. Some lipid accumulation was observed. Figs. 12 and 13 shows the aspects of the VP 7d and 14d after castration, under penicillamine plus vitB6 and Cu^{2+}. The histological organization is similar to non-treated, castrated rats at the same experimental points. Epithelial structures are reduced and the stroma is dense in cells, but collagen accumulation is still reduced. Fig. 14 is a detail of the VP under the same experimental condition as in Fig. 13.

Figs. 15 to 18 are ultrastrutuctural aspects of the VP in non-castrated rats treated with penicillamine for 21d. Epithelial cells accumulate some large, electron transparent vesicle associated with developed Golgi apparatuses (Fig. 15). There is an apparent reduction in the area occupied by rough endoplasmic reticulum (RER) and the basal labyrinth is apparently expanded (Figs. 15 and 16). The SMC are normal, with respect to the cytoskeleton and vesicles (Fig. 17). The basal lamina seems disorganised, but association with nerve terminals seems normal (Fig. 17). Fig. 18 shows an aspect of the extracellular matrix, in which collagen fibers are dispersed and not arranged in fibers. Elastic system components are apparently normal, with a predominance of the microfibrillar component. An accumulation of a non-fibrillar component between the collagen fibrils and microfibrils was detected (Fig. 18).

Penicillamine administration to castrated rats for 7d resulted in epithelial cells which showed some RER and microvilli, some of the electron lucent vesicles observed in non-castrated rats under penicillamine treatment, and lipid accumulation in the intercellular

spaces (Fig. 19). Some dense bodies were detected. The extracellular matrix presented dispersed collagen fibers and elastic system components (Fig. 20). Supplementation with vitB6 and Cu^{2+} resulted in epithelial cells which were not so numerous, but with residues of secretion vesicles and RER, few dense bodies and apparently normal microvilli. Some organization of the fibrillar components, in special collagen fibrils were observed in this experimental situation (Figs. 22 and 23). It seems that processes of the epithelial cells, fibroblasts and SMC are actively involved in the process of collagen fibrils reorganization.

Epithelial cell density in the VP of castrated rats treated with penicillamine for 21d was high. The epithelial cells have little RER and Golgi apparatus. The cylindrical aspect is completely lost. Some epithelial cells project into the adjacent stroma but are still wrapped by the basal lamina (Fig. 24). Fig. 25 is an epithelial ductal cell, which shows normal junctions with the neighboring cells and a huge dense body. Some folding of the basal membrane is seen, but no pleating was observed (The foldings were still associated with epithelial cell processes). Folding of the basal lamina of the endothelial cell was observed (Fig. 26). No organization of collagen fibrils in fibers was observed. Elastic system components were dispersed in the stroma. SMC are spinous, but association with collagen fibrils was not as evident as in castrated, untreated VP (Fig. 27).

Supplementation of VitB6 and Cu^{2+} to penicillamine apparently restored the typical aspects of the VP. Epithelial cells are few in the epithelial structures, but are cylindrical and showed a large cell nucleus, some Golgi apparatuses, a large cell nucleus, some lipid accumulation and normal cell junctions. Microvilli are dense but short (Fig. 28). Figs. 29 and 30 show aspects of a fibroblast and a SMC, respectively, in association with groups of collagen fibrils associated in bundles. In Fig. 30, a suggestive participation of a SMC in folding collagen fibers is seen.

To determine whether the high epithelial cell density in the epithelial structures of the VP after penicillamine treatment was due to a decrease or delay in apoptosis, rats were castrated and treated with penicillamine. Control rats were castrated but received no further treatment. The number of morphologically recognizable apoptotic cells was determined in each experimental condition at 72, 96 and 120h. Fig. 31 show the percentual of apoptotic cells in the VP of castrated rats treated or not with penicillamine. It is evident from these results that penicillamine delays the apototic epithelial cell death in the VP of castrated rats.

Discussion

This paper presents results on the effects of penicillamine on the prostatic regression after castration.

Penicillamine is a chelating agent and as such interferes with the function of lysyl oxidase (LO) by reducing the availability of copper an important cofactor of this enzyme. Penicillamine also binds to pyridoxal (vitamin B6), which is another cofactor of LO. By interfering with LO, penicillamine seems to impar the formation of stable cross-links in collagen and elastin (Nimni & Bavetta, 1965; Nimni, 1968; Nimni et al., 1969; Siegel, 1977). Penicillamine also chelates Zn^{2+} and, hence, may have more diverse effects on prostatic physiology.

Penicillamine administration to growing chickens results in increased elastin in the extracellular space of the aortic wall, modifications were also observed in the elastin-associated microfibrils and in collagen fibrils. These latter were larger and with heterogenous cross diameters and less organized (Pasquali-Ronchetti et al., 1986).

It has been apparent from the literature that inhibition of collagen biosynthesis or processing leads to an increase in prostate size, while inhibition of prostatic function, by castration or estrogen administration increases collagen content (Nakada et al., 1994; Suzuki & Nakada, 1996). These results led some authors to suggest a close association between collagen and prostatic physiology. It is not apparent though, whether there is a direct involvement of collagen in controlling the function of the epithelial and/or stromal cells in the normal gland.

The application of penicillamine to castrated rats led to an evident inhibition of collagen accumulation in the regressing VP, as observed in the Masson's trichromic stained tissue sections. Penicillamine treatment influences the organ regression in two main aspects. First, epithelial structures collapses as the amount of secretion in the lumen is diminished by the inhibition of epithelial function. Second, there is a delay in epithelial cell elimination after castration, resulting in highly cellular epithelial structures. The first finding is likely due to a disconnection between the physiology of the epithelium and of the underlying stroma, as the accumulated secretion is eliminated. SMC are apparently not

involved with the constriction of the epithelial structures after castration in penicillamine treated rats, since they showed a normal behavior observed after castration, assuming a more synthetic phenotype (Zhao et al., 1992). As in non-treated castrated rats, the SMC lack the contacts to each other and show a spinous aspect. Their association with collagen fibers is though not so evident as observed in non-treated castrated rats. The second effect, resulting in highly cellular epithelial structures is not readly understood. The increased number of epithelial cells could result from a delay in epithelial cell loss by apoptosis. To test this, we counted the number of apoptotic cells in penicillamine treated and not treated VP of castrated rats at 72, 96 and 120h. Penicillamine significantly reduced the number of morphologically recognised apoptotic cells at 72h, but not at 96 and 120h. This effect results in a delay of epithelial loss in the penicillamine treated castrated rats, and in the decreased VP weight. Using another drug which interferes with collagen metabolism, β-aminoproprionitrile, Izumiya and Nakada (1997) demonstrated a decrease in collagen content coupled to an increase of prostate weight in young rats. However, it was not ascertained whether it was related to a higher number of epithelial cells and/or epithelial structures.

An interesting observation was the projection of some epithelial cells into the adjacent stroma, observed 21 days after castration under penicillamine treatment. These cells show more compact nuclei and are dissociated from the typical epithelial cells. However, they are kept inside the basal membrane (Figs. 10 and 24). Whether they represent basal epithelial cells remains to be determined. This behavior is clearly induced by the penicillamine administration, since it was not observed in either non-treated castrated rats and was not so evident in penicillamine treated rats with simultaneous administration of vitB6 and Cu^{2+}.

VitB6 and Cu^{2+} supplementation to penicillamine treated rats partially reverted the effects of penicillamine. The amount and organization of collagen fibrils and fibers approached those of the VP from non-treated castrated rats at the different experimental points. Apparently the stromal cells succeded in reorganizing collagen fibrils in fibers, and the spinous SMC were observed in association with the convoluted collagen fibers, as seen in non-treated castrated rats. After 21d, the regressing prostate in this experimental condition was similar to that of non-treated rats.

The basal membrane folding and pleating was not so extensive in either treatment as observed in non-treated, castrated rats 21d after castration (Carvalho & Line, 1996). Folding of the basal lamina of endothelial cells was observed.

As a conclusion, penicillamine reduces the accumulation of collagen in the regressing prostate either by inhibiting the *de novo* synthesis of this fibrillar component or by inhibiting its reorganization by the stromal cells, and causes a delay in the apoptotic elimination of epithelial cells, resulting in highly cellular epithelial structures (acini and ducts). These effects are at least partially reverted by the simultaneous administration of vitB6 and Cu^{2+}.

It remains to be determined whether the epithelial behavior corresponds to a direct effect of penicillamine on the epithelial cells or to the absence of a coordinated support from the affected stroma. We have previously suggested that elastic systems components are important in the contraction of the gland after the loss of epithelial cells following castration (Carvalho et al., 1997a). The modifications associated with the elastic system components are similar to those observed for the chicken aorta (Pasquali-Ronchetti et al., 1986) and will be reported elsewhere.

These results permit the speculation that though not involved in muscular contraction of the epithelial structures, SMC and also the fibroblasts are directly involved in epithelial regression by reorganizing the extracellular matrix, in special the collagen fibers. The reorganized stroma seems important for the coordinated regression of the epithelial structures. Disturbance of the stromal organization, as by the use of penicillamine, leads to the colapse of the epithelial formations as observed in this study.

Since blood supply was suggested to be a major regulator of the prostatic response to castration (Shabsigh et al., 1998, 1999), and penicillamine showed the effects demonstred herein, direct effects of this drug on blood vessel structure and dynamics in the ventral prostate after castration should be studied in the early future.

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Figure legends

Fig. 1. Variation of the relative weight of the VP in rats subjected to different experimental procedures. Weight loss is marked for the castrated animals and in castrated rats treated with penicillamine plus VitB6 and Cu^{2+} (P+C+V). The weight loss in penicilamine treated VP is reduced as compared to the other treatments.

Fig. 2-4. H&E stained sections of the VP after treatment with penicillamine. Fig. 2 is a control prostate in which the epithelium is slightly shorter than untreated control. The stroma is normal with a layer of smooth muscle cells (SMC) underneath the epithelium. Fig. 3 is a histological section of the VP from a castrated rat 7d after castration and treated with penicillamine. The epithelial cells are denser and the number of SMC layers is similar to that of non-castrated rats. Fig. 4 is an aspect of the VP 14d after castration and treatment with penicillamine. Cells are numerous than in the control, and apparently the cell nuclei are more compact. The number of SMC layers is increased. X1 075.

Fig. 5-10. Masson's trichromic stained sections of the VP after treatment with penicillamine. Collagen fibers (COL) are scarce underneath the epithelium in the control prostate at the 14th day of treatment with penicillamine (Fig. 5). Fig. 6 is a low magnification view of the prostate 7d after castration and treated with penicillamine. The epithelial structures are disorganised and colapsed towards the lumen. Fig. 7 shows an aspect of the VP 14d after castration and penicillamine treatment. Epithelial structures are colapsed and folded, the number of SMC is increased but no accumulation of collagen is observed. Some areas of the epithelium are not associated with SMC (arrows). Figs. 8 and 9 are aspects of the VP from castrated rats which received penicillamine for 14d. The SMC show a spinous aspect and their contacts to each other are lost, as seen in non-treated animals. The amount of collagen is low and the collagen fibers are thin and difuse. Fig. 10 is an aspect of the VP of a castrated rat 21d after castration. There is a second layer of cells (arrows), between the eipithelial cells proper and the SMC. These cells have more compact

nuclei. The number of SMC layers is also increased, but the amount of collagen is low. Fig. 5, 8-10, X1 075; Fig. 6, X215, Fig. 7, X430.

Fig. 11-14. Masson's trichromic stained sections of the VP after treatment with penicillamine plus VitB6 and Cu^{2+}. Fig. 11 shows that the effects of penicillamine on the epithelium of non-castrated rats is no longer observed. SMC = smooth muscle cells. D = Dense body; C= capillaries; F= fibroblast. Fig. 12 and 13 are aspects of the VP of a castrated rat 7d and 14d, respectively, after castration. The administration of VitB6 and Cu^{2+} partially restores the aspects of regression observed in non-treated animals, and accumulation of collagen is also partially restored. After 21 days (Fig. 14), epithelial structures are regressed and small, and the stroma is increased and dense in cells but collagen accumulation is not completely restored. A second cell layer below the epithelial cells is present, though not as marked as in penicillamine treated animals. Figs. 11 and 14, X1 075; Figs. 12 and 13, X430.

Fig. 15 and 16. TEM of epithelial cells in the VP of a non-castrated rat, treated with penicillamine for 21d. The cells accumulated a number of vesicles (asterisks), are shorter then untreated controls. Cell junctions are normal and Golgi apparatus (GC) are evident. The area occupied by RER is smaller and the microvilli are shorter. The basal labirynth (BL) is apparently expanded. Fig. 15 and 16, X18 000.

Fig. 17. The SMC are apparently normal with respect to the miofibrils, secretion vesicles, and contacts to each other. The basal membrane is interrupted (arrows). Association with nerve terminals (NT) is also normal. X25 000.

Fig. 18. Aspects of the extracellular matrix in the VP of a penicillamine treated non-castrated rat. Collagen fibrils, elastic system components (mf, microfibrils; e, elastin) are normal, but there is an apparent accumulation of a non-fibrillar component in between those elements (asterisks). X4 050.

Figs. 19 and 20. Ultrastructural aspects the VP of penicillamine treated castrated animals 7d after surgery. In fig. 19, cells are relatively tall and present in great number. The vesicles

observed in penicillamine treated controls are still seen (arrowheads). The RER is present, lipid accumulation in between the cells (arrows) and dense bodies (asterisks) inside the epithelial cells are observed. Folding of the basal membrane is not extensive. Dense and short microvilli are apparent. Fig. 20 shows aspects of the extracellular matrix which are similar to controls, in non-castrated animals treated with penicillamine. Collagen fibrils are not evidently grouped in fibers, but disorganised in the stroma. Ep=epithelial. Fig. 19, X12 470; Fig. 20, X13 940.

Figs. 21-23. Ultrastructural aspects of the VP of castrated animals 7d after surgery and treated with penicillamine, vitB6 and Cu^{2+}. The epithelial cells are much like those observed in penicillamine treated prostate. There is an apparent organization of collagen fibrils in fibers (Fig. 22). Fibroblasts processes (F) are intimately associated with undulating collagen fibers. ep = epithelial cell; RER = rough endoplasmic reituculum. There is an apparent accumulation of non-fibrillar material inbetween the collagen fibrils. Fig. 21, X12 470; Fig. 22, X23 250; Fig. 23, X50 100.

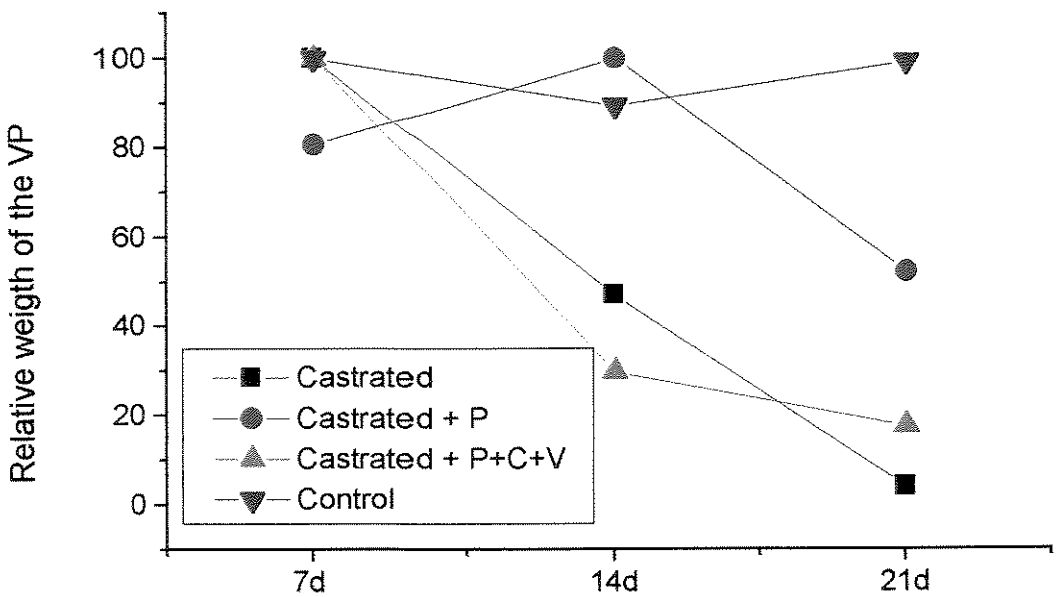
Figs. 24-27. Ultrastructural aspects of the VP of penicillamine treated castrated animals 21d after surgery. Fig. 24 shows aspects of the epithelial cells, which appear in greater number, as compared to non-treated castrated animals. Some epithelial cells (asterisks) project into the stroma but keep their basal membrane. Fig. 25 exhibits a ductal epithelial cell which accumulates a huge dense body (asterisk) and forms junctions with the adjacent cells.
Fig. 26 depicts aspects of the extracellular matrix. Collagen fibrils are not organised in fibers, there is a moderate folding of the basal membrane, but no pleating. Elastic system components are seen (arrowheads). The basal lamina of a vessel shows extensive folding (arrows). Fig. 27 shows a SMC which has extensive folding of the cytoplasm and the basal membrane. Association with the collagen fibrils in not as marked as in non-treated animals. ep, epithelial cell. Fig. 24, X9 055; Fig. 25, 12 000; Fig. 26, X12 600, Fig. 27, X18 000.

Figs. 28-30. Ultrastructural aspects of the VP of castrated animals 21d after surgery and treated with penicillamine, vitB6 and Cu^{2+}. Fig. 28. Epithelial cells a still cylindrical and high, with prominent microvilli. Golgi apparatus is evident. Lipid accumulation is seen .

Fig. 29 shows a fibroblast extending a process which intimately associates with a group of collagen fibrils. Fig. 30 shows a SMC with a spinous aspect, which is associated with collagen fibers. Fig. 28, X10 790; Fig. 29, X18 000; Fig. 30, X9 350.

Fig. 31. Frequency of apoptotic cells in the VP after castration, with or without administration of penicillamine. Values are the mean±sem. The insert shows some aspects of the apoptotic cells (arrows) in the VP after castration. Original magnification of the micrograph, X1 075.

Figure 1


Relative weigth of the VP
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60
40
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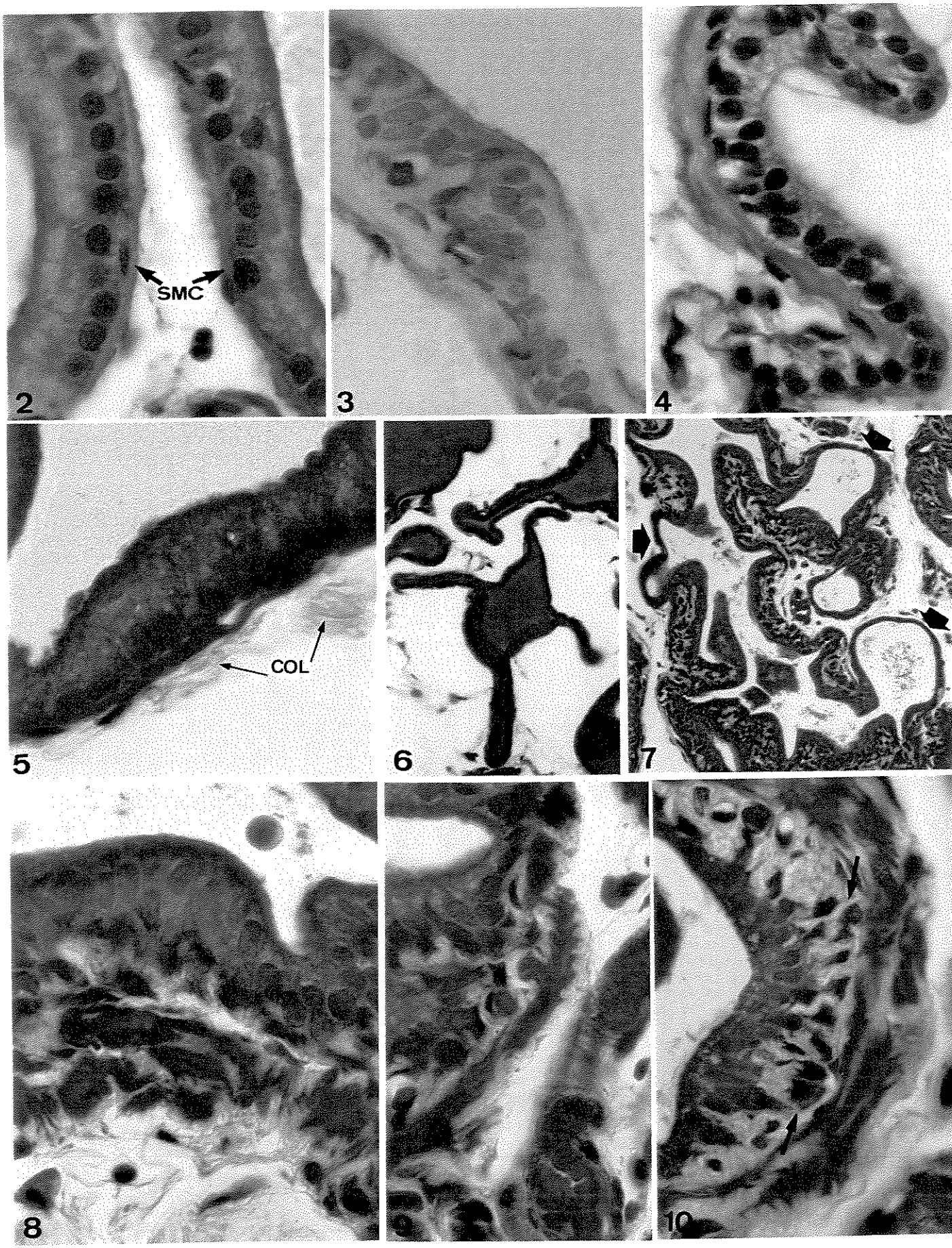
0
7d
14d
21d
Castrated
Castrated + P
Castrated + P+C+V
Control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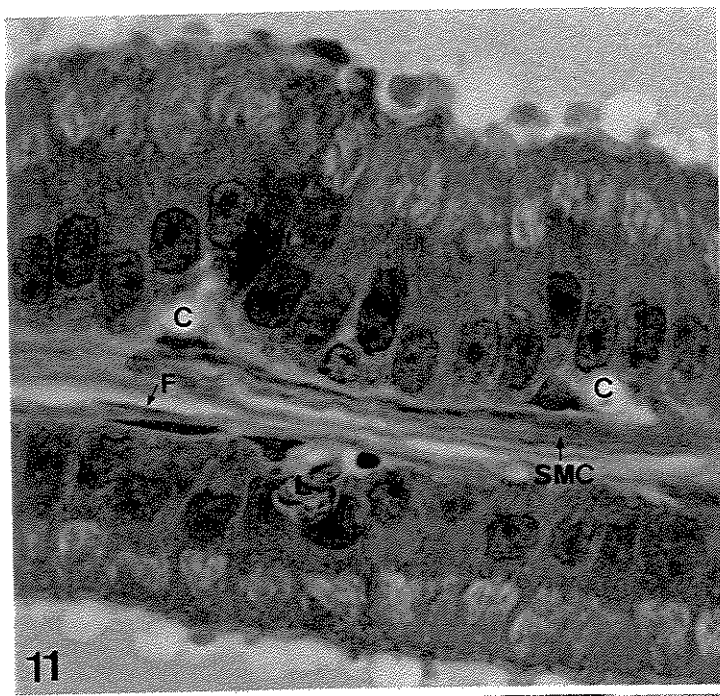

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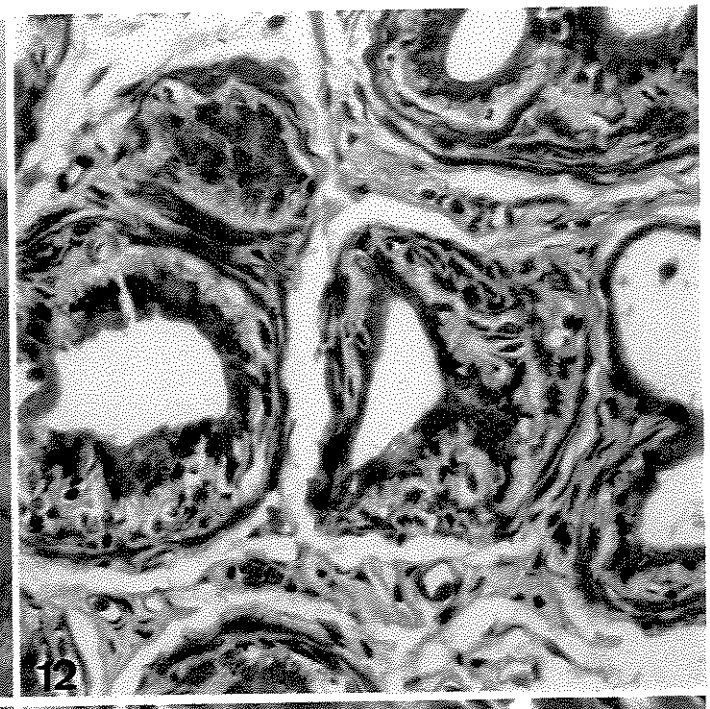
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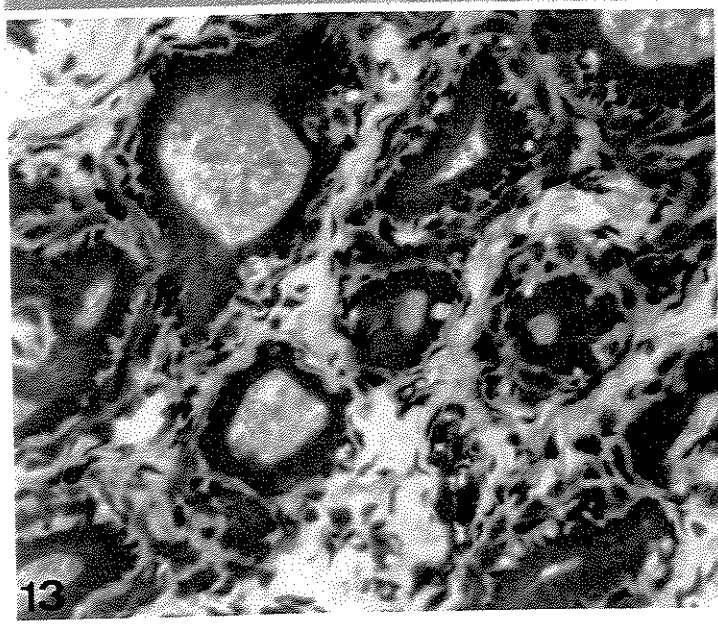
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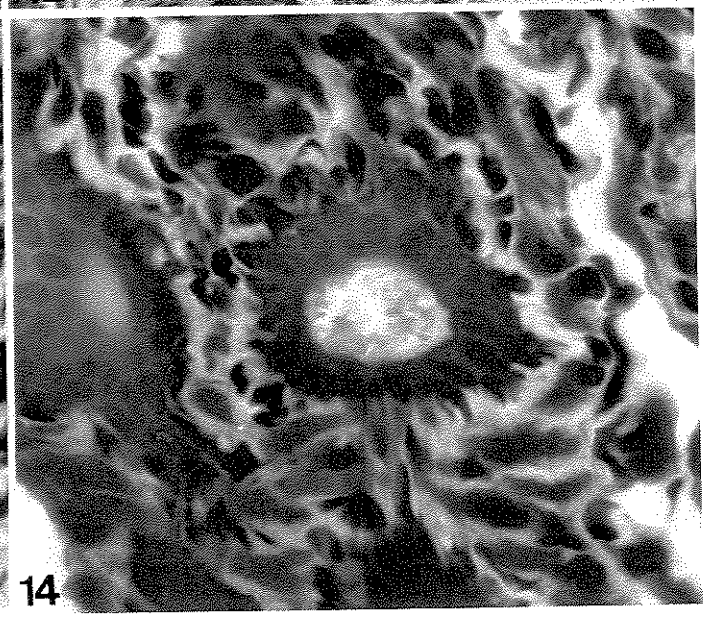
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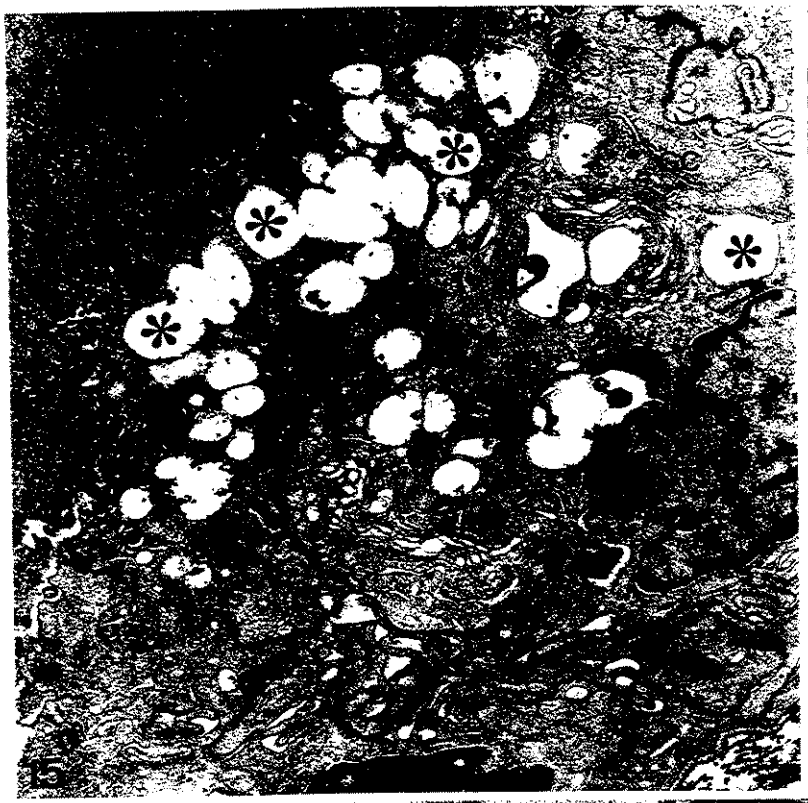


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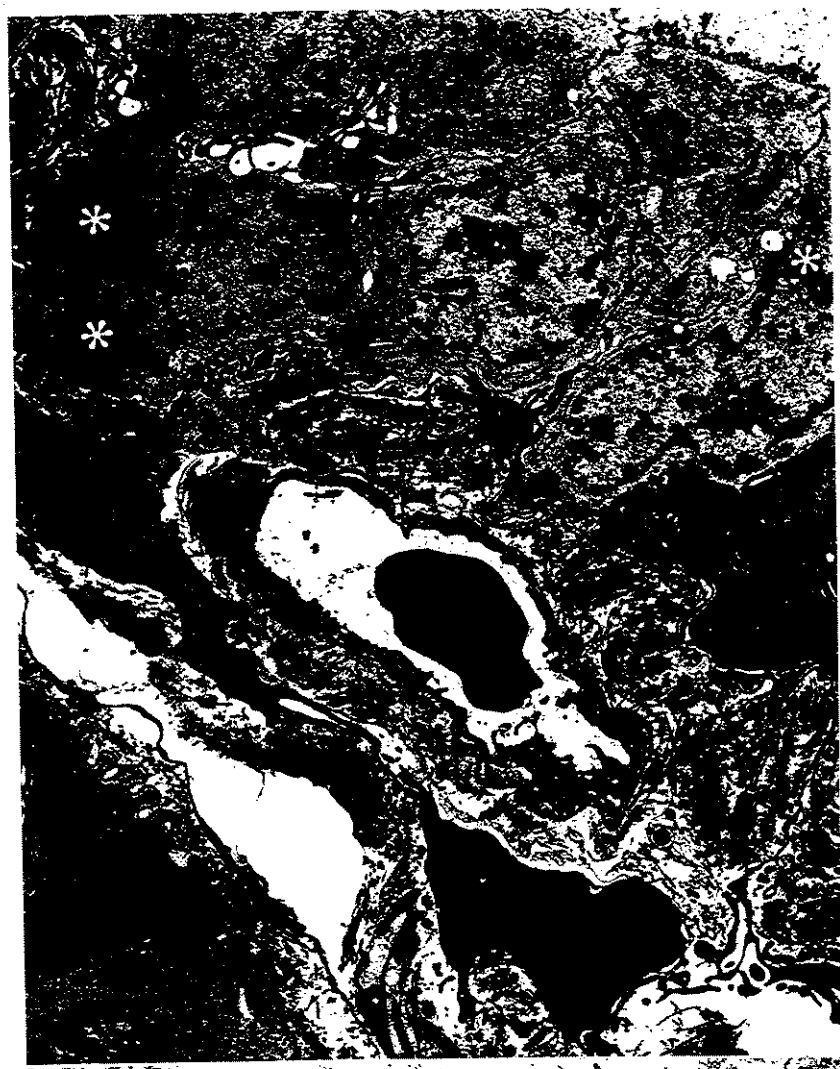
NT
17


e

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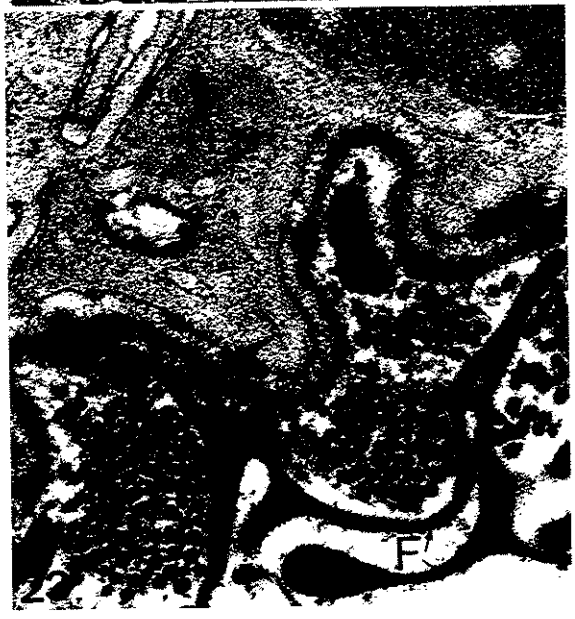
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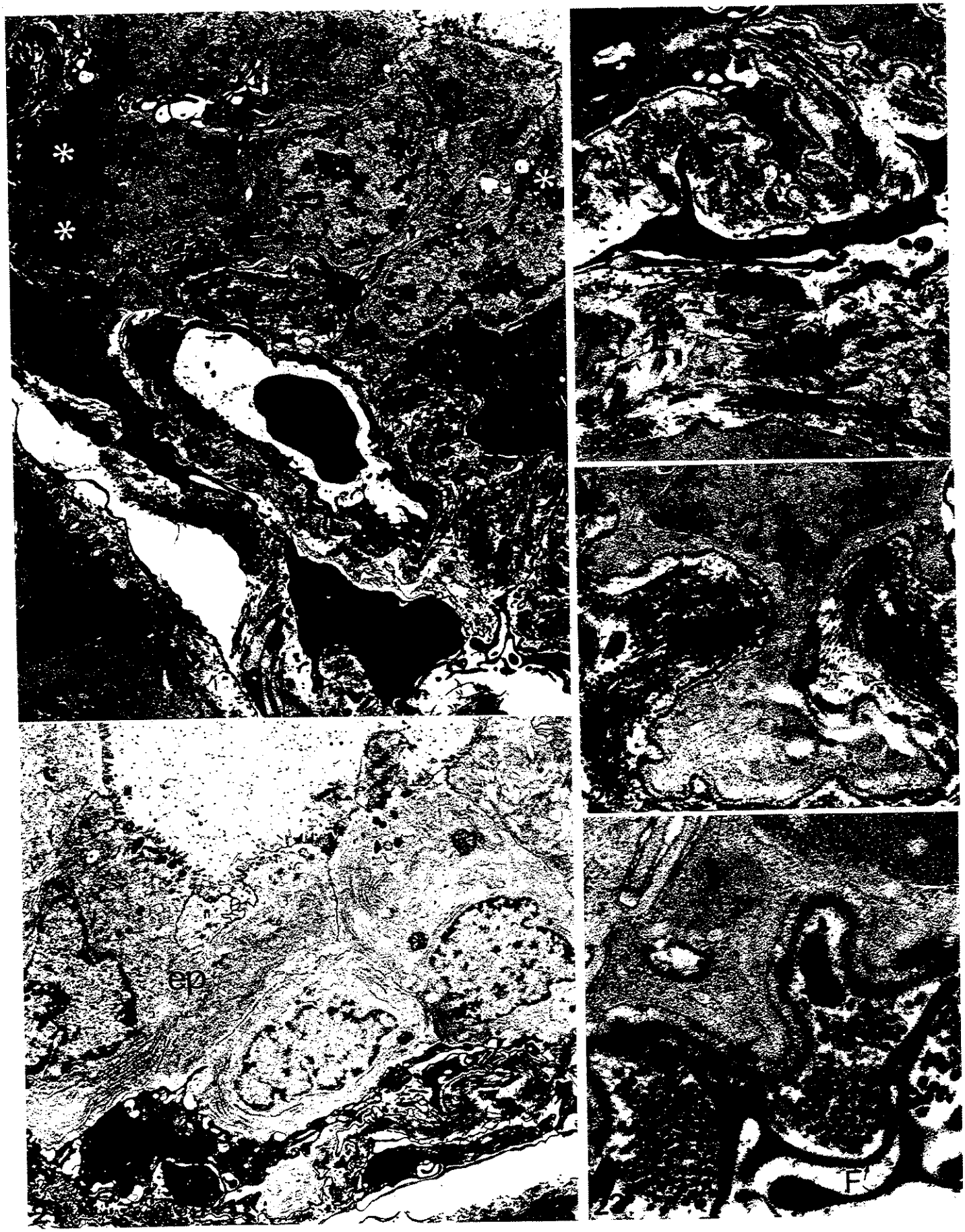
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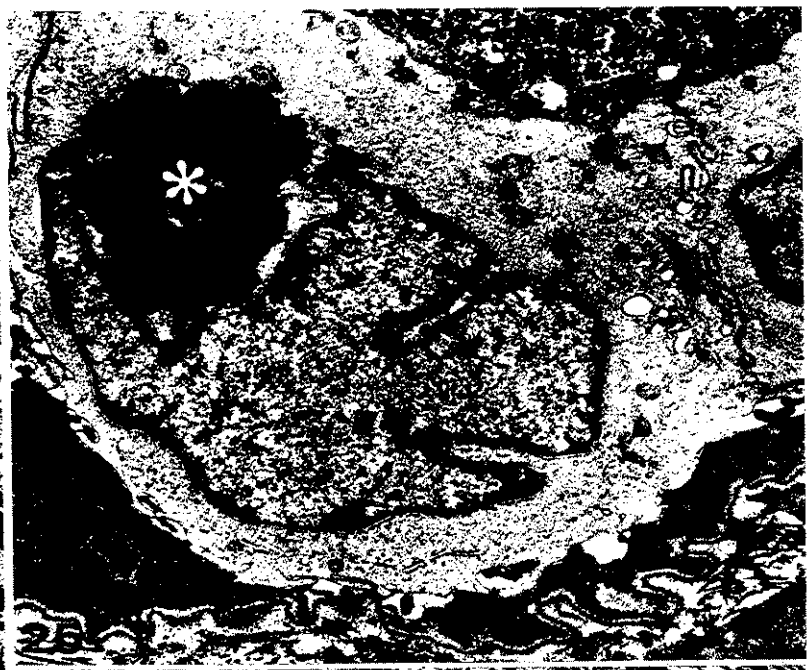
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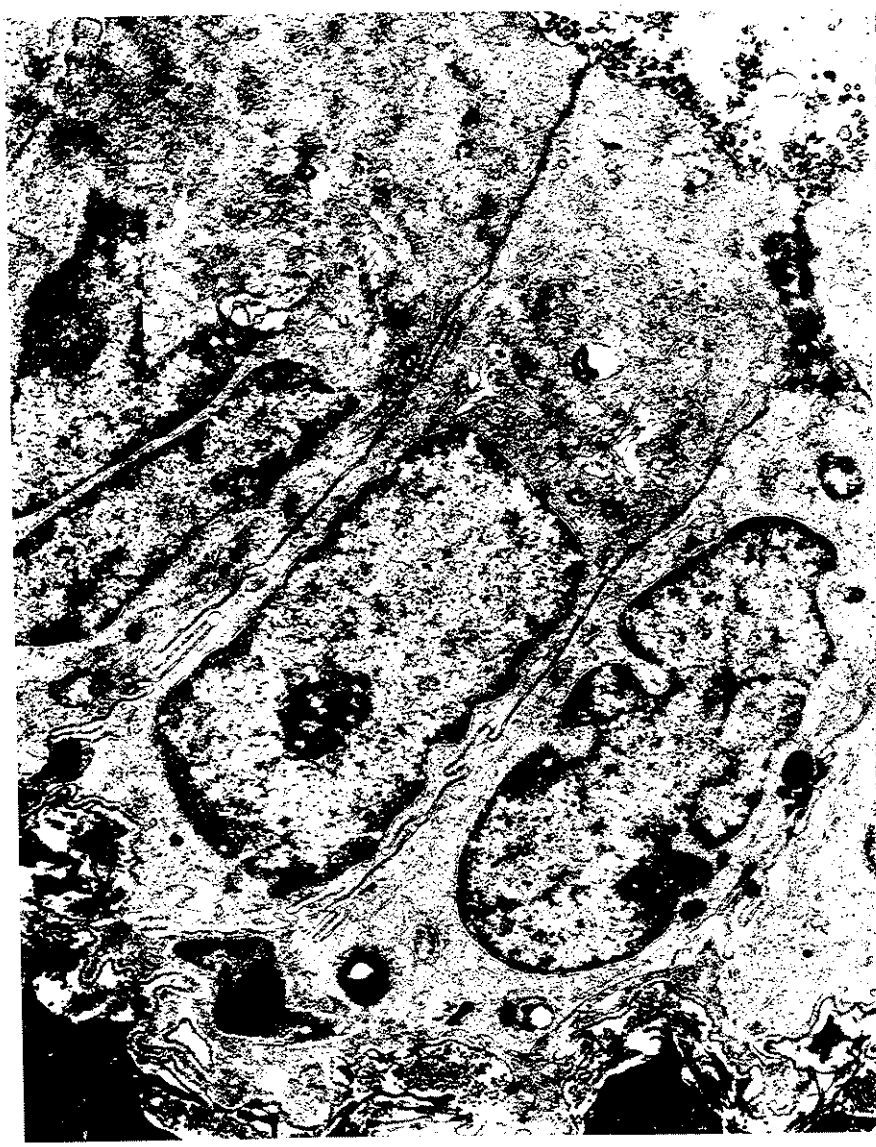
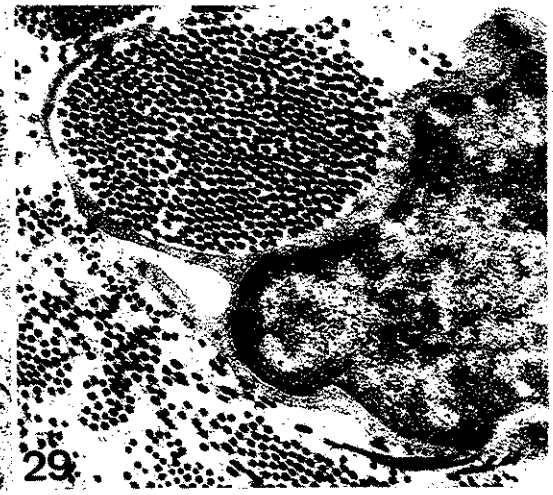
26


SMC
27

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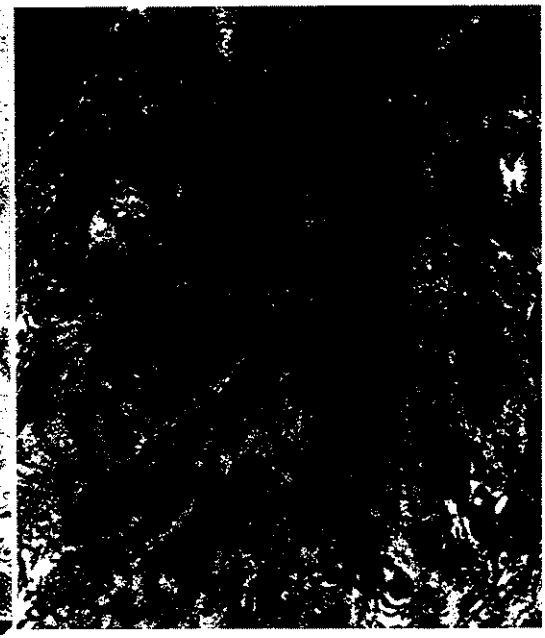
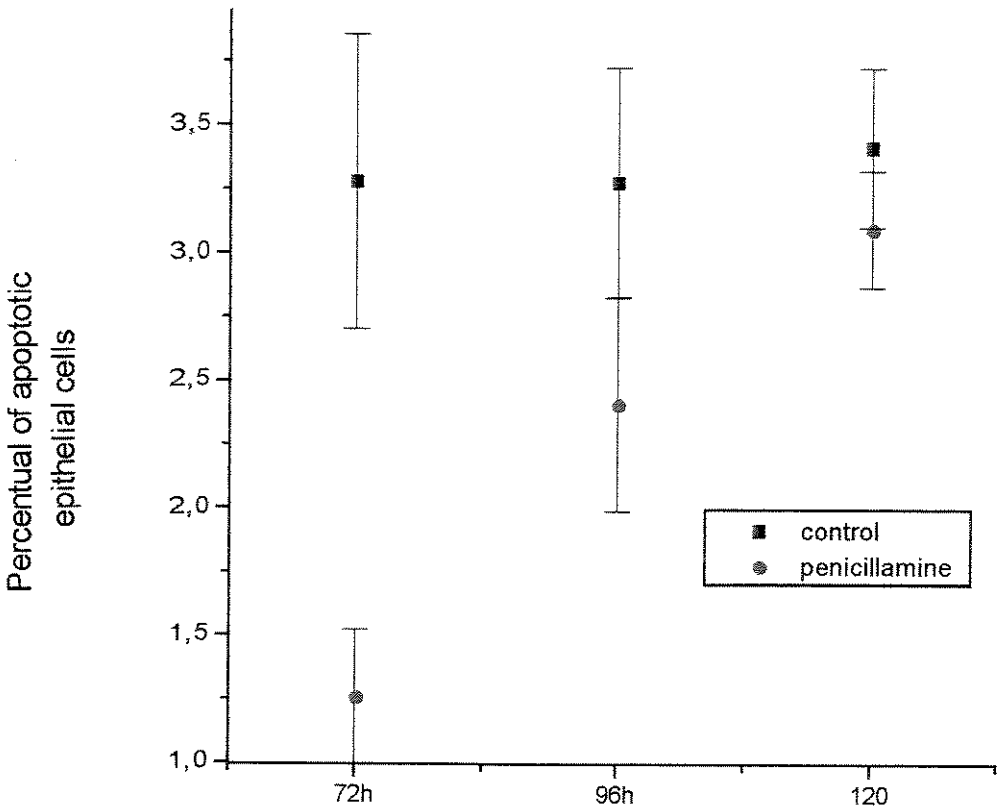
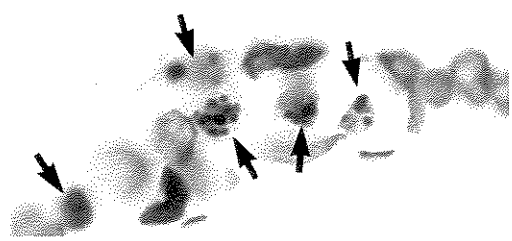



Percentual of apoptotic epithelial cells
3,5
3,0
2,5
2,0
1,5
1,0
72h
96h
120
control
penicillamine



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Conclusões gerais

1. A penicilamina reduz a perda de peso da próstata ventral de ratos após castração e este efeito e bloqueado pela administração simultânea de sulfato de cobre e de vitamina B6.
2. A penicilamina reduz a perda de células epiteliais, por retardar a apoptose.
3. As estruturas epiteliais, principalmente os ácinos, colapsam frente ao tratamento com a penicilamina.
4. Há uma redução na deposição de colágeno na próstata ventral de ratos castrados tratados pela penicilamina.
5. Algumas células epiteliais perdem o contato com as demais e projetam-se em direção ao estroma, sem contudo perder o contato com a membrana basal que se mantém íntegra.
6. As alterações celulares e histológicas observadas são parcialmente revertidos pela administração simultânea de sulfato de cobre e de vitamina B6.

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