

Proliferation and apoptotic rates and increased frequency of p63-positive cells in the prostate acinar epithelium of alloxan-induced diabetic rats

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Summary

The effects of experimental type 1 diabetes were investigated in the acinar epithelium of rat ventral prostate, focusing on the rates of cell proliferation and the frequency of apoptosis and p63-positive cells. Type 1 diabetes was induced in adult male Wistar rats by a single alloxan administration (42 mg/kg b.w.) and its effects were analysed for 1 week and 3 months after the establishment of the disease. A group of diabetic rats was treated daily with 5 IU of insulin during 1 week after diabetes had been diagnosed. Immunocytochemical methods for the localization of cell proliferation antigen (PCNA), androgen receptor (AR) and p63 protein were carried out, and apoptotic cells were identified by TUNEL essay. In diabetic rats, testosterone levels reduced drastically after 1 week and in a lower degree after 3 months. In short-term diabetic rats, cell proliferation decreased, and in medium-term, epithelial apoptotic rates increased. In both periods after the onset of diabetes, the frequency of p63-positive cells doubled. Insulin treatment was effective in preventing testosterone decrease, p63-positive cell increase and apoptotic rates, but did not interfere in cell proliferation. This investigation shows that, soon after diabetes onset there are important modifications in cell proliferation within the prostatic epithelium, and in longer term there is a marked impact on kinetics of differentiation and cell death. These changes may initially be attributable to an androgenic fall, but is also probably because of other factors related to diabetes, since such data are considerably different from those resulting from castration.

Keywords

androgen receptor, apoptosis, cell proliferation, diabetes, p63, prostate

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The prostate is the organ most affected by malignant lesions in men, and prostate cancer is the second most commonly diagnosed cause of death among men in many countries

(Andreoni *et al.* 2001; Jemal *et al.* 2008; Ellison & Wilkins 2009). Prostate cancer arises from progressive transformation of acinar epithelium into prostate intra-epithelial neo-

plasias (PINs), justifying investigations into epithelial behaviour (De Marzo *et al.* 2007). The homeostatic maintenance of the prostatic epithelium requires a dynamic equilibrium between cell proliferation, differentiation and death (Isaacs 1987). Disturbances in some of these processes have a negative impact on epithelial kinetics and may be directly involved in the occurrence of intra-epithelial neoplasia and the subsequent development of malignancy (Cunha *et al.* 2004; De Marzo *et al.* 2007).

At least six cell types with distinct biological and phenotypic characteristics are found in the prostate secretory epithelium: stem cells, basal cells, transit-amplifying cells (TACs), intermediate cells, luminal or secretory cells and neuroendocrine cells (Isaacs 1987; Isaacs & Coffey 1989; Bonkhoff *et al.* 1994; De Marzo *et al.* 1998; Schalken & van Leenders 2003; Singh *et al.* 2006). TAC and intermediate cells correspond to intermediate stages, which exhibit an intense proliferative capacity, and the luminal secretory cells exist at terminally differentiated stage showing an increased secretory capacity (Isaacs & Coffey 1989; De Marzo *et al.* 1998; Singh *et al.* 2006). Stem cells are rare and correspond to less than 1% of the number of basal cells in the prostatic epithelium (Richardson 2004). In normal tissues, they are slow-cycle cells that rarely divide; however, deregulation of stem cell self-renewal is a likely requirement for the development of cancer (Lam & Reiter 2006). Several markers have been used for the identification of epithelial stem cells, but so far, none has proven completely reliable (Kelly & Yin 2008). The transcription factor p63, in particular the γ isoform, has been indicated as a good marker of this cell population in different stratified epithelia such as mammary, salivary and lachrymal glands (Yang *et al.* 1999; Kurita *et al.* 2004). This protein belongs to the family of p73 and p53 but, despite its structural similarity, it does not present tumour suppression activity (Kaghad *et al.* 1997; Yang *et al.* 1998). Signoretti *et al.* (2001) showed that the expression of p63 protein is required for the normal development of prostate cancer in rats, suggesting that p63-positive basal cells represent prostatic stem cells. This latter observation is in agreement with that observed for other tissues such as skin and the corneal limbus, where p63 protein is supposed to be expressed by the TAC (Pelegrini *et al.* 2001).

Androgens are probably the main trophic factor in the prostatic acinar epithelium being essential for cellular proliferation and differentiation. Therefore, their action is essential for both the induction of epithelial differentiation during embryonic development and postnatal maturation and the maintenance of secretory activity and normal differentiation (Cunha *et al.* 1992, 2004; Marker *et al.* 2003; Yan & Brown 2008; Yuan & Balk 2009). Thus, it is known that

androgen deprivation, caused by castration, leads to massive cell death in the secretory luminal epithelium and regression of the gland (Kerr & Searle 1973; Isaacs 1984; Staack *et al.* 2003). Experimental type 1 diabetes impairs androgen biosynthesis by Leydig cells and reduces androgen uptake and retention in the prostate, thereby provoking regression of the gland (Tesone *et al.* 1976, 1980; Ikeda *et al.* 2000). Structural analyses have shown that diabetes-induced prostatic regression involves stromal remodelling, a decrease in the acinar profile and significant alterations in the acinar epithelium accomplished by atrophy (Cagnon *et al.* 2000; Carvalho *et al.* 2003; Ribeiro *et al.* 2006, 2009). An increase in PIN incidence has also been described in type 1 diabetes, and recent results suggest that alloxan-induced diabetes might promote malignancy in the medium-term (Ribeiro *et al.* 2006, 2008). Considering previous evidence showing significant morphological and physiological changes in the secretory acinar epithelium as a result of diabetes, this report evaluates the alterations in cell proliferation and apoptotic rates and the frequency of p63- and AR-positive cells, as well as the putative influence of insulin treatment on the acinar epithelium of short- and medium-term alloxan-induced diabetes.

Material and methods

Groups and treatments

Male adult Wistar rats (3 months old, 400–500 g) were purchased from Central Animal Breeding, São Paulo University, Ribeirão Preto, Brazil. Rats were kept at 25 °C on a 12 h light/dark cycle and had free access to food and water. They were kept in the Animal Breeding of the Department of Zoology and Botany of IBILCE-UNESP for 1 week before the beginning of the experiments. Experiments with animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and were approved by the Institutional Committee for Ethics in Animal Experimentation (UNESP, Protocol no. 17/07-CEEA). Diabetes induction was carried out after 24 h of fasting by endovenous injection of 0.1 ml of physiological solution (NaCl 0.9%) with 42 mg/kg b.w. of alloxan (5,6 Dioxiuracil monohydrate; Sigma, St Louis, MO, USA), in the penian vein. Control rats ($n = 12$) were injected with physiological solution. After 30 min of alloxan treatment, groups were fed and blood glucose levels were daily measured in the tail tips using the glucose monitor Accu-Chek (Roche Diagnostics, Mannheim, Germany). Only rats presenting severe diabetes (glucose level up to 250 mg/dl in all tests, with loss of weight and

increased urine debit) were utilized. Water and food intake were monitored during the experiment. Rats were killed 1 week (D1, $n = 6$) or 3 months (D2, $n = 6$) after the diagnosis of diabetes. Some short-term diabetic rats were daily treated with 5 IU of insulin (Humulin, Biobrás, MG, Brazil) (D1 + I, $n = 6$) for 1 week. All groups were killed by CO₂ inhalation followed by decapitation.

Serum hormone analysis

Blood samples were collected immediately after decapitation. Serum was separated by centrifugation and stored at 20 °C for subsequent assays. Quantification of serum testosterone and oestradiol was performed using the Modular Analyzer for Immunoassay of Chemiluminescence ECI (Johnson and Johnson, Langhorne, PA, USA) (Weeks & Woodhead 1984). Five rats were used from each group, and the test was performed in triplicate. The intra- and inter-assay variations were 4.6% and 4.3% respectively. Serum insulin levels were not measured as it is widely accepted that alloxan administration drastically decreases insulin release from the pancreas and consequently, serum insulin levels after damage to the pancreatic beta cell, which, ultimately, lead to type 1 diabetes mellitus (Jansson & Sandler 1992; Soto *et al.* 2004; Lenzen 2008).

Tissue preparation

After the excision of the prostatic complex and the extraction of adipose tissue, ventral lobes were separated, weighed and transversally sectioned. Prostatic fragments were fixed by immersion in 4% formaldehyde in a phosphate buffer 0.1 M, pH 7.2 and embedded in paraffin for immunocytochemical analysis and TUNEL assay. Some of them were fixed in Karnovsky solution (2.5% formaldehyde, 2.5% glutaraldehyde in a phosphate buffer 0.2 M pH 7.2) for 24 h and embedded in plastic resin (Historesin Embedding Kit; Leica, Nussloch, Germany) or paraffin for histological purposes for 12–24 h.

The analysis of results was conducted in a Zeiss-Jenamed light microscope (Jena, Germany) coupled with a CCD camera and a semi-automatic image analysis system (Image-Pro Plus Media Cybernetics version 4.5 for Windows software, Bethesda, MD, USA).

Detection of apoptotic cells

Apoptotic cells were detected *in situ* using the DNA fragmentation assay associated to cell death based in TUNEL reaction (TdT-Fragel-Calbiochem; CN Biosciences, La Jolla,

CA, USA), following the manufacturer's instructions. Briefly, after digestion with proteinase k (1:100 in 10 mM Tris pH 8.0) at room temperature for 23 min, slides were immersed in a solution of 3% H₂O₂ in methanol for 5 min to block endogenous peroxidases. In the next step, they were incubated with biotinylated TdT followed by enzyme deoxynucleotidyl terminal transferase (TdT), for 1 h at 37 °C. At the end of the reaction, the biotinylated nucleotides were detected by streptoavidin conjugated to peroxidase, and the reaction was revealed using diaminobenzidine (0.07% in distillate water). Slides were finally stained with haematoxylin. Negative controls were obtained by omitting the incubation with TdT enzyme.

Immunocytochemical reactions

Histological sections were submitted to antigen retrieval in citrate buffer pH 6.0, for 20 min and treated for a further 30 min in 3% H₂O₂ in methanol to block endogenous peroxidases. They were subsequently treated with a background sniper blocker to eliminate unspecific bindings (Biocare Medical, Concord, CA, USA), during 15 min. Sequentially, slides were incubated with the following primary antibodies (Santa Cruz Biotechnology, Palo Alto, CA, USA) diluted 1:100 in 1% BSA: rabbit anti-human androgen receptor (sc-816, overnight, at 4 °C), mouse anti-human PCNA (sc-56, 1 h, at 37 °C) and mouse anti-human p63 protein (sc-8431, 1 h, at 37 °C). AR detections were realized with biotinylated secondary antibody anti-rabbit, for 45 min, followed by ABC Staining System (sc2018; Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer's instructions. Primary antibodies anti-PCNA and p63 protein were detected by Polymer conjugated to peroxidase (Novolink Polymer; Novocastra, Norwell, MA, USA), for 45 min. The reactions were revealed with diaminobenzidine (0.03% in TBS) and sections were stained with haematoxylin. Negative controls were obtained by omission of the primary antibody.

Quantitative and statistical analysis

The quantification of PCNA-, p63- and TUNEL-positive cells in the acinar epithelium of the intermediate region of the ventral prostate was performed by using 20 microscopic fields at 400×, randomly selected from two different histological sections per animal. Five rats were employed, resulting in 100 fields per group. The index of immunoreaction was calculated as the number of positive nuclei in epithelial cells divided by the total number of epithelial cell nuclei counted expressed as a percentage. Nuclei were counted in a

total of approximately 2000 epithelial cells. The relative frequency of AR-positive epithelial cells (%) was estimated employing the system of point counting (Weibel 1963) based on the application of a multipurpose graticule M134. Thus, the AR frequency was calculated by counting the number of grid intersections falling on the positive cells.

The quantitative data were analysed by the parametric Student's *t*-test using STATISTICA 6.0 (Statsoft Inc., Tulsa, OK, USA) and confronting the groups C1 *vs.* C2; C1 *vs.* D1; D1 *vs.* D1 + I and C2 *vs.* D2. Values of *P* < 0.05 were considered statistically significant.

Results

Biometrics and hormonal parameters

The biometric data, glucose and hormonal levels detected in the different groups of animals are shown in Table 1. Diabetic rats, in both experimental groups (D1, D2), exhibited blood glucose levels greater than 400 mg/dl. The insulin-treated group (D1 + I) showed 250 mg/dl of blood glucose levels, which was considered the limit for inclusion in diabetic group in this study. The relative weight of the prostate decreased 32% in D1, 27% in D1 + I and 40% in D2, when compared with their respective controls. Testosterone levels decreased 80% after 1 week and 68% after 3 months of diabetes. The oldest control (C2) also exhibited a sharp reduction in testosterone, showing less than half the levels noted in C1. Oestradiol serum levels were neither affected by diabetes in the short or medium-term, nor by insulin treatment. All these data, except for oestrogen serum levels, were statistically significant, as shown in Table 1.

Ventral prostate morphology

There were no drastic changes in the prostatic histology in most of the D1 rats, especially in the intermediary region of

the gland (Figure 1). In both control groups, the prostatic acinar epithelium was fully differentiated, containing abundant secretory columnar cells with a prominent Golgi complex area and few basal cells (Figure 1a,e). Short-term diabetes-induced pronounced wrinkling and epithelial disorganization besides reduction in the acinar lumen and stromal disorganization (Figures 1c,d). The prostate of the insulin-treated group was very similar to that of the control, exhibiting a columnar epithelium with a discrete reduction in epithelial height (Figure 1b). In the medium-term, diabetes promoted a drastic prostatic atrophy, showing a reduced acinar lumen and epithelial height that acquired a pavimentous aspect, making it difficult to distinguish between luminal cells and basal cells (Figure 1f).

Immunocytochemical and cell death analysis of acinar epithelium

The frequency of PCNA-positive cells in the acinar epithelium of the prostate decreased dramatically with ageing, as noted by immunocytochemistry (Figures 2a–e and 4B). The frequency of PCNA-positive cells also showed a significant reduction, notably after 1 week of diabetes, regardless of insulin treatment (Figures 2a–c and 4b). On the other hand, 3 months after diabetes, the frequency of PCNA-positive cells did not change significantly compared with their controls (Figures 2e and 4b).

A tendency to increase was observed in apoptosis levels in the acinar epithelium of D1 (Figure 2f,g). These levels tripled after 3 months of diabetes (Figures 2i,j and 4B). Interestingly, levels of apoptotic cells were lower in the insulin-treated group compared with those of the control, although this difference was not statistically significant (Figures 2h and 4B).

Immunocytochemistry demonstrated that in young rats (C1), most of the cells in the prostatic acinar epithelium were AR-positive (Figure 3a), showing a significant decrease in the older group (C2) (Figures 3d,e and 4A). Diabetes does

Table 1 Variation of biometric and physiological parameters (Average ± Standard Error) in 1 week diabetic (D1), insulin-treated diabetic (D1 + I) and 3 months diabetic (D2) rats compared with their respective controls (C1, C2)

	C1	D1	D1+I	C2	D2
Body weight (g)	523 ± 19.8	359 ± 21.9*	465 ± 21.9‡	444 ± 12.9†	227 ± 18.8*
Relative weight of prostate (×10 ³)	1.3 ± 0.11	0.89 ± 0.09*	0.95 ± 0.05‡	1.3 ± 0.08	0.78 ± 0.08*
Blood glucose (mg/dl)	121 ± 8.01	497 ± 33.04*	250 ± 48.5‡	89.3 ± 3.81	412 ± 15.17*
Serum testosterone (ng/dl)	178.7 ± 37.8	34.21 ± 9.4*	158.6 ± 29.35‡	82.2 ± 21.1†	26.4 ± 6.34*
Serum oestradiol (mg/dl)	31.85 ± 3.31	33.8 ± 7.95	28.7 ± 8.18	28.65 ± 0.82	30.7 ± 7.2

*Statistically significant compared with control.

†Statistically significant between ages.

‡Statistically significant compared with D1.

P < 0.05.

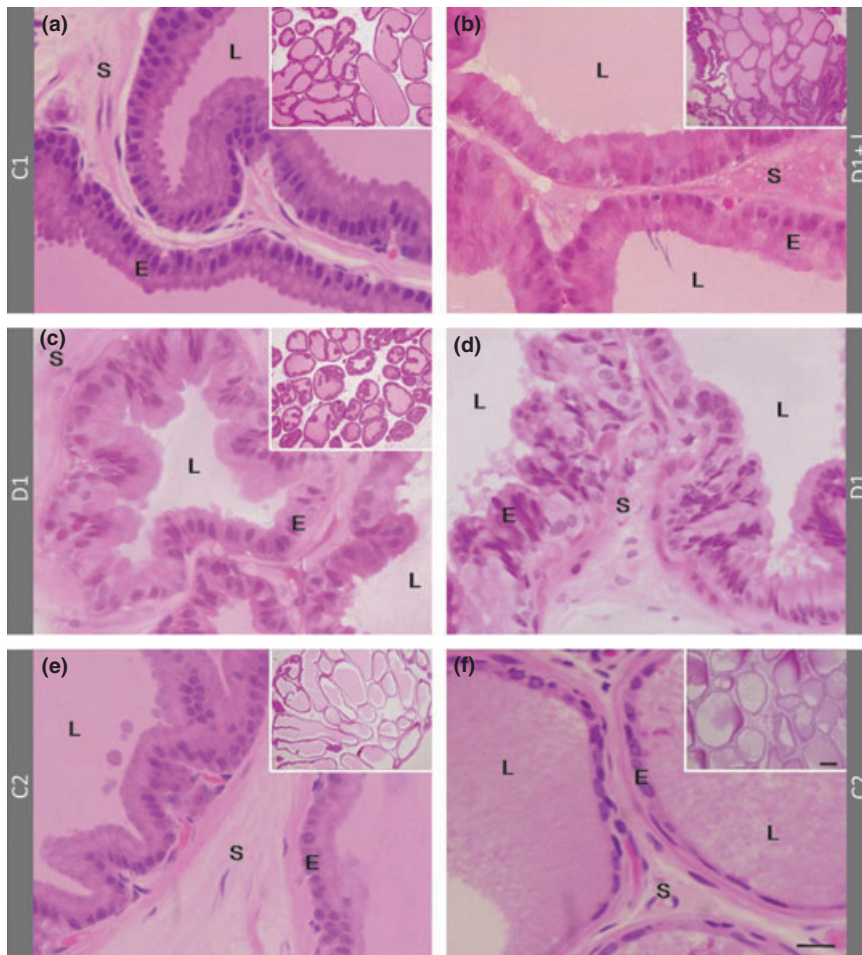


Figure 1 Histological sections in historesin stained with H&E showing general aspect (small images) and details of acinar epithelium (large images) of the ventral prostate in control, C1 (a) and C2 (e), 1 week diabetic insulin-treated (b), 1 week diabetic without treatment (c and d) and 3 months diabetic rats (f). In control groups, the acinar epithelium shows columnar luminal cells and large luminal area (a, e). After 1 week of diabetes, there is a slight reduction in epithelial height (c) and intense acinar disorganization in some glandular regions (d). In medium-term diabetic group, epithelial cells appear cubic or flattened, indicating the intense epithelial atrophy (f). Arrows: areas of Golgi complex. E: Prostatic acinar epithelium; L: acinar lumen; S: Stroma. Scale bars are the same for all small and large images in the figure: 100 and 20 μm respectively.

not interfere with the frequency of AR-positive cells when compared with their respective controls, but the intensity of reaction for this receptor in the cell nuclei is lower 1 week after diabetes (Figures 3b and 4A).

Intense p63 protein immunolabelling was detected in the nuclei of basal cells in the prostate acinar epithelium from control groups (Figures 3f–j and 4A). The frequency of p63-positive cells doubled after 1 week and 3 months of diabetes, but did not change significantly in the diabetic group treated with insulin.

Discussion

This study evaluated the influence of short- and medium-term alloxan-induced diabetes and the possible implications of circulating androgen and insulin on proliferation and cell death in acinar prostatic epithelium. A summary of our data is shown in Table 2. Marked differences were found in the epithelial kinetics of the control groups at different ages as

older rats showed fewer AR-positive cells and lower cell proliferation rates. Cell proliferation was determined here by immunocytochemistry for PCNA protein and is highly expressed in the nuclei during the S-phase of cell cycle (Bravo & Macdonald-Bravo 1987; Kelman 1997; Gulbahar *et al.* 2005). The decrease in cell proliferation in older rats paralleled the 50% reduction in testosterone levels. This indicates that ageing itself affects androgen availability and consequently the mechanisms of cell proliferation in the prostatic epithelium and confirms the auto-regulation of AR in the ventral prostate (Prins & Birch 1993; Prins *et al.* 1996).

In the short-term, diabetes led to a significant reduction in androgen levels, together with an eight-fold reduction in cell proliferation levels in the acinar epithelium. A lower decrease in testosterone was observed in medium-term diabetes, as these levels are already low in older rats, and no significant variations in cell proliferation rates were found. The positive correlation between testosterone levels and

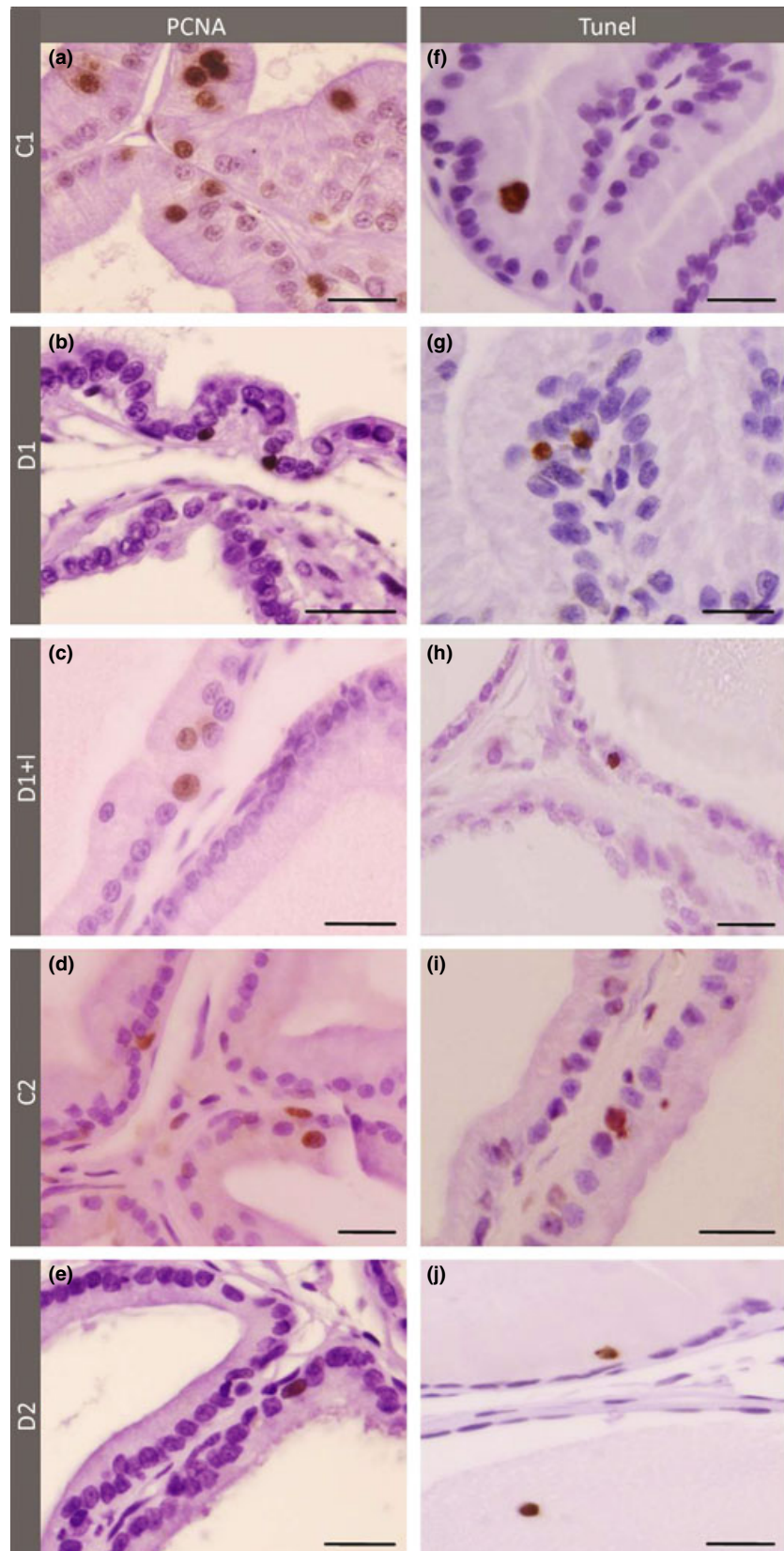


Figure 2 PCNA Immunocytochemistry (a–e) and TUNEL method (f–j) in the ventral prostate of 1 week control (a and f), 1 week diabetic (b and g), 1 week diabetic insulin-treated (c and h), 3 months control (d and i) and 3 months diabetic rats (e and j). Note that proliferation in prostate epithelium decreases significantly in older group (d) and also in diabetic groups, especially in short-term (b). The levels of apoptosis increased in prostate after diabetes (g and j), mainly in long-term rats (j). Insulin treatment completely reverses apoptotic levels but not cell proliferation in prostatic epithelium. Scale bar: 20 μ m.

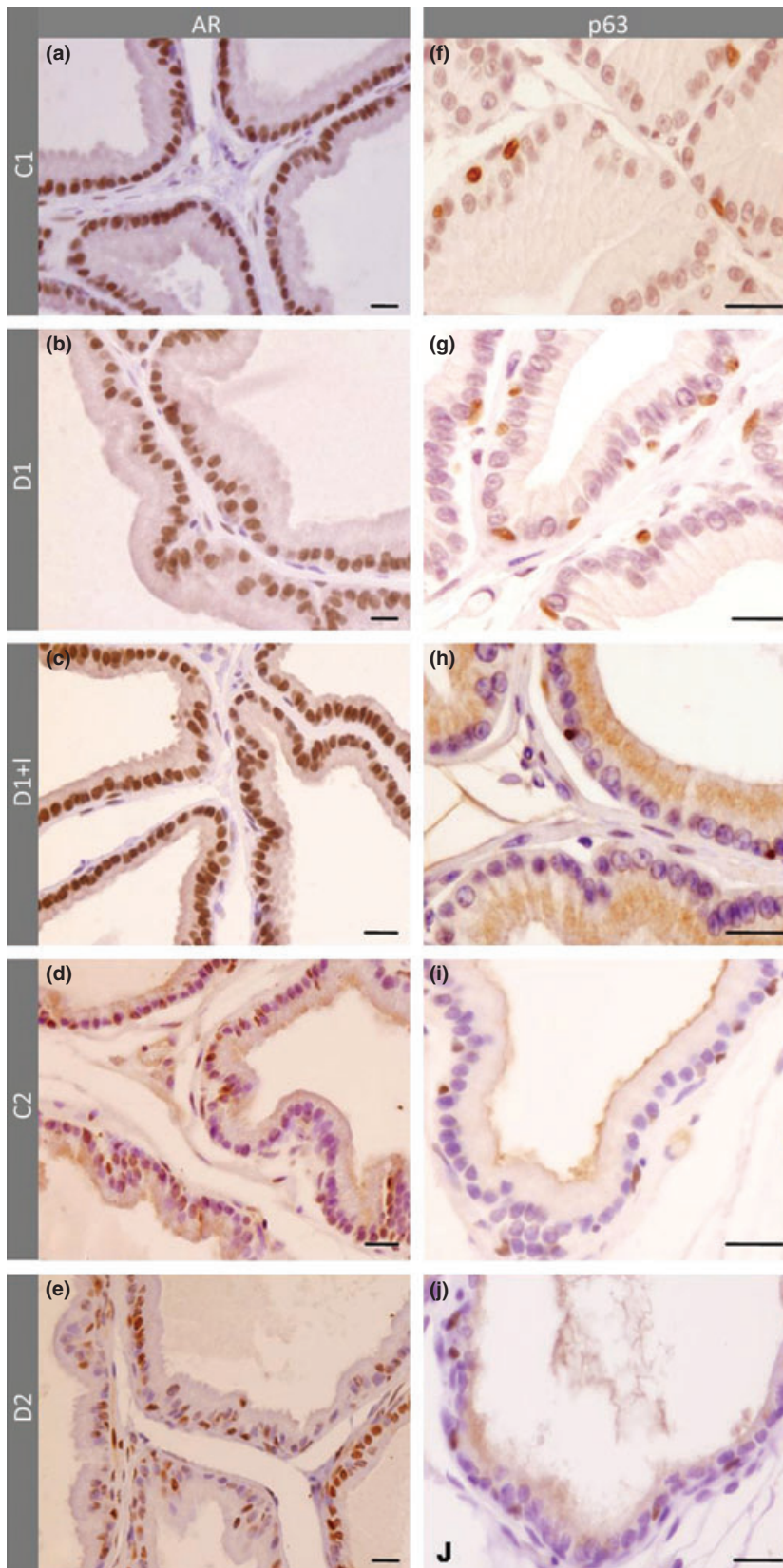


Figure 3 Immunocytochemistry for androgen receptor AR (a–e) and p63 (f–j) in the ventral prostate of 1 week control (a and f), 1 week diabetic (b and g), 1 week diabetic insulin-treated (c and h), 3 months control (d and i) and 3 months diabetic rats (e and j). The frequency of AR significantly decreased because of ageing (d), but not because of diabetes (b and e), although a lower intensity of immunoreaction could be observed in short-term diabetic group (b). The number of p63-positive cells is significantly greater in diabetic rats (g and j) and insulin treatment reduced its expression levels close to those observed in control group (h). Scale Bar: 20 μ m.

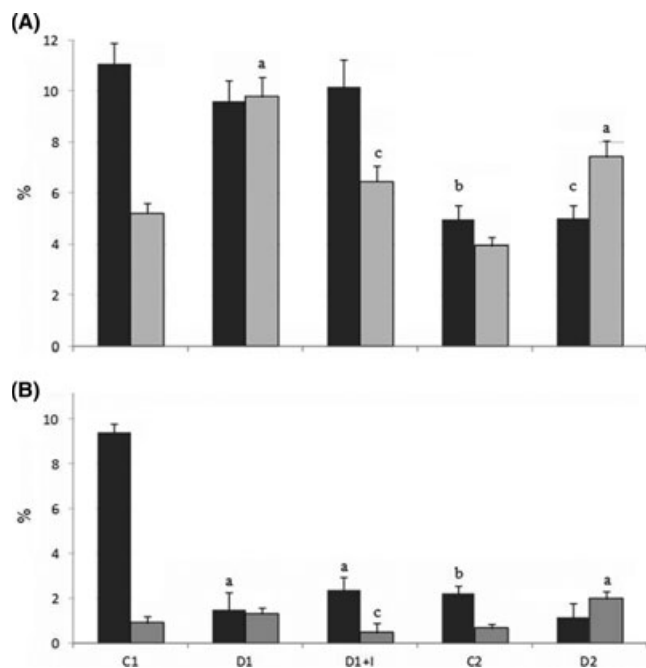


Figure 4 (A) Relative frequency (%) of immunoreactions for androgen receptor (black bars) and p63-positive cells (grey bars). (B) Relative frequency (%) of immunoreactions for cell proliferation (black bars) and apoptotic cells (grey bars). (a) Significantly different from respective control, (b) Significantly different between ages, (c) Significantly different from short-term diabetic group (D1). $P < 0.05$.

epithelial proliferation during ageing and in diabetes was expected, as androgens are the major growth factor for prostate cells. Under normal physiological conditions, the proliferation in the acinar epithelium is indirectly controlled via androgenic stimulation of stromal cells, which secrete growth factors such as FGF-7, FGF-10 and IGF-I. These andromedins activate the proliferation of TAC and are also essential for the survival of luminal cells (Cunha *et al.* 2004; Uzgaré *et al.* 2004). On the other hand, the direct action of AR in epithelial cells results in the differentiation of luminal epithelial cells and regulates the transcription of protein products that are required for prostate function, such as prostate specific antigen (Sugimura *et al.* 1986; Cunha *et al.* 1998; Gao & Isaacs 1998; Yuan & Balk 2009). Alloxan-induced diabetes and testosterone decrease did not interfere in the frequency of AR-positive cells in the acinar epithelium, but in the short-term, there was a notable lower intensity of immunolabelling per nuclei and, in the medium-term, marked epithelial atrophy and lumen reduction. These findings indicate that in the short-term diabetes, andromedin secretion is probably affected impairing cell proliferation, whereas in the medium-term, it affected secretory activity.

Thus, although diabetes did not interfere in the frequency of AR-positive cells as analysed by immunocytochemistry, the signalling pathway involved in the stimulation of epithelial cell differentiation and secretion is probably affected.

Insulin is also an important stimulator of cell proliferation and is usually employed *in vitro* for a better growth of prostate cell lineages (Straus 1981; Cheatham & Kahn 1995; Antonioli 2003). Insulin treatment avoided testosterone reduction, but it was not able to avoid the reduction of cell proliferation in the acinar epithelium, indicating that other factors beyond insulin and androgen should be considered in the regulation of this process. As the insulin treatment employed here did not re-establish normal glycaemia, it is reasonable to presume that tissue alterations related to hyperglycaemia must be implicated. Another putative factor that could be involved is TGF β 1, which has been shown to act as a negative regulator of epithelial cell proliferation under conditions of co-culture of normal prostatic cells (Blanchere *et al.* 2001), and which increases in diabetes (Lamers *et al.* 2007).

Apoptosis, combined with decreased cell proliferation, is one of the main mechanisms leading to prostate regression in situations of androgen deprivation. Ten days after castration, the rat ventral prostate regresses completely, with the loss of 80% of androgen-dependent epithelial cells (Kyprianou & Isaacs 1988). In this situation, high levels of apoptosis occur 3–4 days after castration, but they normalize after prolonged periods, as the surviving cells are androgen-independent (Kyprianou & Isaacs 1988; Staack *et al.* 2003). Our data showed that apoptotic levels were not very elevated 1 week after diabetes. However, the marked reduction of serum testosterone and the decrease of about 32% in prostate weight as well as the increase in relative frequency of epithelial basal cells are strong indicators of massive loss of luminal epithelial cells. Thus, it is possible that the peak of apoptosis occurred before the seventh day as has been

Table 2 Summary of results on frequency of positive cells after immunocytochemistry for androgen receptor (AR), PCNA, p63 and TUNEL assay. The decrease or increase in cell frequency is shown, respectively, by arrow down and arrow up

	Age (C1/C2)	Short-term diabetes (C1/D1)	Medium-term diabetes (C2/D2)	Insulin treatment (D1/D1 + I)
AR	↓	0	0	0
PCNA	↓	↓	0	↓
p63	0	↑	↑	0
TUNEL	0	0	↑	0

Zero indicates no variation.

observed in castration, and therefore, the cellular debris has already been digested and is undetectable. Previous reports with non-obese diabetic mice have pointed to increased rates of apoptosis in the prostatic acinar epithelium after 20 days of diabetes (Favaro *et al.* 2008). Consistent with these results, the epithelial apoptotic rates tripled 3 months after alloxan-induced diabetes. Considering that androgen levels were similar in both diabetic groups, we assume that diabetes has a prolonged effect on the apoptosis of prostatic epithelial cells. Additionally, this effect is not exclusively dependent on androgen as, after 3 months of androgen reduction, most androgen-dependent cells might have died. The injury of the immune system together with the ageing process, which is associated to a greater accumulation of lesions in DNA, could possibly be responsible for the higher apoptotic levels in medium-term diabetes. It is also probable that the increase in products of advanced glycation (AGE) and the consequent increase in oxidative stress, because of hyperglycaemia are involved in increased apoptosis. Such alterations have been reported in other organs affected by diabetes, and a direct correlation between an increase in AGE and oxidative stress and the death of retinal pericytes has been shown (Chen *et al.* 2006). Interestingly, the normalization of androgen levels after insulin treatment resulted in apoptotic rates lower than control, suggesting a protective effect of insulin against the apoptosis of prostatic epithelial cells. Similar data have been found in experiments with retinal cells *in vitro*, showing that insulin prevents cell death in a dose-dependent pathway (Kobayashi & Puro 2007).

Short- or medium-term diabetes doubled the frequency of p63-positive cells in the rat ventral prostate, and insulin treatment prevented it. Considering previous reports, it is clear that the p63 protein, which is restricted to basal compartment, has an important role in regulating the development and differentiation of epithelial cells (Senoo *et al.* 2007), in addition to controlling cell proliferation, apoptosis and senescence (Yang *et al.* 1998; Senoo *et al.* 2007). Basal stem cells, which are able to survive in an environment with low levels of androgens are responsible, but not essential, for the reconstitution of the prostate epithelium after the administration of testosterone in castrated animals (Bonkhoff 1996; Bonkhoff & Remberger 1996; Kurita *et al.* 2004; Grisanzio & Signoretti 2008). Studies in other epithelia have also linked the expression of p63 protein with diabetes, and higher levels of p63 were reported by Galkowska *et al.* (2003) in keratinocytes obtained from diabetic patients. The increase in frequency of p63-positive cells in the prostate of diabetic rats is probably explained by the massive death of luminal cells, as observed after castration. However, the possibility that alterations in the hormonal/paracrine envi-

ronment may have stimulated the activation of stem cells or implemented the proliferation of TACs cannot be excluded. Anyway, the preservation of basal cells in diabetes explains the maintenance of the structural integrity of the prostate, as these cells and p63 protein are essential in the ductal structure.

In conclusion, the epithelial response to alloxan-induced diabetes differed in the short- and medium-term not only because the epithelial kinetic varies according to age, but also because the processes affected are distinct. In the short-term, diabetes reduces cell proliferation, whereas in the medium-term, it increases apoptotic levels. This study shows that diabetes led to a fast reduction in testosterone, affecting the proliferative behaviour of the epithelium and inducing a drastic prostate atrophy. Insulin treatment attenuates these effects, but it is not sufficient to prevent them completely, particularly with regard to cell proliferation. This finding suggests the joint action of insulin, testosterone and other factors associated to diabetes, such as high glucose levels may be the cause of epithelial alterations induced by this disease. Furthermore, this investigation provide evidences that the prostatic epithelial atrophy observed in diabetic individuals is accompanied by marked changes in the kinetic of differentiation of epithelial cells, which may have implications for prostatic homeostasis and the incidence of diseases of epithelial origin.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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