UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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EXPRESSÃO E REPOSIÇÃO ESTROGÊNICA E ANDROGÊNICA NO LOBO VENTRAL DA PRÓSTATA DE CAMUNDONGOS DIABÉTICOS (NOD) FRENTE A TERAPIA INSULÍNICA

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

Orientadora: Profa. Dra. Valéria Helena Alves Cagnon Quitete

Campinas, 2009

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

F989e

Fávaro, Wagner José
Expressão e reposição estrogênica e androgênica no lobo ventral da próstata de camundongos diabéticos (NOD) frente a terapia insulínica / Wagner José Fávaro. – Campinas, SP: [s.n.], 2009.

Orientadora: Valéria Helena Alves Cagnon Quitete.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Diabetes. 2. Hormônios esteróides. 3. Morfologia. 4. Imunohistoquímica. 5. Insulina. I. Cagnon, Valéria Helena Alves, 1967-. II. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

(rcdt/ib)

Título em inglês: Androgen and estrogen replacement and expression in the ventral lobe of the prostate of non-obese diabetic mice (NOD) following insulin therapy.
Palavras-chave em inglês: Diabetes; Steroid hormones; Morphology; Immunohistochemistry; Insulin.
Área de concentração: Anatomia.
Titulação: Doutor em Biologia Celular e Estrutural.
Banca examinadora: Valéria Helena Alves Cagnon Quitete, Athanase Billis, Silvana Martinez Baraldi Artoni, Edson Rosa Pimentel, Classius de Oliveira.
Data da defesa: 31/07/2009.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 31 de Julho de 2009.

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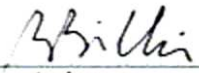

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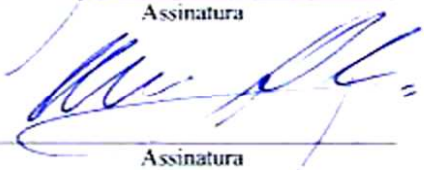

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DEDICATÓRIA

A DEUS...

"O Senhor é meu rochedo, minha fortaleza e meu libertador.

Meu Deus é minha rocha, onde encontro o meu refúgio,

Meu escudo, força de minha salvação e minha cidadela" (Salmo 31:2-4)

AOS AMORES DE MINHA VIDA

MEUS PAIS, ADILSON E CARMEN,

"A minha gratidão pela educação que me proporcionaram, e por termos percorridos juntos mais uma parte do longo caminho de minhas conquistas. Pelo amor único e infinito; fraterno, amigo e confidente que sempre zela e se oferece por inteiro."

AOS MEUS IRMÃOS, MARCO E PATRÍCIA,

"Pelo exemplo, compreensão e incentivo. Esta conquista é de todos nós."

AGRADECIMENTOS

À **Profa. Dra. Valéria Helena Alves Cagnon Quitete**, orientadora e amiga sempre, que com disponibilidade, bravura e capacidade soube me conduzir no caminho de crescimento intelectual. Minha eterna gratidão. Que o Grande Arquiteto do Universo ilumine sempre seu caminho e de sua família.

À **CAPES/PROEX**, **CNPq** e **FAPESP** pelos auxílios, o que tornou possível à execução e finalização deste trabalho.

Aos Mestres **Profa. Dra. Maria Júlia Marques**, **Prof. Dr. Humberto Santo Neto** e **Prof. Dr. José Ângelo Camilli**, por guiarem meu início e incentivo constante. Muito obrigado.

Aos Professores **Dr. Edson Rosa Pimentel** e **Dra. Laurecir Gomes**, pela amizade e pelo grande apoio durante todo esse período.

Aos Professores **Dr. Ubirajara Ferreira** e **Dr. Athanase Billis**, pelas valiosas contribuições durante a execução desse trabalho.

Aos meus "filhos" e "filhas" do Laboratório de Biologia da Reprodução, **Amanda Cia Hetzl**, **Eduardo Marcelo Cândido**, **Fábio Montico**, **Leslie Cristina Pinto** e **Raísa Mistieri Lorencini**.

Ao amigo, **Leonardo Oliveira Reis**, pela dedicação e apoio.

Á todos do **Programa de Pós Graduação em Biologia Celular e Estrutural**.

Ao **Prof. Dr. Carlos Roberto Padovani**, pelo auxílio na execução da análise estatística.

À **Profa. Dra. Luciana Bolsoni Lourenço Morandini,** ao **Prof. Dr. Wagner Eduardo Matheus** e ao **Prof. Dr. José Ângelo Camilli** pelas pertinentes considerações no exame de qualificação.

Ao **Prof. Dr. Athanase Billis**, ao **Prof. Dr. Edson Rosa Pimentel**, à **Profa. Dra. Patrícia Fernanda Felipe Pinheiro**, ao **Prof. Dr. Classius de Oliveira e ao Prof. Dr. Humberto Santo Neto**, pela indicação dos nomes, pelas correções e lapidação deste trabalho.

À **Sra. Ana Floriano Rodrigues**, pela atenção que me dispensou durante todo o período no Departamento de Anatomia.

Em especial a **Sra. Lilian Alves Senne Panagio**, sempre presente, amiga e que com grande maestria dispensou-me atenção durante todo o Doutorado.

Aos **Srs. Norivaldo Celestino** e **Marco Aurélio Ribeiro de Paula**, pela disposição em ajudar durante a parte experimental deste trabalho.

À **Sra. Marlene Lima Francisco**, pela manutenção e cuidados com os animais utilizados neste trabalho.

Aos **Srs. Paulo Afonso Bernardes**, **Carlos Roberto Gonçalves**, **Paulo Francisco dos Santos** e **Toni Donizeti dos Santos**, pelo apoio durante a realização deste trabalho.

Aos **colegas do Curso de Pós-Graduação**, por ensinarem um pouco mais sobre a convivência em grupo.

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RESUMO

Estudos clínicos e experimentais indicam que o diabetes provoca alterações no eixo hipotálamo-hipófise-gonadal, prejudicando o funcionamento prostático. Assim, os objetivos do presente estudo foram caracterizar os aspectos morfológicos, proliferativos e imunológicos dos compartimentos epitelial e estromal do lobo ventral da próstata de camundongos diabéticos após controle glicêmico prolongado e reposição hormonal. Além disso, correlacionaram-se os resultados obtidos a possíveis processos de patogênese prostática. Um total de 40 camundongos machos da linhagem Nod (diabético não-obeso) e 08 camundongos controles BALB/c/ Uni, com 18 semanas de idade, foram divididos em seis grupos, após 20 dias de diabetes: Grupo Controle recebeu injeções subcutâneas de 5mL/Kg de soro fisiológico 0,9% diariamente por 20 dias; Grupo Diabético recebeu o mesmo tratamento que o grupo controle; Grupo Diabético-Insulina recebeu injeções subcutâneas de 4-5UI de insulina NPH diariamente por 20 dias; Grupo Diabético-Testosterona recebeu injeções subcutâneas de 5mg/Kg de Cipionato de Testosterona em dias alternados por 20 dias; Grupo Diabético-Estrógeno recebeu injeções subcutâneas de 25μg/Kg de 17β-estradiol em dias alternados por 20 dias; Grupo Diabético-Insulina-Testosterona-Estrógeno recebeu tratamento simultâneo com insulina, testosterona e estrógeno, nas mesmas concentrações que nos grupos diabético-insulina, diabético-testosterona e diabético-estrógeno. Após 20 dias de tratamento todos os animais foram sacrificados e amostras do lobo ventral da próstata foram coletadas para análises morfológicas, morfométricas, imunohistoquímicas, Western Blotting e hormonais. Os resultados mostraram marcantes desorganizações estruturais e diminuição das proteínas de adesão, α e β distroglicanas, as quais foram mais intensas no grupo diabético em relação aos demais grupos. O estado diabético mostrou uma taxa de proliferação e apoptose duas vezes maior em relação ao grupo controle. Ainda, os resultados mostraram diminuição dos níveis séricos de testosterona e seus receptores, sendo que o grupo diabético apresentou menor valor para essa variável seguido em ordem crescente pelos grupos diabético-insulina, diabético-testosterona, diabético-estrógeno e diabético-insulina-testosterona-estrógeno. Os

níveis séricos de estradiol e seus receptores tiveram relação oposta à testosterona. O receptor de IGF-1 apresentou intensa localização no grupo diabético. Assim, pode-se concluir que o diabetes comprometeu o balanço hormonal e a interação epitélio-estroma, causando prejuízo morfológico e funcional desse órgão caracterizado pela diminuição da imunolocalização das proteínas de adesão. A associação entre insulina e reposição de hormônios sexuais esteróides foi determinante para a recuperação estrutural e hormonal. Além disso, o aumento da imunolocalização de IGF-1 sugeriu que o diabetes pode ser considerado um fator deflagrador de processo mitogênico na próstata. Contudo, a reposição hormonal não recuperou os níveis normais de IGFR-1. Por outro lado, a reposição de insulina e hormônios esteróides indicou recuperação parcial dos níveis desse receptor. Um bom entendimento da relação entre esses fatores poderá melhorar as atuais terapias para o tratamento de doenças prostáticas, bem como dos processos diagnósticos nesse órgão.

ABSTRACT

Diabetes adversely affects prostate morphology and function through alterations in the hypothalamic-hypophyseal-gonadal axis. Thus, the aims of this study were to characterize morphological, proliferative and immunological features of the prostate of diabetic mice after long term glycemic control and hormonal replacement, and as well as to relate these parameters to prostate pathogenesis. A total of 40 mice Nod (Non-obese diabetic) and 8 control mice (BALB/c/Uni), 18 weeks old, were divided into six groups after 20 days of diabetes: the control group received a daily dose of 0.9% NaCl (5 mL/kg, s.c.) for 20 days, as did the diabetic group. The diabetic-insulin group received daily doses of NPH insulin (4-5 IU, s.c.), the diabetic-testosterone group received a supraphysiological dose of testosterone cypionate (5 mg/kg, s.c.) every other day for 20 days, the diabetic-estrogen group received a supraphysiological dose of 17β-estradiol (25 μg/kg, s.c.) every other day for 20 days and the diabetic-insulin-testosterone-estrogen group received insulin, testosterone and estrogen, simultaneously, at the same concentrations given to the other groups. The mice were sacrificed after 20 days of treatment and samples from the prostatic ventral lobe were processed for morphological, morphometrical, immunological, western blotting and hormonal analyses. The results showed structural disorganization and diminished adhesion proteins, α and β dystroglycans, which were more intense in the diabetic group than in the other groups. The diabetic state showed a proliferation and apoptosis rate that was two times higher than that found in the control group. Also, there was a decrease in serum testosterone levels (diabetic mice and diabetic-insulin-testosterone-estrogen mice had the greatest and smallest decreases, respectively) and in the level of androgen receptor immunolocalization. The serum estrogen level and its receptor showed changes opposite to those of testosterone and its receptor. The greatest IGF receptor localization occurred in diabetic mice. Thus, it could be concluded that diabetes disturbed the prostatic hormonal balance and the stroma-epithelium interaction, leading to morphological and functional imbalance of this organ characterized by the decrease immunolocalization of the adhesion proteins. Concomitant treatment with insulin and

steroid hormone therapy was determinative for glandular structural and hormonal restoration. Furthermore, the increased immunolocalization of IGF-1 suggested that diabetes may be an important factor to the mitogenic process. However, hormonal therapy did not restore the distribution of IGF-1 to normal. On the other hand, concomitant treatment with insulin and steroid hormone therapy showed partial recovery of the IGFR-1 levels. A proper understanding of the relationship between these two factors could improve the current therapies for treating prostate diseases as well as diagnostics.

I-INTRODUÇÃO

I.1- Diabetes Mellitus e Generalidades

O diabetes Mellitus é uma doença que promove alterações do metabolismo de proteínas, carboidratos e gordura levando à hiperglicemia proveniente da deficiência insulínica (Mokdad, 2001; Conget, 2002). Essa doença é causada por deficiência na secreção pancreática de insulina e/ ou incapacidade dos tecidos em responder eficientemente à insulina, gerando hiperglicemia e afetando todos os órgãos (Öztürk, 1996; Robbins et al., 1996).

Dois tipos de diabetes são descritos, o tipo I e o tipo II. O diabetes do tipo I (infanto-juvenil) ou Diabetes Mellitus Dependente de Insulina (IDDM) é uma doença crônica que se manifesta em crianças e adolescentes, caracterizada pela destruição das células beta do pâncreas produtora de insulina, resultando em severa ou absoluta falta de insulina, além de alta tendência a cetoses (Ritz, 2002). Os portadores deste tipo de diabetes necessitam de doses diárias de insulina para minimizar os efeitos sistemáticos da doença e promover melhorias significativas na qualidade de vida (Stefan, 1996). O diabetes do tipo II (adulto) ou Diabetes Mellitus Não Dependente de Insulina (NIDDM), que é caracterizado por estabilidade e relativa insensibilidade à insulina com ausência de cetoses. A obesidade é uma das características marcantes do diabetes tipo II, levando a diminuição do número de receptores de insulina nas células alvo tornando a quantidade de insulina ineficiente (Robbins et al., 1996; Stefan, 1996; Ritz, 2002).

A incidência mundial do diabetes tem aumentado substancialmente nos últimos anos, com cerca de 164 milhões de portadores (Sociedade Brasileira de Diabetes, 2009; Ministério da Saúde, 2009). Dados recentes mostraram que o diabetes é a terceira causa de morte no mundo, superada apenas pelas doenças cardiovasculares e os diferentes tipos de cânceres (Sociedade Brasileira de Diabetes, 2009). Estima-se que, o Diabetes afeta 6,8% da população norte americana e 7,6% dos brasileiros (IDF Diabetes e-Atlas, 2009; Ministério da Saúde, 2009). No Brasil, cerca de 20662 pacientes diabéticos foram registrados no

Sistema Único de Saúde (SUS) somente no período de janeiro de 2009 a abril de 2009 (Ministério da Saúde, 2009). Além disso, o diabetes é uma doença que debilita os portadores levando-os a várias complicações como: deficiências vasculares, renais, oftálmicas, neurológicas, bem como perda de peso, problemas digestivos, alteração no processo de cicatrização de lesões, sendo que na maioria dos casos leva o indivíduo ao afastamento das atividades de trabalho (Ciardullo et al., 2004).

Apesar de se conhecer os malefícios provenientes do estado diabético ainda persistem dúvidas, especialmente, pela variedade de perfis clínicos apresentados por essa doença, além da idade de manifestação da doença e a incidência nos diferentes grupos étnicos e geográficos (Creutzfeld et al., 1976). Assim, freqüentemente são realizadas tentativas para elucidar quais os efeitos deletérios nos sistemas orgânicos e, se esses efeitos podem ser revertidos através de tratamentos e/ ou dieta. Baseado nessas questões, a utilização de animais que expressem o diabetes, seja por indução química através de drogas ou por espontaneidade vem sendo freqüente e de grande utilidade. Para obtenção de um animal diabético por indução química são utilizadas drogas como estreptozotocina e aloxana, especificamente, diabetogênicas (Perez et al., 1998; Kovacs et al., 1998; Palomar-Morales et al., 1998; Saprykina et al., 1998; Avedano et al., 1999; Orie & Anyaegbu, 1999; Ravikumar & Anuradha, 1999; Yildirim et al., 1999). Os animais mais utilizados nesses experimentos são cães e roedores. Alguns roedores, como o camundongo da linhagem C57BL/Ksj homozigotos diabéticos (db/db), o Wistar Chinês e Nod (diabético não obeso) (Kikutani & Makino, 1992), apresentam síndrome semelhante ao diabetes humano tipo II e I, respectivamente. O camundongo NOD foi descoberto no Japão em 1974, o qual exibia poliúria, severa glicosúria e rápida perda de peso, sendo por isso chamado de não obeso (Makino et al., 1985). As características fisiológicas apresentadas pelos animais NOD eram muito semelhantes aos sintomas clínicos do diabetes tipo I (polidipsia, poliúria, polifagia, emaciação, retinopatias, nefropatias e distúrbios sexuais). Além disso, conforme ocorre em humanos, o diabetes desenvolvido nesses animais é uma conseqüência de um defeito genético no sistema imune, sendo, portanto, uma doença autoimune (Homo-Delarche, 2001).

I.2- Diabetes Mellitus e Próstata

No sistema genital masculino, diferentes estudos experimentais com a indução do diabetes tipo I quimicamente, ou com animais os quais desenvolveram espontaneamente esta doença demonstraram complicações como ejaculação retrógrada (Ellenberg, 1980), disfunções na bexiga urinária (Buck et al., 1976; Stefan, 1996), impotência sexual, diminuição do número de espermatozóides no líquido seminal (Frenkel et al., 1978 e Mcculloch et al., 1984). Também, Jackson & Hutson (1984) e Saito et al. (1996) constataram redução dos pesos dos órgãos reprodutivos masculinos como testículos, próstata, vesícula seminal e glândula de coagulação de ratos diabéticos. Posteriormente, Cagnon et al. (2000) e Ribeiro et al. (2006) observaram na próstata ventral de camundongos espontaneamente diabéticos atrofia das células epiteliais secretoras, hipertrofia estromal, processos inflamatórios, neoplasia intraepitelial prostática (NIP) e dilatação das organelas envolvidas no processo secretor glandular. Em adição, Carvalho et al. (2003) constataram modificações na glândula coaguladora de camundongos diabéticos, tais como: espaçamento entre os ácinos, infiltrado inflamatório na região estromal e hipertrofia dos componentes da matriz extracelular.

Também, outros estudos revelaram diminuição dos níveis séricos de testosterona com conseqüente redução dos níveis de receptores androgênicos na próstata de animais diabéticos devido ao desequilíbrio no metabolismo desse hormônio (Okasen, 1975; Tesone et al., 1976). Ainda, Wang et al. (2000) verificaram que o controle do nível glicêmico de ratos diabéticos, através da administração de insulina, não restaurou o peso da próstata. Contudo, a utilização simultânea de testosterona e insulina em ratos diabéticos foi necessária para recuperação morfofuncional da próstata, embora as condições morfofisiológicas prostáticas não foram similares aos padrões de normalidade (Sufrin & Scott, 1972; Tesone et al., 1980; Ho, 1991). Segundo Soudamani et al. (2005) roedores que expressaram o Diabetes mellitus tipo I apresentaram comprometimento no desenvolvimento e diferenciação da próstata ventral, sendo que a administração de insulina exógena minimizou parcialmente os efeitos dessa doença.

I.3- Generalidades e Morfologia da Próstata

A próstata é uma glândula sexual acessória masculina presente em todas as ordens de mamíferos (Setchell & Brooks, 1988; Marker et al., 2003; Untergasser et al., 2005). Na espécie humana, encontra-se localizada ao redor da uretra, inferiormente à bexiga urinária onde são descritas três regiões glandulares: zona periférica, zona central e região pré-prostática, envoltas por uma fina camada fibromuscular (Setchell & Brooks, 1988). A secreção prostática é formada por diferentes constituintes como ácido cítrico, ácido siálico, espermina e prostaglandinas, enzimas como amilase, fibrinogenase, aminopeptidase, transglutaminase, fosfatases ácida e alcalina, uma cascata de fibrinolisinas e zinco (Blandy & Lytton, 1986; Lin & Bissel, 1993; Bull et al., 2001), as quais propiciam condições favoráveis para a capacitação e sobrevivência dos espermatozóides (Guyton & Hall, 1996; Lin & Bissel, 1993; Bull et al., 2001).

Nos roedores, a próstata divide-se em três pares de lobos: ventral, lateral e dorsal de acordo com a localização ao redor da uretra prostática e um par de glândulas coaguladoras ou próstata anterior localizadas na face côncava das vesículas seminais (Jesik et al., 1982; Sugimura et al., 1986; Aumuller & Seitz, 1990). Esses lobos estão conectados à uretra por uma série de ductos e são funcionalmente similares à próstata humana. (Jesik, et al.1982; Slayter et al., 1994). Os diferentes lobos prostáticos diferem quanto à morfologia, aos tipos de produtos secretados e à resposta hormonal (Colombel & Buttyan, 1995; Costello & Franklin, 1994). De maneira geral, os lobos prostáticos são compostos por um conjunto de estruturas túbulo-alveolares, onde o epitélio secretor simples encontra-se envolvido pelo estroma (Aumüller & Ader, 1979). A membrana basal está localizada entre o epitélio e o estroma, tendo como seus principais componentes o colágeno tipo IV e a laminina (Knox et al., 1994).

O epitélio prostático simples é formado por células colunares, com citoplasma apresentando complexo de golgi, retículo endoplasmático granular, grânulos de secreção e mitocôndrias. Também, entremeadas às células epiteliais evidenciam-se células basais, as quais estão intimamente relacionadas ao transporte e distribuição de substâncias entre os compartimentos epitelial e estromal (McNeal et al., 1988; Abate-Shen & Shen, 2000; Garraway et al., 2003).

O estroma prostático é formado por um arranjo complexo de células estromais e matriz extracelular associado a fatores de crescimento, moléculas reguladoras e enzimas de remodelação, as quais provêm sinais biológicos gerais e exercem influências mecânicas sobre as células epiteliais (Tuxhorn et al., 2001; Cunha & Matrisian, 2002). Também, vasos sanguíneos, terminações nervosas e células imunes constituem partes integrais do estroma (Tuxhorn, et al., 2001). Os fibroblastos e as células musculares lisas são importantes tipos celulares do estroma prostático. A principal função destas é sintetizar componentes estruturais e reguladores da matriz extracelular. A matriz extracelular é uma rede de proteínas fibrilares, glicoproteínas adesivas e proteoglicanos (Lin & Bissel, 1993; Kreis & Vale, 1999; Tuxhorn, et al., 2001), sendo um reservatório de fatores de crescimento ativos e latentes (Taipale & Keski-Oja, 1997; Tuxhorn, et al., 2001). Além disso, componentes estruturais como colágeno e fibras elásticas, proporcionam rigidez mecânica e flexibilidade ao tecido. Os proteoglicanos regulam a estrutura e a permeabilidade da matriz extracelular, ligando-se a fatores de crescimento, proteases e inibidores de proteases, modulando a atividade destes (Taipale & Keski-Oja, 1997; Kreis & Vale, 1999 e Tuxhorn, et al., 2001). Assim, em associação, células estromais e matriz extracelular criam um microambiente que regula o crescimento e diferenciação funcional das células adjacentes, desempenhando cada um desses, importante papel na manutenção da forma e função tecidual (Labat-Robert et al., 1990; Tuxhorn et al., 2001; Cornell et al., 2003).

Diferentes processos biológicos na glândula prostática tais como regulação da proliferação e diferenciação celular, atividade mitogênica, processos secretores e crescimentos tumorais são regulados e/ ou influenciados por diferentes polipeptídeos como os fatores de crescimento homólogos a insulina (IGF), fatores de crescimento fibroblásticos (FGF), fatores de crescimento do endotélio vascular (VEGF) e pelos fatores de crescimento transformadores (TGF) (Ryan et al., 1992; Kerr et al., 1995; Djavan et al., 2001; Takahashi et al., 2003; Zhao et al., 2004; Marszalek et al., 2005). Os IGF na próstata são produzidos pelas células estromais e atuam como fatores de crescimento parácrinos no epitélio prostático normal (Djavan et al., 2001). Existem dois tipos de receptores transmembrana de IGF na glândula prostática, a saber, o receptor tipo 1 (IGFR-1), que é expresso nas células epiteliais e estromais, e o receptor tipo 2 (IGFR-2) o qual não foi

encontrado tanto nas células epiteliais quanto no estroma prostático (Djavan et al., 2001). Os efeitos proliferativos e mitogênicos dos IGF nas células prostáticas são modulados pelas proteínas ligantes de IGF (IGF-BPs) (Takahashi et al., 2003). A expressão acentuada dos IGF e das IGF-BPs pode estimular a proliferação de células cancerosas e suas metástases (Denley et al., 2005; Neuvians et al., 2005). Estudos clínicos e epidemiológicos demonstraram que a elevação dos níveis séricos de IGF-1 constitui um potente fator de risco para o início da hiperplasia benigna e da carcinogênese prostática (Djavan et al., 2001; Takahashi et al., 2003; Pandini et al., 2005).

As distroglicanas (DGs) são proteínas integrais de membranas expressadas por uma variedade de tecidos, interagindo com proteínas extracelulares. Dentre estas, podemos citar a laminina, a perlecam, a agrina e as proteínas de membrana (Sugita et al., 2001). De maneira geral, as DGs são moléculas de adesão responsáveis pela crucial interação entre matriz extracelular e o compartimento citoplasmático. As DGs são formadas por duas subunidades α e β, que interagem para formar um complexo funcional não-covalente, sendo codificadas pelo mesmo gene (Brennan et al., 2004). A DG-α é extracelular e a DG-β é transmembrânica, sendo que a α-DG se liga às proteínas da matriz extracelular e proteoglicanos, como a laminina e as agrinas. Já a DG-β ancora a DG-α para a membrana e é ligada ao citoesqueleto de actina via distrofina (Sgambato et al., 2003). Assim, as distroglicanas e outras glicoproteínas como as sarcoglicanas constituem juntamente com a distrofina importante complexo responsável pela integridade estrutural da matriz extracelular e do citoesqueleto (Brennan et al., 2004).

Por muito tempo, o interesse pelas DGs limitou-se ao seu papel nas doenças musculares. Contudo, apesar das DGs terem sido descobertas em músculos esqueléticos, como um componente do complexo glicoproteína-distrofina, elas são expressas em muitos tipos celulares diretamente adjacentes à membrana basal, como nervo periférico e epitélio de revestimento (Durbeej et al., 1998). Assim, considerando-se a importante participação das DGs na interação célula e matriz extracelular, esta vem sendo estudada em uma variedade de tecidos, dentre eles as células epiteliais. O papel das diferentes moléculas de adesão, como as DGs e seus ligantes não foram completamente elucidados. É evidente que essas proteínas têm múltiplas funções como: conectar as células epiteliais à lâmina basal e

contribuir para a troca de informações com o microambiente extracelular, conectar e organizar o citoesqueleto e participar no processo de sinalização e transdução. Portanto, as moléculas de adesão são importantes no crescimento tecidual, diferenciação e manutenção da citoarquitetura de diferentes órgãos, incluindo a próstata (Brennan et al., 2004).

A interação epitélio-estromal tem papel primordial na manutenção da estrutura e funcionamento da glândula prostática (Ekman, 2000). Baseando-se em aspectos morfológicos, funcionais e embriológicos, esta interação pode ser considerada como única unidade funcional (Aumuller & Seitz, 1990; Hayward & Cunha, 2000). A membrana basal é o ponto de união dessa interação oferecendo suporte mecânico e fisiológico ao epitélio secretor (Knox, et al., 1994; Hayward & Cunha, 2000). O desequilíbrio da interação epitélio-estromal na glândula prostática favorece a formação do carcinoma prostático (Cunha et al., 2002). As células estromais associadas às células tumorais respondem aos andrógenos e fatores de crescimento levando a interrupção da homeostase epitélio-estromal, o que desencadeia processos de crescimento, migração, angiogênese, apoptose e metástases tumorais (Wong et al., 2000; Cunha et al., 2001; Cunha et al., 2003; Cornell et al; 2003).

I.4- Próstata e Hormônios

A morfogênese, a manutenção da atividade funcional e da morfologia, a proliferação e a diferenciação das células da próstata são reguladas por andrógenos (Leav et al., 2001; Cunha et al., 2002; Imamov et al., 2005). O desenvolvimento andrógeno-dependente do sistema urogenital masculino ocorre via interações epitélio-estroma no qual o andrógeno orienta o desenvolvimento epitelial através de mecanismos parácrinos, mediados por receptores androgênicos no estroma glandular (Cunha et al., 1992). Os andrógenos expressam seus efeitos biológicos através da interação com receptores intracelulares específicos sendo que, o complexo receptor-hormônio associado à cromatina nuclear, regula a expressão do gene específico (Prins et al., 1991).

A testosterona e a dihidrotestosterona (DHT) são os principais andrógenos a induzir a diferenciação prostática (Hsing, 2002, Toorians et al., 2003). A DHT é resultante da conversão da testosterona através da enzima 5α-redutase (Prins et al., 1991; Toorians et al.,

2003). Embora, a testosterona e a DHT utilizem o mesmo receptor de andrógeno (AR) para atuarem no tecido prostático, essas ações parecem estar associadas a diferentes funções teciduais (Prins et al., 1991; Toorians et al., 2003). Tanto a testosterona quanto a DHT são capazes de manter a atividade prostática, porém a DHT é 10 vezes mais potente que a testosterona, devido a sua dissociação do receptor de andrógeno ser mais lenta (Droller, 1997).

Apesar da próstata ser, primariamente, regulada por andrógenos, o seu desenvolvimento, tanto em humanos como em roedores, é sensível a outros hormônios, como os estrógenos, que atuam sinergicamente à testosterona, influenciando tanto as funções normais do órgão quanto às alterações patológicas (Weihua et al., 2001; Cunha et al., 2002). Os estrógenos possuem efeitos anti-androgênicos e regulam negativamente o eixo hipotálamo-hipófise-gonada, com redução da produção de andrógenos pelas células de Leydig e decorrente involução do epitélio prostático e crescimento estromal em animais adultos (Weihua et al., 2002).

A biossíntese de estrógenos ocorre a partir de um substrato androgênico, através da aromatização desse hormônio pela enzima aromatase (Fishman & Goto, 1981; O' Donnell et al., 2001; Risbridger et al., 2003). Os efeitos estrogênicos na próstata são resultados da ligação desses hormônios em receptores estrogênicos específicos α e β (ERα, ERβ), os quais são predominantemente expressos no estroma e no epitélio, respectivamente (Risbridger et al., 2001; Cunha et al., 2002). Segundo Risbridger et al. (2001), utilizando tecido prostático recombinante de animais adultos *knockout* para os receptores estrogênicos α e ou β, demostraram que a resposta estrogênica completa no tecido prostático requer mecanismos parácrinos, tanto mediados por receptores α do estroma bem como receptores β do epitélio.

Os efeitos estrogênicos na próstata são complexos e podem envolver tanto ações diretas, através dos receptores, como indiretas, através do eixo hipotálamo-hipófise-gonadal (Cunha et al., 2002).

As ações diretas de estrógenos na próstata foram avaliadas através de um modelo de camundongos hipogonadal (hpg), os quais têm deficiência pós-natal em gonadotrofinas e testosterona, mas são sensíveis a hormônios. Nesse estudo, verificou-se resposta

proliferativa direta aos estrógenos nos lobos ventral e anterior da próstata e vesícula seminal desses animais. Tais alterações aberrantes foram demonstradas através de proliferação de fibroblastos no estroma e metaplasia das células epiteliais basais, além de processo inflamatório. Contudo, evidenciou-se redução das células musculares lisas e das células epiteliais secretoras (Bianco et al., 2002). Os resultados também mostraram que as mudanças frente à administração de estrógenos foram lobo-específicas, sendo que o lobo anterior da próstata mostrou-se mais sensível a ação estrogênica, quando comparado ao lobo ventral. Contudo, os autores destacaram que a resposta direta estrogênica pode depender de outras variáveis adicionais como a dosagem administrada, tempo de exposição e a presença de andrógenos (Bianco et al., 2002). Outro estudo utilizando camundongos estrógeno-modulados, os quais eram *Knockout* para enzima aromatase, demonstrou que doses elevadas de andrógenos têm efeitos morfológicos similares a doses elevadas de estrógenos. Os resultados exibiram doses periféricas e intraprostáticas elevadas de andrógenos, aumento dos receptores androgênicos, além da expansão dos volumes dos compartimentos estromal, epitelial e luminal indicando efeito proliferativo glandular (Jarred et al., 2002). Assim sendo, esses trabalhos evidenciaram que tanto os estrógenos como os andrógenos são elementos proliferativos para a próstata, porém em diferentes caminhos.

Além disso, na próstata existe um mecanismo denominado *imprinting* estrogênico ou estrogenização no desenvolvimento que se caracteriza pela exposição perinatal (do 1º ao 5º dia após o nascimento) ao estrógeno, a qual leva ao bloqueio da diferenciação normal das células epiteliais, cujos efeitos manifestam-se na adolescência e vida adulta (Prins & Birch, 1997; Prins et al., 2001). Em estudo com ratos neonatais, demonstrou-se que durante o desenvolvimento, a exposição desses animais a elevadas doses de estrógenos resulta em múltiplas mudanças no crescimento morfológico prostático, além de reduzir a sensibilidade à exposição de andrógenos na vida adulta, o qual inclui a aceleração da degradação dos receptores androgênicos prostáticos (Prins et al., 1991; Jarred et al., 2000). Também, outros efeitos da estrogenização na próstata incluem a ocorrência de atipia celular ao avançar da idade, processo inflamatório, hiperplasia epitelial e a emergência de lesões displásicas (Jarred et al., 2002; Bianco et al., 2002).

Estudos envolvendo os ERβ estrogênicos têm adicionado mais um nível de complexidade nos mecanismos de ações dos estrógenos na próstata (Weihua et al., 2002). Experimentos caracterizaram importante envolvimento dos ERβ nos mecanismos prostáticos, conjuntamente as ações exercidas pelos ERα, sendo os efeitos estrogênicos produto de um balanço dinâmico entre ERα e ERβ (Adams et al., 2002; Weihua et al., 2002). Os ERβ são expressos especialmente nas células epiteliais basais da próstata normal, sendo que essa subpopulação de células mostra importante propriedade biológica, com potencial efeito proliferativo sobre as células epiteliais além de envolvimento na carcinogênese (McNeal et al., 1995; Bonkhoff et al., 1999; Weihua et al., 2002; Imamov et al., 2005). Ainda, estudos têm postulado efeito antiproliferativo dos ERβ na glândula prostática (Chang & Prins, 1999; Imamov et al., 2004). Segundo Weihua et al. (2002), camundongos *Knockout* para os ERβ demonstraram focos de hiperplasia epitelial celular no lobo ventral da próstata aos 5 meses de idade, sendo que com 1 ano de idade esses animais desenvolveram neoplasias. Portanto, esse experimento confirmou a capacidade antiproliferativa relacionada aos ERβ. Em adição, outros estudos destacaram que ERβ podem estar envolvidos não só à um processo antiproliferativo epitelial mas também à diminuição do processo apoptótico epitelial glandular (Imamov et al., 2004). Esta afirmação baseou-se no fato de camundongos *Knockout* para os ERβ apresentar aumento da proliferação epitelial e decréscimo da apoptose (Imamov et al., 2004). Segundo Adams et al. (2002), os receptores estrogênicos β em conjunto com os hormônios androgênicos podem mediar diversos efeitos sobre a proliferação epitelial prostática, primeiramente promovendo a proliferação celular em períodos iniciais gestacionais e após isso agir de forma a limitar o crescimento celular em períodos tardios gestacionais em fetos humanos. Ainda, diversos estudos demonstraram que o ERβ é supra-regulado por andrógenos. (Adams et al., 2002; Asano et al., 2003).

Ainda, diversos autores têm demonstrado a relação dos estrógenos no desenvolvimento de doenças autoimunes, como lúpus eritematoso sistêmico, artrite reumatóide e Síndrome de Sjogren (Beeson, 1994). Os estrógenos, provavelmente, bloqueiam a destruição das células B auto-reativas imaturas na medula óssea de

camundongos resultando em autoimunidade (Bynoe et al., 2000). Contudo, o tratamento com estrógeno de camundongos com lúpus eritematoso sistêmico revelou aumento da incidência de doenças autoimunes, enquanto que o tamoxifeno e os antagonistas dos receptores estrogênicos parecem suprimir essa doença (Wu et al., 2000). Os efeitos dos estrógenos são determinados pelo balanço entre ERα e ERβ com as diferentes células do sistema imune (Shim et al., 2003). Na ausência total de estrógenos, o sistema imune é diferente na deficiência sistêmica de ERα e ERβ (Shim et al., 2004). Assim, os efeitos terapêuticos do ERα e ERβ e seus agonistas e antagonistas seletivos nas diferentes doenças autoimunes precisam ser amplamente investigados.

I.5- Lesões Prostáticas

A morfologia e a fisiologia da próstata têm sido examinadas com particular atenção devido às condições patológicas que atingem esse órgão. Dentre essas, podem-se destacar a hiperplasia benigna (HBP) e o câncer prostático (Marcelli & Cunningham, 1999; Leav et al., 2001). O câncer de próstata é a segunda maior causa de mortes por câncer na população ocidental masculina, embora sua etiologia não esteja totalmente estabelecida (Wong et al., 2000). A HBP caracteriza-se por uma predominante proliferação estromal e, embora um aumento substancial do epitélio também ocorra, a integridade regional da glândula é mantida (Droller, 1997). O câncer de próstata, em contraste, é considerado uma doença epitelial e, freqüentemente, estende-se além dos limites normais do órgão (Droller, 1997). O desenvolvimento do carcinoma prostático é de natureza endócrina e a possibilidade de ocorrer aumenta com a idade (Davies & Eaton, 1991).

Atualmente, a importância dos hormônios androgênicos e estrogênicos tem sido demonstrada no desenvolvimento de lesões prostáticas a partir de estudos clínicos e experimentais (Marcelli & Cunningham, 1999; Cunha et al., 2002; Tuxhorn et al., 2002; Risbridger et al., 2003).

Várias hipóteses têm sido correlacionadas à manifestação do carcinoma prostático. Estudos epidemiológicos consideram os hormônios estrogênicos como tendo papel fundamental na carcinogênese prostática (Montie & Pienta, 1994). É conhecido que, excessiva exposição aos estrógenos em diferentes etapas do desenvolvimento pode levar à

neoplasia prostática (Weihua et al., 2001). Walsh & Wilson (1976) confirmaram a relação de estrógenos e andrógenos usando modelo canino e demonstraram que os estrógenos podem causar o desenvolvimento de hiperplasia glandular.

Segundo Bianco et al. (2002), estudando experimentalmente as ações de andrógenos e estrógenos, verificaram que as atuações independentes de andrógenos e estrógenos têm potencial para iniciar mudanças na próstata, incluindo a hiperplasia e displasia, mas não a malignescência no órgão. Por outro lado, como esses dois hormônios agem em sinergismo para induzir a carcinogênese não é conhecido (Bianco et al., 2002). Também, Risbridger et al. (2003), através de estudos com animais *knockou*t para a enzima aromatase demonstraram que as alterações malignas na próstata são dependentes tanto das respostas estrogênicas como androgênicas e que nenhum desses hormônios isoladamente é capaz de deflagrar aberrantes modelos de crescimento, resultando em malignescência.

Segundo Prins et al. (1998), a regulação hormonal do RNA mensageiro para a expressão dos ERβ, sugere que ERβ no epitélio prostático pode ter importante papel na regulação do crescimento glandular. Estudos anteriores têm destacado que não há correlação entre a expressão dos ERβ na HBP (Weihua et al., 2002). Contudo, quando há ocorrência de câncer prostático verificou-se decréscimo do mesmo, com aumento da expressão em estágios avançados da doença incluindo metástase (Weihua et al., 2002).

Diferentes autores tem relacionado às alterações na expressão das DGs à cânceres de mama, cólon e próstata, dentre outros (Losasso et al., 2000; Henry et al., 2001; Sgambato et al., 2003; Brennam et al., 2004). Diversos tumores de mama e cólon demonstraram heterogeneidade na expressão da β-DG e baixa ou ausente expressão da α-DG, quando comparados aos tecidos epiteliais normais (Sgambato et al., 2003). Segundo Henry et al. (2001), no câncer de próstata de alto grau tem-se acentuada redução na expressão das DGs, levando à anormal interação da célula prostática com a matriz extracelular, o que certamente acarreta em progressão de metástases. Segundo Sgambato et al. (2007), a superexpressão de DGs inibe o crescimento e a tumorgenecidade de células prostáticas. Assim sendo, estes estudos indicam que as anormalidades na expressão das DGs podem ser fatores determinantes na patogênese prostática.

Estudos clínicos e epidemiológicos demonstraram que a elevação dos níveis séricos de IGF-1 constitui potente fator de risco para o início da hiperplasia benigna e da carcinogênese prostática (Djavan et al., 2001; Takahashi et al., 2003; Pandini et al., 2005). Ohlson et al. (2007) verificaram que a baixa expressão de receptores androgênicos associado à manutenção de síntese de IGF-1 no estroma prostático, pode resultar em redução da morte celular tumoral, após terapia de castração em casos de cânceres prostáticos.

Na clínica, tem sido sugerido que pacientes diabéticos tem poucas chances de desenvolver o câncer de próstata (Wedeirpass et al., 2002). Por outro lado, alguns autores já apontaram o diabetes como possível fator etiológico para o desenvolvimento do câncer de próstata (Ilic et al., 1996). Além disso, dados epidemiológicos demonstraram que indivíduos diabéticos por um período de cinco anos ou mais têm maior incidência de câncer prostático do que homens não diabéticos (Will et al., 1999). Somado a isso, foi sugerido que o rápido surgimento da HBP e sua progressividade em diabéticos é um fator de risco para o desenvolvimento clínico do câncer de próstata (Hammarsten & Hogstedt, 2002). Além disso, estudos com homens apresentando câncer de próstata, os quais receberam terapia de privação androgênica, demonstraram alto risco para o desenvolvimento de resistência à insulina e ocorrência de hiperglicemia (Basaria et al., 2006).

II-JUSTIFICATIVA E OBJETIVOS

A partir da literatura especializada é conhecido que a condição diabética causa complicações morfofisiológicas da glândula prostática levando a deficiência funcional. Também, é relatado que a administração de insulina nos portadores do diabetes tipo I é prática rotineira para minimização dos efeitos sistemáticos da doença, permitindo aos portadores melhorias significativas na qualidade de vida. Entretanto, é sabido que mesmo havendo controle glicêmico os hormônios sexuais como andrógenos, apresentam-se alterados comprometendo a homeostase dos órgãos que deles dependem, como a próstata. Também, a relação do tratamento insulínico, bem como a reposição hormonal são assuntos pouco conhecidos e que certamente contribuirão para a manutenção da estrutura e funcionamento da glândula prostática em indivíduos diabéticos. Assim sendo, a caracterização dos mecanismos hormonais na glândula prostática quando da ocorrência do diabetes e as relações da morfofisiologia prostática no desencadeamento de lesões desse órgão certamente levarão à melhoria das condições de vida nos indivíduos diabéticos e acrescentarão ao conhecimento da glândula prostática.

Assim, o presente trabalho teve como objetivos:

a) Caracterizar o comportamento estrutural epitelial e estromal e a distribuição das distroglicanas na glândula prostática de camundongos diabéticos frente ao controle glicêmico prolongado;

b) Caracterizar o comportamento estrutural epitelial e estromal em animais diabéticos frente a associação das reposições de insulina, testosterona e estrógeno;

c) Estabelecer correlações entre os eventos celulares, moleculares e a reatividade hormonal glandular, provenientes do diabetes experimental, à patogênese prostática;

d) Localizar e identificar a ocorrência das proteínas específicas para os receptores androgênicos, estrogênicos α e β, e IGF na próstata de camundongos diabéticos frente a reposição insulínica e hormonal;

III-MATERIAIS E MÉTODOS

No presente trabalho foram utilizados 40 camundongos machos da linhagem Nod (diabético não obeso) e 08 camundongos controles BALB/c/ Uni, com 18 semanas de idade, obtidos no Centro de Bioterismo da Unicamp. O nível de glicose no sangue foi aferido por glicemia capilar, utilizando aparelho Optium Advanced Diabetes Management System (MediSense, Abingdon, UK). Os camundongos (NOD) diabéticos foram aqueles que apresentaram glicemia capilar ≥ 300 mg/dl (Shirai et al., 1998). Após 20 dias de diabetes, os animais foram divididos em seis grupos (08 animais cada): Grupo Controle (BALB/ c/Uni) recebeu injeções subcutâneas de 5mL/Kg de soro fisiológico 0,9% diariamente por 20 dias (Fresenius Kabi, São Paulo, Brasil); Grupo Diabético recebeu o mesmo tratamento que o grupo controle; Grupo Diabético-Insulina recebeu injeções subcutâneas de 4-5UI de insulina NPH diariamente por 20 dias (Biobrás, Montes Claros, Minas Gerais, Brasil) (Anderson, 1983); Grupo Diabético-Testosterona recebeu injeções subcutâneas suprafisiológicas de 5mg/Kg de Cipionato de Testosterona (Deposteron-Sigma Pharma, São Paulo, Brazil), diluídos em 5mL de óleo de amendoim em dias alternados por 20 dias (Franck-Lissbrant et al., 1998); Grupo Diabético-Estrógeno recebeu injeções subcutâneas de 25μg/Kg de 17β-estradiol (Sigma Chemical Co., St Louis, MO, USA), diluídos em 25μL de óleo de amendoim em dias alternados por 20 dias (Prins et al., 2001); Grupo Diabético-Insulina-Testosterona-Estrógeno recebeu tratamento simultâneo com insulina, testosterona e estrógeno, nas mesmas concentrações que os grupos diabético-insulina, diabético-testosterona e diabético-estrógeno. As administrações de testosterona, 17β-estradiol e insulina foram interrompidas 24 horas antes do sacrifício. Após 20 dias de tratamento todos os animais foram pesados em balança semi-analítica Marte AS 5500 e sacrificados. Amostras do lobo ventral da próstata e de sangue foram coletadas para análises morfológicas, morfométricas, imunohistoquímicas, Western Blotting e de sorologia hormonal. Todos os procedimentos experimentais estavam de acordo com a Comissão de Ética em Experimentação Animal do Colégio Brasileiro de Experimentação Animal (COBEA/ protocolo 1472-1).

III.1- Dosagens Séricas

Amostras de sangue foram obtidas através de punção cardíaca, 24 horas após a administração da última dose dos hormônios em cada grupo experimental. O plasma foi separado por centrifugação (10000 rpm, -4°C por 10 minutos) e armazenado a – 20º C para subseqüentes análises. As concentrações de testosterona, estradiol e glicose foram mensuradas por radioimunoensaio usando os *kits* Coat-a-Count total Testosterone/ Estrogen (Diagnostic Products Corporation, Los Angeles, CA, USA), sendo as concentrações hormonais séricas expressas em ng/dL e pg/dL. A concentração sérica de glicose foi determinada por quimioluminescência e expressas em mg/dL.

III.2- Microscopia de Luz

Amostras do lobo ventral da próstata foram coletadas de quatro animais, de cada grupo, e fixadas em solução de Bouin por doze horas e/ ou paraformoldeído 4%. Após a fixação, os tecidos foram lavados em álcool etílico à 70%, com posterior desidratação em uma série crescente de álcoois. Posteriormente, os fragmentos foram diafanizados com xilol por 2 horas e inclusos em parafina e polímeros plásticos (Paraplast Plus, ST. Louis, MO, USA). Em seguida, os materiais foram seccionados em micrótomo (Biocut – Modelo 1130) com espessura de 5 micrômetros, com posterior coloração em Hematoxilina-eosina, Tricrômico de Masson (Junqueira et al., 1979), Reticulina de Gömori (Vilamaior et al., 2000) e fotografados no fotomicroscópio (Nikon Eclipse E-400) e submetidos à análises morfométricas.

III.3- Microscopia Eletrônica de Transmissão

Um total de quatro animais de cada grupo experimental foi perfundido com solução de Karnovsky (Sprando, 1990). A seguir as próstatas ventrais foram coletadas e pós-fixadas em tetróxido de ósmio a 1% por duas horas. Os fragmentos foram inclusos em resina plástica (Araldite, Polyscience, Niles, IL, USA) e seccionados com 0,5 micrômetro de espessura no ultramicrótomo LKB 8800 ultratome III e corados com azul de metileno. Posteriormente, os blocos foram trimados e seccionados no ultramicrótomo Ultracult UCT 020 Leica. Os cortes obtidos foram montados em telas de cobre de 200 Mesh e contrastados

pelo acetato de uranila e pelo citrato de chumbo, examinados e fotografados no microscópio eletrônico de transmissão LEO 906 no Laboratório de Microscopia Eletrônica do Instituto de Biologia/UNICAMP.

III.4- Análises Morfométricas

III.4.1- Morfometria em Microscopia de Luz

Para o estudo morfométrico foram utilizadas amostras da próstata ventral, coradas com hematoxilina e eosina (4 animais por grupo). Posteriormente, as imagens celulares obtidas foram digitalizadas no fotomicroscópio (Nikon Eclipse E-400) e submetidas às mensurações do volume nuclear, volume citoplasmático e porcentagem da área relativa do epitélio, lúmen e estroma glandular.

Para quantificação do volume nuclear foram medidos os diâmetros de 20 núcleos de células epiteliais do lobo ventral da próstata de cada animal. A escolha dos núcleos foi casual, priorizando os com limites celulares definidos. As medidas foram realizadas com auxílio da ocular graduada (10X) acoplada ao microscópio de luz Nikon Eclipse E-400, fixando-se as observações com a objetiva de 100X. A calibragem da ocular foi realizada através de lâmina especial (0,01mm, 10 μm) (*Carl Zeiss*). A partir desses valores foram calculadas as médias dos volumes dos núcleos através da seguinte fórmula: $V=4/3\ \pi\ (d/2)^2 D/2$, sendo "d" diâmetro menor, "D" diâmetro maior. Além disso, foram realizadas medidas para determinação quantitativa das frações de volume (Vv) ocupadas pelo núcleo e pelo citoplasma das células epiteliais da próstata ventral. Essas mensurações foram feitas através de uma ocular 10X contendo um retículo de integração quadrilátero com 100 pontos acopladas ao microscópio de luz Nikon Eclipse E-400 e objetiva de 100X. Através desse retículo foram observados e contados os pontos localizados sobre os núcleos e sobre o citoplasma das células de vinte campos previamente definidos. A fração de volume ocupada pelo núcleo em relação ao citoplasma foi calculada usando-se a seguinte fórmula:

$$Vv = p/P$$

Vv = densidade de volume ou fração de volume (%)

p = número de pontos sobre o núcleo

P = número total de pontos ou soma dos pontos sobre os núcleos e citoplasmas nos diferentes campos.

Para a determinação, em porcentagem, das áreas relativas do epitélio, lúmen e estroma glandular foram utilizados 25 campos por animal, escolhidos aleatoriamente, usando objetiva de 20X. As áreas foram medidas com o auxílio do programa NIS-Elements*: Advanced Research (*USA).

III.4.2- Morfometria em Nível de Microscopia Eletrônica de Transmissão

Eletromicrografias da próstata ventral, 4 animais por grupo, foram obtidas no microscópio eletrônico de transmissão LEO 906. As eletromiocrografias apresentaram aumento de 3597X e ampliação de 3,5X. Dez células, por grupo as quais apresentaram núcleo visível, lâmina basal e ápice evidentes foram analisadas. Posteriormente, as imagens celulares obtidas foram digitalizadas e submetidas às mensurações da área ocupada pelas vesículas secretoras e vacúolos digestivos nas células epiteliais secretoras prostáticas. As medidas foram realizadas através de um sistema de análise de imagens computadorizado, programa NIS-Elements*: Advanced Research (*USA).

III.5- Imunomarcação dos receptores androgênicos (AR), estrogênicos alfa e beta (ERα, ERβ), IGFR-1, Distroglicanas alfa e beta (DG-α e DG-β) e Ki-67

Amostras do lobo ventral foram coletadas de quatro animais de cada grupo experimental, os mesmos destinados à microscopia de luz. A seguir foram obtidos cortes com cinco micrômetros de espessura no micrótomo (Biocut – Modelo 1130), coletados em lâminas silanizadas. Os cortes foram desparafinizados em xilol, hidratados em uma série decrescente de álcoois e lavados em água destilada. A recuperação antigênica foi realizada por incubação dos cortes em tampão citrato (pH 6.0) a 100ºC por 15 minutos em microondas (potência de 750 W) ou tratamento com proteinase K, a depender das características de cada um dos anticorpos avaliados. O bloqueio das peroxidases endógenas foi obtido com H_2O_2 (0,3% em metanol) por 15 minutos. Para diminuir as ligações inespecíficas proteína-proteína, os cortes foram incubados em solução bloqueadora com

albumina soro bovino (3%), em tampão TBS-T por 1 hora em temperatura ambiente. Posteriormente, os antígenos, AR, ERα, ERβ, IGFR-1, DG-α, DG-β e Ki-67 foram localizados através dos anticorpos: policlonal *rabbit* AR-N20 (sc-816) (Santa Cruz Biotchenollogy, USA) para AR, monoclonal *mouse* clone 1D5 (Dako Cytomation Inc., Carpenteria, CA, USA) para ERα, policlonal *rabbi*t 06-629 (Upstate, USA) para ERβ, policlonal *rabbit* N-20 (sc-720) (Santa Cruz Biotchenollogy, USA) para IGFR-1, policlonal *rabbit* H-300 (sc-28534) (Santa Cruz Biotchenollogy, USA) para DG-α, policlonal *mouse* (NCL-b-DG) (Novocastra, USA) para DG-β e policlonal *rabbit* H-300 (sc-15402) (Santa Cruz Biotchenollogy, USA) para Ki-67 diluídos (1:50) em BSA 1% e armazenados *overnight* a 4 ºC. O *kit* Envision HRP (Dako Cytomation Inc., Carpenteria, CA, USA) foi usado para detecção dos antígenos, de acordo com as instruções do fabricante. Após lavagem com tampão TBS-T, os cortes foram incubados com anticorpo secundário HRP conjugado proveniente do *kit* Envision (Dako) por 40 minutos e, posteriormente revelados com diaminobenzidina (DAB). Para a contra-coloração destes foram utilizados verde metil (*Methyl Green*) e Hematoxilina de Harris. As lâminas foram desidratadas, montadas e avaliadas no fotomicroscópio Nikon Eclipse E-400. A intensidade da imunolocalização foi graduada como intensa (+++), moderada (++) e fraca (+) (Markopoulos et al., 2000).

A imunolocalização dos antígenos DG-α e DG-β foi mensurada em quatro animais de cada grupo experimental. Cinco campos microscópicos, com área total de 90304.7 μm², foram medidos com objetiva de X40 e as quantificações de DG-α e DG-β basearam-se nas quantificações das áreas de imunolocalização positiva expressa em relação à porcentagem de área total examinada.

III.6- Extração de Proteínas e Western Blotting

Amostras do lobo ventral foram coletadas de quatro animais de cada grupo experimental e pesadas, homogeneizadas através do homogeneizador Polytron (Kinematica) por 1 min em 50 μl/mg de tampão de extração contendo 100 mM Tris pH 7.5, 10mM EDTA, 10% (v/v) Triton X-100 and 10 μl/ml de inibidores de proteases (Sigma-Aldrich, St. Louis, Mo., USA). Os extratos dos tecidos foram obtidos por centrifugação durante 20 minutos a 14000 rpm a 4^{o}C. Uma alíquota de cada amostra foi usada para

determinação da concentração de proteínas, usando o reagente de Bradford (Bio-Rad Laboratories, Hercules, Calif.,USA). As amostras foram misturadas (1:1) com tampão de amostra 3X (100mM Tris-HCl pH 6.8, 10%β-mercaptoetanol, 4% SDS and 20% glycerol),incubadas em banho seco a 95ºC por 5 minutos, rapidamente congeladas e armazenadas a -70ºC até o momento do uso. O correspondente a 50 microgramas de proteínas foi aplicado no gel de SDS-poliacrilamida. Após a eletroforese, o material foi transferido eletricamente (Sistema Hoefer) para membranas de nitrocelulose (Amersham) a 70 V por 3 horas. A qualidade da transferência assim como da quantificação de proteínas foi analisada pela coloração das membranas com *Ponceau* S. As membranas foram então bloqueadas com 3% BSA diluído em TBS-T por uma hora e incubadas com os anticorpos primários policlonal *rabbit* AR-N20 (sc-816) (Santa Cruz Biotchenollogy, USA) para AR, monoclonal *mouse* clone 1D5 (Dako Cytomation Inc., Carpenteria, CA, USA) para ERα, policlonal *rabbi*t 06-629 (Upstate, USA) para ERβ e policlonal *rabbit* N-20 (sc-720) (Santa Cruz Biotchenollogy, USA) para IGFR-1, nas diluições 1:1000; 1:350; 1:500; 1:500, respectivamente. Após lavagem com tampão TBS-T, as membranas foram incubadas por 2 horas com os anticorpos secundários *anti-rabbit e anti-mouse* HRP conjugados na diluição de 1:2000 em 1% BSA. Após nova série de lavagens com TBS-T, a atividade peroxidásica foi revelada com o cromógeno diaminobenzidina (DAB). Anticorpo para β actina foi usado como controle endógeno. A intensidade da marcação obtida nas diferentes situações foi determinada por densitometria através do programa de análise de imagens NIS-Elements*: Advanced Research* (USA).

III.7- Contagem de Células Ki-67 Positivas

Os índices proliferativos foram obtidos por contagem das células Ki-67 positivas e Ki-67 negativas (coradas com verde metil). Amostras do lobo ventral de quatro animais de cada grupo experimental, os mesmos destinados a imunomarcação para Ki-67 foram utilizadas. A seguir, dez campos de cada espécime foram analisados com objetiva de 40X e o número total de células Ki-67 positivas foi expresso em porcentagem do total de células.

III.8- Detecção da Apoptose e Determinação do Índice Apoptótico

Amostras do lobo ventral de quatro animais de cada grupo experimental e fixadas em paraformoldeído a 4% foram seccionadas e submetidas às reações de detecção da fragmentação do DNA. A fragmentação do DNA foi detectada utilizando o sistema de detecção fluorescente para apoptose (Promega, Madison, WI, USA), de acordo com as instruções do fabricante, e Feulgen (Garcia-Florez, 2005). Os núcleos apoptóticos foram identificados e fotografados através do microscópico Nikon TS-100, equipado com fluorescência (DXM 1200F, Nikon).

Para a reação de Feulgen, os cortes foram submetidos à hidrólise com 4 N HCl por 75 minutos e tratados com o reagente de Schiff por 40 minutos. Depois de extensa lavagem, os cortes foram desidratados e montados em lâminas. A seguir, dez campos de cada animal foram analisados com objetiva de 40X e o índice apoptótico foi determinado dividindo-se o número de núcleos apoptóticos pelo número total de núcleos encontrados nos campos microscópicos. Os núcleos apoptóticos foram identificados por características como picnose e/ ou fragmentação nucleolar, de acordo com Kerr & Searle (1973).

III.9- Análises Estatísticas

Os parâmetros quantificados (volumes nuclear e citoplasmático; áreas do epitélio, lúmen, estroma, das vesículas secretoras e vacúolos digestivos; dosagens séricas de testosterona, estradiol e glicose; porcentagens de imunomarcação das áreas positivas para DG-α e DG-β; e análises de Western Blotting) foram analisados estatisticamente para os diferentes grupos experimentais. Para a análise estatística foram empregados o Test-T e a análise de variância (ANOVA), seguida pelo teste de Tukey para comparação entre médias. Todas as análises foram realizadas com nível de significância de 1% e 5% (Zar, 1999).

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IV- ARTIGOS CIENTÍFICOS

IV.1- **"Ultrastructural and proliferative features of the ventral lobe of the prostate in Non-obese diabetic mice (NOD) following androgen and estrogen replacement associated to insulin therapy"**. Publicado no Periódico *Tissue & Cell* (**FÁVARO, W.J.; PADOVANI, C.R.; CAGNON, V.H. *Tissue & Cell*; vol.41, pp.119-132, 2009**).

IV.2- **"Dystroglycan patterns on the prostate of non-obese diabetic mice submitted to glycaemic control"**. Publicado no Periódico *International Journal of Experimental Pathology* (**CAGNON, V.H.; FÁVARO, W.J. *International Journal of Experimental Pathology*; vol.90, pp.156-165, 2009**).

IV.3- **"IGF-1 signaling and its interaction with androgen and oestrogen receptors in the ventral prostate of Non-obese diabetic (Nod) mice following hormonal therapy"**. Submetido ao Periódico *The Journal of Urology*; abril de 2009.

"Ultrastructural and proliferative features of the ventral lobe of the prostate in Non-obese diabetic mice (NOD) following androgen and estrogen replacement associated to insulin therapy"

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Key words: Ventral Prostate; Diabetes Mellitus; Ultrastructural; Steroid Hormones; Apoptosis; Proliferation

This work was supported by FAPESP and CNPq.

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ABSTRACT

Diabetes causes harmful effects on prostatic function. Thus, the aims of this study were to characterize morphological and proliferative features of the prostate of diabetic mice after long term glycemic control and testosterone and estrogen replacement. A total of 48 mice (Nod and BALBc) were used. After twenty days in a diabetic state, the mice were divided into six groups: the control group received a 5 mL/Kg dose of peanut oil; the diabetic group received the same treatment as the control group; the diabetic-Insulin group received 4 IU doses of insulin; the diabetic-testosterone group received a 5 mg/Kg dose of testosterone cypionate; the diabetic-estrogen group received a 25 μg/Kg dose of 17β-estradiol; the diabetic-insulin-testosterone-estrogen group received insulin, testosterone and estrogen at the same concentration as the other groups. After 20 days, the ventral lobe was processed for morphological and immunological analyses. The results showed structural disorganization, which was more intense in the diabetic group than in the other groups. The diabetic state showed a proliferation and apoptosis rate that was two times higher than that found in the control group. To conclude, diabetes disturbed the prostatic secretory activity and the association of insulin, testosterone and estrogen was crucial for glandular structural restoration, characterizing the complex activity of the prostate. The imbalance verified between the proliferation process and apoptosis in diabetic mice showed diabetes to be a triggering factor for prostatic pathogenesis.

INTRODUCTION

Diabetes Mellitus is a disease which causes protein, carbohydrate and lipid metabolism alterations leading to hyperglycemia due to insulin deficiency (Mokdad, 2001; Conget, 2002). Also, experimental and clinical studies have shown alterations in different organic systems, including the urogenital system (Ciardullo et al., 2004).

Jackson and Hutson (1984) and Saito et al. (1996), verified that there was a weight reduction of different male reproductive organs such as the prostate, the testes, the seminal vesicle and the coagulating gland in diabetic rats. Afterwards, Cagnon et al. (2000), Ribeiro et al. (2006) observed atrophied prostatic secretory epithelial cells, stromal hypertrophy, inflammatory cells, prostatic intraepithelial neoplasia and dilated organelles in the secretory

process of spontaneous diabetic mice. Also, Carvalho et al. (2003) characterized thickness components of extracellular matrix and inflammatory cells in the coagulating gland of diabetic mice. Moreover, other studies observed diminished testosterone serum levels and their receptors in the prostate of diabetic rodents due to androgen imbalance (Okasen, 1975; Tesone et al., 1976).

Wang et al. (2000) verified that the glycemic level control of diabetic rats with insulin treatment did not recover prostate weight. However, simultaneous testosterone and insulin treatment in rats was useful for all morphofunctional retrieval, despite the prostatic structure not being similar to that observed in normal morphological and physiological conditions (Sufrin and Scott, 1972; Tesone et al., 1980; Ho, 1991). According to Soudamani et al. (2005), diabetic rats showed disturbed development and differentiation of the ventral lobe of the prostate, whereas exogenous insulin administration led to a reduction of the diabetic effects in the prostate.

The prostate is an accessory sex gland present in different mammals which is divided into three pair of lobes: ventral, dorsal and lateral, distributed around the prostatic urethra in rodents (Setchell and Brooks, 1988; Marker et al., 2003; Untergasser et al., 2005). In general, the prostatic lobes exhibit tubule-alveolar structures with simple epithelium surrounded by stroma (Aumüller and Ader, 1979). The prostatic epithelium shows columnar cells intermingled with basal cells (McNeal et al., 1988; Abate-Shen and Shen, 2000; Garraway et al., 2003). The prostatic stroma is a complex net with stromal cells and extracellular matrix associated to growth factors; regulatory molecules; and enzyme restructuring, which provide biological signals and lead to mechanical influence on the epithelial cells (Tuxhorn et al., 2001; Cunha and Matrisian, 2002).

Morphogenesis, functional activity and morphology maintenance, cellular proliferation and differentiation are regulated by androgens (Leav et al., 2001; Cunha et al., 2002; Imamov et al., 2005). Testosterone and dihydrotestosterone (DHT) are the main androgens that induce prostatic differentiation (Hsing, 2001; Toorians et al., 2003). Despite the prostate being primarily regulated by androgens, its development in both human and rodents is sensitive to other hormones such as estrogens which act in association with the

latter, influencing not only normal prostatic function but also pathological processes (Weihua et al., 2001; Cunha et al., 2002).

Clinical studies have pointed diabetes out as being an important etiological factor towards the development of prostatic cancer (Ilic et al., 1996). Furthermore, epidemiological data demonstrated that men who have been diabetic for five years or more showed high occurrence levels of prostatic cancer in relation to those who did not present this disease (Will et al., 1999). According to Hammarsten and Hogstedt (2002), the beginning of benign prostatic hyperplasia (BPH) and its quick advance in the diabetic patient could be considered a risk factor for the clinical development of prostatic cancer. On the other hand, a study analyzing prostatic cancer in men treated with androgen withdrawal therapy, showed an increased risk of developing resistance to insulin and hyperglycemia (Basaria et al., 2006).

Despite there being signs of an association between the occurrence of prostatic diseases and diabetes, there are still many doubts in this field. Furthermore, the effects originated from insulin treatment as well as hormonal replacement through the administration of testosterone and estrogen are subjects which need more studies, considering the complex prostatic mechanism. Therefore, taking into consideration the crucial role of estrogen and testosterone in maintaining normal prostatic function, in addition to following the development and progression of diseases in this organ, it is fundamental to know much more about all these topics which will certainly help to maintain prostatic activities in diabetic patients.

Therefore, the aims of this present study were to characterize morphological and proliferative features of the prostatic epithelium and stroma of diabetic experimental mice after long term glycemic control and testosterone and estrogen replacement. Also, the association between the present data and the occurrence of possible prostatic pathogenesis was analyzed.

ANIMALS AND TISSUE PREPARATION

A total of 40 mice (NOD) and 8 control mice (BALB/c/Uni), (18 weeks old), from The Bioterism Center/Unicamp were used. The blood glucose level was measured by

capillary glycemia, utilizing the Optium Advanced Diabetes Management System (MediSense, Abingdon, UK). Thus, all Nod mice which presented blood glucose level >300 mg/dl were considered diabetic (Shirai et al., 1998). After twenty days in a diabetic state, the mice were divided into six groups: The control group received a 5 mL/Kg dose of peanut oil subcutaneously daily for 20 days (All Chemistry, São Paulo, Brazil); The diabetic group received the same treatment as the control group; The diabetic-Insulin group received 4-5 IU doses of NPH insulin subcutaneously daily for 20 days (Biobrás, Montes Claros, Minas Gerais, Brazil) (Anderson, 1983); The diabetic-testosterone group received a 5 mg/Kg supraphysiological dose of testosterone cypionate (Deposteron-Sigma Pharma, São Paulo, Brazil), diluted in 5mL peanut oil, subcutaneously every other day for 20 days (Franck-Lissbrant et al., 1998); The diabetic-estrogen group received a 25 μg/Kg supraphysiological dose of 17β-estradiol (Sigma Chemical Co., St Louis, MO, USA), diluted in 25 μL peanut oil, subcutaneously every other day for 20 days (Prins et al., 2001); The diabetic-insulin-testosterone-estrogen group received insulin, testosterone and estrogen, simultaneously, at the same concentration administered to the other groups. Testosterone, 17β-estradiol and insulin administrations were interrupted 24 hours prior to the mice being sacrificed. After 20 days of experimental treatment, the animals were anesthetized with a 0.25 mL/100 g body weight dose of Francotar/Virbaxyl (1:1, Vibra® Roseira, SP, Brazil), and samples of the intermediate and distal regions from the ventral lobe of the prostate were collected under a DF Vasconcellos Steromicroscopy and processed for morphometrical, ultrastructural, structural and immunological analyses. Hormonal analyses were also carried out on the blood samples.

Samples of the ventral prostate were collected from four animals in each group for histological analysis and then fixed by immersion in either Bouin's solution or 4% paraformaldehyde from each one of the animals, embedded in paraplast (Paraplast Plus, Brazil), cut into 5-μm thick sections and submitted to the following staining procedures: hematoxylin-eosin (Behmer et al., 1976), Gomori's silver impregnation for reticulin (Behmer et al., 1976) and Masson's trichrome (Junqueira et al., 1979). The slides were photographed with a Nikon Eclipse E-400 photomicroscope.

Four animals from each experimental group were anesthetized and perfused with Karnovsky's solution (Sprando, 1990) for ultrastructural analysis. Next, samples of the ventral prostate were immersed in the same fixative for 2 h, followed by 1 h in cacodylate buffer and 1% osmium tetroxide. After that, all the material was embedded in resin (Polysciences, Niles, IL), cut with an ultramicrotome (Ultracult UCT 020 Leica), mounted on copper grids (Sigma Chemical Company, St. Louis, MO, USA), and counterstained with uranyl acetate and lead citrate. The specimens were then examined and photographed under a LEO 906 transmission electron microscope.

Serum testosterone, estradiol and glucose levels

At the end of the 20-day treatment, blood samples were collected from all animals in each group. Blood samples were collected by means of a cardiac puncture, 24 hours after administrating the last dose of hormones to each mouse in the experimental groups. The serum concentrations of testosterone and estradiol were determined by radioimmunoassay using Coat-a-Count total testosterone/estrogen kits (Diagnostic Products Corporation, Los Angeles, CA, USA). The serum hormone concentrations were expressed in ng/dL and pg/mL. The serum concentration of glucose was determined by quimioluminiscence and expressed in mg/dL.

Stereological and Morphometrical Procedures

Samples of the ventral prostate were collected from four animals in each group for stereological and morphometrical analyses. Four 5-μm thick cuts from each animal in the different experimental groups were obtained and stained with hematoxylin-eosin.

Cellular, cytoplasm and nuclear volumes were measured and the groups were randomly chosen so that there would be no indication from which group these samples originated. So as to quantify nuclear volume, the diameters of 20 randomly chosen epithelial cell nuclei, with well-defined limits, were measured, reaching a total of 80 nuclei per group. A graduated ocular lens (X10) coupled to an X100 objective was used to determine these measurements. Based on these values, the average volume of the nuclei were calculated based on the following formula: $V= 4/3.\pi.(d/2)^2.D/2$, where “d” is the smallest diameter and “D” the greatest diameter. Apart from this, measurements to

quantitatively determine the volume fractions (Vv) taken up by the epithelial cell nucleus and the cytoplasm, were carried out. An ocular lens (X10) with a 100 grid coupled to an X100 objective was used to determine these measurements. The Points on the nucleus and on the cytoplasm were counted in 20 areas and taken at random per animal, resulting in 80 fields per group. A fraction of the volume taken up by the nucleus in relation to the cytoplasm was calculated by the following formula: Vv= p/P (Vv= volume fraction, p= number of points on the nucleus, P= total number of points or the sum of the points on the nucleus and cytoplasm in different fields). These data and the nuclear volume were used to estimate the cytoplasmic volume of each animal. The cellular volume was calculated by the addition of the nuclear and cytoplasmic volumes (Weibel, 1979).

Epithelial, luminal and stromal areas in the ventral lobe of the prostate were measured (25 fields per animal were randomly taken). The microscopic field was analyzed with an X20 objective. The areas were measured using the NIS-Elements*: Advanced Research* (USA) computerized image analysis system.

So as to determine autophagic and secretory vesicle areas, electronmicrographs of the ventral prostate from four animals in each group taken with a LEO 906 transmission electron microscope were used. Ten cells from each experimental group were measured and the final magnification electronmicrographs were measured using the following proportion: x3597 X x3.5. The cells used were those which presented accurate limits, basal membrane and cellular apex. The areas were measured using the NIS-Elements*: Advanced Research* (USA) computerized image analysis system.

Detection of apoptosis and determination of the apoptotic index

Samples of the ventral prostate were collected from four animals in each group and processed for DNA fragmentation by means of a fluorescein apoptosis detection system (Promega, Madison, WI, USA) according to manufacturer instructions. The apoptotic nuclei were identified using a Nikon TS-100 microscope equipped for fluorescence (DXM 1200F, Nikon), in addition to Feulgen's reaction (Garcia-Florez, 2005).

The sections were subjected to hydrolysis with 4 N HCl for 75 min and then treated with Schiff's reagent for 40 min., for Feulgen's reaction. After extensive washing, the sections

were dehydrated and mounted. Ten microscopic fields were taken at random and analyzed per animal, resulting in 40 fields per group, through a Nikon Eclipse E-400 photomicroscope with an X40 objective. The number of nuclei counted was from 1290 to 2657 in the different experimental groups. The apoptotic index was determined by dividing the number of apoptotic nuclei by the total number of nuclei found in the microscope field. Apoptotic nuclei were identified by the characteristic pyknosis and/or nuclear fragmentation, as reported by Kerr and Searle (1973). Only the epithelium was considered for these counts.

Counting of Immunolabeled Ki-67

Samples of the ventral lobe prostate were removed from four animals in each group fixed by immersion in 4% paraformaldehyde, embedded in paraplast (Paraplast Plus, Brazil) and cut into 6 µm thick sections. The sections were deparaffinized in xylene, hydrated through graded alcohol concentrations and rinsed under tap water. Antigens were retrieved by boiling the sections in 10 mM citrate buffer, pH 6.0, three times for 5 min in a microwave oven. After that, the sections were incubated in 0.3% H_2O_2 for 15 min to block endogenous peroxidase. Nonspecific binding was blocked by incubating the sections in blocking solution for 1h at room temperature. Primary rabbit H-300 (sc-15402) (Santa Cruz Biotchenollogy) for the Ki-67 antibody was diluted in 1% BSA (1:50) and applied to the sections overnight at 4ºC. The Envision HRP Kit (Dako Cytomation Inc., Carpenteria, CA, USA) was used to visualize the bound antibody according to manufacturer instructions. The sections were washed for 15 min. with TBS-T and secondary labeled polymer from the Envision HRP Kit (Dako) was applied for 40 min. at room temperature. After washing in TBS-T, peroxidase activity was detected using a diaminobenzidine chromogen kit from Envision HRP Kit (Dako) for 10 min. Sections were lightly counterstained with methyl green dehydrated in an increasing ethanol series and xylene, mounted in Entellan (Merck, Darmstadt, Germany) and photographed with a Nikon Eclipse E-400 photomicroscope.

The immunolocalization of the Ki-67 was measured in four animals in each experimental group. Ten fields were taken at random and measured per animal, resulting in 40 fields per group with an X40 objective lens and the total number of Ki-67 staining

positive cells was expressed as the percentage of these total cells, including luminal, basal epithelial and stromal cells. The number of nuclei counted was 1290 to 2657 for the different experimental conditions.

Statistical Analyses

Average data for cell volume, autophagic and secretory vesicle areas, serum levels, apoptotic index and immunolabeled Ki-67 were compared between groups and analyzed statistically by analysis of variance and Tukey multiple range test, with the level of significance set at 1% and 5% (Montgomery, 1991; Zar, 1999).

RESULTS

Serum Testosterone, estradiol and glucose levels

The average glucose level of the animals from the diabetic group was over 300mg/dL. In contrast, the levels observed in the animals from the control, diabetic-insulin and diabetic-insulin-testosterone-estrogen groups were lower than the value verified in the diabetic group (Table 1).

The average values of serum testosterone levels were higher in the control group than those found in the other experimental groups. Moreover, the diabetic, diabetic-insulin, and diabetic-estrogen showed no significant differences in relation to each other (Table 1). The diabetic-insulin-testosterone-estrogen group presented lower testosterone average levels than those found in the control group. However, these levels were higher than those shown in the other experimental groups (Table 1). In relation to estradiol levels, these levels were reduced in the control group in comparison to other experimental groups (Table 1).

Transmission Electron and Light Microscopies and Morphometrical Analyses

The Control Group

The ventral lobe of the prostate showed different sizes and folded mucosa of the acini (Figures 1a and 1b). The secretory epithelium presented tall columnar cells with basal

nuclei (Figure 1a). In the cellular cytoplasm, flattened rough endoplasmic reticulum cisternae and developed Golgi complex in the supranuclear and perinuclear (Figures 1a and 2b) were observed. Also, secretory vesicles with granules, showing different eletrondensity in the apical cytoplasm were presented (Figures 2a and 2b). The area taken up by secretory vesicles was 7.8 times greater than that of the digestory vacuoles (Figures 2a and 2b and Graph 1). The microvilli were short and sparse (Figure 2a).

The prostatic stroma showed thin collagen fibers underlying the secretory epithelium and intermingled with smooth muscle cells, as well as reticular fibers, especially underlying the epithelium, in addition to blood vessels (Figures 1a, 1b and 2c). The muscular layer presented long smooth fibers underlying the epithelium (Figure 2c).

The epithelial area was greater than that of the stromal area, representing 36.6% and 21.3%, respectively (Graph 2).

The Diabetic Group

The prostatic acini presented less folded mucosa than that observed in the control group (Figures 1c, 1d and 1n). Atrophied secretory epithelium with cuboidal cells was verified (Figures 1c and 1n), showing decreased total cell volume with nuclei occupying a large cytoplasmic portion (Figures 1c and 1n and Table 2). Prostatic intraepithelial neoplasia (PIN) was identified in some specific places, characterized by increased size nuclei and altered nuclei (Figures 1m and 2h). Dilated rough endoplasmic reticulum and Golgi complex cisternae were observed in the cellular cytoplasmic (Figures 2e and 2f). Occasional vesicles containing secretion of low electrondensity material was verified in the apical region cell (Figures 2d and 2e). The secretory vesicle area was 2.56 times greater than that occupied by digestory vacuoles (Figures 2d and 2e and Graph 1). Rupture of mitochondrial crista was verified (Figure 2e). Also, lipidic droplets were widespread in the cytoplasm (Figure 2f). Short and scarce microvilli covered the cellular surface (Figure 2d).

In the prostatic stroma, thick collagen fibers not only underlying the epithelium but also in all the stromal area (Figures 1c, 1n, 2f and 2g) was verified, characterizing the hypertrophied stroma. The stromal area was greater than the epithelial one, 35.7% and

21.8%, respectively (Graph 2). The smooth muscle cells showed folded sarcolemma with espinous features and small secretory vesicles (Fig. 2g). A great amount of reticular fibers was observed in all the cytoplasmatic area (Figure 1d). Inflammatory cells were seen in the prostatic stroma (Figures 1n and 2f).

The Diabetic-Insulin Group

The prostatic acini demonstrated less folded mucosa than that observed in the control group, however this folding was intensified in relation to those verified in the diabetic group (Figures 1e and 1f). Atrophied secretory epithelium with cuboidal cells and basal nuclei was seen (Figure 1e), showing total volume greater than those verified in the diabetic group and smaller than those found in the control group (Table 2). The rough endoplasmic reticulum was dilated; nevertheless there were flattened points of this organelle in the supranuclear cytoplasm (Figure 3c). Dilated Golgi cisternae were verified (Figure 3b). Rupture of mitochondrial crista was observed (Figures 3b and 3c). Areas of secretory vesicles with different electrondensities were observed in the apical cytoplasm, which were smaller than those verified in the control group. However, those areas were greater than the secretory vesicle area found in the diabetic group (Figures 3a and 3b and Graph 1). An inverse proportion could be seen in the digestory vacuoles (Graph 1).

Hypertrophied stroma was verified in relation to animals of the control group, however, there appeared to be a lower occurrence of thick collagen fibers than that verified in the diabetic group with a similarity of distribution of reticular fibers, dispersed to all the stromal area (Figures 1e, 1f and 3c). Smooth muscle cells showed spinous features with increased secretory vesicles (Figure 3c). Occasional inflammatory cell foci were found (Figure 1e). The glandular epithelial area was around 1.4 times smaller than the stromal area (Graph 2).

The Diabetic-Testosterone Group

The prostatic acini showed lightly folded mucosa (Figures 1g and 1h). The secretory epithelium presented columnar cells, however these cells were smaller than those observed in the animals of the control group. In contrast, these same cells had an

increased total volume when they were compared to the cells of the animals in the diabetic and diabetic-insulin groups (Figure 2g and Table 2). Nevertheless, decreased total cellular volume was found in relation to that observed in the control group (Table 2). Dilated rough endoplasmic reticulum and Golgi complex cisternae were verified in the cellular cytoplasm (Figure 3e). Ruptured mitochondrial cristae were observed in this group, as was observed in other groups such as the diabetic group with or without hormonal replacement (Figure 3d). Increased secretory vesicle cellular area was verified which showed secretory granules of different electron densities in the apical cytoplasm in relation to diabetic and diabetic-insulin groups (Figures 3d and 3e and Graph 1). However, the numerical values of these vesicles were lower than those observed in the animals of the control group (Graph 1). The areas occupied by digestory vacuoles were lower than those found in the diabetic and diabetic-insulin groups. However, these same areas were greater than those areas found in the control group (Graph 1). Short and scarce microvilli covered the cell surface in addition to an occurrence of lipidic droplets (Figures 3d, 3f and 3g).

Hypertrophied stroma was found, showing thick collagen fibers distributed underlying the epithelium and among the smooth muscles (Figures 1g, 3f and 3g). Increased stromal area was verified in relation to the epithelial area, 33.7% and 24.7%, respectively (Graph 2). The reticular fibers in this group seemed to have increased, which was similar to the fibers observed in the diabetic group, described earlier, (Figure 1h). Thick smooth muscle cells were found with folded sarcolemma, representing a spinous feature with small secretory vesicles (Figures 3f and 3g). The occurrence of occasional stromal inflammatory cells was verified in relation to the diabetic and diabetic-insulin groups (Figure 3g).

The Diabetic-Estrogen Group

The soft folded glandular acini and mucosa were verified in this experimental group, however these aspects were more intense than those observed in the diabetic, diabetic-insulin and diabetic-testosterone groups and less intense than those found in the control group (Figures 1i and 1j). The secretory epithelium showed columnar cells with

basal nuclei (Figure 1i) and an increased total volume in relation to the diabetic, diabetic-insulin and diabetic-testosterone groups. Nevertheless, this volume was lower than in the control group (Table 2). Occasional points of dilation of the rough endoplasmic reticulum were identified in the cellular cytoplasm (Figure 3i). Dilated Golgi complex cisternae were observed. However this feature was less obvious than that in the diabetic, diabetic-insulin and diabetic-testosterone groups (Figure 3i). Increased secretory vesicle area which showed secretory granules with different electrondensities in the apical cytoplasm in relation to diabetic, diabetic-insulin and diabetic-testosterone groups was observed (Figures 3h and 3i and Graph 1). Nevertheless, it was reduced in relation to the control group. Short and scarce microvilli covered the cellular surface (Figure 3h). Rupture of the mitochondrial cristae were verified (Figure 3i). Moreover, vacuolization of the supranuclear cytoplasm and lipidic droplets were characterized (Figures 3h, 3i and 3j).

The collagen fibers appeared to be increased in the prostatic stroma, underlying the epithelium (Figures 1i and 3j), however, this increase was noticeably lower than that verified in the diabetic, diabetic-insulin and diabetic-testosterone groups which was evident in relation to the control group. Corrugated and increased reticular fibers were verified to be widespread in the stromal area (Figure 1j). The smooth muscle cells presented corrugated nuclear membrane and occasional secretory vesicles (Figure 3j). Occasional inflammatory cells were found in the prostatic stroma (Figure 1i).

An increased stromal area could be observed when related to that of the epithelial, following a 30.2% and 27.2% proportion, respectively (Graph 2). Nevertheless, this rate decreased in relation to that observed in the diabetic-testosterone group as well as other diabetic experimental groups.

The Diabetic-Insulin-Testosterone-Estrogen Group

The structural, ultrastuctural and morphometrical features were similar to those verified in the control group (Figures 1k, 1l, 3k, 3l and 3m). The total epithelial cell volume was greater than that observed in the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups. However, this volume was lower than that

verified in the control group (Table2). Occasional points of dilatation of the rough endoplasmic reticulum were verified (Figure 3l). The mitochondria cristae were also seen to have ruptured (Figure 3l) and lipidic droplets (Figure 3m) were also observed.

Moderate hypertrophy was verified in the prostatic stroma with collagen and reticular fibers which was similar to that of the control group (Figures 1k, 1l and 3m). Smooth muscle cells were seen, showing occasional secretory vesicles (Figure 3m).

Detection of apoptosis and determination of the apoptotic index

The apoptotic index revealed different kinetics for cell death for each treatment (Figures 4b, 4d, 4f, 4h, 4j, 4l and 4m). This index was significantly increased in the animals from the diabetic, diabetic-insulin, diabetic-estrogen and diabetic-testosterone groups in relation to the control group (Figure 4m). The diabetic-insulin-testosterone-estrogen group presented significantly lower average values of apoptotic index than those found in the diabetic, diabetic-insulin, diabetic-estrogen and diabetic-testosterone groups, although these values were significantly higher than those found in the control group (Figure 4m).

Counting of Immunolabeled Ki-67

The proliferative response from luminal, epithelial, basal and stromal cells was measured by immunolocalization of the Ki-67 antigen (Figures 4a, 4c, 4e, 4g, 4i, 4k and 4m). The proliferative activity was significantly increased in the animals from the diabetic group in relation to the other experimental groups (Figure 4m). Estrogen treatment presented significantly higher average values of proliferative activity than those found in the diabetic-insulin, diabetic-testosterone and diabetic-insulin-testosterone-estrogen groups (Figure 4m). However, the diabetic-insulin-testosterone-estrogen group showed significantly lower average values of proliferative activity than those found in the control group (Figure 4m).

DISCUSSION

This work showed high serum glucose levels, in particular, in animals from diabetic, diabetic-testosterone and diabetic-estrogen groups. However, these values were close to the

normal status in the diabetic animals under glycemic control. Similar results were characterized in the diabetic-insulin-testosterone-estrogen group a decrease of serum glucose levels. Several studies observed high serum glucose levels in diabetic rodents (Tesone et al., 1980; Ho, 1991; Saito et al., 1996; Caldeira et al., 2005). According to Ohta et al. (2007) insulin replacement to diabetic rats led to unchanged blood glucose levels, showing values similar to those of healthy animals. Based on these considerations, the present results confirmed the diabetic state of the experimental animals and the recovery of the normal serum glucose levels after insulin administration.

The results showed that the diabetic state led to decreased serum testosterone levels and increased serum estradiol levels. Nevertheless, the exogenous insulin was not able to restore the normal status of serum testosterone and estradiol levels, as well as the association of these three hormones. Clinical and experimental studies demonstrated that diabetes caused altered gonadotrophic hormones, resulting in reduced testicular hormone secretion (Tesone et al., 1980; Saito et al., 1996; Ho, 1991; Ballester et al., 2004). Daubresse et al. (1978) verified that diabetic men showed low testosterone levels and gonadotrophic hormones as a result of negative feedback of the hypothalamic-hypophyseal-gonodal axis. In addition, Jackson and Hutson (1984) emphasized that the changes in the accessory sex glands from diabetic rodents occurred due to two aspects; decreased testosterone levels and lack of insulin which could alter the cellular mechanism, damaging the normal androgen action.

Literature characterized that despite the prostate being primarily androgen regulated, there are other hormones such as estrogens which act with androgen, influencing the development and maintenance of the prostate activities, as well as the occurrence and progression of diseases in this organ (Marcelli and Cunnuingham, 1999; Weihua et al., 2001; Cunha et al., 2002; Tuxhorn et al., 2002; Mcpherson et al., 2007). Naslund et al. (1988) observed that hormonal imbalance is one of the main etiological factors involved in the development of prostatitis in rats. Estrogens have anti-androgen effects and negatively regulate the hypothalamic-hypophyseal-gonodal axis, with a reduction of androgen production by Leydig cells, resulting in atrophied prostatic epithelium and stromal growth in adult animals (Weihua et al., 2002). Bianco et al. (2002) and Risbridger et al. (2003)

demonstrated that both estrogens and androgens could lead to morphological and functional prostate alterations, however, each one of them *per se* is not able to cause carcinogenesis of this gland. Thus, it could be concluded that diabetes altered the estrogen and testosterone levels, which caused an imbalance of the hypothalamic-hypophyseal-gonodal axis and the exogenous administration of these hormones recovered the stability of hormonal parameters.

The animals from the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups showed structural disorganization in both glandular secretory epithelium and stroma such as inflammatory cells, microacini and prostatic intraepithelial neoplasia (PIN), whereas PIN was only identified in the diabetic group. These alterations were much more intense in the diabetic group than in the other groups which become less intense in the gradual stages from the diabetic-insulin group to the diabetic-testosterone group and the diabetic-estrogen group. Nevertheless, there was structural recovery in both stromal and epithelial regions of the prostatic ventral lobe in the group which was treated with the three hormones simultaneously. In general, the cellular proliferation and apoptosis events were increased in all diabetic groups, despite being more intense in the diabetic group without receiving either testosterone or estrogen. In contrast, despite these events having increased in the diabetic state, the proliferative process was much higher, 10 times higher than the increase shown for the apoptosis process, which was just 5 times higher. Also, a proliferation and apoptosis rate close to 1.2 was verified in the control animals, which in the diabetic animals was 2.2, reaching intermediate numbers and/or above these numbers when testosterone, estrogen and insulin were administrated alone. Rates closer to normal numbers were verified in mice which received the three hormones simultaneously.

Cagnon et al. (2000) and Carvalho et al. (2003) verified atrophied secretory cells, hypertrophied stroma, occurrence of inflammatory cells, prostatic intraepithelial neoplasia and dilated secretory organelles in both prostatic ventral lobe and coagulating gland from Nod mice, concluding that diabetes caused harmful consequences to the sex accessory glands and was a risk factor for glandular pathogenesis. In addition, Ribeiro et al. (2006) observed increased collagen fibers, underlying the basal membrane; thin reticular fibers; and folded plasmatic membrane of smooth muscle cells in the prostatic ventral lobe from

Nod mice. Other studies have shown that diabetes led to changed smooth muscle cells in various organs, reducing the nucleus/cytoplasm rate (Öztürk et al., 1996).

According to Wang et al. (2000), the glycemic control of diabetic rats did not restore the prostate weight. Nevertheless, the simultaneous administration of testosterone and insulin led to the structural partial recovery of the sex accessory glands (Santi and Johanson, 1973; Tesone et al., 1980; Ho, 1991). Makino et al. (1980) stated that NOD mice are rodents which present an autoimmune genetic factor. However, different authors have shown that the autoimmune factor is not responsible for triggering glandular morphological changes because these animals develop moderate autoimmune alterations (Humprhreys-Beher et al., 1998; Yamano et al., 1999). According to Hu et al. (1992), the autoimmune effects could be considered to increase the first changes verified as a result of metabolic disorder provoked by type I diabetes.

The inflammatory process is a result of the prostatic secretion flow in the stroma, after blocking up its ducts (Billis, 2000). Also, other studies demonstrated the relation to inflammatory infiltrate and tumor progression (Wilson and Balkwill, 2002; Lin & Pollard, 2004). Wang et al. (2008) mentioned that prostatitis plays an important role in prostatic diseases such as PBH by affecting the proliferation and apoptosis of the prostatic cells. Thus, the simultaneous occurrence of inflammatory cells and PIN as well as stromal hypertrophy, suggested active participation of these cells in the stromal-epithelium disorganization of diabetic animals. Moreover, it could be concluded that the administration of the three hormones, insulin, testosterone and estrogen, carried out separately, led to a smaller amount of inflammatory cells and no occurrence of PIN. The association of these same three hormones caused morphological recovery of the prostate, without demonstrating proliferative features and presence of inflammatory cells. The active role of hormonal interaction on the restoration of the prostatic pathogenesis was clear in the animals from the diabetic-insulin-testosterone-estrogen group.

The prostatic stroma is an important compound for glandular activity influencing the maintenance of the glandular homeostasis and acting on the development of diseases such as benign prostatic hyperplasia and prostatic cancer (Ekman, 2000; Donjacour et al., 2003; Zhang et al. 2003). The imbalance of the stromal-epithelium interaction benefits the

development of prostatic cancer (Cunha et al., 2002). The stromal microenvironment changes, which are caused by different cells and extracellular matrix elements, could point to the beginning of the development and progression of cancer in the prostate (Cunha et al., 2003). According to Cornell et al. (2003), proliferation regulation of prostatic cancer is a complex event which involves extracellular matrix elements and endocrine, paracrine and autocrine mechanisms. The increased extracellular matrix element production, specially, collagen fibers, growth factors and stromal element reorganization is called reactive stroma, creating a positive microenvironment for tumoral growth (Tuxhorn et al., 2002; Rowley, 1998). The reactive stroma alters the stroma-epithelium interaction benefiting genetically modified epithelial cell extension (Grossfeld et al., 1998; Hayward et al., 2001). Also, other studies showed the phenotype changes of smooth muscle cells as a result of different lesions, such as prostatic cancer (Vilamaior et al., 2000; Wong et al., 2000; Tuxhorn et al., 2001; Fávaro and Cagnon, 2006).

Also, prostatic intraepithelial neoplasia is pointed out as being a lesion tissue which comes prior to invasive adenocarcinoma, tending to become malignant (Davidson et al., 1995; Häggman et al., 1997; Xie et al., 2000; Alberts and Blute, 2001).

In addition, the apoptosis is an intrinsic active cellular mechanism, which destroys cells during tissue restructuring and development processes (Kerr et al., 1972; Kerr and Searle, 1973). This event is characterized by morphological changes, including loss of plasmatic membrane and condensation of cytoplasm and nucleus (Quarrie et al., 1995; Strange et al., 1995; Sohn et al., 2001). This physiological mechanism guarantees tissue organization during cellular development and balance in the mature organ, which is very often related to tissue involution process (Rauch et al., 1997). Then, the control mechanism of tissue homeostasis is a balance between proliferation and cellular death (Quarrie et al., 1995; Strange et al., 1995; Sohn et al., 2001).

Thus, it could be concluded that diabetes led to proliferation/apoptosis rate imbalance, showing prevalence of the proliferative process. Moreover, administration of insulin, testosterone and estrogen alone did not restore the balance between these two processes. However, the association of these three hormones was a fundamental element to establish proliferation and cellular death.

Finally, it also could be concluded that diabetes disturbed the prostatic secretory activity. Furthermore, glycemic control and testosterone and estrogen administration alone were not effective on the structural recovery as well as in the proliferation and apoptosis balance of the prostate. In contrast, the association of insulin, testosterone and estrogen was determinative for glandular structural restoration. Finally, the imbalance verified between the proliferation process and apoptosis as a result of diabetes, was certainly an indication of this disease as a triggering factor for prostatic pathogenesis.

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FIGURE LEGENDS

Figures 1a and 1b: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the control group.

1a: Folded prostatic acini. Secretory epithelium (Ep) with columnar and basal cells. Lumen (L). Chromophobe areas taken up by Golgi complex and its vesicles (G). Stroma with collagen fibers (col) and blood vessels (arrow). X150. Masson's trichrome. Inset: Secretory cells with clear nucleoli (Nu) and basal cells (bc). X1000. Hematoxylin-eosin.

1b: Reticular fibers (rf) underlying the epithelium (Ep). Lumen (L). X150. Gömori reticulin.

Figures 1c, 1d, 1m and 1n: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic group.

1c: Atrophied secretory cells (Ep). Intra-luminal secretion (L). Hypertrophied stroma (St). X150. Masson's trichrome. Inset: Nuclei of the epithelial cells occupy a large area in the cellular cytoplasm with one or two nucleoli (Nu). Stroma with collagen fibers (col) and smooth muscle cells (smc). X1000. Hematoxylin-eosin.

1d: Increased reticular fibers (rf), distributed in all the stromal area (St). Epithelium (Ep). Lumen (L). Blood vessels (bv). X150. Gömori reticulin.

1m: Prostatic intraepithelial neoplasia (arrow). Epithelium (Ep). Stroma (St) with collagen fibers (col) and blood vessels (bv). Lumen (L). X150. Hematoxylin-eosin. Inset: Foci of epithelial stratification with increased nuclei (N) and irregular nucleoli. X1000. Hematoxylin-eosin.

1n: Secretory epithelium (Ep) with decreased cells and intraluminal secretion (L). Stroma (St). Increased collagen fibers and blood vessels (bv). Inflammatory cells (asterisk). X150. Hematoxylin-eosin. Inset: Inflammatory cells (arrows). Collagen fibers (col). X1000. Masson's trichrome.

Figures 1e and 1f: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic-insulin group.

1e: Secretory epithelium (Ep) with basal cells. Lumen (L). Collagen fibers (col) and inflammatory cells (arrows) in the stroma (St). X150. Masson's trichrome. Inset: Epithelial cells showing evident nuclei (Nu). Basal cell (bc). Golgi complex (G). X1000. Hematoxylin-eosin.

1f: Increased reticular fibers (rf), distributed in the stroma area (St). Glandular epithelium (Ep) and intraluminal secretion (L). X150. Gömori reticulin.

Figures 1g and 1h: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic-testosterone group.

1g: Secretory epithelium (Ep) with columnar cells, lumen (L). Golgi complex area (G). Increased collagen fibers (col). Blood vessels (bv). X150. Masson's trichrome. Inset: Nuclei showing clear nucleoli (Nu). Golgi complex area (G). Collagen fibers (col) and smooth muscle cells (smc). X1000. Hematoxylin-eosin.

1h: Undulated reticular fibers (rf) distributed in all the stroma (St). Secretory epithelium (Ep). Lumen (L). X150. Gömori reticulin.

Figures 1i and 1j: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic-estrogen group.

1i: Secretory epithelium (Ep) with columnar cells, lumen (L). Collagen fibers (col) in the stroma (St). X150. Masson's trichrome. Inset: Nuclei of epithelial cells (Ep) with clear nucleoli. X1000. Hematoxylin-eosin.

1j: Increased reticular fibers (rf) distributed in all regions of the stroma (St). Secretory epithelium (Ep). Lumen (L). X150. Gömori reticulin.

Figures 1k and 1l: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic-insulin-testosterone-estrogen group.

1k: Secretory epithelium (Ep) with tall columnar cells. Golgi complex areas (G). Stroma (St) showing collagen fibers (col). X150. Masson's trichrome. Inset: Basal nuclei with nucleoli (Nu). Collagen fibers (col). X1000. Hematoxylin-eosin.

1l: Stroma (St) with reticular fibers (rf) underlying the epithelium (Ep). Lumen (L). X150. Gömori reticulin.

Figures 2a, 2b and 2c: Electronmicrographs of the ventral lobe of the prostate from the control group.

2a: Apical region. Secretory vesicles (Vs). Lumen (L). X10000. Inset: Short and scattered microvilli (Mv). Lumen (L). X12930.

2b: Supranuclear region. Rough endoplasmic reticulum cisternae (RER) and paralell and flattened Golgi complex cisternae (arrow) with secretory vesicles (Vs). Mitochondria (M). Cellular nuclei (N). X10000.

2c: Secretory epithelium (Ep) with basal nuclei (N). Rough endoplasmic reticulum (RER). Basal lamina (arrow). Stroma (St) with collagen fibers (col) underlying and smooth muscle cells (sm). X10000.

Figures 2d, 2e, 2f 2g and 2h: Electronmicrographs of the ventral lobe of the prostate from the diabetic group.

2d: Apical region. Discontinuity of microvilli. Rare secretory vesicles (Vs). Lumen (L). X10000.

2e: Supranuclear region. Nuclei (N). Dilated Golgi cisternae (arrow). Secretory vesicles (Vs). Digestive vesicles (asterisks). X10000. Inset: Mitochondria (M) with ruptured cristae. X12930.

2f: Nuclei (N) of the secretory cell. Stroma (St) and inflammatory cells (arrows), blood vessels (bv), smooth muscle cells (smc). Nucleus of the endothelial cell (ec). X7750. Inset: Nuclei of the epithelial cells (N). Lipidic droplets (arrows) and digestive vesicles (asterisk) in the cytoplasm. Dilated rough endoplasmic reticulum cisternae (RER). X7750.

2g: Irregular contour of the nuclei (N). Digestive vesicles (arrows). Basal lamina (bm). Increased collagen fibers (col). Smooth muscle cells (smc), showing secretor phenotype with secretory vesicles (v). Blood vessels (bv). X10000. Inset: Smooth muscle cells (smc), showing spinous aspect (arrow). X10000.

2h: Prostatic intraepitelial neoplasia (PIN). Prostatic stroma (St). X10000.

Figures 3a, 3b and 3c: Electronmicrographs of the ventral lobe of the prostate from the diabetic-insulin group.

3a: Apical region. Discontinuity of microvilli (Mv). Occasional secretory vesicles (Vs). Junctional complex (J). Lumen (L). X10000.

3b: Supranuclear region. Nuclei (N). Dilated Golgi cisternae (arrows). X10000. Inset: Mitochondria (M) with ruptured cristae. Rough endoplasmic reticulum (RER). X12930.

3c: Nuclei (N). Mitochondria (M). Digestive vesicles (arrows). Basal lamina (bm). Collagen fibers (col). Smooth muscle cells (smc), showing secretory phenotype (v). Fibroblast (fb). X10000. Inset: Digestory vacuoles (asterisk) and occasional lipidic droplets (arrow). Dilated rough endoplasmic reticulum cisternae (RER). Epithelial cell nuclei (N). X10000.

Figures 3d, 3e, 3f and 3g: Electronmicrographs of the ventral lobe of the prostate from the diabetic-testosterone group.

3d: Apical region. Short scattered microvilli. Occasional secretory vesicles (Vs). Mitochondria (M) with ruptured cristae. Lumen (L). X10000. Inset: Mitochondria (M) with ruptured cristae. X12930.

3e: Supranuclear region. Nuclei (N). Dilated Golgi cisternae (arrows). Occasional secretory vesicles (Vs). X10000. Inset: Nuclei (N) of the epithelial cell. Dilated rough endoplasmic reticulum cisternae (arrows). Stroma (St). X10000.

3f: Nuclei (N) of the secretory cell. Lipidic droplets (arrows). Collagen fibers (col). Smooth muscle cell (sm) with and small secretory vesicles (v). X10000. Inset: Smooth muscle cells (smc), showing spinous aspect (arrow). X10000.

3g: Lipidic droplets (arrows) and secretory epithelium (Ep). Collagen fibers (col) placed underlying the epithelium and among the smooth muscle cell (sm) and fibroblast (fb). Inflammatory cell (thick arrow). X7750. Inset: Inflammatory cell, probably mastocyte, with central nuclei (Nic) and vesicles distributed in the cytoplasm. X7750.

Figures 3h, 3i and 3j: Electronmicrographs of the ventral lobe of the prostate from the diabetic-estrogen group.

3h: Apical region. Short and scatttered microvilli. Secreory vesicles (Vs). Vacuolization of the cytoplasm (asterisk). Lumen (L). X10000.

3i: Supranuclear region. Nuclei (N). Dilated Golgi cisternae (arrows). Vacuolization of the cytoplam (asterisk). X10000. Inset: Nuclei (N). Mitochondria (M) with ruptured cristae. Rough endoplasmic reticulum cisternae (RER) with some points of dilatation.

3j: Nuclei (N) of the epithelial cells. Basal lamina (bm). Digestory vesicles (asterisks). Rough endoplasmic reticulum cisternae (RER) with some points of dilatation. Collagen fibers (col) and smooth muscle cells (smc). Irregular nuclear membrane of smooth muscle cells (smc) and occasional secretory vesicles (arrows). X10000. Inset: Nuclei of the epithelial cell (N). Digestive vesicles (asterisks) and lipidic droplets (arrows) in the cytoplasm. X7750.

Figures 3k, 3l and 3m: Electronmicrographs of the ventral lobe of the prostate from the diabetic-insulin-testosterone-estrogen group.

3k: Apical region. Short and scattered microvilli (Mv). Vesicles (Vs) with granules of different electrondensity. Lumen (L). X10000.

3l: Supranuclear region. Nuclei (N). Mitochondria (M). Golgi complex cisternae (arrow). Rough endoplasmic reticulum cisternae (asterisk) with some points of dilatation. X10000. Inset: Mitochondria (M) with ruptured cristae. X12930.

3m: Nuclei of epithelial cells (N). Lipidic droplets (arrows) and digestive vacuoles (asterisks). Basal membrane (bm). Collagen fibers (col). Smooth muscle cells (smc) with some secretory vesicles (v). Blood vessels (bv). X10000.

Figure 4a: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the control group: Weak immunoreactivity for Ki-67 (arrows) in the secretory and basal epithelial cells (Ep) and stromal cells (St). Lumen (L).

Figure 4b: Ventral lobe of the prostate. Detection of apoptotic cell from the control group: Weak DNA fragmentation (arrow) in the secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4c: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the diabetic group: Strong immunoreactivity for Ki-67 (arrows) in the secretory and basal epithelial cells (Ep) and stromal cells (St). Lumen (L).

Figure 4d: Ventral lobe of the prostate. Detection of apoptotic cell from the diabetic group: Strong DNA fragmentation (arrows) in the secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4e: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the diabetic-insulin group: Moderate immunoreactivity for Ki-67 (arrows) in the secretory and basal epithelial cells (Ep) and stromal cells (St). Lumen (L).

Figure 4f: Ventral lobe of the prostate. Detection of apoptotic cells from the diabetic-insulin group: Moderate DNA fragmentation (arrows) in the secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4g: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the diabetic-testosterone group: Moderate immunoreactivity for Ki-67 (arrows) in the secretory and basal epithelial cells (Ep) and stromal cells (St). Lumen (L).

Figure 4h: Ventral lobe of the prostate. Detection of apoptotic cell from the diabetic-testosterone group: Moderate DNA fragmentation (arrows) in the secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4i: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the diabetic-estrogen group: Strong immunoreactivity for Ki-67 (arrow) in secretory and basal epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4j: Ventral lobe of the prostate. Detection of apoptotic cell from the diabetic-estrogen group: Strong DNA fragmentation (arrows) in secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4k: Ventral lobe of the prostate. Immunolocalization of the Ki-67 antigen from the diabetic-insulin-testosterone-estrogen group: Weak immunoreactivity for Ki-67 (arrows) in secretory and basal epithelial cells (Ep) and stromal cells (St). Lumen (L).

Figure 4l: Ventral lobe of the prostate. Detection of apoptotic cell from diabetic-insulin-testosterone-estrogen group: Weak DNA fragmentation (arrow) in secretory epithelial cells (Ep). Stroma (St). Lumen (L).

Figure 4m: Percentage of immunolabeled of Ki-67 and apoptotic index of the ventral lobe of the prostate. Inset: Secretory epithelial cell (Ep) with apoptotic nuclei (arrow). The apoptotic nuclei was identified by Feulgen's reaction. Stroma (St). Lumen (L). X1000.

Table 1: Serum glucose (mg/dl), testosterone (ng/ml) and estradiol (pg/ml) levels of the mice in the different experimental groups.

Variants	Groups					
	Control	**Diabetic**	**Diabetic-Insulin**	**Diabetic-Testosterone**	**Diabetic-Estrogen**	**Diabetic-Insulin-Testosterone-Estrogen**
Glucose	185.7±5.3 a	910.2±80.1 e	373.7±41.5 bc	629.5±131.8 d	484.0±101.7c	317.2±54.7b
Testosterone	34.1±6.1 d	6.7±2.0 a	9.4±0.7a	18.5±0.8 b	8.9±1.0 a	24.9±1.2 c
Estradiol	24.9±1.6 a	49.0± 4.7 d	36.7±1.7 c	38.5±0.7 c	52.4±1.0 d	29.4± 1.6 b

Two averages, followed by the same small letter are not different from each other ($P < 0.05$) Tukey's test.

Table 2: Mean and standard deviation of the nuclear (NCL) and cytoplasmic (CYT) volumes (μm^3) of the epithelial cells of the ventral lobe of mice from six experimental groups.

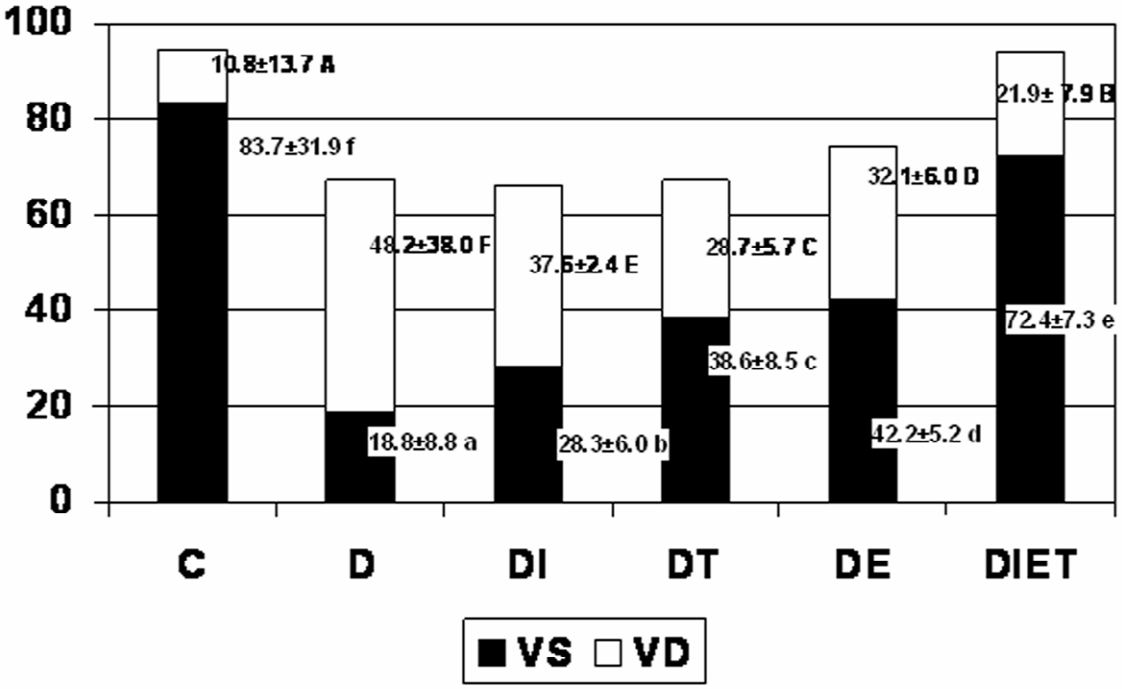
Variants	Groups					
	Control	**Diabetic**	**Diabetic-Insulin**	**Diabetic-Testosterone**	**Diabetic-Estrogen**	**Diabetic-Insulin-Testosterone-Estrogen**
NCL Vol	82.1± 5.2 d	64.6±3.3 a	72.5±4.1 b	77.0±2.0c	78.6±1.6 cd	82.1±1.3d
CYT Vol	359.1±29.7 f	152.4±2.9 a	167.6±1.6 b	197.9±1.1 c	219.7±2.8 d	326.5±2.9 e
Total Vol	441.2±7.4 f	216.9±2.2 a	240.0±3.5 b	274.9±2.3 c	298.3±1.9 d	408.6±3.6
NCL%	18.6±0.2 a	29.7±1.0 b	30.2±0.5 b	27.9±0.3 ab	26.5±0.6 ab	20.0±0.2 a
CIT%	81.4±0.2 b	70.3±1.0 a	69.8±0.5 a	72.0±0.3 ab	73.5±0.6 ab	80.0±0.2 b

Abbreviations: NCL Vol.—nuclear volume; CYT Vol.—cytoplasmic volume; Total Vol.—total volume; NCL%—nuclear percentage; CYT%—cytoplasmic percentage.

Two averages, followed by the same small letter are not different from each other ($P < 0.05$) Tukey's test.

Graph 1

Graph 1: Mean and standard deviation of the areas (µm2) of the secretory vesicle (VS) and digestory vacuoles (VD) of the epithelial cells of the ventral lobe of mice from six experimental groups.

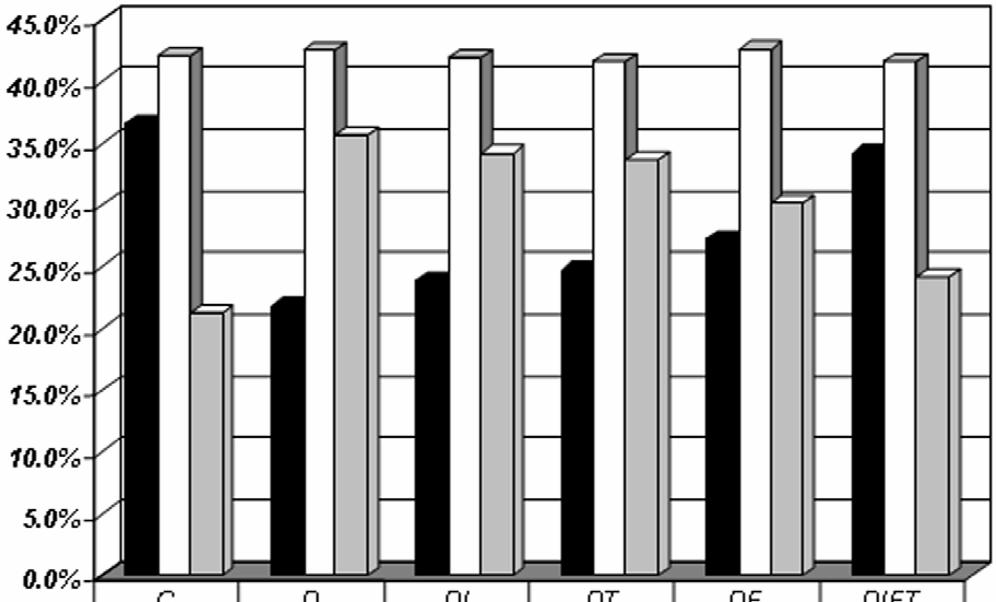


Two averages, followed by the same small letter are not different each other ($P > 0.05$) Tukey's test.
Two averages, followed by the same capital letter are not different each other ($P > 0.05$) Tukey's test.

Abbreviations: Groups: C- control; D- diabetic; DI- diabetic-insulin; DT- diabetic-testosterone; DE- diabetic-estrogen; DIET- diabetic-insulin-testosterone-estrogen.

Graph 2

Graph 2: Mean of the areas (%) of the epithelium, lumen and stroma of the ventral lobe of the prostate in the six experimental groups.



	C	D	DI	DT	DE	DIET
Epithelium	36.6%	21.8%	23.9%	24.7%	27.2%	34.2%
Lumen	42.1%	42.5%	41.9%	41.6%	42.6%	41.6%
Stroma	21.3%	35.7%	34.2%	33.7%	30.2%	24.2%

Abbreviations: Groups: C- control; D- diabetic; DI- diabetic-insulin; DT- diabetic-testosterone; DE- diabetic-estrogen; DIET- diabetic-insulin-testosterone-estrogen.

Figure 1

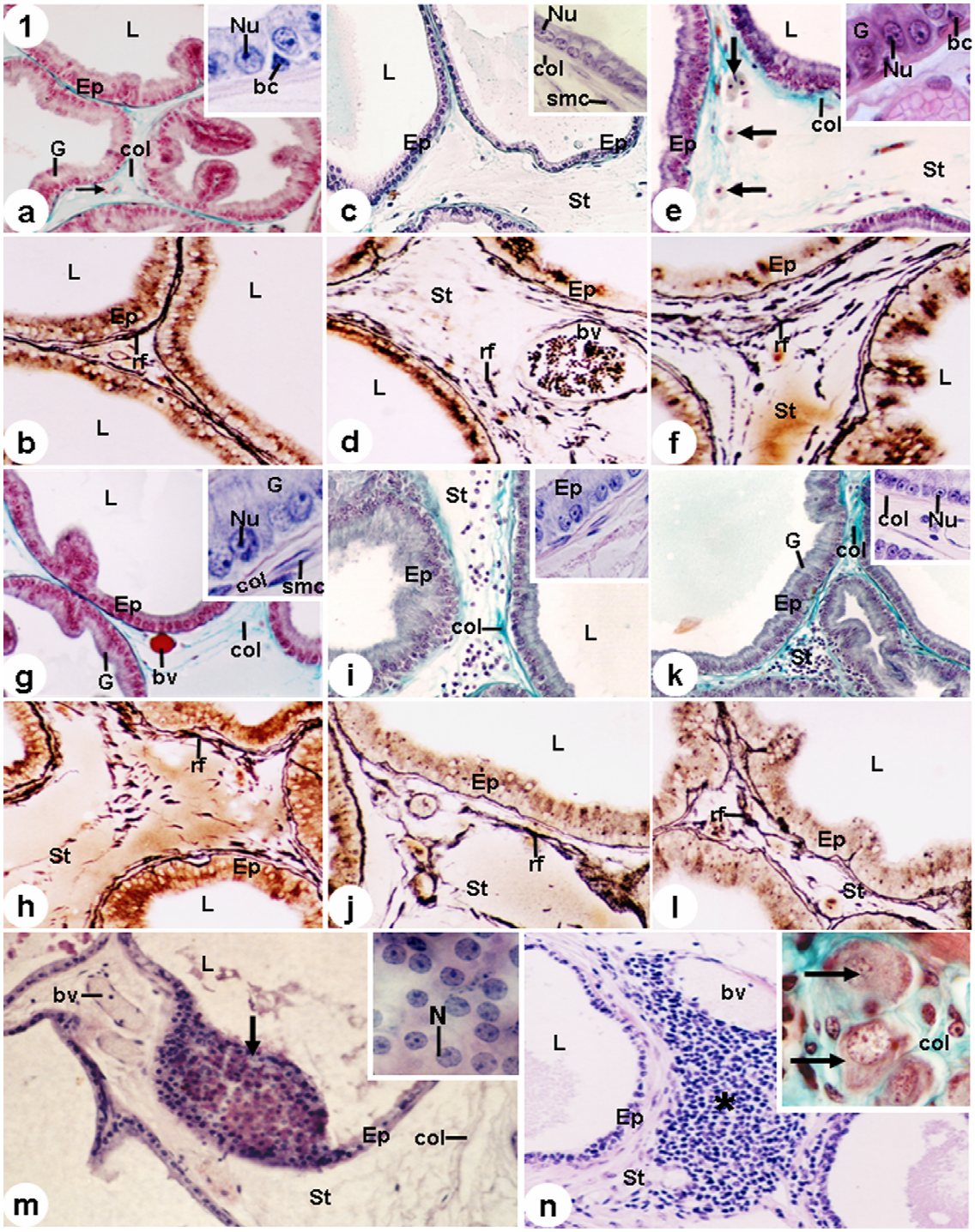

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Figure 2

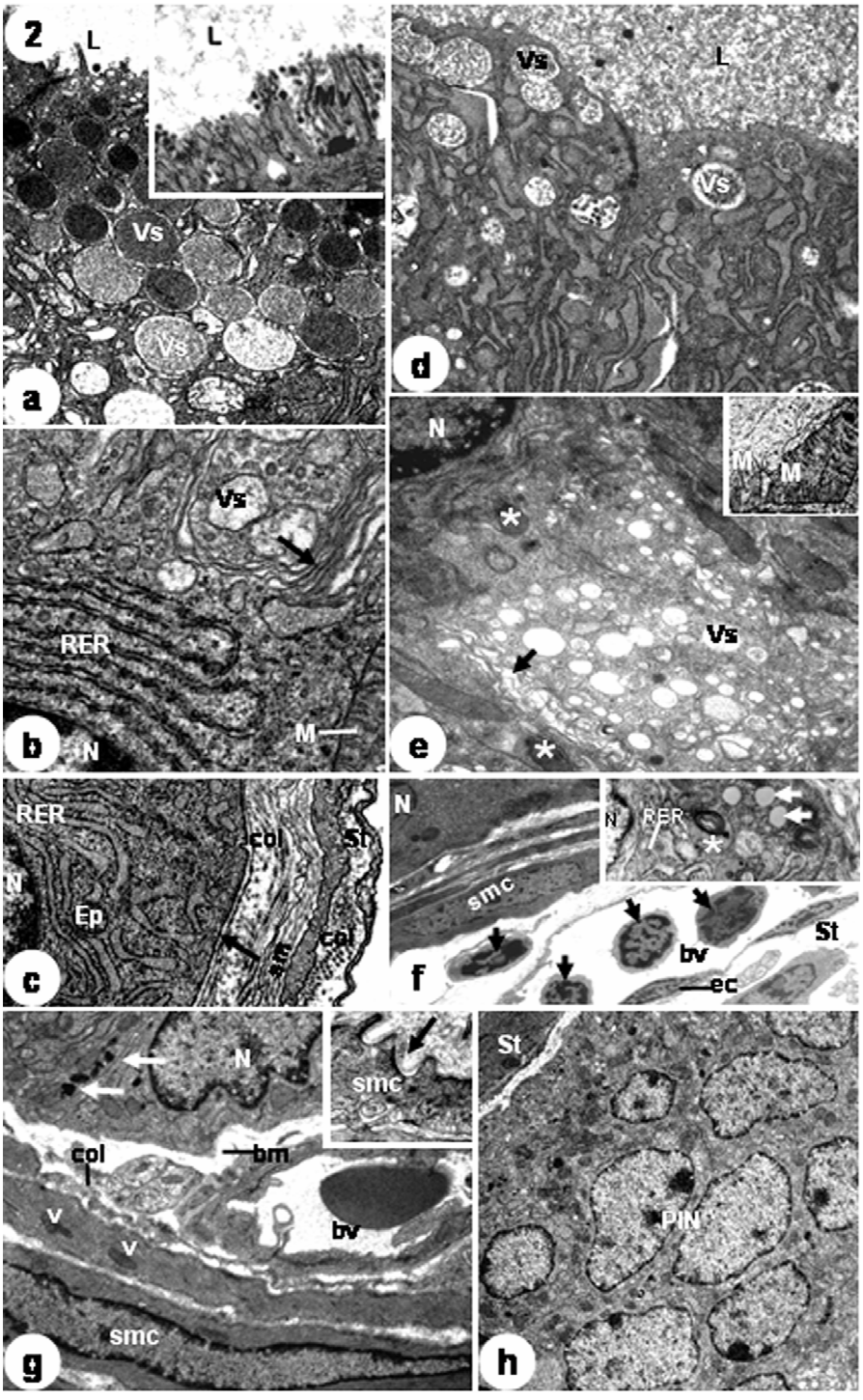
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Figure 3

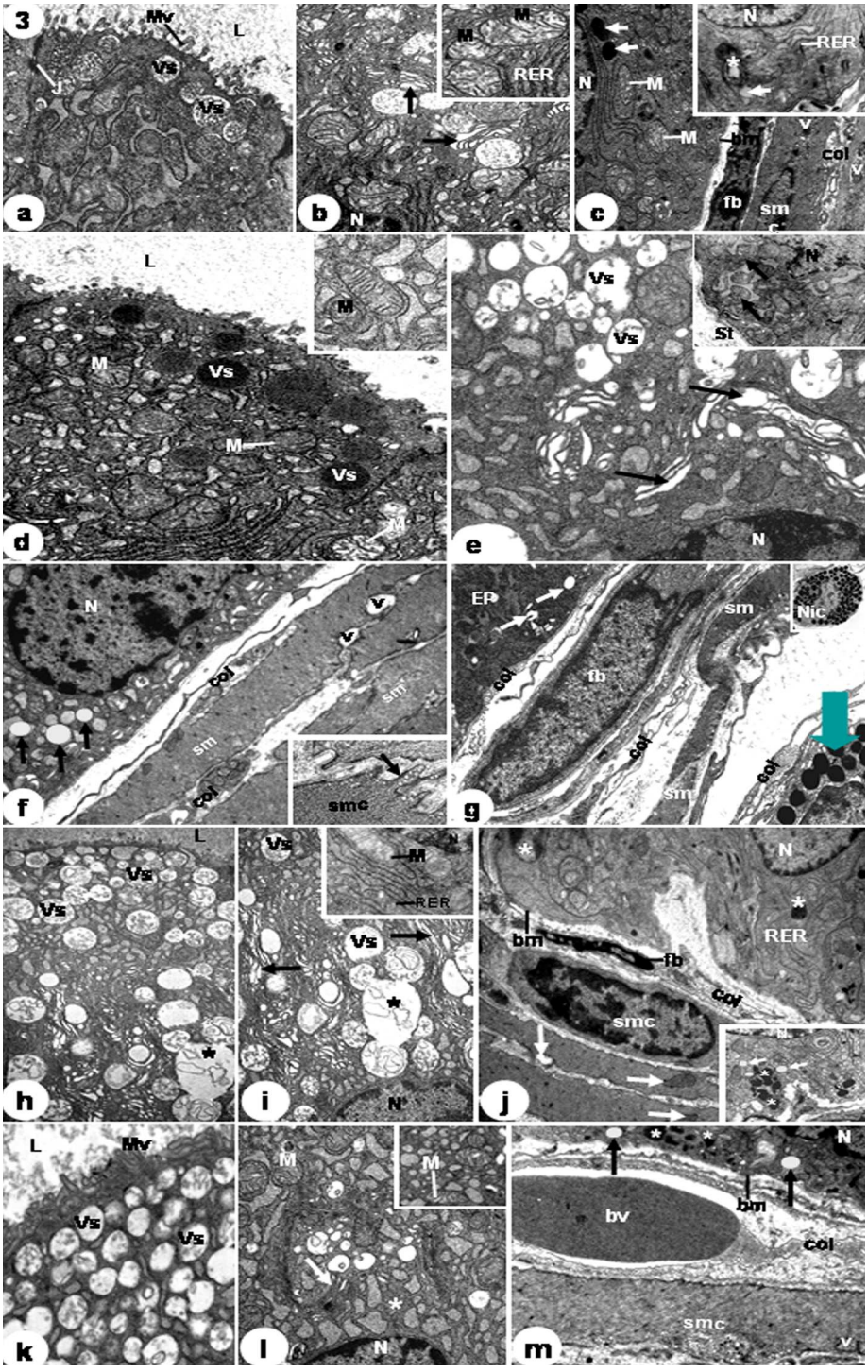
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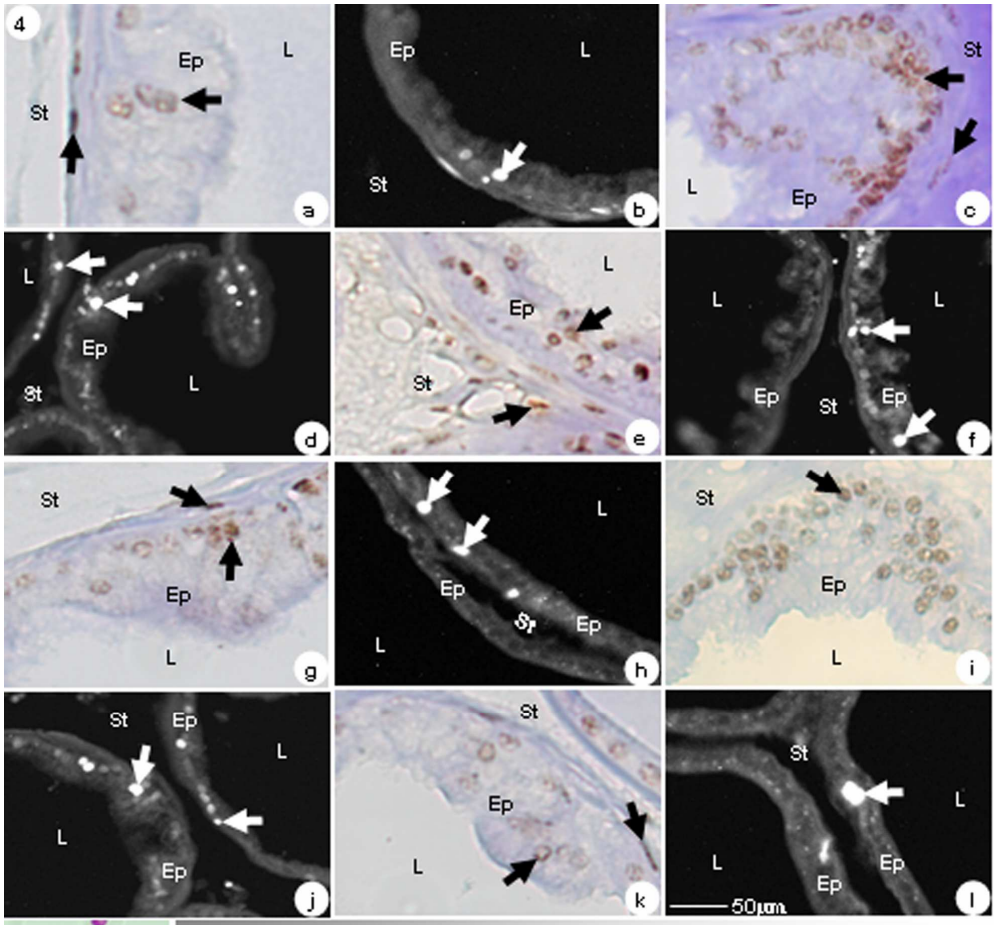
Figure 4



Averages, followed by the same letter, are not different each other (P>0.01) "Tukey's test".

"Dystroglycan patterns on the prostate of non-obese diabetic mice submitted to glycemic control"

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Short title: Dystroglycan and diabetes on the prostate

Key words: Ventral Prostate; Diabetes Mellitus; Dystroglycan; Immunohistochemistry; Insulin

This work was supported by FAPESP and CNPq.

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ABSTRACT

Dystroglycan (DG) is an adhesion protein which plays a crucial role in maintenance of tissue integrity. Diabetes has been pointed out as a disease which causes harmful effects on prostate function. Therefore, the main objective of this work was to verify DG distribution and structure features in diabetic mice with and without glycemic control, and as well as to relate these parameters to prostate pathogenesis. Thirty mice (Nod and BALB/c) were divided into three groups, after twenty days of diabetic state: the control group received a 5 mL/Kg dose of physiological saline daily for 20 days; the diabetic group had the same treatment as the control group; the diabetic-Insulin group received 4-5 IU doses of NPH insulin daily for 20 days. After 20 days of treatment, all animals were sacrificed and samples from the ventral prostate were processed for immunological and light microscopy analyses. The results showed diminished β and α DG receptors in the diabetic group. However, there was a recovery of both β and α DG receptor immunolocalization after insulin administration. The epithelial and stromal morphological changes were verified in the diabetic group which also presented recovery after insulin treatment. Thus, it could be concluded that diabetes disturbed the prostate structure integrity and altered the occurrence of α and β-DG receptors pointing to decreased cell-matrix extracellular and cell-basal membrane attachment. However, insulin treatment at least partially restored glandular homeostasis. The decrease of epithelial-stromal interaction, certainly, predisposes this gland in diabetic mice to be a prostate disease target.

INTRODUCTION

Diabetes mellitus is a disease which causes protein, carbohydrate and lipid metabolism alterations leading to hyperglycemia due to insulin deficiency (Mokdad, 2001; Conget, 2002). Moreover, diabetic patients showed various alterations in their organic system such as vascular; urogenital; neuronal; digestive disorders; among others (Ciardullo et al., 2004).

Different experimental studies in the male reproductive system, which exhibited type 1 diabetes mellitus due to either chemical induction or spontaneous development presented retrograde ejaculation (Ellenberg, 1980); urinary bladder dysfunction (Buck et

al., 1976; Stefan, 1996); sexual impotence; and decreased spermatozoid number in the seminal fluid (Frenkel et al., 1978; Mcculloch et al., 1984). Cagnon et al. (2000) and Ribeiro et al. (2006) verified atrophied secretory cells; stromal hypertrophy; inflammatory processes; prostatic intraepithelial neoplasia; and dilated secretory organelles in the ventral lobe of the prostate in non obese diabetic mice. Also, Carvalho et al. (2003) observed morphological changes in the coagulating gland of diabetic mice such as; acini thickening; inflammatory cells in the stroma, and hypertrophied extracellular matrix elements. Other studies revealed diminished testosterone serum levels and a low androgen receptor rate in the diabetic animals due to imbalanced hormone metabolism (Okasen, 1975; Tesone et al., 1976). Furthermore, Wang et al. (2000) stated that the glycemic control of diabetic rats, by means of insulin administration, did not recover prostate weight. Also, Suthagar et al. (2008) verified that the diabetic state caused diminished testosterone and estrogen receptor levels, and insulin replacement was a recovery factor for these steroid hormone levels. In clinical observation, diabetes was pointed out as being a feasible etiological factor in prostate cancer development (Ilic et al., 1996). According to Hammarsten & Hogstedt (2002), the increase of HBP as well as its quick progression in diabetic men could be considered a risk factor towards development of clinical cancer. On the other hand, there are clinical studies which demonstrated that diabetic patients had a reduction in the risk of prostate cancer (Kasper et al., 2008). In addition, another study with men, prostate cancer and who received androgen suppression therapy, showed high risk of developing insulin resistance and the occurrence of hyperglycemia (Basaria et al., 2006).

The prostate is one of the most important accessory sex glands, with its secretion being fundamental for the male reproductive process (Setchell & Brooks, 1988; Marker et al., 2003; Untergasser et al., 2005).

The rodent prostate is divided into three pairs of lobes: ventral, dorsal and lateral according to their anatomic positions in relation to the urethra (Jesik et al., 1982; Sugimura et al., 1986; Aumuller & Seitz, 1990). These lobes show different characteristics in relation to morphology, secretion and hormone dependence and the ventral lobe is primarily regulated by androgens (Colombel & Buttyan, 1995; Costello & Franklin, 1994).

In general, the prostate lobes show a simple epithelium surrounded by stroma (Aumüller and Adler, 1979). The prostate stroma is made up of a complex net of stromal cells and extracellular matrix associated to growth factors; regulatory molecules; and remodelation enzymes, which provide biological signals and have mechanical influence on the epithelial cells (Tuxhorn et al., 2001; Cunha & Matrisian, 2002). The fibroblasts and smooth muscle cells are important cellular types in the prostate stroma, synthesizing structural and regulatory compounds of the extracellular matrix. The extracellular matrix is a net of fibrillar proteins, adhesion glycoproteins and proteoglycans (Lin & Bissell, 1993; Kreis & Vale, 1999; Tuxhorn, et al., 2001). Thus, the association of stromal cells with extracellular matrix provides a microenvironment which regulates the growth and functional differentiation of the adjacent cells, where each of these elements plays a crucial role in the structure maintenance and tissue function (Tuxhorn et al., 2001). The stroma-epithelium interaction has an important commitment in prostatic structural maintenance and function (Ekman, 2000). The basal membrane is an interaction link, offering mechanical and physiological support which is specially made up of collagen type IV and laminin (Knox et al., 1994). The imbalance of the stroma-epithelium interaction leads to different prostate diseases including prostate cancer (Wong et al., 2000; Cunha et al., 2001; Cunha et al., 2003; Cornell et al.; 2003).

Nowadays, different studies suggest that dystroglycan (DG), which is an adhesion protein, plays a role in epithelial and neuronal cell development; formation of basal membrane; and maintenance of tissue integrity (Henry & Campbell, 1998). DG, despite having first been discovered in the skeletal muscle as a compound of the dystrophin-glycoprotein complex, is found in many other non-muscle tissues such as smooth muscle, epithelia and peripheral nerves (Ibraghimov et al., 1992; Henry et al., 2001). Dystroglycan is linked to extracellular matrix proteins such as laminin; perlecan; and agrin; proteoglycans, in addition to cytosolic proteins (Losasso et al., 2000; Sugita et al., 2001). DG is formed by two protein subunits, β and α, interacting to form a non-covalent complex which is identified by the same gene (Brennan et al., 2004). α-DG links extracellular molecules, whereas the transmembrane β-DG anchors α-DG to the cell membrane (Sgambato et al., 2003).

DG expression changes have been verified in the occurrence of cancer in different organs, including the prostate (Losasso et al., 2000; Henry et al., 2001; Sgambato et al., 2003; Brennam et al., 2004). According to Henry et al. (2001), decreased DG expression was observed in high grade prostatic cancer, leading to abnormal prostate cell interaction with the extracellular matrix, causing metastasis. Thus, these studies indicated that changes in the DG expression are a determining factor for prostate pathogenesis.

Thus, based on the negative influence of diabetes mellitus on the prostate function as well as the fundamental DG participation in the cell-extracellular matrix interactions; in the connection between epithelial cells and basal lamina; and in the cytoskeleton organization, the main objective of this work was to characterize DG distribution and structure features in diabetic mice with and without glycemic control, and to verify the possibility of association of these parameters on prostate pathogenesis.

ANIMALS AND TISSUE PREPARATION

A total of 20 mice (Nod) and 10 control mice (BALB/c/Uni), all eighteen weeks old, from The Bioterism Center/Unicamp were used. The blood glucose level was measured by capillary glycemia, utilizing the Optium Advanced Diabetes Management System (MediSense, Abingdon, UK). Thus, the animals showing blood glucose level >300 mg/dl were considered diabetic (Shirai et al., 1998). Twenty days into diabetic state, the mice were divided into three groups: The control group received a 5 mL/Kg dose of 0.9% physiological saline subcutaneously daily for 20 days (Fresenius Kabi, São Paulo, Brazil); The diabetic group received the same treatment as the control group; The diabetic-insulin group received 4-5 IU doses of NPH insulin subcutaneously daily for 20 days (Biobrás, Montes Claros, Minas Gerais, Brazil) (Anderson, 1983). Insulin administrations were interrupted 24 hours prior to the mice being sacrificed. After 20 days of experimental treatment, the animals were anesthetized with a 0.25 mL/100 g body weight dose of Francotar/Virbaxyl (1:1, Vibra® Roseira, SP, Brazil), and samples of the intermediate and distal regions from the ventral lobe of the prostate were collected under a DF Vasconcellos Steromicroscopy which allows the withdrawal of prostatic samples from these specific

regions to be processed for morphometrical, structural and immunological analyses. Hormonal dosages were also carried out on the blood samples.

Serum testosterone and glucose levels

At the end of the 20-day treatment, blood samples from all animals in each group were collected. The blood samples were collected through a cardiac puncture, 24 hours after administrating the last dose of insulin. The serum concentrations of testosterone were determined by radioimmunoassay using Coat-a-Count total testosterone kit (Diagnostic Products Corporation, Los Angeles, CA, USA) and expressed in ng/dL. The serum concentration of glucose was determined by chemiluminescense and expressed in mg/dL.

Light Microscopy: Immunolabelled α-DG and β-DG and Morphometrical Analyses

Samples of the ventral prostate were collected from ten animals in each group for histological and immunological analyses and then fixed by immersion in Bouin's solution, embedded in paraplast (Paraplast Plus, Brazil), cut into 5-6 μm thick sections. These samples were submitted to the following staining procedures: hematoxylin-eosin (Behmer et al., 1976), Picrossirius red (Behmer et al., 1976) and immunostaining for α and β DGs. The slides were photographed with a Nikon Eclipse E-400 photomicroscope. Epithelial, luminal and stromal areas in the ventral lobe of the prostate were measured (25 fields per animal) for structural analyses in the sections stained for light microscopy in ten animals per group, using X200 magnification. The photomicroscope Nikon Eclipse E-400 and the NIS-Elements*: Advanced Research* (USA) computerized image analysis system were used.

For immunological proceeding, the sections were deparaffinized in xylene, hydrated through graded alcohol concentrations and rinsed under tap water. Antigens were retrieved by boiling the sections in 10 mM citrate buffer, pH 6.0, three times for 5 min in a microwave oven. After that, the sections were incubated in 0.3% H_2O_2 for 15 min to block endogenous peroxidase. Nonspecific binding was blocked by incubating the sections in a blocking solution for 1h at room temperature. Primary rabbit H-300 (sc-28534) (Santa Cruz Biotchenollogy) for the α-DG and mouse (NCL-b-DG) (Novocastra) for the β-DG antibodies were diluted in 1% BSA (1:50) and applied to the sections overnight at 4ºC. The

Envision HRP Kit (Dako Cytomation Inc., Carpenteria, CA, USA) was used to visualize the bound antibody according to the manufacturer's instructions. The sections were washed for 15 min. with TBS-T and secondary labeled polymer from the Envision HRP Kit (Dako) was applied for 40 min. at room temperature. After washing in TBS-T, peroxidase activity was detected using a diaminobenzidine chromogen kit from Envision HRP Kit (Dako) for 10 min. Sections were lightly counterstained with methyl green dehydrated in an increasing ethanol series and xylene, mounted in entellan (Merck, Darmstadt, Germany).

The immunolocalization of the α-DG and β-DG were measured in ten animals in each experimental group. Five microscopic fields per animal were measured with a X40 objective lens, and corresponded to a total area of 90304.7 μm². α-DG and β-DG were quantified based on the area of positive immunostaining expressed as a percentage of the total area examined. In addition, staining intensity was graded as strong, moderate or weak according to concentration and distribution of the receptor in the sectioned tissues (Markopoulos et al., 2000).

Statistical Analysis

The serum glucose and testosterone levels; the percentage of α-DG and β-DG positive stained areas; epithelial, luminal and stromal areas were compared between groups and analyzed statistically by means of analysis of variance and Tukey multiple range test, with the level of significance set at 1% (Zar, 1999).

RESULTS

Serum testosterone and glucose levels

The glucose average level of the animals from the diabetic group was over 300mg/dL. In contrast, the levels observed in the animals from the control and diabetic-insulin groups were lower than the value verified in the diabetic group (Table 1).

The average serum testosterone levels were higher in the control group than those found in the other experimental groups. Moreover, the diabetic and diabetic-insulin groups did not show any significant differences in relation to each other (Table 1).

Light Microscopy: Immunolabelled α-DG and β-DG and Morphometrical Analyses

The Control Group

The ventral lobe of the prostate showed folded acini mucosa (Figures 1a and 1b). The secretory epithelium presented tall columnar cells with basal nuclei (Figures 1a and 1b). The prostatic stroma showed thin collagen fibers underlying the secretory epithelium and intermingled with smooth muscle cells (Figures 1a and 1b). The epithelial area was greater than that of the stromal one, representing 39.6% and 18.0%, respectively (Figure 1g).

The secretory epithelium and smooth muscle cells showing intense α-DG immunolocalization, representing 37.6% of the total area (Figures 2a and 2d). The β-DG immunoreactivity was observed in 43.9% of the total measured area found in the periacinal prostatic stroma (Figures 3a and 3d).

The Diabetic Group

The prostate acini presented less folded mucosa than that observed in the control group (Figures 1c and 1d). Atrophied secretory epithelium with cuboidal cells was verified (Figures 1c and 1d). Also, hypertrophied stroma was found, representing an area of 36.6% in contrast to 19.3% of the epithelial area (Figures 1c, 1d and 1g).

The secretory epithelium and smooth muscle cells showed weak α-DG immunolocalization, representing 7.8% (Figures 2b and 2d). The β-DG immunoreactivity was observed in 13.6% of the total measured area found in the periacinal prostatic stroma (Figures 3b and 3d).

The Diabetic-Insulin Group

The prostatic acini demonstrated less folded mucosa than that observed in the control group, however this folding was intensified in relation to that verified in the diabetic group (Figures 1e and 1f). Atrophied secretory epithelium with cuboidal cells and basal nuclei was seen (Figures 1e and 1f). Hypertrophied stroma was verified in relation to the control group, however there was a lower occurrence of collagen fibers than that verified in the diabetic group (Figures 1e and 1f). Some inflammatory cell foci were found

(Figure 1e). The glandular epithelial area was approximately 1.5 times smaller than the stromal area (Figure 1g).The secretory epithelium and smooth muscle cells showed a moderate α-DG immunolocalization, containing 19.9% of the immunoreactivity (Figures 2c and 2d). Also, moderate β-DG immunoreactivity was observed, occupying 29.5% of the total measured area in the periacinal prostatic stroma (Figures 3c and 3d).

DISCUSSION

This work showed high serum glucose levels in diabetic mice. Nevertheless, these values were close to the normal parameter in the diabetic animals submitted to insulin treatment. Early studies have showed that hyperglycemia in diabetic rodents is a determining characteristic in both diabetic rodents by chemical induction and spontaneously developed diabetes (Tesone et al., 1980; Makino et al., 1980; Ho, 1991; Saito et al., 1996; Ader et al., 1998; Ribeiro et al., 2006; Ohta et al., 2007; Caldeira & Cagnon, 2008). Also, another study verified that insulin administration in diabetic rats by chemical induction led to unchanged blood glucose levels with values similar to those of healthy rats (Ohta et al.; 2007). Thus, in the present study, the glycemic levels showed that there was an effective diabetic state in the analyzed mice as well as confirmed insulin action on glycemic control.

Another result of this work is that the prostate exhibited significant molecular and structural changes on the prostate of diabetic animals. The diabetic mice presented atrophied epithelium, hypertrophied stroma, low α and β-DG receptor localization and low serum testosterone level. In contrast, recovery of these structural alterations, α and β-DG receptor immunolocalization and testosterone levels were observed in the diabetic animals which received insulin treatment.

Clinical and experimental studies demonstrated that diabetes led to reduced gonadotrophic hormones by means of hypothalamic-hypophyseal-gonodal axis imbalance which cause physiopathological changes in male reproductive organs, including the prostate (Tesone et al., 1980; Saito et al., 1996; Ho, 1991; Ballester et al., 2004). Other studies observed low serum testosterone levels with diminished androgen receptor on the prostate of diabetic animals as a result of hormone metabolism imbalance, provoked by a

negative feed-back of the hypothalamic-hypophyseal-gonadal axis (Okasen, 1975; Tesone et al., 1976; Daubresse et al., 1978). Nevertheless, Jackson & Hutson (1984) emphasized that the changes in the accessory sex glands of diabetic rodents occurred due to two aspects; decreased testosterone levels and lack of insulin which could alter the cellular mechanism, damaging the normal androgen action.

In addition, Cagnon et al. (2000) and Ribeiro et al. (2006) showed atrophied epithelium, hypertrophied stroma, inflammatory cells and occurrence of prostatic intraepithelial neoplasia in the prostate ventral lobe of Nod mice. In another experimental study, Carvalho et al. (2003) detected coagulating gland alterations in diabetic mice such as inflammatory cells and atrophied acini. In contrast, clinical studies, by means of epidemiological evidence suggested that diabetes mellitus is associated with the decrease in prostate cancer risk. However, these same authors believe that these findings are based on a small number of observations and require further investigation (Pierce et al., 2008; Kasper et al., 2008). According to Kasper et al. (2008), the IGF-1R levels appear to be decreased in diabetic patients when compared with non-diabetic ones. These same authors thought that the possible reduction of prostate cancer in diabetic patient could be related to the hypothesis of varying hormonal profiles. Nevertheless, other epidemiological data showed that men that have been diabetic for five years or more have more chances of developing prostate cancer than healthy men (Will et al., 1999).

In another way, Soudamani et al. (2005) verified that type I diabetes mellitus compromised the differentiation development of the ventral prostate during sexual maturation and exogenous insulin minimized the harmful diabetic effects. Wang et al. (2000) verified that glycemic control in diabetic rats by means of insulin replacement did not restore prostate weight. However, the simultaneous administration of insulin and testosterone was much more efficient in the restoration of morphological and functional prostate characteristics (Sufrin & Scott, 1972; Tesone et al., 1980; Ho, 1991). According to Suthagar et al. (2008) diabetes mellitus altered the biochemical parameters of the prostate as well as androgen and estrogen expression receptors. In addition, the insulin replacement partially or completely minimized, these changes, pointing to insulin as being essential for maintaining prostate functional integrity.

Regarding physiological and molecular aspects of the prostate, this gland is an androgen-mediated organ which has been widely studied, due to its fundamental role in the male reproductive system as well as the high occurrence of pathologies (Leav et al., 2001; Cunha et al., 2002). The cellular and molecular prostate complexity, especially in relation to paracrine events signaling the stroma-epithelium interaction, has pointed to different molecules, which are involved in the various prostate biological processes (Marker et al., 2003).

Dystroglycan (DG) is a non-integrin adhesion molecule expressed by a variety of tissues, interacting with extracellular proteins including laminin, perlecan, agrin (Winder, 2001). The DG biosynthesis is complex, which is the product of a single gene and the primary peptide is post-translationally cleaved, resulting in two protein subunits α and β DG (Winder, 2001). DG was initially studied in skeletal muscles and its role has been limited to muscle physiopathology for a long time (Brennan et al., 2004). Nevertheless, nowadays the adhesion protein role such as DG has shown multiple functions which are involved in connecting cells to the basal lamina and in the exchange of information with the extracellular environment (Hood & Cheresh, 2002; Lyons & Jones, 2007). In addition, it is well known that the precise contact between epithelial cells and their underlying basement membrane is crucial to the maintenance of tissue architecture and function (Weir et al., 2006). Thus, various studies verified that different types of cancers such as breast, colon and prostate demonstrated heterogeneity of the β DG expression and a low or absent α DG one when compared to normal epithelial tissue (Sgambato et al., 2003). Moreover, reduced α DG was associated with tumor progression (Henry et al., 2001). According to Sgambato et al. (2007), the DG overexpression inhibits the growth and tumorigenesis of these cells. Also, these same authors analyzed the DG in patients with a diagnosis of prostate cancer who were submitted to radical prostectomy and anti-androgen treatment and who demonstrated a dose and time dependent decrease of the DG expression. Nevertheless, the molecular relationship between androgen and DG is not clear. Raz (2004) showed that reduced DG expression caused loss of function leading to aberrant cell-extracellular matrix interaction, resulting in an increase of invasion properties. This author suggested the DG as being a positive element towards tumorigenesis in the epithelial cells. Also, other results

suggested that lack of DG could have early effects on the carcinogenesis events, much more than neoplasia transformation (Sgambato et al., 2003; Sgambato & Brancaccio, 2005).

Regarding the animal model, it would be appropriate to state that the NOD mouse is a polygenic model of human insulin-dependent diabetes which presents an autoimmune genetic factor (Makino et al., 1980; Martin et al., 1997). In addition, different authors have shown that the autoimmune factor is not responsible for triggering glandular morphological changes because these animals develop moderate autoimmune alterations (Humprhreys-Beher et al., 1998; Yamano et al., 1999). According to Hu et al. (1992), the autoimmune effects could intensify the changes caused by the metabolic disorder provoked by type I diabetes.

Finally, it can be concluded that diabetes disturbed the prostate structure integrity and the altered occurrence of α and β-DG receptors, probably decreased the cell-matrix extracellular and cell-basal membrane attachment. However, insulin treatment at least partially restored glandular homeostasis. Also, it is possible to suggest that DG could be regulated by testosterone. However, the relation between DG and testosterone is still not clear and new studies in this field will be necessary. Moreover, DG analysis is another important step towards knowledge of the prostate complexity and could certainly be a useful molecule as a marker in prostate diseases.

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FIGURE LEGENDS

Figures 1a, 1b: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the control group.

1a: Folded prostatic acini. Secretory epithelium **(Ep)** with columnar and basal cells. Lumen **(L)**. Stroma **(St)** collagen fibers underlying the epithelium. Hematoxylin-eosin.

1b: Collagen fibers **(col)** underlying the epithelium **(Ep)**. Lumen **(L)**. Stroma **(St)** with blood vessel **(bv)**. Picrossirius red.

Figures 1c, 1d: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic group.

1c: Acini showing poorly folded mucosa and intra-luminal secretion **(L)**. Atrophied secretory cells **(Ep)**. Hypertrophied stroma **(St)**. X150. Hematoxylin-eosin.

1d: Increased collagen fibers **(col)**, distributed in all the stromal area **(St).** Epithelium (**Ep)**. Lumen **(L)**. Picrossirius red.

Figures 1e, 1f: Photomicrographs of the secretory epithelium of the ventral lobe of the prostate from the diabetic-insulin group.

1e: Secretory epithelial cells **(Ep)** with basal cells intermingled with cuboidal cell. Lumen **(L)**. Collagen fibers **(col)** in the stroma **(St)**. Inflammatory cells **(arrow)** in the fibrillar elements. Hematoxylin-eosin.

1f: Increased collagen fibers **(col)**, distributed in the stroma area **(St)**. Glandular epithelium **(Ep)** and intraluminal secretion **(L)**. Picrossirius red.

Figure 2a: Ventral lobe of the prostate. α-DG immunolocalization from the control group: Strong α-DG immunoreactivity **(arrows)** in the secretory epithelium **(Ep)** and smooth muscle cells. Stroma **(St)**. Lumen **(L)**.

Figure 2b: Ventral lobe of the prostate. β-DG immunolocalization from the control group: Strong β-DG immunoreactivity **(arrows)** in the periacinal prostatic stroma. Epithelium **(Ep)**. Stroma **(St)**. Lumen **(L)**.

Figure 2c: Ventral lobe of the prostate. α-DG immunolocalization from the diabetic group: Weak α-DG immunoreactivity **(arrows)** in the secretory epithelium **(Ep)** and smooth muscle cells. Stroma **(St)**. Lumen **(L)**.

Figure 2d: Ventral lobe of the prostate. β-DG immunolocalization from the diabetic group: Weak β-DG immunoreactivity **(arrows)** in the periacinal prostatic stroma. Secretory epithelium **(Ep)**. Stroma **(St)**. Lumen **(L)**.

Figure 2e: Ventral lobe of the prostate. α-DG immunolocalization from the diabetic-insulin group: Moderate α-DG immunoreactivity (**arrows)** in the secretory epithelium **(Ep)** and smooth muscle cells. Stroma **(St)**. Lumen **(L)**.

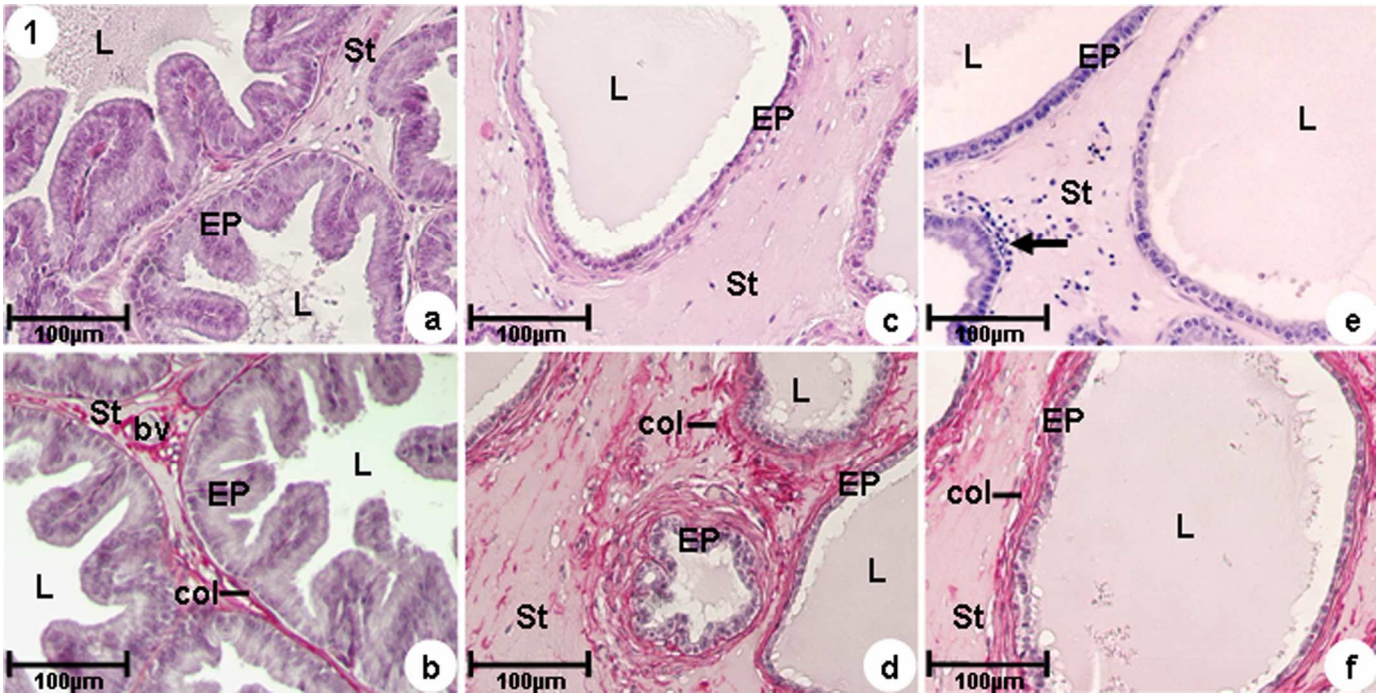
Figure 2f: Ventral lobe of the prostate. β-DG immunolocalization from the diabetic-insulin group: Moderate β-DG immunoreactivity **(arrows)** in the periacinal prostatic stroma. Secretory epithelium **(Ep)**. Stroma **(St)**. Lumen **(L)**.

Table 1: Serum glucose (mg/dL) and testosterone (ng/mL) levels of mice in the different experimental groups.

Variants	Groups		
	Control	**Diabetic**	**Diabetic-Insulin**
Glucose	180.1± 6.1 a	839.1 ± 40.0 b	365.4 ± 9.5 c
Testosterone	33.3 ± 2.1 a	6.6 ± 0.6 b	11.1 ± 1.5 b

Two averages, followed by the same small letter are not different from each other ($P > 0.01$) Tukey's test.

Figure 1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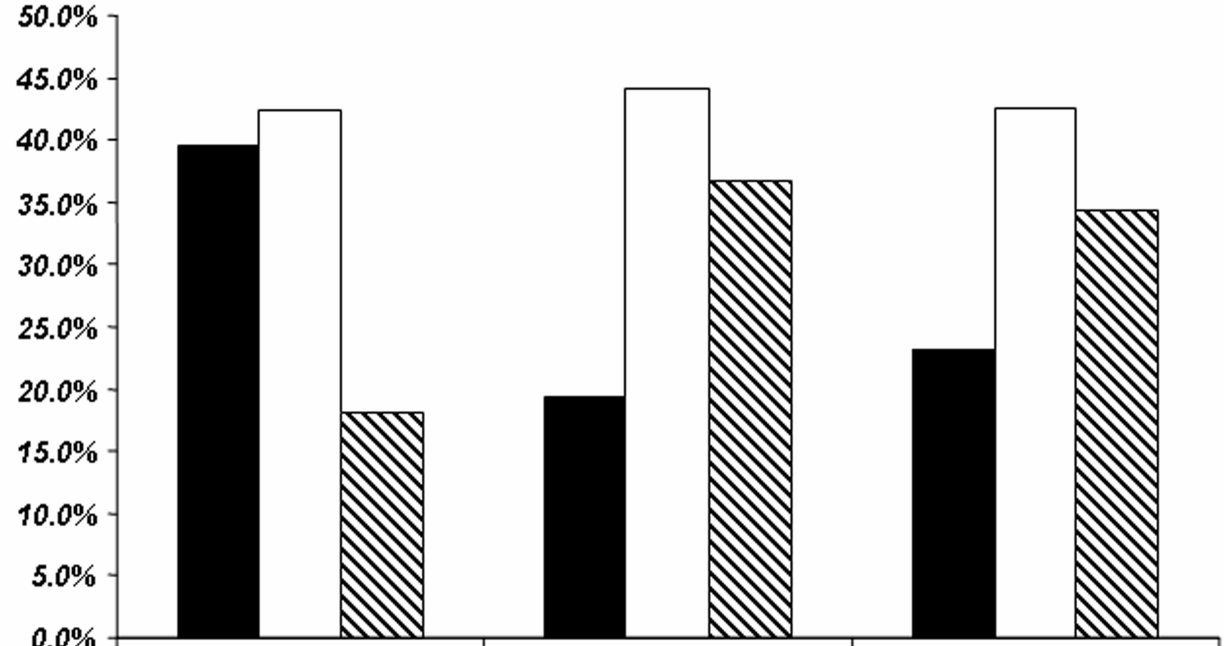
Graph 1

Graph 1: Mean and standard deviation of the areas (%) of the epithelium, lumen and stroma of the ventral lobe of the prostate in the experimental groups.

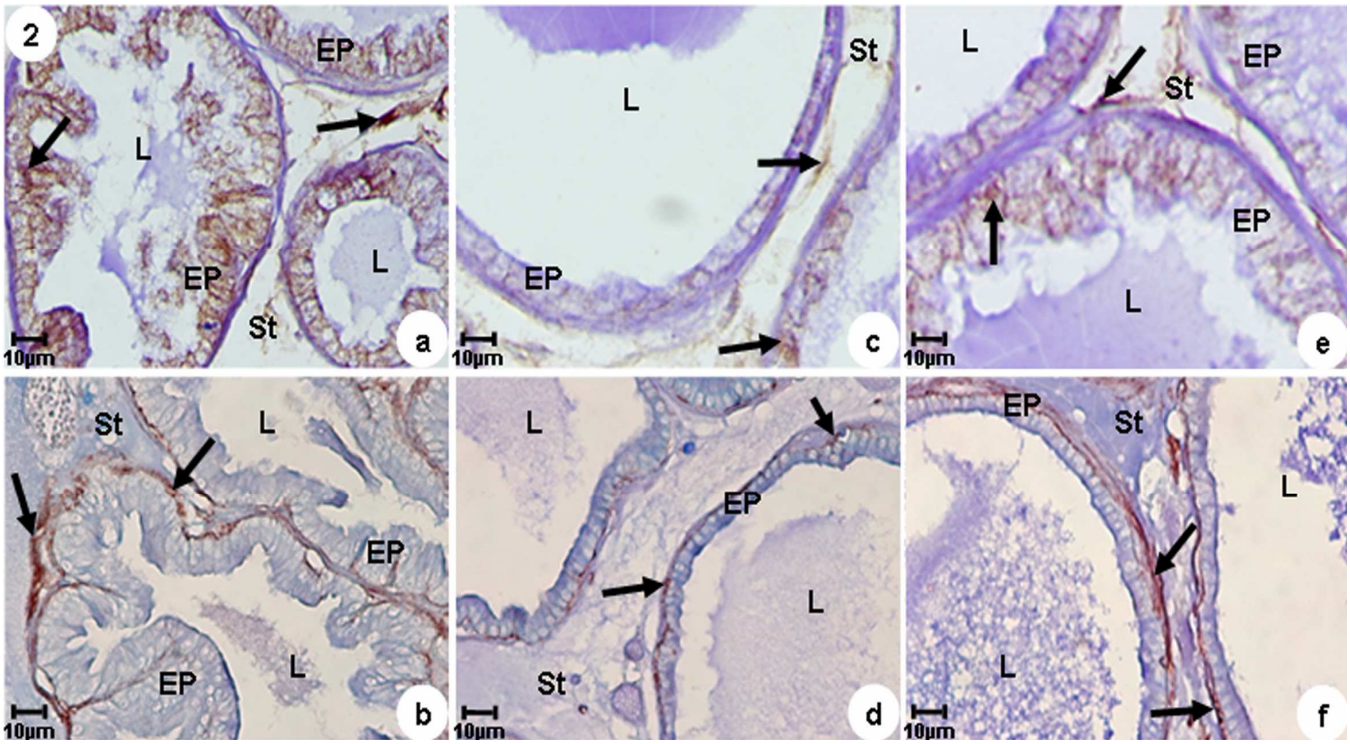


	C	D	DI
■ *Epithelium*	39.6 ± 1.1 a	19.3 ± 2.3 b	23.1 ± 1.1 b
□ *Lumen*	42.4 ± 0.5 a	44.1 ± 0.8 a	42.6 ± 0.6 a
▨ *Stroma*	18.0 ± 1.5 a	36.6 ± 1.9 b	34.3 ± 1.4 b

Averages, followed by the same letter, are not different each other (P>0.01) "Tukey's test".

Abbreviations: Groups: C- control; D- diabetic; DI- diabetic-insulin

Figure 2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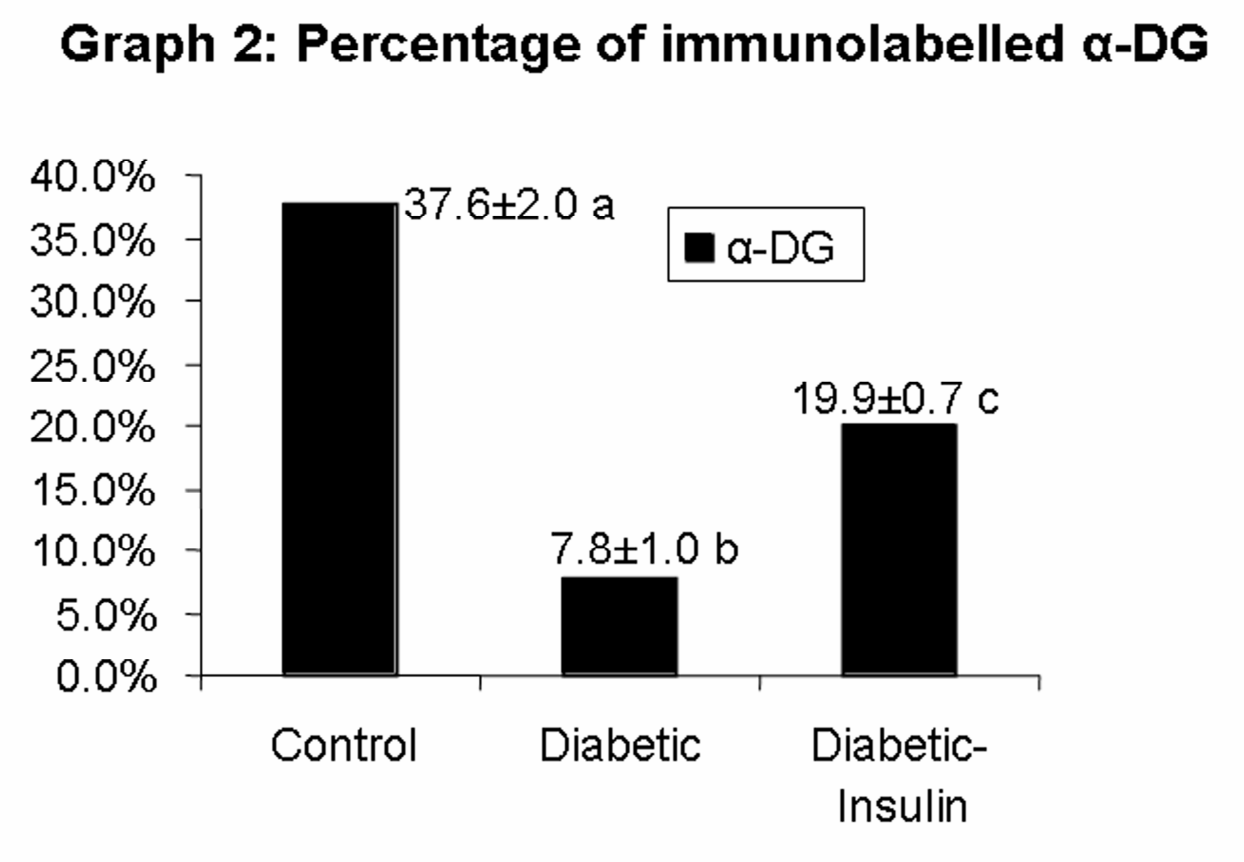
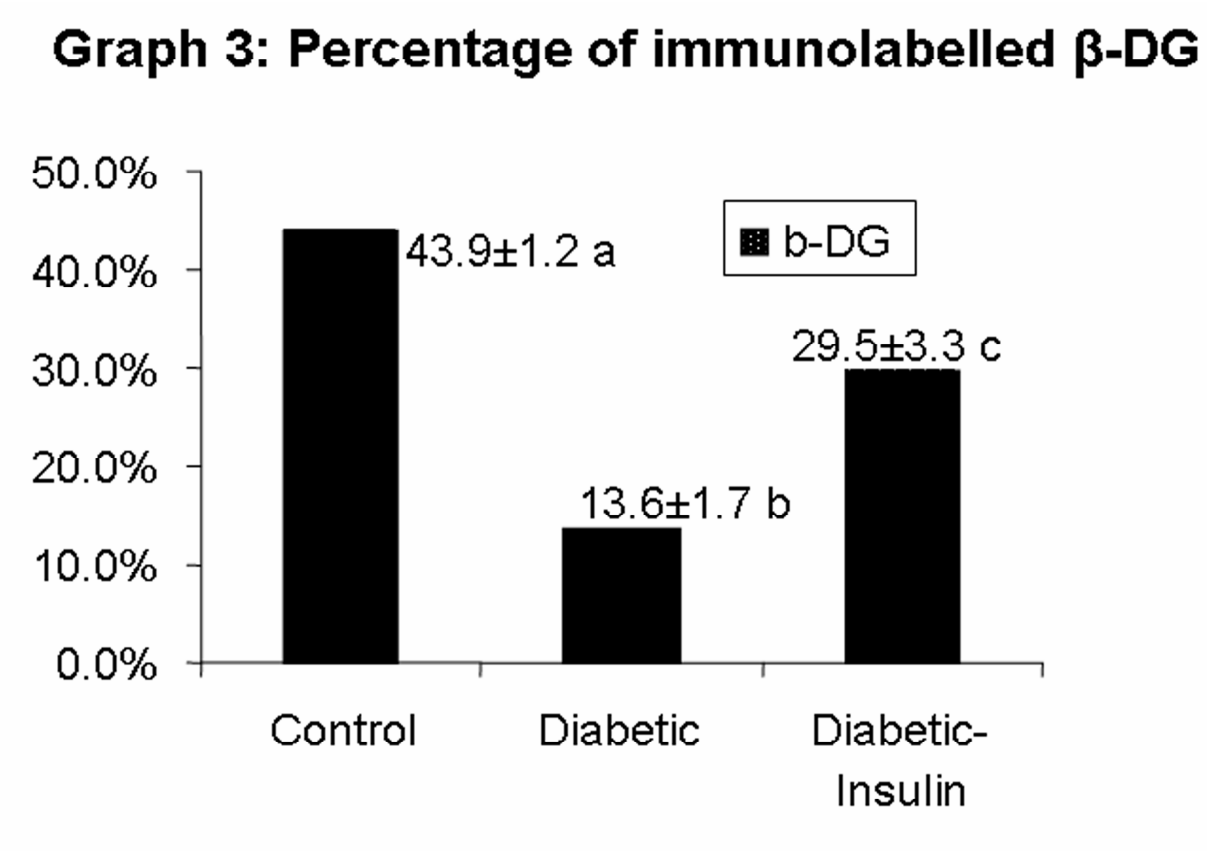
Graph 2


Graph 2: Percentage of immunolabelled α-DG
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
37.6±2.0 a
7.8±1.0 b
19.9±0.7 c
α-DG
Control
Diabetic
Diabetic-Insulin

Graph 3


Graph 3: Percentage of immunolabelled β-DG
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
43.9±1.2 a
13.6±1.7 b
29.5±3.3 c
b-DG
Control
Diabetic
Diabetic-Insulin

"IGF-1 signaling and its interaction with androgen and oestrogen receptors in the ventral prostate of Non-obese diabetic (Nod) mice following hormonal therapy"

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Key words: Ventral Prostate; Diabetes Mellitus; Steroid Hormones; Immunohistochemistry; Insulin; IGF-1

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ABSTRACT

Purpose: The aim of this study was to identify the estrogen, androgen and insulin-like growth factor-1 (IGF-1) receptor immunolocalization in non-obese diabetic (Nod) mice after long-term glycemic control and hormonal therapy. As well as, it was examined possible relation to diabetes and prostatic disorders. *Materials and Methods:* Thirty mice were divided into six groups after 20 days of diabetes: the control group received daily doses of 0.9% NaCl, as did the diabetic group. The diabetic-insulin group received daily doses of NPH insulin, the diabetic-testosterone group received doses of testosterone cypionate, the diabetic-estrogen group received doses of 17β-estradiol and the diabetic-insulin-testosterone-estrogen group received insulin, testosterone and estrogen, simultaneously, at the same concentrations given to the other groups. After 20 days, the ventral lobe was processed for immunohistochemistry, Western blotting and hormonal analyses.

Results and Conclusions: There was a decrease in serum testosterone levels (diabetic mice and diabetic-insulin-testosterone-estrogen mice had the greatest and smallest decreases, respectively) and in the level of testosterone receptor immunolocalization. The serum estrogen level and its receptor showed changes opposite to those of testosterone and its receptor. The greatest IGF receptor localization occurred in diabetic mice. Thus, diabetes disturbed the prostatic hormonal balance and affected the functioning of this organ. Concomitant treatment with insulin and steroid hormone therapy partially restored the hormonal imbalance caused by diabetes. The increased expression of IGF-1 suggested that diabetes may be an important factor in prostatic cellular proliferation. However, hormonal therapy did not restore the distribution of IGF-1 to normal.

INTRODUCTION

Diabetes mellitus causes changes in various organs, including the urogenital system [1]. Experimental studies have shown that type 1 diabetes mellitus causes reduction in the weight of the testes, prostate and coagulating gland in diabetic rats [2]. More recently, Ribeiro et al. [3] observed atrophied secretory cells, hypertrophied stroma, inflammatory cells, prostatic intraepithelial neoplasia and dilation of organelles involved in glandular

secretion in the ventral prostate of spontaneously diabetic mice. Several studies have also reported a reduction in serum testosterone levels and receptor expression in the prostate of diabetic mice as a consequence of disturbances in hormone metabolism [4].

In diabetic patients, insulin therapy has a fundamental role in attenuating the systemic effects of the disease and improving the quality of life. However, Wang et al. [5] showed that controlling the glucose level of diabetic mice by administering insulin did not restore the prostate weight. In contrast, the concomitant administration of testosterone and insulin in diabetic rats resulted in partial morphophysiological recovery of the prostate [6].

The prostate is a hormone-regulated male accessory sex gland that is fundamental for reproduction [7]. The ventral lobe of the prostate has a simple epithelium with high columnar cells and stroma characterized by stromal cells, extracellular matrix, growth factors, regulatory molecules and enzymes involved in tissue remodeling [8]. The stromal cells and extracellular matrix regulate the growth and development of the glandular cells that have crucial morphological and functional roles [8]. The basal membrane is located between the epithelium and stroma [9].

Proteins such as insulin-like growth factor (IGF) are important mitogenic factors for maintaining prostatic function [10]. IGFs are produced by prostatic stromal cells and act as paracrine growth factors in the glandular epithelium. IGFs act via transmembrane receptors, of which two types are known, namely, IGF-1, which is expressed in prostatic stromal and epithelial cells, and IGF-2, which has not been detected in these two regions [10]. The overexpression of IGFs may be an important factor in stimulating the proliferation and metastasis of cancer cells in the prostate [11].

The androgen-mediated development of the prostate involves paracrine interactions between the epithelium and mesenchyme, with androgen receptors inducing morphogenesis and epithelial proliferation, in addition to modulating the expression of specific secretory proteins and epithelial androgen receptors [8]. The biological actions of androgens are mediated by interaction with specific intracellular receptors that modulate gene expression by binding to nuclear chromatin [8]. Testosterone and dihydrotestosterone (DHT) are the main circulating androgens [12], although estrogen also contributes to prostate function. The biosynthesis of estrogen involves hydroxylation of the androgen precursor

and is catalyzed by an enzyme complex known as aromatase [13]. This enzyme is critical in regulating the equilibrium between androgen and estrogen, and in determining the circulatory and tissue levels of these hormones [13]. The effects of estrogen in the prostate are mediated by the binding of this hormone to specific α and β estrogen receptors (αER, βER) that are expressed in the stroma and epithelium, respectively [14, 7].

Thus, there still are doubts about the occurrence of various diseases associated to diabetes. Also, the relation to insulin treatment as well as hormonal therapy is an unclear subject which will certainly contribute to structural and functional maintenance of the prostate in diabetic patient. Then, the aim of the present study was to characterize and quantify the distribution of steroid receptors and of IGF-1 in the experimental diabetes after insulin therapy and testosterone and estrogen therapy. Moreover, the intention was to establish the relationship between cellular, molecular events and the glandular hormonal reactivity, originated from experimental diabetes, to prostate pathogenesis.

MATERIALS AND METHODS

Experimental Proceedings

Twenty non-obese diabetic (Nod) mice and four control mice (BALB/c/Uni), all 18 weeks old, were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP. Blood glucose levels were measured by capillary glycemia using an Optium Advanced Diabetes Management System (MediSense, Abingdon, UK). Nod mice with a blood glucose level >300 mg/dl were considered diabetic [15]. After 20 days of diabetes, the Nod and BALB/c/Uni mice were divided into six groups (4 mice per group): the control group (CT) received a daily dose of 0.9% physiological saline (5 mL/kg, s.c.; Fresenius Kabi, São Paulo, SP, Brazil) for 20 days, the diabetic group (D) received the same treatment as the control group, the diabetic-insulin group (DI) received daily doses of NPH insulin (4-5 IU, s.c.; Biobrás, Montes Claros, MG, Brazil) for 20 days [16], the diabetic-testosterone group (DT) received a supraphysiological dose of testosterone cypionate (5 mg/kg, s.c., diluted in 5 mL of peanut oil; Deposteron-Sigma Pharma, São Paulo, SP, Brazil) every other day for 20 days [17], the diabetic-estrogen group (DE) received a supraphysiological dose of 17β-estradiol (25 μg/kg, s.c., diluted in 25 μL of peanut oil;

Sigma Chemical Co., St Louis, MO, USA) every other day for 20 days [18] and the diabetic-insulin-testosterone-estrogen group (DITE) received insulin, testosterone and estrogen, simultaneously, at the same doses as given to the other groups. The testosterone, 17β-estradiol and insulin administrations were interrupted 24 h prior to killing the mice. After 20 days of treatment, the mice were anesthetized and sacrified with 10% ketamine (60 mg/kg, i.m.; Vibra® Roseira, São Paulo, Brazil) and 2% xylazine (5 mg/kg, i.m.; Vibra® Roseira, São Paulo, Brazil), and samples of the intermediate and distal regions of the ventral lobe of the prostate were collected using a stereomicroscope and processed for immunological and hormonal analyses. This study was approved by the Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol no. 1472-1) and the experiments were done according to the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Quantification of serum testosterone, estradiol and glucose levels

Blood samples were collected by cardiac puncture from anesthetized mice immediately before killing and 24 h after the last dose of hormones in each group. Serum testosterone and estradiol concentrations were determined by radioimmunoassay using Coat-a-Count total testosterone/estrogen kits (Diagnostic Products Corporation, Los Angeles, CA, USA), and the concentrations were expressed in ng/dL and pg/mL, respectively. Serum glucose was quantified by chemiluminescence and the concentration expressed in mg/dL.

Immunodetection of androgen (AR), α-estrogen (αER), β-estrogen (βER) and IGF-I receptors

Samples of the prostate ventral lobe obtained from all of the mice in each group were fixed in Bouin solution, embedded in paraplast (Paraplast Plus, São Paulo, Brazil) and cut into 6 µm thick sections. The sections were deparaffinized in xylene, hydrated in a graded alcohol series and rinsed in tap water. Different protocols were used for antigen retrieval. After, the sections were incubated in 0.3% H_2O_2 for 15 min to block endogenous peroxidase. Nonspecific binding was blocked by incubating the sections in blocking

solution for 1 h at room temperature. Primary rabbit AR-N20 antibody for the AR (sc-816; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse clone 1D5 antibody (Dako Cytomation Inc., Carpenteria, CA, USA) for the αER, rabbit antibody 06-629 (Upstate, Temecula, CA, USA) for the βER, and rabbit N-20 antibody (sc-720; Santa Cruz Biotechnology) for the IGF-1 receptor were diluted in 1% BSA (1:50) and applied to the sections overnight at 4ºC. Bound antibody was detected with an Envision HRP kit (Dako Cytomation Inc., Carpenteria, CA, USA) according to the manufacturer´s instructions. The sections were subsequently washed for 15 min with TBS-T and secondary labeled polymer (Envision HRP kit) was applied for 40 min at room temperature. After washing in TBS-T, peroxidase activity was detected using a diaminobenzidine chromogen mixture (Envision HRP kit) for 10 min. Sections were lightly counterstained with methyl green and Harris' hematoxylin, dehydrated in an increasing ethanol series and xylene, and mounted in entellan (Merck KGaA, Darmstadt, Germany) prior to photographing with a Nikon Eclipse E-400 photomicroscope. Sections that were not incubated with primary antibody for AR, αER and βER, IGF-1 were used as negative controls. The intensity of staining was scored as weak (+), moderate (++) or strong (+++) based on the concentration and distribution of the receptors.

Western blotting

Samples of prostate ventral lobe from all of the mice in each group were weighed and homogenized (Polytron, Kinematica GmbH, Luzern, Switzerland) for 1 min in 50 µl of lysis buffer/mg of tissue. The tissue homogenates were centrifuged (14,000 x g, 20 min, 4°C) and a sample of each extract was used for protein quantification with Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). The supernatants were subsequently mixed (1:1, v/v) with 3X sample buffer, transferred to a dry bath at 95°C for 5 min, rapidly frozen on ice and stored at -70°C until used. Aliquots containing 50 µg of protein were separated by SDS-PAGE on 12% polyacrylamide gels under reducing conditions. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, IL., USA) at 70 V for 3 h. The membranes were blocked with TBS-T containing 1% BSA for 60 min, rinsed in TBS-T and

incubated at 4°C overnight with rabbit primary antibody AR-N20 for the AR, mouse clone 1D5 for the αER receptor, rabbit 06-629 antibody for the βER receptor and IGF-1 N-20 for the IGF-1 (diluted 1:1,000; 1:350, 1:500 and 1:500, respectively in 1% BSA). The membranes were then incubated for 2 h with rabbit or mouse secondary HRP-conjugated antibodies (diluted 1:2000 in 1% BSA; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in TBS-T, peroxidase activity was detected by incubation with a diaminobenzidine chromogen (Sigma Chemical Co., St Louis, MO, USA) for 10 min. β-Actin was used as an internal control. The immunoreactive bands were analyzed densitometrically with a computerized image analysis system (NIS-Elements, Advanced Research; Nikon, Tokyo, Japan).

Statistical analysis

The results were expressed as the mean ± standard deviation. The serum glucose, testosterone and estradiol levels and the Western Blotting were statistically compared among the groups by analysis of variance followed by the Tukey's test, with the level of significance set at 1% .

RESULTS

Serum testosterone, estradiol and glucose levels

The glucose levels of diabetic, diabetic-testosterone and diabetic-estrogen mice were >300 mg/dL, whereas those of control, diabetic-insulin and diabetic-insulin-testosterone-estrogen mice were lower than in diabetic mice (Table 1). The mean serum testosterone levels were higher in control mice than in the other experimental groups, but there were no significant differences in the levels of this hormone among the diabetic, diabetic-insulin and diabetic-estrogen groups (Table 1). The diabetic-insulin-testosterone-estrogen group had lower testosterone levels than the control group. However, these levels were higher than those shown in the other experimental groups. The estradiol levels were lower in the control group compared to the other experimental groups (Table 1).

Immunolocalization of AR, αER, βER and IGF-1 and Western Blot analysis

Control group

The secretory epithelium and stromal cells of control prostatic tissues showed intense staining for AR, representing 146% of protein level (Figures 1a and 1g; Table 2). In contrast, αER protein level was significantly lower (15.3%) than those found in the other experimental groups, showing weak immunolocalization in the periacinal prostatic stroma (Figures 2a and 2g; Table 2). The epithelial cells also stained intensely for βER, representing 143.1% of protein level (Figures 3a and 3g; Table 2). IGF-1 protein level was detected in 8.7% of the stromal cells (Figures 4a and 4g; Table 2).

Diabetic group

AR protein level was detected in only 43.2% of the cells, showing weak immunoreactivity (Figures 1b and 1g; Table 2). The αER protein level represented 86.9%, which was significantly higher than those verified in other experimental groups. Intense immunoreactivity was observed in the periacinal prostatic stroma (Figures 2b and 2g; Table 2). Weak immunoreactivity for βER was observed in epithelial cells and represented 22.4% of the normal protein level (Figures 3b and 3g; Table 2). IGF-1 protein level was detected in 36.5% of the stromal cells (Figures 4b and 4g; Table 2).

Diabetic-insulin group

The secretory epithelium and stromal cells showed weak staining for AR that corresponded to 56.9% of the normal protein level (Figures 1c and 1g; Table 2). Intense staining for αER was observed in the periacinal prostatic stroma, with the level of protein level (73.8%) being significantly higher than in the control group. However, this level was still lower than in the diabetic group (Figures 2c and 2g; Table 2). The immunolocalization for βER was weak compared to the control group, with the level of protein expression being 31.5% of normal, but still higher than in the diabetic group (Figures 3c and 3g; Table 2). IGF-1 protein level was detected in 35.6% of the stromal cells, and was higher than in the control group but not significantly different from the diabetic group (Figures 4c and 4g, Table 2).

Diabetic-testosterone group

The secretory epithelium and stromal cells showed moderate staining for AR that corresponded to 83.5% of the normal protein level (Figures 1e and 1g; Table 2). Moderate immunoreactivity for αER was seen in the periacinal prostatic stroma, although the level of protein expression (37.3%) was significantly lower than in the diabetic and diabetic-insulin groups (Figures 2d and 2g; Table 2). Epithelial cells showed moderate staining for βER and had a protein expression that was 80.9% of the normal level; this level was significantly higher than in the diabetic and diabetic-insulin groups (Figures 3d and 3g; Table 2). IGF-1 protein level was detected in 36.6% of the stromal cells, and was not significantly different from the diabetic and diabetic-insulin groups (Figures 4d and 4g; Table 2).

Diabetic-estrogen group

AR protein level was detected in only 70.4% of the cells, showing moderate immunoreactivity (Figures 1d and 1g; Table 2). Intense staining for αER was observed in the periacinal prostatic stroma, although the level of protein expression (69.7%) was significantly lower than in the diabetic and diabetic-insulin groups but significantly higher than in the diabetic-testosterone group (Figures 2e and 2g; Table 2). Epithelial cells showed weak immunolocalization for βER that corresponded to 65.4% of the normal level of βER protein expression; this value was significantly lower than in the diabetic-testosterone group (Figures 3e and 3g; Table 2). IGF-1 protein level was detected in 38.0% of the stromal cells, a value not significantly different from the diabetic, diabetic-insulin and diabetic-testosterone groups (Figures 4e and 4g; Table 2).

Diabetic-insulin-testosterone-estrogen group

The secretory epithelium and stromal cells showed intense immunoreactivity for AR that corresponded to 110.1% of the normal level of protein expression. This level was significantly higher than in the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups but significantly lower than in the control group (Figures 1f and 1g; Table 2). Moderate staining for αER was observed in the periacinal prostatic stroma, with a level of protein expression (25.4%) that was significantly higher than in the control group but

significantly lower than in the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups (Figures 2f and 2g; Table 2). The immunolocalization for βER was less intense than in the control group, but greater than in the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups, with a protein expression that was 107.5% of the normal level (Figures 3f and 3g; Table 2). IGF-1 protein level was detected in 19.6% of the stromal cells, which was lower than in the diabetic, diabetic-insulin, diabetic-testosterone and diabetic-estrogen groups (Figures 4f and 4g; Table 2).

DISCUSSION AND CONCLUSION

As shown here, diabetes resulted in a decrease in serum testosterone concentrations and in the immunoreactivity of AR and βER compared to normal, non-diabetic (control) mice and to diabetic-insulin, diabetic-testosterone, diabetic-estrogen and diabetic-insulin-testosterone-estrogen mice. In contrast, serum estradiol concentrations and αER immunolocalization showed responses that were opposite to those of testosterone. The administration of exogenous insulin in association with steroid hormones partially restored the normal level of receptor expression. The IGF-1 receptor was highly characterized in diabetic mice, as well as in mice treated with insulin, testosterone or estrogen, but was attenuated in the control and diabetic-insulin-testosterone-estrogen groups.

Clinical and epidemiological studies have shown that diabetes reduces the levels of gonadotrophic hormone, leading to decreased levels of testicular hormones [6, 2, 19]. Daubresse et al. [20] observed low testosterone and hormone gonadotrophic levels in diabetic patients and attributed this to negative feedback of the hypothalamus-pituitary-gonadal axis. These observations suggested that insulin can alter cellular function and compromise normal androgenic actions. In addition, other hormones such as estrogen act in association with androgen to maintain the normal hormonal balance, thereby influencing prostate development and function, as well as the incidence and progression of prostate diseases [21].

Several studies showed the important role of a correct androgen and estrogen balance on the development of prostate pathologies [13, 22]. Naslund et al. [23] reported that a hormonal imbalance is one of the most important etiological factors in the development of

prostatitis in rats. Estrogens have anti-androgen effects and negatively regulate the hypothalamus-pituitary-gonadal axis, thereby reducing androgen production by Leydig cells [24]. Androgen and estrogen can initiate morphological and functional changes in the prostate, including displasia and hyperplasia, but not malignancy [13]. The prostatic action of estrogen is mediated by interaction with αER and βER, which are expressed in the stroma and epithelium, respectively [13, 22]. The βER mediates antiproliferative effects in the prostate epithelium and is influenced by the serum androgen levels or activation of the αER [25].

Men who have had diabetes for ≥5 years have more chances of developing prostate cancer than healthy men [26]. In contrast, other studies have suggested that diabetes mellitus is associated with a reduced risk of prostate cancer [27], although these findings are based on a small number of observations and require confirmation.

Several investigations have indicated that a high serum IGF-1 level may be an important risk factor for benign hyperplasia and carcinogenesis [10, 28]. Ohlson et al. [29] observed that low AR expression and the maintenance of IGF-1 synthesis after castration reduced cell death in prostate cancer. In addition, Pandini et al. [30] reported that 17β-estradiol up-regulated IGFR-1 mRNA and protein expression in AR-positive (LNCap cells) and AR-negative (PC-3 cells) prostate cancer cells. According to these authors, these effects were specific for IGF-1 and involved the stimulation of αER and βER.

In conclusion, the results of this study indicated that diabetes altered the testosterone/estrogen ratio by increasing the levels of estrogen and stimulating the αER signaling pathway. Androgen or estrogen therapy in experimental diabetes did not restore the normal levels of AR, αER and βER, although an association of these hormones was much more efficient in maintaining the glandular hormonal balance. Experimental diabetes enhanced the characterization of IGF-1 in the prostate, which suggested that the activation of this receptor was an important pathway in cellular proliferation and possibly in the development of prostatic cancer. Concomitant treatment with testosterone, estrogen and insulin did not restore IGF-1 expression to the levels seen in healthy mice. This finding indicated that there was no direct correlation between steroid hormone levels and IGF-1 expression. A proper understanding of the relationship between these two factors could improve the current therapies for treating prostate diseases.

Acknowledgments

This work was supported by FAPESP (number: 06/55645-2) and CNPq.

This work was presented in the American Urological Association (AUA)/ 2009.

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FIGURE LEGENDS

Figure 1: AR Immunolocalization. (a) Control Group: intense AR immunoreactivity **(arrows)** in the nuclei of secretory epithelial cells and stromal cells. (b) Diabetic Group: weak AR immunoreactivity **(arrows)** in secretory epithelial cells and stromal cells. (c) Diabetic-Insulin Group: weak AR immunoreactivity **(arrows)** in secretory epithelial cells and stromal cells. (d) Diabetic-Estrogen Group: moderate AR immunoreactivity **(arrows)** in secretory epithelial cells and stromal cells. (e) Diabetic-Testosterone Group: moderate AR immunostaining **(arrows)** in secretory epithelial cells and stromal cells. (f) Diabetic-Insulin-Testosterone-Estrogen Group: intense AR immunostaining **(arrows)** in secretory epithelial cells and stromal cells. In **a-f**, **L –** lumen, **Ep –** secretory epithelium and **St –** stroma. (g) Representative western blots and quantitative determination of AR expression in prostatic ventral lobe. β-actin was used as an internal control.

Figure 2: αER Immunolocalization. (a) Control Group: weak αER immunoreactivity in the periacinal prostatic stroma **(arrows)**. (b) Diabetic Group: intense αER immunoreactivity **(arrows)** in the glandular stroma. (c) Diabetic-Insulin Group: Intense αER immunoreactivity **(arrows)** in the glandular stroma. (d) Diabetic-Testosterone Group: moderate αER immunoreactivity **(arrows)** in the prostatic stroma. (e) Diabetic-Estrogen Group: intense αER immunoreactivity **(arrows)** in the glandular stroma. (f) Diabetic-Insulin-Testosterone-Estrogen Group: moderate αER immunoreactivity **(arrows)** in the prostatic stroma. In **a-f**, **L –** lumen, **Ep –** secretory epithelium and **St –** stroma. (g)

Representative western blots and quantitative determination of αER expression in prostatic ventral lobe. β-actin was used as an internal control.

Figure 3: βER Immunolocalization. (a) Control Group: intense βER immunoreactivity **(arrows)** in the nuclei of epithelial cells. (b) Diabetic Group: weak βER immunoreactivity **(arrows)** in the nuclei of the epithelial cells. (c) Diabetic-Insulin Group: weak βER immunoreactivity **(arrows)** in the nuclei of the epithelial cells. (d) Diabetic-Testosterone Group: moderate βER immunoreactivity **(arrows)** in secretory epithelial cells. (e) Diabetic-Estrogen Group: weak βER immunoreactivity **(arrows)** in the nuclei of the epithelial cells. (f) Diabetic-Insulin-Testosterone-Estrogen Group: intense βER immunoreactivity **(arrows)** in the nuclei of the epithelial cells. In **a-f**, **L –** lumen, **Ep –** secretory epithelium and **St –** stroma. (g) Representative western blots and quantitative determination of βER expression in prostatic ventral lobe. β-actin was used as an internal control.

Figure 4: IGF-1 Immunolocalization. (a) Control Group: weak IGF-1 immunoreactivity in the prostatic stroma **(arrow)**. (b) Diabetic Group: intense IGF-1 immunoreactivity **(arrow)** in the glandular stroma. (c) Diabetic-Insulin Group: intense IGF-1 immunoreactivity **(arrow)** in the glandular stroma. (d) Diabetic-Testosterone Group: intense IGF-1 immunoreactivity **(arrow)** in the prostatic stroma. (e) Diabetic-Estrogen Group: intense IGF-1 immunoreactivity **(arrow)** in the glandular stroma. (f) Diabetic-Insulin-Testosterone-Estrogen Group: moderate IGF-1 immunoreactivity **(arrow)** in the prostatic stroma. In **a-f**, **L –** lumen, **Ep –** secretory epithelium and **St –** stroma. (g) Representative western blots and quantitative determination of IGF-1 expression in prostatic ventral lobe. β-actin was used as an internal control.

Table 1: Mean and standard deviation of the serum glucose (mg/dL), testosterone (ng/mL) and estradiol (pg/mL) levels of the mice in the different experimental groups.

Variants	Groups					
	Control	Diabetic	Diabetic-Insulin	Diabetic-Testosterone	Diabetic-Estrogen	Diabetic-Insulin-Testosterone-Estrogen
Glucose	181.8±2.8 a	876.7±26.7 e	351.0±23.0 bc	627.3±12.2 d	487.0±6.6c	306.9±15.3b
Testosterone	35.8±2.5 d	6.4±0.7 a	10.9±1.2a	19.2±2.6 b	8.1±0.7 a	24.3±0.8 c
Estradiol	22.4±1.8 a	49.8± 1.3 d	37.0±1.5 c	37.5±1.7 c	50.6±2.0 d	28.0± 1.4 b

Means with different letters are significantly different (P< 0.01) from each other.

Table 2: Immunolabeled receptor intensity of the different experimental groups in the prostate.

Receptors	Groups					
	Control	Diabetic	Diabetic-Insulin	Diabetic-Testosterone	Diabetic-Estrogen	Diabetic-Insulin-Testosterone-Estrogen
AR	+++	+	+	++	++	+++
αER	+	+++	+++	++	+++	++
βER	+++	+	+	++	+	+++
IGF-1	+	+++	+++	+++	+++	++

Intense (+++), Moderate (++), Weak (+)

Figure 1

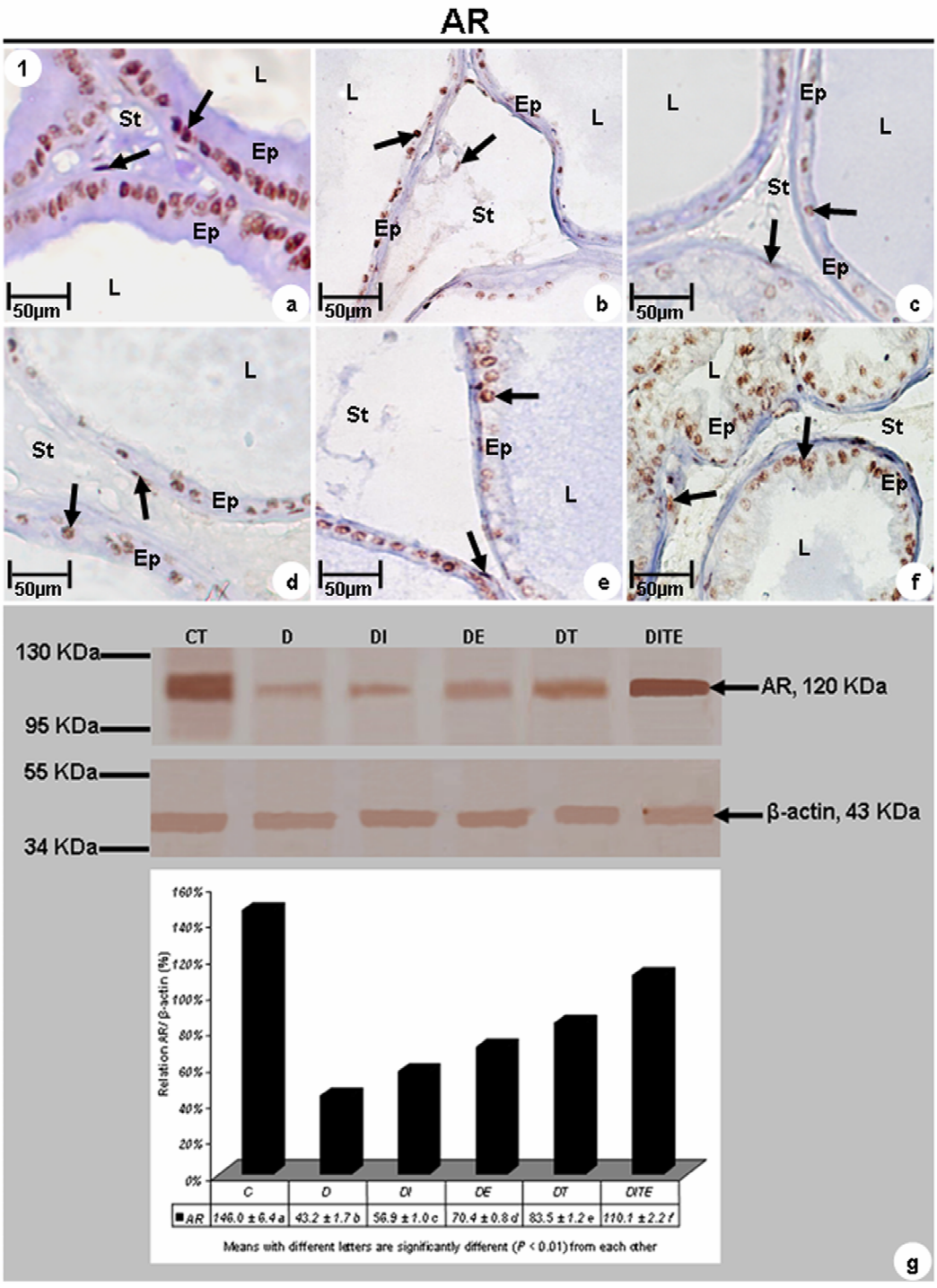
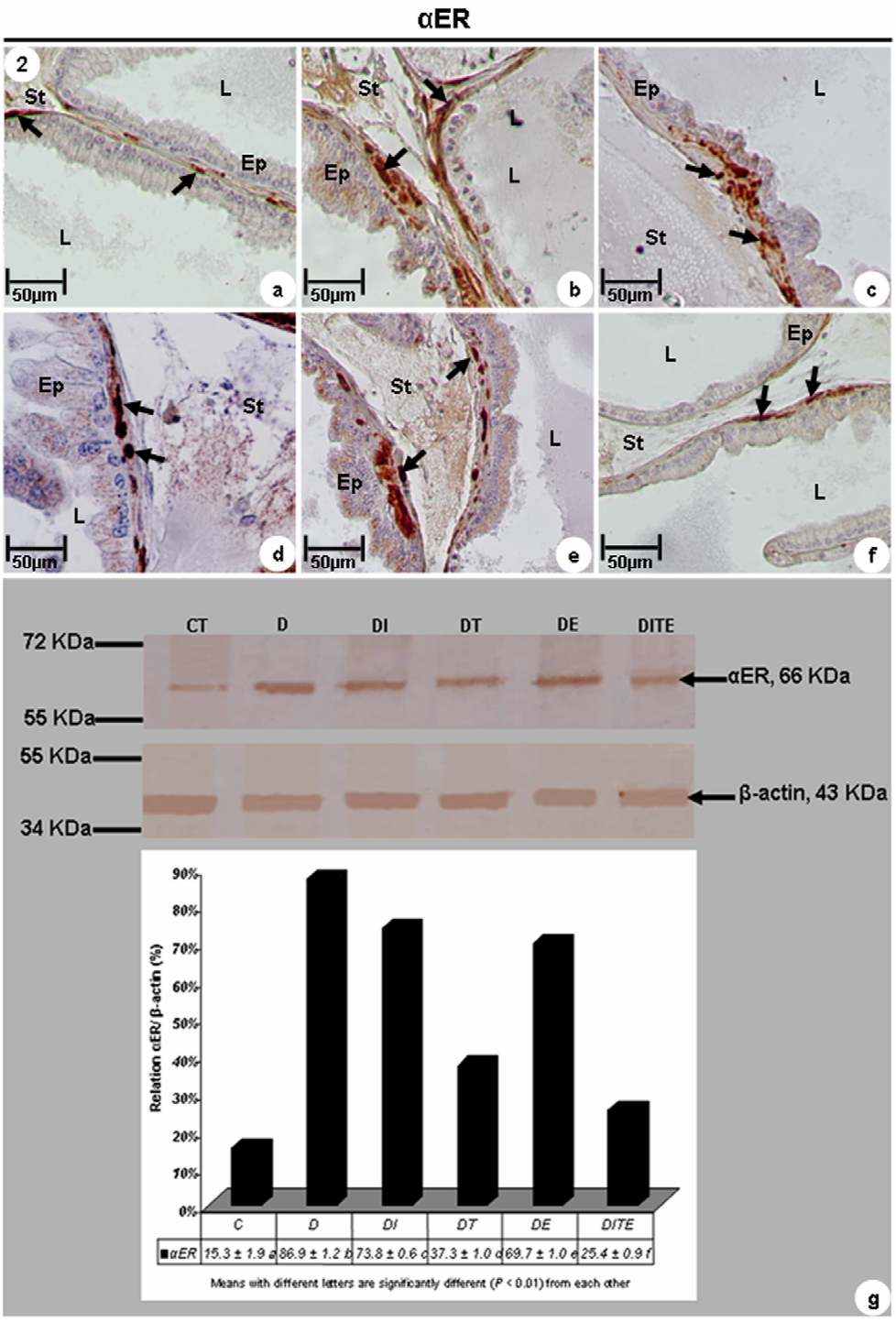

AR
1
L
St
Ep
50µm
a
b
c
d
e
f
CT
D
DI
DE
DT
DITE
130 KDa
95 KDa
55 KDa
34 KDa
AR, 120 KDa
β-actin, 43 KDa
Relation AR/ β-actin (%)
160%
140%
120%
100%
80%
60%
40%
20%
0%
C
D
DI
DE
DT
DITE
AR
146.0 ± 6.4 a
43.2 ± 1.7 b
56.9 ± 1.0 c
70.4 ± 0.8 d
83.5 ± 1.2 e
110.1 ± 2.2 f
Means with different letters are significantly different (P < 0.01) from each other
g

Figure 2


αER
2
St
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50µm
a
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c
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CT
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DI
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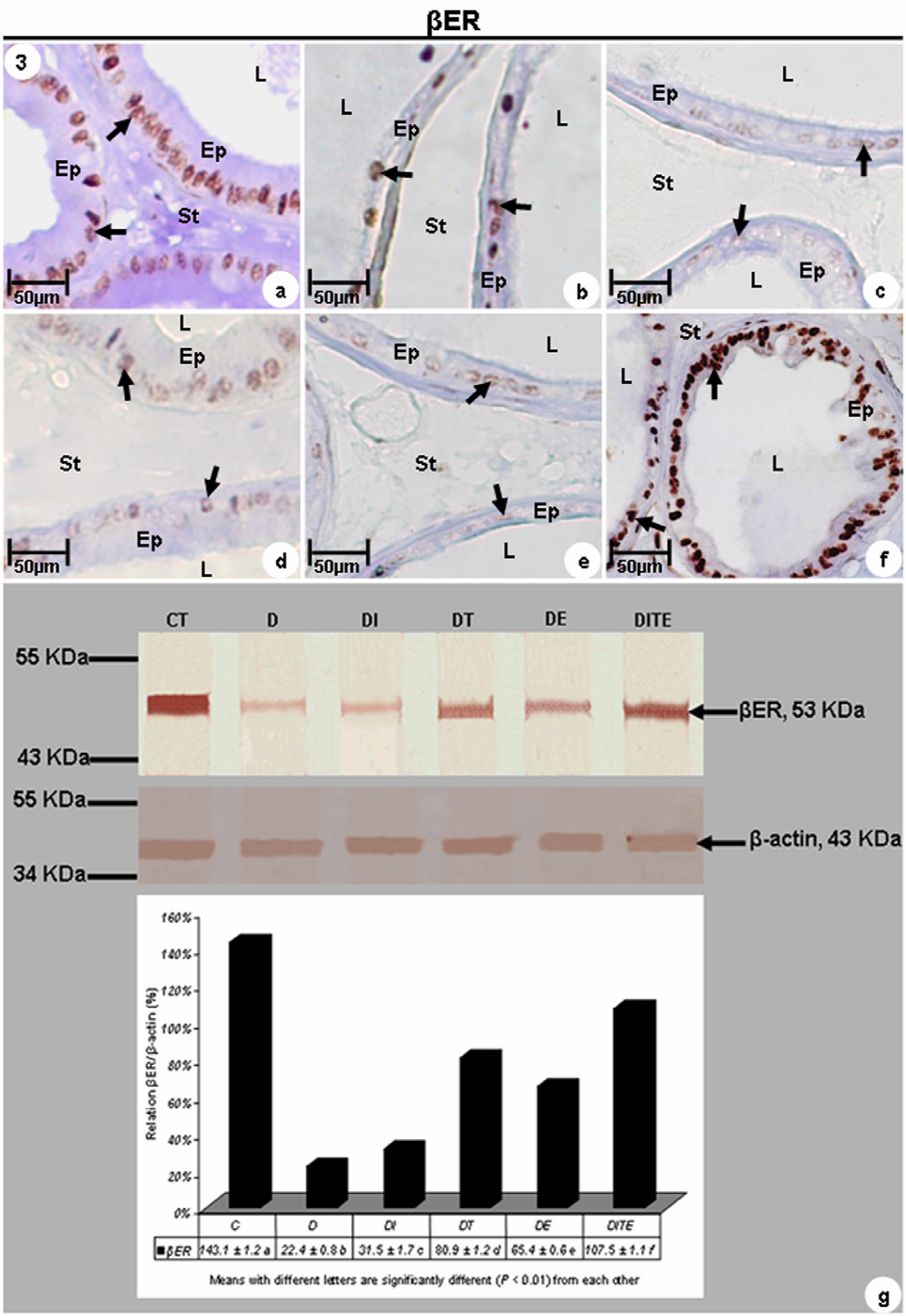
DE
DITE
72 KDa
55 KDa
αER, 66 KDa
β-actin, 43 KDa
34 KDa
Relation αER/ β-actin (%)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C
D
DI
DT
DE
DITE
αER
15.3 ± 1.9 a
86.9 ± 1.2 b
73.8 ± 0.6 c
37.3 ± 1.0 d
69.7 ± 1.0 e
25.4 ± 0.9 f
Means with different letters are significantly different (P < 0.01) from each other
g

Figure 3


βER
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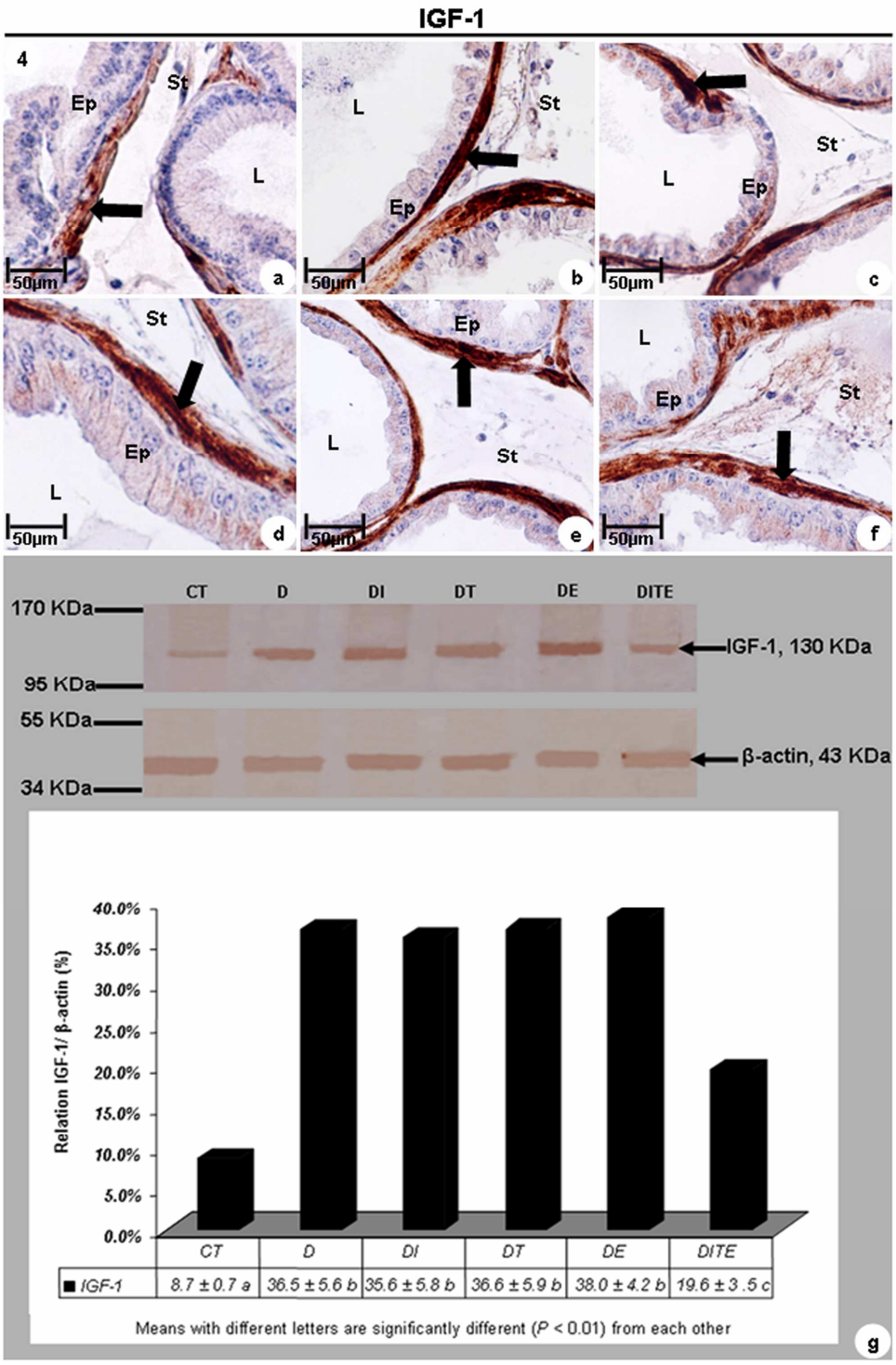
55 KDa
43 KDa
βER, 53 KDa
55 KDa
34 KDa
β-actin, 43 KDa
Relation βER/β-actin (%)
160%
140%
120%
100%
80%
60%
40%
20%
0%
C
D
DI
DT
DE
DITE
βER
143.1 ± 1.2 a
22.4 ± 0.8 b
31.5 ± 1.7 c
80.9 ± 1.2 d
65.4 ± 0.6 e
107.5 ± 1.1 f
Means with different letters are significantly different (P < 0.01) from each other
g

Figure 4


IGF-1
4
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e
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CT
D
DI
DT
DE
DITE
170 KDa
95 KDa
55 KDa
34 KDa
IGF-1, 130 KDa
β-actin, 43 KDa
Relation IGF-1/ β-actin (%)
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
IGF-1
8.7 ± 0.7 a
36.5 ± 5.6 b
35.6 ± 5.8 b
36.6 ± 5.9 b
38.0 ± 4.2 b
19.6 ± 3.5 c
Means with different letters are significantly different (P < 0.01) from each other
g

V- CONSIDERAÇÕES E CONCLUSÕES

1) O diabetes provocou drásticas alterações morfológicas dos compartimentos epitelial e estromal contribuindo para a ruptura da homeostase glandular. Entretanto, a associação do controle glicêmico prolongado com a reposição hormonal foi efetiva na minimização dessas alterações.

2) O diabetes alterou a ocorrência da alfa e beta distroglicanas, levando a diminuição da interação célula-matriz extracelular e célula-membrana basal. Entretanto, o controle glicêmico recuperou parcialmente a localização dessas proteínas de adesão.

3) O estado diabético levou ao desbalanço entre os processos de proliferação e apoptose, o que certamente aponta essa doença como fator deflagrador de lesões prostáticas.

4) O diabetes causou desequilíbrio da relação testosterona/estrógeno, prevalecendo altos níveis de estrógeno caracterizados pela via de ação do ERα.

5) A reposição de estrógeno e testosterona frente ao diabetes experimental não restaurou os níveis de normalidade de AR, ERα e ERβ, sendo que a associação desses hormônios e insulina foi mais eficaz para o equilíbrio hormonal glandular, demonstrando a complexidade de interações hormonais da próstata e sinalizando a via de atuação dos estrógenos através do receptor ERβ, o qual indica ser supra-regulado por testosterona.

6) O diabetes experimental sensibilizou a ocorrência dos níveis de IGFR-1 no lobo ventral da próstata, o que certamente favoreceu o processo mitogênico bem como a patogênese glandular.

7) A reposição simultânea de testosterona, estrógeno e insulina apesar de apontar a uma tendência de equilíbrio da localização dos receptores esteróides AR, ERα e ERβ, não demonstrou a normalidade da localização de IGFR-1, sem relação diretamente proporcional entre os receptores dos hormônios esteróides e o IGFR-1, embora esses níveis tenham sido sensíveis à ação hormonal conjunta.

8) A relação dos receptores esteróides com o IGFR-1 e distroglicanas pode ser uma via importante para novas tendências terapêuticas no tratamento de doenças prostáticas, bem como de diagnóstico.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada **Expressão e reposição estrogênica e androgênica no lobo ventral da próstata de camundongos diabéticos (NOD) frente a terapia insulínica**:

( ) não se enquadra no § 3° do Artigo 1° da Informação CCPG 01/08, referente a bioética e biossegurança.

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

(X) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n°1472-1);

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°_____);

( ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar: _____

Wagner José Fávaro
Aluno: Wagner José Fávaro

Orientadora: Profa. Dra. Valéria Helena Alves Cagnon Quitete

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

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