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MÍRIAM DAMBROS

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Campinas, 31 de outubro de 2001.

Prof. Dr. PAULO CÉSAR RODRIGUES PALMA – Orientador

INFLUÊNCIA DA OOFORECTOMIA E DA REPOSIÇÃO DE ESTRADIOL NO VOLUME DAS FIBRAS COLÁGENAS E NO SISTEMA ELÁSTICO DA BEXIGA, EM RATAS

CAMPINAS

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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Mestre em Cirurgia, área de Cirurgia.

ORIENTADOR: *PROF. DR. PAULO CÉSAR RODRIGUES PALMA*

CO-ORIENTADORA: *PROF. DRA. VIVIANE HERRMANN RODRIGUES*

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Data:

DEDICATÓRIA

... aos meus pais Odila e Sivietto que me deram o bem mais valioso que possuo: a vida.

... aos meus irmãos Mara e Márcio pelo carinho e amizade incontestáveis.

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À Deus, que está presente em todos os dias da minha vida, iluminando-me e mostrando-me o caminho.

Se as coisas são inatingíveis... ora!

Não é motivo para não querê-las...

Que tristes os caminhos, se não fora

A presença distante das estrelas!

Mário Quintana

PÁG.

β	beta
μg	micrograma
Kg	quilograma
ml	mililitro
p	significância estatística
g	grama
mg	miligrama
μm	micrômetro
mm^3	milímetro cúbico
pg	picograma
%	percentagem

RESUMO

As fibras colágenas e o sistema elástico, principais componentes do tecido conjuntivo, estão presentes em todas as camadas da parede vesical e encontram-se intimamente relacionados com a complacência vesical. Estudos prévios demonstraram que mudanças estruturais e quantitativas das fibras do tecido conjuntivo são induzidas por determinadas condições, como a idade e os níveis de estrogênio, por exemplo.

A despeito dos vários estudos experimentais e clínicos tratando sobre a influência dos hormônios produzidos pelo ovário na estrutura vesical, os achados quanto à ação do estrogênio no tecido conjuntivo têm sido contraditórios.

Com a finalidade de investigar os possíveis efeitos da ooforectomia e a reposição do estrogênio sobre o tecido conjuntivo vesical, realizou-se análise morfométrica da parede vesical em ratas.

Sessenta ratas *Wistar* (idade média de três meses) foram dispostas em seis grupos com o mesmo número de animais (10): controle (grupo 1), ooforectomia bilateral e sacrifício após quatro semanas (grupo 2), procedimento *sham* e sacrifício após quatro semanas (grupo 3), ooforectomia bilateral e, após quatro semanas, iniciado reposição de 17β-estradiol (10μg/Kg/dia, via subcutânea, durante 12 semanas) (grupo 4), procedimento *sham* e, após quatro semanas, iniciado administração de óleo de sésamo (0,2ml/dia, via subcutânea, durante 12 semanas) (grupo 5) e ooforectomia bilateral e, após quatro semanas, iniciado administração de óleo de sésamo (0,2ml/dia, via subcutânea, durante 12 semanas) (grupo 6).

As fibras colágenas e o sistema elástico foram fixados em paraplast e os cortes corados com *Sirius red* e *Weigert'* resorcina-fucsina, respectivamente. O volume absoluto e a densidade volumétrica das fibras foram determinados através da contagem de pontos, utilizando-se o sistema teste M-42. Diferenças nos achados estereológicos dos seis grupos foram testadas com o teste não-paramétrico de Kruskal-Wallis. Foi adotado como critério de significância estatística o valor de $p < 0,05$.

O exame macroscópico não evidenciou diferenças destoantes na espessura das bexigas-controles e ooforectomizadas com ou sem reposição hormonal. O grupo de animais ooforectomizados e o grupo de ooforectomizados que recebeu estrogênio não apresentaram efeitos no volume vesical. A comparação dos achados estereológicos dos seis grupos não mostrou diferenças significativas no volume absoluto ($p = 0,12$) e na densidade volumétrica ($p = 0,24$) das fibras colágenas. A análise do sistema elástico evidenciou ausência de diferença estatística no volume absoluto ($p = 0,32$) e na densidade volumétrica ($p = 0,09$) entre os grupos.

Os baixos níveis de estrogênio durante longo período, seguidos de reposição hormonal, não alteraram significativamente a concentração dos principais elementos do tecido conjuntivo vesical, o que permite inferir que o estrogênio não influencia o padrão quantitativo das fibras colágenas e do sistema elástico na parede vesical de ratas.

1. INTRODUÇÃO

1.1. PREVALÊNCIA DAS DISFUNÇÕES VESICAIS NO PERÍODO PÓS MENOPAUSA

O período pós-menopausa está relacionado com sintomas miccionais como polaciúria, urgência e, principalmente, incontinência urinária (THOMAS *et al.*, 1980). Urge-incontinência aumenta com a idade e acomete entre 33% e 61% das mulheres acima de 65 anos. Dados epidemiológicos mostram que 10% a 50% das mulheres idosas têm dificuldade no controle da continência (DIOKNO, BROCK, BROWN, 1986). Em parte, estes sintomas podem ser ocasionados pela atrofia urogenital que ocorre nesse período, atingindo entre 10% e 40% das mulheres (GREENDALE & JUDD, 1993).

A prevalência da incontinência urinária varia, segundo diferentes autores. YARNELL *et al.* (1981) verificaram-na em 42% das mulheres entre 18 e 64 anos, atingindo até 59% das mulheres com idade superior a 75 anos.

Estudos recentes indicaram que a incidência de incontinência urinária em idosas é de 10% a 20%, nas que vivem em comunidade, elevando-se este índice para 50% nas institucionalizadas (BROCKLEHURST, 1990).

É comum infecção do trato urinário em mulheres idosas e tem sido estimado que em torno de 13% destas, com idade acima de 60 anos, há infecção urinária recorrente (BERGMAN, KARRAM, BHATIA, 1990). Após a menopausa, os baixos níveis de estrogênio levam a alterações no pH e na flora vaginal, aumentando o risco de infecções recorrentes (CARDOZO, BENNESS, ABBOTT, 1998).

Não obstante as variações encontradas, há consenso a respeito do aumento da prevalência das disfunções vesicais após a menopausa.

1.2. DISTRIBUIÇÃO DOS RECEPTORES PARA ESTROGÊNIO NO TRATO URINÁRIO INFERIOR FEMININO

O trato urinário inferior feminino é alvo de ação do estrogênio (BATRA & IOSIF, 1983). Esta teoria é baseada nas mudanças observadas nos órgãos urinários após administração de hormônio (BERGMAN *et al.*, 1990). Entretanto, o significado da presença destes receptores no trato geniturinário necessita ser esclarecido.

IOSIF *et al.* (1981) foram os primeiros a demonstrarem a presença de receptores específicos para estrogênio na uretra e na bexiga humanas. Estes pesquisadores detectaram receptores no detrusor, na uretra e no trígono, sendo que a concentração no trígono e no detrusor foi consideravelmente menor que na uretra. INGELMAN-SUNDBERG *et al.* (1981) confirmaram a presença destes receptores no tecido urogenital feminino. Quantidades semelhantes destes receptores foram encontradas na bexiga, no epitélio uretral e vaginal, e na musculatura pubococcígena. A análise funcional da musculatura detrusora evidenciou aumento da resposta contrátil mediante ação dos receptores alfa-agonistas, colinomiméticos e prostaglandinas (LEVIN, SHOFER, WEIN, 1980; WATANABE & CONSTANTINOU, 1996).

Os esteróides atuam através de receptores intracelulares, cuja expressão é regulada através de genes específicos. A presença de receptores estrogênicos é considerada um pré-requisito para que um tecido reconheça os estrógenos e manifeste uma resposta à terapia hormonal (MENDONÇA, VINHA, ARAÚJO, 1992).

Os receptores encontrados no trato urinário são potencialmente sensíveis à ação estrogênica em todas as fases do ciclo menstrual, em contraste com o endométrio e a vagina, que apresentam expressão cíclica dos receptores (SJOBERG, RYLANDER, VON SCHOULTZ, 1989). Este dado sugere que o trato urinário permanece receptivo à influência hormonal, independentemente dos seus níveis.

Um estudo conduzido por ROBINSON, REGISTER, CARTER (1998) demonstrou que os receptores do trato urogenital de macacos permaneceram sensíveis ao estímulo estrogênico após dois anos de ooforectomia, sugerindo que os efeitos da terapia hormonal no trato urinário pode ser identificada, mesmo transcorridos alguns anos da menopausa.

Os receptores para estrogênio encontram-se distribuídos nos tecidos do trato urinário inferior, que contém epitélio escamoso, incluindo as células transicionais no trígono e na uretra proximal com mudanças metaplásicas, entretanto não foram detectados no epitélio transicional normal (BLAKEMAN, HILTON, BULMER, 2000).

MAKELA *et al.* (2000), investigando o epitélio da bexiga e da uretra em ratas, verificaram que estes locais expressavam receptores do subtipo beta mRNA, enquanto o tecido conjuntivo vesical expressava receptores do subtipo alfa mRNA, dando a entender que a ação hormonal apresenta diferenças de acordo com o tecido analisado.

A caracterização precisa das regiões onde os receptores estão presentes, associada ao conhecimento dos subtipos de receptores, pode auxiliar no entendimento das diferentes manifestações da ação hormonal no trato urogenital.

1.3. CARACTERÍSTICAS ESTRUTURAIS E FUNCIONAIS DAS FIBRAS QUE FORMAM O TECIDO CONJUNTIVO DA BEXIGA

A configuração das fibras que formam o tecido conjuntivo da bexiga é essencial para a manutenção da função vesical (MURAKUMO *et al.*, 1995). Estas fibras estão divididas em dois sistemas: o colagenoso (cujas fibras contêm basicamente colágeno) e o sistema elástico (inclui fibras elásticas, elauninas e oxitalânicas).

Diferentes tipos de fibras do sistema elástico e colagenoso foram descritos nos tecidos e nos órgãos do sistema urogenital (MONTES, 1996). Este sistema apresenta características funcionais e estruturais distintas que se relaciona à distribuição qualitativa e quantitativa variada dos elementos que formam o tecido conjuntivo.

Em determinados sítios anatômicos, o tecido conjuntivo tem função predominantemente mecânica, apresentando uma combinação apropriada de dois atributos principais: a capacidade de resistir a grandes forças de tensão e de compressão, e a propriedade de recuperar a forma e a estrutura, quando estas forças cessam. Assim sendo, é importante entender como seus constituintes químicos – colágenos, glicosaminoglicanos, elastina, sais minerais e água – conferem ao tecido seus atributos mecânicos, ainda que seja difícil determinar as contribuições individuais destes diversos componentes (EWALT *et al.*, 1992).

Se por um lado as fibras que contêm colágeno conferem resistência ao tecido, por outro as do sistema elástico e os proteoglicanos são essenciais na manutenção das propriedades elásticas da matriz. Os componentes do sistema elástico têm sido descritos na matriz extracelular do tecido conjuntivo de tecidos e de órgãos que requerem a habilidade de deformar repetidamente e reversivelmente como a bexiga (KOO *et al.*, 1998).

A organização molecular presente nas fibras elásticas permite que elas se estirem aproximadamente 1,5 vez o seu comprimento em repouso. As características biomecânicas das fibras elásticas indicam que elas são responsáveis pela distensão reversível do tecido conjuntivo, uma vez que exibem deformação elástica e também são capazes de armazenar energia elástica para restaurar à sua configuração original o tecido deformado (GREENLEE, ROSS, HARTMAN, 1966; ROSS, 1973; PANIAGUA *et al.*, 1983).

A elastina é considerada um componente estático da matriz extracelular, apresentando um *turnover* muito lento em tecidos normais (MECHAN, 1981).

As fibras colágenas e elásticas estão distribuídas entre as camadas da parede vesical, formando um padrão de organização que sugere que cada região apresenta diferentes propriedades mecânicas e participa de forma variada na função de enchimento e de esvaziamento vesical.

Na mucosa vesical, o colágeno está arranjado densamente, atuando como suporte para o epitélio transicional. Nesta região, as fibras colágenas são incapazes de se expandirem, proporcionando proteção ao epitélio transicional da superdistensão. As fibras elásticas encontram-se dispostas de forma esparsa na mucosa, sobretudo na parede de grandes vasos e na muscular da lâmina própria (MURAKUMO *et al.*, 1995).

Na camada muscular, o tecido conjuntivo fica em torno dos fascículos, possibilitando que os mesmos se contraiam de forma independente, impedindo superestiramento das fibras musculares. Além disso, as fibras colágenas provavelmente mantêm a localização original dos fascículos independentemente da contração/distensão da parede vesical. Em contraste, as fibras do sistema elástico circundando os fascículos podem prover excelente elasticidade.

Diferente das camadas acima descritas, a serosa consiste de fibras de colágeno arranjadas de forma ondulante, permitindo que a serosa acompanhe de forma coordenada os movimentos da musculatura. As fibras elásticas encontradas nesta região correspondem à lâmina elástica da parede vesical, descrita por MONSON *et al.* (1988). Provavelmente estas fibras não contribuam para a complacência vesical, pois distribuem-se esparsamente, formando uma fina camada entre a musculatura e a serosa.

1.4. PAPEL DO ESTROGÊNIO NO TRATO URINÁRIO INFERIOR FEMININO

A influência dos estrógenos no trato urinário tem sido estudada há vários anos. SALMON, WALTER, GEIST (1941) relataram melhora da incontinência e dos sintomas irritativos urinários em 12 pacientes tratadas com injeções intramusculares de benzoato de estradiol e dipropionato de estradiol.

A mucosa vaginal, uretral e trigonal são sensíveis ao estrogênio e apontam variações de suas estruturas, dependendo dos níveis séricos deste hormônio que promove proliferação da mucosa vaginal e aumento do fechamento uretral, para evitar perdas urinárias (MIODRAG, CASTLEDEN, WALLANCE, 1988).

Os baixos níveis de estrogênio levam a um aumento da incontinência urinária, urgência, polaciúria e infecção urinária de repetição, provocadas por mudanças no PH da uretra e vagina, além de atrofia urogenital (KLTKE & BERGMAN, 1995; WEHBA *et al.*, 1997). MIODRAG *et al.* (1988) demonstraram diminuição no volume das fibras colágenas no tecido conjuntivo parauretral e nos ligamentos do assoalho pélvico, contribuindo para o decréscimo da pressão uretral e fraqueza ligamentar, promovendo incontinência urinária e prolapso uterovaginal nas mulheres pós-menopausa.

HASHIMOTO, ISHIGOOKA, ZERMAN (1999) detectaram, em coelhas castradas, diminuição da densidade do tecido conjuntivo da bexiga. Estes achados divergem dos resultados encontrados por DIEP *et al.* (1998), que, analisando as fibras colágenas da parede vesical em coelhas em relação aos níveis de estrogênio, observaram que a ooforectomia não induziu a modificações na concentração do tecido conjuntivo. Ainda contrariando os achados acima, SUSSET *et al.* (1978) detectaram aumento significativo do conteúdo de colágeno vesical em mulheres acima de 50 anos. Desta forma, verificou-se que a literatura demonstra ampla variabilidade de mudanças estruturais e funcionais induzidas pelo estrogênio, dificultando a caracterização precisa da ação deste hormônio sobre o trato urogenital feminino.

Estudos experimentais em ratas provaram que a ooforectomia promoveu redução do fluxo urinário máximo, aumento da pressão uretral de abertura durante a micção (DIEP *et al.*, 1998) e redução da estimulação colinérgica na musculatura detrusora (DIEP *et al.*, 1999), comprometendo a micção. Os baixos níveis de estrogênio levaram à redução da força contrátil da musculatura estriada do assoalho pélvico, estrutura responsável por um terço da pressão de fechamento uretral. Concomitantemente, houve declínio do tônus muscular liso em decorrência da diminuição da sensibilidade dos receptores alfa-adrenérgicos e da densidade das fibras nervosas na musculatura uretral e vesical (DIEP *et al.*, 1999).

Uma metanálise de 72 artigos mostrou um número pequeno de estudos controlados e randomizados utilizando terapia com estrogênio na incontinência urinária (ZULIO, OLIVA, FALCONI, 1998). Foram encontrados resultados diferentes quando comparadas as respostas objetivas e subjetivas que, segundo os autores, poderiam ser explicados por uma ação estrogênica fugaz e não registrada por parâmetros clínicos e instrumentais ou utilização de metodologia deficiente para coletar os dados subjetivos (BROWN, GRADY, OUSLANDER, 1999).

SARTORI, BARACAT, GIRÃO (1995), em estudo clínico, demonstraram, em um grupo de mulheres no período pós-menopausa, recebendo durante três meses estrogênios conjugados, aumento da capacidade cistométrica máxima e do fluxo médio. Estas pacientes também apresentaram um decréscimo da urina residual e da freqüência miccional diurna e noturna. FANTIL, BUMP, ROBINSON (1996), em estudo semelhante, não constataram diferenças entre os sintomas clínicos de incontinência urinária e qualidade de vida após três meses com estrogenoterapia.

Como as disfunções miccionais ocorrem mais freqüentemente em mulheres idosas e este período resulta em deficiência de estrogênio, a reposição hormonal tem sido caracterizada como medida eficaz no tratamento destes sintomas. Porém, mais estudos serão necessários para esclarecer o papel do estrogênio, pois os dados existentes divergem na indicação e efeitos do hormônio.

1.5. MODELO EXPERIMENTAL

A utilização de ratas ooforectomizadas para estudo da ação dos baixos níveis de estrogênio sobre o trato urinário feminino representa modelo experimental amplamente reconhecido (SAVILLE, 1969; DIEP *et al.*, 1998). Este fato, associado à facilidade de aquisição/alojamento dos animais e à necessidade de um elevado número de ratas para o desenvolvimento desta pesquisa, levou à opção pela utilização destes animais.

O tamanho da amostra foi determinado de acordo com critérios estereológicos, com o propósito de que a análise dos resultados não fosse comprometida por um número insuficiente de animais (HALLY, 1964). A dose, a freqüência e a via de administração da medicação baseou-se em estudos prévios, embora vários esquemas, e com tempo de uso variados, tenham sido encontrados (SARTORI, 1998; DIEP *et al.*, 1999).

O método de escolha para obter a estimativa morfométrica das fibras foi a estereologia, devido a maior sensibilidade do método em relação aos sistemas de avaliação quantitativa através da análise de imagem ou de programas de computador (MATHIEU *et al.*, 1981; COTRIM *et al.*, 1990). A densidade volumétrica é um parâmetro estereológico cujos resultados são confiáveis, em decorrência da pequena variação dos mesmos, independentemente do preparo histológico do material e da experiência do pesquisador (CRUZ-ORIVE & WEIBEL, 1990).

Para a análise das fibras colágenas, foram obtidos cortes de 7μm e foi utilizada coloração com *Sirius red* e luz polarizada, pois este é um procedimento histológico específico para detecção e quantificação de colágeno (MONTES, 1996). As fibras que formam o sistema elástico são avaliadas preferencialmente com cortes de 5μm e coradas com *Weigert's* resorcina-fucsina com prévia oxidação. A oxidação com solução de oxone a 10% é necessária para que todos os componentes do sistema elástico sejam corados e possam ser quantificados (MONTES, 1996).

1.6. JUSTIFICATIVA DO ESTUDO

O período após a menopausa é associado a um aumento da incidência das disfunções do trato urinário inferior (CARDOZO & KELLEHER, 1995). A identificação de receptores estrogênicos no trato urinário tem levado à prática da administração do hormônio no tratamento destes sintomas.

O depósito aumentado de tecido conjuntivo entre os fascículos musculares da parede vesical promove alterações nas propriedades funcionais da bexiga, levando a distúrbios miccionais principalmente em mulheres acima de 50 anos (SUSSET *et al.*, 1978; CARDOZO & KELLEHER, 1995).

Desta forma, é possível supor que os baixos níveis de estrogênio induzam ao aumento da concentração dos componentes do tecido conjuntivo da bexiga, fundamentalmente o colágeno, principal componente, e que a reposição hormonal reverteria este padrão. Contudo, os efeitos dos baixos níveis de estrogênio na estrutura vesical são contraditórios, tornando-se fundamental que hajam mais estudos com o objetivo de elucidar esta hipótese.

2. OBJETIVO

Determinar a influência da ooforectomia e da reposição de estradiol na quantificação das fibras colágenas e no sistema elástico na parede vesical de ratas, através de parâmetros estereológicos.

3. ARTIGO

Campinas, 10th September , 2001.

To,

The Editor-in – Chief : Jay Y. Gillenwater,

The Journal of Urology

The undersigned authors hereby submit the article "**The effect of ovariectomy and estradiol replacement on collagen and elastic fibers in the bladder of rats**" to be evaluated by the journal's board of editors .

The authors confirm that the article has been read and each author's contribution has been approved by the appropriate author. The authors have no substantial direct or indirect commercial financial incentive associated with publishing the article.

The article is original, is not under consideration by any another publication and has not been previously published.

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Best regards,

Míriam Dambros*

Paulo C.R. Palma

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THE EFFECT OF OVARIECTOMY AND ESTRADIOL REPLACEMENT ON COLLAGEN AND ELASTIC FIBERS IN THE BLADDER OF RATS

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Running Title: Ovariectomy effects on bladder extracellular matrix

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ABSTRACT

Purpose: Experiments conducted on animals to investigate the effect of ovarian hormones on bladder structure as well as the effect of estrogen on the extracellular matrix have presented contradictory results. The authors quantified the collagen and elastic fibers in the bladder wall of ovariectomized rats with and without estradiol replacement.

Materials and Methods: This study was conducted on 60 Wistar rats (3 months old). Group 1: remained intact; Group 2: underwent bilateral ovariectomy and were sacrificed after 4 weeks; Group 3: sham operated and sacrificed after 4 weeks; Group 4: underwent bilateral ovariectomy and after 4 weeks daily replacement of 17β-estradiol for 12 weeks; Group 5: sham operated and after 4 weeks daily replacement of sesame oil for 12 weeks. Group 6: underwent bilateral ovariectomy and after 4 weeks daily replacement of sesame oil for 12 weeks. Sirius red and Weigert's resorcin-fuchsin were used to stain collagen and elastic fibers on paraffin rat bladder sections. The M-42 grid system was used to quantitatively analyze the fibers.

RESULTS: Ovariectomy had no effect on the volumetric density and absolute volume of the collagen and elastic fibers in the bladder wall of rats as well as on the weight of the bladder. Estradiol replacement in castrated animals did not demonstrate any significant difference in the stereological parameters when compared to the castrated group without hormonal replacement.

CONCLUSION: Surgical castration performed on rats did not induce any alterations in the quantification of the main elements of the bladder extracellular matrix and estradiol treatment did not have any significant effect on the bladder wall.

Key Words: extracellular matrix; rats; estradiol; ovariectomy; bladder.

INTRODUCTION

The presence of estrogen receptors has been demonstrated in the human lower urinary tract[1]. Estrogen receptors are present in the bladder trigone and urethra[2]. However, the concentration of receptors in the urethra is less than the concentration in the uterus and lesser still in the bladder body. This differentiated distribution of estrogen receptors makes the urethra more sensitive than the bladder to estrogen influence of depletion and/or repletion[3].

The postmenopausal period is associated with a high incidence of symptoms in the lower urinary tract[4]. The main urological bladder symptoms are urge incontinence and recurring urinary tract infections. It is hard to separate the influence of aging from that of menopause when studying the etiology of dysfunction in elderly women. The most significant result of menopause is estrogen deprivation. This fact has led to the use of hormonal replacement in postmenopausal women with lower urinary tract dysfunction. However, the best treatment in terms of type, dosage and method of estrogen administration has still not been defined[5].

Previous investigations performed in animals regarding the influence of ovarian hormones on bladder function and structure presented contradictory results[4,6]. The choice of treatment in these studies varied. Some studies compared only intact controls and intact animals treated with estrogen. Others used ovariectomized animals with and without estrogen treatment, but without intact controls. Moreover, in most of the studies, the animals were castrated and/or treated with hormones for only short periods, and therefore their relevance to long-term hormonal changes that occurred with aging was not clear.

Collagen and elastin are important components of the bladder wall and participate in bladder function. Collagen provides tensile strength, but over accumulation may inhibit bladder contractility and conduction of electrical impulses through the wall[7]. Elastin provides tissue elasticity, which helps in compliance[8]. The collagen content of the detrusor muscle in a postmortem study was found to increase significantly in females above the age of 50 than in younger females or males at same age[9].

Our knowledge on connective tissue alterations in the genitourinary tract and their hormonal regulation potential is limited. Since bladder wall connective tissue is crucial for the mechanical properties of the genitourinary regions, it is important to characterize the possible changes related to estrogen status. We analyzed the effects of ovariectomy and estradiol replacement on the extracellular matrix in the urinary bladder of rats.

MATERIALS AND METHODS

Animals

This study was conducted on 60 female virgin Wistar rats, 3 months old and weighing between 250g - 300g. The animals were maintained in a controlled environment (25 ± 2°C; exposed to daily light cycle for 12 hours) and had *ad libitum* access to water and Purina® ration. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Health Institute (NHI Publication n° 85-23, revised 1985) and the Animal protection Committee of the State University of Campinas approved the protocols.

The animals were randomly divided into six groups and underwent the following procedures: Group 1- remained intact; Group 2 - bilateral ovariectomy and sacrificed after 4 weeks; Group 3 - sham operated and sacrificed after 4 weeks; Group 4 - bilateral ovariectomy and after 4 weeks, subcutaneous injection of 17β - estradiol [10μg/Kg body weight (Galenica Laboratory, São Paulo, Brasil)] for 12 weeks; Group 5 - sham operated and after 4 weeks, subcutaneous sesame oil replacement [0,2ml per day (Galenica Laboratory)] for 12 weeks; Group 6 - bilateral ovariectomy and after 4 weeks, subcutaneous sesame oil replacement (0,2ml per day) for 12 weeks. Ovariectomy was performed using the anesthesia sodium thiopental (40mg/Kg weight) bilaterally via the dorsolumbar region.

Estradiol Dosage

The estradiol serum dosage was measured just before the animals were sacrificed [Veterinary Protocol, (Immunotech Cat # 2464, radioimmunoassay Kit)].

Tissue Sample Collection

An intraperitoneal injection of sodium thiopental was used to sacrifice the animals (60mg/Kg body weight). The bladder was sectioned at the neck and the anterior wall was longitudinally opened from the neck to the bladder fundus. The organ was then weighed using the Scherle method, suspended by a thread and immersed in physiological solution in a vessel on the plate of the scale[10], after which it was spread out on a cork sheet and held down with pins so that a microscopic examination could be performed. The bladders were then immediately fixed in a 10% formaldehyde tamponade (pH=7.2) for 48 hours. Subsequently, the material was dehydrated in a series of increasing concentrations of alcohol solution and diaphanized in xylol. In order to get a cross section of the bladder wall, 5µm and 7µm sections were obtained from the paraffin embedded tissue samples. The 7µm sections were stained with Sirius red and analyzed under a polarized light and the 5µm sections were stained using Weigert's resorcin-fuchsin technique, with and without previous peracetic acid oxidation[8].

Morphometric Quantification

The stereological count of the absolute volume and the volumetric density of the collagen and elastic fibers was determined by superimposing the M-42 grid system on the morphological image[11]. The volumetric density is the relative density occupied by these structures in the tissue and the absolute volume is the total volume of these structures. The stereological method determines three-dimensional quantitative parameters of anatomic structures based on two - dimensional sections. Hence, it is based on Delesse's principle, which states that the relationship between the areas of a determined structure at the surface where the organ was sectioned is the same as the relationship that exists between the volume of the structure and the total volume of the organ[12].

The formula Vv = Pp/Pt x 100% was used to calculate the volumetric density of collagen and elastic fibers, where Vv is volumetric density; Pp is the number of points on the structure being studied (collagen or elastic fibers) and Pt the point test number (42 in this case). The absolute volume was obtained using the formula: V (collagen or elastic fibers) = Vv (collagen or elastic).V (bladder). The V (bladder) was considered as the same as bladder weight W (bladder) because the specific gravity (g) of the saline solution was approximately 1.0048 and the bladder weight was checked using the Scherle method which is V (bladder) = W (bladder)/g.

The quantitative measurements were obtained using the Olympus BX 50 microscope. Quantification was performed at a final magnification of 400X using an M-42 test (Tonbridge®). Ten microscopic fields were randomly analyzed in each group so that the collagen fibers could be analyzed[13]. Quantification of the elastic fibers involved the analysis of 50 random fields in each group because of the small quantity of elastic fibers intermingled with the smooth muscle[13].

Statistical Analysis

As the stereological results presented a discreet variability, the Kruskal-Wallis nonparametric test was used to check the differences between the independent samples (Statistics for Windows. Statsoftt, Inc, 1995). A probability of $p < 0,05$ was taken as the criterion of significance.

The program Origin 5.0 was used for the graphs.

RESULTS

General Characteristics

A quick look at the results showed that there were no major differences regarding the thickness and macroscopy of the control bladders and the ovariectomized bladders with or without estradiol replacement.

All the animals showed an increase in weight during the experiment. The average weight gained was 62mg for all the groups.

Ovariectomy and estradiol treatment had no effect on bladder volume. The Table demonstrates that despite the varied procedure, the volume remained the same for all the groups.

The distribution of the connective tissue components within the bladder wall remained essentially unchanged in all the groups. The collagen fibers were found arranged in all the layers of the bladder wall and were the main components of the extracellular matrix. The sections stained with Weigert's resorcin-fuchsin showed that the elastic fibers were present mainly in the walls of the blood vessels and a smaller number intermingled with smooth muscle fibers and collagen. Therefore it was necessary to analyze a greater number of microscopic fields.

Estradiol Serum

The concentrations just before sacrifice in the ovariectomized groups without estradiol replacement were below 17pg/ml, which is the lower limit of sensitivity of the method applied. In the non-ovariectomized and ovariectomized groups that underwent hormonal replacement, the average hormonal concentration was 76pg/ml (varying between 66pg/ml -87pg/ml).

Stereological Parameters

The Table presents the stereological results. The volume density (Vv) and the absolute volume (V) of the collagen and elastic fibers did not show a significant difference when the 4 week ovariectomized animals (Group 2) were compared with the control group of the same age (Group 3) and when the 16 week ovariectomized animals (Group 6) were compared with their respective control group (Group5).

Estradiol replacement for 12 weeks (Group 4) did not alter the Vv and V of the fibers in comparison with the sham operated and castrated groups of the same age (Groups 5 and 6, respectively).

Figures 1 and 2, respectively, visually demonstrate the Vv collagen and elastic fiber results.

DISCUSSION

Estrogen receptors have been detected in the urethra, bladder tissues and in the pelvic floor muscles of animals[14] and humans[2]. These observations have led to the hypothesis that hypoestrogenism plays a role in urethral and bladder dysfunctions. Evidence has been found of the direct influence of estrogen on bladder function and structure[1]. However the results obtained are contradictory and the hormonal effect is more evident in the urethra[3].

The extracellular matrix is made up of fibrous and amorphous components. The fibers are of two types: collagen fibers and elastic fibers. Collagen is a source of tensile strength for the tissues, while elastin is essential for matrix resiliency[8]. It has been suggested that the extracellular bladder matrix has an effect on the passive properties of the bladder wall and that the collagen and elastic fibers, probably, also play an important role in intercellular active force transmission[7]. Therefore, a change in the extracellular matrix concentration might, also affect the contractile properties of the smooth muscle. The most common collagen types found in the bladder are: type I, which supplies resistance against tension and type III responsible for structural maintenance of the expansible organs[8]. It has been assumed that loss of compliance is due to bladder fibrosis resulting from excessive or abnormal deposit of collagen[8]. The effect of estrogen levels on the extracellular matrix is still not clear. While some studies show an increased deposit of collagen fiber among the bladder muscle fascicles[1], others associate the postmenopausal period to an increased collagen turnover resulting in reduction[15].

The purpose of this study is to contribute information on the behavior of the bladder connective tissue in relation to estrogen levels. The experimental model used in this study was previously used in studies concerning the influence of sexual hormones on the lower urinary tract in rats[16], but in this study the animals were deprived of hormones for long periods because some studies demonstrated a change in the bladder function and structure only 4 weeks after castration[17]. Estradiol replacement was maintained for 12 weeks so that any transitory reactions to low hormonal levels did not influence the final assessment of the stereological parameters, although some studies suggest that after five days of estradiol replacement, modifications occur in bladder function[16]. The dose, frequency and method of administering the medication were based on previous studies, although various schemes and periods of use are available[1,16].

The results of our study demonstrate that ovariectomy in rats did not affect the concentration of collagen and elastic fibers in the bladder wall.

Stereology was chosen to obtain these morphometric fiber estimates. Point counting is the best method for estimating the volumetric density of a cell type within a tissue[18]. The use of an automatic image analyzer or of a computer assisted tracing system is very rarely an advantage[19]. The Sirius red polarization method was used to evaluate the collagen fibers because it is a specific histochemical procedure for the detection of collagen in tissue sections[8], while the Weigert's resorcin-fuchsin oxidation method is more sensitive to elastic fibers[8]. The size of the sample was in accordance with the stereological criteria so that an insufficient number of animals did not affect the result analysis[13]. Therefore, we do not believe that the absence of statistical significance in our study is due to the experimental method adopted or the inadequate size of the sample.

The use of estradiol did not affect the quantitative behavior of the main elements of the connective tissue in castrated animals. It was observed that the animals that remained under the influence of low hormonal levels for long periods presented characteristics that were statistically similar to the pattern of collagen and elastic fibers deposited in the group of rats exposed to normal estradiol levels. Hence, there was no relationship found between estrogen serum levels and bladder extracellular matrix in rats.

A wide variety of estrogen induced structural changes are reported in literature. A hypothesis that could explain these changes is that the methodologies used as well as the multiple tissues investigated for the effect of estrogen are not standardized. A recent study on rats showed that epithelium of the bladder and the urethra express subtype beta mRNA receptors while the connective tissue in the bladder expresses sub-type alpha mRNA receptors[14]. Therefore, the hormone may present differing actions according to the tissue being analyzed and the animal species being studied. A study on rabbits analyzed the collagen fibers in the bladder wall regarding the estrogenic status and observed that ovariectomy had no effect on collagen tissue concentrations[4]. A similar research on postmenopausal women detected that estrogen replacement therapy resulted in a lower concentration of collagen in the paraurethral connective tissue[20]. A study on rabbits detected a significant association between low estrogen levels and the response pattern of the detrusor muscle to nerve reactions, electric stimulus and a variety of medication but alterations in the mechanical properties or structural composition of the bladder did not occur[4]. It has been suggested that the effect of estrogens on the lower urinary tract may be mediated by a variety of alterations involving neurotransmitters and modifications in the hormonal receptor response in virtue of the low levels of circulating estrogen.

Therefore the discrepancy between the clinical and experimental study results and the evidences that demonstrate a variable estrogenic action in accordance with the organ and animal being studied demand extreme caution when extrapolating research results with regard to the human being.

CONCLUSION

The conclusion reached is that estrogen depletion had no effect on collagen and elastic fiber concentration in the bladder wall of rats. Estrogen treatment did not influence any of the parameters studied.

As clinical and experimental studies show a discrepancy in results concerning the influence of estrogen on the lower urinary tract, there is reason to believe that changes associated with decreased estrogen levels occurring after menopause have a subtle effect on the urinary bladder. However, in order to prove this hypothesis, there is a need for clinical and laboratory experimentation that include functional and structural parameters symptoms of the lower urinary tract, objective status documentation and appropriate determination of the outcome.

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Table - Descriptive Stereological Parameter Statistics of the extracellular matrix in the bladder wall of rats.

Groups	Vv (collagen fibers) %		Vv (elastic system) %		V (bladder) mm3		V (collagen fibers) mm3		V (elastic system) mm3	
	Median	CI 95%	Median	CI 95%	Median	CI 95%	Median	CI 95%	Median	CI 95%
1	44,04	35,30 - 55,63	4,32	3,71 - 4,80	62,00	58,41 - 67,58	27,56	21,35 - 36,44	2,70	2,43 - 3,01
2	42,85	35,64 - 52,44	4,81	4,38 - 5,12	77,00	70,77 - 80,62	31,76	26,14 - 40,91	3,12	2,90 - 3,41
3	44,04	37,03 - 52,00	4,99	4,52 - 5,38	67,50	60,23 - 71,76	29,94	23,39 - 36,34	2,82	2,50 - 2,98
4	39,28	34,51 - 51,19	5,02	4,79 - 5,33	71,50	58,68 - 76,31	28,36	21,89 - 32,40	2,69	2,29 - 3,03
5	40,47	36,89 - 48,34	4,48	4,21 - 4,78	61,50	55,68 - 64,71	23,17	20,15 - 28,30	2,23	3,98 - 2,48
6	40,12	31,97 - 51,34	4,73	4,49 - 4,91	67,50	62,88 - 84,11	28,88	21,85 - 32,41	2,79	2,38 - 3,02
p value	0,24		0,09		0,23		0,12		0,32	

Vv: volumetric density; V: volume; CI 95%: confidence intervals; p: probability (Kruskal-Wallis test)

Figure 1 – Volumetric densities of collagen fibers in the bladder wall of rats as determined on sections stained with Sirius red. Values represent medians and quartiles of 10 rats in each group (p = 0,24).

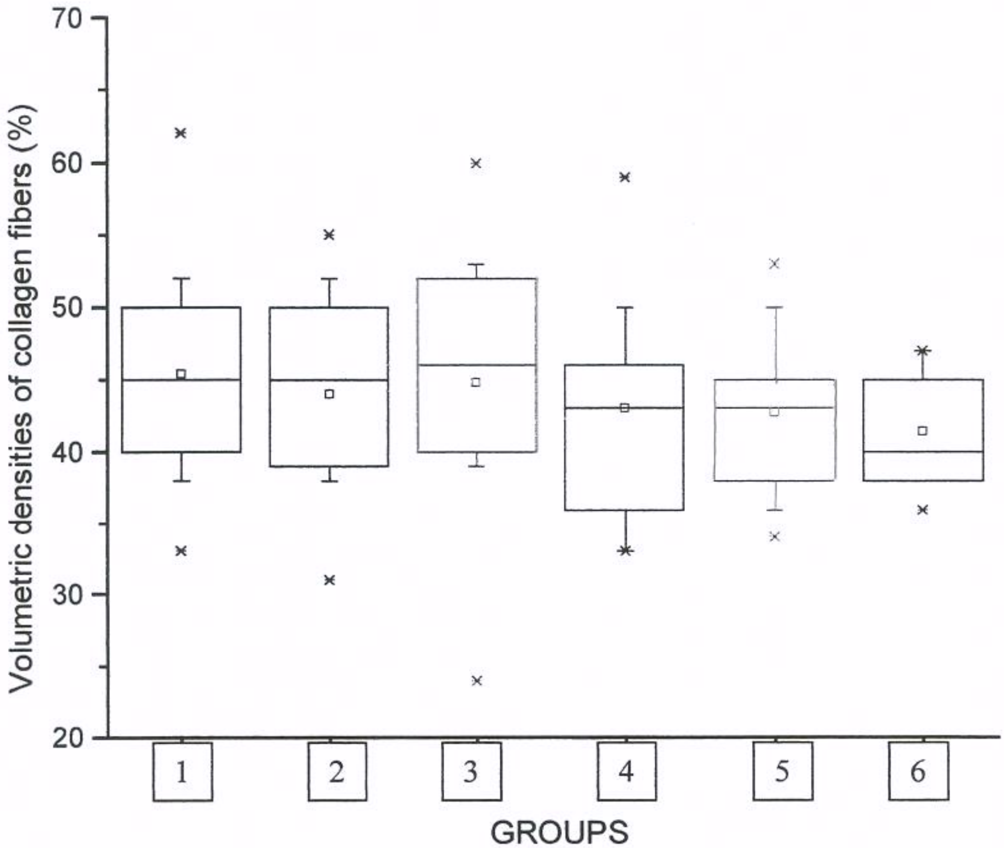
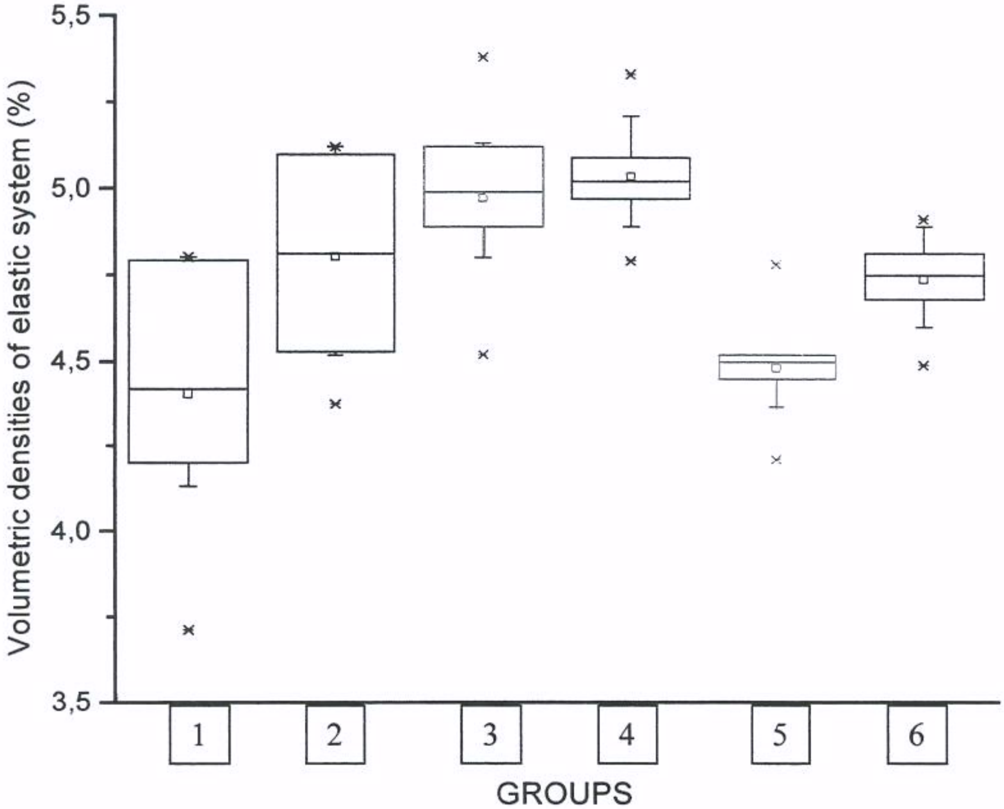


Figure 2 – Volumetric densities of elastic fibers in the bladder wall of rats as determined on sections stained with Weigert's resorcin-fuchsin. Values represent medians and quartiles of 10 rats in each group (p = 0,09).



4. CONCLUSÕES

A ooforectomia bilateral, em ratas, não induziu a alterações no volume das fibras colágenas e no sistema elástico da parede vesical.

A administração de estradiol no grupo castrado não apresentou diferenças significativas nos parâmetros estereológicos analisados, em relação aos animais castrados sem reposição.

5. SUMMARY

Collagen and elastic fibers, which form the major components of the connective tissue, are present throughout the urinary bladder wall and are closely related to bladder compliance. Previous studies have focused mainly on the structural and quantitative alterations of the extracellular matrix fibers of the muscle layer related to conditions like aging and hormonal status. Experiments performed on animals to investigate the effect of ovarian hormones on bladder structure obtained contradictory results regarding the effect of estrogen on connective tissue.

We carried out quantitative morphometric analysis of rat bladders to investigate the possible effects of ovariectomy and successive estrogen replacement therapy on bladder wall structure.

Sixty female Wistar rats (3 month old) were divided into six groups: Group 1- remained intact; Group 2 - bilateral ovariectomy; Group 3 - sham operated; Group 4 - bilateral ovariectomy followed by subcutaneous injection of 17β-estradiol (10mg/Kg per day for 12 weeks); Group 5 - sham operated followed by sesame oil replacement (0,2ml per day, s.c, for 12 weeks); Group 6 - bilateral ovariectomy followed by sesame oil replacement (0,2ml per day, s.c, for 12 weeks). All the rats rested for 4 weeks following ovariectomy. Collagen and elastic fibers in rat bladder paraffin sections were stained using Sirius red and Weigert's resorcin-fuchsin, respectively. The M-42 test grid system was used to determine the absolute volume and volumetric density of the fibers on the paraffin sections. The Kruskal-Wallis nonparametric test was used to test the differences in the stereological findings of the six groups. A probability of $p < 0,05$ was used as the criterion of significance.

A quick examination showed that there were no major differences in the thickness and macroscopy of the control bladders and ovariectomized bladders with or without estradiol replacement. Ovariectomy as well as ovariectomy followed by estradiol treatment had no effect on bladder volume. A comparison of the stereological findings of the four groups showed no significant differences in the absolute volume ($p= 0,12$) and volumetric density ($p=0,24$) of the collagen fibers. Analysis of the elastic system showed that there were no significant differences in the absolute volume ($p=0,32$) and volumetric density ($p=0,09$) of the groups.

Long-term estrogen deprivation did not have a significant influence on extracellular matrix of the bladder. Ovariectomy and successive estrogen supplementation did not affect the collagen and elastic fiber concentration in the bladder wall of rats.

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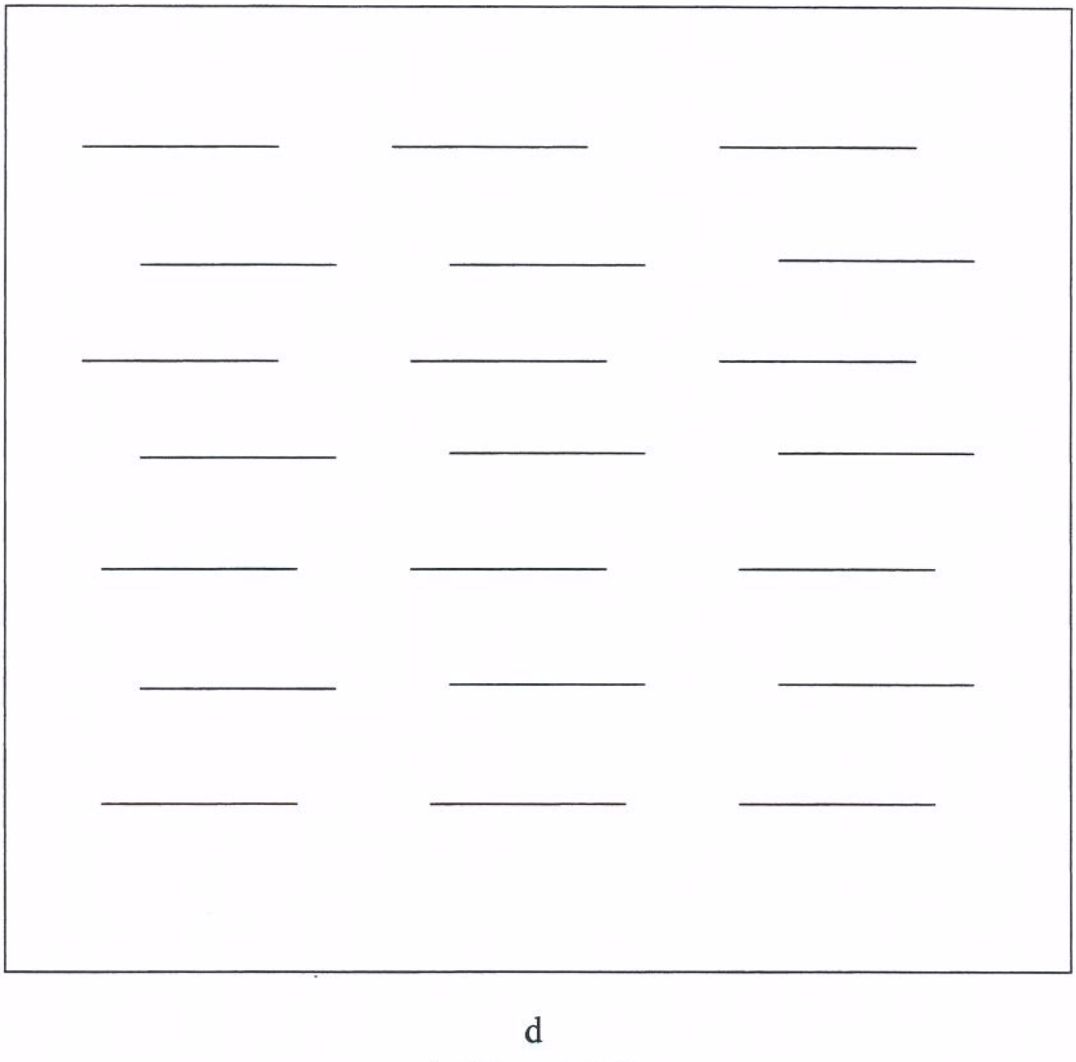
7. APÊNDICES

SISTEMA TESTE M-42



Desenho de construção geométrica do sistema teste M-42 com base em WEIBEL *et al.* (1966). O valor de "d" é estabelecido pelo pesquisador de acordo com o microscópico e aumento utilizados para análise histológica.

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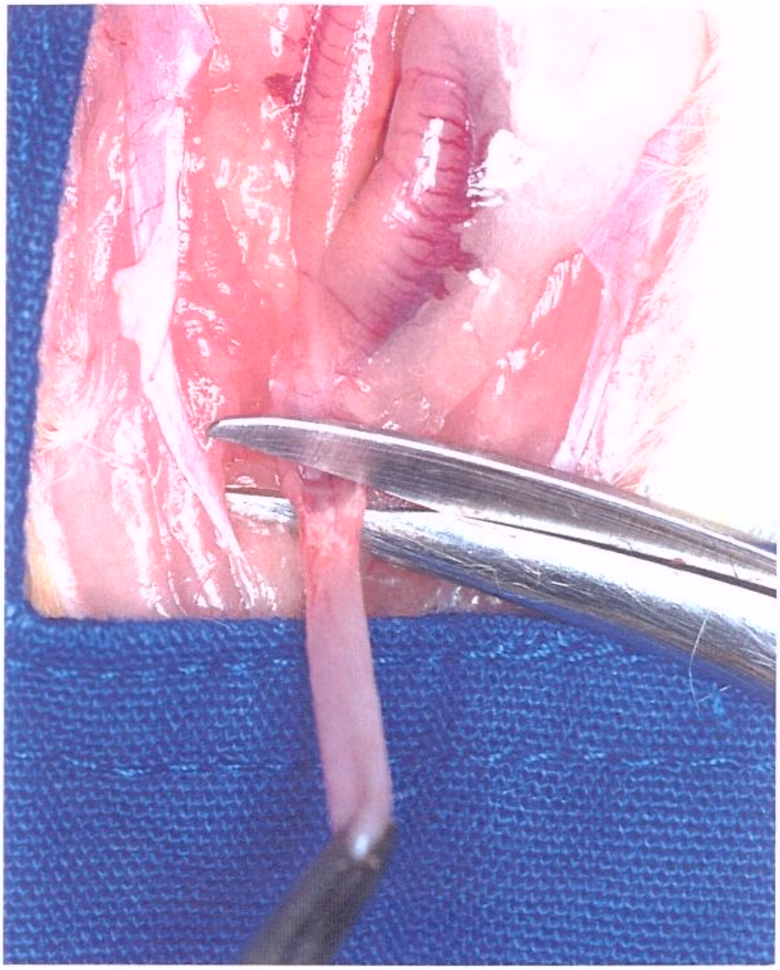

Figura 1: Exérese da bexiga ao nível do colo vesical.

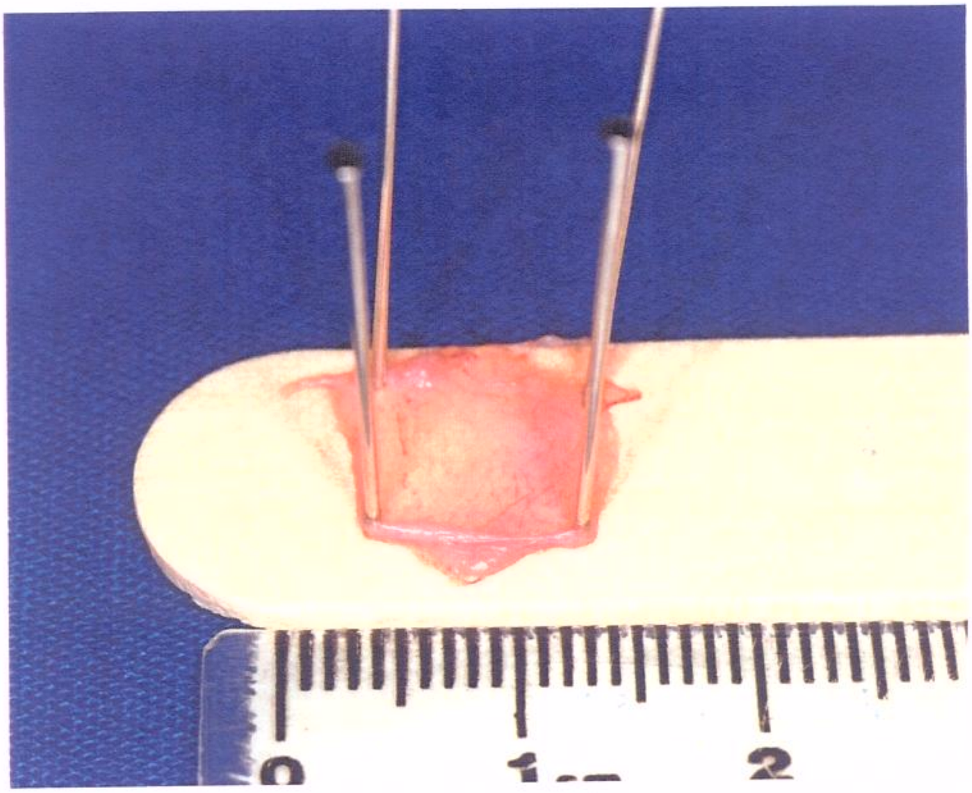

Figura 2: A bexiga foi estendida sobre uma espátula de madeira e presa com alfinetes, para facilitar a fixação em formalina a 10%.

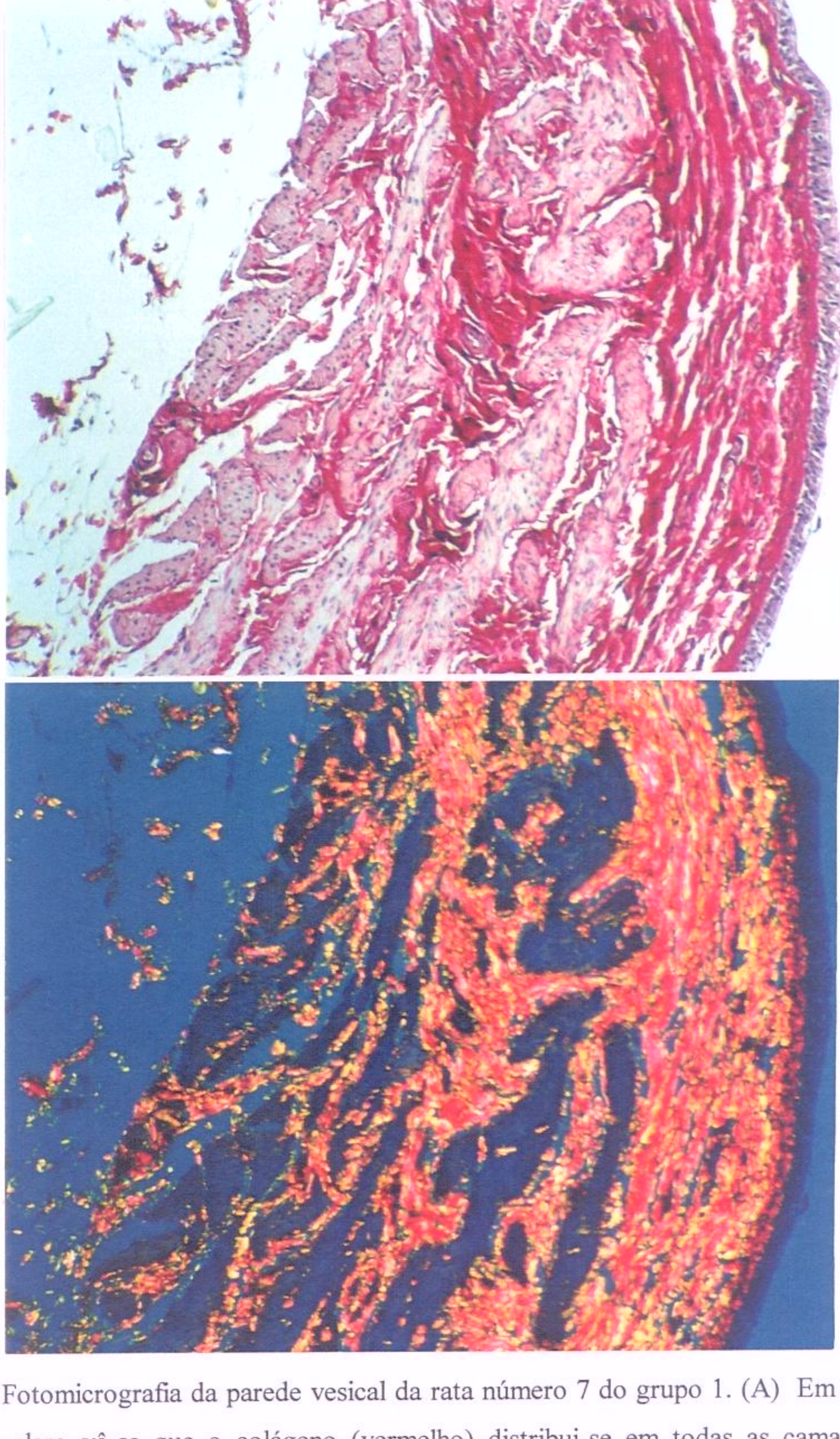

Figura 3: Fotomicrografia da parede vesical da rata número 7 do grupo 1. (A) Em campo claro vê-se que o colágeno (vermelho) distribui-se em todas as camadas da bexiga. (B) Em campo escuro (luz polarizada) vê-se o mesmo campo da figura anterior, porém a birrefringência do colágeno permite a identificação mais precisa das fibras (*Sirius red*, 350 vezes).

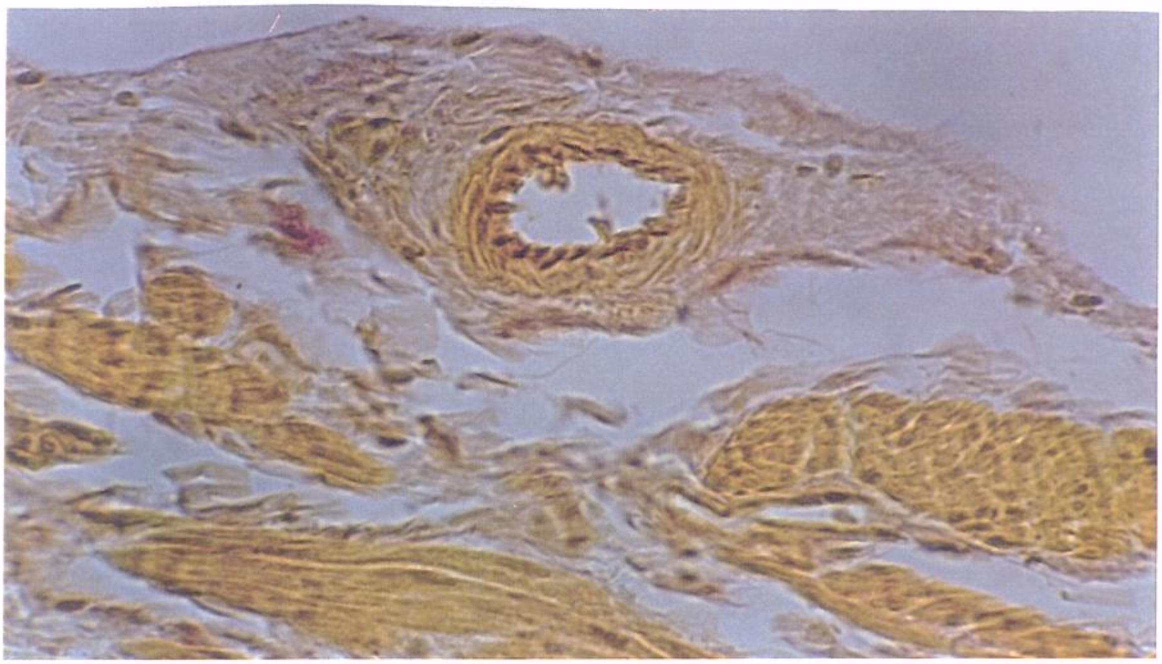

Figura 4: Sistema elástico da parede vesical da rata número 5 do grupo 1. Observa-se poucas fibras (verde acastanhado) dispostas principalmente na parede do vaso sangüíneo. (*Weigert'* resorcina-fucsina, 700 vezes).