Prostatic stromal microenvironment and experimental diabetes

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The diabetes causes alterations in various organ systems, including the male accessory sex glands. The prostate is very important in the reproductive process and it is a frequent target of malignant changes. The aim of this work was to demonstrate the histochemical and ultrastructural alterations in the prostate of diabetic animals. Two groups of animals were utilized: control and non-obese diabetic mice (NOD). Twelve days after the characterization of diabetic status the ventral prostate was collected, fixed in Karnovsky and paraformaldehyde, processed for histochemistry and TEM associated to stereology. The results showed reduction of the epithelial area and increasing of the stromal area with muscular and collagen hypertrophy in the prostatic gland. It was characterized the development of prostatic intraepithelial neoplasia, inflammatory processes and dilation of the organelles involved in the secretory process. It was concluded that diabetes besides damaging the reproductive process, affects the glandular homeostasis favoring the development of prostatic pathologies.

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

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Diabetes mellitus is one of the most important diseases of modern society and it 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 affected by the disease (Ritz, 2002). In the United States, 6.8% of the population has diabetes, which is responsible for 2% of the deaths in the country (Parker, 1997; IDF Diabetes Atlas, 2005). In Brazil, this number is even higher, with 7.6% of the population suffering from the disease (Brazilian Society of Diabetes, 2005).

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 non-insulin-dependent diabetes mellitus, in which obesity is a risk factor (Robbins, 1989). Tissue alterations caused by diabetes affect different organic systems, including the male reproductive system, especially the 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 the diminution of low sperm quality, by alterations in tests and accessory sex glands (Frenkel *et al*., 1978); with a reduction in the weight of accessory sex organs (Saito *et al*., 1996) and with 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 animals. Atrophy of the ventral prostate secretory epithelium 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). Besides that, it can negatively influence the 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 placed above a visible basement membrane with stroma consisting of smooth muscle cells, fibroblasts and extracellular matrix (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 (Hayward and 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 benign hyperplasia (Cunha *et al.*, 1996).

In the literature, there are several studies showing that diabetes impairs the sexual function and changes the prostatic morphology in animals, but these works didn't describe the importance of stroma in prostatic functioning or in diseases. There is no study about the effects of diabetic state on the stromal components or on the epithelial- stromal interaction. Thus, the objective of the present study was to analyze the influence of diabetes type I on stromal components and on epithelial-stromal interaction in 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.

Materials and Methods

Experimental design

Twenty-two adult male mice aged 16-20 weeks were divided into two groups of 11 animals each: control (Balb/C) and diabetic (NOD). All animals received as solid diet Purina® ration in the 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 (Urofita 10® Biobrγs/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.

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), Gömöri reticulin (Vilamaior *et al.*, 2000), and Weigert's resorcin-fuchsin (Carvalho *et al.*, 1997).

Transmission electron microscopy

The five remaining animals offrom each group were perfused (Sprando, 1990) with heparinized saline solution, 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 (Polyscience/USA). Ultrathin sections were cut with an LBB ultramicrotome, counterstained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and analyzed under a LE0906 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 compart-

ments 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 (μm^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 (μm^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 (Zar, 1999; Johnson and Wichern, 1992).

Results

Urine analysis

Diabetic animals showed elevated mean glucose levels (1000 mg/dL), while no glycosuria (0 mg/dL) was observed in 100% of animals of the control group.

Nutritional assessment and weight

Mean daily food and water intake was significantly higher in diabetic mice than in the control ones. However, despite this higher food intake, diabetic animals showed a remarkable loss in mean daily body weight. In contrast, control mice gained weight throughout the experimental period (Table 1).

Histochemistry and stereology

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

Table 1. Mean and standard error of food and water intake and variation (Δ) in body weight in the two experimental groups.

	Control	*Diabetic*	*Test*
Solid (g/day)	5,47±0,27[a]	13,64±0,69[b]	$p<0,05$
Liquid (mL/day)	4,28±0,12[a]	24,27±1,80[b]	$p<0,05$
Δ weight (g/day)	0,09±0,02[a]	-0,10±0,09a	$p>0,05$

[a,b] *Mean comparison between experimental groups.*

Table 2. Mean and standard error of cellular cytoplasmic and nuclear volumes (μm^3) of control and diabetic animals.

	Control	*Diabetic*	*Test*
Cellular volume	360,5±12,1[a]	254,9±17,2[b]	$p<0,05$
Citoplasmatic volume	280,3±12,8[a]	184,2±13,8[b]	$p<0,05$
Nuclear volume	80,2±1,3[a]	70,7±3,7[a]	$p>0,05$

[a,b] *Mean comparison between experimental groups.*

Diabetic animals showed remarkable atrophy of secretory epithelial cells, with cells tending to be cuboid, and loss of mucosa folds (Table 2 and Figure 1E), 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 (Figure 1F), were frequently observed in the prostate, characterizing prostatic intraepithelial neoplasias (PINs) of the pseudocribriform type (Figure 1G and H). 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), in which fibers were abundantly distributed throughout the stromal space (Figure 1I). Reticular fibers were significantly increased and showed a predominantly basal location and an undulated aspect (Figures 1J and 2B). In contrast, a decrease was observed in the relative frequency of elastic fibers, which were found to be grouped into dense bundles placed under the basement membrane (Figures 1K and 2B).

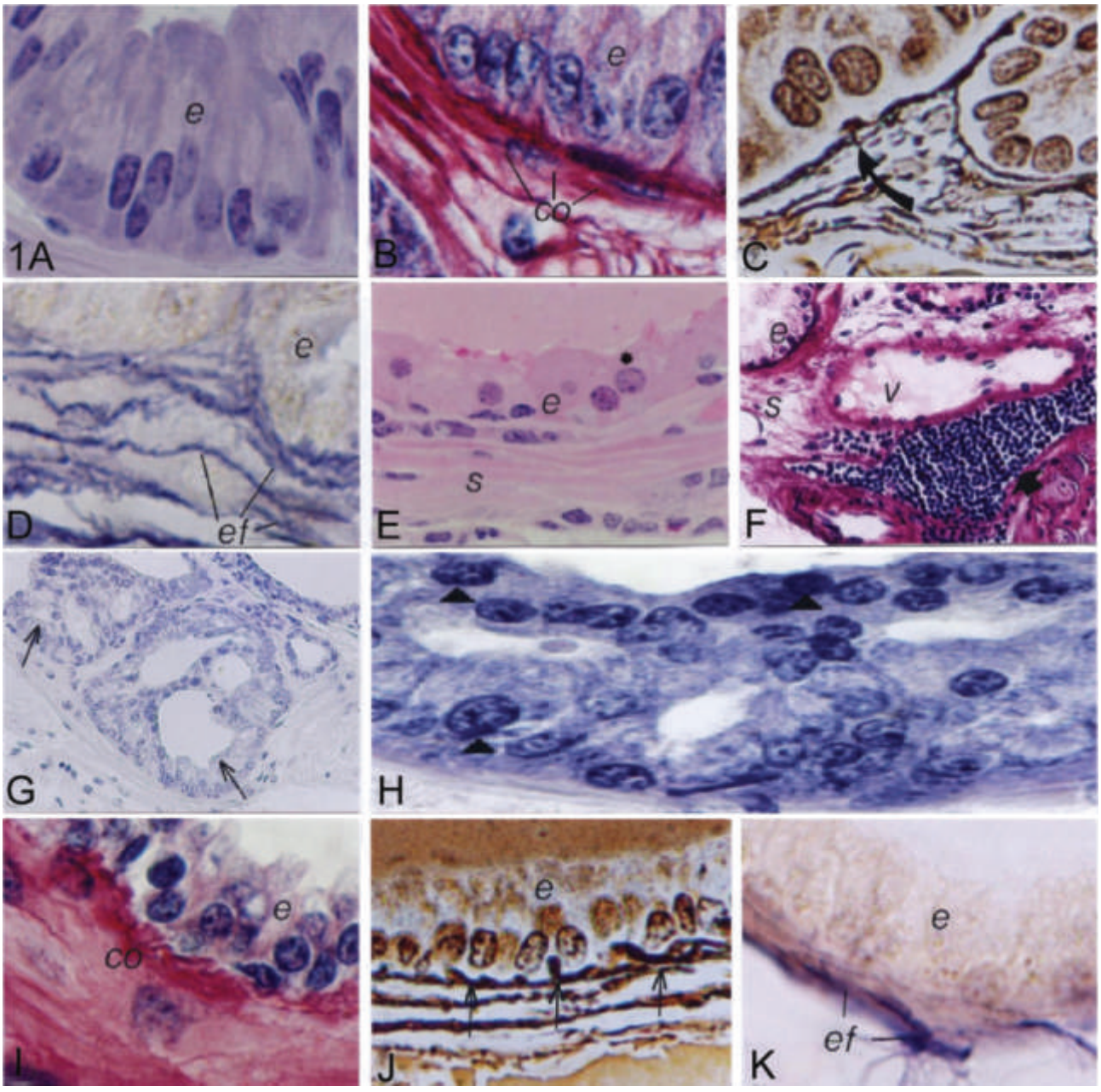
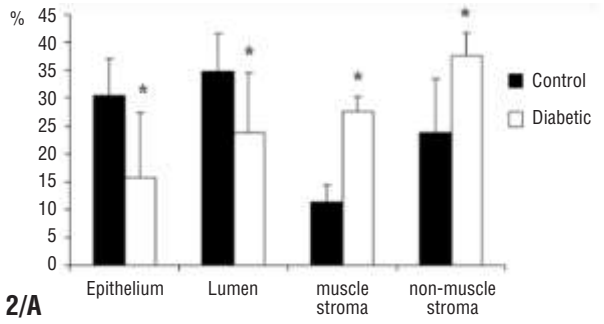


Figure 1. Photomicrographs of the ventral prostate of control (A-D) and diabetic mice (E-K). A: Secretory epithelium (e). HE: X1250. B: Distribution of collagen fibers (co) underlying the basement membrane. Epithelium (e). Picrosirius-hematoxylin: X1250. C: Stromal location of thin and straight reticular fibers (arrow) underlying the epithelium (e). Gomori reticulin: X1250. D: Distribution of elastic fibers (ef) between with muscle cells underlying the secretory epithelium (e). Weigert's resorcin-fuchsin: X1250. E: Atrophied secretory epithelium (e) showing very short cells (*). Stroma (s). HE: X1250. F: Inflammatory infiltrate (arrows) in the prostatic stroma (s). Blood vessel (bv). Epithelium (e). Picrosirius-hematoxylin: X250. G and H: Acinus with PIN (arrows) showing benign cells with strongly labeled nuclei of variable sizes and clearly visible nucleoli (arrow heads). HE: X1250. I: Collagen fibers (co) distributed throughout the stroma. Epithelium (e). Picrosirius-hematoxylin: X1250. J: Reticular fibers showing a thick and undulated aspect (arrows). Epithelium (e). Gömöri reticulin: X1250. K- An aggregated of elastic fibers (ef) located beneath the epithelium (e). Weigert's resorcin- resorcin-fuchsin with peracetic acid oxidation: X1250.



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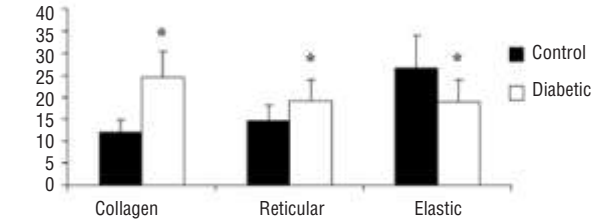

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Figure 2. Graphics showing the mean relative frequency (%) of prostatic stromal components (epithelium, lumen, muscle and non-muscle stroma) (A), and the mean relative frequency (%) of stromal fibers (collagen, elastic and reticular) (B) of control and diabetic animals. * indicate the level of significance between the groups studied.

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 parallel and 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 a large part of the cytoplasm. Collagen was placed under the basement membrane and distributed between muscle fibers (Figure 3D). The smooth muscle cells showed a typical contractile pattern, possessing high myofibrillar fraction and some secretory vesicles (Figure 3D, E).

In the diabetic group, intensely atrophied epithelial cells were observed, characterized by a nucleus occupying most of the cytoplasm (Figure 4A) and a thick and folded basal lamina (Figure 4B). The basal cytoplasm of the epithelial cell was characterized by the accumulation of digestive vacuoles close to the basal lamina (Figure 4C). 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 4D). Focal epithelial proliferations were frequent, as also observed by light microscopy (Figure 4E). The stroma presented smooth muscle cells (SMC) 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 5A). The secretory activity of these cells seemed to be altered due to the increased presence of secretory vesicles (Figure 5A). In addition to this, there was a remarkable increase in collagen concentration between muscle fibers (Figure 5B). Some eventual muscle cells demonstrated an alteration of the membrane system, forming myelin type figures and characterizing signs of an initial cell degeneration process (Figure 5C).

Discussion

The present study demonstrated a reduction in the body weight of diabetic animals 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 numerical results showed drastic atrophy of the glandular secretory epithelium in diabetic animals. Besides that, 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 indicated a possible deficiency in the secretory process in diabetic animals, which was especially associated with morphophysiological modifications of the prostate gland (Jackson and Hutson, 1984; Cagnon *et al.*, 2000; Carvalho *et al.*, 2003). In contrast, no occurrence of PINs has

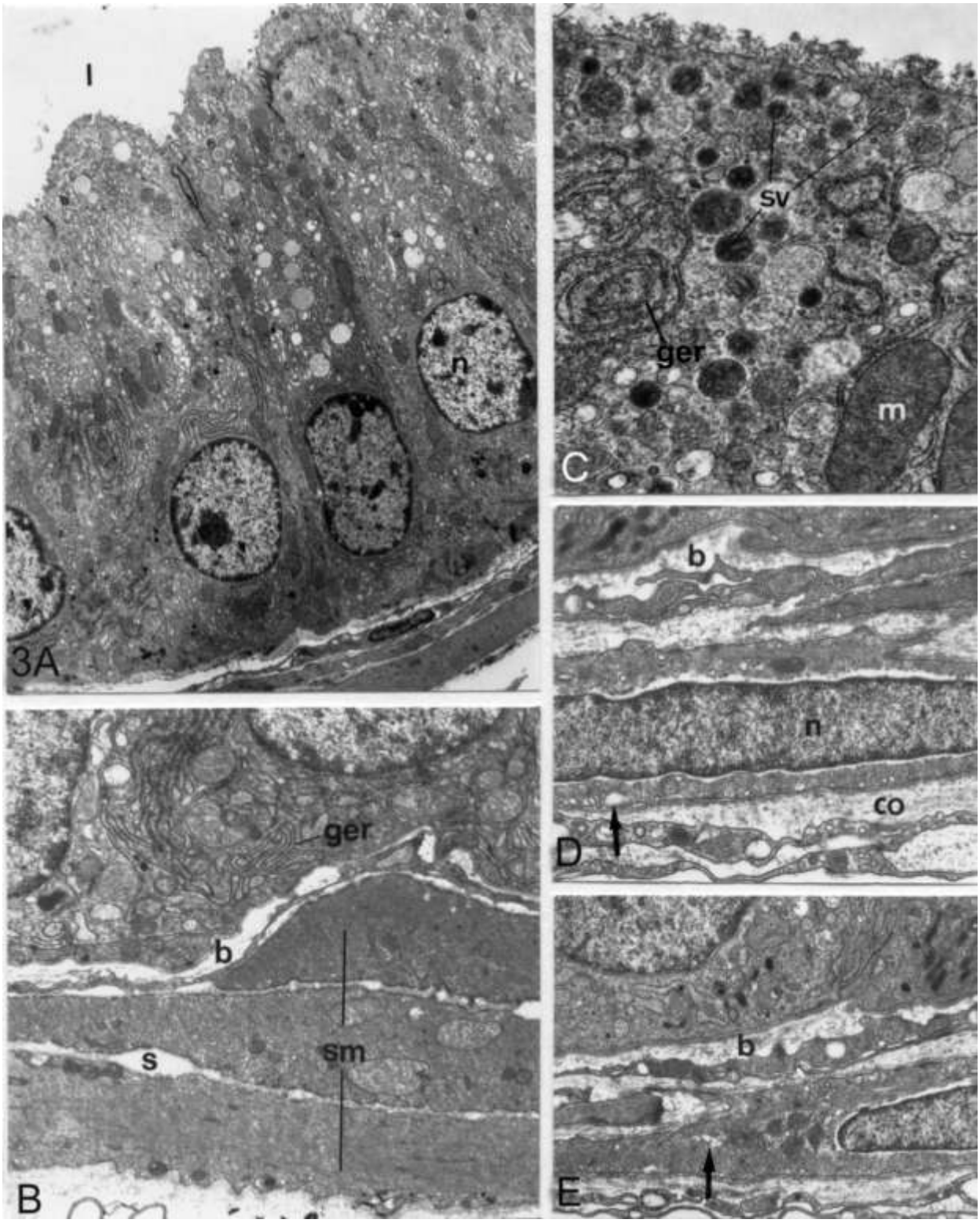

Figure 3. Electron micrographs of the ventral prostate of control mice. A: Simple epithelium characterized by tall columnar cells with basally located elongated nucleus (n). Lumen (l). X4312. B: Basal region. Prominent granular endoplasmic reticulum (ger) and intact basal lamina (b). In the stroma (s), note the thin layer of muscle fibers (sm). X20000. C: Apical region, secretory vesicles (sv), elongated mitochondria (m) and a granular endoplasmic reticulum (ger) . 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.

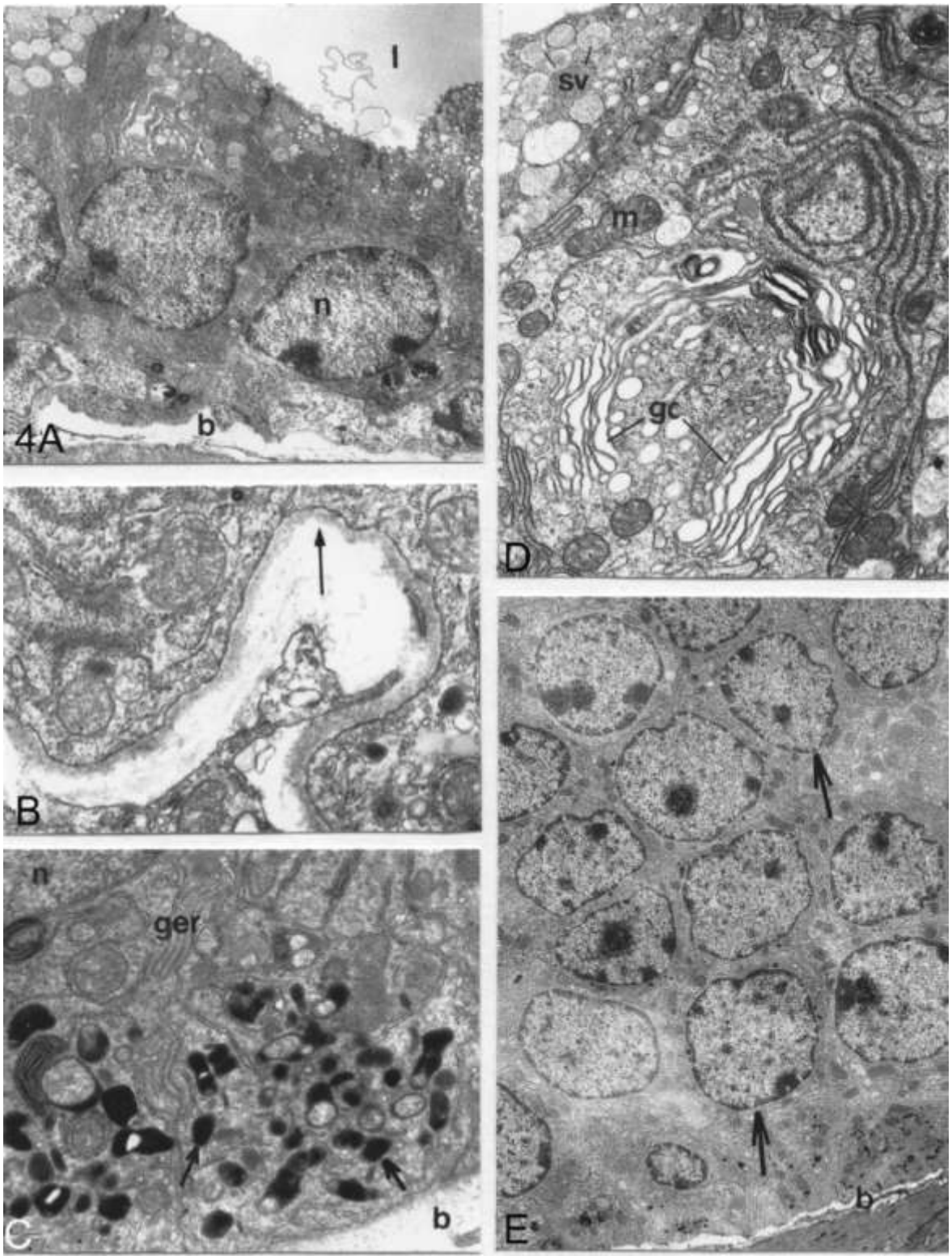

Figure 4. Electron micrographs of the epithelium of the ventral prostate of diabetic mice. A: Atrophied secretory epithelium consisting of cuboid cells with nucleus occupying a large part of the cytoplasm (n). Lumen (l) and basal lamina (b). X4312. B: Acentuated folded basal lamina (arrow). X20000. C: 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. 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.

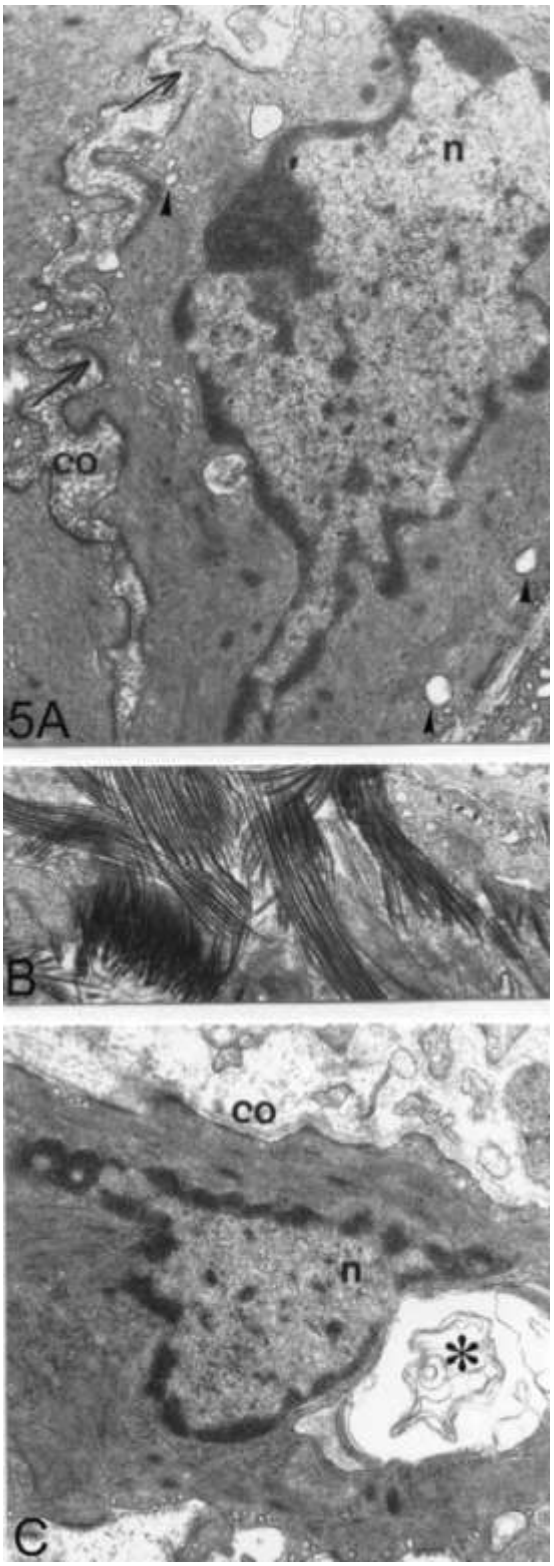

Figure 5. Electron micrographs of the stroma of the ventral prostate of diabetic mice. A: Muscle cell with an altered phenotype. Note the extensively folded cell membrane (arrows) and the nucleus with spinous aspect (n). Collagen (co). Secretory vesicles (arrowheads). X25860. B: Collagen fibers between muscle cells. X25860. C: Muscle cell showing "myelin type figures" with membrane deposits (*). Nucleus (n). Collagen (co). X20000.

been reported when studying the prostate of diabetic rodents. Thus, it may conclude that diabetes disturbs the structure of the prostatic epithelium, causing impairment of the reproductive process in the animals studied and it can result in evident premalignant lesions.

Structural and quantitative modifications in fibrillar and muscular elements were observed in the prostatic stroma of the 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. 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. The association between these stromal elements provides the plasticity necessary during muscle fiber contraction and restoration (Carvalho *et al*., 1997). It may therefore be concluded that diabetes led 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 diabetic animals, such as smaller myofibrillar extension and extensive folding of the plasma membrane showing numerous secretory vesicles, indicate an intense synthetic and non-contractile activity of these cells. 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 castrated rats, characterizing it with a spinous aspect. Hypertrophy of the muscle layer, as well as the synthetic phenotype acquired by SMC and the accumulation of collagen between these cells, suggests an active role of SMC 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 pre-

dominance of lymphocytes were observed in the glandular stroma of 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 and 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 considered 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 tumor stroma frequently differs from normal stroma by showing an elevated expression of growth factors and collagen, and the replacement of smooth muscle cells with myofibroblasts (Cunha *et al*., 2002). 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 and by 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 signaling between the epithelium and stroma that leads to changes in prostate homeostasis. Thus, besides damaging the reproductive process, diabetes may possibly result in pathogenesis on prostate.

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