UNIVERSIDADE ESTADUAL DE CAMPINAS

JUSSARA KIYA HUARANGA FERNANDES

Caracterização dos glicosaminoglicanos da próstata ventral, glândula de coagulação e vesícula seminal de ratos: Efeitos da castração

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Biologia Celular

Orientador: Prof. Dr. Hernandes Faustino de Carvalho

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Fernandes, Jussara Kiya Huaranga

F391c Caracterização dos glicosaminoglicanos da próstata ventral, glândula de coagulação e vesícula de ratos: efeitos da castração/ Jussara Kiya Huaranga Fernandes. - - Campinas-SP. [s.n.], 2000.
43 f: ilus.

Orientador: Hernandes Faustino de Carvalho
Dissertação (mestrado) – Universidade Estadual de Campinas. Instituto de Biologia.

1. Próstata. 2. Castração. 3. Glicosaminoglicanos. I. Carvalho, Hernandes Faustino de. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título

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Data de Defesa: 31 de Outubro de 2000

Banca Examinadora:

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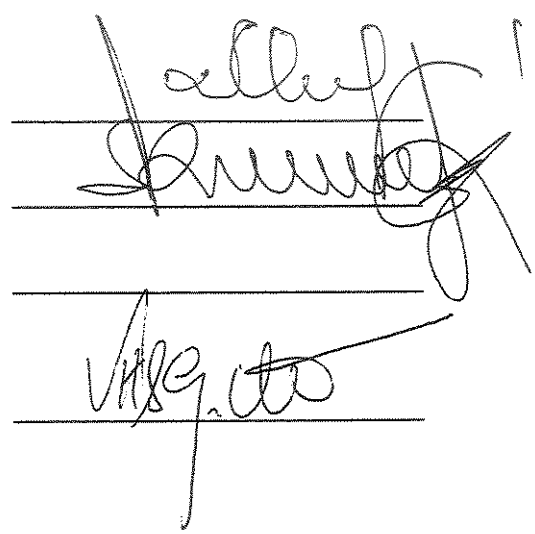

Em especial

A Deus,
Louvores ti dou ó Meu Senhor
Por todo sua Onipresença, Onipotência e Onisciência em meu viver,
Por ser a mão a me carregar sobre os mares de dificuldades,
Por todo zelo e infinito amor de um Pai tão misericordioso e bom

Àquela que nunca faltou
Estando em cada passo dado,
Cada fronteira alcançada
Sempre pronta nos momentos dificeis
O meu espelho de luta e persistência
O incentivo constante de minha felicidade
A tí Mainha,
A minha imensa gratidão

To my Angel of the Sun
Even our bodies are apart
You made me laugh when I wanted to cry
You cheered me up with your kind and hope words
You shared with me each step with patience,
comprehension and incentive
You prayed to Lord for my success
Even though our bodies are apart, you were all this time
in my heart, soul and mind.

Agradecimentos

A Hernandes por compartilhar comigo o seu legado de conhecimento, sempre empenhado em ajudar-me na construção de um pensamento científico e crítico.

Aos professores Fernanda Ramos Gadelha, Valéria Helena Alves Cagnon Quitete, Sebastião Roberto Taboga pelas valiosas sugestões após a análise inicial do manuscrito.

A todos do lab, por tudo que vocês fizeram por mim.

Aos doces corações - Soninha, Edna, Silvia e Verinha por todo apoio, edificação, compreensão, crítica e amizade.

As minhas lindas e abençoadas princesas- Lene, Fer, Rafa, Kate e Paty pelo prazer da convivência, pelo partilhar de todo o decorrer da minha tese e minha estada aqui em Campinas.

Aos meus amados e valiosos amigos Sue, Paulinha, Beta, Kent e Carl, Tia Marilucia e Família e Tia Eglantine e Família pela torcida por minha vitória.

As minhas adoráveis e grandiosas famílias - o Clã dos Fernandes e dos Hagen - por torcerem a todo momento pelo meu sucesso.

À Suely Ferreira Chavante, minha orientadora de iniciação científica, pelo incentivo durante a graduação.

Ao Prof. Dr. Edson Rosa Pimentel e ao aluno Sérgio Luis Felisbino pela prontidão a qualquer momento e pelo exemplo de gentileza e respeito - Meu muitíssimo obrigado.

À Líliam sempre presente e disposta em me ajudar.

A todos os funcionários, professores e colegas da Pós-Graduação por toda ajuda prestada.

À CAPES pelo auxilio financeiro concedido.

SUMÁRIO

Resumo

Os glicosaminoglicanos (GAGs) têm sido implicados em diversos aspectos da fisiologia celular. Uma vez que as glândulas acessórias do aparelho reprodutor masculino são dependentes de andrógenos, o presente trabalho foi realizado para examinar o efeito da privação androgênica sobre os tipos e conteúdo de GAGs na próstata ventral, glândula de coagulação e vesícula seminal de ratos com três meses de idade. Os animais foram castrados e sacrificados 7, 14 e 21 dias após a cirurgia. Ratos não castrados foram utilizados como controles. Os glicosaminoglicanos foram extraídos e quantificados pelo método do azul de dimetilmetileno. Os tipos de GAGs foram identificados por eletroforese, digestão enzimática e degradação com ácido nitroso. A incorporação *in vivo* de [^{35}S] foi acompanhada por autoradiografia de cortes histológicos. O conteúdo total de GAGs sulfatados aumenta progressivamente após castração até o 21°. dia após cirurgia. Condroitim sulfato (CS), dermatan sulfato (DS) e heparam sulfato foram encontrados nas diferentes glândulas de animais controles. DS é o GAG predominante tanto nos controles como nos animais castrados, aparecendo em concentração crescente nestes últimos. Este aumento de DS é o principal responsável pelo aumento dos GAGs totais, sendo acompanhado por um ligeiro aumento de HS. Por outro lado a concentração de CS diminui após castração. A marcação com [^{35}S] mostrou aspectos semelhantes, mas revelou uma marcante alteração da expressão de CS para HS na próstata ventral de animais castrados, quando comparados com os controles. A autoradiografia demonstrou que os GAGs encontram-se difusos no estroma e concentrados na membrana basal das estruturas epiteliais e das células musculares lisas. Aspectos similares foram observados nos animais controles e castrados. Os resultados demonstram que as variações na organização tecidual que ocorrem após a castração são associadas a alterações nos GAG sulfatados, provavelmente refletindo uma reprogramação celular, não somente do estroma mas também das células epiteliais.

Abstract

Glycosaminoglycans have been implicated in several aspects of cellular physiology. Since the male accessory sex glands are highly dependent on androgens, the present study was undertaken to examine the effect of androgen deprivation on sulfated glycosaminoglycan (GAG) types and content in the ventral prostate, coagulating gland and seminal vesicle of 3-month-old rats. The animals were castrated and sacrificed 7, 14 and 21 days after surgery. Non-castrated animals were used as controls. The sulfated GAGs were extracted and quantified by the dimethylmethylene blue method. GAG types were identified by electrophoresis, enzymatic digestion and nitrous acid degradation. *In vivo* ^{35}S incorporation was followed by autoradiography of tissue sections. Chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) were found in the different glands of the control animals. DS is the predominant GAG in both control and castrated groups. The total amount of GAG increased progressively after castration up to the 21th day, representing absolute increases in DS and HS. On the other hand, the CS amount decreased after castration. [^{35}S] labeling showed similar results, but a more prominent change in the expression of CS to HS in the prostate of castrated animals as compared to controls. *In situ* labeling showed the GAG to be diffuse in the stroma and concentrated at the basement membrane of epithelial structures and of smooth muscle cells. Similar aspects were observed in castrated and control animals. These results demonstrated that the variations in tissue organization following castration are associated with changes in the sulfated GAGs, most likely reflecting a reprogramming of both the stromal and epithelial cells.

Introdução

A próstata, a vesícula seminal e a glândula de coagulação são as principais glândulas acessórias do aparelho reprodutor masculino de roedores. Elas são responsáveis pela produção de grande parte dos componentes que formam o sêmen (MANN, 1975).

No curso evolutivo, essas glândulas foram selecionadas por contribuírem para o sucesso da fecundação interna. Deste modo, o sêmen funciona como um microambiente de proteção e fornecimento de nutrientes para os espermatozóides, auxiliando também na sua mobilidade e transporte dentro dos tratos genitais (GUYTON, 1984). Enquanto a próstata é encontrada em todos os mamíferos (PRICE, 1963), a vesícula seminal aparece apenas em alguns mamíferos (SETCHEEL & BROOKS, 1988) e a glândula de coagulação tem sido encontrada em ratos, hamster, cobaias e macacos (PRICE, 1965).

Nos últimos anos, a próstata tem sido foco de muitos estudos por ser origem de diversas doenças no homem, dentre elas destacando-se o câncer da próstata, a hiperplasia prostática e as prostatites. Anatomicamente, no homem, a próstata, que é do tamanho de uma castanha (HAM & CORMACK, 1991), envolve a uretra inferiormente à bexiga (ROSAI, 1996). Em roedores, essa glândula é formada de três pares de lobos: ventral, dorsal e lateral (HAYASHI et al., 1991). O lobo ventral localiza-se anteriormente à uretra, enquanto o lobo dorsolateral circunda-o dorsolateralmente. Já a vesícula seminal e a glândula de coagulação são encontradas dorsolateralmente (PRICE, 1963; SUGIMURA et al., 1986). Esses lobos são compostos de dutos que emergem da uretra e arborizam-se distalmente. Eles diferem quanto aos tipos celulares (CUNHA et al., 1987), padrão de dutos (HAYASHI et al., 1991), tipo de secreção (PRINS, 1991; CHAN, 1999), resposta a hormônios (PRINS et al., 1992; BANERJEE et al., 1995), padrão de expressão gênica (TAKEDA et al.,1990) e de síntese protéica (LEE et al.,1990; PRINS et al., 1992; BANERJEE et al., 1993).

A próstata ventral é composta de um conjunto de ácinos no qual as células epiteliais encontram-se envolvidas por um estroma (AÜMULLER, 1979). Na próstata ventral de rato, cada lobo prostático consiste de 8 conjuntos de dutos que se originam a partir da uretra como uma simples estrutura tubular dos quais emergem ramos e sub-ramos. Esse conjunto de dutos é dividido em três regiões morfológica e funcionalmente distintas denominadas de

distal, intermediária e proximal em relação à uretra (LEE, 1990; SHABSIGH et al., 1999). Na região distal são encontradas células epiteliais colunares altas de núcleo apical, indicando atividade proliferativa enquanto as células musculares lisas se apresentam como uma camada esparsa e descontínua associadas a uma abundante camada de tecido fibroso (NEMETH & LEE, 1996). Na região intermediária, as células epiteliais também são colunares altas porém quiescentes, apresentando características de células secretoras. Nesta região, a camada de células musculares lisas é fina e contínua. Na região proximal, as células epiteliais são cúbicas baixas, muitas das quais sofrem apoptose. Tanto na região intermediária como na proximal, o tecido fibroso está presente no espaço entre os dutos e ocasionalmente intercalando a camada de células musculares lisas. Outros tipos celulares também são encontrados como mastócitos, células endoteliais e pericitos, além de outros elementos tais como terminações nervosas e gânglios sensitivos. Cada célula desempenha um papel importante e específico na manutenção e função secretora na próstata ventral. Como em glândulas, a atividade das células epiteliais é fortemente influenciada pelos componentes estromais, as diferenças fenotípicas das células epiteliais encontradas ao longo dos dutos prostáticos parecem estar relacionadas com a distribuição diferenciada do tecido fibroso e muscular liso (PRINS, 1991; LEE, 1990; SENSIBAR et al., 1991; NEMETH et al., 1996).

A interação epitélio-estroma desempenha um papel fundamental na regulação da atividade funcional e manutenção da próstata (LEE, 1996). Durante a embriogênese, sob ação de andrógenos, células invaginam da uretra na base da bexiga para formar o mesênquima do sino urogenital (TIMMS, 1994). Esse tecido induz a formação dos dutos e suas ramificações, a proliferação epitelial, a expressão de receptores de andrógenos e secreção de proteínas específicas da próstata (CUNHA et al, 1987; CUNHA et al., 1992; HAYWARD et al., 1997). Na idade adulta, a manutenção da fisiologia da próstata continua dependente de andrógenos.

Dentre os componentes que atuam na interação estroma-epitélio, estão aqueles que constituem a matriz extracelular. Ela é composta de colágeno, elastina, proteoglicanos, fibronectina, laminina e outras proteínas não colagênicas (LABAT-ROBERT et al., 1990). O colágeno tipo VI e o sistema de microfibrilas associado à elastina parecem estar envolvidos no controle de alguns aspectos do comportamento celular e em desempenhar um papel

estrutural, mantendo a integridade do órgão (CARVALHO et al., 1997). ILIO et al. (2000) observaram que a laminina está localizada ao longo do sistema de dutos prostáticos enquanto o colágeno tipo IV é eventualmente encontrado na membrana basal na região distal e intermediária, porém está quase ausente na região proximal e não é detectado após a castração, mesmo embora outros autores não tenham observado essa diferenciação regional e fisiológica (CARVALHO & LINE, 1996). Os proteoglicanos (PG) são componentes abundantes na matriz, consistindo de um esqueleto protéico no qual cadeias de glicosaminoglicanos (GAGs) e oligossacarídeos estão ligados (HASCALL & HASCALL, 1981). Os GAGs são um grupo heterogêneo, formado de unidades dissacarídicas repetitivas composto de uma hexosamina e um açúcar não aminado- exceto o queratam sulfato que apresenta uma galactose substituindo o ácido urônico. Os principais tipos são: ácido hialurônico (AH), condroitim sulfato (CS), dermatam sulfato (DS), queratam sulfato (KS), heparam sulfato (HS) e heparina (Hep). O ácido hialurônico é o único que não está ligado covalentemente ao esqueleto protéico dos PG e não apresenta grupamentos sulfatos em sua cadeia.

Um grande espectro de funções tem sido atribuído aos proteoglicanos, estando eles envolvidos na manutenção das propriedades estruturais, mecânicas e biológicas da matriz extracelular; na manutenção das funções celulares (HAY, 1984; BISSEL et al., 1992); na regulação da proliferação e mobilidade celular e nas interações célula-célula e célula-matriz (HOOOK et al., 1984). Devido a seu caráter polianiônico, os GAGs são capazes de interagir com uma variedade de componentes presentes tanto na matriz extracelular quanto na superfície celular. Assim, estudos têm mostrado que esses polímeros ou regiões específicas dele podem inibir ou regular a passagem de outras moléculas através da membrana basal e controlar o acesso de macromoléculas - como fatores de crescimentos e hormônios – à superfície celular (OBRINK et al., 1975; KANWAR, 1984; LERNER & TORCHEA, 1986; ROBERTS et al., 1988; TAPALES & KESKI-OJA, 1997).

Os trabalhos iniciais enfocando proteoglicanos na próstata mostraram bioquimicamente que o CS e o DS são os GAGs predominantes na próstata normal humana (SATO & GYORKEY, 1972). DeKLERK (1983), DeKLERK et al. (1984) e DeKLERK & HUMAN (1985), estudando a próstata em diferentes estágios do desenvolvimento, identificaram quatro tipos de GAGs: HA, CS, DS e HS. O HS não foi

detectado em tecido fetal. Dentre eles, o DS é o GAG mais abundante na próstata normal e hiperplástica, concentrando-se na fração epitelial e estromal, enquanto que o HS e o AH localizam-se na fração estromal. Além disso, KLERK & HUMAN (1985) mostraram que ocorre uma mudança marcante em termos quantitativos e qualitativos de GAGs no estroma durante o desenvolvimento da próstata. Na idade adulta, o conteúdo de DS e HS parece aumentar, sugerindo que a proliferação epitelial pode estar associada a mudanças nos níveis de GAGs durante a puberdade. As mudanças no conteúdo de GAGs no lobo dorsal de próstata em cobaias durante o desenvolvimento também foram estudadas (HORSFALL et al., 1994), sendo aparentemente dependentes da regulação hormonal.

Muitos trabalhos têm mostrado os efeitos da castração na próstata que incluem: diminuição de vacúolos secretores (HEYNS, 1990), aumento dos níveis de enzimas degradativas como ribonucleases (ENGEL et al., 1980), alteração no sistema elástico (CARVALHO et al.,1997) e no colágeno VI (CARVALHO et. al., 1997) e expressão de vários componentes que levam à apoptose, tais como a catepsina D (SENSIBAR et al., 1991) e o ativador de plasminogênio do tipo uroquinase (FREEMAN et al., 1990).

Alterações de GAGs na próstata são observadas após castração. KOFOED et al. (1971) observaram que as concentrações de GAGs na vesícula seminal e na próstata de rato são alteradas após castração e por administração de testosterona. A castração causa atrofia nos lobos prostáticos e alterações nos níveis qualitativos e quantitativos dos GAGs, mas que podem ser revertidos com a reposição de andrógenos (KOFOED et al., 1990). TERRY e CLARK (1996a), estudando a influência hormonal nos GAGs dos três lobos prostáticos de ratos observaram uma diminuição no conteúdo de GAGs após a castração que foi revertido com a administração de testosterona. Comparando os diferentes lobos, os autores observaram uma mudança no conteúdo de GAGs mais pronunciada para a próstata ventral do que para os lobos dorsais e laterais, sugerindo com isso, que esses dois últimos lobos podem apresentar um mecanismo regulatório distinto em comparação com a próstata ventral. Horsfall et. al (1994) observaram que os GAGs sulfatados no estroma de cobaias são influenciados por hormônios durante o desenvolvimento. Ocorre um aumento na quantidade de GAGs do estroma durante o desenvolvimento mas uma diminuição da concentração de GAG por mg de tecido. Por outro lado, a utilização de testosterona levou a

uma redução dos níveis de GAGs do estroma enquanto não foram observadas alterações quando seguido do tratamento com estradiol (HOSFALL et. al., 1994).

Estudos mostraram que as interações epitélio-estroma desempenham um papel fundamental na etiologia e na progressão da hiperplasia prostática (McNEAL, 1990). O conteúdo de GAGs é bastante variado em diferentes tumores prostáticos. Os tumores de origem epitelial são geralmente associados com aumento nos níveis de CS e AH e diminuição de DS e HS (IOZZO et al., 1981). A quantidade de CS se encontra aumentada na próstata hiperplástica e em carcinoma quando comparada com a próstata normal, enquanto o conteúdo de HS e de seu grau de sulfatação parecem diminuir no carcinoma prostático (DeKLERK et al., 1984). BONKHOFF et al. (1991) observaram que a imunoreatividade de um proteoglicano de heparam sulfato (HSPG) foi acentuado na membrana basal de próstata fetal e cancerosa, porém fraca em tecidos normais e hiperplásticos, sugerindo com isso que mudanças nos níveis de HS podem estar relacionados com o crescimento da próstata cancerígena e em processos de diferenciação celular. Outros estudos têm revelado que o aumento de condroitim sulfato tem relação com a hiperplasia, enquanto que o aumento na relação CS: DS parece estar associado com o desenvolvimento de tumores malignos (ILDA et al., 1997).

Histoquimicamente, os GAGs sulfatados parecem concentrar-se na interface estroma-epitélio de próstata (KLERK et al., 1985). CHAN e WONG (1989 a, b), usando corantes catiônicos e degradação com GAG-liases específicas, detectaram três diferentes classes de PGs na próstata lateral de cobaias: aqueles próximos à membrana basal são ricos em heparam sulfato, os próximos às fibrilas de colágeno são ricos em dermatam sulfato e aqueles presentes no espaço intersticial são ricos em condroitim sulfato. TERRY e CLARK (1996b) demonstraram por técnicas imunocitoquímicas que o CS está localizado preferencialmente na membrana basal da próstata ventral de rato de onde desaparece com a privação de andrógenos, embora continue presente na matriz extracelular. Curiosamente, o conteúdo de CS do lobo lateral não é alterado após castração, reforçando as diferenças de resposta a andrógeno que são lobo-específicas (TERRY & CLARK, 1996b) .

Pouco se conhece sobre os GAGs presentes na vesícula seminal e na glândula de coagulação. A vesícula seminal consiste de tubos com várias dobras formando divertículos irregulares, situando-se entre a face posterior da bexiga e o reto e lateralmente à ampola do

duto deferente (SETCHEEL & BROOKS,1988) . O epitélio é pseudoestratificado, no qual as células basais se encontram dispostas inferiormente e intercalando as células epiteliais secretoras colunares altas. Os ácinos são envolvidos por um tecido muscular organizado em duas camadas: longitudinal externa e circular interna enquanto o tecido conjuntivo é constituído de terminações nervosas e vasos sanguíneos. A glândula de coagulação (originalmente denominado de lobo prostático anterior ou craniodorsal) localiza-se na concavidade interna da vesícula seminal (CAVAZOS, 1975). Os ácinos são compostos de células epiteliais colunares secretoras e basais lentiformes, sendo circundados por densas camadas (2-4) de células musculares lisas. O estroma se encontra adjacente a essa camada muscular contendo vasos, terminações nervosas e tecido connectivo esparso. A glândula de coagulação recebe este nome devido ao seu grande conteúdo de transglutaminase, enzima cuja função é a formação de pontes cruzadas entre os aminoácidos lisina e glutamina de glicoproteínas secretadas pela vesícula seminal, causando aglutinação das mesmas e contribuindo assim com um aumento na viscosidade do sêmen. Além da transglutaminase, outras enzimas tais como como vesiculase (SAMUEL & FLICKINGER, 1986) e as proteínas dorsal 1 e dorsal 2 (WILSON & FRENCH, 1980) também participam na aglutinação das proteínas provenientes da vesícula seminal. Estudos iniciais realizados por RZESZOWSKA (1966) mostraram que os GAGs se encontram no epitélio e na membrana basal da vesícula seminal, e que são reduzidos após castração. Esses resultados corroboram aqueles obtidos por KOFOED et al. (1971), que indicam que a concentração de GAGs na vesícula seminal de rato é alterada após castração e administração de testosterona. KIPLESUND et al. (1988), comparando a dependência das glândulas aos hormônios testiculares, observaram que a glândula de coagulação é menos dependente de andrógenos do que as demais glândulas. CHAN (1991; 1992), através de microscopia de luz e eletrônica, constatou que os GAGs na vesícula seminal de cobaias apresentam a mesma distribuição daqueles observados na próstata lateral de cobaias, indicando que a interface estroma-epitélio nesta glândula é também rica em GAGs sulfatados.

Apesar desses trabalhos já descritos, os estudo dos GAGs nas glândulas acessórias masculinas tem sido ainda insuficiente para a compreensão de como essas moléculas atuam na interação epitélio-estroma e como se encontram distribuídas no tecido.

Objetivos

O objetivo deste trabalho foi identificar bioquímica e citoquimicamente os tipos de glicosaminoglicanos sulfatados presentes na próstata ventral de ratos, determinar suas concentrações e variações decorrentes da deprivação de andrógenos ocasionada pela castração, em comparação com a glândula de coagulação e a vesícula seminal. Além disto, procurou-se iniciar um estudo sobre o metabolismo dos glicosaminoglicanos nas diferentes condições hormonais e localizar por imunocitoquímica o condroitim sulfato.

Hormonal regulation of the sulfated glycosaminoglycan types and contents in the rat ventral prostate, coagulating gland and seminal vesicle

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Short title: Glycosaminoglycans of the male sex accessory glands

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Grant sponsor: FAPESP; CAPES

BACKGROUND. Glycosaminoglycans have been implicated in several aspects of cellular physiology. Since the male accessory sex glands are highly dependent on androgens, the present study was undertaken to examine the effect of androgen deprivation on sulfated glycosaminoglycan (GAG) types and content in the ventral prostate, coagulating gland and seminal vesicle of 3-month-old rats.

METHODS. The animals were castrated and sacrificed 7, 14 and 21 days after surgery. Non-castrated animals were used as controls. The sulfated GAGs were extracted and quantified by the dimethylmethylene blue method. GAG types were identified by electrophoresis, enzymatic digestion and nitrous acid degradation. *In vivo* ^{35}S incorporation was followed by autoradiography of tissue sections.

RESULTS. Chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) were found in the different glands of the control animals. DS is the predominant GAG in both control and castrated groups. The total amount of GAG increased progressively after castration up to the 21th day, representing absolute increases in DS and HS. On the other hand, the CS amount decreased after castration. [^{35}S] labeling showed similar results, but a more prominent change in the expression of CS to HS in the prostate of castrated animals as compared to controls. *In situ* labeling showed the GAG to be diffuse in the stroma and concentrated at the basement membrane of epithelial structures and of smooth muscle cells. Similar aspects were observed in castrated and control animals.

CONCLUSIONS. These results demonstrated that the variations in the tissue organization following castration are associated with changes in the sulfated GAGs, resulted not only by changes in the stromal compartment, but also in the epithelium.

Key words: castration, chondroitin sulfate, glycosaminoglycans, stroma, ventral prostate

INTRODUCTION

The male sex accessory organs secrete several components which form the semen and improve sperm cell performance. The development and maintenance of tissue integrity and functional activities of these glands are strongly influenced by androgens but the stromal-epithelial interactions seem also to be involved.

Despite the hormonal regulation and the cross-talk between the epithelium and the different cell types of the prostatic stroma (i.e. smooth muscle cells, fibroblasts and endothelial cells amongst others), the extracellular matrix is responsible for the tissue organization and creation of specific environments that guarantee proper cell differentiation and function, migration and access to hormones and growth factors.

The proteoglycans have been associated with in each of these functions. They are versatile macromolecules consisting of a protein core to which at least one glycosaminoglycan (GAG) chain is attached. The main GAGs are chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS) and heparin. Hyaluronan (HA) is an exception to this group as it is not covalently attached to a protein core and is not sulfated.

Early studies have shown the presence of GAGs in normal and hyperplastic human prostate and that DS is the main GAG present in either these conditions [1]. Changes in the content and types of GAGs during prostatic development were also reported for humans [2] and Guinea pigs [3]. However, the level of CS is increased in benign hyperplasic prostate and prostate gland carcinoma as compared to the normal prostate [2,4]. The content of HS and its sulfation level are decreased in the prostate gland carcinoma [2,5].

Androgen ablation either by surgical or chemical castration has been widely used to understand the physiology of the prostate gland and apparently, the male sex glands are differently affected by castration, the coagulating gland being less dependent on testicular hormones than the other glands [6]. Some studies have described that the GAGs present in male sex organs are also influenced by androgens [7,8,9]. This differential influence is also observed in the prostatic lobes [9]. Kofoed et al. [7] observed that GAGs concentration in seminal vesicle and prostate of rats changed after castration and testosterone administration. The castration induced an increase of uronic acid concentration in ventral prostate in rats

[8]. However, Terry and Clark [9] have shown a decrease in the GAG content following the regression of three prostatic lobes in rats after castration. Horsfall [3] has also suggested that sulfated GAGs in the prostatic stroma of the Guinea pig are influenced by steroid hormones.

To improve our understanding on the involvement of GAGs in the biology of the prostate gland, we investigated the variations in GAG type and content in the ventral prostate, seminal vesicle and coagulating gland of rats and checked their modifications at different times after surgical castration, by using biochemistry, immunocytochemistry and in *vivo* and in *vitro* ^{35}S-labeling. The results demonstrated differences between the three glands in GAGs and in the response to androgen ablation, and that the remodelling of the sex glands after castration involves changes in the GAGs.

MATERIALS AND METHODS

Animals

Three-month-old Wistar male rats were used. Animals were divided into four experimental groups (10 animals per group): control rats (non-operated), 7 days, 14 days and 21 days after castration. They were sacrificed by ether inhalation. The ventral prostate, coagulating gland and seminal vesicles were dissected out and the adhering fat and connective tissue were carefully removed.

Extraction and quantitation of GAGs

The tissue was homogenized, delipidated in acetone, dried and weighed. The samples were digested in 10 volumes of 1mg/mL papain in 0.5M sodium acetate buffer pH 5.8, containing 10mM EDTA and 5mM cysteine hydrochloride at 60°C for 36hr. GAGs were isolated by precipitation with 2 volumes of cold ethanol overnight at 4°C. The precipitates containing the GAGs were air dried, resuspended in water and quantitated by the dimethylmethylene blue (DMMB) method [10]. Contaminating nucleic acid was

eliminated by sequential digestion with deoxiribonuclease I and RNase (Sigma Chemical Co., St. Louis MO, USA) as described by Jaques [11].

The extracted GAGs were identified by electrophoresis in 1,3 diaminopropane (DAP) buffer system and DAP/barium acetate discontinuous buffer system [12]. Standard GAGs were included in each run. The GAGs in the gel were fixed by 0.1% cetavlon plus 0.1M NaCl during 1 hour and after washed with 1% cetavlon each 15 minutes during 2 hours. The individual bands were visualized by toluidine blue staining and quantified on a densitometer. Further identification of the individual GAGs was done by enzymatic digestion with chondroitinase AC and ABC (Seikagaku America, Falmouth, MA, USA)[13] or by nitrous acid hydrolysis [14].

[^{35}S]-labeling

Individual glands were placed on 24 well plates in 1ml of Eagle culture medium containing 10% fetal calf serum and 100μCi/ml of $Na_2[^{35}S]O_4$ plus penicillin and streptomycin. The incubation was for 4 hours in a humidified atmosphere of 5% CO_2 - 95% air. After incubation, the GAGs were analyzed using the above described protocol. Cold GAGs were added as carriers during the ethanol precipitation. Labeled GAGs were visualized by radiography on Kodak X-Omat film for 7days at –70°C.

Autoradiography

In this experiment 2mCi/ 350g of body weight were injected intraperitoneally in rats 24 hours before the sacrifice. Control and castrated (21days after surgery) rats were used. The samples were formalin fixed and paraffin embedded. Six μm thick section were obtained by routine procedures. Following dewaxing, the slides were covered with the Amersham's emulsion for 5 seconds and exposed for 7 days at 4°C, protected from the light. The material was developed with the Kodak D19B developer and fixed with 30% sodium tiosulfate. The sections were counter stained with cresyl violet, mounted under glass coverslips and observed by dark field microscopy.

Immunohistochemistry

Tissue sections were dewaxed with xylene and treated with 3% hydrogen peroxide in water for 15min to block endogenous peroxidase activity. After rinsing with TBS containig 0.1% Tween 20 (TBS-T), non-specific binding sites were blocked with 3% bovine serum albumin (Sigma Chemical Co.) in TBS-T for 30min. Sections were incubated with a mouse anti-chondroitin sulfate monoclonal antibody (Clone C56; Sigma Chemical Co.), diluted 1:500 in 1% BSA in TBS-T overnight at 4°C. The sections were then washed and incubated with a biotinylated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz CA, USA.) diluted 1:125 in 1% BSA in TBS-T for 2 h. The peroxidase activity was revealed with the 3,3'-diaminobenzidine reaction. The sections were counter stained with methyl green and mounted in Entellan.

RESULTS

A marked reduction of the dry weight of three glands was observed after castration (Fig. 1). This was more evident for the ventral prostate (with a 75% reduction in the first seven days after castration) than for the other glands. The coagulating gland and seminal vesicle showed similar reduction to about 50% of controls 7 days after castration. There was a less pronounced reduction in weight loss after the 14^{th} day.

There was a progressive increase of the concentration of the sulfated GAGs solubilized by papain-digestion and ethanol precipitation after castration (Fig. 2). This increase in the content of total GAGs was more evident in the ventral prostate, while the coagulating gland and seminal vesicle showed similar increment in the content of sulfated GAGs.

Three types of GAGs (CS, DS and HS) were found in the three glands of control rats (Fig. 3). The individual types were identified by comparison with the electrophoretic behavior of standard GAGs and either enzymatic and nitrous acid degradation (Fig. 4). DS was the most abundant GAG and this predominance was sustained after castration (Fig. 5). It was the main responsible for the increase in the total GAGs content. The proportion of

CS decreased after castration while the content of HS was comparable to the controls, except for the seminal vesicles, in which the content of HS also decreased (Fig. 5).

Sulfated labeling revealed changes in the proportion of the GAGs synthesized under the different experimental situations (Figs. 6 and 7). DS represented 55-60% of the total GAGs synthesized in a 4-hour *in vitro* pulse of ^{35}S, in the three glands of control animals. CS and HS were found in equivalent amounts (~25% each) n the ventral prostate of control rats. After castration, there was an evident tendency to the synthesis of heparan sulfate, while a striking reduction of CS was noted (Fig. 7). The presence of sulfated proteins was indicated by the identification of ^{35}S labeling of protease-sensitive material at the origin of the agarose gels. The proteolysis of this material did not result in increase of the GAG band intensity, demonstrating that it is not due to incomplete cleavage of proteoglycans by papain. Furthermore, SDS-PAGE of these samples demonstrated that they correspond to low molecular weight (papain resistant fragments) of sulfated proteins (not shown).

Likewise, the coagulating gland and seminal vesicle showed a tendency to an increase in the synthesis of HS as compared to CS. However, this tendency was not as pronounced as in the ventral prostate.

Autoradiography demonstrated the incorporation of ^{35}[S] predominantly at the basal membrane around the epithelial structures and around smooth muscle cells in all glands analyzed (Fig. 8). As expected, mast cells were strongly labeled. In the ventral prostate of control rats, the epithelial components occupy most of the gland while the stromal compartment was scarce and limited to a small area around the epithelium. Following the remarkable regression of the epithelium seen 21 days after castration, there was an increased labeling of the epithelium, specially at the apical surface of the cells (Figs. 8a-d).

On the other hand, in the coagulating gland and seminal vesicle of non-castrated rats, the stromal portion is more prominent than in the ventral prostate, and is represented by a large number of smooth muscle cells. The association of the ^{35}[S] labeling and the smooth muscle cells in these two glands was evident (Figs. 8e-l).

The immunoperoxidase staining of chondroitin sulfate showed it to be concentrated at the basis of the epithelium and around smooth muscle cells of the ventral prostate in control rats (Fig. 9a). This pattern was maintained in the castrated animals, 21 days after surgery, though there seems to be an increased reactivity. A weak and diffuse staining was

found in the stroma. Chondroitin sulfate immunoreactivity was located to basis of the epithelial structures and around smooth muscle cells also in the other two glands (Fig. 9d-k). Labeling was intense in association with the blood vessel wall (Fig. 9e). A clear concentration of chondroitin sulfate was observed in the seminal vesicle and reflected an atrophy of the smooth muscle cells (Fig. 9h e j).

DISCUSSION

The male sex accessory glands show different responses to androgen manipulation. These differences are seen at different levels, as observed by biochemical and cytochemical parameters [15,16,17].

The characterization of the GAGs in the rat accessory glands seems to be relevant since these molecules are involved in different physiological events. The ability of the GAGs to perform diverse functions arise from their heterogeneous chemical and structural aspects [18].

The effect of androgen deprivation on the sex accessory glands is easily observed by the striking reduction in weight following castration. The reduction in weight of the ventral prostate confirms an earlier report from our lab [19] and is more prominent than that observed for the other two glands. This behavior might reflect either a larger proportion of the epithelial compartment in the ventral prostate, as compared to the coagulating gland and seminal vesicle or a true differential responsiveness to testicular hormones.

There is a marked increase in the concentration of total sulfated GAGs in the three glands following castration. The ventral prostate accumulate as much as 8-fold sulfated glycosaminoglycan after 21 days of androgen deprivation. The GAGs found in the control glands are CS, DS and HS. DS was the predominant GAG irrespective of the hormonal situation in each gland, but there were modifications in the content of GAGs in the three glands after castration. They involve an increase in DS, a reduction in CS and a slight increase in HS. After castration, the increase in the content of DS is striking and it is responsible for most of the increase in total sulfated GAGs. This increase in DS is probably

associated with the concentration of collagen fibers around the epithelial structures that takes place after castration [20], since an intimate association between these two components of the extracellular matrix has been suggested. Other studies have observed that decorin and fibromodulin which contein DS chain may be involved in the concentration of collagen fibers [21].

The increase observed in HS, though much smaller than that observed for DS, is consistent in the three glands. It was observed that the basement membrane accumulates after castration and if folded and pleated beneath the epithelium [22]. This accumulation in basement membrane as well as a marked reduction in the epithelial compartment may be responsible for the observed increase in HS. It was shown that HS increases during puberty and it was correlated to a more differentiated situation of the epithelial cells [1]. HS also diminishes in prostatic carcinomas, further indicating a correlation between the the differentiated state of the epithelium and the accumulation of HS [5]. However, no clear correlation exists between the prostatic cells during differentiation or maturation (occurring at puberty), and the dedifferentiation that occurs in cancer and in the regressing prostate. Furthermore, the presence of other cell types, as the smooth muscle cells and other basement membrane-containing structures, such as nerves and blood vessels must also be taken into account, when considering variations in HS content.

Surprisingly, the content of CS progressively diminished after castration and was not detected by the biochemical procedures employed in this work 14 and 21 days after castration in the prostate and coagulating gland, though it was detected by immunohistochemistry. Furthermore, the content of CS was reported to decrease in the ventral prostate following castration [9,23]. These contradictions are explained first by the concept that immunohistochemistry has no quantitative aspect and that a single CS chain seems to have multiple antibody binding sites, resulting in an enhancement of the reaction. Secondly, there are specific differences between the methodology those used in the present investigation and that carried out by Terry & Clark [9,23]. These authors first extracted the prostatic tissue with guanidine hydrochloride and then solubilized the GAG by papain digestion. Separation of the GAGs was attained by electrophoresis on cellulose acetate. We attempted to reproduce the guanidine extraction and examine the GAGs present in the

unsoluble residue and observed that some DS and HS was not solubilized by the guanidine treatment (not shown).

Autoradiography showed a similar aspect of the incorporation of [^{35}S] in the three glands. There was a conspicuous labeling of the basement membrane of the epithelium and around smooth muscle cells, irrespective of the hormonal condition. There was some labeling of the secretion in the ventral prostate, and the apical border of the epithelial cells was also intensely labeled. This is likely associated with the presence of sulfated mucins in the glycocalix. Sulfated papain-resistant protein fragments were detected in the three glands. However, their specific nature is unknown. These sulfated proteins must also contribute to the autoradiograph labeling, but their specific location rely on the selective removal of the GAGs *in situ*. These two aspects will be investigated in the future. [^{35}S] labeling also demonstrated changes in the biosynthesis of the glycosaminoglycans and that these GAGs synthesized *de novo* also contributed to the observed increase in total GAGs. The most dramatic change occurred in the ventral prostate, in which equivalent amounts of CS and HS are produced in the control conditions. Following castration, there was an evident increase in HS and a decrease in CS. However, the reasons for this change is not understandable at the moment.

These alterations in the GAGs observed in this work probably reflect a differential biosynthetic activity of the cells, which is more evident in the ventral prostate than in the other two glands, in association with a putative differential degradation of the GAGs.

Although it is known that mast cell are found, we were not able to find heparin in our preparations. Other protocols for heparin purification are being tested for the establishment of a correlation between the heparin content and the number of mast cells in the prostate in the different experimental conditions.

ACKNOWLEDGMENTS

The authors are thankful to Dr. Shirlei M. Recco-Pimentel and Dr. Olga M. Toledo for supplying them with the nucleases employed in this research. They also express their gratitute to Dr. Telma M. T. Zorn for helpful comments on the autoradiographs.

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Legends of the figures

Fig. 1. Dry weight variation of the accessory glands at the different experimental points. Control (Ct), and 7d, 14d and 21 days after castrastion (7d, 14d e 21d). Ventral prostate (VP); coagulating gland (CG); seminal vesicle (SV). Values are the mean ± standard deviation.

Fig. 2. Content of total sulfated GAGs in the three glands at the different experimental points. Control (Ct), and 7d, 14d and 21 days after castration (7d, 14d e 21d). Ventral prostate (VP); coagulating gland (CG); seminal vesicle (SV). Values are the mean ± standard deviation.

Fig. 3. Agarose gel electrophoresis of the GAGs extracted from each of the three glands. Origen (OR); Comercial standards (St); Control (Ct), and 7d, 14d and 21 days after castration (7d, 14d e 21d). Ventral prostate (VP); coagulating gland (CG); seminal vesicle (SV). Heparine standard (Hep).

Fig. 4. Representative agarose gel electrophoresis of the GAGs extracted from the ventral prostate prior (1) and after digestion with chondroitinase ABC (2) or nitrous acid (3). Heparin standard (H); heparin standard + nitrous acid (H+). Origin (OR); Comercial standards (St)

Fig. 5. Relative (A) and absolute (B) concentration of the individual GAGs for the three glands. Chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS). Ventral prostate (VP); coagulating gland (CG); seminal vesicle (SV).

Fig. 6. Autoradiography of the GAGs separated by agarose gel electrophoresis after [^{35}S] *in vitro* labeling. Ventral prostate (VP); coagulating gland (CG); seminal vesicle (SV). Origin (OR). Control (Ct) and castrated animals 7, 14 and 21 days after surgery (7d, 14d and 21d, respectively).

Fig. 7. Relative content of the individual [^{35}S]-labeled GAGs from the three glands, as determined by densitometry of the bands shown in figure 6. Chondroitin sulfate (CS),

dermatan sulfate (DS) e heparan sulfate (HS). Ventral prostate (VP), coagulating gland (CG) and seminal vesicle (SV). Values are the mean of two different sets of experiments.

Fig. 8. Autoradiographs of tissue sections stained with crezyl violet of the glands after *in vivo* administration of [^{35}S]. **a-d**, ventral prostate; **e-h**, coagulating gland; **i-l**, seminal vesicle. **a** and **b**; and **f**, **i** and **j** are glands from control animals. **e** and **d**, **g** and **h**, **k** and **l** are glands from 21th days castrated animals. Bright and dark field pairs are presented for each gland from control and castrated animals. Labeling is concentrated at the basement membrane of the epithelial structures, around the smooth muscle cells, and diffuse in the stroma. Strong labeling of the mast cells (arrows) is evident in thre ventral prostate (**a-d**). Epithelium (**ep**)

Fig. 9. Chondroitin sulfate immunolocalization in the three glands of control (**a, d, g, h**) and castrated (**b, c, e, f, i-k**) rats. Chondroitin sulfated concentrated at the basis of the epithelial structures and is diffuse in the stroma of the the ventral prostate (**a** and **b**). It is also intimately associated with the surface of the smooth muscle cells in each gland (**a-k**). A clear concentration of the immunoreativity is seen in the seminal vesicle is observed and clearly results from the atrophy of the smooth muscle cells (**h-j**). The negative controls for the reaction are shown (**c,f,k**). Epithelium (**ep**) and smooth muscle cells (**smc**).

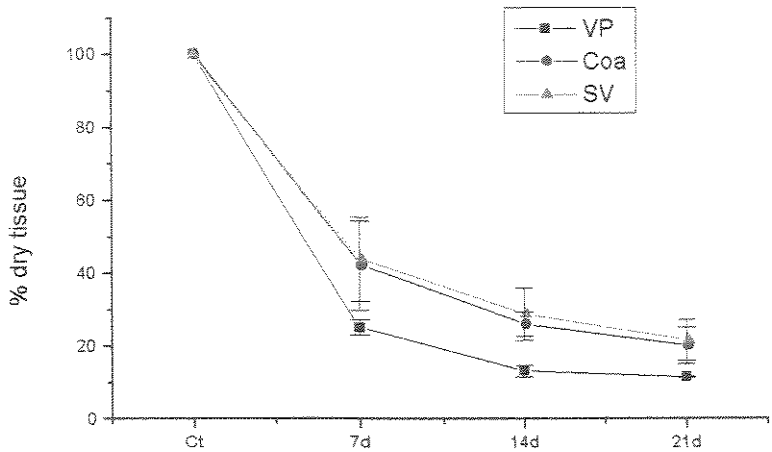
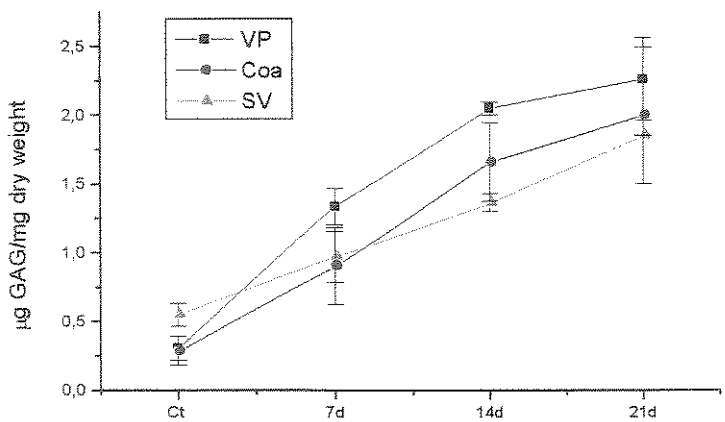

VP
Coa
SV
% dry tissue
100
80
60
40
20
0
Ct
7d
14d
21d

Fig. 1


VP
Coa
SV
µg GAG/mg dry weight
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2,0
1,5
1,0
0,5
0,0
Ct
7d
14d
21d

Fig. 2

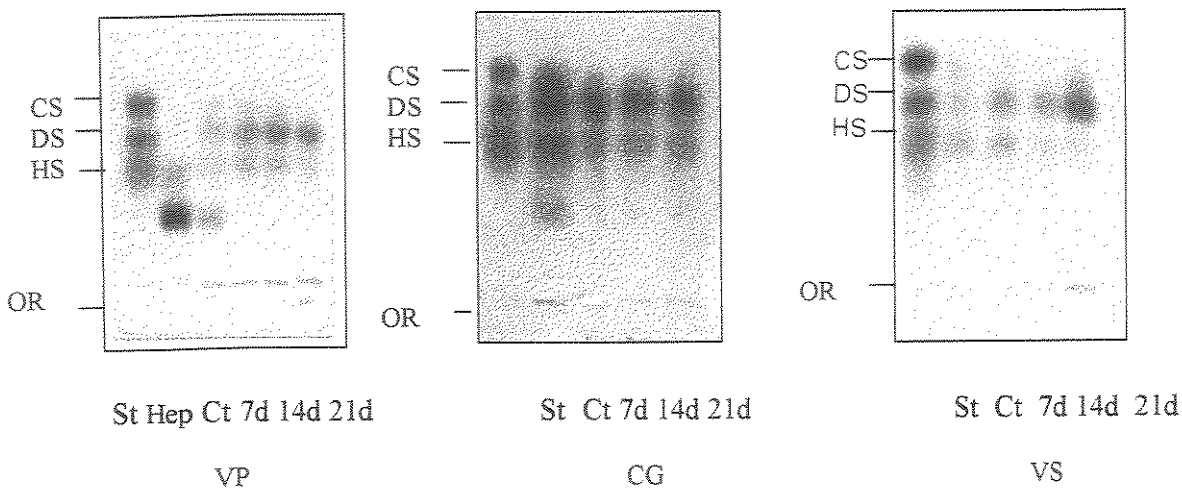
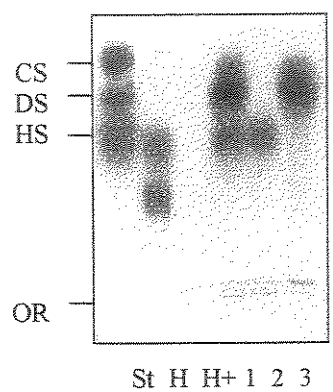

CS
DS
HS
OR
St Hep Ct 7d 14d 21d
VP
CS
DS
HS
OR
St Ct 7d 14d 21d
CG
CS
DS
HS
OR
St Ct 7d 14d 21d
VS

Fig. 3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DS
HS
OR
St H H+ 1 2 3

Fig. 4

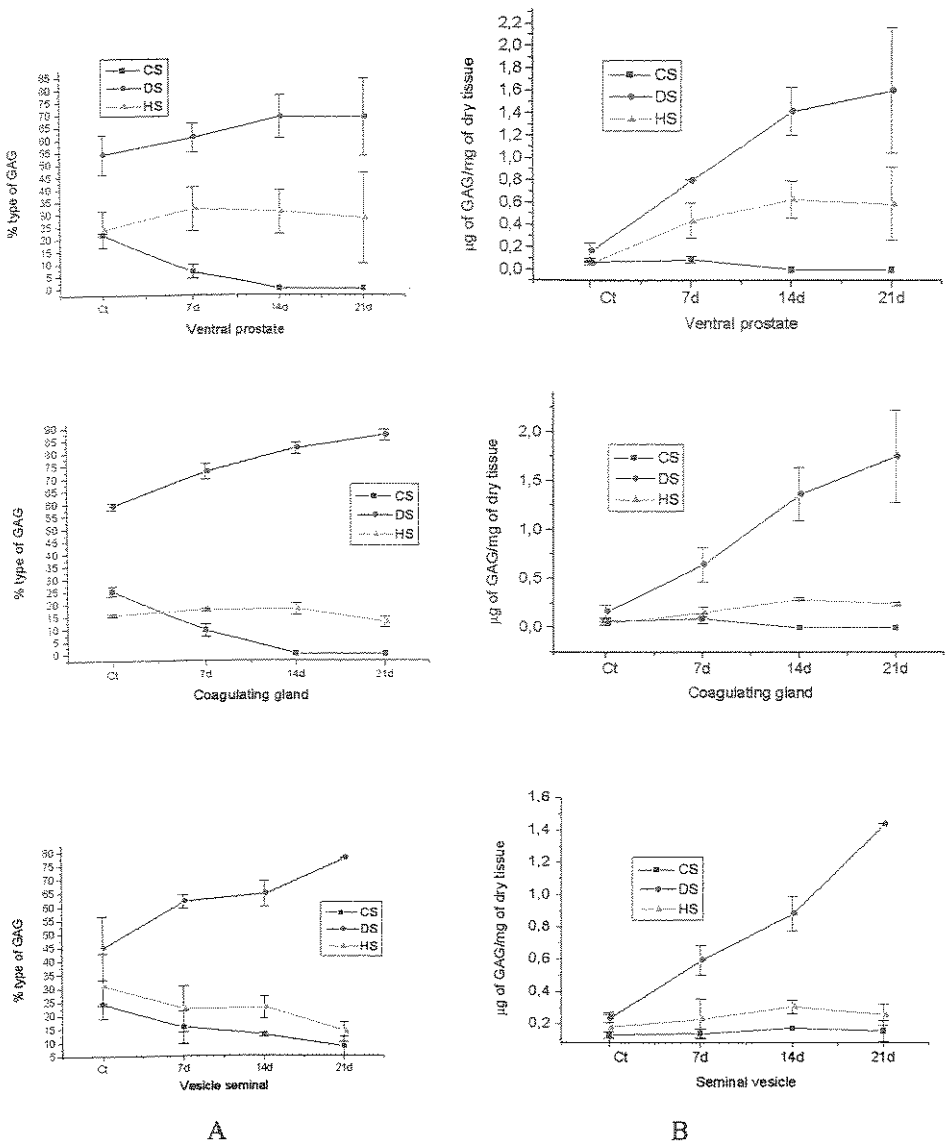

% type of GAG
CS
DS
HS
Ct
7d
14d
21d
Ventral prostate
µg of GAG/mg of dry tissue
Coagulating gland
Vesicle seminal
Seminal vesicle
A
B

Fig. 5

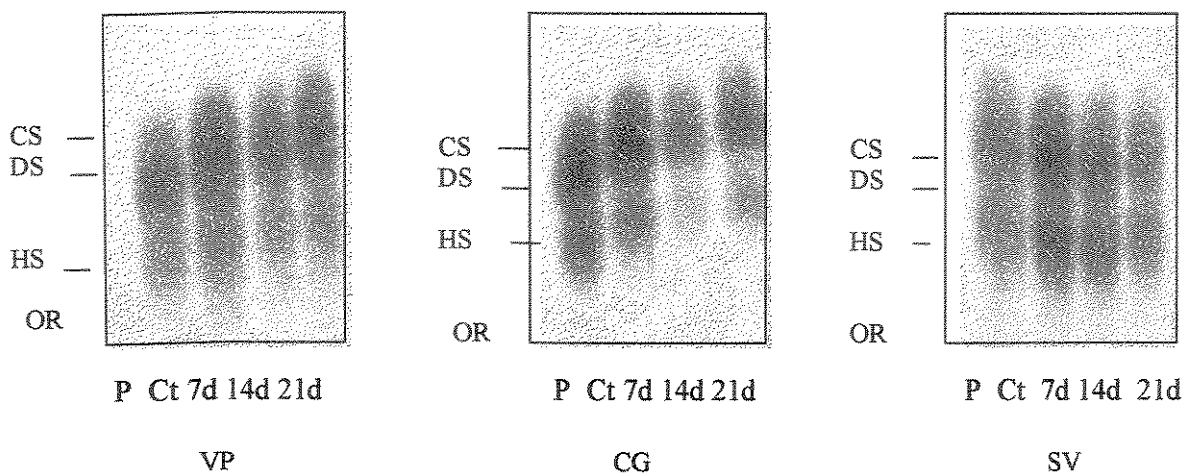
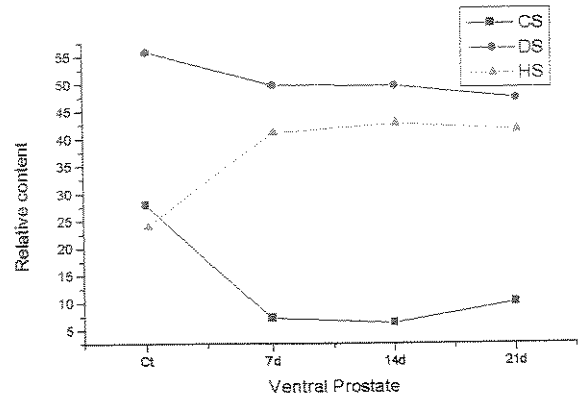


Fig. 6


CS
DS
HS
Relative content
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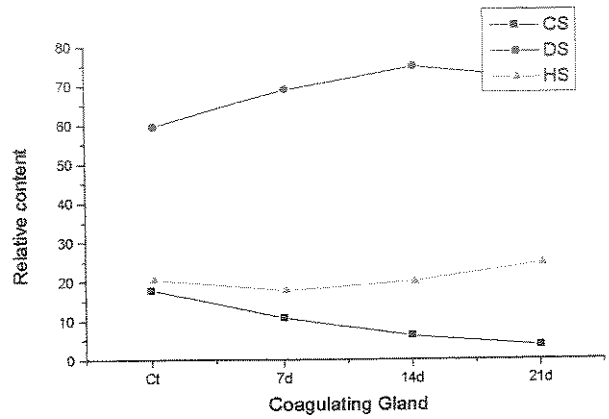
50
45
40
35
30
25
20
15
10
5
Ct
7d
14d
21d
Ventral Prostate


CS
DS
HS
Relative content
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70
60
50
40
30
20
10
0
Ct
7d
14d
21d
Coagulating Gland

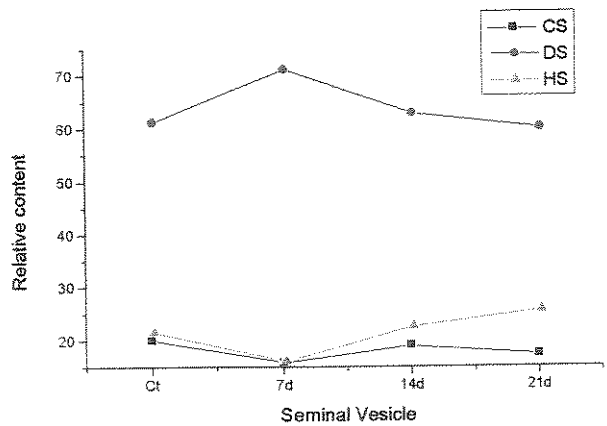
CS
DS
HS
Relative content
70
60
50
40
30
20
Ct
7d
14d
21d
Seminal Vesicle

Fig. 7

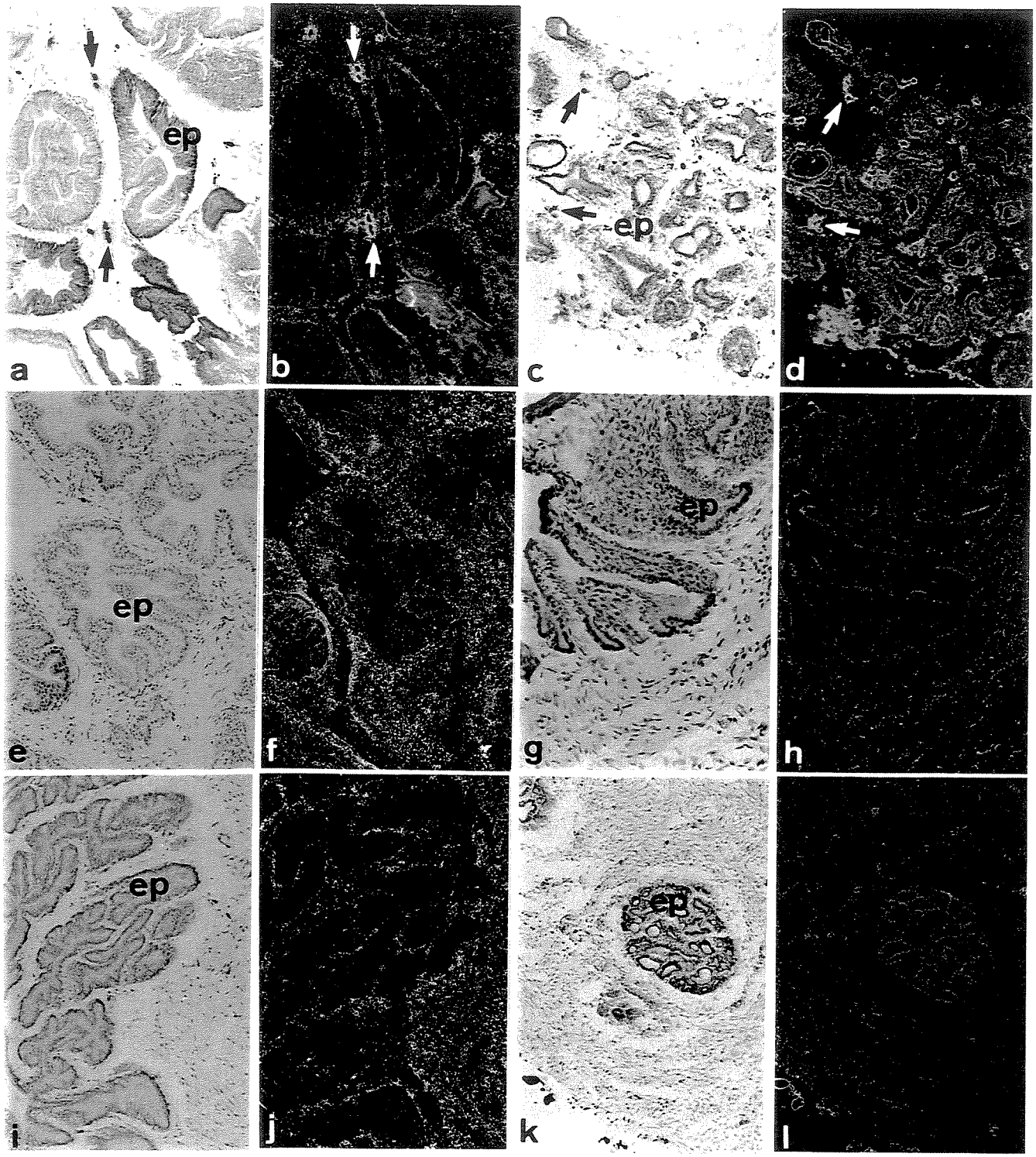

fig.8

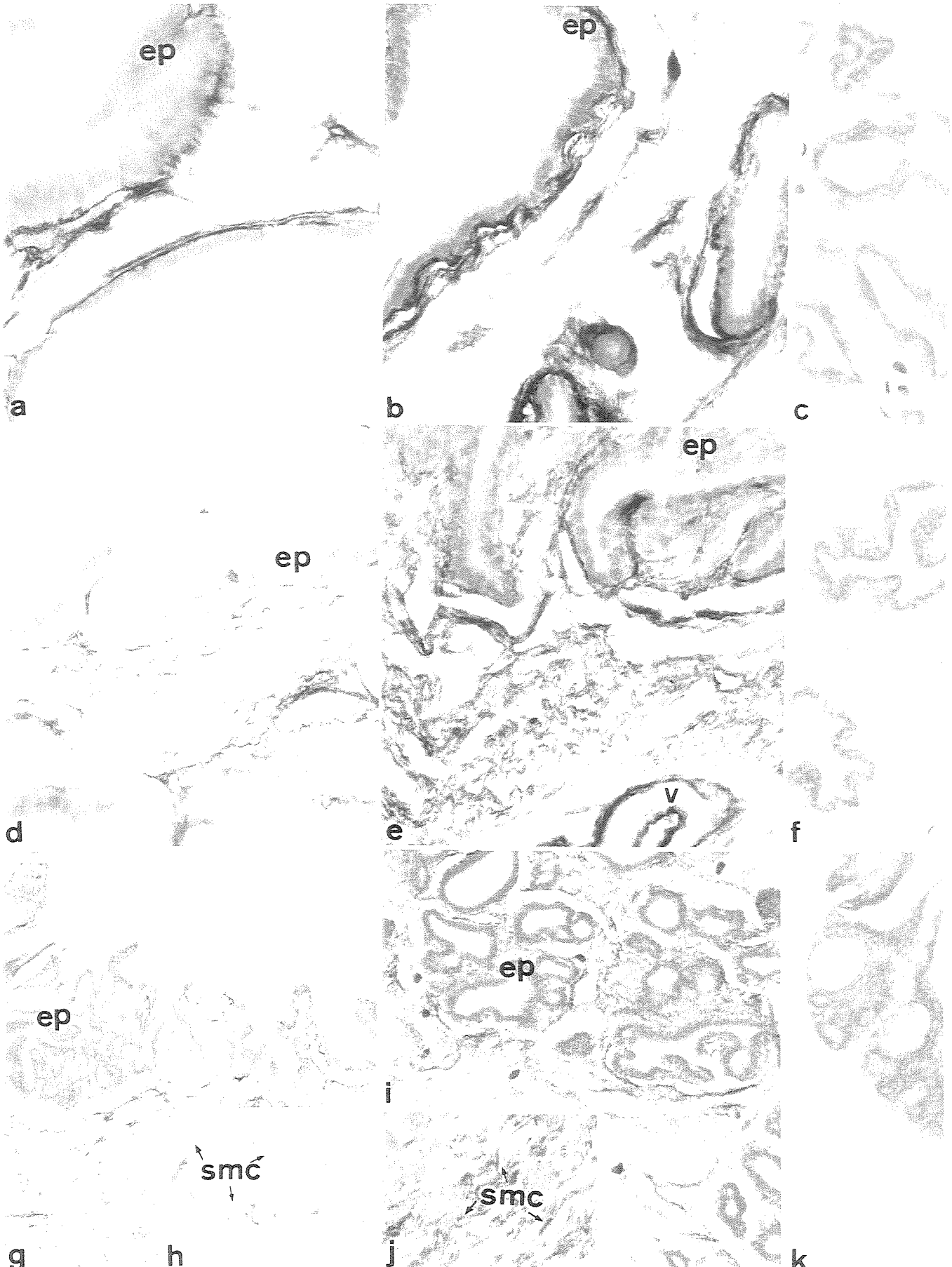

fig.9

Conclusões Gerais

- A regressão tecidual observada após castração é mais pronunciada para a próstata ventral do que para a glândula de coagulação e a vesícula seminal.
- Há modificações no conteúdo de GAGs presentes nas diferentes glândulas em resposta à castração.
- Há um aumento progressivo na concentração de glicosaminoglicanos sulfatados nas três glândulas. O aumento da quantidade de DS tem grande contribuição para o aumento da quantidade dos GAGs totais.
- Há uma tendência geral a uma diminuição do CS, enquanto a concentração de HS tende a aumentar. Na próstata ventral este efeito é mais visível.
- A localização do condroitim sulfato por imunocitoquímica demonstrou a presença deste glicosaminoglicano em associação com a membrana basal do epitélio e das células musculares lisas, além de uma distribuição difusa pelo estroma.
- Além de haver incorporação de [^{35}S], nos sítios de localização dos glicosaminoglicanos, há também marcação da secreção da próstata ventral assim como da superfície apical das células epitheliais, sugerindo a presença de mucinas sulfatadas.

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