ARTICLE
Journal of Cellular Biochemistry 113:2223–2233 (2012)

Journal of Cellular Biochemistry

Oxidative Stress Markers and Apoptosis in the Prostate of Diabetic Rats and the Influence of Vitamin C Treatment

Marina Guimarães Gobbo,[1,2] Daniele Lisboa Ribeiro,[3] Sebastião Roberto Taboga,[2] Eduardo Alves de Almeida,[4] and Rejane Maira Góes[2*]

[1] *Department of Cell Biology, Institute of Biology-UNICAMP, Campinas, São Paulo, Brazil*

[2] *Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, Univ Estadual Paulista—UNESP, São José do Rio Preto, São Paulo, Brazil*

[3] *Histology Sector, Institute of Biomedical Sciences, Federal University of Uberlandia—UFU, Uberlândia, MG, Brazil*

[4] *Department of Chemistry and Environmental Sciences, Institute of Biosciences, Languages and Exact Sciences, Univ Estadual Paulista—UNESP, São José do Rio Preto, São Paulo, Brazil*

ABSTRACT

Negative consequences of diabetes on the prostate such as involution are associated with diminished testosterone, insulin deficiency, and hyperglycemia. The contributions of oxidative damage, which usually increases with diabetes, are unknown for these alterations. This study evaluated the impact of streptozotocin-induced diabetes on the biomarkers of the antioxidant system of rat ventral prostate, the influence of vitamin C supplementation on these biomarkers, and on the balance between cell proliferation and death. Diabetes (D) was induced in Wistar male rats by streptozotocin (5 mg/100 g b.w., i.p.). Control animals (C) were injected with a vehicle. Vitamin C (150 mg/kg b.w./day) supplementation was introduced by gavage in diabetes (D + V) as well as control (C + V) groups. Thirty days after diabetes onset, the rats were killed and the ventral prostates were analyzed using light microscopy, immunocytochemistry, and biochemical assays for biomarkers of oxidative stress. In comparison to control groups, the levels of circulating testosterone, proliferating, and androgen receptor-positive cells decreased in diabetic groups regardless of vitamin C treatment whereas apoptosis was increased. The levels of superoxide dismutase and glutathione peroxidase did not change, but the levels of glutathione-*S*-transferase (GST) were increased in diabetic prostate. Vitamin C supplementation normalized GST activity and recovered the apoptotic rates in the prostate. In conclusion, GST is a good indicator of compensatory oxidant defense in the prostate at earlier stages of diabetes and vitamin C improves its activity and attenuates apoptosis in the gland. J. Cell. Biochem. 113: 2223–2233, 2012.

KEY WORDS: EXPERIMENTAL DIABETES; OXIDATIVE STRESS; PROSTATE; VITAMIN C SUPPLEMENTATION; APOPTOSIS

Diabetes is a complex disease with multiple etiologies characterized by hyperglycemia and insufficient insulin secretion or action. Male reproductive function is impaired in diabetes, and there is increasing evidence to indicate that the prostate gland is also negatively affected. Experimental studies demonstrated that diabetes leads to prostatic involution [Cagnon et al., 2000; Ikeda et al., 2000; Ribeiro et al., 2006], a kinetic imbalance of proliferation and cell death in the secretory epithelium [Burke et al., 2006; Fávaro et al., 2008], extracellular matrix remodeling, and functional and phenotypic changes in stromal cells [Ribeiro et al., 2006; Arcolino et al., 2010]. Such studies indicated that short-term histopathological alterations caused in prostate by spontaneously developed or drug-induced diabetes are related to testosterone decrease; however, other factors, such as impaired insulin action and the upregulation of TGF-β and IGF, have also been demonstrated [Ikeda et al., 2000; Ribeiro et al., 2006; Fávaro et al., 2008; Arcolino et al., 2010]. Regardless of the experimental model, diabetes increases apoptotic rates in the prostate [Fávaro

Grant sponsor: National Research Council (CNPq); Grant sponsor: Fellowship for R.M. Góes; Grant number: 302693/2008-4; Grant sponsor: São Paulo State Research Foundation (FAPESP); Grant numbers: 2006/03873-1, 2008/05341-2; Grant sponsor: Fellowships for D.L. Ribeiro; Grant number: 2008/00542-0; Grant sponsor: Samantha Yuri Maeda; Grant number: 2009/05078-2; Grant sponsor: M.G. Gobbo; Grant number: 2009/06885-9.

*Correspondence to: Prof. Dr. Rejane Maira Góes, Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, SP, Brazil. E-mail: remagoes@ibilce.unesp.br

Manuscript Received: 6 July 2011; Manuscript Accepted: 31 January 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 February 2012

DOI 10.1002/jcb.24092 • © 2012 Wiley Periodicals, Inc.

et al., 2008; Arcolino et al., 2010] but an inhibitory [Arcolino et al., 2010] or stimulatory effect on cell proliferation have been reported [Fávaro et al., 2008]. These discrepancies on cell proliferative activity might be explained by differences in experimental models of diabetes, disease states, and analytical methods. However, not even the associated administration of testosterone and insulin was able to normalize the cell proliferation levels in NOD mice [Fávaro et al., 2008]. Thus, more information is necessary to understand the impact of diabetes on the proliferative activity of the prostate.

Increases in hyperglycemia and end-products of advanced non-enzymatic glycation (AGE) are directly associated with the formation of reactive oxygen species (ROS) and the prooxidant state [Baynes et al., 1989]. Studies in other organs have provided compelling evidence that the prooxidant state might lead to apoptosis and carcinogenesis [Denis et al., 2002; Yatoh et al., 2006; Kotrikadze et al., 2008; Thotala et al., 2009]. However, little is known about the contribution of oxidative stress in development of the prostate diseases and the behavior of the antioxidant system under diabetes [Oberley et al., 2000; Parsons et al., 2001; Kotrikadze et al., 2008; Sarafinovska et al., 2009]. In addition, the association between diabetes and prostate cancer in clinical studies is still a matter of debate [González-perez et al., 2005; Turner et al., 2011]. The protective role of some antioxidants, such as carotenoids, vitamin E, and selenium, against malign transformations in the prostate has been proven [Chan et al., 2000, 2005; Matos et al., 2006] but the influence of vitamin C supplementation has not been evaluated. The vitamin C or ascorbic acid is a water-soluble vitamin present in a variety of foods. Several studies report that the vitamin C metabolism is abnormal in diabetes, decreasing its plasma concentration what might contribute to enhanced oxidative stress [Yasser et al., 2005].

The present study evaluated if streptozotocin-induced diabetes at medium-term (1) affects the antioxidant system of rat ventral prostate based on activity of oxidative stress biomarkers; (2) the consequences on cell proliferation and death; and (3) the influence of vitamin C supplementation on these parameters.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The institutional Ethical Committee for Animal Experimentation approved the experiments with animals (UNESP, Protocol no. 006/09-CEEA) in accordance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The adult male Wistar rats (3-month old) were provided by the breeding house of São Paulo State University (Botucatu, SP, Brazil), and they were kept at 24°C on a 12 h light/dark cycle. The animals had free access to food (Labina, Purina) and water for 1 week before the beginning of the experiments. The rats were randomly divided into four groups (n = 15 per group): control (C), control treated with vitamin C (C + V), diabetic (D), and diabetic treated with vitamin C (D + V).

Diabetes was induced after 24 h of fasting by an intra-peritoneal injection of 5 mg/100 g body weight of streptozotocin (Sigma, St. Louis, MO) diluted in citrate buffer 0.01 M, pH 4.5. The control animals were injected only with citrate buffer. The blood glycemia was evaluated in the tail tips using the glucose monitor Accu-chek (Roche Diagnostics, Mannheim, Germany). Only animals showing blood glucose levels above 200 mg/dl were included in the diabetic groups.

Vitamin C (ascorbic acid; Sigma Chemical Co) was administered to the control and diabetic animals by gavage (150 mg/kg body weight/day) diluted in water (2 ml/day/animal), during 30 days, according to Fernandes et al. [2011]. Food intake, water intake, and body weight were monitored during the experiments. The rats were sacrificed using CO_2 inhalation and decapitation 1 month after diabetes diagnosis and treatment.

HORMONE ANALYSIS

The blood was collected in polyethylene tubes containing heparin immediately after the decapitation of rats and centrifuged to separate the plasma. The plasma testosterone and estradiol levels were measured using the Modular Analyzer for Immunoassay of Chemiluminescence ECL (Johnson and Johnson, Langhorne, PA) as described by Weeks and Woodhead [1984]. Ten rats were randomly selected from each group and used to hormone analysis. The tests were performed in triplicate and intra- and inter-assay variations were 4.6% and 4.3%, respectively.

LIGHT MICROSCOPY

After removing and weighing the ventral prostate, the right ventral lobe was isolated and some fragments were fixed by immersion in Karnovsky solution (2.5% freshly prepared formaldehyde, 2.5% glutaraldehyde in Sörensen phosphate buffer pH 7.2) for 12–24 h for paraffin processing and Historesin embedding (Historesin Embedding Kit-Leica). The histological sections were stained with hematoxylin–eosin for general morphological studies. Other fragments were fixed by immersion in 4% formaldehyde freshly prepared in phosphate buffer pH 7.2 and methacarn (1:3:6 of acetic acid, chloroform, and methanol) and used for immunocytochemical analysis. The samples were observed under a bright field microscope (Olympus CH30) coupled with a charge-coupled device camera, and the digitization of selected microscopic fields and the quantitative analyses were performed using an image analysis system (Image-Pro Plus Media Cybernetics, version 6.0 for Windows software, Bethesda, MD).

IMMUNOCYTOCHEMISTRY

Immunocytochemical reactions for the identification of the AGE CML [Nε-(carboxymethyl) lysine] (ab-30917, Abcam), the proliferating cell nuclear antigen (PCNA, sc-56; Santa Cruz Biotechnology, Santa Cruz, CA) and the androgen receptor (AR; sc-816, Santa Cruz Biotechnology) were conducted. The sections were subjected to antigen retrieval in a citrate buffer (pH 6) for 20–40 min in a vegetable steamer and immersed in 3% H_2O_2 in methanol to block endogenous peroxidase. Subsequently, to eliminate unspecific binding, the sections were treated with 3% BSA in PBS for the identification of CML and AR or with Background Sniper (Biocare Medical, Concord, CA) for the PCNA reaction. After washing in PBS, the slides were incubated with primary antibodies diluted in 1% BSA as follows: mouse anti-human CML (1:125, overnight, at 4°C), mouse anti-human PCNA (1:50, 1 h at 37°C), and rabbit anti-human

AR (1:100, overnight at 4°C). The detection of primary antibody was performed using a peroxidase-conjugated polymer (Polymer Novolink, Novocastra, Norwell, MA) following the incubation with diaminobenzidine (0.03% in TBS). The sections were stained with hematoxylin. The ventral prostates from rats 3 months after diabetes onset were used as a positive control for CML. The negative control for all reactions was achieved by omitting the primary antibody.

DETECTION OF APOPTOTIC CELLS

Apoptotic cells were detected in situ using the DNA fragmentation assay associated with cell death based on a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction (TdT-Fragel-Calbiochem, CN Biosciences, La Jolla, CA) following the manufacturer's instructions. The negative controls were obtained by omitting the incubation with TdT enzyme and the slides were stained with hematoxylin.

QUANTIFICATION OF CML POSITIVE AREAS, PCNA, TUNEL, AND AR POSITIVE CELLS

The images of the immunoreactions and the TUNEL method were digitalized using the image analysis system previously described. Five animals per group and three prostate fragments per animal were used for estimation of CML positive areas, PCNA, TUNEL, and AR positive cells. The CML positive areas were estimated using the point counting method of Weibel [1963] with a 220-point reticulum and 20 contiguous microscopic fields per tissue section using the 40× objective. The quantification of PCNA-, AR-, and TUNEL-positive cells in the acinar epithelium of the intermediate region of the ventral prostate was determined using 20 contiguous microscopic fields per each tissue section in 20× objective. The numeric data were expressed as relative frequency (%), that is, number of positive nuclei divided by total number of epithelial cell nuclei.

ACTIVITY OF ANTIOXIDANT ENZYMES

The left ventral lobes were weighed and homogenized with 1:5 vol. of buffer (Tris–HCl 50, 1 mM EDTA, 1 mM DTT, 0.5 M sucrose, 0.15 M KCl, pH 7.4) and centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was centrifuged at 40,000*g* for an additional 60 min at 4°C, and the supernatant fraction was collected and used for the measurement of catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities.

The blood samples were collected in polyethylene tubes containing heparin immediately after sacrificing the animals. For the determination of CAT activity, the blood samples were diluted 50 times in distilled water, while this dilution was diluted 20 times in a hemolyzing solution for the determination of GST and GPx activities.

The total protein content in the samples was determined by the modified Lowry method, using bovine serum albumin as a standard [Peterson, 1977].

The CAT activity was quantified at 240 nm by the H_2O_2 decomposition according to the method of Beutler [1975]. The GST activity was determined by measuring the increase in absorbance at 340 nm after incubating reduced glutathione (GSH) and 1-chloro-2,4-dinithrobenzene (CDNB) as substrates according to Keen et al. [1976]. The GPx activity was assayed by the oxidation of NADPH (associated with GSSG reduction by excess glutathione reductase) at 340 nm as described by Sies et al. [1979]. The SOD activity was measured at 550 nm by the method of Mccord and Fridovich [1969] based on a system that generates superoxide (xanthine/xanthine oxidase) associated with the reduction of cytochrome c by this radical. Thus, the SOD in the samples competes with cytochrome, inhibiting its superoxide reduction. The molar extinction constant (ε) was used to calculate the activity levels of the antioxidant enzymes as U/mg protein ($\varepsilon = 0.071$ for CAT; $\varepsilon = 6.22$ for GST; and $\varepsilon = 9.6$ for GPx).

DETERMINATION OF LIPID PEROXIDATION LEVELS

The malondialdehyde (MDA) level, an indicator of free radical generation that increases at the end of lipid peroxidation, was estimated using the double heating method of Draper and Hadley [1990]. For the MDA quantification in the ventral prostate, 100 μl of the resulting solution from the tissue homogenization in buffer (1:5 vol.) prepared as described in item 7 was used. The quantification of lipid peroxidation in the blood was performed on the serum that was separated by centrifugation. Next, 300 μl of 0.4% thiobarbituric acid solution diluted in 0.2 M HCl was added to the samples and incubated for 60 min at 90°C. Subsequently, the colored derivative of the MDA–TBA complex was extracted with 1 ml of *n*-butanol followed by centrifugation and quantification at 532 nm. The data were expressed as nmol TBARs/g tissue and TBARs/ml plasma.

STATISTICAL ANALYSIS

The data were presented as the mean ± standard deviation. The statistical analyses were performed using ANOVA followed by Tukey test for multiple comparisons using the Statistica 6.0 Software (Copyright©StatSoft Inc., Tulsa, OK). The *P*-values <0.05 were considered statistically significant.

RESULTS

BIOMETRIC AND PHYSIOLOGICAL PARAMETERS

The biometric and physiological data are shown in Table I. The diabetic rats showed a marked decrease in body weight ($P \leq 0.05$), regardless of treatment with vitamin C (C = 444.22 ± 53.74, C + V = 397.8 ± 17.24, D = 264.66 ± 47.1, and D + V = 252.73 ± 42.92 g). The same was observed for the prostate weight (C = 535.11 ± 84.2, C + V = 420.3 ± 137.63, D = 269.57 ± 68.53, and D + V = 295.26 ± 113.11 mg; $P \leq 0.05$); thus, the relative prostate weight did not vary statistically between the groups. The glucose levels were approximately four times higher in the D (483.94 ± 64.53 mg/dl) and D + V (491.75 ± 86.26 mg/dl) groups in comparison with the control groups (C = 114.42 ± 18.41 and C + V = 112.55 ± 22.17 mg/dl; $P \leq 0.05$). The food intake did not differ between the groups, but the water intake was much higher for the diabetic groups (C = 47.42 ± 9.28; C + V = 51.38 ± 20.34, D = 174.93 ± 54.97, and D + V = 125.27 ± 53.33 ml/day/animal; $P \leq 0.05$).

TABLE I. Mean Values and Standard Deviation of Body Weight, Prostate Relative Weight, Blood Glucose Levels, Ratio, Water Intake, and Median Values and Standard Deviation for Serum Testosterone and Estradiol Levels of the Control Group (C), Control Treated With Vitamin C (C + V), Non-Treated Diabetic (D), and Diabetic Treated With Vitamin C (D + V)

	C	C + V	D	D + V
Body weight (g)	444.22 ± 53.74^{a}	397.8 ± 17.24^{a}	264.66 ± 47.1^{b}	252.73 ± 42.92^{b}
Prostate weight (mg)	$535.11 \pm 84{,}2^{a}$	420.3 ± 137.63^{a}	269.57 ± 68.53^{b}	295.26 ± 113.11^{b}
Prostate relative weight ($\times 10^{3}$)	1.23 ± 0.30^{a}	1.055 ± 0.34^{a}	1.0 ± 0.18^{a}	1.17 ± 0.48^{a}
Blood glucose (mg/dl)	114.42 ± 18.41^{a}	112.55 ± 22.17^{a}	483.94 ± 64.53^{b}	491.75 ± 86.26^{b}
Food intake (g/day/animal)	26.49 ± 8.97^{a}	31.24 ± 12.34^{a}	35.46 ± 11.32^{a}	29.06 ± 13.27^{a}
Water intake (ml/day/animal)	47.42 ± 9.28^{a}	51.38 ± 20.34^{a}	174.93 ± 54.97^{b}	125.27 ± 53.33^{c}
Serum testosterone (ng/dl)	125 ± 44.2^{a}	128.4 ± 45.4^{a}	12.6 ± 5.6^{b}	3.4 ± 1.2^{b}
Serum estradiol (mg/dl)	25.1 ± 8.87^{a}	26.1 ± 9.21^{a}	21.8 ± 7.26^{a}	22.9 ± 6.61^{a}

The mean differences between the values bearing different superscript letters are statistically significant (≤ 0.05).

HISTOLOGY AND IMMNUNOCYTOCHEMISTRY

The tissue alterations in the ventral prostate caused by experimental diabetes with or without the vitamin C treatment are summarized in Figure 1. After 1 month of streptozotocin-induced diabetes, the prostate atrophy was evident because most of the acini exhibited reduced lumen, acinar epithelium with cubic cells, and signs of epithelial shrinkage (Fig. 1E–G). The treatment with vitamin C did not affect the histology of the prostate, control, or diabetic groups (Fig. 1A,B,H,I,J).

The immunocytochemical reaction for CML, the most abundant AGE in vivo, showed strong labeling in the ventral prostate of the D group compared to the C and C + V groups (Fig. 2A–D). Despite the relative frequencies of CML-positive areas doubled in diabetes, these were not statistically different among the experimental groups in this study (Fig. 2F). In diabetic groups this AGE was predominantly accumulated in the acinar epithelium, particularly in the nuclei of the epithelial cells (Fig. 2C,D). The positive control for this immunoreaction was provided by the prostate from rats with 3 months of diabetes without insulin therapy in which CML was more abundant and diffuse (Fig. 2E). In this case, the CML deposition was also observed in the apical surface of acinar epithelium and in the vascular endothelium.

OXIDATIVE STRESS MARKERS

Figure 3 shows the changes in the biomarkers of oxidative stress in tissue samples and blood. No alterations were observed in the blood, except for CAT activity in the D + V group which was higher than D and C + V groups ($C = 9.55 \pm 1.96$, $C + V = 6.93 \pm 1.59$, $D = 8.06 \pm 1.86$, and $D + V = 12.32 \pm 3.98$ U/mg protein; $P \leq 0.05$). The CAT activity was slightly increased in the prostate tissue of diabetic rats ($C = 1.38 \pm 0.56$, $C + V = 0.68 \pm 0.36$, $D = 1.91 \pm 0.63$; $D + V = 2.42 \pm 0.97$ U/mg protein; $P \leq 0.05$), independently of vitamin C administration (Fig. 3A). Treating diabetic rats with vitamin C ameliorated the levels of GST activity in the prostate ($C = 0.33 \pm 0.99$, $C + V = 0.31 \pm 0.08$, and $D + V = 0.45 \pm 0.18$ U/mg protein; $P \leq 0.05$), which were elevated by diabetes ($D = 0.63 \pm 0.10$ U/mg protein). The prostatic levels of GPx and SOD activities (Fig. 3E,G) were not discrepant between groups, which were not influenced by diabetes and treatment with vitamin C. The lipid peroxidation, evaluated by MDA levels, showed a discrete increase in the prostate of diabetic rats, although it was not significant (Fig. 3H).

CELL PROLIFERATION, APOPTOSIS, AND AR SENSITIVITY

There was a reduction ($P \leq 0.05$) in the relative frequency of AR-positive cells in the D and D + V groups (Fig. 4). As expected, diabetes reduced markedly the circulating testosterone which were 10% in D group ($D = 12.6 \pm 5.6$ ng/dl) and a 2.7% in D + V group ($D + V = 3.4 \pm 1.2$ ng/dl) from levels found in C group ($C = 125 \pm 44.2$ ng/dl; $P \leq 0.05$), as shown in Table I and Figure 5A. The plasma estradiol levels did not vary greatly between the groups (Table I, Fig. 5B).

The TUNEL assay and quantitative analyses (Fig. 6A–D) showed that apoptotic cells were more abundant in diabetic rats ($P \leq 0.05$), where they were usually found in detached epithelial fragments in the acinar lumen (Fig. 6A,C,I). The treatment of the diabetic rats with vitamin C restored partially apoptosis to levels similar to those of the control rats (Fig. 6A,D,I). The immunocytochemistry for PCNA indicated a marked reduction in cell proliferation levels ($P \leq 0.05$) in the acinar epithelium of the ventral prostate after 1 month of diabetes (Fig. 6E–H,J). Furthermore, this reduction was not influenced by vitamin C supplementation (Fig. 6J).

DISCUSSION

The various effects from diabetes-induced oxidative stress are well known in several organs [Denis et al., 2002; Armagan et al., 2006; Yatoh et al., 2006; Shrilatha and Muralidhara, 2007ab; Thotala et al., 2009], but it has not been examined specifically in the prostate. The major finding of this study is that diabetes at medium-term induced apoptosis and increased GST activity in rat prostate tissue and these effects were attenuated by vitamin C supplementation. Diabetes stimulates apoptosis in several organs [Denis et al., 2002; Thotala et al., 2009], including the prostate [Fávaro et al., 2008; Arcolino et al., 2010]. Fávaro et al. [2008] detected higher apoptotic rates in comparison with those observed here in NOD mice after 20 days of diabetes, which exhibited high levels of glycemia (~910 mg/dl). Furthermore, not even treatment with insulin, testosterone, and estrogen was able to restore glycemia or apoptotic rates in spontaneously developed diabetes [Fávaro et al., 2008]. Previous data from our laboratory comparing apoptosis in the rat ventral prostate after 1 week and 3 months of experimental diabetes revealed that apoptotic rates increase with disease progression [Arcolino et al., 2010]. Based on these data we raised the hypothesis that such alterations might be due to the progressive complications

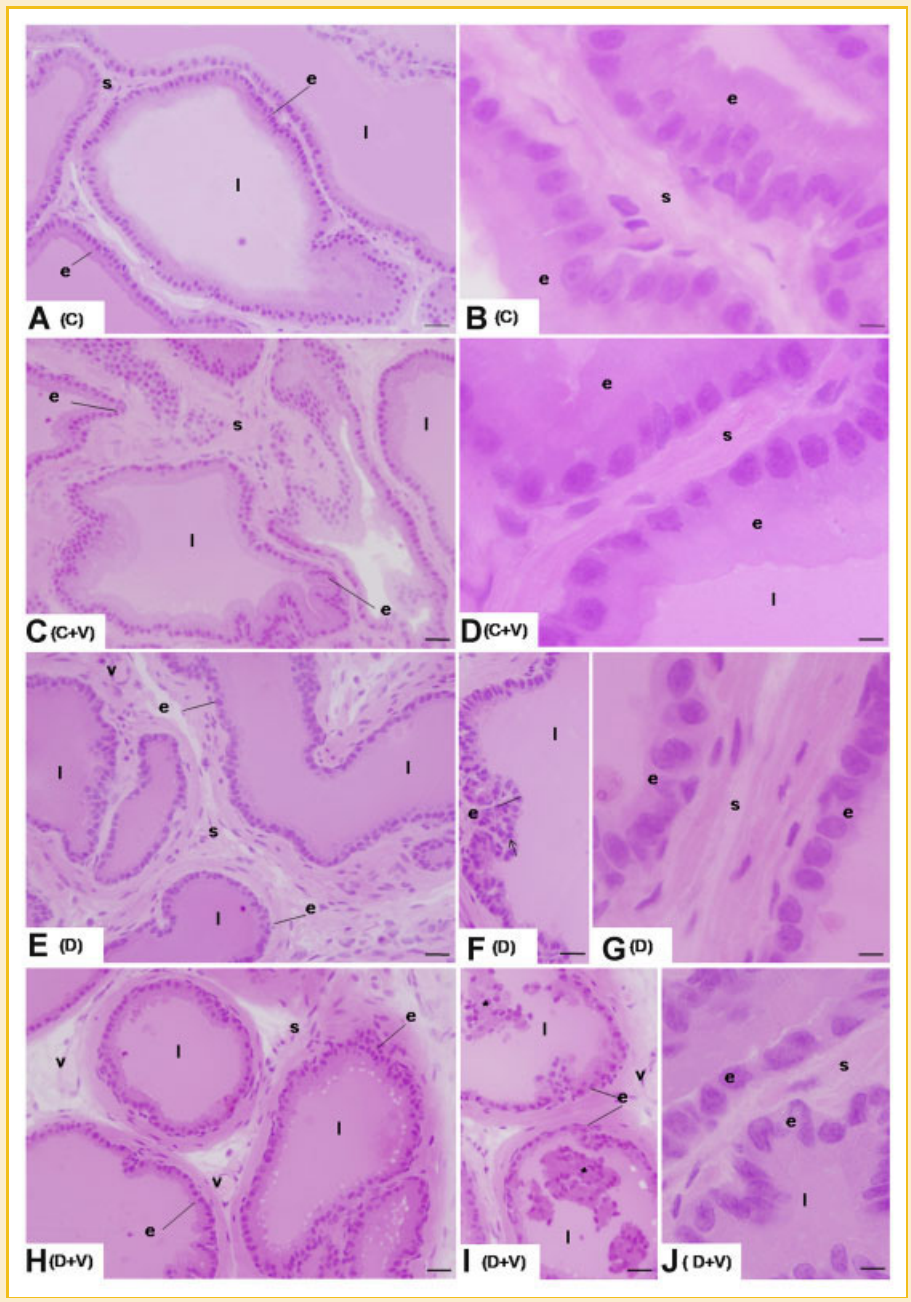

Fig. 1. Histological sections in the historesin of rat ventral prostate stained with H&E. A,B: Control group (C); C,D: control group treated with vitamin C (C + V); E–G: untreated diabetic group (D); and H–J: diabetic group treated with vitamin C (D + V). Signs of acinus shrinkage (F) and epithelial fragments in the lumen were found in the diabetic animals (I). e, epithelium; l, lumen; s, stroma; v, blood vessel; arrowhead, epithelial shrinkage; *, epithelium fragments. A,C,E,F,H,I: 200×, bars = 50 μm; B,D,G,J: 1000×, bars = 10 μm.

of this disease, such as increase of AGEs, and in the oxidant condition. In the present study immunocytochemistry indicated a higher intensity of CML immunolabeling in localized points of the diabetic prostate which widespread in the gland in higher periods of diabetes. In addition, biochemical data not evidenced a marked induction of oxidative stress, except for GST and a trend of increase in MDA. It is important to note that although these data do not indicate significant changes in markers of oxidative stress, the influence of these factors in the imbalance of apoptosis in the prostate cannot be ruled out because it is very possible that at medium-term the changes have affected individual or groups of cells and could not be detected in biochemical analysis of crude prostate samples. Studies using double staining for markers of oxidative stress and apoptosis in the gland are being done to clarify this point. Moreover, previous studies have demonstrated that the binding of AGE, such as CML, to receptors for advanced glycation

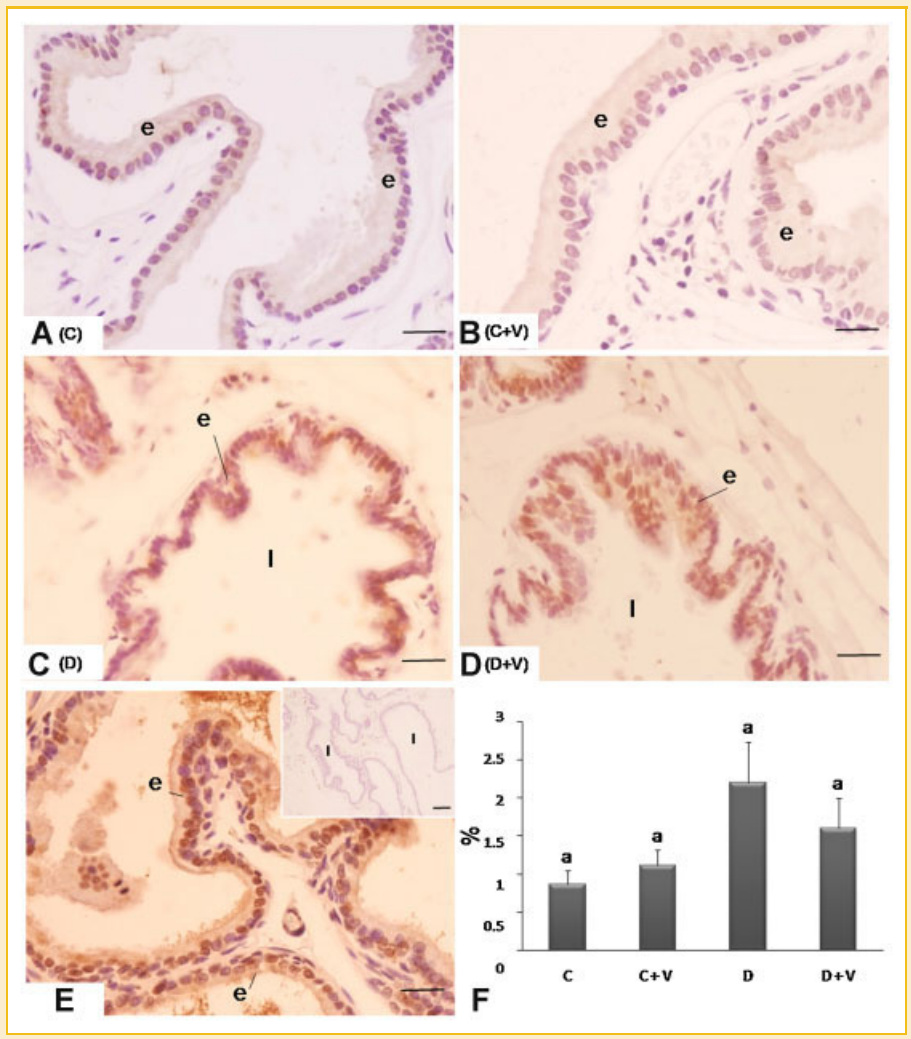

Fig. 2. Immunohistochemical localization (A–E) of Nε-carboxymethyl lysine (CML) and relative frequency (F) of the CML immunostaining areas in tissue sections of ventral prostate of (A) control (C); (B) control treated with vitamin C (C + V); (C) untreated diabetic (D); (D) diabetic treated with vitamin C (D + V) rats. (E) shows the positive control of CML staining of rat ventral prostate after 3 months of untreated diabetes. Inset shows negative control. 400×, bars = 25 μm. (F) The values of mean relative CML-positive areas (%) were higher in diabetic in comparison with control prostates but not statistically different.

end-products (RAGE) initiates a cascade of signal transduction and leads to the generation of ROS, the activation of Bax, and the expression of pro-inflammatory and pro-apoptotic genes, such as the c-Jun N-terminal kinase [Du et al., 1998; Wautier et al., 2001; Buccellato et al., 2004].

Biochemical analyses indicated that 1 month of diabetes was not sufficient to alter the biomarkers of oxidative stress in the blood of rats, but a significant increase was noted in GST and a marginal incremental increase in lipid peroxidation in the prostate. The increase in GST activities may indicate a rise in the antioxidant defense in the gland during diabetes, which might represent an attempt to reverse the oxidative stress levels related to an initial phase of AOS disturbance in the rat ventral prostate. It should be noted that studies of long duration with experimental diabetes are difficult to execute because of the low survival rates of animals without proper insulin therapy, which, in turn, normalizes blood glucose levels, and, as a consequence, reduces the injuries caused by oxidative stress. Literature data about the AOS in other genital organs, such as the testis and epididymis of rodents with similar conditions of diabetes (4 weeks), are very discrepant; a decrease of the main enzymatic markers (CAT, GPx, and SOD) was observed in some cases [Shrilatha and Muralidhara, 2007a], and an increase was reported in other studies [Armagan et al., 2006; Shrilatha and Muralidhara, 2007b]. Comparing our prostate data with that of above-mentioned studies, we conclude that there is no pattern of response among the main biomarkers of AOS in the genital organs under experimental diabetes, and such a response is probably tissue-specific. In spite of these discrepancies, there seems to be a consensus in the literature for the GST behavior, whose activity increases in the testis, epididymis [Shrilatha and Muralidhara, 2007ab], and prostate after 1 month of diabetes. The GSTs are an important class of enzymes with a crucial role in the detoxification of intracellular reactive electrophiles and products of oxidative stress by catalyzing the conjugation of these compounds with

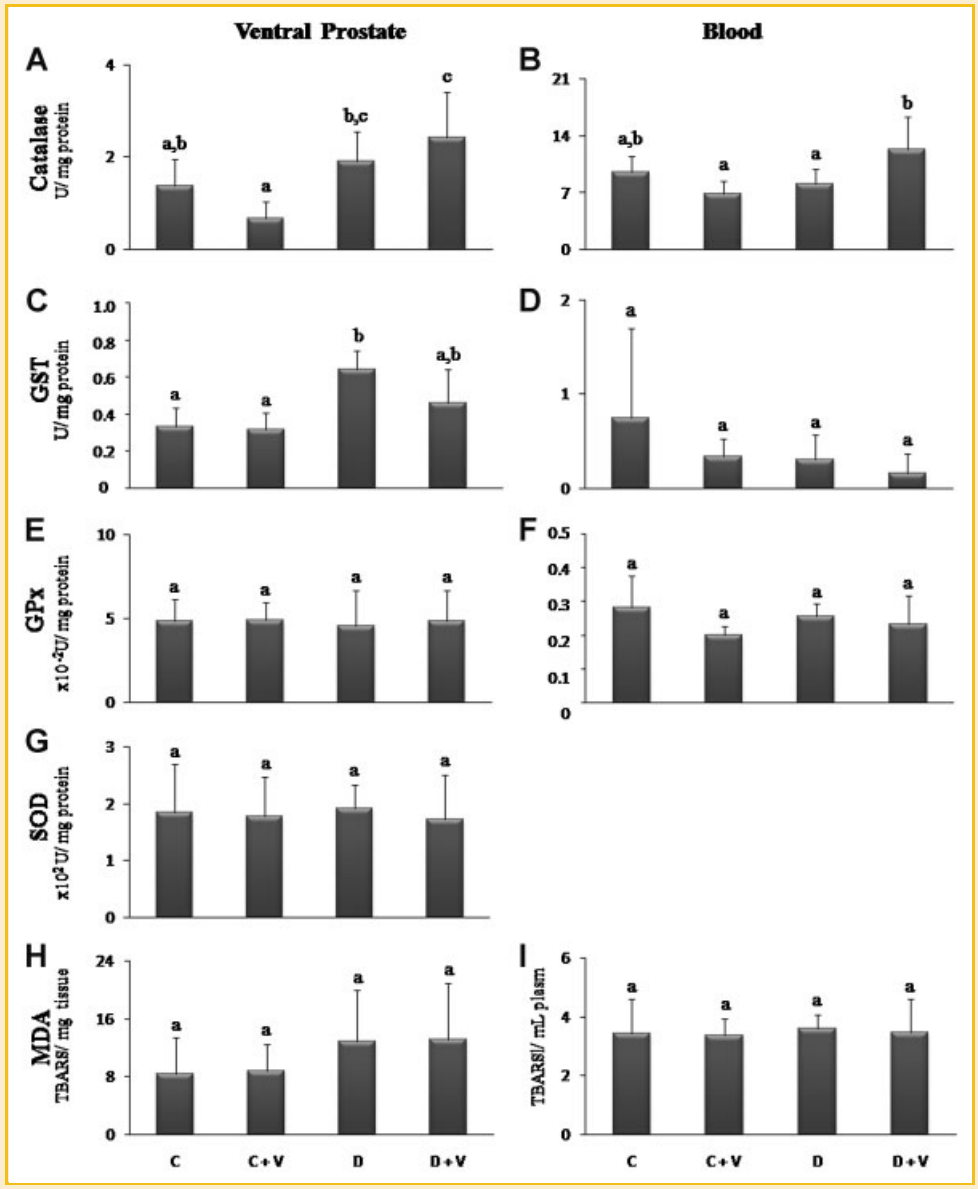

Fig. 3. Levels of catalase (A,B), GST (C,D), GPx (E,F), and SOD (G) activities (U/mg protein) and MDA (H,I) (TBARS/mg tissue and TBARS/ml plasma) in ventral prostate extracts and blood samples of control group (C), control treated with vitamin C (C + V), untreated diabetic (D), and diabetic treated with vitamin C (D + V) rats. The values are presented as the mean ± standard deviation. The mean differences between the values bearing different superscript letters are statistically significant ($\leq$0.05). Seven animals per group were used for each biomarker of oxidative stress.

glutathione [Hayes and Pulford, 1995]. Several reports have indicated that GST acts as a protector against neoplasic transformation [Hayes and Pulford, 1995; Parsons et al., 2001; De Marzo et al., 2007]. The relationship between inflammation and prostate cancer was examined in several studies [De Marzo et al., 1999, 2007; Putzi and De Marzo, 2000; Van Leenders et al., 2003]. Contrary to PIN and in neoplasic lesions, GST expression (GSTP1 and GSTA1) is high in PIA, suggesting that these lesions are subject to increased oxidative stress, which has a relevant role in malignance [Parsons et al., 2001; Nakayama et al., 2003; De Marzo et al., 2007; Karaivanov et al., 2007]. It is necessary to mention that the investigations attempting to elucidate the cause–effect connection between prostate cancer and oxidative damage are based on the biochemical analysis of biomarkers in the blood and not in the gland itself [Kotrikadze et al., 2008; Sarafinovska et al., 2009]. In this context, our experimental data with the rodent allowed us to examine the behavior of antioxidant enzymes in the gland during the development of diabetes sequelae. Correlating the present histological and biochemical data with previous results from our laboratory [Ribeiro et al., 2008] in which a alloxan-induced diabetes resulted in an incidence ~64% of prostate atrophy and ~35% of neoplasic lesions, we suggest that during the progression of diabetes,

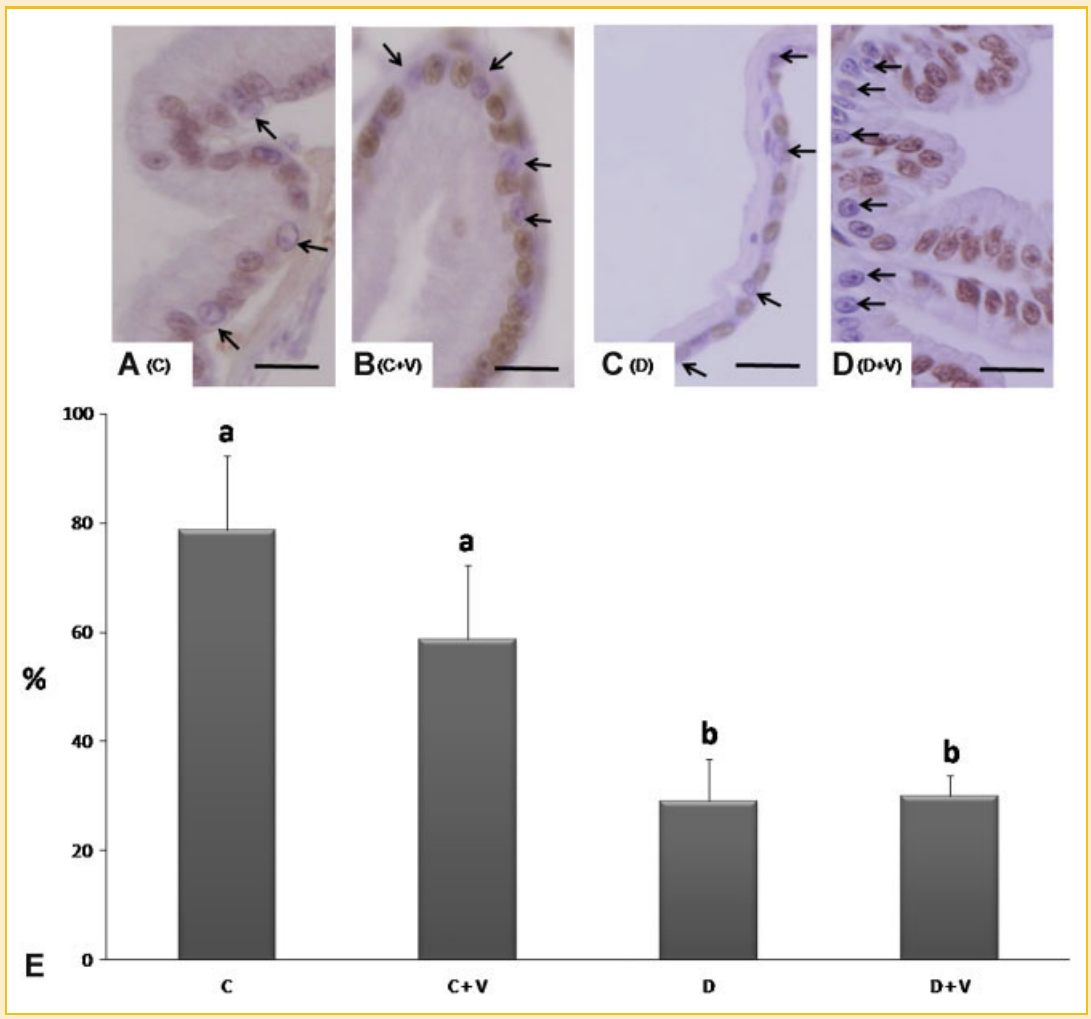

Fig. 4. Immunocytochemistry (A–D) for androgen receptor (AR) and quantification of AR-positive cells (E) in the acinar epithelium of rat ventral prostate of: (A) control (C); (B) control treated with vitamin C (C + V); (C) untreated diabetic (D); (D) diabetic treated with vitamin C (D + V) groups. A–D: The arrows indicate the AR-negative cells (blue nuclei). 400×, bars = 25 μm. E: The values represent the mean relative frequency (%) of AR-positive cells in total cells of epithelium for above-mentioned groups. The mean differences between the values bearing different superscript letters are statistically significant (≤0.05).

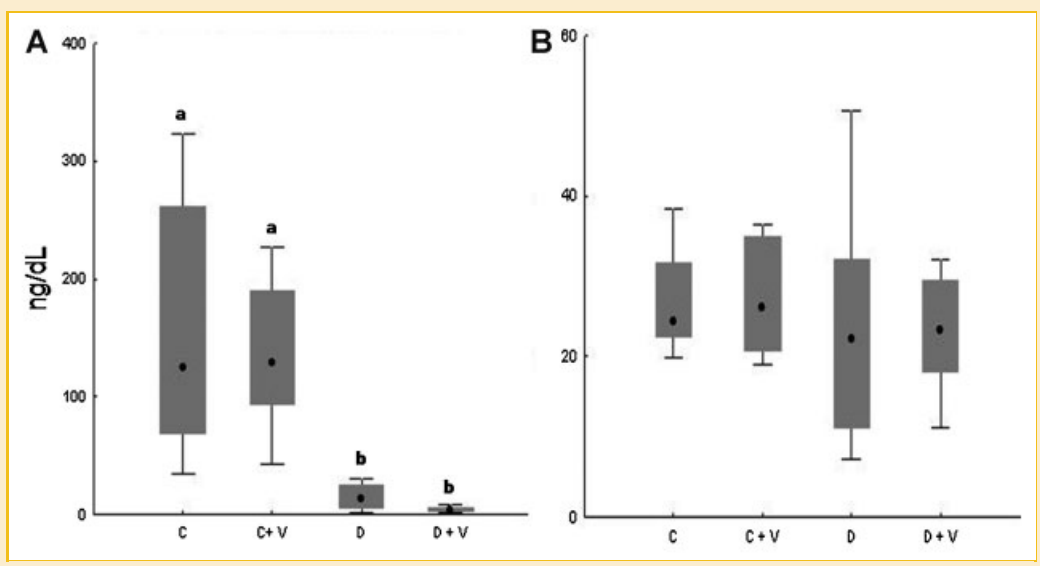

Fig. 5. Variations in testosterone (A) and estradiol (B) serum levels (ng/dl). Control (C), control treated with vitamin C (C + V), untreated diabetic (D), and diabetic treated with vitamin C (D + V). The mean differences between the values bearing different superscript letters are statistically significant (≤0.05).

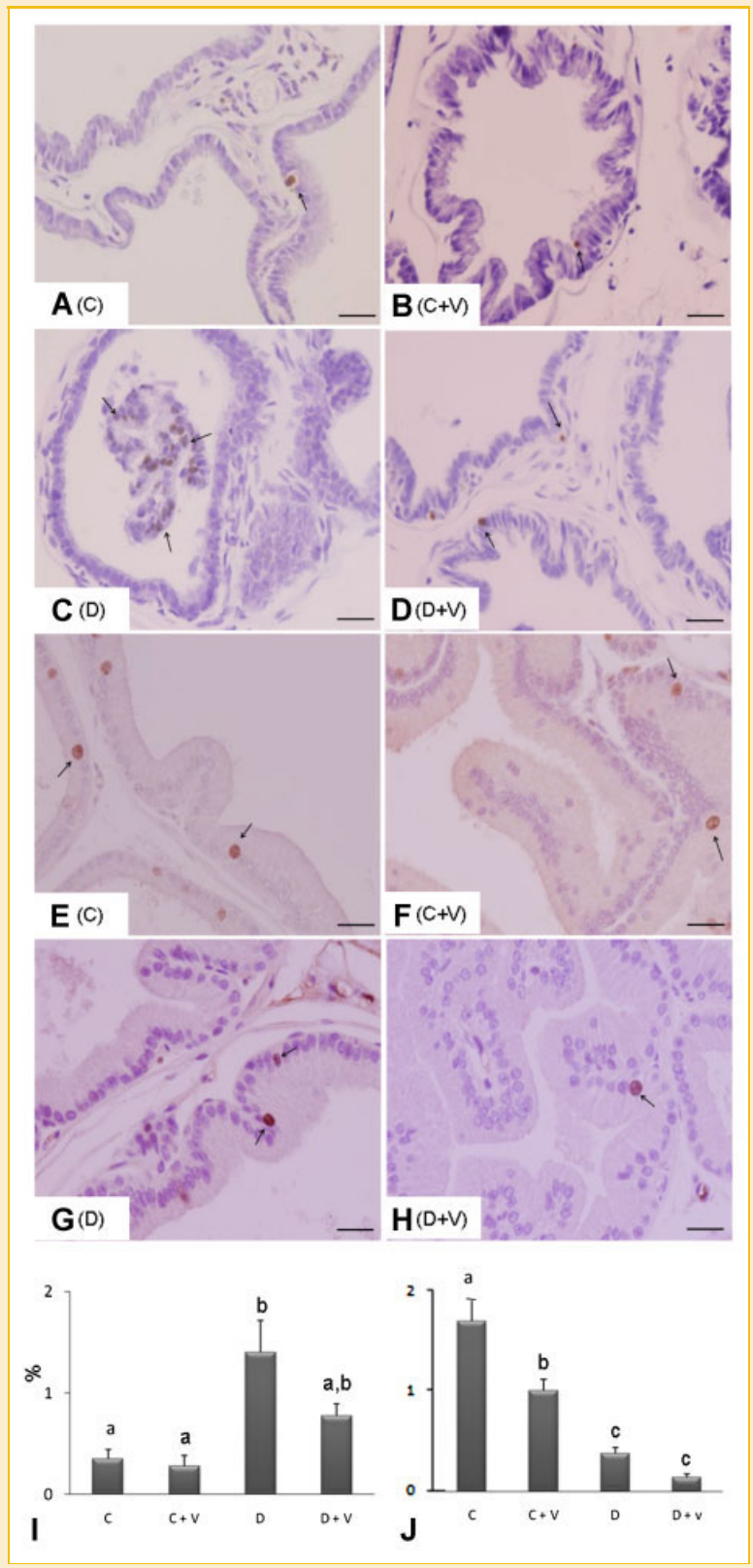

Fig. 6. Identification (arrows) of apoptotic (A–D) and proliferating (E–H) cells and variations in their relative frequencies (I,J) in the acinar epithelium of rat ventral prostate following TUNEL method (A–D,I) and immunocytochemistry for PCNA (E–H,J) in control (C); control treated with vitamin C (C + V); untreated diabetic (D); and diabetic treated with vitamin C (D + V) groups. 400×, bars = 25 μm. The values in I and J are the mean relative frequency (%) of apoptotic and proliferating cells in total cells of epithelium and statistically significant differences (≤0.05) are indicated by different superscript letters.

the increase in GST activity in the prostate precedes the PIA and may influence the development of these lesions and other pathological alterations. Such suggestion is reinforced by previous data from the literature showing an ongoing oxidative insult may result in the upregulation of GST in PIA [De Marzo et al., 1999; Parsons et al., 2001] and downregulation of GST and other oxidative stress markers during malignant progression [Oberley et al., 2000]. Our data also indicate that the GST family, in addition to being a valuable marker for pathological lesions in the prostate, is an important component of protective mechanisms against prostate oxidative damage in diabetes.

Several lines of evidence denote the protective effects of supplementation with some antioxidants against prostate cancer, but their actions on AOS in other disorders, like diabetes, have not been carefully examined. Vitamin C has an advantage of having a low inherent toxicity in humans and rodents even at high doses [Cadenas et al., 1997; Gupta et al., 2004; Yasser et al., 2005]. Our research demonstrated that vitamin C treatment in control rats did not interfere with the activity of antioxidant enzymes and lipid peroxidation levels in the prostate and blood. However, it partially restored apoptotic rates and GST activity in the prostate of the diabetic group. Therefore, it is possible that vitamin C had a protective effect, not requiring an increase in GST activity and leading to protection against prostatic lesions observed in diabetes.

As expected, the atrophic alterations observed in the prostate after 1 month of STZ-induced diabetes were intermediate between those described for shorter and longer times, with the acinar cells exhibiting a cubical shape and a transitional phenotype between the normal and the high atrophic aspect detected after 3 months of diabetes [Ribeiro et al., 2008]. Our findings on the relative weight of the prostate were discrepant from other studies [Scarano et al., 2006; Bal et al., 2011; Fernandes et al., 2011].

Since we found no significant differences between experimental groups and similar to those of Porto et al. [2011]. It should be noted that the mean prostate relative weight of the diabetic group decreased approximately 20% compared with the control and recovered after treatment with vitamin C (Table I). It is likely that this reduction was not statistically significant due to the variation of this value in the control group and marked body weight loss observed here for the diabetic animals, which certainly reflects their high glycemia. Similarly to the short-term studies, our data showed a drastic androgen reduction in the D and D + V groups which paralleled the reduction of androgen-responsive and proliferating cell levels, indicating testosterone drift is the main factor responsible for gland atrophy and the disturbance of proliferation and cell death. They are also consistent with previous studies, which demonstrate that prostate involution after experimental diabetes requires both an increase in apoptosis and a reduction in cell proliferation [Baynes et al., 1989; Ikeda et al., 2000]. Additionally, we found that the negative effect on the proliferation activity of prostate acinar epithelium is not an immediate response to experimental diabetes but persists at medium-term. Our data indicated that antioxidant treatment reduced the cell proliferation both in control as in diabetic groups. These findings together with low impact of medium-term diabetes in AOS, difficult the discrimination of mechanism affecting cell proliferation in diabetes.

In conclusion, the present study indicates the unbalanced cell proliferation and death in the rat prostate at medium-term streptozotocin-induced diabetes. No marked induction of oxidative stress was observed in the prostate at this period of diabetes but the incremental increase in GST activity suggests that it is also an important component in the defense against oxidative damage in the prostate under diabetes. Additionally, vitamin C supplementation of diabetic rats improved the antioxidant defense concerning GST and recovered the apoptotic rates in the prostate.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Mr. Luiz Roberto Faleiros Jr. and Ms. S.Y. Maeda.

REFERENCES

Arcolino FO, Ribeiro DL, Gobbo MG, Taboga SR, Góes RM. 2010. Aloxan-induced diabetes increases apoptosis and frequency of p63 positive cells in prostate acinar epithelium. Int J Exp Pathol 91:144–154.

Armagan A, Uz E, Yilmaz HR, Soyupek S, Oksay T, Ozcelik N. 2006. Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic rat testis. Asian J Androl 8(5):595–600.

Bal R, Türk G, Tuzcu M, Yilmaz O, Ozercan I, Kuloglu T, Gür S, Vitor SN, Tykhomyrov GV, Andrievsky GV, Baydas G, Naziroglu M. 2011. Protective effects of nanostructures of hydrated C60 fullerene on reproductive function in streptozotocin-diabetic male rats. Toxicology 282:60–81.

Baynes W, Watkins NG, Fischer CI, Hull CJ, Patrick JS, Ahmed MU, Dunn JA, Thorpe SR. 1989. The Amadori product on protein: Structure and reactions. Prog Clin Biol Res 304:43–67.

Beutler E. 1975. Red cell metabolism: A manual of biochemical methods. New York: Grune and Straton.

Buccellato LJ, Tso M, Akinci OI, Chandel NS, Budinger GR. 2004. Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. J Biol Chem 279(8):6753–6760.

Burke JP, Jacobson DJ, Mcgree M, Roberts RA, Girman C, Lieber MM, Jacobsern SJ. 2006. Diabetes and benign prostatic hyperplasia progression in Olmsted Country, Minnesota. Adult Urol 67:22–25.

Cadenas S, Barja G, Poulsen HE, Loft S. 1997. Oxidative DNA damage estimated by oxo8dG in the liver of guinea-pigs supplemented with graded dietary doses of ascorbic acid and α-tocopherol. Carcinogenesis 18:2373–2377.

Cagnon VHA, Camargo AM, Rosa RM, Fabiani CR, Padovani CR, Martinez FE. 2000. Ultrastructural study of the ventral lobe of the prostate of mice with streptozotocin induced diabetes (C57bl/6j). Tissue Cell 32:275–283.

Chan FL, Choi HL, Chen ZY, Chan PS, Huang Y. 2000. Induction of apoptosis in prostate cancer cell lines by a flavonoid, baicalin. Cancer Lett 160(2):219–228.

Chan JM, Elkin EP, Silva SJ, Broering JM, Latini DM, Carroll PR. 2005. Total and specific complementary and alternative medicine use in a large cohort of men with prostate cancer. Urology 66(6):1223–1228.

De Marzo AM, Marchi VL, Epstein JI, Nelson WG. 1999. Proliferative inflammatory atrophy of the prostate: Implications for prostatic carcinogenesis. Am J Pathol 155(6):1985–1992.

De Marzo AM, Nakai Y, Nelson WG. 2007. Inflammation, atrophy, and prostate carcinogenesis. Urol Oncol 25(5):398–400.

Denis U, Lecomte M, Paget C, Ruggiero D, Wiemsperger N, Lagarde M. 2002. Advanced glycation end-products induce apoptosis of bovine retinal pericytes in culture: Involvement of diacylglycerol/ceramide production and oxidative stress induction. Free Radical Biol Med 33:236–247.

Draper HH, Hadley M. 1990. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 186:421–431.

Du X, Stotcklauser-farber K, Rosen P. 1998. Generation of reactive oxygen intermediates, activation of NF-kappaB, and induction of apoptosis in human endothelial cells by glucose: Role of nitric oxide synthase? Free Radic Biol Med 27:752–763.

Fávaro WT, Padovani CR, Cagnon VHA. 2008. Ultrastructural and proliferative features of the ventral lobe of the prostate in non-obese diabetic mice (NOD) following androgen and estrogen replacement associated to insulin therapy. Tissue Cell 2:119–132.

Fernandes GS, Fernandez CDB, Campos KEC, Damascenos DC, Anselmo-Francis JA, Assumpção TA, Campos KE, Damasceno DC, Pereira OC, Kempinas WG. 2011. Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. Reprod Biol Endocrinol 9:1–9.

González-perez A, García Rodríguez LA. 2005. Prostate cancer risk among men with diabetes mellitus. Cancer Cause Control 16:1055–1058.

Gupta R, Dhakal B, Thakur A, Ahnn J. 2004. Vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. Mol Cells 17:132–139.

Hayes JD, Pulford DJ. 1995. The glutathione *S*-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 30:445–600.

Ikeda K, Wada Y, Foster He JR, Wang Z, Weiss RM, Latifpour J. 2000. Experimental diabetes-induced regression of the rat prostate is associated with an increased expression of transforming growth factor-β. J Urol 164: 180–185.

Karaivanov M, Todorova K, Kuzmanov A, Hayrabedyan S. 2007. Quantitative immunohistochemical detection of the molecular expression patterns in proliferative inflammatory atrophy. J Mol Histol 38(1):1–11.

Keen JH, Habig WH, Jakoby WB. 1976. Mechanism for several activities of the glutathione *S*-transferases. J Biol Chem 251:6183–6188.

Kotrikadze N, Alibegashvili M, Zibzibadze M, Abashidze N, Chigogidze T, Managadze L, Artsivadze K. 2008. Activity and content of antioxidant enzymes in prostate tumors. Exp Oncol 30(3):244–247.

Matos HR, Marques SA, Gomes OF, Silva AA, Heimann JC, Di mascio P, Medeiros MH. 2006. Lycopene and beta-carotene protect in vivo iron-induced oxidative stress damage in rat prostate. Braz J Med Biol Res 39(2):203–210.

Mccord JM, Fridovich I. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244(22):6049–6055.

Nakayama M, Bennett CJ, Hicks JL, Epstein JI, Platz EA, Nelson WG, De Marzo AM. 2003. Hypermethylation of the human GSTP1 CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: A detailed study using laser-capture microdissection. Am J Pathol 163:923–933.

Oberley TD, Zhong W, Szwedal LI, Oberley LW. 2000. Localization of antioxidant enzymes and oxidative damage products in normal and malignant prostate epithelium. Prostate 44:144–155.

Parsons JK, Nelson CP, Gage WR, Nelson WG, Kensler TW, De Marzo AM. 2001. GSTA1 expression in normal, preneoplastic, and neoplastic human prostate tissue. Prostate 49:30–37.

Peterson GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Porto EM, Santos SAA, Ribeiro LM, Lacorte LM, Rinaldi JC, Justulin LA, Jr., Felisbino SL. 2011. Lobe variation of experimental diabetes and insulin replacement on rat prostate. Microsc Res Tech 74(11):1040–1048.

Putzi MJ, De Marzo AM. 2000. Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia. Urology 56:828–832.

Ribeiro DL, Candido EM, Manzato AJ, Taboga SR, Cagnon VHA. 2006. Prostatic stromal microenvironment and experimental diabetes. Eur J Histochem 5:51–60.

Ribeiro DL, Marques SF, Alberti S, Spadella CT, Manzato AJ, Taboga SR, Dizeyi N, Abrahamsson PA, Góes RM. 2008. Malignant lesions in the ventral prostate of alloxan-induced diabetic rats. Int J Exp Pathol 89(4):276–283.

Sarafinovska ZA, Eken A, Matevska N, Erdem O, Sayal A, Savaser A, et al. 2009. Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer. Clin Biochem 42:1228–1235.

Scarano WR, Messias AG, Oliva SU, Klinefelter GR, Kempinas WG. 2006. Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. Int J Androl 29(4):482–488.

Shrilatha B, Muralidhara. 2007a. Occurrence of oxidative impairments, response of antioxidant defences and associated biochemical perturbations in male reproductive milieu in the streptozotocin-diabetic rat. Int J Androl 30:508–518.

Shrilatha B, Muralidhara. 2007b. Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. Reprod Toxicol 23:578–587.

Sies H, Koch OR, Martino E, Boveris A. 1979. Increased biliary glutathione disulfide release in chronically ethanol treated rats. FEBS Lett 103:287–290.

Thotala D, Chetyrkin S, Hudson B, Hallahan D, Voziyan P, Yazlovitskaya E. 2009. Pyridoxamine protects intestinal epithelium from ionizing radiation induced apoptosis. Free Radic Biol Med 47(6):779–785.

Turner EL, Lane JA, Donovan JL, Davis MJ, Metcalfe C, Neal DE, Hamdy FC, Martin RM. 2011. Association of diabetes mellitus with prostate cancer: Nested case–control study (prostate testing for cancer and treatment study). Int J Cancer 128(2):440–446.

Van Leenders GJ, Gage WR, Hicks JL. 2003. Intermediate cells in human prostate epithelium are enriched in proliferative inflammatory atrophy. Am J Pathol 162:1529–1537.

Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. 2001. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab 280:685–694.

Weeks I, Woodhead JS. 1984. Chemiluminescence immunoassays. J Clin Immunoassays 7:82–89.

Weibel ER. 1963. Principles and methods for the morphometric study of the lung and other organs. Lab Invest 12:131–155.

Yasser HA, Abdel-Wahab Finbarr PM, O'Harte Mark H, Mooney Christopher R, Barnett Peter R, Flatt PR. 2005. Vitamin C supplementation decreases insulin glycation and improves glucose homeostasis in obese hyperglycemic (ob/ob) mice. Metabolism 51(4):514–517.

Yatoh S, Mizutani M, Yokoo T, Kozawa T, Sone H, Toyoshima H, et al. 2006. Antioxidants and an inhibitor of advanced glycation ameliorate death of retinal microvascular cells in diabetic retinopathy. Diab Metab Res Rev 22(1):38–45.