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FACULDADE DE CIÊNCIAS MÉDICAS

FÁBIO THADEU FERREIRA

EFEITOS DA REPOSIÇÃO DE TESTOSTERONA NO REMODELAMENTO PENIANO RELACIONADO AO ENVELHECIMENTO

Effects of Testosterone Supplementation on Age-Related Penile Remodeling

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Tese de Doutorado apresentada do Programa de Cirurgia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas - UNICAMP como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciências.

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Orientadora: Profa. Dra. Miriam Dambros Lorenzetti

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Dedico este trabalho...

à minha querida filha Milena, que me lembra o porquê da vida valer a pena;

à Camila, companheira de todas as horas, desde meus primeiros passos;

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RESUMO

Introdução: A disfunção erétil é presente em 46,2% dos homens entre 40 e 70 anos de idade. Vários fatores já foram apontados como causa da disfunção erétil masculina, incluindo as alterações neurológicas, vasculares arteriogênicas, vasculares venogênicas, hormonais e psicológicas. Além disso, a incidência da DE é mais acentuada conforme aumenta a idade dos indivíduos, podendo chegar a 60-70% na sétima década de vida. A disfunção erétil (DE) deve ser encarada como uma doença multifatorial e seu tratamento deve ser realizado da mesma forma. Alguns estudos demonstram uma substituição no músculo detrusor por tecido colágeno devido a privação de testosterona; é natural que se faça tal analogia também para trato genital masculino. Observa-se uma clara associação entre o envelhecimento masculino, o decréscimo de testosterona sérica e a disfunção erétil, porém existem raros estudos na que avaliem a estrutura histológica do pênis em tal situação. **Objetivos:** Este estudo tem como objetivo avaliar os efeitos da reposição de testosterona sobre a fibrose dos corpos cavernosos de pênis de ratos senis. **Metodologia:** 16 ratos Wistar idosos foram divididos em 2 grupos: Tratamento (receberam suplementação padronizada de testosterona - 50mg/Kg) e controle (receberam dose de solução salina equivalente). Testosterona foi dosada no D0 e D56 do estudo. Os pênis dos ratos foram preparados em parafina e suas lâminas coradas com picrosirius; estereologia foi aplicada para determinar a densidade volumétrica das fibras colágenas. **Resultados:** Os testes de ANOVA demonstraram que a reposição de testosterona foi efetiva, enquanto a privação androgênica se manteve no grupo controle ($p < 0,01$). O grupo com reposição de testosterona obteve uma densidade volumétrica (Vv) de 20,6%, menor que o do controle (47,8%); Teste-t e Kruskal-Wallis ($p < 0,001$). A correlação de Pearson demonstrou uma inversa relação entre os níveis de testosterona e Vv ($p < 0,001$). **Conclusões:** Este é um dos estudos pioneiros na demonstração de alterações estruturais sobre a musculatura do corpo cavernoso causadas pela deprivação de testosterona em ratos idosos. Estes achados implicam que os níveis de testosterona podem influenciar, não só a libido, mas também, na função erétil.

Palavras–Chave: Envelhecimento, Disfunção Erétil, Fibrose, Testosterona

ABSTRACT

Introduction: *Erectile dysfunction (ED) develops among 46,2% of men between 40-70 years. Many factor are implicated on the male ED, including neurology alterations, artheriogenic vascular pathologies, and venogenics vascular pathologies, hormonal and physiologic. Besides, the incidence of ED is more common with the aging, reaching over 60-70% on the 7th decade of life. ED must be faced as one multifactorial disease and its treatment must be performed as such. Some studies demonstrated detrusor muscle substitution by collagens fibers due to testosterone deprivation; it is natural to make similar analogy to the male genial tract. There is a clear association between aging, lower levels of testosterone and ED, however, there are few studies evaluating the histologic penile structure on this situation.* ***Objetives:*** *This study aims to evaluate the effects of testosterone supplementation over the corpora corpus fibroses in pênis of elderly rats.* ***Methodology:*** *16 senescent Wistar rats were divided in 2 groups: treatment (receiving standard supplementation testosterone dose -* 50mg/Kg*) and control (receiving equivalent saline solution). Testosterone was dosed on D0 and D56 of study. All penises were prepared with picrosirius colored histology; stereology was applied to determine the volumetric density of collagen fibers (Vv).* ***Results:*** *ANOVA demonstrated testosterone group´s replacement therapy was effective, while the androgenic decline continued by the time of experiment in control group ($p<0.05$). Testosterone group had Vv of 20,6%, lower than control group (47,8%); t-test and Kruskal Wallis test ($p<0.001$). Pearson's correlation demonstrated an inverse correlation between the Vv and testosterone's levels ($p<0,001$).* ***Conclusions:*** *This is a pioneer study on demonstration of structural alterations over the cavernous corpora muscle caused by deprivation of testosterone on elderly rat. These finding implicate that the testosterone levels can influence, not only the libido, but also the erectile function.*

Keywords: Aging, Erectile Dysfunction, Fibrosis, Testosterone

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LISTA DE ABREVIAÇÕES E SIGLAS

AII – Angiotensina II

ANOVA – Análise de Variância

ART – Artéria

ATPase – Adenosina Tri-Fosfatase

CEUA – Comitê de Ética no Uso de Animais

COBEA – Colégio Brasileiro de Experimentação Animal

DAEM – Distúrbio Androgênico do Envelhecimento Masculino

DE – Disfunção Erétil

DHT – Dihydrotestosterone

DM2 – Diabetes Mellitus Tipo 2

ED – Erectile Dysfunction

g - grama

HAS – Hipertensão Arterial Sistêmica

mcm - micrômetro

mmHg – Milímetros de Mercúrio

Músc – Músculo

n - número amostral

NAD – Nicotinamida Dinucleotideo

NADH – Nicotinamida Dinucleotídeo Reduzida

NO – Óxido Nítrico

Nv – Densidade numérica

O_2 – Oxigênio

p – nível de significância

PDE-5 – Fosfodiesterase-5

PDE-5i – Inibidor de Fosfodiesterase-5

$PGF_{2\alpha}$ – Fator de Crescimento Plaquetário 2α

Pp – Pontos positivos

Ps – Pontos Possíveis

TGU – Trato Gênito-Urinário

TU – Undecanoato de Testosterona

LISTA DE ABREVIAÇÕES E SIGLAS

USA – United States of America

Vv – Densidade Volumétrica / Volumetric Density

Vol – Média volumétrica

“Uma paixão forte por qualquer objeto assegurará o sucesso, porque o desejo pelo objetivo mostrará os meios”

(William Hazlitt)

SUMÁRIO

Epidemiologia da Disfunção Erétil

É de senso comum que as populações do mundo enfrentam um processo, talvez irreversível, de envelhecimento. Além de todos os desafios sociais conhecidos, enfrenta-se uma enorme gama de doenças, cada vez mais prevalentes, associadas à idade. As patologias do Trato Gênito-Urinário (TGU) também se encontram nesta situação. Cerca de 10-20% dos homens com mais de 40 anos, atualmente, são diagnosticados com alguma patologia urológica (Abdo, 2006; Lue, 2012). Nesta população masculina, o processo de envelhecimento está intimamente associado ao declínio da produção endógena de testosterona e/ou de sua ação inadequada em receptores específicos nos diversos órgãos. Tal situação se expressa no Distúrbio Androgênico do Envelhecimento Masculino (DAEM), que além de sintomas pouco específicos como disforia, ondas de calor, alteração de sono e apetite, alteração do padrão de crescimento de pelos faciais e corporais; tem nos sintomas sexuais, tanto a diminuição de libido como a disfunção erétil, aqueles que mais incomodam os pacientes (Morales, 2012).

A disfunção erétil (DE) figura entre as patologias que mais transtornos causam na saúde masculina, não somente no bem-estar físico e biológico, mas também em seus aspectos sociais e afetivos. Segundo os últimos levantamentos realizados, cerca de 20% dos homens com mais de 20 anos enfrentará sintomas relacionados à disfunção erétil alguma vez na vida, com uma incidência potencial de 325 milhões de homens em 2025 (Lue, 2012). No Brasil, estima-se uma incidência de 46,2% entre os homens com 40 a 70 anos de idade (Abdo, 2006).

Dados atuais mostram uma relação positiva entre DE e outras patologias, como Diabetes Mellitus tipo 2 (DM2), obesidade, doença cardiovascular, dislipidemia, depressão e hiperplasia benigna da próstata. Esta associação corrobora a premissa de existir uma doença endotelial, oclusão arterial e/ou inflamação sistêmica que leva a DE (Glina, 2002; Kue, 2012). Observa-se ainda, que os medicamentos da classe dos inibidores de fosfodiesterase-5 (PDE-5), desenhados para o tratamento deste mal, estão entre os mais vendidos no mundo, nas últimas duas décadas.

Vários fatores já foram apontados como causa da disfunção erétil masculina, incluindo as alterações *neurológicas* (acidentes vasculares cerebrais, trauma raquimedular, Doença de Parkinson, Doença de Alzheimer, etc.), *vasculares arteriogênicas* (tabagismo crônico etc.), *vasculares venogênicas* (doenças veno-oclusivas parciais ou totais), *hormonais* (diabetes mellitus, hipotireoidismo, hiperprolactinemia, etc.) e *psicológicas* (transtornos de ansiedade, depressão, hiperatividade, etc.) (Martini, 2012). Além disso, a incidência da DE é mais acentuada conforme aumenta a idade do grupo estudado, podendo chegar a 60-70% na sétima década de vida (Lue, 2012; Morales, 2012). Atualmente, a DE é encarada como uma doença multifatorial e que seu tratamento deve ser realizado da mesma forma.

Fisiologia da Ereção

A ereção ocorre por meio de uma fina sincronia endócrina, vascular e nervosa, que em última instância, preenche os corpos cavernosos com sague e o mantém represado até o final do processo.

Anatomicamente, o pênis é composto por três estruturas cilíndricas: um par de corpos cavernosos e um corpo esponjoso (que abriga a uretra), recobertos por uma camada de tecido conjuntivo e pele **(Figura 1)**. Os corpos cavernosos são revestidos por tecido conjuntivo especial conhecido como Túnica Albugínea, que se inserem nos ramos púbicos da pelve e estendem-se até a glande peniana, e tem como principal característica seu grande potencial elástico, graças sua composição de várias camadas de fibras elásticas entremeadas por uma trama de colágeno. Internamente, essa trama de tecido conjuntivo, forma um espaço irregular, composto pelos pilares intracavernosos, que se inundam com sangue proveniente das artérias cavernosas e ramos das artérias dorsais, fazendo com que o pênis se enrijeça **(Figura 2)** (Lue, 2012).

Ainda participam de sua estrutura fibras de tecido muscular liso, que possuem ação ativa no mecanismo de ereção. O corpo esponjoso e a glande peniana possuem características estruturais similares aos corpos cavernosos, exceto pelos espaços sinusoidais internos mais largos, desprovidos de estrato cortical e um tecido de revestimento mais fino e delicado, não participando ativamente no processo de ereção, mas importantes para a proteção uretral e sensibilidade peniana.

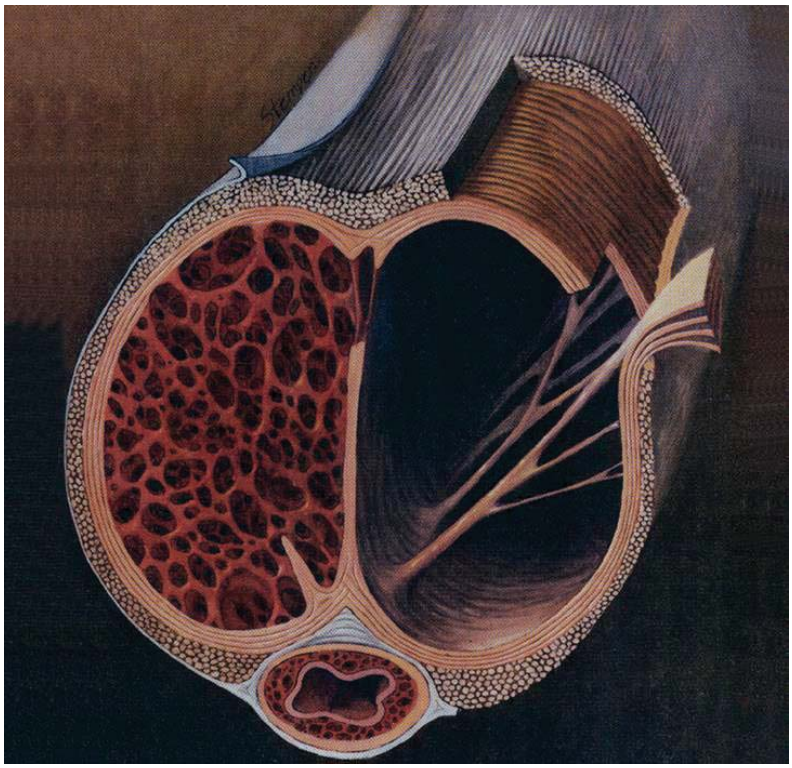


Figura 1 – Estrutura anatômica macroscópica do pênis. (Fonte: Urology, Campbell-Walsh, 10th Edition, Souders-Elsevier, 2012)

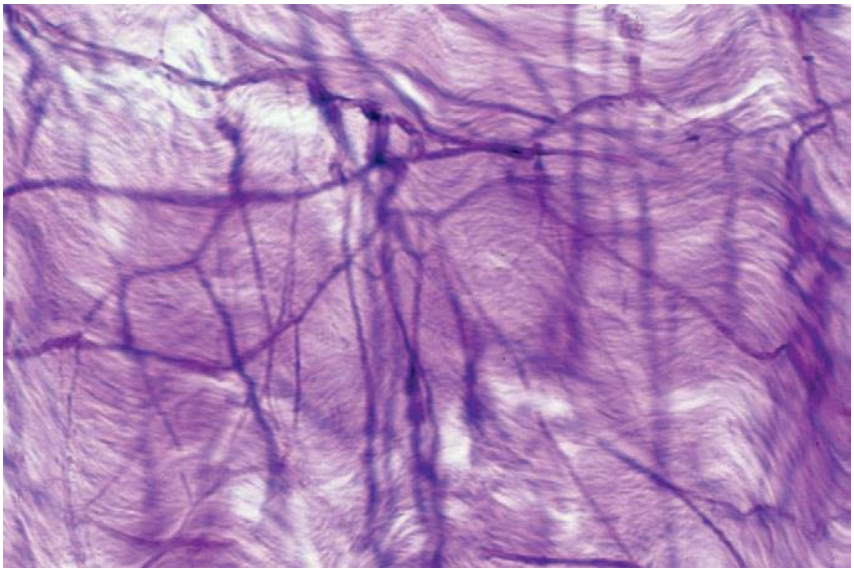


Figura 2 – Estrutura anatômica microscópica na túnica albugínea, demonstrando sua estrutura de fibras colágenas. Aumento de 100x (Fonte: Urology, Campbell-Walsh, 10th Edition, Souders-Elsevier, 2012)

A fonte de suprimento arterial para o pênis é derivada da artéria pudenda interna (ramo da artéria ilíaca interna), entretanto, variações são possível e ramos das artérias ilíacas externas, obturatória, vesical e femoral, podem participar da irrigação peniana **(Figura 3).** Já a drenagem venosa se origina de pequenas vênulas imediatamente abaixo da túnica albugínea, compondo um plexo venoso subtunical antes de tributarem nas veias emissárias. Fora da túnica albugínea, o sangue pode percorrer diversos caminhos **(Figura 4)** (Netter, 2000; Lue, 2012).

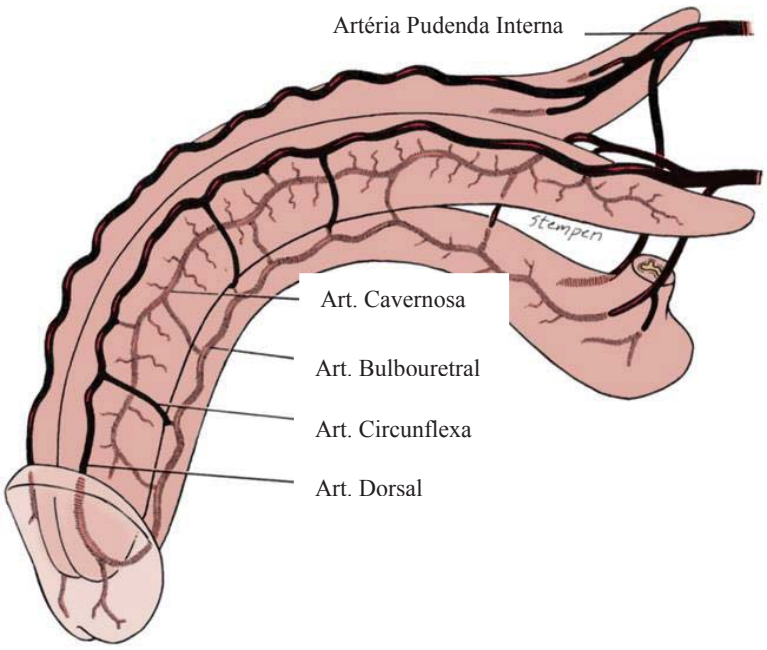


Figura 3. Irrigação Arterial do Pênis (Fonte: Urology, Campbell-Walsh, 10th Edition, Souders-Elsevier, 2012)

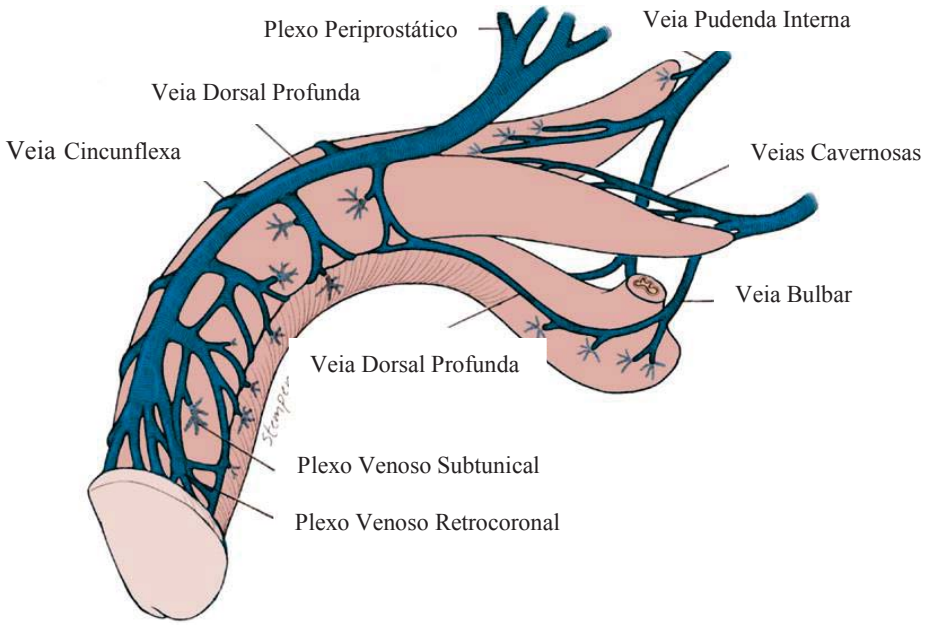


Figura 4. Drenagem venosa peniana (Fonte: Urology, Campbell-Walsh, 10th Edition, Souders-Elsevier, 2012)

As estruturas anatômicas do genital masculino, bem como suas funções no mecanismo da ereção estão descritos na **Tabela1**.

Tabela 1. Componentes Penianos e suas Funções na Ereção

Corpo Cavernoso	Suporte do corpo esponjoso e glande Estrutura erétil – presença de sinusóides
Túnica Albugínea	Protege a estrutura erétil e participa dos mecanismos veno-oclusivo
Musculatura Lisa	Regula o fluxo de sangue para os sinusóides
Músc. Isquiocavernoso	Bombeia o sangue em sentido distal para prolongar a manter a ereção
Músc. Bulbocavernoso	Comprime o bulbo para emitir o sêmen
Corpo Esponjoso	Pressiona e contrai a uretra para emitir o sêmen
Glande	Coxim protetor, porção mais sensível e facilita e penetração durante o ato sexual

Fisiologicamente, o pênis quando em estado flácido tem as fibras musculares lisas dos corpos cavernosos em contração tônica, permitindo um fluxo sanguíneo arterial suficiente apenas para a perfusão e nutrição do tecido peniano. O estímulo sexual desencadeia a liberação de uma série de neurotransmissores que levam a um relaxamento desta musculatura lisa que culmina com uma dilatação das arteríolas e artérias, levando ao aumento do fluxo sanguíneo, tanto na fase sistólica como na diastólica; o aprisionamento do sangue afluído através da expansão dos sinusóides; compressão do plexo subtunical venoso, reduzindo o refluxo venoso; o estiramento da túnica albugínea ao máximo, ocluindo as veias emissárias e reduzindo ainda mais a evasão do sangue venoso; um aumento da pressão de oxigênio (O_2) (cerca de 90mmHg) e aumento da pressão intracavernosa (cerca de 100mmHg), levando o pênis ao estado de rigidez; um maior aumento da pressão peniana devido a contração dos músculos isquiocavernosos (fase rígido-erétil). Após a fase de ereção e ejaculação, o pênis entra no estágio conhecido como detumescência, em que os processos retornam ao estado inicial, levando a fuga venosa e um estado de flacidez peniana (**Figura 5)** (Lue, 2012).

A atividade contrátil espontânea da musculatura lisa peniana ocorre devido ao aumento do cálcio intracelular e à alteração do balanço de Nicotinamida Dinucleotídeo Reduzida/Nicotinamida Dinucleotídeo (NADH/NAD). Há atuação da enzima Adenosina Tri-Fosfatase (ATPase) na fase de contração e durante a fase refratária ou de flacidez há a liberação endotelial de Fator de Crescimento Plaquetário 2α ($PGF_{2\alpha}$) (Lue, 2012).'

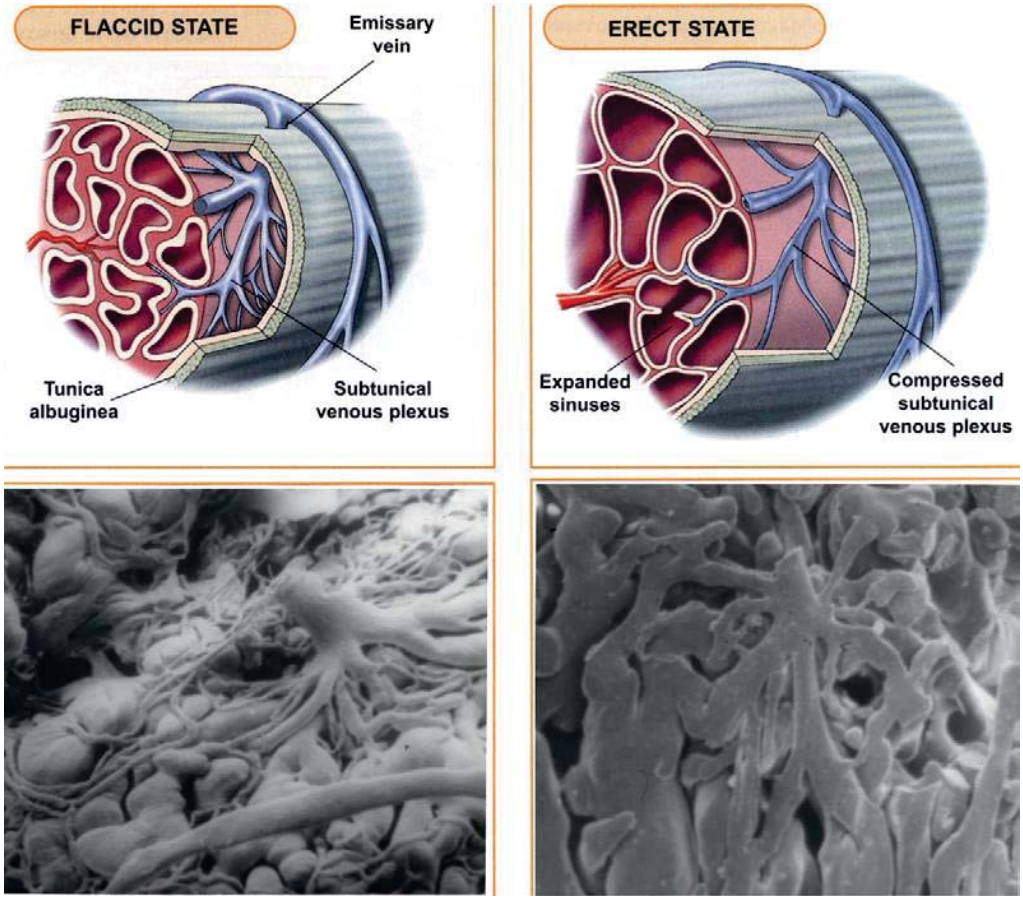


Figura 5 – Mecanismos anatômicos do processo de ereção peniana. Microscopia eletrônica (Fonte: Urology, Campbell-Walsh, 10th Edition, Souders-Elsevier, 2012)

Distúrbio Androgênico do Envelhecimento Masculino (DAEM)

O DAEM, decorrente do decréscimo na produção endógena de testosterona e/ou na resistência tecidual a sua ação, é uma das causas mais recorrentes de disfunção erétil, especialmente na população idosa, com prevalência de 12,3/1.000 homens entre 40 e 69 anos (Feldman, 1994). Esta patologia refere-se às alterações físicas, psíquicas, cognitivas e sociais relacionadas à queda dos níveis dos hormônios androgênicos em decorrência do envelhecimento, com forte impacto na qualidade de vida do idoso. Ressalta-

se que estas alterações não são universais entre os homens e ainda não são completamente entendidas.

O conjunto de sinais e sintomas relacionados à diminuição dos níveis de testosterona é bastante variado, tais como fogachos, diminuição de pelos, queda de libido, etc., e corresponde diretamente às suas inúmeras funções ativas ou de manutenção no corpo (Abdo, 2006). O declínio androgênico parece permear direta ou indiretamente vários processos que definem o envelhecimento, sendo um denominador comum das doenças relacionadas à idade no homem, como por exemplo: a obesidade, disfunção erétil e alterações miccionais (Morales, 2012).

O processo de envelhecimento pode trazer consigo uma série de patologias que, em maior ou menor grau, influenciam no surgimento da disfunção erétil. O DM2, hipertensão arterial (HAS), Hipertrigliceridemia, Dislipidemias (agrupadas no que se conhece como Síndrome Metabólica) são os principais fatores de risco estudados (Glina, 2002). A obesidade, em toda sua complexidade fisiopatológica, funciona como um fator de risco isolado para o surgimento de hipoandrogenismo. Os intricados mecanismos hormonais associados às respostas inflamatórias exacerbadas que o paciente obeso desenvolve, leva não só a um descontrole na produção de andrógenos, mas também a uma lesão endotelial, que pode ser irreversível, o que contribui, de forma significativa, para o desenvolvimento de disfunção erétil, normalmente, de difícil manejo clínico (Meldrum, 2014).

Além disso, há evidências demonstrando que o processo de envelhecimento e tal declínio androgênico contribui para o acúmulo de danos oxidativos em diferentes células e moléculas do organismo (Cabelof, 2006).

O envelhecimento decorre de um desequilíbrio nos delicados processos pró e anti-apoptose. A apoptose, definida como o conjunto de mudanças bioquímicas e morfológicas em diferentes níveis celulares, elimina células indesejadas e preserva os tecidos circunjacentes (Wyllie, 2010). Ela executa um papel importante em vários tecidos mitóticos, como o fígado e leucócitos, na medida em que evita a tumorigênese e mantém o controle das células imunocompetentes (Costa, 2004). Em tecidos pós-mitóticos como a musculatura detrusora (Higami, 1993; Warner, 1997), a apoptose apresenta um efeito deletério, havendo destruição de células essenciais para a função tecidual, eventualmente, de forma irreversível (Warner, 1997; Warner, 1999).

A Testosterona e o Trato Gênito-Urinário

Vários tecidos sofrem ação do decréscimo de testosterona, especialmente em sua arquitetura morfo-funcional, observando uma substituição de tecido normal funcionante por fibras colágenas não funcionantes. A próstata também sofre efeitos diretos da testosterona, tanto em seu volume, quanto em sua fisiologia, alterando a produção de antígeno prostático específico (Holgmän, 1993). Estudando bexigas de ratos jovens orquiectomizados e ratos senis observou que a intensidade da fibrose da parede vesical foi maior no grupo orquiectomizado, decorrente provavelmente da queda abrupta e não adaptativa dos níveis de testosterona, consequentemente perdendo a capacidade de diferenciação do estroma em musculatura lisa e do poder anti-inflamatório. Entretanto a apoptose na musculatura detrusora foi mais evidente nos senis, talvez pelo maior envolvimento de radicais livres quando a queda androgênica é lenta (Lorenzetti, 2009).

A Testosterona e a Disfunção Erétil

Sabe-se que a influência hormonal na diferenciação sexual intra-uterina até a sua maturação na puberdade é inconteste, desde o desenvolvimento do pênis até a maturação dos caracteres sexuais secundário. Os hormônios androgênicos desempenham um papel importante na fisiologia da ereção, visto que estudos em animais mostram que a privação de testosterona leva a alterações da função erétil (Wespes, 2010). Observa-se que uma concentração mínima de testosterona é necessária para que haja uma ereção adequada, fato corroborado na melhora clínica de paciente hipogonádicos que recebem reposição de testosterona exógena (Shabsing, 2006).

Traish et al, realizou uma revisão de literatura questionando a importância das concentrações de testosterona na função erétil do homem adulto. Apesar de não demostrar os mecanismos fisiológicos de forma detalhada, tampouco, esclarecer os alvos da ação hormonal, observou-se algumas questões relevantes e que inferem a importância da testosterona nos mecanismos de ereção: a perda de função erétil em pacientes tratados cirurgicamente ou através de castração por conta de neoplasias prostáticas; melhora das ereções noturnas em homens em terapia de reposição androgênica; melhora da função erétil em paciente em terapia de reposição hormonal e que apresentaram pouca, ou nenhuma resposta com o uso de inibidores de fosfodiesterase 5 (PDE-5i) (Traish, 2006).

Além disso, a produção de óxido nítrico (NO) no trato urinário depende de regulação da testosterona, e seu potencial efeito de relaxamento da musculatura detrusora e do colo vesical pode ficar prejudicado naquele paciente

hipogonádico (Pradidarcheep, 2008). A rigidez peniana durante a ereção depende, basicamente, do “inflow” maximizado e o “outflow” minimizado dentro dos corpos cavernosos. O NO atua como principal mediador vascular para isto ocorra (Morales, 2012). Visto que há uma relação entre a concentração de NO e a ação da testosterona no trato urinário, é natural que se faça tal analogia também para trato genital masculino, extrapolando estes princípios para o adequado funcionamento dos mecanismos de ereção.

Observa-se, na literatura, uma clara associação entre o envelhecimento masculino, o decréscimo de testosterona sérica e a disfunção erétil, porém poucos são os estudos que avaliam a estrutura histológica do pênis em tal situação.

Avaliar os efeitos da reposição de testosterona sobre a fibrose dos corpos cavernosos de pênis de ratos senis.

A metodologia deste trabalho seguiu as recomendações dos protocolos do Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pelo Comitê de Ética no Uso de Animais da Unicamp (CEUA).

A amostra de 16 ratos senis da raça Wistar, com idade entre 18 e 20 meses, foram divididos em dois grupos distintos, de forma randomizada e duplo-cega (animais identificados por códigos): Grupo Controle (6 animais) e Grupo Testosterona (10 animais), foi estatisticamente calculada de modo que se atingisse uma magnitude de 2,1%, ou seja, 99% dos indivíduos do grupo experimental excederia os valores médios do grupo controle.

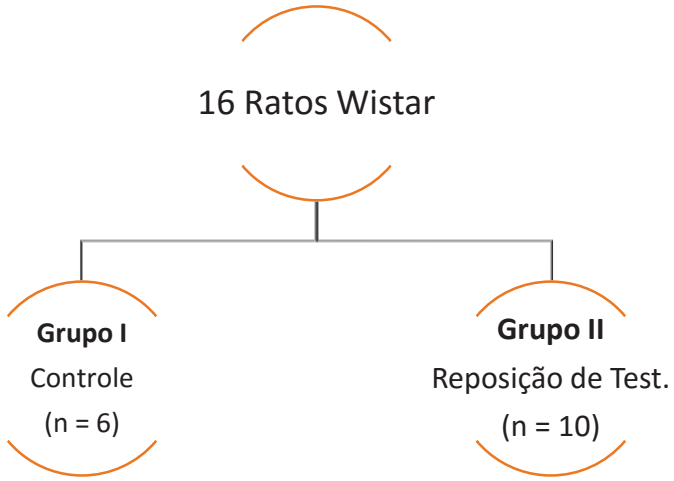


Figura 6 – Distribuição dos animais nos dois grupos de estudo

Inicialmente (D0), todos os animais foram anestesiados com um composto de Xylasine (0,87mg/Kg) e Ketamine (43,3mg/Kg) por via intraperitoneal. A seguir, foram pesados e tiveram seu sangue coletado através de punção com agulha fina do plexo venoso retro-orbital, transferindo-se o sangue diretamente para um tubo de Eppendorf. Estas amostras foram centrifugadas e congeladas a -20º C.

Os ratos do Grupo Testosterona receberam uma dose de Undecanoato de Testosterona (50mg/Kg), por via intramuscular, em seus dorsos, no primeiro dia do experimento (D0) e no 28º. dia (D28). Os animais do Grupo Controle receberam injeção semelhante com o mesmo volume de uma solução salina a 0,9%.

No 56º dia (D56) de estudo, foi feita uma nova coleta de amostra sanguínea e foram congeladas, em procedimento semelhante àquele realizado

no D0. Os animais foram sacrificados, através de aprofundamento do nível anestésico e injeção de Cloreto de Potássio. Após a eutanásia, os pênis de todos os ratos foram seccionados em suas bases, conservados em formalina por 48 horas, em seguida, colocados em solução de álcool a 70% e posteriormente parafinizados.

Os blocos de parafina foram seccionados em lâminas finas de 5 micrômetros, corados com uma gota de Picrosirus (corante usado para melhor evidência fibras de colágeno) e fixados.

As lâminas coradas foram analisadas em microscópio óptico digital, com luz polarizada e magnificação de 40x. Quatro imagens de cada lâmina foram adquiridas para análise estereológica.

Após a aquisição das imagens digitais, uma placa M-42 digitalizada e vetorizada foi sobreposta às lâminas e foi feita a avalição estereológica dos pontos de concentração de fibras colágenas. Esta análise foi realizada por dois observadores independentes. A equação $Vv = \frac{Ps}{Pp} * 100$ foi usada para calcular a densidade volumétrica das fibras de colágeno, onde: Vv = densidade volumétrica, Ps = número de pontos na estrutura estudada e Pp = número de pontos totais possíveis.

Os níveis de testosterona das amostras coletadas em D0 e D56 foram analisados através de um Kit de radioimunoensaio DSL-4100 Testerorene ®, produzido por Diagnostic Systems Laboratories Inc (EUA) e importado pela Genisis Diagnostic Product LTDA. Todas as amostras foram dosadas de forma duplicada, com uma sensibilidade de detecção de 0,05ng/mL.

Os dados obtidos, foram tabulados e a análise estatística foi feita pelo software SigmaPlot 12.0 ®. Foram utilizados os testes de Análise de Variância (ANOVA), Regressão Linear de Pearson, teste t não paramétrico e o teste não paramétrico de Kruskal-Wallis, este para avaliação das amostras independentes. O critério de significância estatística utilizado foi de 5% ($p < 0,05$).

Método Estereológico

O método estereológico determina parâmetros quantitativos tridimensionais de estruturas anatômicas a partir de cortes bidimensionais, conseguindo-se, desta forma, uma avalição global da estrutura. Sendo esta, a ciência das relações geométricas entre uma estrutura que existe em três dimensões e as imagens daquela estrutura, fundamentalmente bidimensionais. Trata-se de uma metodologia extremamente eficiente, visto que é necessário menor número de objetos analisados para se obter resultados com grande relevância estatística (Lacerda, 2000). Em estereologia, a significância estatística (p) pode ser expresso através da seguinte equação: $p = \left(\frac{1}{2}\right)^{\text{eventos}}$, por exemplo, para um "n" de 5 indivíduos, obtém-se um p = 0,03. Obviamente que quanto maior a amostra do estudo, maior será seu poder estatístico, mas este princípio demonstra claramente os reduzidos números de sujeitos com os quais a estereologia é capaz de trabalhar (Lacerda, 2000).

Também é de grande importância, determinar quantos campos microscópicos devem ser analisados. Em trabalhos de Orive e Weibel, raros foram os casos em que foi necessária uma análise de mais de 200 pontos. Utilizando-se uma placa M-42, isto significa a avaliação de no máximo 5 campos por indivíduos do estudo (Cruz-Orive, 1990).

O Princípio de Cavalieri, fundamentado por Hamilton, 1995 e Mandarim de Lacerda, 1995, diz que em uma série de cortes, pode-se avaliar o volume total da estrutura sem que seja necessário a análise de todos os cortes e sem prejuízo na acurácia do método. Da mesma forma, este método é adequado para se determinar a proporção e concentração das estruturas em análise. Sendo assim, três parâmetros são de relevante importância para a análise esterológica: *densidade volumétrica (Vv), densidade numérica (Nv)* e *volume médio (Vol).*

O **princípio de Delesse** versa que a quantidade relativa de pontos que tocam a estrutura é comparável à quantidade de área desta estrutura contida na área-teste, ou a quantidade de volume dessa estrutura no volume-teste (Mandarim de Lacerda, 1998). Sendo assim, a equação $Vv = \frac{Ps}{Pp} * 100$ é usada para calcular a **densidade volumétrica** das fibras de colágeno, onde: Vv =

densidade volumétrica, Ps = número de pontos na estrutura estudada e Pp = número de pontos totais possíveis.

O aumento da microscopia é indiferente para os resultados, sendo que se deve utilizar aquele que for mais confortável para a avalição da estrutura em questão, desde que não haja variações. No caso deste trabalho, optou-se por um aumento de 40x em microscopia óptica com luz polarizada.

O cálculo da **densidade numérica** se baseia na análise em dois planos distintos, considerando apenas as estruturas que se mostram em um plano separado, para isso é necessário conhecer a espessura dos cortes utilizados para a confecção das lâminas. Esta espessura deve ser de cerca de 1/3 do diâmetro médio da estrutura estudada (Gundersen, 1988). A **densidade numérica** pode ser expressa na seguinte fórmula:

$$Vol[dissector] = e.At$$

$$Nv = \frac{\sum Qa}{Vol[dissector]}\ 1/mm^3$$

Onde, e = espessura que separa s dois planos, At = área pré-determinada por um sistema-teste, Qa = número de estruturas encontradas em At.

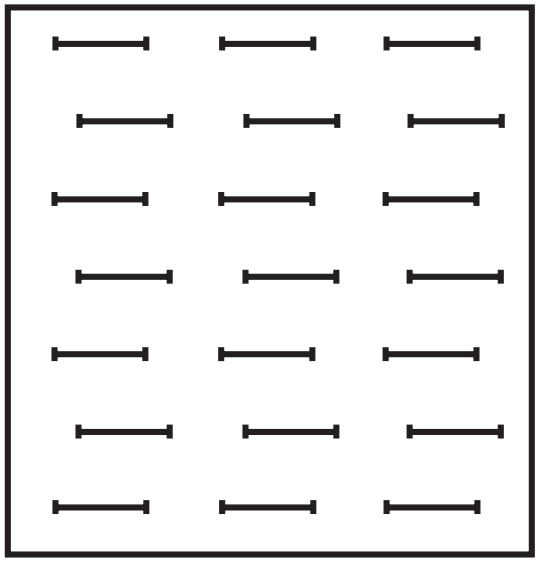
Por fim, uma vez sabido o Vv e o Nv, o cálculo do **volume médio** é simples e pode ser expresso da seguinte maneira:

$$Vol = \frac{Vv}{Nv}\ mcm^3$$

Dentre as vantagens inerentes ao método estereológico, pode-se citar:

- Possibilidade de se obter resultados na forma quantitativa
- Necessidade de um número reduzido de sujeitos a serem analisados, sem que se haja prejuízo da análise estatística
- Fácil reprodutibilidade e replicabilidade
- Possui sólidas bases teóricas.

No presente estudo, a análise da densidade volumétrica das fibras foi feita pela sobreposição uma grade M-42 sobre as lâminas já coradas (figuras abaixo). Foram analisadas quatro lâminas para cada indivíduo, representando 168 pontos para cada análise.



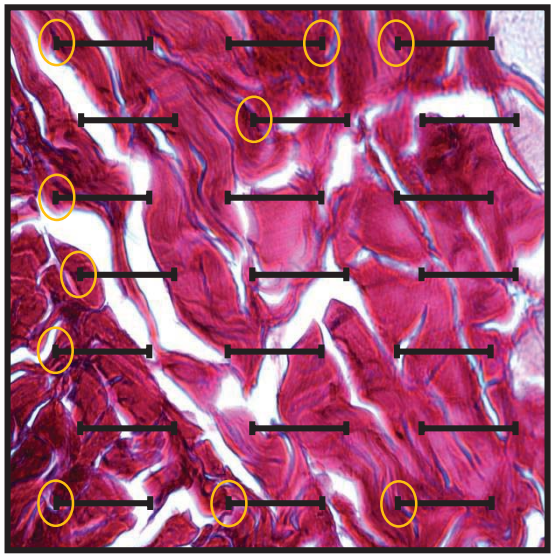


Figura 7 – Grade esterológica vetorizada

Figura 8 – Pênis de ratos preservados em parafina, corados com Picrosirus (aumento de 42x) – grade estereológica M42, vetorizada, com marcações em pontos de interesse.

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ORIGINAL ARTICLE

Effects of testosterone supplementation on prevention of age-related penile remodeling

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Abstract

Erectile dysfunction develops among 46,2% of men between 40-70 years. Studies demonstrated substitution on detrusor muscle by collagen due testosterone deprivation. This study aims to demonstrate the collagen substitution over the muscle fibers on muscle structure of rat's penis and the effects of testosterone supplementation. 16 senescent Wistar rats were divided in 2 groups: treatment (receiving standard supplementation testosterone dose) and control (receiving equivalent saline solution). Testosterone was dosed on D0 and D56 of study. All penises were prepared with picrosirius colored histology; stereology was applied to determine the volumetric density of collagen fibers (Vv). ANOVA demonstrated testosterone group´s replacement therapy was effective, while the androgenic decline continued by the time of experiment in control group ($p<0.05$). Testosterone group had Vv of 20,6%, lower than control group (47,8%); t-test ($p<0.001$). Pearson's correlation demonstrated an inverse correlation between the Vv and testosterone's levels ($p<0,001$). This is a pioneer study on demonstration of structural alterations over the cavernous corpora muscle caused by deprivation of testosterone on elderly rat. These finding implicate that the testosterone levels can influence, not only the libido, but also the erectile function.

Introduction

Erectile dysfunction is directed linked to age and it is the main well established risk factor for the appearance of erection difficulties in men[1]. Its prevalence is 46,2% on Brazilian male population between 40 and 70 years old, and it causes a great social, psychological and physical impact[2]. Age, expressed as Androgen Deficiency of the Adult Male and plurimetabolic syndrome, expressed on obesity, are the most known organic causes[3]. However, such clinical finding may be explained the alteration of cavernous corpora muscle fiber composition and distribution during the aging process[4]. A similar process can be observed over the aged bladder caused by the deprivation of testosterone[5].

Disequilibrium in the pro and anti-apoptosis mechanism is critical in the aging process[6]. Apoptosis plays a role in various mitotic tissues, as in the liver and leucocytes, as it prevents tumorigenesis and keeps track of immunocompetent cells[7]. However, in post-mitotic cells, as those in the penile muscles and bladder detrusor muscle, it has a negative effect since there is destruction of essential[8,9] and sometimes irreplaceable cells[10].

Recent studies have shown that oxidative stress can alter the structure and function of the bladder in mice. Dambros et al. (2005) showed that bladder strips subjected to repeated electrical stimulation are involved with reduction of contractile force and no apparent increase of oxidants, suggesting that hydrogen peroxide forms induced cell damage depending on the concentration of the oxidants generated[11]. Frequently, antioxidant mechanisms are able to limit or prevent the adverse effects of hydrogen peroxide, but with age, these mechanisms decrease, thereby making oxidative damage more prevalent with aging[12].

Lorenzetti (2009) showed that fibrosis of the bladder wall was higher within the orchiectomy group, probably resulting from the sharp drop and not adaptive levels of testosterone, thus losing the ability to differentiate into smooth muscle stroma and anti-inflammatory power. However, apoptosis in the detrusor muscle was stronger in senescent rats, perhaps by greater involvement of free radicals when androgen decline is slow[13].

The genitourinary tract is especially sensitive to changes in serum testosterone levels. The versatility of testosterone and its interference in various

chains of action in the prostate was first observed by Holgman (1993)[14]. Studies in the basic science demonstrated the close relation between Dihydrotestosterone (DHT) receptor in the bladder muscle and suppression of detrusor activity[15, 16].

The importance of testosterone on bladder physiology and its decrease with aging have encouraged research on the impact of these phenomena on the genitourinary tract detrusor muscle structure apoptosis. Nakazawa (2007), demonstrated, for the first time, increased expression of angiotensin-converting enzyme and angiotensin receptor type II (AII) in the bladder of rats undergoing bilateral orchiectomy[17]. Fraga-Silva (2013) showed that the use of one Angiotensin Inhibitor reduces penile fibrosis associated with attenuation of oxidative stress[18]. On 2013, Kilarkaje demonstrated that Angiotensin II signaling is involved in Diabetes induced structural changes on penile structure[19]. Since angiotensin type II acts through the rho/rho kinase via, this means that there are increasing pro-apoptotic factors associated with declining levels of testosterone.

Also, it is known that the production of Nitric Oxide (NO) in the urinary tract depends on the regulation of testosterone, and its potential relaxing effect of the detrusor muscle and the bladder neck can be impaired in hypogonadic patients[20]. Sánches (2012) demonstrated that nitrergic dysfunction and impaired neural NO signalling due to oxidative stress and nNOS uncoupling in penile arteries under conditions of insulin resistance[21].

There are few bases in the literature that describe the changes over the male penis muscle architecture during the aging process or over its effects on the physiopathology of erectile dysfunction.

Regarding what was previous discussed, the objective of this work is to study the relationship between late onset hypogonadism and structural remodeling of the senescent penile muscle structure, and whether this process can be influenced by exogenous testosterone replacement. Once there is enough information about the changes over the bladder detrusor muscle caused by oxidative stress, decreasing of testosterone and, by consequence, aging, it is logical to think that alterations of this nature can be observed in penile muscle structure and could lead to erectile dysfunction.

Materials and Methods

Sixteen male senescent Wistar senile rats (18-20 months old), weighing 380-530g were kept in a controlled environment (25 ± 2 °C) with exposure to light for 12 hours a day, water available *ad libitum* and Labina® animal chow (Purina®). The study was carried out according to the guidelines of the Brazilian College for Animal Experimentation (COBEA) under approval of the Institutional Committee for Ethics in Animal Research.

Two groups were formed, double blinded, as follows: Testosterone Group - 10 senile animals subjected to testosterone supplementation: Control Group – 6 senile animals subjected to intramuscular injection of 0.9% saline solution. This sample was statistically calculated with a magnitude of effect of 2,1 (99% of the subjects in the experimental group will exceed the mean value of the control group).

All animals were anesthetized with a solution composed of Xylazine (0.87 mg/kg) and Ketamine (43.3 mg/kg) injected intraperitoneally. After anesthesia was reached, they were weighed and later the venous blood was held through puncture of the retro-orbital venous plexus with a Pasteur pipette[22], transferring the blood sample directly to an Eppendorf tube. Subsequently, the sample was centrifuged and only the serum was frozen at - 20 °C.

Testosterone undecanoate (TU) was administered intramuscularly (50mg/kg) into the animal's dorsum using a fine insulin type needle, on the first and 28th day of the experiment. In the control group, 0.9% saline was administered at the same volume of drug solution in the testosterone group, on the same days. On the last day of the experiment (56th day), new blood sample was collected and the penis was removed by a section over its base. After removal, the penile were stored in formalin for 48 hours and then in 70% alcoholic solution for further paraffinization and study of stereological collagen fibers.

Stereology was the method chosen in order to evaluate morphometrically the muscle and collagen fibers of penis. The fiber analysis was performed using preparations from five-micrometer thin sections[23]. The modified picrosirius red staining technique was used. The slides were analyzed via optical microscopy with polarized light at a 40x magnification. Ten fields per slide and ten slides per

animal were evaluated. The volumetric density of the collagen and muscle fibers was analyzed by overlaying the M-42 grid system on the digital morphological image of the slides. The volumetric density was the relative density taken up by fibers in the tissue under examination. The stereological method determined quantitatively the parameters of the anatomical structural base on the two-dimensional thin sections, in three dimensions[24].

Stereology has advantages inherent to the method as turning the results into numeric values, with easy reproducibility and comparison between groups and, most importantly, presents a well define theoretic basis.

The equation $Vv = \frac{Ps}{Pp} * 100$ was used to calculate the volume density of the collagen fibers, where: Vv = volumetric density, Ps = the number of structure points studied (collagen), and, Pp = the number of possible test points (42 in this case).

Levels of testosterone were evaluated using the radioimmunoassay kit DSL-4100 Testosterone®, manufactured by Diagnostics Systems Laboratories Inc. (USA) and imported by Genesis Diagnostics Products Ltda. All samples were analyzed in duplicate with theoretical sensitivity or 0.05 ng/mL minimum detection limit.

The data obtained were analyzed using the SigmaPlot 12.0 software. The results were validated by analysis of variance (multivariate analysis). Pearson's Regression, non-parametrical t test and the nonparametric Kruskal-Wallis test were used to assess the differences between the independent samples. The significance criterion used was two-sided $p < 0.05$.

Results

The mean body weights of rats between the two groups throughout the duration of the experiment were compared using the Kruskal-Wallis test at 95% of probability, showing that there was no change in body weight between the groups (Table 01).

The mean serum testosterone of the testosterone group on the first day (D0) of experiment was 1,196 ng/mL. After hormone replacement therapy, this index reached a value of 3,240 ng/mL, measured on the 56th day (D56). On the

other hand, the control group had an average of 1.173 ng/mL on D0, which decreased to 0.635 ng/mL of serum testosterone at the end of the study (D56). According to the analysis of variance (ANOVA), by Tukey test, the testosterone group replacement therapy was effective and satisfactory, while the androgenic decline continued by the time of experiment in the control group. This showed that there was a significant hormone deficit at the time of sacrificing the control animals ($p < 0.05$) (Figure 01).

Table 01 – Average mice body weight (mean) in grams over time and groups involved in the experiment.

Groups	**Average weights (g)**		
	D_0	D_{28}	D_{56}
Testosterone	438 *	427,9 *	418,9*
Control	449,5 *	420*	405*

* ($p > 0,05$), (Kruskal-Wallis Test)

Table 2 presents the descriptive analysis of the volumetric density of the collagen fibers in the penile cavernous corpora muscle in both groups of the study. It was observed that the testosterone group had a volumetric density of collagen of 20,6%, which was lower than control group (47,8%) showing statistical significance, t test ($p<0.001$ and $r^2 = 0,771$) (Figure 2).

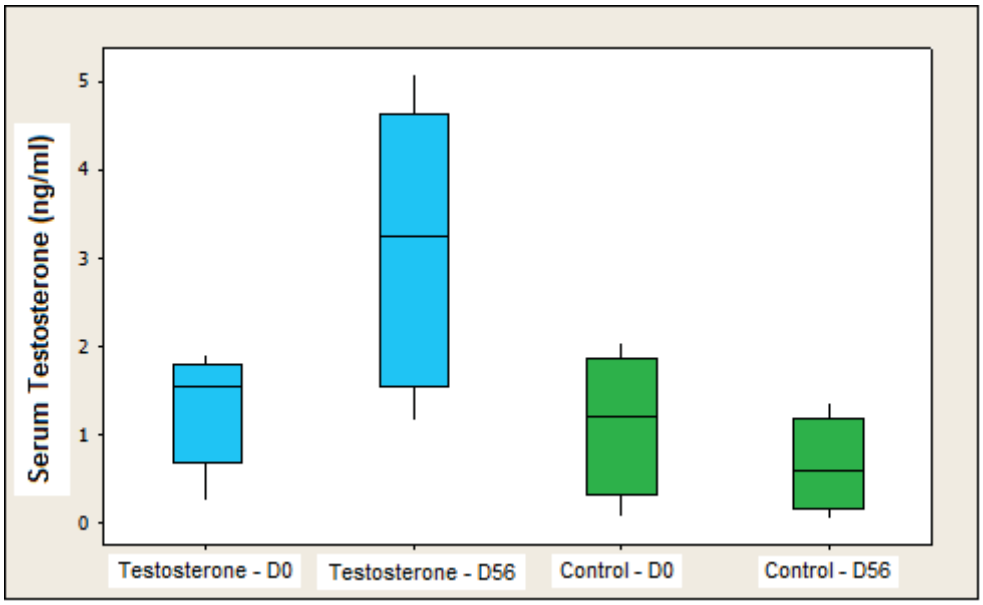


Figure 01 – Box-plot of the values of serum testosterone in the testosterone and control groups on D0 (beginning of the experiment) and D56 (animal sacrifice).

Table 2 – Descriptive analysis on collagen fibers of penile in both groups.

	Volumetric density (Vv) - %	
	Testosterone	***Control***
Mean	**20,6**	**47,8**
SD	**7,49**	**7,93**
Minimum	**9,52**	**35,12**
Median	**21,43**	**48,81**
Maximum	**31,55**	**59,52**

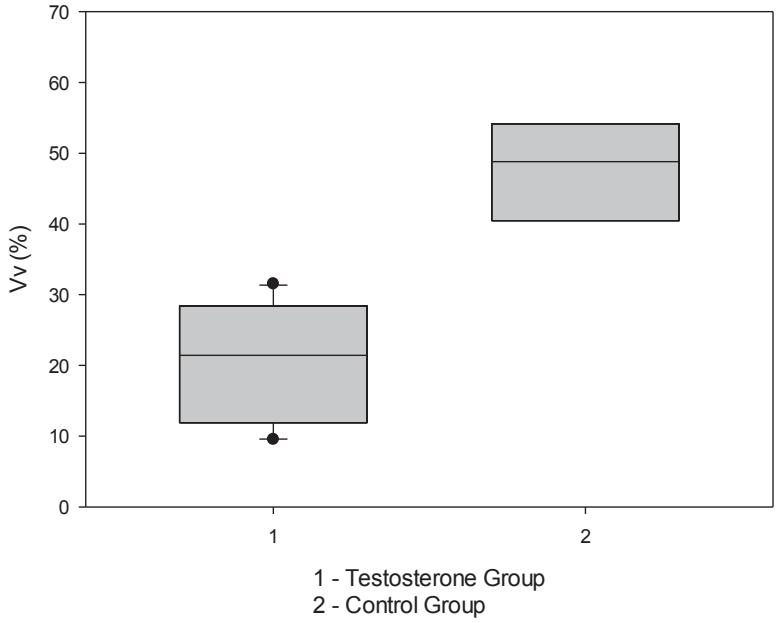


Figure 2 – Box Plot of volumetric density of the collagen fibers of the bladder wall in different groups

The linear regression, with Pearson's correlation demonstrated an inverse and statistical significant correlation between the density of collagens fiber and level of testosterone on both groups ($p < 0,001$ and $r^2 = 0,538$) (Figure 3).

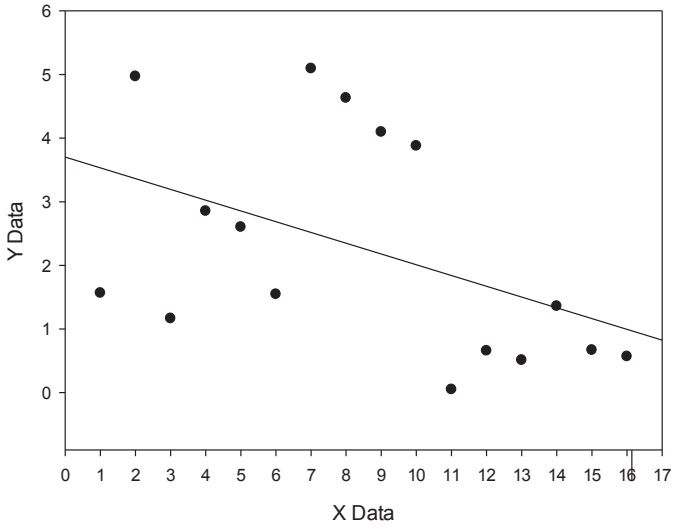


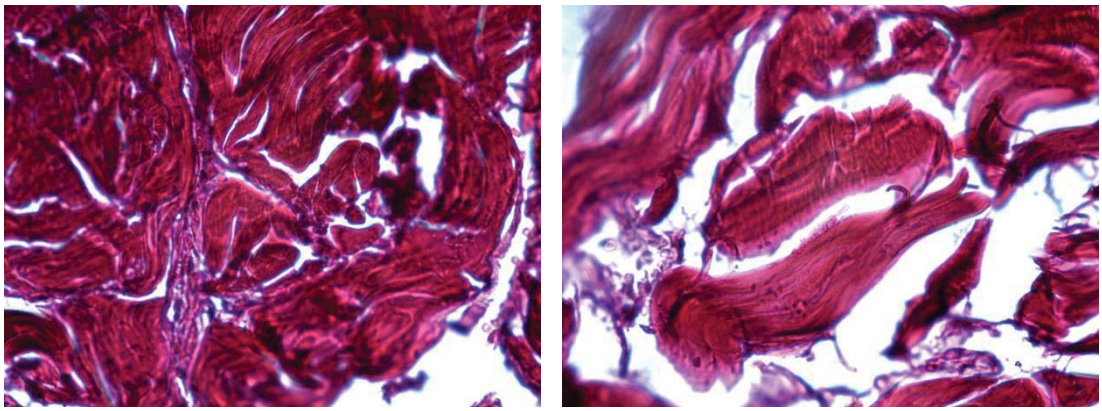
Figure 3 – Linear regression between the density of collagens fiber and level of testosterone

Figure 4 shows picrosirius stained cavernous corpora highlighting collagen fibers distributed among muscle fibers, ranging mainly from an orange to intense red. In the testosterone group, the presence of less collagen fibers between the detrusor is observed when compared to the control group.

Discussion

The presence of androgen receptor in rat penile and the modulation of autonomic pelvic plexus by testosterone[25] indirectly reinforce the influence of androgenic hormone in the lower genital and urinary tract.

A. **B.**



Figures 4 – A. High levels of collagen replacement on penile muscle structure (Control Group). B. Low levels of collagen replacement on penile muscle structure (Testosterone replacement group). Collored by picrosirius, 40x.

Takyu (1993) observed that castration decreased the function of alpha1-adrenergic and muscarinic receptors, restoring their functions after testosterone replacement[26]. Moreover, the existence of angiotensin II (AII) receptors in the bladder, especially type 2, are related to inflammatory and apoptotic stimuli. Nakazawa et al. (2007), using castrated rats, observed increased expression of caspase-3 in the bladder mediated by type 2 AII receptor, when compared to control and testosterone replacement groups[17]. Fraga-Silva e Kilarkaje found out

similar processes on penile structure, mediated by angiotensin receptors system, causing penile fibrosis[18].

Fillipi et al. (2007) found that the expression of phosphodiesterase-5 (PDE 5) in the bladder – an important enzyme that inhibits the NO cycle – is dependent on the levels of circulating androgens[27] and Sánches (2013) demonstrated the effects of NO signaling over the oxidative stress process in penis[21]. Furthermore, the RhoA/Rho-kinase system has been investigated for years as a cause of urinary tract disorders, including participation in the overactive bladder, due to its activation of actin-myosin complex that causes muscle contractions, regardless of the levels of cytosolic free calcium. This pathway is also modulated by sex hormones and the ratio estrogen/testosterone imbalance, as occurs in aging and obesity[3], which seems to be the most important factor in the stimulation RhoA/Rho-kinase via [28].

Bhasin et al. (2003) and Singh et al. (2003 and 2006) believe that androgens stimulate concomitantly the pluripotent cells to differentiate into muscle lineages including smooth muscle, inhibiting strains for the differentiation of adipocytes[29, 30,31]. To support this finding, Lorenzetti (2009), in assessing the influence of testosterone on the bladder wall fibrosis in rats, observed that the sudden drop of testosterone by castration of young rats caused more muscle replacement by collagen fibers when compared to senile rats, whose process of hormonal decline is slow and gradual[13]. Although the orchiectomy model is usually used to assess the influence of testosterone on the bladder, it does not seem appropriate to reproduce the process of late onset hypogonadis. Therefore, we decided to use only senile animals (18-28 months) in this work, which represents more risks and higher costs, but portrays adequately the physiological aging process, avoiding the sample selection bias.

Baseline levels of testosterone in both groups were below the levels described by Kinoshita el al. (1985) in seven-month-old rats (2.2±0.3 ng/dL average testosterone), but very similar to the 21-month-old rats (1.1±0.2 ng/dL average testosterone)[32]. This fact demonstrates the physiologic decrease of testosterone in rats in this study. Early studies using the replacement model with 50mg/kg of testosterone undecanoate per month have been conducted for about twelve years, showing that this choice is simple, reproducible and effective for hormone replacement in rats[33, 34]. Another variation of the experimental model

of hormone supplementation, described more recently, uses 100 mg/kg of TU, in a single dose, and its effects can be evaluated after two months of administration[35]. Monthly dosage was chosen in this study for its consolidation in literature. This method of supplementation caused a mean serum testosterone of about 3.2 ng/dL in the group that underwent hormone replacement therapy, which was superior as the values of young rats (2.2 ± 0.3 ng/dL average testosterone)[32].

For quantitative analysis of fibrotic reaction in the penile cavernous muscle, it was performed the stereological study of collagen fibers in both groups[24]. The volume density (Vv), magnitude used in this study is a stereological parameter that produces reliable results with minimal variation. At the same time, it is not dependent on histologic complex blade or experience of the researcher, being employed in the quantification of fibrous component of the extracellular matrix, particularly in ace collagen and elastic fibers of various tissues[36,37,38].

Under polarized light, type I collagen fibers appear as being thick, strongly birefringent, with colors ranging from yellow to intense red[39]. The use of polarized light for analysis of Picrosirius Red stained samples is a special procedure for histological analysis of type-specific collagen[40], and it is not necessary to evaluate fibrotic processes, especially due to the prevalence of type I collagen in these processes. Nevertheless, Picrosirius Red polarization facilitates the identification and counting of collagen mass in relation to the detrusor muscle, improving the accuracy of the method[39]. The time between intervention and evaluation of the final result on this issue was two months and this period of time has been the most used to assess changes in rat muscles. [35,41,42].

Ludwig et al. (2011) found oxidative stress and apoptosis increased in the bladder of rats subjected to castration; however, it could be reduced by supplementation with alpha-tocopherol[42]. Still, it was not clear whether the rise of the apoptotic process increases the risk of bladder dysfunction. In fact, oxidative stress is one of the most important factors involved in the pathogenesis of age-related detrusor dyskinesia[43] and may be aggravated by diabetes mellitus[44] and by the hypogonadism[42]. On the other hand, vitamin E produces protective effect against free radicals, especially when used in an early stage of tissue injury[46]. Helmy (2012) proved that antioxidant therapy with vitamin E ameliorates the age-associated erectile dysfunction[45] and Zhang et al correlated an antioxidant dietary with the improvement of arteriogenic erectile dysfunction [46].

Cayan et al. (2008) showed that rats with bilateral orchiectomy, testosterone replacement associated with estradiol had better results than androgen replacement alone regarding the preservation of the muscle/collagen ratio, demonstrating the stroma modulating role of testosterone, regardless of gender[47]. Tek et al. (2010) used the model of castrated ten-month-old rats to evaluate the effect of testosterone replacement (100mg/kg testosterone undecanoate dose) on bladder function and histology. They concluded that hormone replacement in these animals produced an improvement in the smooth muscle/collagen ratio, including the development of bladder capacity[35].

Our study was a pioneer in demonstrating that testosterone, even lower than the doses used in the works mentioned above, favored the gradual development of smooth muscle cells in penis of rats with senile hormonal decline and testosterone supplementation in aged rats protected them against the process of remodeling/fibrosis of the penis. These finding can implicate that the testosterone levels can influence, not only the libido, but also the erectile function in elderly population.

Conflicts of Interests: The authors report no conflict of interests.

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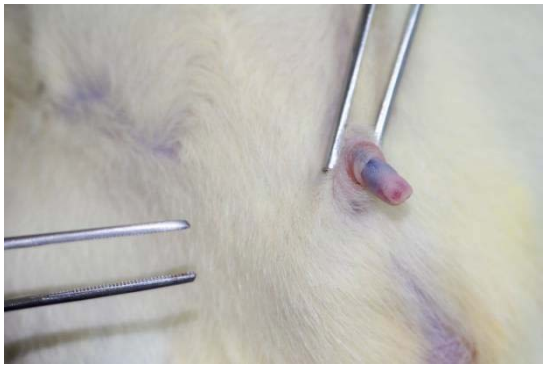
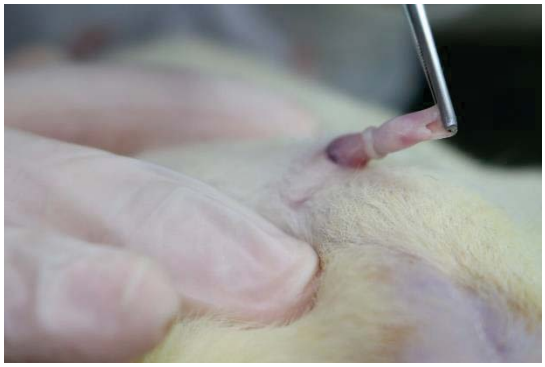
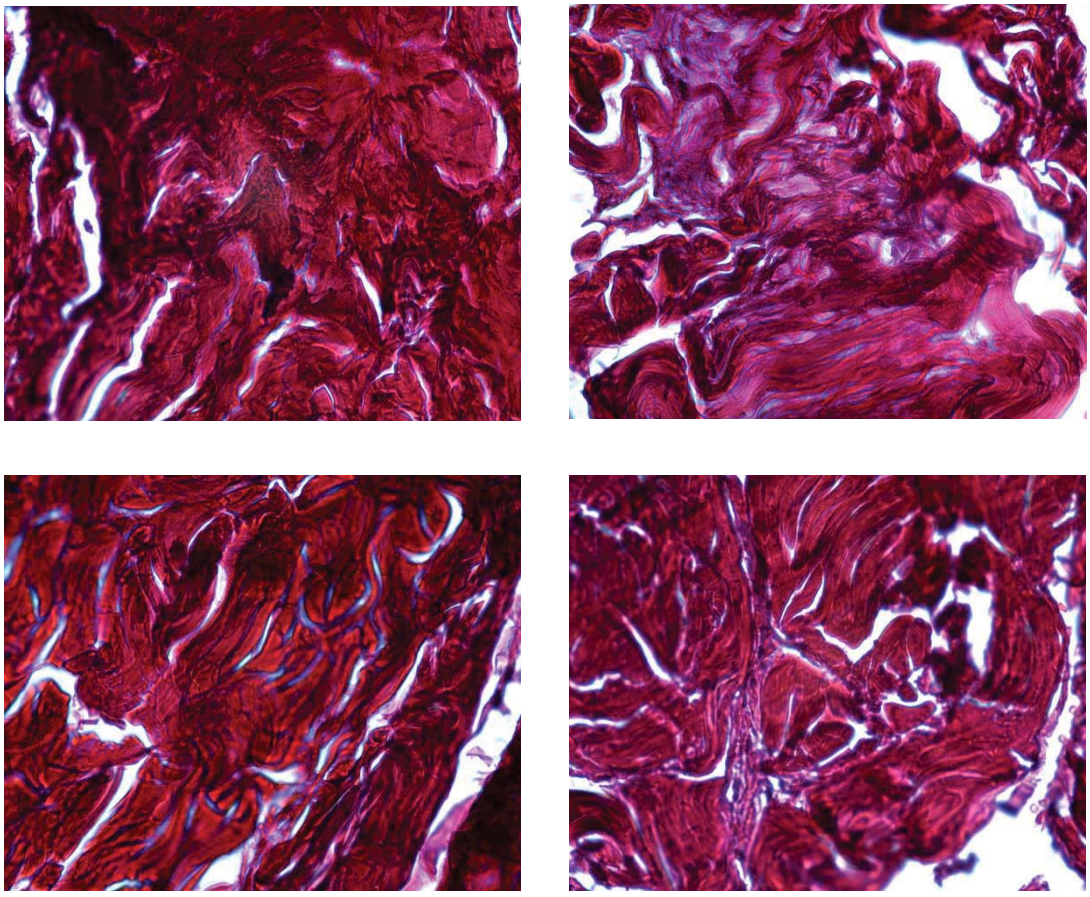
APÊNDICE 1 – Manejo dos Animais





APÊNDICE 2 – Painel de Lâminas

Lâminas – Pênis de ratos preservados em parafina, corados com Picrosirus (aumento de 42x) – Grupo I – Reposição.



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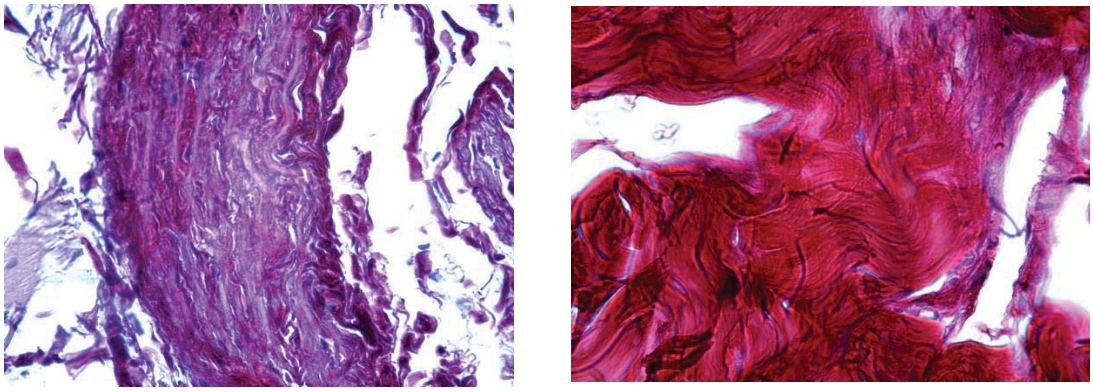

APÊNDICE 3 – Tabela I – Pontos Coletados em Grade Estereológica

Código	*Lâmina 1*	*Lâmina 2*	*Lâmina 3*	*Lâmina 4*	*Total de Pontos*	*Média*
FCX1R1	11	11	17	14	53	13,25
FCX1R2	35	20	15	30	100	25
FCX1R3	15	24	8	6	53	13,25
FCX2R2	8	12	22	5	47	11,75
FCX2R3	18	20	10	11	59	14,75
FCX3R2	21	10	17	28	76	19
FCX4R2	3	2	7	4	16	4
FCX4R3	30	20	6	16	72	18
FCX5R1	6	11	9	11	37	9,25
FCX5R2	3	8	12	14	37	9,25
FCX5R3	10	15	12	13	50	12,5
FCX6R1	3	3	7	8	21	5,25
FCX6R2	18	25	24	21	88	22
FCX6R3	17	19	15	20	71	17,75
FCX7R1	2	2	6	7	17	4,25
FCX7R2	8	9	9	19	45	11,25

APÊNDICE 4 – Tabela II – Dados Laboratoriais – Divididos por Grupos

Grupos	*Animal*	*Código*	*Total de Pontos*	*Média*	*Vv (%)*	*Test. D0*	*Test. D56*
Grupo I	Animal 1	FCX1R3	35	8,75	20,83	0,683	4,971
Grupo I	Animal 2	FCX1R1	53	13,25	31,55	1,868	1,564
Grupo I	Animal 3	FCX2R2	47	11,75	27,98	0,38	1,166
Grupo I	Animal 4	FCX4R2	16	4,00	9,52	1,152	2,853
Grupo I	Animal 5	FCX5R1	37	9,25	22,02	1,781	2,603
Grupo I	Animal 6	FCX5R3	50	12,50	29,76	1,594	5,093
Grupo I	Animal 7	FCX6R1	21	5,25	12,50	0,254	4,632
Grupo I	Animal 8	FCX5R2	37	9,25	22,02	1,578	1,545
Grupo I	Animal 9	FCX7R1	17	4,25	10,12	1,909	4,097
Grupo I	Animal 10	FCX7R2	34	8,50	20,24	0,761	3,879
			Média	8,68	20,65	1,196	3,240
			Mediana	9,00	21,43	1,365	3,366
			Desvio Padrão	3,14	7,49	0,601	1,416
			Máximo	13,25	31,55	1,91	5,09
			Mínimo	4,00	9,52	0,25	1,17

Grupos	*Animal*	*Código*	*Total de Pontos*	*Média*	*Vv (%)*	*Test. D0*	*Test. D56*
Grupo II	Animal 1	FCX3R2	76	19,00	45,24	2,049	0,511
Grupo II	Animal 2	FCX1R2	100	25,00	59,52	0,064	0,05
Grupo II	Animal 3	FCX2R3	59	14,75	35,12	1,364	0,657
Grupo II	Animal 4	FCX4R3	88	22,00	52,38	1,055	1,358
Grupo II	Animal 5	FCX6R2	88	22,00	52,38	1,13	0,668
Grupo II	Animal 6	FCX6R3	71	17,75	42,26	1,375	0,567
			Média	20,08	47,82	1,173	0,635
			Mediana	20,50	48,81	1,247	0,612
			Desvio Padrão	3,33	7,93	0,590	0,384
			Máximo	25,00	59,52	2,05	1,36
			Mínimo	14,75	35,12	0,06	0,05

1. Certificado de aprovação da pesquisa pelo Comitê de Ética no Uso de Animais (CEUA) – Unicamp
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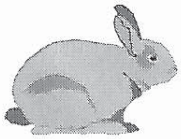

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C E R T I F I C A D O

Certificamos que o projeto **"AVALIAÇÃO QUANTITATIVA ESTEREOLOGICA DO PROCESSO FIBRÓTICO EM PÊNIS DE RATOS SENIS SUBMETIDOS À REPOSIÇÃO DE TESTOSTERONA"** (protocolo nº **3202-1**), sob a responsabilidade de **Prof. Dr. PAULO CESAR RODRIGUES PALMA / FÁBIO THADEU FERREIRA**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **03 de fevereiro de 2014**.

Campinas, 03 de fevereiro de 2014.

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ORIGINAL ARTICLE

Effects of testosterone supplementation on prevention of age-related penile remodeling

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Abstract

Erectile dysfunction develops among 46.2% of men between 40 and 70 years. Studies demonstrated substitution on detrusor muscle by collagen due testosterone deprivation. It is clear the correlation among aging and oxidative stress, accelerating apoptosis process in many tissues. This study aims to demonstrate the collagen substitution over the muscle fibers on muscle structure of rat's penis and the effects of testosterone supplementation. Sixteen senescent Wistar rats were divided into two groups: treatment (receiving standard supplementation testosterone dose) and control (receiving equivalent saline solution). Testosterone was dosed on D0 and D56 of study. All penises were prepared with picrosirius colored histology; stereology was applied to determine the volumetric density of collagen fibers (Vv). Analysis of variance demonstrated testosterone group's replacement therapy to be effective, while the androgenic decline continued by the time of experiment in control group ($p < 0.05$). Testosterone group had Vv of 20.6%, lower than control group (47.8%); t-test ($p < 0.001$). Pearson's correlation demonstrated an inverse correlation between the Vv and testosterone's levels ($p < 0.001$). This is a pioneer study on demonstration of structural alterations over the cavernous corpora muscle caused by deprivation of testosterone on elderly rat. These finding implicate that the testosterone levels can influence, not only the libido, but also the erectile function.

Keywords

Aging, erectile dysfunction, fibrosis, testosterone

History

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Introduction

Erectile dysfunction is directed linked to age and it is the main well established risk factor for the appearance of erection difficulties in men [1]. Its prevalence is 46.2% on Brazilian male population between 40 and 70 years old, and it causes a great social, psychological and physical impact [2]. Age, expressed as androgen deficiency of the adult male and plurimetabolic syndrome, expressed on obesity, are the most known organic causes [3]. However, such clinical finding may be explained the alteration of cavernous corpora muscle fiber composition and distribution during the aging process [4]. A similar process can be observed over the aged bladder caused by the deprivation of testosterone [5].

Disequilibrium in the pro- and anti-apoptosis mechanism is critical in the aging process [6]. Apoptosis plays a role in various mitotic tissues, as in the liver and leucocytes, as it prevents tumorigenesis and keeps track of immunocompetent cells [7]. However, in post-mitotic cells, as those in the penile muscles and bladder detrusor muscle, it has a negative effect since there is destruction of essential [8,9] and sometimes irreplaceable cells [10].

Recent studies have shown that oxidative stress can alter the structure and function of the bladder in mice. Dambros et al. [11] showed that bladder strips subjected to repeated electrical stimulation are involved with reduction of contractile force and no apparent increase of oxidants, suggesting that hydrogen peroxide forms induced cell damage depending on the concentration of the oxidants generated. Frequently, antioxidant mechanisms are able to limit or prevent the adverse effects of hydrogen peroxide, but with age, these mechanisms decrease, thereby making oxidative damage more prevalent with aging [12].

Lorenzetti [13] showed that fibrosis of the bladder wall was higher within the orchiectomy group, probably resulting from the sharp drop and not adaptive levels of testosterone, thus losing the ability to differentiate into smooth muscle stroma and anti-inflammatory power. However, apoptosis in the detrusor muscle was stronger in senescent rats, perhaps by greater involvement of free radicals when androgen decline is slow.

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The genitourinary tract is especially sensitive to changes in serum testosterone levels. The versatility of testosterone and its interference in various chains of action in the bladder was first observed by Holgman [14]. Studies in the basic science demonstrated the close relation between dihydrotestosterone (DHT) receptor in the bladder muscle and suppression of detrusor activity [15,16].

The importance of testosterone on bladder physiology and its decrease with aging have encouraged research on the impact of these phenomena on the genitourinary tract detrusor muscle structure apoptosis. Nakazawa et al. [17], demonstrated, for the first time, increased expression of angiotensin-converting enzyme and angiotensin receptor type II (AII) in the bladder of rats undergoing bilateral orchiectomy. Fraga-Silva et al. [18] showed that the use of one angiotensin inhibitor reduces penile fibrosis associated with attenuation of oxidative stress. Kilarkaje et al. [19] demonstrated that Angiotensin II signaling is involved in diabetes-induced structural changes on penile structure. Since angiotensin type II acts through the rho/rho kinase via, this means that there are increasing pro-apoptotic factors associated with declining levels of testosterone.

Also, it is known that the production of Nitric Oxide (NO) in the urinary tract depends on the regulation of testosterone, and its potential relaxing effect of the detrusor muscle and the bladder neck can be impaired in hypogonadic patients [20]. Sánches et al. [21] demonstrated that nitrergic dysfunction and impaired neural NO signalling due to oxidative stress and nNOS uncoupling in penile arteries under conditions of insulin resistance.

There are data in the literature that describe the changes over the male penis muscle architecture during the aging process or over its effects on the physiopathology of erectile dysfunction.

Regarding what was previous discussed, the objective of this work is to study the relationship between late onset hypogonadism and structural remodeling of the senescent penile muscle structure, and whether this process can be influenced by exogenous testosterone replacement. Once there is enough information about the changes over the bladder detrusor muscle caused by oxidative stress, decreasing of testosterone and, by consequence, aging, it is logical to think that alterations of this nature can be observed in penile muscle structure and could lead to erectile dysfunction.

Materials and methods

Sixteen male senescent Wistar senile rats (18–20 months old), weighing 380–530 g were kept in a controlled environment ($25 \pm 2\,^{\circ}C$) with exposure to light for 12 h a day, water available ad libitum and Labina® animal chow (Purina®). The study was carried out according to the guidelines of the Brazilian College for Animal Experimentation (COBEA) under approval of the Institutional Committee for Ethics in Animal Research.

Two groups were formed, double-blinded, as follows: Testosterone Group – 10 senile animals subjected to testosterone supplementation: Control Group – 6 senile animals subjected to intramuscular injection of 0.9% saline solution. This sample was statistically calculated with a magnitude of effect of 2.1 (99% of the subjects in the experimental group will exceed the mean value of the control group).

All animals were anesthetized with a solution composed of Xylazine (0.87 mg/kg) and Ketamine (43.3 mg/kg) injected intraperitoneally. After anesthesia was reached, they were weighed and later the venous blood was held through puncture of the retro-orbital venous plexus with a Pasteur pipette [22], transferring the blood sample directly to an Eppendorf tube. Subsequently, the sample was centrifuged and only the serum was frozen at $-20\,^{\circ}C$.

Testosterone undecanoate (TU) was administered intramuscularly (50 mg/kg) into the animal's dorsum using a fine insulin type needle, on the 1st and 28th day of the experiment. In the control group, 0.9% saline was administered at the same volume of drug solution in the testosterone group, on the same days. On the last day of the experiment (56th day), new blood sample was collected and the penis was removed by a section over its base. After removal, the penile were stored in formalin for 48 h and then in 70% alcoholic solution for further paraffinization and study of stereological collagen fibers.

Stereology was the method chosen in order to evaluate morphometrically the muscle and collagen fibers of penis. The fiber analysis was performed using preparations from 5-μm thin sections [23]. The modified picrosirius red staining technique was used. The slides were analyzed via optical microscopy with polarized light at a $40\times$ magnification. Ten fields per slide and 10 slides per animal were evaluated. The volumetric density of the collagen and muscle fibers was analyzed by overlaying the M-42 grid system on the digital morphological image of the slides. The volumetric density was the relative density taken up by fibers in the tissue under examination. The stereological method determined quantitatively the parameters of the anatomical structural base on the two-dimensional thin sections, in three dimensions [24].

Stereology has advantages inherent to the method as turning the results into numeric values, with easy reproducibility and comparison between groups and, most importantly, presents a well define theoretic basis.

The equation $Vv = \frac{Ps}{Pp} \times 100$ was used to calculate the volume density of the collagen fibers, where: Vv = volumetric density, Ps = the number of structure points studied (collagen), and, Pp = the number of possible test points (42 in this case).

Levels of testosterone were evaluated using the radioimmunoassay kit DSL-4100 Testosterone®, manufactured by Diagnostics Systems Laboratories Inc. (Webster, TX, USA) and imported by Genesis Diagnostics Products Ltd. (São Paulo, Brazil). All samples were analyzed in duplicate with theoretical sensitivity or 0.05 ng/ml minimum detection limit.

The data obtained were analyzed using the SigmaPlot 12.0 software. The results were validated by analysis of variance (multivariate analysis). Pearson's Regression, nonparametrical *t* test and the nonparametric Kruskal–Wallis test were used to assess the differences between the independent samples. The significance criterion used was two-sided $p < 0.05$.

Results

The mean body weights of rats between the two groups throughout the duration of the experiment were compared using the Kruskal–Wallis test at 95% of probability, showing that there was no change in body weight between the groups (Table 1).

The mean serum testosterone of the testosterone group on the first day (D0) of experiment was 1.196 ng/ml. After hormone replacement therapy, this index reached a value of 3.240 ng/ml, measured on the 56th day (D56). On the other hand, the control group had an average of 1.173 ng/ml on D0, which decreased to 0.635 ng/ml of serum testosterone at the end of the study (D56). According to the analysis of variance (ANOVA), by the Tukey test, the testosterone group replacement therapy was effective and satisfactory, while the androgenic decline continued by the time of experiment in the control group. This showed that there was a significant hormone deficit at the time of sacrificing the control animals ($p < 0.05$) (Figure 1).

Table 2 presents the descriptive analysis of the volumetric density of the collagen fibers in the penile cavernous corpora muscle in both groups of the study. It was observed that the testosterone group had a volumetric density of collagen of 20.6%, which was lower than control group (47.8%) showing statistical significance, t test ($p < 0.001$ and $r^2 = 0.771$) (Figure 2).

The linear regression, with Pearson's correlation demonstrated an inverse and statistical significant correlation between the density of collagens fiber and the level of testosterone in both groups ($p < 0.001$ and $r^2 = 0.538$) (Figure 3).

Figure 4 shows picrosirius-stained cavernous corpora highlighting collagen fibers distributed among muscle fibers, ranging mainly from an orange to intense red. In the testosterone group, the presence of less collagen fibers between the detrusor is observed when compared to the control group.

Discussion

The presence of androgen receptor in rat penile and the modulation of autonomic pelvic plexus by testosterone [25] indirectly reinforce the influence of androgenic hormone in the lower genital and urinary tract.

Takyu [26] observed that castration decreased the function of α1-adrenergic and muscarinic receptors, restoring their functions after testosterone replacement. Moreover, the existence of angiotensin II (AII) receptors in the bladder, especially type 2, are related to inflammatory and apoptotic stimuli. Nakazawa et al. [17], using castrated rats, observed increased expression of caspase-3 in the bladder mediated by type 2 AII receptor, when compared to control and testosterone replacement groups. Fraga-Silva et al. [18] found out similar processes on penile structure, mediated by angiotensin receptors system, causing penile fibrosis.

Fillipi et al. [27] found that the expression of phosphodiesterase-5 (PDE 5) in the bladder – an important enzyme that inhibits the NO cycle – is dependent on the levels of circulating androgens and Sánches et al. [21] demonstrated the effects of NO signaling over the oxidative stress process in penis. Furthermore, the RhoA/Rho-kinase system has been investigated for years as a cause of urinary tract disorders,

Table 1. Average mice body weight in grams over time and groups involved in the experiment.

	Average weights (g)		
Groups	D_0	D_{28}	D_{56}
Testosterone	438*	427.9*	418.9*
Control	449.5*	420*	405*

*($p > 0.05$), (Kruskal–Wallis test).

Table 2. Descriptive analysis on collagen fibers of bladder wall in both groups.

	Volumetric density (Vv) – %	
	Testosterone	Control
Mean	20.6	47.8
SD	7.49	7.93
Minimum	9.52	35.12
Median	21.43	48.81
Maximum	31.55	35.12

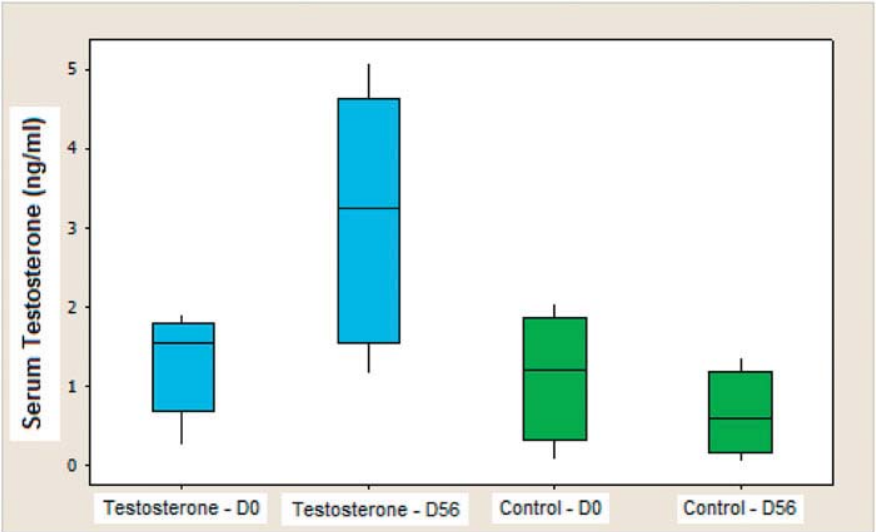


Figure 1. Box-plot of the values of serum testosterone in the testosterone and control groups on D0 (beginning of the experiment) and D56 (animal sacrifice).

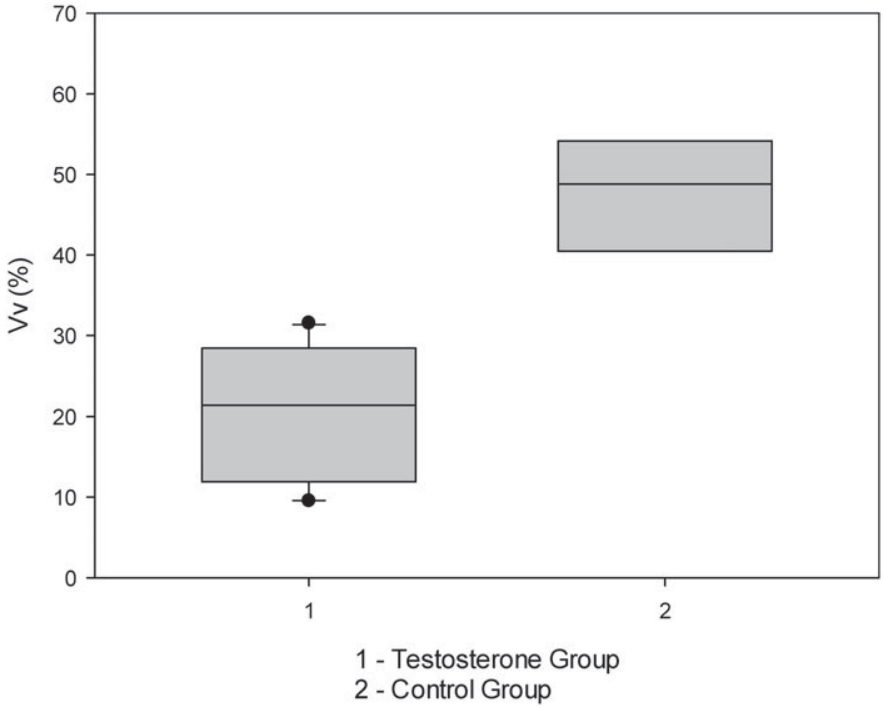


Figure 2. Box-plot of volumetric density of the collagen fibers of the bladder wall in different groups.

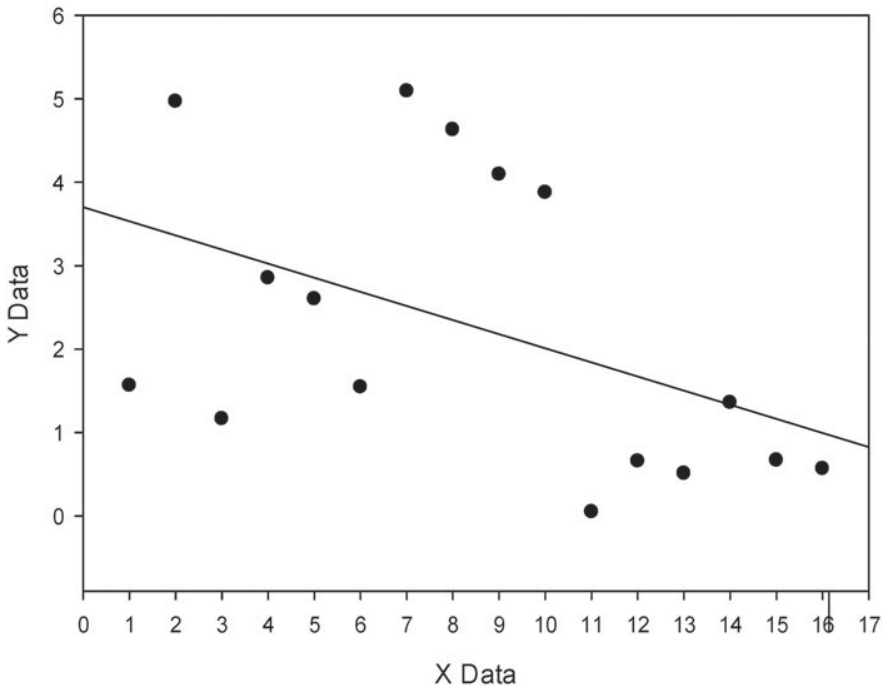
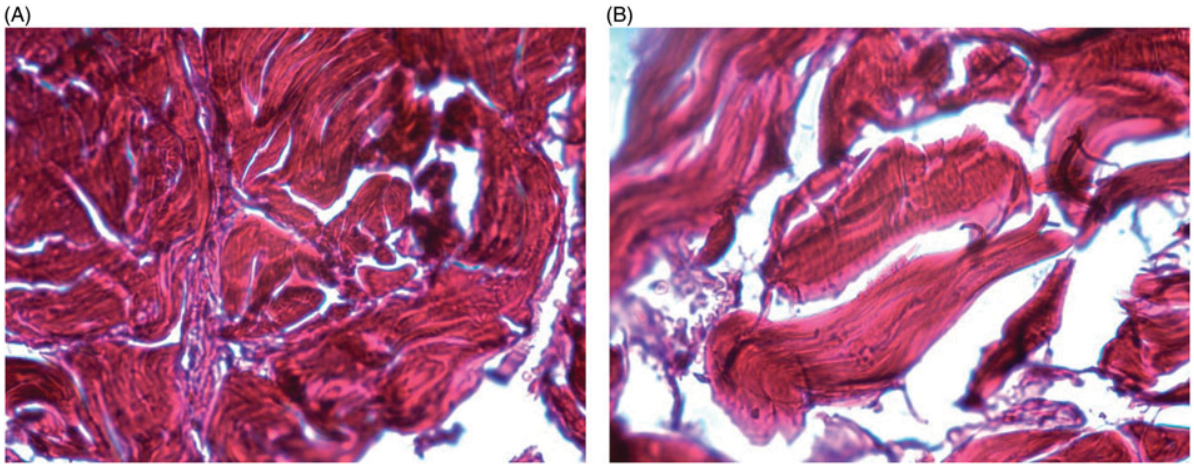


Figure 3. Linear regression between the density of collagens fiber and level of testosterone.

including participation in the overactive bladder, due to its activation of actin–myosin complex that causes muscle contractions, regardless of the levels of cytosolic free calcium. This pathway is also modulated by sex hormones and the ratio estrogen/testosterone imbalance, as occurs in aging and obesity [3], which seems to be the most important factor in the stimulation of RhoA/Rho-kinase [28].

Bhasin et al. [29] and Singh et al. [30,31] believe that androgens stimulate concomitantly the pluripotent cells to differentiate into muscle lineages including smooth muscle, inhibiting strains for the differentiation of adipocytes. To support this finding, Lorenzetti [13], in assessing the influence of testosterone on the bladder wall fibrosis in rats, observed that the sudden drop of testosterone by castration of young rats caused more muscle replacement by collagen fibers when compared to senile rats, whose process of hormonal decline is slow and gradual. Although the orchiectomy model is usually used to assess the influence of testosterone on the bladder, it does not seem appropriate to reproduce the process of late onset hypogonadis. Therefore, we decided to use only senile animals (18–28 months) in this work, which represents more risks and higher costs, but portrays adequately the physiological aging process, avoiding the sample selection bias.

Baseline levels of testosterone in both groups were below the levels described by Kinoshita et al. [32] in 7-month-old



Figures 4. (A) High levels of collagen replacement on penile muscle structure (Control Group). (B) Low levels of collagen replacement on penile muscle structure (Testosterone replacement group). Colored by picrosirius, 40×.

rats (2.2 ± 0.3 ng/dl average testosterone), but very similar to the 21-month-old rats (1.1 ± 0.2 ng/dl average testosterone). This fact demonstrates the physiologic decrease of testosterone in rats in this study. Early studies using the replacement model with 50 mg/kg of TU per month have been conducted for about 12 years, showing that this choice is simple, reproducible and effective for hormone replacement in rats [33,34]. Another variation of the experimental model of hormone supplementation, described more recently, uses 100 mg/kg of TU, in a single dose, and its effects can be evaluated after 2 months of administration [35]. Monthly dosage was chosen in this study for its consolidation in literature. This method of supplementation caused a mean serum testosterone of about 3.2 ng/dl in the group that underwent hormone replacement therapy, which was not too superior as the values of young rats (2.2 ± 0.3 ng/dl average testosterone) [32].

For quantitative analysis of fibrotic reaction in the penile cavernous muscle, it was performed the stereological study of collagen fibers in both groups [24]. The volume density (Vv), magnitude used in this study is a stereological parameter that produces reliable results with minimal variation. At the same time, it is not dependent on histologic complex blade or experience of the researcher, being employed in the quantification of fibrous component of the extracellular matrix, particularly in ace collagen and elastic fibers of various tissues [36–38].

Under polarized light, type I collagen fibers appear as being thick, strongly birefringent, with colors ranging from yellow to intense red [39]. The use of polarized light for analysis of picrosirius red stained samples is a special procedure for histological analysis of type-specific collagen [40], and it is not necessary to evaluate fibrotic processes, especially due to the prevalence of type I collagen in these processes. Nevertheless, picrosirius red polarization facilitates the identification and counting of collagen mass in relation to the detrusor muscle, improving the accuracy of the method [39]. The time between intervention and evaluation of the final result on this issue was 2 months and this period of time has been the most used to assess changes in rat muscles [35,41,42].

Ludwig et al. [42] found oxidative stress and apoptosis increased in the bladder of rats subjected to castration; however, it could be reduced by supplementation with α-tocopherol. Still, it was not clear whether the rise of the apoptotic process increases the risk of bladder dysfunction. In fact, oxidative stress is one of the most important factors involved in the pathogenesis of age-related detrusor dyskinesia [43] and may be aggravated by diabetes mellitus [44] and by the hypogonadism [42]. On the other hand, vitamin E produces protective effect against free radicals, especially when used in an early stage of tissue injury [46]. Helmy and Senbel [45] proved that antioxidant therapy with vitamin E ameliorates the age-associated erectile dysfunction and Zhang et al. correlated an antioxidant dietary with the improvement of arteriogenic erectile dysfunction [46].

Cayan et al. [47] showed that rats with bilateral orchiectomy, testosterone replacement associated with estradiol had better results than androgen replacement alone regarding the preservation of the muscle/collagen ratio, demonstrating the stroma modulating role of testosterone, regardless of gender [47]. Tek et al. [35] used the model of castrated 10-month-old rats to evaluate the effect of testosterone replacement (100 mg/kg TU dose) on bladder function and histology. They concluded that hormone replacement in these animals produced an improvement in the smooth muscle/collagen ratio, including the development of bladder capacity.

Our study was a pioneer in demonstrating that testosterone, even lower than the doses used in the works mentioned above, favored the gradual development of smooth muscle cells in penis of rats with senile hormonal decline and testosterone supplementation in aged rats protected them against the process of remodeling/fibrosis of the penis. These finding can implicate that the testosterone levels can influence, not only the libido, but also the erectile function in elderly population.

Declaration of interest

The authors report no conflict of interests.

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Effects of testosterone supplementation on prevention of age-related penile remodeling - Abstract

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Erectile dysfunction develops among 46.2% of men between 40 and 70 years.

Studies demonstrated substitution on detrusor muscle by collagen due testosterone deprivation. It is clear the correlation among aging and oxidative stress, accelerating apoptosis process in many tissues. This study aims to demonstrate the collagen substitution over the muscle fibers on muscle structure of rat's penis and the effects of testosterone supplementation. Sixteen senescent Wistar rats were divided into two groups: treatment (receiving standard supplementation testosterone dose) and control (receiving equivalent saline solution). Testosterone was dosed on D0 and D56 of study. All penises were prepared with picrosirius colored histology; stereology was applied to determine the volumetric density of collagen fibers (Vv). Analysis of variance demonstrated testosterone group's replacement therapy to be effective, while the androgenic decline continued by the time of experiment in control group ($p < 0.05$). Testosterone group had Vv of 20.6%, lower than control group (47.8%); t-test ($p < 0.001$). Pearson's correlation demonstrated an inverse correlation between the Vv and testosterone's levels ($p < 0.001$). This is a pioneer study on demonstration of structural alterations over the cavernous corpora muscle caused by deprivation of testosterone on elderly rat. These finding implicate that the testosterone levels can influence, not only the libido, but also the erectile function.

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