

UNIVERSIDADE ESTADUAL DE CAMPINAS



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**HISTOQUÍMICA E ULTRA-ESTRUTURA DO ESTROMA DO LOBO VENTRAL DA  
PRÓSTATA DE CAMUNDONGOS ESPONTANEAMENTE DIABÉTICOS (NOD)**

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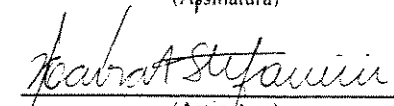
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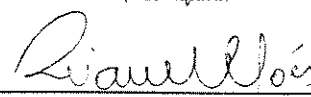
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*Dedico:*

*À minha grande família, meu porto seguro...*

*Ao Mauricio, meu amor.*

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*“Nossas dúvidas são traidoras e nos fazem  
perder, aquilo que certamente poderíamos  
ganhar, por simples medo de arriscar.”*

*Shakespeare*

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O diabetes é uma doença de grande importância social, devido à alta incidência populacional e ao considerável índice de mortalidade. A deficiência de insulina pode levar à complicações em diversos sistemas do corpo, dentre eles, o sistema reprodutivo, envolvendo glândulas acessórias e gônadas. Estudos revelaram que a insulina atua simultaneamente à testosterona durante o processo de manutenção do estado funcional prostático, entretanto não há informações sobre a ação da insulina no tecido estromal. O presente trabalho objetivou caracterizar histoquímica e ultra-estruturalmente o estroma prostático de camundongos espontaneamente diabéticos. Três grupos experimentais foram considerados: Grupo I - animais controles, Grupo II - animais NOD diabéticos negativos e Grupo III - animais NOD diabéticos positivos. A glicemia foi determinada através de fita reagente colocada em contato com a urina dos animais. Após 12 dias da constatação do diabetes no grupo III, a próstata ventral foi removida fixada em Karnovsky e processada para histoquímica e microscopia eletrônica de transmissão associadas à estereologia. Os resultados mostraram atrofia celular no epitélio secretor dos animais diabéticos, o que pode prejudicar o processo reprodutivo dos mesmos. O estado diabético promoveu alterações no microambiente prostático, tais como, desenvolvimento de neoplasias intraepiteliais, modificações no fenótipo da célula muscular e remodelação dos elementos fibrilares da matriz extracelular. Pode-se concluir que essas alterações afetam as interações epitélio-estromais, podendo favorecer o desenvolvimento de patologias prostáticas.

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## II- ABSTRACT

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The diabetes causes alterations in various organic systems, encompassing the male accessory sex glands. The prostate is very important in the male reproductive process and it is a frequent target of malignant changes. The aim of this work was to demonstrate the histochemistry and ultrastructural alterations in the prostate of the animals submitted to experimental diabetes. It was attempted to associate this results to cellular events involved in prostatic diseases. Three groups of animals were utilized: control, non-obese diabetic positive mice (NOD+) and non-obese diabetic negative mice (NOD-). The diabetic status was characterized using Bio Bras reagents strips. Twelve days after the characterization of diabetic status the ventral lobe was collected, fixed in karnovsky solution and paraformaldehyde and processed for histochemistry and TEM associated to stereology. The results showed reduction of the epithelial and luminal areas and increasing of the stromal area with muscular layer hypertrophy and collagen and reticular fibers in the prostatic gland. Also, it was characterized development of prostatic intraepithelial neoplasia, inflammatory process and dilation of the organelles involved in the secretory process. Then, it was concluded that the diabetes besides damaging the reproductive process, affect the glandular homeostasis favoring the development of prostatic pathologies.

O *diabetes mellitus* é uma doença crônica que afeta o metabolismo de carboidratos, proteínas e lipídios. A maior característica do diabetes é a hiperglicemia, reflexo da utilização ineficiente de carboidratos (glicose), devido a uma anormalidade na secreção de insulina ou a uma ação defeituosa desta sobre os tecidos (Ellenberg & Rifkin, 1962, Robbins, 1989, Cotran, 2000, Conget, 2002).

O pâncreas é uma glândula mista, de secreção tanto endócrina quanto exócrina. A porção endócrina é formada pelas ilhotas de Langerhans e a porção exócrina apresenta-se como uma glândula acinosa de secreção serosa, produzindo as enzimas digestivas (Byrne *et al.*, 1996). As ilhotas de Langerhans se dispõem sob a forma de aglomerados arredondados de células, sendo as principais as  $\alpha$  e  $\beta$ . As células  $\alpha$  sintetizam glucagon e as células  $\beta$  são as responsáveis pela síntese de insulina e correspondem a 70% de todas as células da ilhota. A síntese de insulina é estimulada pela concentração de glicose no sangue (Nattras & Halle, 1988). A insulina é um polipeptídeo composto por duas cadeias de aminoácidos. Esse hormônio tem importante papel na regulação de várias doenças do metabolismo, incluindo o de carboidratos, o armazenamento de glicogênio, a síntese de ácidos graxos, o transporte de aminoácidos e a síntese de DNA, RNA e proteínas. (Anderson *et al.*, 1994). Todavia, quando as células  $\beta$  deixam de executar suas funções, seja por fatores hereditários ou relacionados à obesidade, surge uma disfunção patológica denominada *diabetes mellitus* (Öztürk *et al.*, 1996).

Duas formas clínicas da doença são conhecidas:

- *Diabetes Mellitus* dependente de insulina (IDDM) ou tipo I, onde ocorre uma infiltração das células do sistema imune nas ilhotas pancreáticas, destruindo seletivamente as células  $\beta$  produtoras de insulina, resultando na produção deficiente desse hormônio. Dentre as características do diabetes tipo I, podem-se destacar a presença de cetoses e a dependência diária de insulina para o controle glicêmico.
- *Diabetes mellitus* não dependente de insulina (NIDDM) ou tipo II, que se caracteriza por uma resistência à insulina em certos tecidos, como adiposo, muscular e

hepático. Essa resistência impede que a glicose seja metabolizada pela insulina, causando hiperglicemia e hiperinsulinaemia. Esse tipo de diabetes é de menor severidade ao organismo, não levando à cetoses e a obesidade é um fator de risco (Byrne *et al.*, 1996).

Dados recentes mostram que o diabetes é a terceira causa de morte no mundo, superada apenas pelas doenças cardiovasculares e os diferentes tipos de cânceres (Sociedade Brasileira do Diabetes, 2003). Estima-se que, essa doença afete 6,8% da população norte americana e 7,6% dos brasileiros (IDF Diabetes e-Atlas, 2004 ). 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 do conhecimento das várias complicações que o indivíduo diabético apresenta, dúvidas persistem sobre a etiologia dessa doença devido aos fatores como os perfis clínicos, a idade com que a doença se manifesta e a incidência nos diferentes grupos étnicos e geográficos. Dessa forma, várias tentativas têm sido realizadas para elucidar os diferentes efeitos deletérios nos sistemas orgânicos e a possibilidade da reversão dos mesmos através de tratamento e/ou dieta. Assim, a utilização de modelos animais que desenvolvam o diabetes é comum, tanto na forma induzida (quimicamente) quanto na forma espontânea. A indução do diabetes pode ser feita através de drogas como a estreptozotocina e a aloxana, as quais agem diretamente nas células  $\beta$ , destruindo-as e provocando nos animais estado diabético irreversível (Perez *et al* 1998; Kovacs *et al* 1998; Palomar-Morales *et al.*, 1998; Saprykina *et al.*, 1998; Orie & Anyaegbu, 1999; Ravikumar & Anuradha, 1999; Yildirim *et al.*, 1999; Avedano *et al.*, 1999). O diabetes também pode ocorrer espontaneamente, em roedores, como no rato Wistar chinês (BB), nos camundongos C57BL/ksj homozigotos diabéticos (db/db) e nos NOD (diabéticos não obesos) (Kikutani & Makino, 1992).

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 consequência de um defeito genético no sistema imune sendo, portanto, uma doença autoimune (Homo-Delarche, 2001). Desse modo, em 1980 a linhagem NOD foi estabelecida como modelo experimental do diabetes tipo I (Kikutani & Makino, 1992).

Diferentes pesquisadores têm descrito alterações no sistema reprodutor masculino, decorrentes do diabetes. Algumas complicações como ejaculação retrógrada (Ellenberg & Weber, 1966) e disfunções na bexiga urinária (Buck *et al.*, 1976) são descritas na literatura. A correlação da infertilidade em indivíduos diabéticos foi descrita por Oksanen (1975) que constatou redução do peso dos testículos, do diâmetro do ducto deferente e da fertilidade. Frenkel *et al.* (1978) e McCulloch *et al.* (1984) relataram impotência sexual e prejuízo à fertilidade causada pela diminuição do número de espermatozóides no líquido seminal em roedores diabéticos. Posteriormente, Tsuno *et al.* (1991) descreveram o enrijecimento da vesícula seminal e ducto deferente em ratos diabéticos. Também, Jackson & Hutson (1984) e Saito *et al.* (1996) constataram a redução dos pesos dos órgãos reprodutivos masculinos como testículo, próstata, vesícula seminal e glândula de coagulação de ratos. Cagnon *et al.* (2000) observaram na próstata ventral de camundongos espontaneamente diabéticos atrofia das células epiteliais secretoras, diminuição do pregueamento da mucosa e aumento da região estromal glandular. Ultra-estruturalmente, esses autores caracterizaram a atrofia do citoplasma, dilatação das cisternas do Golgi, acúmulos de corpos densos e vacuolização do citoplasma celular. 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. Além disso, existem estudos relatando a diminuição dos níveis séricos de testosterona e a redução do conteúdo de receptores androgênicos na próstata de animais diabéticos (Ho, 1991). Todos esses trabalhos indicaram que o diabetes provoca alterações no eixo hipotálamo-hipófise-gônada e assim como a castração, ocasiona desequilíbrio hormonal, prejudicando a manutenção do estado funcional prostático.

A próstata é um órgão sexual acessório do sistema reprodutor masculino, sendo considerada a glândula mais volumosa e de maior expressividade funcional desse sistema (Netter, 1965; Marker *et al.*, 2003). A próstata humana é freqüentemente descrita como uma glândula compacta, com formato semelhante ao de uma “castanha”, pesando aproximadamente 20g. Ela está localizada ao redor da uretra, inferiormente à bexiga urinária. Nela são descritas três regiões glandulares, sendo elas as zonas periférica, central e pré-prostática, também chamada de zona de transição (Setchell & Brooks, 1988).

A secreção prostática é um líquido alcalino composto por ácido cítrico, cálcio, fosfatases, proteínas e diferentes enzimas como as coaguladoras (Luchter-Wasyl & Ostrowski, 1974, Lin *et al.* 1993). Esta composição propicia aos espermatozóides condições ideais antes e após a ejaculação, garantindo o sucesso da fertilização (Costello & Franklin, 1994; Bull *et al.*, 2001).

Nos roedores, a glândula prostática divide-se em três pares de lobos distintos sendo chamados de ventral, lateral e dorsal, de acordo com a sua localização em relação à uretra prostática, formando o complexo prostático. Esses lobos estão conectados à uretra por uma série de ductos e são funcionalmente similares a próstata humana (Brandes, 1966; Jesik *et al.* 1982; Aumüller & Seitz, 1990).

Microscopicamente, a próstata é uma glândula túbulo-alveolar revestida por epitélio secretor simples assentado em nítida membrana basal (Bourne & Danielli, 1966). O estroma é formado por tecido conjuntivo, fibras musculares lisas, vasos sangüíneos e fibras nervosas (Price, 1963; Jesik *et al.*, 1982; Zanetoni & Taboga, 2001). Periféricamente, nota-se uma delgada cápsula fibroelástica que envolve a glândula penetrando através de septos (Dahl *et al.* 1973).

As células das unidades secretoras e seus ductos são geralmente cilíndricas com complexo de Golgi proeminente entre seu núcleo e a superfície luminal. Essas unidades secretoras, além de produzirem a secreção, também podem armazená-la devido ao extenso pregueamento do epitélio secretor (McNeal *et al.*, 1988).

As fibras musculares lisas e a matriz extracelular, uma intrincada rede de macromoléculas que circunda os ácinos, são os componentes do estroma prostático (Cunha

*et al.*, 1985; Aumüller & Seitz, 1990; Prins *et al.*, 1991; Lin & Bissel, 1993). A matriz extracelular é composta basicamente por fibras (colágenas, reticulares e elásticas), proteoglicanos e glicoproteínas estruturais. As fibras colágenas conferem resistência ao tecido, enquanto a elastina é fundamental para a elasticidade da matriz (Montes, 1992). De acordo com a variabilidade na distribuição do sistema elástico nos diferentes tecidos, é de se esperar que os componentes desse sistema influenciem sobremaneira no processo de reparo, remodelagem e rearranjo dos tecidos (Carvalho *et al.*, 1997; Vilamaior *et al.*, 2000).

As interações parácrinas que ocorrem entre o epitélio e o estroma da próstata são responsáveis pelo comportamento dinâmico glandular e permitem respostas aos agentes exógenos através de modificações dos componentes estromais (Chung & Davies, 1996, Hayward & Cunha, 2000). Dessa forma, durante um desequilíbrio hormonal glandular, o estroma exhibe mudanças como resposta do próprio tecido, tais como modificações nos componentes da matriz extracelular, alteração no fenótipo das células musculares lisas e fibroblastos (Cunha *et al.* 1996).

A próstata é um órgão dependente de andrógenos, tanto para sua diferenciação, quanto para seu desenvolvimento pós-natal e funcionamento na vida adulta (Marker *et al.*, 2003). Os andrógenos desempenham papel chave na regulação das interações epitélio-estromais e também estão relacionados com a progressão de doenças benignas ou malignas na próstata (Chung & Davies, 1996; Cunha *et al.*, 1996, 2003; Taboga & Vidal, 2003).

A testosterona é o principal hormônio envolvido no processo homeostático da próstata e é sintetizado pelas células de Leydig do testículo pelo estímulo do hormônio luteinizante (LH) da hipófise que, por sua vez, é dependente de um estímulo hipotalâmico, através do hormônio de liberação do hormônio luteinizante (LHRH) (Wright *et al.*, 1999). A testosterona circulante entra nas células prostáticas, onde é convertida em um andrógeno intracelular ainda mais potente, a diidrotestosterona (DHT), pela ação da enzima 5-alfa redutase. Essa conversão é necessária, pois a testosterona tem pouca afinidade aos receptores androgênicos, ao contrário do que acontece com a DHT que se liga com maior facilidade a esses receptores (Prins, 1989). Além da testosterona, outros hormônios como estrógeno e prolactina interferem no desenvolvimento prostático. O estrógeno exerce efeito

direto na próstata por estimular o estroma e induzir a metaplasia no epitélio; e indireto por suprimir a secreção de gonadotrofinas reprimindo a secreção de testosterona pelos testículos (Sinowatz *et al.*, 1995; Bodker *et al.*, 1999). A prolactina induz a proliferação e diferenciação das células prostáticas através da ligação direta nos receptores de prolactina distribuídos por todo tecido. Essa ligação ao receptor estimula a atividade da 5  $\alpha$  redutase e potencializa a ação dos andrógenos (Reiter *et al.*, 1999).

A próstata é uma glândula muito estudada por ser acometida por patologias de impacto mundial, como a hiperplasia benigna e o câncer (Zhang *et al.*, 2003). Tais patologias atingem homens acima de 50 anos e prejudicam, substancialmente, sua qualidade de vida. Estudos vêm demonstrando que um microambiente estromal alterado, atuando através de mecanismos parácrinos é fator decisivo na carcinogênese prostática (Gerdes *et al.*, 2004). Isso conseqüentemente, leva à perda da homeostase na interação epitélio-estromal, o que é fundamental na progressão do tumor (Cunha, 1994, Cunha *et al.*, 2003; Ayala *et al.*, 2003).

O câncer de próstata é uma das malignecências mais comuns e a segunda causa de morte por câncer entre a população ocidental (Parker, 1995). Apesar da alta incidência e prevalência, sua etiologia é ainda pouco conhecida (Wong *et al.*, 2000). Fatores hormonais, genéticos, metabólicos, incluindo o diabetes têm sido apontados como possíveis fatores etiológicos para o desenvolvimento do câncer de próstata (Ilic *et al.*, 1996). Resultados de um estudo sobre prevenção do câncer indicaram que homens que tiveram diabetes (não controlado por insulina) por cinco anos ou mais têm maior incidência de câncer de próstata do que homens que nunca tiveram diabetes (Will *et al.*, 1999).

Nos últimos anos, estudos clínicos vêm demonstrando que homens diabéticos e portadores de hiperplasia benigna da próstata (HBP) possuem maior crescimento da região prostática hiperplásica, do que aqueles pacientes portadores de HBP sem a ocorrência do diabetes (Boon *et al.*, 2001; Hammarsten & Hogstedt, 2001). Além disso, foi descrito em um estudo epidemiológico que a severidade dos sintomas do HBP está significativamente associada à presença do diabetes (Michel *et al.*, 2000).

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#### IV- JUSTIFICATIVAS E OBJETIVOS

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O estroma desempenha papel essencial na função glandular prostática, portanto estudos relacionados à interação epitélio-estroma da próstata são de fundamental importância para o conhecimento tanto do funcionamento como dos fenômenos celulares e moleculares que envolvem o equilíbrio morfofuncional desse órgão. Além disso, em decorrência das alterações hormonais presentes no quadro de diabetes tipo I, tornam-se necessários estudos que avaliem, sistematicamente, e por metodologia de avaliação *in situ*, os efeitos da deficiência hormonal provenientes do diabetes sobre o estroma prostático. Como o diabetes é uma doença com grande incidência mundial é fundamental o estudo dos efeitos dessa sobre os componentes fibrilares e celulares do estroma prostático, associados ao comportamento do epitélio secretor, podendo esta patologia ser um fator de risco para a ocorrência de patologias prostáticas.

Assim, o objetivo deste trabalho foi caracterizar os efeitos do diabetes espontâneo sobre os componentes fibrilares e celulares estromais e epiteliais da glândula prostática de camundongos espontaneamente diabéticos, além de correlacionar esses eventos celulares à possíveis processos patológicos neste órgão.

### **Delineamento Experimental**

No presente estudo foram utilizados 33 camundongos machos, sendo 11 camundongos BALB/C (grupo controle), 11 camundongos NOD diabéticos positivos (grupo NOD+) e 11 camundongos NOD diabéticos negativos (grupo NOD-), na faixa etária de 16 a 20 semanas, pesando em média 30 gramas, provenientes do Centro de Bioterismo/Unicamp. Os animais Balb/C foram controles negativos do grupo NOD+, comprovadamente diabético e os animais do grupo NOD- foram também controles negativos, sendo considerados predispostos ao diabetes, porém sem o desenvolvimento da doença.

Nos animais pertencentes aos três grupos experimentais, foi realizado monitoramento diário da urina, onde foram avaliados os níveis de glicose (mg/dl) e pH. A urina desses animais foi coletada através de compressão manual na região da bexiga urinária desprezando-se a primeira gota. O concentrado de urina foi colocado em contato com a fita reagente (Urofit 10 BioBrás). A leitura do resultado realizou-se após trinta segundos à aplicação desta fita reagente, relacionando-se com a tabela fornecida pelo fabricante para as mensurações dos níveis dos elementos citados acima. Após 12 dias da caracterização do estado diabético nos animais do grupo NOD+, os camundongos de todos os grupos foram anestesiados com Francotar/Virbaxil (1:1), na dosagem de 0,25ml para cada 100g de peso corpóreo e se procedeu a coleta do lobo ventral da próstata, para análises de microscopias de luz e eletrônica de transmissão e estereológica. A vesícula seminal e glândula de coagulação foram retiradas somente para pesagem. Todos animais estudados receberam como dieta sólida ração Purina®, na forma de grãos e água *ad-libitum*. Ao longo do período experimental, foram realizadas mensurações do consumo de líquido e sólido dos animais dos três grupos estudados. Os pesos corpóreos (g) dos animais foram verificados no início e no fim do experimento.

## **Microscopia de Luz**

As amostras da próstata ventral de 6 animais de cada grupo foram fixadas em Bouin, por 24 horas, e posteriormente processadas para inclusão em parafina e historesina (*Historesin Embedding Kit, Leica*). Os cortes histológicos foram submetidos às colorações pela Hematoxilina-eosina (H&E) para estudo morfológico geral (Behmer *et al.*, 1976); Picrossírius-hematoxilina (Junqueira *et al.*, 1979) para fibras colágenas; Resorcina fucsina de Weigert para fibras elásticas (Carvalho *et al.*, 1997) e Reticulina de Gömöri para fibras reticulares (Vilamaior *et al.*, 2000). Nos cortes em historesina foram realizadas as colorações H&E e reticulina de Gömöri. As análises do material, fotodocumentação das áreas ocupadas pelo epitélio e estroma foram efetuadas em microscópio Zeiss-Jenaval.

## **Microscopia Eletrônica de Transmissão**

Os 5 animais restantes foram perfundidos com solução salina heparinizada (Sprando, 1990) seguida de solução fixadora Karnovsky (Karnovsky, 1965). Os fragmentos de próstata ventral foram fixados por imersão na mesma solução fixadora durante 6 horas e submetidos à pós-fixação em tetróxido de ósmio a 1% em tampão fosfato 0,1M pH 7,4, por duas horas. O contraste dos fragmentos foi realizado com o acetato de uranila a 0,5% em solução aquosa de sacarose por 12 horas. Depois de desidratados em série crescente de acetona, os fragmentos foram incluídos em resina araldite (Polyscience). Os cortes ultra-finos foram feitos em ultramicrotomo LKB 8800 ultratome III, com navalha de diamante e posteriormente contrastados com acetato de uranila (Watson, 1958) e citrato de chumbo (Reynolds, 1963). A seguir, o material foi fotografado no microscópio eletrônico de transmissão LEO-906 e realizada análise dos componentes celulares do epitélio secretor (avaliação geral das organelas citoplasmáticas) e do estroma (padrão de organização, distribuição e integridade das fibras estromais e da membrana basal).

## Avaliação Estereológica

Para o estudo estereológico foram utilizados 5 animais de cada grupo (controle, NOD+ e NOD-) e cortes histológicos corados com H&E.

Na quantificação do volume nuclear, foram medidos os diâmetros de 20 núcleos das células epiteliais, de cada animal, totalizando 100 núcleos em cada grupo estudado. A escolha dos núcleos foi casual, priorizando aqueles com limites bem definidos. As medidas foram realizadas com auxílio de ocular graduada (10X) acoplada ao microscópio de luz Olympus CBB, fixando-se as análises na objetiva 100X. A seguir, foram calculadas as médias dos volumes nucleares através da seguinte fórmula:

$$V_n = 4,19 (d^2/2) (D/2),$$

sendo “d” o diâmetro menor e “D” o diâmetro maior do núcleo.

Além disso, foram realizadas medidas para determinação quantitativa das frações volumétricas ocupadas pelo núcleo e pelo citoplasma das células epiteliais da próstata ventral. Essas mensurações foram feitas pelo sistema de contagem de pontos de Weibel (1979) utilizando uma ocular 10X reticulada acoplada no mesmo microscópio e objetiva. Através desse retículo foram contados os pontos localizados sobre os núcleos e sobre o citoplasma das células de dez campos para cada animal de cada grupo. As frações volumétricas foram calculadas para cada animal da seguinte forma:

$$\Sigma \text{Pontos citoplasma (Pc)} + \Sigma \text{Pontos núcleos} = \text{Pontos totais (pt)}$$

$$\% \text{ citoplasma (\%c)} = Pc/Pt \times 100$$

$$\% \text{ nuclear} = \%c - 100$$

A fração volumétrica real do núcleo já foi calculada através da contagem dos diâmetros nucleares e para a fração volumétrica do citoplasma foi feita uma regra de três, onde:

$$\% \text{ nuclear} \text{ ----- } V_n$$

$$\% \text{ citoplasma} \text{ ----- } X \text{ (volume citoplasmático)}$$

O volume celular foi calculado pela soma dos volumes nuclear e citoplasmático.

A quantificação da frequência relativa (%) dos compartimentos prostáticos: epitélio, lúmen, estroma muscular (camada de células musculares lisas que circunda o ácino) e estroma não

muscular (matriz extracelular e fibroblastos) e das fibras estromais: colágenas, elásticas e reticulares foi realizada através do sistema analisador digital Image Pro Plus (*Media Cybernetics*) com auxílio de câmara CCD-IRIS (*Sony*) acoplados ao microscópio Olympus. Através desse sistema foram capturadas imagens de 20 campos histológicos de cada grupo experimental, corados com H&E para análise dos compartimentos prostáticos e picrossírius- hematoxilina, reticulina de Gömöri e resorcina-fucsina de Weigert para as fibras colágenas, reticulares e elásticas respectivamente. Foram examinadas 5 lâminas por grupo (uma por animal), das quais foram coletadas 4 imagens de cada, no aumento de 100X. Realizou-se a contagem dos pontos que tocavam as variáveis acima (Weibel, 1979) e os valores obtidos foram transformados em porcentagem para a obtenção da frequência relativa.

### **Análise Estatística**

Para as seguintes variáveis: volume ( $\mu\text{m}^3$ ) citoplasmático das células epiteliais da próstata ventral e frequência relativa (%) dos compartimentos prostáticos: epitélio, lúmen, estroma muscular e estroma não muscular e das fibras estromais: reticulares e elásticas foi realizado o teste paramétrico, que corresponde à análise de variância para comparação de médias entre os grupos e no caso de haver significância estatística, aplicou-se o método de Tukey para comparações múltiplas das médias. Os testes não paramétricos de Kruskal Wallis, complementado com o teste de comparações múltiplas de Levy foi utilizado para as variáveis: frequência relativa (%) das fibras colágenas; volume ( $\mu\text{m}^3$ ) celular e nuclear; consumo de ração (g/dia) e água (mL/dia); variação de peso corpóreo (g/dia); peso (g) da vesícula seminal e glândula de coagulação. Todas as análises foram realizadas com 5% de significância (Levy, 1979; Johnson & Wichern, 1992; Zar, 1999).

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## **“PROSTATIC STROMAL MICROENVIRONMENT AND EXPERIMENTAL DIABETES”**

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Short title: Effects of diabetes on prostate.

Key words: prostate, strom, diabetes, NOD mice, histochemistry and ultrastructure.

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## **ABSTRACT**

The diabetes causes alterations in various organic systems, encompassing the male accessory sex glands. The prostate is very important in the male reproductive process and it is a frequent target of malignant changes. The aim of this work was to demonstrate the histochemistry and ultrastructural alterations in the prostate of the animals submitted to experimental diabetes. It was attempted to associate this results to cellular events involved in prostatic diseases. Three groups of animals were utilized: control, non-obese diabetic positive mice (NOD+) and non-obese diabetic negative mice (NOD-). The diabetic status was characterized using Bio Bras reagents strips. Twelve days after the characterization of diabetic status the ventral lobe was collected, fixed in karnovsky solution and paraformaldehyde and processed for histochemistry and TEM associated to stereology. The results showed reduction of the epithelial and luminal areas and increasing of the stromal area with muscular layer hypertrophy and collagen and reticular fibers in the prostatic gland. Also, it was characterized development of prostatic intraepithelial neoplasia, inflammatory process and dilation of the organelles involved in the secretory process. It was concluded that the diabetes besides damaging the reproductive process, affect the glandular homeostasis favoring the development of prostatic pathologies.

## INTRODUCTION

*Diabetes mellitus* is one of the most important diseases of modern society and represents not only a medical problem but also a social one because it impairs the quality of life of affected individuals (Mokdad, 2001). The world incidence of diabetes has increased substantially over the last few years, with about 160 million people being affected by the disease (Ritz, 2002). In the United States, 6.8% of the population have diabetes, which is responsible for 2% of deaths in the country (Parker, 1997; IDF Diabetes Atlas, 2004). In Brazil, this number is even higher, with 7.6% of the population suffering from the disease (Sociedade Brasileira do Diabetes, 2003).

Diabetes is a metabolic disease caused by a deficiency in the pancreatic secretion of insulin and/or by the inability of tissues to efficiently respond to insulin, events that cause hyperglycemia and affect all organs (Öztürk *et al.*, 1996). The main types of diabetes are insulin-dependent *diabetes mellitus* or type I, characterized by a total lack of insulin and the development of ketosis, and non-insulin-dependent *diabetes mellitus*, in which obesity is a risk factor (Robbins, 1989; Byrne *et al.*, 1996). Tissue alterations caused by diabetes affect different organic systems, including the male reproductive system, especially accessory sex glands and gonads. Studies have suggested that reproductive dysfunction is one of the common secondary effects of diabetes, which is associated with sexual impotence (Daubrese *et al.*, 1978), infertility caused by low sperm quality and alterations in testes and accessory sex glands (Frenkel *et al.*, 1978), a reduction in the weight of accessory sex organs (Saito *et al.*, 1996), and a decline in serum androgen levels (Ho, 1990). Crowe *et al.* (1987) observed smooth muscle atrophy and enlargement of the acinar lumen of the ventral prostate in diabetic rats. Atrophy of the secretory epithelium of the ventral prostate and of the coagulating gland has also been described experimentally on diabetic mice (Cagnon *et al.*, 2000; Carvalho *et al.*, 2003). Studies have suggested that diabetes may be associated with the occurrence of prostate cancer (Ilic *et al.*, 1996; Will *et al.*, 1999), in addition to negatively influencing clinical manifestations of benign prostatic hyperplasia (Michel *et al.*, 2000).

The prostate is an androgen-dependent accessory sex gland, which plays a fundamental role in the reproductive process (Netter, 1965; Marker *et al.*, 2003). It is formed by simple secretory epithelium resting above a visible basement membrane, with the stroma consisting of smooth muscle cells, fibroblasts and extracellular matrix (Price, 1963; Jesik *et al.*, 1982). The stroma is involved in the development of the prostate, as well as in the maintenance of its adult functional state, through paracrine interactions with the epithelium (Cunha *et al.*, 1992; Hayward & Cunha, 2000). An imbalance in this epithelial-stromal interaction results in drastic changes in the prostate, which can even be related to the development and progression of malignant diseases such as cancer or of benign hyperplasia (Cunha *et al.*, 1996; Grossfeld *et al.*, 1998; Tuxhorn, 2002).

Although the importance of the stroma in both prostate functioning and diseases has been recognized, little is known about the effects of the diabetic state on the stroma or on the epithelial-glandular stromal interaction. Thus, the objective of the present study was to analyze the influence of diabetes type I on stromal and epithelial components of the prostate of spontaneously diabetic mice, as well as to establish morphological correlations between cellular alterations resulting from diabetes and the occurrence of diseases in this organ.

## **MATERIAL AND METHODS**

### *Experimental Design*

Thirty-three adult male mice (Balb/C and NOD) aged 16-20 weeks were divided into three groups of 11 animals each: control (Balb/C), positive diabetic (NOD+) and negative diabetic (NOD-). All animals received as solid diet Purina® ration in granular form and water *ad libitum*. Food and water intake and body weight of the animals were measured throughout the experiment. The diabetic state was characterized using reagent strips (Urofit 10® Biobrás-Brazil), which determine glucose levels (mg/dl) and pH in urine. Twelve days after the characterization of diabetes, the animals were anesthetized with 0.25 ml/0.1 kg ketamine/xylazine (1:1) (Francotar®/Virbaxil® Virbac-Brazil) and

sacrificed. The seminal vesicle and coagulating gland were collected from all animals and weighed on a Sartorius 2434 analytical scale.

### *Light Microscopy*

The ventral prostate of six animals from each group was fixed in Bouin solution for 24 hours and embedded in paraffin (Paraplast Plus/ Brazil) and methacrylate resin (Historesin Embedding Kit Leica/ USA). The slides were stained with hematoxylin-eosin, Picrosirius-hematoxylin (Junqueira *et al.*, 1979), Gomori reticulin (Vilamaior *et al.*, 2000), and Weigert's resorcin-fuchsin (Carvalho *et al.*, 1997).

### *Transmission Electron Microscopy*

The five remaining animals from each group were perfused with heparinized saline solution (Sprando, 1990), followed by Karnovsky fixative (Karnovsky, 1965). Prostate fragments were fixed in 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, for 3 hours, and postfixed in 1% osmium tetroxide in the same buffer for 2 hours. The material was then dehydrated in an increasing acetone series and embedded in plastic resin (Poliscience/USA). Ultrathin sections were cut with an LBB ultramicrotome, counterstained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and analyzed under an LEO906 transmission electron microscope.

### *Stereologic Analysis*

The variables analyzed were cellular, cytoplasmic and nuclear volumes of the prostate epithelium obtained from tissue samples processed for light microscopy. Volumes were measured by the point-counting method of Weibel (1979) using a graded eyepiece at 100x magnification. Nuclear volume was determined by measuring the minor and major diameters of 20 nuclei per experimental group. The relative frequency of the prostate compartments and stromal fibers was determined using the Image Pro Plus digital analysis system (Media Cybernetics/USA), in which 20 histological fields were captured per experimental group. Next, using the method of Weibel, which involves fields containing

168 points and 84 lines, the points relative to the following prostate compartments were counted: epithelium, lumen, muscle (muscle cell layer involving the acini) and non-muscle stroma (extracellular matrix and fibroblasts), and stromal fibers (collagen, elastic and reticular fibers). These values were transformed into percentages in order to obtain the relative frequency.

### *Statistical Analysis*

A parametric analysis of variance was used for analysis of cytoplasmic volume ( $\mu\text{m}^3$ ) and relative frequency (%) of the epithelium, lumen, muscle and non-muscle stroma and reticular and elastic fibers, complemented by the Tukey multiple comparisons test. The nonparametric Kruskal-Wallis test was applied to the following variables: relative frequency (%) of collagen fibers, variation in body weight (g/day), seminal vesicle weight (g), coagulating gland weight (g), water (ml/day) and food (g/day) intake, and cellular and nuclear volumes ( $\mu\text{m}^3$ ). The Levy multiple comparisons test was used in this nonparametric analysis (Levy, 1979). The level of significance was set at 5% in all analyses (Johnson & Wichern, 1992; Zar, 1999).

## **RESULTS**

### *Urine Analysis*

Positive diabetic animals (NOD+) showed elevated mean glucose levels (1000 mg/dl), while no glycosuria (0 mg/dl) was observed in 100% of animals of the control and negative diabetic (NOD-) groups. The pH was similar in the different experimental groups, with a pH of 6.0 being observed for the control group and of 7.0 for the NOD+ and NOD- groups, values considered to be neutral.

### *Nutritional Assessment and Weight of the Male Genital Organs*

Mean daily food and water intake was significantly higher in positive diabetic mice than in control and negative diabetic animals, which did not differ from one another.

However, despite this higher food intake, positive diabetic animals showed a marked loss in mean daily body weight. In contrast, control and negative diabetic mice gained weight throughout the experimental period (Table 1).

Mean weight of the seminal vesicle showed a significant decrease in positive diabetic animals, while no significant reduction in coagulating gland weight was observed, with these mean values showing a progressive reduction from the control to the NOD- and NOD+ groups (Table 1).

### *Histochemistry and Stereology*

In the control group, the ventral prostate showed a tubuloacinar structure containing acini with unfolded mucosa and simple epithelium consisting of tall columnar cells and basal nucleus (Figure 1A). In the stroma, collagen fibers underlying the basement membrane were noted (Figure 1B), in addition to thin and straight reticular fibers showing moderate undulation (Figure 1C) and elastic fibers distributed homogenously between smooth muscle cells (Figure 1D).

In the negative diabetic group, most glandular characteristics were similar to those of the control group. However, some alterations were observed. The secretory epithelium was characterized by short columnar cells, indicating discrete atrophy of the organ (Table 2, Figures 1E and 2A). The relative frequency of the stroma was significantly higher, in addition to a significant increase in the muscle layer (Figure 2A). Still in the stroma, the relative frequency and distribution of collagen fibers was similar to that observed for the control group, with fibers mainly being located below the basement membrane (Figures 1F and 2B). Reticular fibers were found to be significantly increased (Figure 2B) and presented a more undulate aspect but with predominance of thin and straight fibers (Figure 1G). The relative frequency of the elastic system was significantly reduced compared to the control group (Figure 2B), with fibers being concentrated beneath the basement membrane (Figure 1H).

In positive diabetic animals, marked atrophy of secretory epithelial cells was observed, with atrophic cells and loss of mucosa folds (Table 2 and Figure 1I), demonstrating

a significant reduction in relative epithelial and luminal frequency of the ventral prostate lobe (Figure 2A). Areas of focal epithelial stratification, in addition to inflammatory cells, were frequently observed in the prostate, characterizing prostatic intraepithelial neoplasias (PINs) of the pseudocribiform type (Figure 1J and K). The relative stromal frequency was significantly increased, especially in portions involving muscle stroma (Figure 2A), and fibrillar elements demonstrated visible morphological alterations. The frequency of collagen fibers was significantly higher in this group (Figure 2B) and the fibers were wide distributed throughout the stromal space (Figure 1M). Reticular fibers were significantly increased and showed a predominantly basal location and an undulated aspect (Figures 1N and 2B). In contrast, a decrease was observed in the relative frequency of elastic fibers, which were found to be grouped into dense bundles underlying the basement membrane (Figures 1O and 2B).

### *Ultrastructure*

In the control group, the secretory epithelium was characterized by tall columnar cells resting on a clearly visible and continuous basal lamina (Figure 3A and B). In the basal cytoplasm, the granular endoplasmic reticulum demonstrated flattened cisternae (Figure 3B). On the luminal cell surface secretory vesicles in different stages of maturation were detected (Figure 3C). In the stroma, the muscle layer was evidenced (Figure 3B) by well-developed and extensive smooth muscle fibers containing a flattened nucleus that occupied large part of the cytoplasm. Collagen was underlying the basement membrane and distributed between muscle fibers (Figure 3D). The smooth muscle cells did not show the typical secretory pattern, possessing few organelles involved in the secretory process and secretory vesicles (Figure 3E).

Negative diabetic animals showed a cellular ultrastructure close to that of the control group but with moderate alterations. The secretory epithelium was discretely reduced in height, containing cells with a round nucleus and a secretory product (Figure 4A). The basal lamina was continuous. The basal region of the cytoplasm of secretory cells contained eventual digestive vacuoles (Figure 4B). Secretory vesicles of different electron

densities were observed in the supranuclear region, and discrete dilatation of Golgi cisternae was noted (Figure 4C). In the stroma, collagen was located between muscle cells, which were found to be extensive and to contain a flattened nucleus and few secretory vesicles, similar to the findings obtained for the control group (Figure 4D).

In the positive diabetic group, intensely atrophied epithelial cells were observed, characterized by a nucleus occupying most of the cytoplasm (Figure 5A) and a thick and folded basal lamina (Figure 5B). The basal cytoplasm of the epithelial cell was characterized by the accumulation of digestive vacuoles close to the basal lamina (Figure 5C). In the apical region, secretory vesicles with a flocculent aspect and different electron densities were observed, in addition to the clearly visible dilatation of Golgi cisternae (Figure 5D). Focal epithelial proliferations- NIPs, as also observed by light microscopy, were frequent (Figure 5E). The stroma presented smooth muscle cells of altered morphology, including an extensively folded cytoplasmic membrane, causing cell shortening and conferring a spinous aspect to the cell as a result of the folding of the nuclear envelope (Figure 6A). The secretory activity of these cells seemed to be altered due to the increased presence of secretory vesicles (Figure 6A). In addition, a marked increase in collagen concentration between muscle fibers was noted (Figure 6B). Unstructuring of the membrane system was observed in eventual muscle cells, forming myelin type figures and characterizing signs of an initial cell degeneration process (Figure 6C).

## DISCUSSION

The present study demonstrated elevated glucose levels in the urine of diabetic animals. Glycosuria is one of the determining factors in the identification of diabetes type I. Different studies have reported glycosuria in both chemically induced diabetic rats and spontaneously NOD animals (Makino, 1980; Ader *et al.*, 1998), pointing to the diabetic condition of the animals studied. In addition, diabetic animals showed a reduction in body weight even after ingestion of high amounts of ration and water. An imbalance in food intake and poor utilization of food have been reported in the literature for both diabetic

humans and experimentally diabetic animals (Daubrese *et al.*, 1978; Seethalakshmi *et al.*, 1987). Thus, the present findings are related to the diabetic condition and demonstrate that diabetes compromises the organic metabolism of these animals, leading to body imbalance. The numeric results showed drastic atrophy of the glandular secretory epithelium in positive diabetic animals (NOD+). In addition, alterations in the membrane system of different organelles involved in the prostatic secretion process were observed, as well as the frequent occurrence of PINs. PINs are considered to be premalignant lesions and carry the risk of evolving to histological carcinoma due to the presence of mutant cells that proliferate in an uncontrolled manner (Bogliolo, 2000).

Previous studies investigating different accessory sex glands have revealed a possible deficiency in the secretory process in diabetic animals, which was especially associated with morphophysiological modifications of the prostate gland (Jackson & Hutson, 1984; Cagnon *et al.*, 2000; Carvalho *et al.*, 2003). In contrast, no occurrence of PINs has been reported when studying the prostate of diabetic rodents. Thus, it may be concluded that diabetes disturbs the structure of the prostatic epithelium, causing impairment of the reproductive process in the animals studied, in addition to resulting in evident premalignant lesions. It is important to note that negative diabetic animals also showed significant epithelial atrophy; however, these alterations were moderate in relation to those observed in the NOD+ group. NOD mice were developed in Japan in 1980 and are considered to be a good model for the study of type I autoimmune insulin-dependent *diabetes mellitus* in view of its clinical similarity to human diabetes type I, including signs such as hypercholesterolemia, polydipsia, polyphagia and polyuria (Makino, 1980; Tochino, 1987; Kolb, 1987). In addition, the literature reports that NOD mice are an excellent model for the study of different alterations resulting from the diabetic state, since the latter primarily develops a lymphocyte inflammatory infiltrate in the pancreas, more precisely in the pancreatic islets with concomitant destruction of beta cells, a fact resulting in a severe reduction in insulin and an imbalance in blood glucose levels (Humphreys-Beher *et al.*, 1998). On the other hand, NOD mice are also known to develop autoimmune sialoadenitis resembling Sjögren's syndrome in humans (Skarsten *et al.*, 1995). However,

most findings in salivary glands have demonstrated that the autoimmune genetic factor is not responsible for the onset of glandular morphofunctional changes (Humphreys-Beher *et al.*, 1998). Moreover, Yamano *et al.* (1999) considered NOD not to be a perfect model for the study of autoimmune Sjögren's disease because of moderate autoimmune complications. In addition, other investigators studying the effects of diabetes on salivary glands in NOD animals suggested that autoimmune effects act synergistically with primary alterations that occur due to metabolic disorders resulting from diabetes type I (Hu *et al.*, 1992). Therefore, one may infer that the drastic alterations observed in confirmed diabetic rats might have been intensified by the autoimmune factor of these animals, and that the moderate changes identified in the prostate gland of NOD animals are related to this factor.

Structural and quantitative modifications in fibrillar and muscular elements were observed in the prostatic stroma of the positive diabetic group, including an expressive increase in relative stromal frequency with accumulation of collagen underlying the basement membrane, thickening and undulation of reticular fibers, and aggregation of elastic fibers. Negative diabetic animals showed fibrillar morphological characteristics similar to those of the control group. However, alterations in the relative frequency of these components were observed. No studies were found in the literature concerning the behavior of the prostatic extracellular matrix of diabetic animals. On the other hand, these modifications resemble those observed in castrated animals. According to Carvalho *et al.* (1996, 1997) and Vilamaior *et al.* (2000), castration leads to fibrillar reorganization of extracellular matrix components such as collagen and elastic fibers. Collagen secreted by fibroblasts and smooth muscle cells plays a structural role and guarantees prostate strength, whereas elastic fibers are involved in tissue extensibility and deformation. The association between these elements provides the plasticity necessary during muscle fiber contraction and restoration (Carvalho *et al.*, 1997). It can therefore be concluded that diabetes leads to effective remodeling of the glandular stroma similar to the event of castration, with androgen deficiency probably being the main factor in these alterations.

The differentiated phenotypic characteristics observed in smooth muscle cells of positive diabetic animals, such as smaller myofibrillar extension and extensive folding of

the plasma membrane showing numerous budding secretory vesicles, indicate a marked synthetic and non-contractile activity of these cells. Smooth muscle cells present in the stroma of negative diabetic cells showed a morphology similar to that of the control group. Previous studies have reported that diabetes causes alterations in smooth muscle of diverse organs, such as a reduction in the nucleus/cytoplasm ratio and in the size of the endoplasmic reticulum (Öztürk *et al.*, 1996). In experimental models involving castration, Vilamaior *et al.* (2000) observed this cellular phenotype in the prostate of rats, characterizing it with a spinous aspect. Hypertrophy of the muscle layer, as well as the synthetic phenotype acquired by smooth muscle cells and the accumulation of collagen between these cells, suggests an active role of these cells in the constant remodeling of extracellular matrix, an event that might occur in the stroma during prostatic involution resulting from diabetes.

In addition, inflammatory infiltrates with a predominance of lymphocytes were observed in the glandular stroma of positive diabetic animals. This type of inflammation is a typical characteristic of non-bacterial prostatitis (Kwon *et al.*, 2001). Prostatitis is a common condition in men above the age of 60 and a frequent finding in cases of benign prostatic hyperplasia. Foster & Bostwick (1998) suggested both autoimmune mechanisms and extravasation of secretion into the stroma due to obstruction caused by muscle fiber hypertrophy as the origin of prostatic inflammatory processes.

The prostatic stroma has been regarded as the principal compartment in glandular functioning due to its role in the maintenance of prostate homeostasis and its morphophysiological involvement in diseases such as benign prostatic hyperplasia and cancer (Donjacour *et al.*, 2003; Zhang *et al.*, 2003). The literature has shown that the stroma plays a role in both the onset and progression of carcinogenesis, since tumor stroma frequently differs from normal showing an elevated expression of growth factors and collagen, and the replacement of smooth muscle cells with myofibroblasts (Cunha *et al.*, 2002). In addition, the regulation of proliferation of prostatic carcinoma is a complex phenomenon that involves many extracellular matrix components and endocrine, paracrine and autocrine mechanisms, in which the stroma plays a fundamental role since it acts as a

three-dimensional matrix where interactions with the tumor occur (Wang *et al.*, 2001; Cornell *et al.*, 2003). Modifications in the stromal microenvironment consisting of cells and extracellular matrix are the first step in the development of prostate cancer (Cunha *et al.*, 2003). This activation has been described as “reactive stroma” and is characterized by an increased production of extracellular matrix, especially collagen and growth factors, and the reorganization of stromal components, creating a microenvironment favorable to tumor growth (Rowley, 1998; Tuxhorn, 2002). Reactive stroma alters the epithelial-stromal interaction, favoring the expansion of genetically altered epithelial cells (Grossfeld *et al.*, 1998; Hayward *et al.*, 2001). Thus, diabetes-induced stromal alterations indicate abnormal signalization between the epithelium and stroma that leads to changes in prostate homeostasis. In this respect, diabetes definitely favors the occurrence of diseases such as benign hyperplasia, prostatitis and even cancer.

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## FIGURE LEGENDS

**FIGURE 1** – Photomicrographs of the ventral prostate of control (A-D), negative diabetic (E-H) and positive diabetic rats (I-O). **A-** Secretory epithelium showing tall columnar cells (e) and basally located nucleus. HE: X1250. **B-** Distribution of collagen fibers (co) underlying the basement membrane. Epithelium (e). Picrosirius-hematoxylin: X1250. **C-** Stromal location of thin and straight reticular fibers (arrow) underlying the epithelium (e). Gomori reticulin: X1250. **D-** Distribution of elastic fibers in glandular stroma (ef) intermingled with muscle cells underlying the secretory epithelium (e). Weigert's resorcin-fuchsin: X1250. **E-** Secretory epithelium (e) showing slightly atrophied, columnar cells. Stroma (s). HE: X1250. **F-** Distribution of collagen fibers (co) within the stroma, concentrated beneath the secretory epithelium (e). Picrosirius-hematoxylin: X1250. **G-** Reticular fibers underlying the basement membrane and showing a discretely undulated morphology (thick arrow), but with a predominance of thin fibers (thin arrows). Epithelium (e). Gomori reticulin: X1250. **H-** Elastic fibers (ef) distributed beneath the epithelium (e). Weigert's resorcin-fuchsin: X1250. **I-** Atrophied secretory epithelium (e) showing very short cells (\*). Stroma (s). HE: X1250. **J-** Acinus showing intraepithelial neoplasias (PINs) (arrows). HE: X250. **K-** Detail of a PIN showing cells with strongly labeled nuclei of variable sizes and clearly visible nucleoli (arrowheads). HE: X1250. **L-** Inflammatory infiltrate (arrow) in the prostatic stroma (s). Blood vessel (bv). Epithelium (e). Picrosirius-hematoxylin: X250. **M-** Collagen fibers (co) distributed throughout the stroma. Epithelium (e). Picrosirius-hematoxylin: X1250. **N-** Predominant location of reticular fibers beneath the basement membrane showing a thick and undulated aspect (arrows). Epithelium (e). Gomori reticulin: X1250. **O-** Elastic fibers (ef) aggregated in bundles located beneath the epithelium (e). Weigert's resorcin-fuchsin with peracetic acid oxidation: X1250.

**FIGURE 2-** Graphics showing the mean relative frequency (%) of prostatic stromal components (epithelium, lumen, muscle and non-muscle stroma) (**A**), and the mean relative frequency (%) of stromal fibers (collagen, elastic and reticular) (**B**) of control, negative

diabetic (NOD-) and positive diabetic (NOD+) animals. a,b,c indicate the level of significance between the groups studied.

**FIGURE 3** – Electron micrographs of the epithelium and stroma of the ventral prostate of control mice. **A-** Simple epithelium characterized by tall columnar cells with basally located elongated nucleus (n) showing condensed chromatin at the periphery. Lumen (l). X4312. **B-** Basal region. Prominent granular endoplasmic reticulum (ger) and intact basal lamina (b). In the stroma (s), note the thin layer of muscle fibers (sm). X20000. **C-** Apical region. secretory vesicles (sv), elongated mitochondria (m) and granular endoplasmic reticulum (ger). X20000. **D and E-** Elongated smooth muscle cell containing an extensive and flattened nucleus (n). Collagen scattered between muscle fibers (co) and few secretory vesicles (arrows). Basal lamina (b). D: X25860; E: X20000.

**FIGURE 4** – Electron micrographs of the epithelium and stroma of the ventral prostate of negative diabetic NOD mice. **A-** Discretely atrophied epithelium consisting of small columnar cells with small and round nucleus (n) resting on the basal lamina (b). Enlarged fibromuscular layer (sm). Lumen (l). X4312. **B-** Basal region. Nucleus (n). Digestive vacuoles (arrows). Thick basal lamina (b). Muscle fibers (sm). X20000. **C-** Apical region. Secretory vesicles (sv) of different electron densities, discrete dilatation of the Golgi complex (gc) and mitochondria (m). Nucleus (n). X20000. **D-** Elongated smooth muscle cell containing few secretory vesicles and an extensive nucleus (n) with undulations in the plasma membrane (arrow). Basal lamina (b). Collagen distributed between muscle cells (co). X20000.

**FIGURE 5** – Electron micrographs of the epithelium of the ventral prostate of positive diabetic NOD mice. **A-** Atrophied secretory epithelium consisting of cuboid cells with nucleus occupying large part of the cytoplasm (n). Lumen (l) and basal lamina (b). X4312. **B-** Acentuated folded basal lamina (arrow). X20000. **C-** Basal region showing the accumulation of digestive vacuoles (arrows) close to the granular endoplasmic reticulum

(ger). Nucleus (n). Basal lamina (b). X20000. **D-** Apical region. Note the secretory vesicles containing a product with flocculent aspect (sv) and the clearly visible dilatation of the Golgi cisternae (gc). Mitochondria (m). X20000. **E-** Cell proliferation showing nuclei of different sizes (arrows) arranged in various layers of the epithelium. Basal lamina (b). X4312.

**FIGURE 6** – Electron micrographs of the stroma of the ventral prostate of positive diabetic NOD mice. **A-** Muscle cell with an altered phenotype. Note the extensively folded cell membrane (arrows) and the nucleus with spinous aspect (n). Collagen (co). Secretory vesicles (arrowheads). X25860. **B-** Collagen fibers between muscle cells. X25860. **C-** Muscle cell showing “myelin type figures” with membrane deposits (\*). Nucleus (n). Collagen (co). X20000.

## TABLES

**Table 1** – Mean and standard error of food and water intake and variation ( $\Delta$ ) in body, seminal vesicle (SV) and coagulating gland (CG) weight in the three experimental groups.

|                         | Control                      | NOD -                         | NOD +                         | Test   |
|-------------------------|------------------------------|-------------------------------|-------------------------------|--------|
| Ration (g/day)          | 5,47 $\pm$ 0,27 <sup>a</sup> | 5,36 $\pm$ 0,16 <sup>a</sup>  | 13,64 $\pm$ 0,69 <sup>b</sup> | p<0,05 |
| Water (mL/day)          | 4,28 $\pm$ 0,12 <sup>a</sup> | 5,15 $\pm$ 0,16 <sup>a</sup>  | 24,27 $\pm$ 1,80 <sup>b</sup> | p<0,05 |
| $\Delta$ weight (g/day) | 0,09 $\pm$ 0,02 <sup>a</sup> | 0,06 $\pm$ 0,02 <sup>a</sup>  | -0,10 $\pm$ 0,09 <sup>a</sup> | p>0,05 |
| SV weight (g)           | 0,37 $\pm$ 0,03 <sup>a</sup> | 0,27 $\pm$ 0,01 <sup>a</sup>  | 0,24 $\pm$ 0,01 <sup>b</sup>  | p<0,05 |
| CG weight (g)           | 0,08 $\pm$ 0,02 <sup>a</sup> | 0,08 $\pm$ 0,009 <sup>a</sup> | 0,07 $\pm$ 0,003 <sup>a</sup> | p>0,05 |

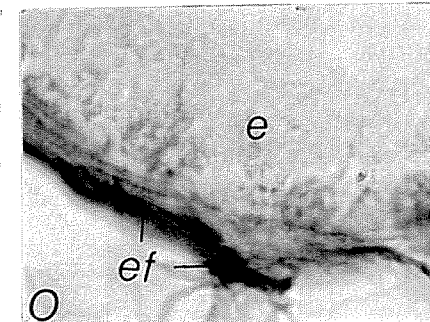
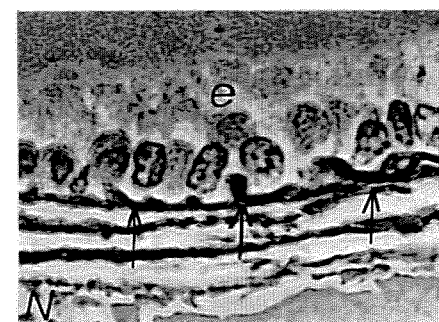
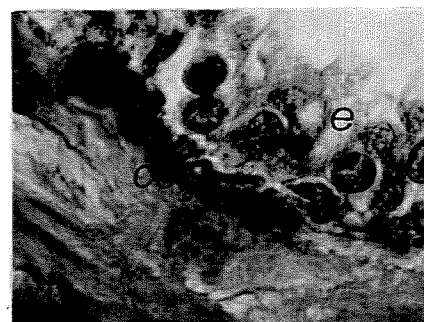
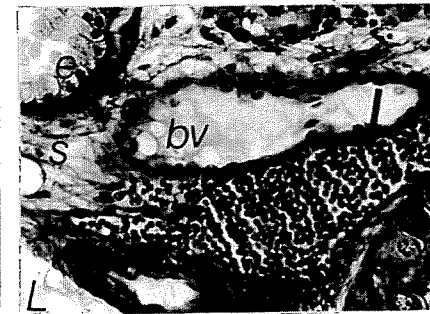
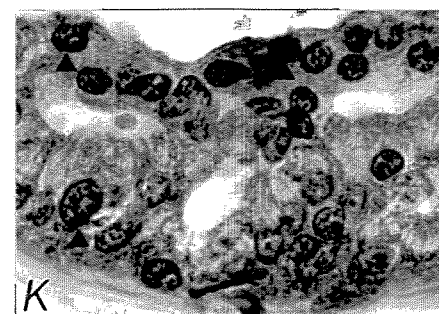
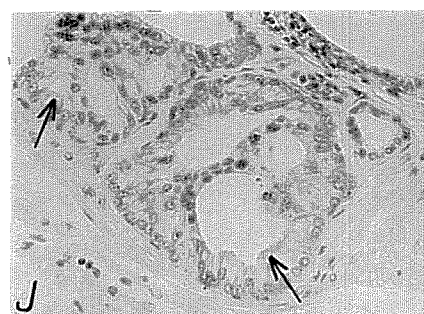
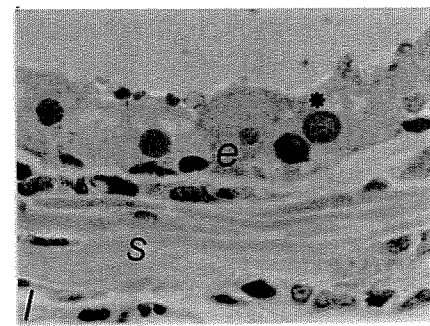
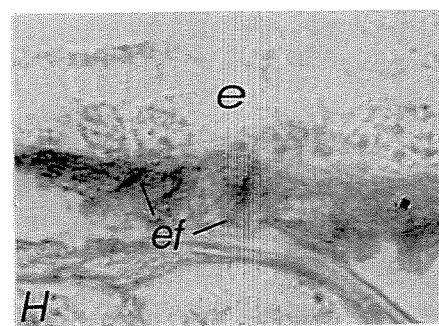
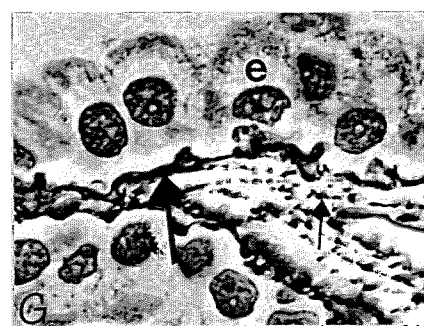
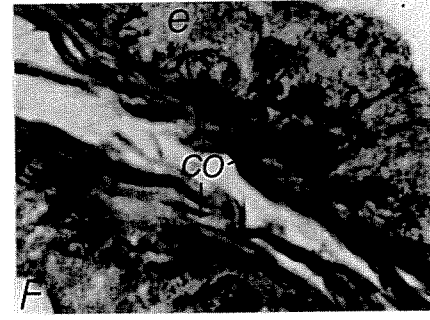
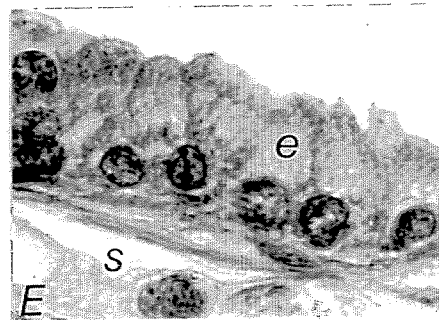
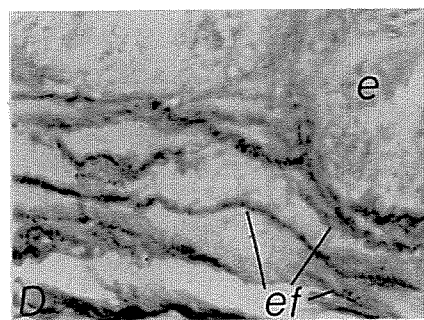
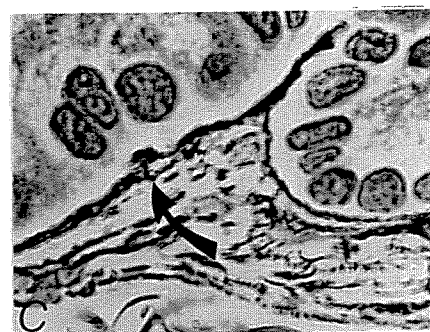
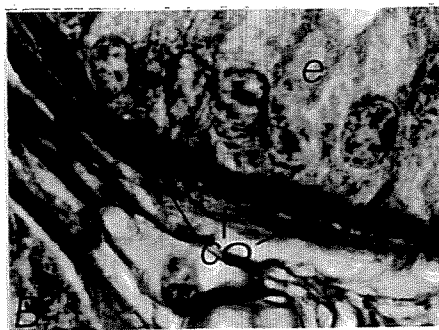
<sup>a,b</sup> Different letters mean significative differences between groups.

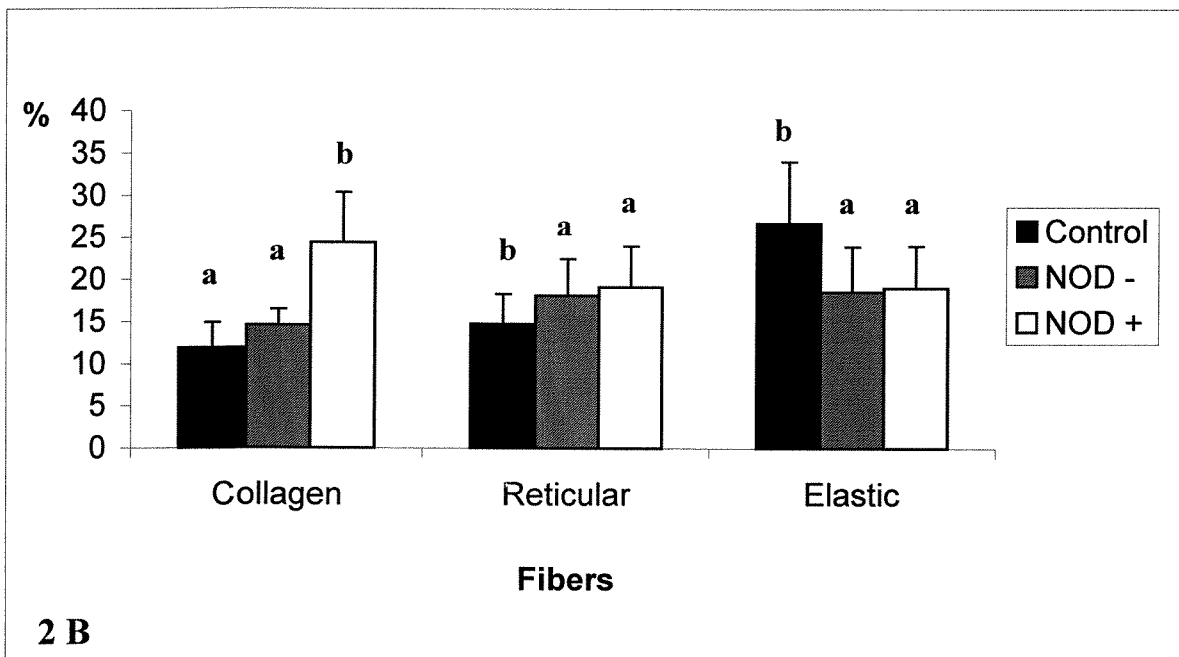
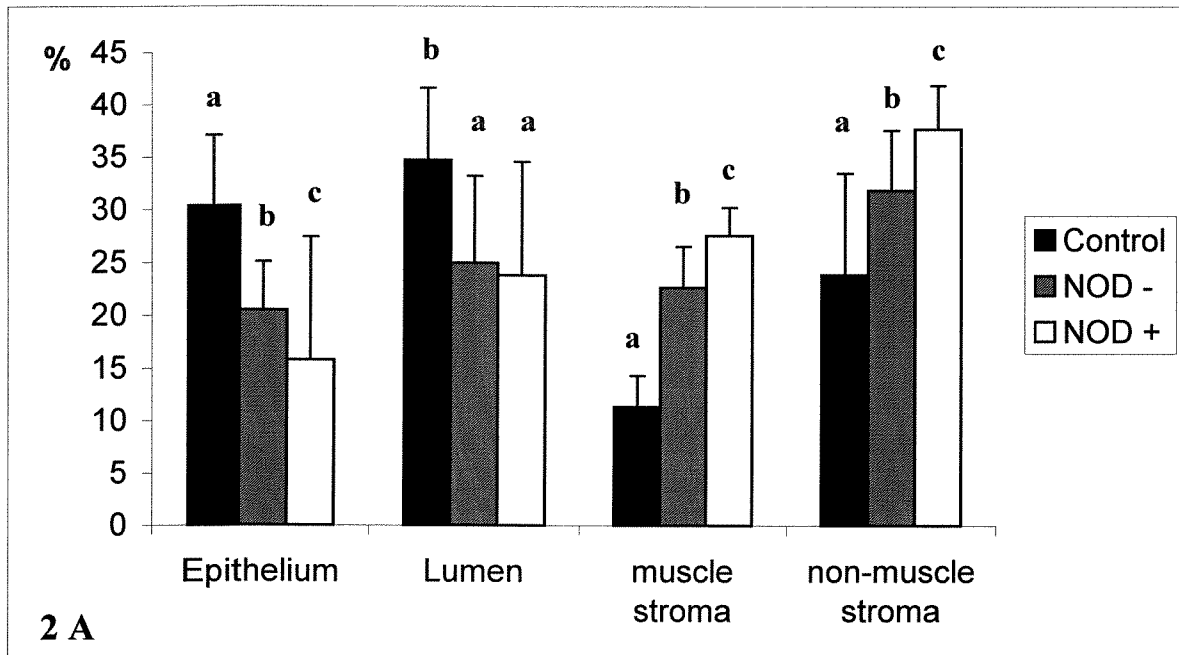
**Table 2-** Mean and standard error of cellular, cytoplasmic and nuclear volumes ( $\mu\text{m}^3$ ) of control, negative diabetic (NOD-) and positive diabetic (NOD+) animals.

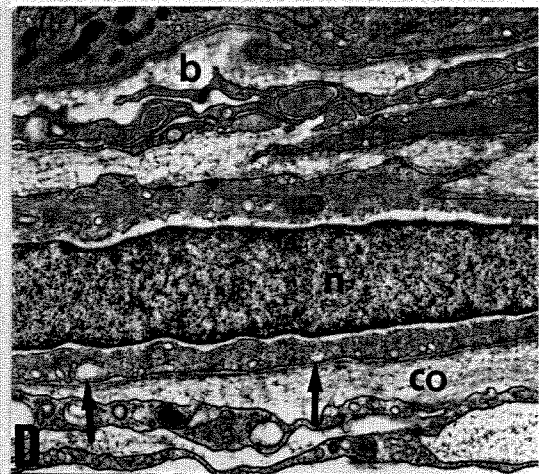
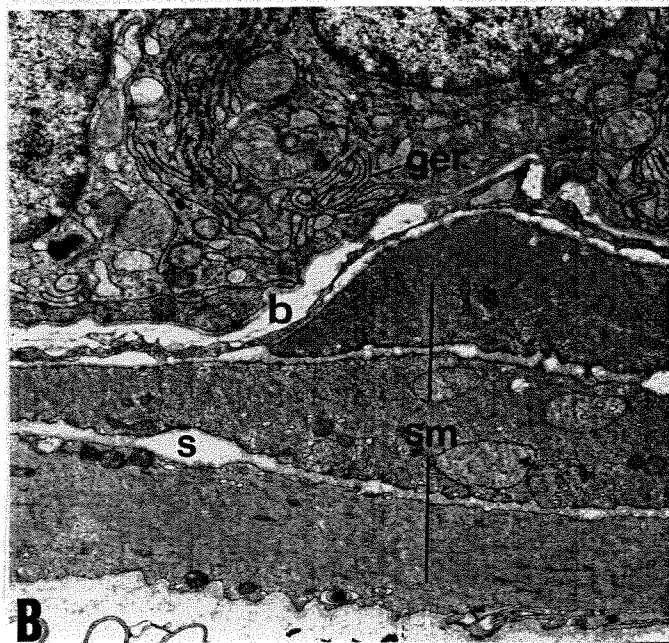
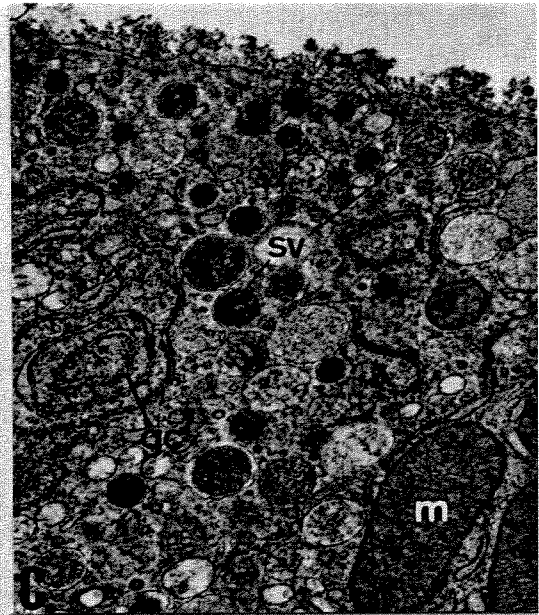
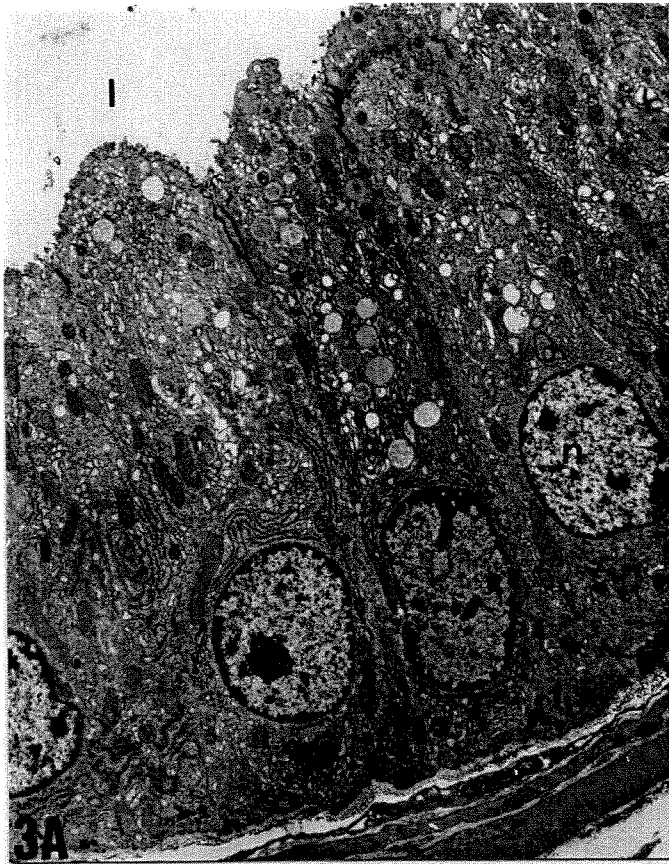
|                    | Control                       | NOD -                          | NOD+                          | Teste  |
|--------------------|-------------------------------|--------------------------------|-------------------------------|--------|
| Celular volume     | 360,5 $\pm$ 12,1 <sup>a</sup> | 321,8 $\pm$ 6,6 <sup>a,b</sup> | 254,9 $\pm$ 17,2 <sup>b</sup> | p<0,05 |
| Cytoplasmic volume | 280,3 $\pm$ 12,8 <sup>a</sup> | 267,2 $\pm$ 15,9 <sup>a</sup>  | 184,2 $\pm$ 13,8 <sup>b</sup> | p<0,05 |
| Nuclear volume     | 80,2 $\pm$ 1,3 <sup>a</sup>   | 74,7 $\pm$ 1,3 <sup>a</sup>    | 70,7 $\pm$ 3,7 <sup>a</sup>   | p>0,05 |

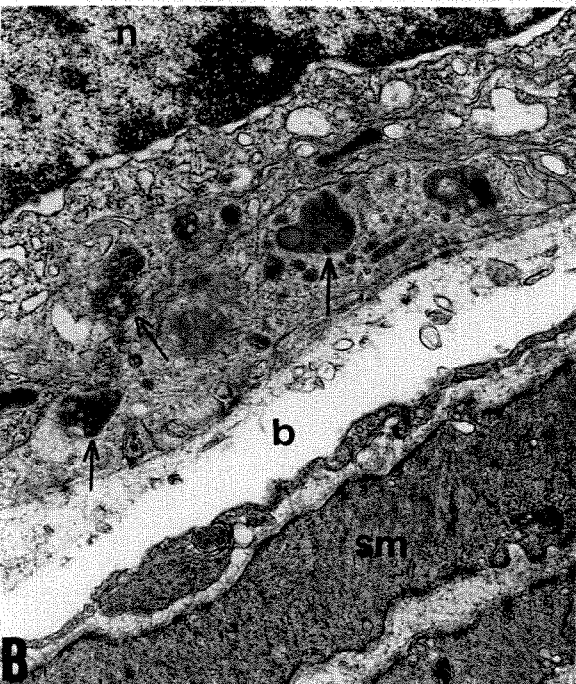
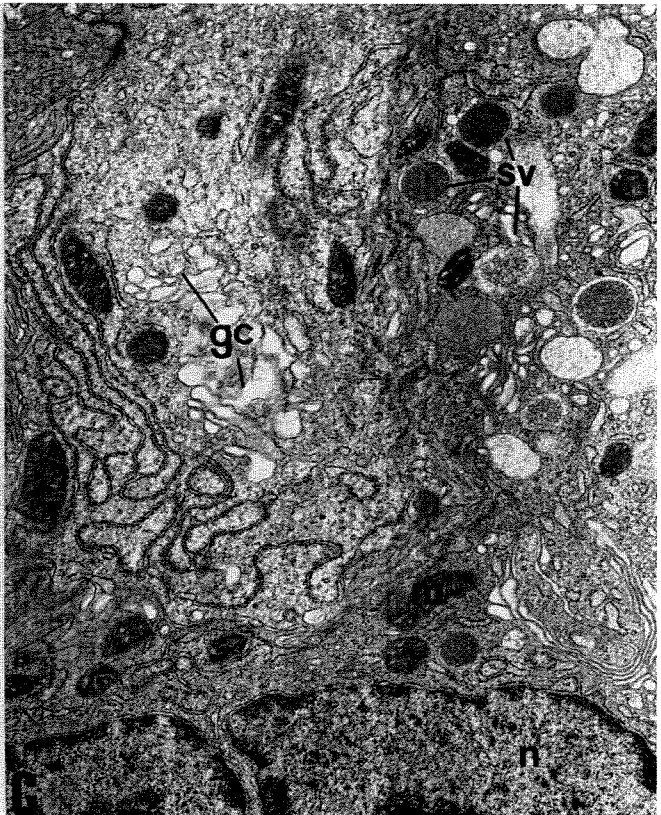
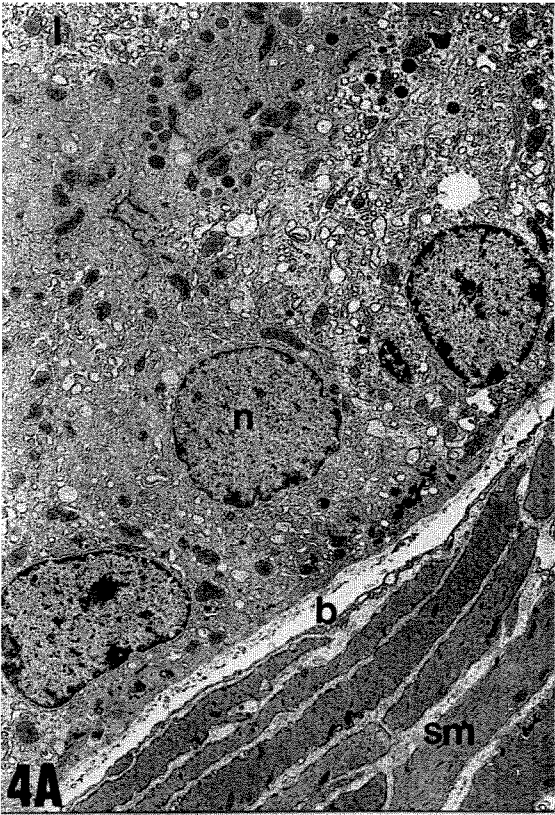
<sup>a,b</sup> Different letters mean significative differences between groups.

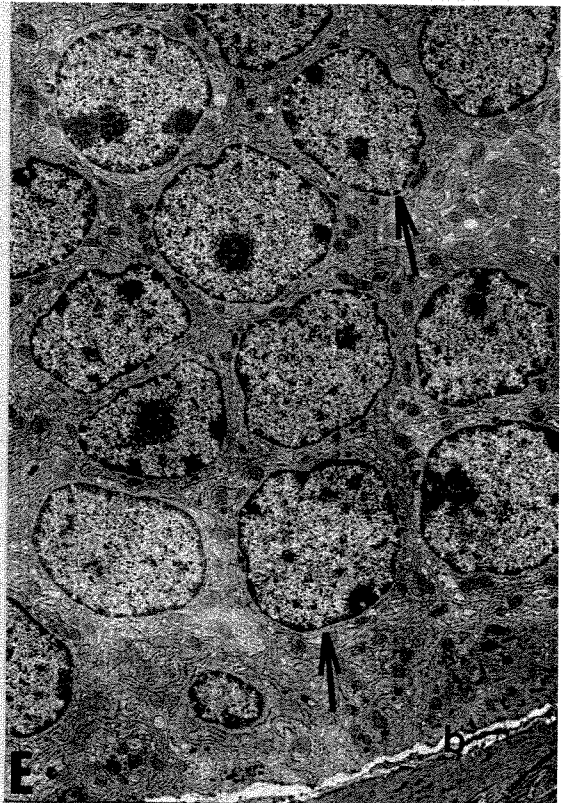
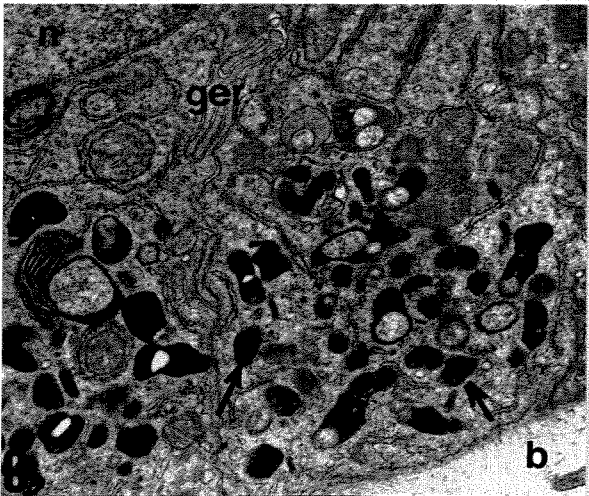
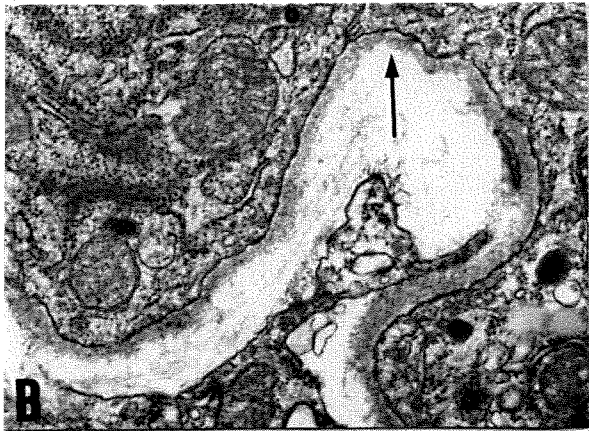
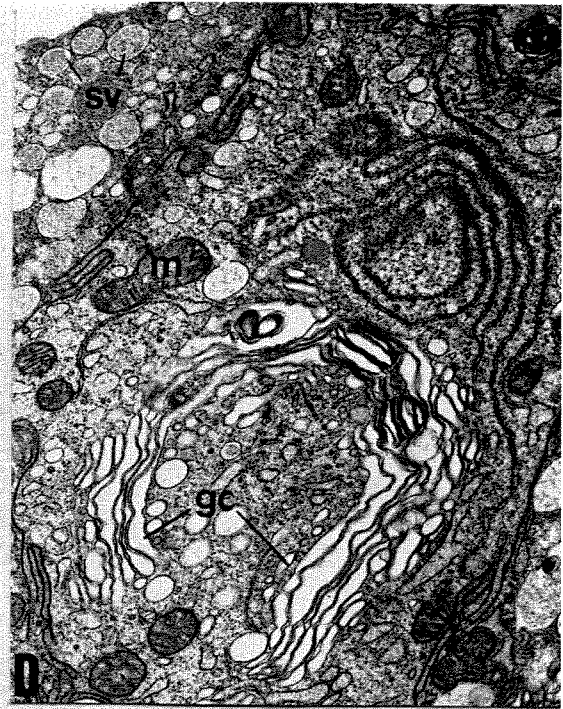
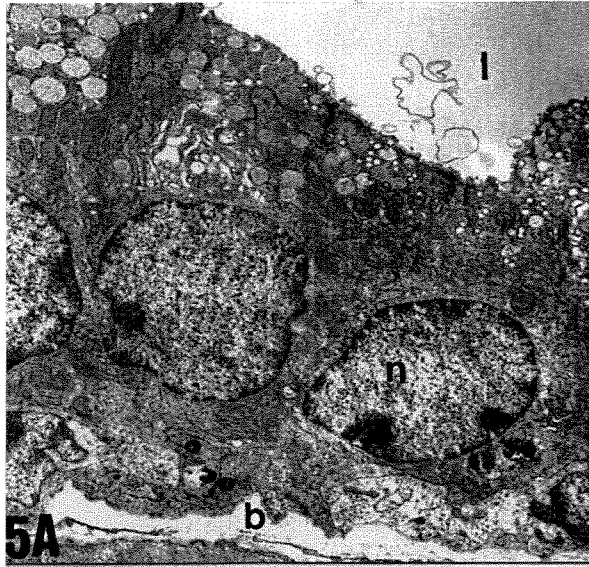
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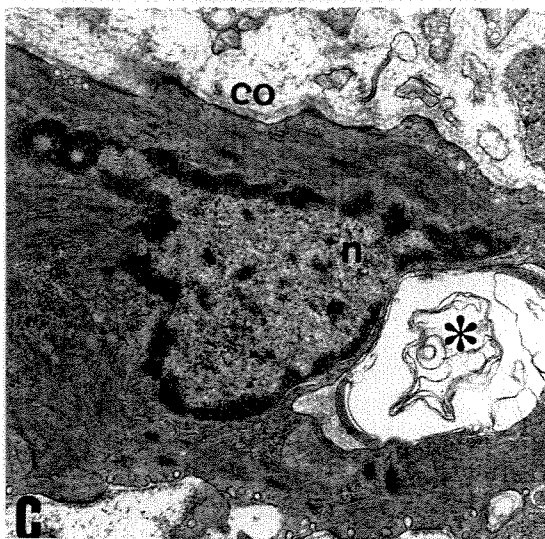
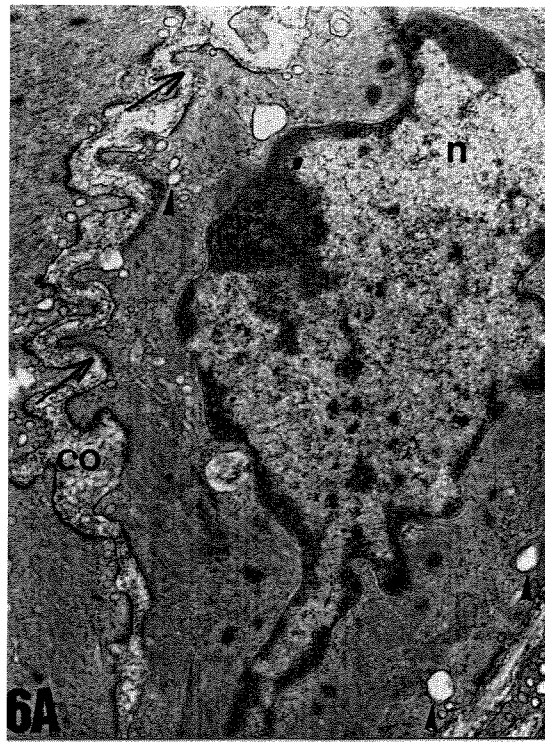












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## VII- CONSIDERAÇÕES E CONCLUSÕES GERAIS

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1. Os níveis de glicose na urina dos animais diabéticos positivos foram elevados em relação aos controles e diabéticos negativos, comprovando o estado diabético dos animais em estudo.
2. O peso corpóreo do grupo diabético positivo mostrou-se diminuído mesmo apresentando alto consumo de ração e água, quando comparado ao grupo controle e diabético negativo. Isso indica que o diabetes prejudica o metabolismo orgânico desses animais e provoca desequilíbrio corpóreo.
3. Os pesos frescos da vesícula seminal e glândula de coagulação foram menores nos animais diabéticos positivos.
4. O diabetes causou drástica atrofia no epitélio secretor da próstata ventral dos camundongos diabéticos positivos. As células do epitélio apresentaram-se com volume significativamente reduzido assim como a frequência relativa do compartimento epitelial. Os animais diabéticos negativos apresentaram modesta atrofia das células epiteliais. Essa involução prostática certamente prejudica o funcionamento normal da glândula e o processo reprodutivo dos animais.
5. No estroma, as fibras colágenas e reticulares tiveram significativo aumento de frequência relativa enquanto as fibras elásticas foram reduzidas nos animais diabéticos positivos e também nos negativos. Essas alterações indicam que o desequilíbrio hormonal decorrente do diabetes promove, assim como na castração, remodelação dos elementos da matriz extracelular no estroma prostático.
6. O diabetes promove alterações no microambiente prostático, tais como, desenvolvimento de neoplasias intraepiteliais, modificações no fenótipo da célula

muscular (que passa a ser secretora) e presença de infiltrados inflamatórios. Pode-se concluir que essas alterações afetam totalmente a interação equilibrada entre epitélio-estroma, podendo favorecer o desenvolvimento de patologias prostáticas, como prostatites e até mesmo adenocarcinomas.

7. O animal NOD negativo não expressou o diabetes e apresentou a maioria das características glandulares similar ao controle. Entretanto, modestas alterações foram detectadas. Provavelmente, tais alterações são decorrentes da autoimunidade presente nos camundongos NOD, que favorece algumas alterações teciduais. Assim, as drásticas alterações observadas nos animais diabéticos positivos foram consequência da ação sinérgica do fator autoimune e do desenvolvimento do diabetes.

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