

Evaluation of PCNA, Caspase 3 and E-cadherin on the Ventral Prostate of Soy Treated Rats

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Abstract: The incidence of certain cancers, including prostate cancer, is considerably higher in western countries than in Southeast Asia. Many studies have linked soy consumption to the lower incidence of prostate cancer in these countries. Twenty male Sprague-Dawley rats were randomized and divided into a control group and a group treated with soy. The ventral prostates of each animal were sectioned and stained with hematoxylin-eosin for morphological description and immunostained to detect PCNA, cleaved caspase 3 and E-cadherin immunoreactivities. Estimates of the number of total epithelial cells and the number of epithelial cells immunoreactive to PCNA and cleaved caspase 3 were calculated using the optical disector technique. Measurement of E-cadherin was carried out by calculating the volume fraction of epithelium immunostained by E-cadherin. Soy treated group showed atrophy in the epithelium and a diminished expression of PCNA, cleaved caspase 3 and E-cadherin, which means there is a reduced cell proliferation, apoptosis through caspases way and cell adherence. It can be concluded that soy treatment induces atrophy in the epithelium by reducing cell proliferation.

Keywords: Apoptosis, cell adherence, cell proliferation, rat prostate, soy, stereology.

INTRODUCTION

The incidence of certain cancers, including prostate cancer, is considerably higher in western countries than in Southeast Asia. There are several epidemiological studies that attribute this lower incidence to environmental factors, because the incidence in Asians that move to western countries is similar to that of the population of said countries [1, 2]. Many studies have linked soy consumption to the lower incidence of prostate cancer in Asian countries [3-8].

These beneficial properties of the soy are associated to organic compounds called isoflavones [9], which appear in significant amounts in soybeans. Isoflavones are diphenolic compounds with a chemical structure similar to estrogens, although they are not steroidal. This is why they are commonly known as phytoestrogens.

Isoflavones protect against cancer by mechanisms not yet well known, but many studies suggest that it is due to their activity as estrogen receptor modulators [4, 5, 10, 11]. Other studies have linked soy consumption with low insulin-dependent growth factor (IGF), which has also been associated with the development of prostate cancer [12]. Other

proposed mechanisms are inhibition of angiogenesis [4, 13], inhibition of cell proliferation, induction of apoptosis [7] and their antioxidant activity [14-16].

Immunohistochemical techniques and quantitative stereological methods were used to study those different mechanisms. The proliferating cell nuclear antigen (PCNA) has been used in numerous studies as a marker of cell proliferative status, associating an increased proliferation to an abnormal cell growth in tumor tissues [17-19].

Caspases are fundamental components of the mammalian apoptotic machinery. Caspase 3 is a prototypical enzyme that becomes activated during apoptosis in a wide variety of tissues [20]. On the other hand, there is evidence of loss of cleaved caspase 3 protein expression in prostate cancer [21]. The quantification of immunoeexpression of cleaved caspase 3 may constitute a good method for measuring apoptotic activity in prostate cancer and its precursors.

E-cadherin is a molecule involved in adhesion between epithelial cells and therefore it has been linked to invasiveness of many malignant tumors [22]. It is thought that the gene that codify for E-cadherin is a suppressor gene, because there have been mutations at these level linked to a lesser immunoeexpression of E-cadherin at immunostainings [23].

The aim of this work is to study morphological and quantitative changes occurring in the ventral prostate of rats treated with soy as well as soy effects on cell proliferation, cell-adherence and apoptosis.

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MATERIALS AND METHODOLOGY

Animals

Twenty male Sprague-Dawley rats were randomized and divided into two groups, according to treatment, that began in all cases at three months old. Soy-enriched chow (Teklad Global 18% Protein Rodent Diet, Harlan Interfauna, Ibérica SL, Barcelona, Spain) was used for feeding the first group. The second group was used as a control and was fed with Pan Lab Chow (Panlab, Barcelona, Spain). All animals were kept in a controlled environment with light/dark cycle 12h/12h, 45-55% humidity, 20-22°C and fed "ad libitum". Animals fed with soy-enriched chow were estimated to have an average intake of 250 mg/kg/day of isoflavones.

During this work, all animal studies were conducted in accordance to local directives ruled by "Real Decreto 223/1988" and "Orden de 13 de Octubre".

Animals were sacrificed at eighteen months old by narcosis with carbon dioxide. Previous studies [24] showed that such aged animals will probably develop prostate damages and so we shall compare damages appearing in treated and non-treated animals.

All animals were weighed and the mean of each group was calculated. The means \pm SD were compared by a t student test, so that we could dismiss a weight loss in animals treated with soy. Immediately after that, prostates were removed from each animal and were also weighed. Total fresh volume of each prostate was determined by water displacement. Then, the ventral lobes were separated from the dorsolateral ones and the volume and weight of each lobe were also determined.

The ventral prostates were embedded in paraffin and the paraffin blocks were used to obtain 10 μ m-thick serial sections. Sections from each animal were stained with hematoxylin-eosin for morphological description. The percentage of cases with at least one dysplastic lesion was also determined in each group of animals, taking into account both mild and severe dysplasia. Mild dysplasia is characterized by a higher number of nuclei appearing piled and with alterations in their shape, size and polarity. Severe dysplasia presents a thicker epithelium, which acquires cribriform structure, and also shows alterations in the shape and size of nuclei. In addition nucleoli and mitotic figures are frequently observed [24].

Immunohistochemical Methods

Slides from each animal of both groups were immunostained. Deparaffined and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% to block endogenous peroxidase. To detect PCNA, caspase 3 and E-cadherin immunoreactivities, sections were incubated with anti-PCNA antibody (Biomeda, Foster City, CA, USA) diluted at 1:400; anti-caspase 3 antibody (Cell Signaling, Boston, USA) diluted at 1:30; and anti-E-cadherin antibody (Novocastra, Newcastle, UK) diluted at 1:150. All incubations with primary antisera were kept overnight at 4°C. Pretreatment of sections by heat in citrate buffer pH 6.0 using a pressure cooker was performed to enhance all the immunostainings [25, 26]. The immunohistochemical method was

performed by an indirect technique using the antibody detection kit Histostain SP (Kit Histostain SP, Zymed Laboratories, Carlsbad, USA). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3',4,4'-Tetraminobiphenyl, Sigma, St. Louis, USA) in PBS, pH 7.4 (200 mL), plus 40 μ L of hydrogen peroxide.

After immunoreactions, sections were counterstained with Harris hematoxylin. All slides were dehydrated in ethanol and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany).

Quantitative Methods

Estimates of the number of total epithelial cells and the number of epithelial cells immunoreactive to PCNA and cleaved caspase 3 were calculated using the optical disector technique, an unbiased stereological method [27, 28]. Measurements were carried out using an Olympus microscope fitted with a motorized stage and equipped with an X100 oil immersion lens (numerical aperture of 1.4) at a final magnification of X1200, employing the stereological software CAST-GRID [29].

In each selected field from PCNA-immunostained sections, the numerical density (N_v) of epithelial cells was estimated. The N_v of epithelial cells immunoreactive to PCNA was simultaneously estimated. In cleaved caspase 3 stained sections, positive epithelial cells were evaluated. In all cases, the unit used for counting was the nucleus eligible according to the Sterio rule [30].

The N_v is determined by the formula: $N_v = Q^- / \Sigma V \text{ dis}$, where: Q^- = number of eligible nuclei, $\Sigma V \text{ dis}$ = Sum of the volumes of the measured disectors.

In this case, the measured disectors were those, whose upper-right corner hit the epithelium, since the reference volume is the epithelium. To determine the volume of the disectors, the area of each disector was multiplied by its height, which in this case is 5 μ m. The absolute number of epithelial cells was then calculated multiplying N_v by the volume of epithelium. These calculations are expressed in number of cells per μm^3 . For convenience, the results were expressed in number of cells/mm³.

Labeling indices for PCNA and cleaved caspase 3 were calculated: $Li \text{ PCNA} = Q^- \text{ PCNA} / Q^-$, where: $Q^- \text{ PCNA}$ = number of nuclei immunoreactive for PCNA, Q^- = total number of nuclei; $Li \text{ casp} = Q^- \text{ casp} / Q^-$, where: $Q^- \text{ casp}$ = number of immunoreactive cells to cleaved caspase 3, Q^- = total number of nuclei.

The volume fraction of epithelial cytoplasm immunostained by E-cadherin will be estimated by previously calculating the volume fractions of epithelium and glandular lumen: $V_v \text{ Ep} = \text{disEp} / \text{disTot}$, $V_v \text{ L} = \text{disL} / \text{disTot}$, where: $V_v \text{ Ep}$ = epithelium volume fraction, $V_v \text{ L}$ = volume fraction of glandular lumen, disEp = number of disectors hitting the epithelium, disL = number of disectors hitting the glandular lumen, disTot = total number of disectors hitting the glands ($\text{disEp} + \text{disL}$). The volume fraction of epithelium immunostained by E-cadherin was calculated as follows: $V_v \text{ cadE} = \Sigma \text{P cadE}^+ / \Sigma \text{P Ep}$, where: $\Sigma \text{P cadE}^+$ = number of points hitting epithelium immunoreactive to E-cadherin, $\Sigma \text{P Ep}$ = number of points hitting both, immunoreactive and non-

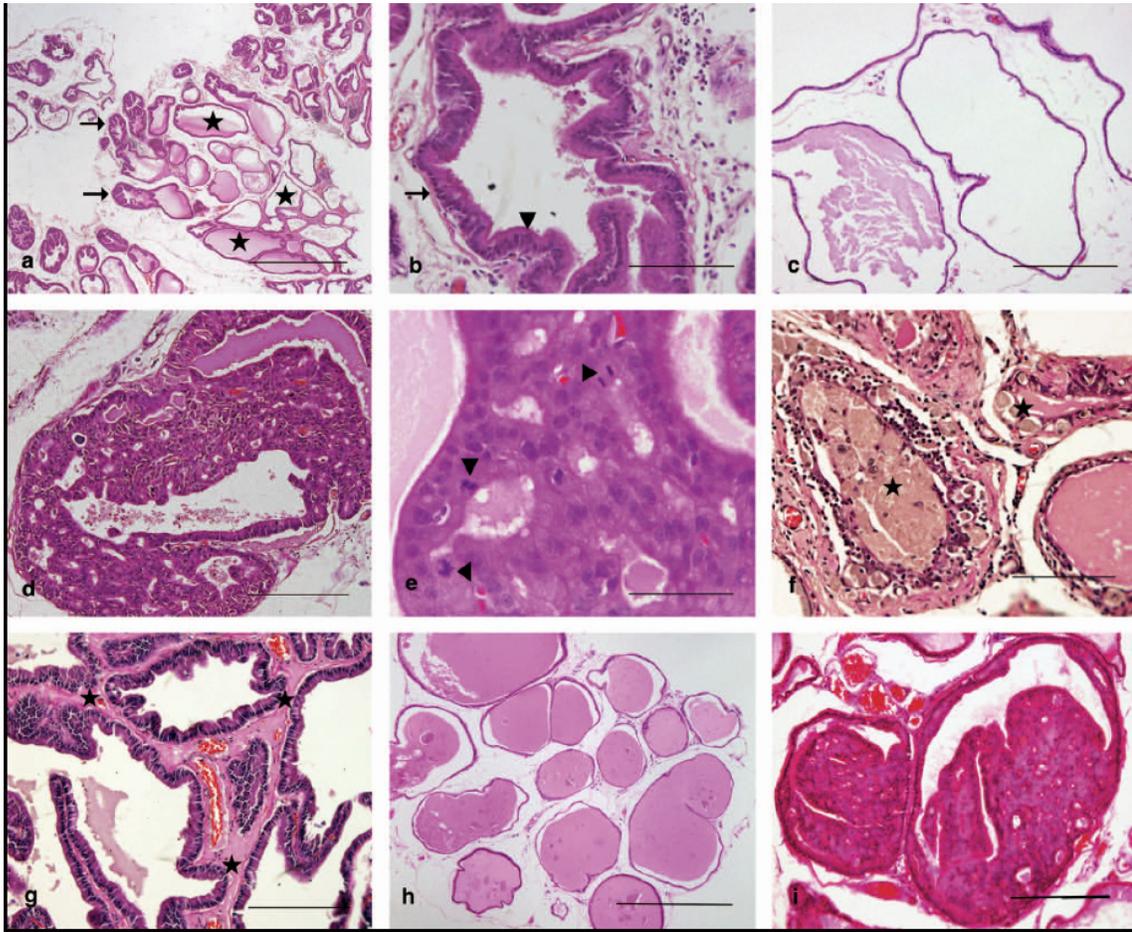


Fig. (1). Histology of the ventral prostate of rats in both groups (hematoxylin-eosin):

a- Peripheral acini with smaller size and a higher epithelium (arrows) and central acini more dilated with flattened epithelium (stars) in a control rat. The bar represents 1mm.

b- Cylindrical monostratified epithelium with nuclei in basal position (arrow) in a control rat. In some areas the epithelium may look pseudostratified (arrowhead). The bar represents 100 μ m.

c- Some acini have atrophic epithelium in control rats. The bar represents 200 μ m.

d- Dysplastic lesion with cribriform structure in a control rat. The bar represents 200 μ m.

e- Some mitotic figures in a dysplastic lesion from a control rat (arrowheads). The bar represents 50 μ m.

f- Prostatitis with invasion of macrophages (stars) in acini and interstitium in a control rat. The bar represents 100 μ m.

g- Fibrosis in the interstitium (stars) of a control rat. The bar represents 200 μ m.

h- Large areas of atrophy in the epithelium of a soy treated animal. The bar represents 500 μ m.

i- Dysplastic lesion with cribriform structure in a soy treated rat. The bar represents 400 μ m.

immunoreactive epithelium. To calculate absolute volumes, volume fractions were multiplied by the ventral prostate volume.

Statistical Analysis

The means and SD were calculated for all the parameters measured and a Student t-test was applied to compare these means between the two groups of animals at a level of significance $p < 0.05$.

RESULTS

Qualitative Results

Average weight of the control group was of 653 ± 18 g, whereas in the soy treated group it was of 644 ± 18 g. There were no significant differences between those means.

Prostates from the control group have peripheral acini with a smaller size than the central ones (Fig. 1a). The epithelium of these acini is cylindrical but sometimes may appear pseudostratified (Fig. 1b) and also have small papillae that protrude to the lumen. The central acini are dilated and show atrophic epithelium in some areas, characterized by a decrease in its height (Fig. 1c). The stroma is loose and well developed.

In this group, dysplasias are generally located in central acini. In all cases, the lesions appear well developed showing a cribriform structure (Fig. 1d, e). Dysplasias are composed mainly of epithelial cells, but they can also have slender axes of connective tissue and small capillaries. Dysplastic cells show alterations in polarity, changes in the size and shape of nuclei and some of them show the appearance of signet ring cells. There are also abundant mitotic figures (Fig. 1e). These dysplasias are detected in 44% of cases.

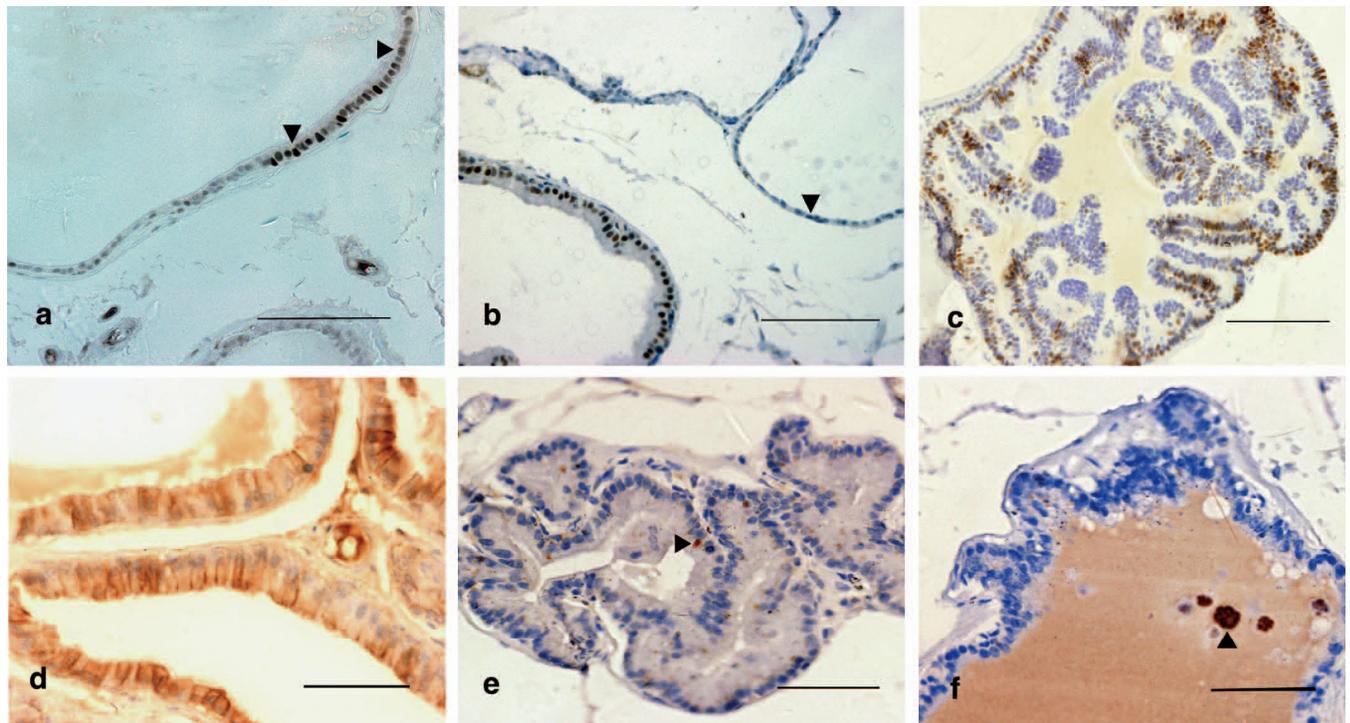


Fig. (2). Immunohistochemical study:

- a-** Immunoreactive nuclei to PCNA (arrowheads) in the prostatic epithelium of a control rat. The bar represents 100 μ m.
b- No immunoreactivity to PCNA in the atrophic epithelium (arrowhead) of a rat treated with soy. The bar represents 100 μ m.
c- Numerous immunostained nuclei for PCNA in a dysplastic acinus of a soy treated rat. The bar represents 100 μ m.
d- Positive E-cadherin immunostaining between the epithelial cells of a control rat. The bar represents 50 μ m.
e- Positive immunostaining for cleaved caspase 3 (arrowhead) in epithelial cells of a control rat. The bar represents 250 μ m.
f- Cleaved caspase 3 immunostained cells inside a control rat acinus (arrowhead). The bar represents 250 μ m.

Prostatitis was detected in 22% of controls, characterized by infiltration of leukocytes and macrophages in acinar lumen and in the interstitium (Fig. 1f). Fibrosis was occasionally found in interstitial areas too (Fig. 1g).

The group fed with soy enriched chow, had the epithelium quite similar to controls but showing extensive areas of atrophy (Fig. 1h). This implicates a reduction on the size of cells, showing a remarkable flattening of the epithelium. The periglandular stroma had no modifications comparing to the control group.

Dysplasias with a cribriform structure appear in 23% of animals (Fig. 1i). Prostatitis was detected in 8% of the cases and fibrosis in 23%.

In all cases, treated or not, there is remarkable immunoreactivity for PCNA in the nuclei of epithelial cells of prostatic acini (Fig. 2a-c). This expression is in particular much less prominent in the atrophic epithelium in animals treated with soy (Fig. 2b).

E-cadherin is expressed in the contact areas between epithelial cells, showing an immunostained band in the apical juncture of epithelial cells. It is abundantly expressed in all animals in the study (Fig. 2d).

The immunoreactivity for cleaved caspase 3 shows a granular expression in the cytoplasm of isolated epithelial cells and is also seen in cells shed from the epithelium to the lumen of the acini (Fig. 2e, f).

Quantitative Results

The total volume (V_t) of the prostate decreased significantly in soy treated animals (Fig. 3a). However, the ventral prostate volume (V_v) presents no significant differences with controls, although it tends to decrease (Fig. 3b). The volume fraction (V_v ep) and absolute volume (V ep) of epithelium present no significant differences, although they are also lower than in controls (Fig. 3c, d). The volume fraction (V_v Lumen) and absolute volume (V Lumen) of glandular lumen do not present significant differences between controls and animals treated with soy (Fig. 3e, f).

The numerical density (N_v) is significantly higher in soy treated animals than in controls. The absolute number (N_t) of epithelial cells presents no significant differences with controls, although it is higher in animals treated with soy (Fig. 4a, b).

The labeling index of PCNA and cleaved caspase 3 are significantly decreased in soy treated animals. The volume fraction and absolute volume of E-cadherin were also significantly lower in treated rats than in controls (Fig. 5a-d). The qualitative results shown in Figs. (3-5), are summarized in Table 1.

DISCUSSION

Since there are no significant weight differences between treated and non-treated rats, there is no weight loss attributable to soy. Therefore, prostate damages, must be associated either to treatment or aging.

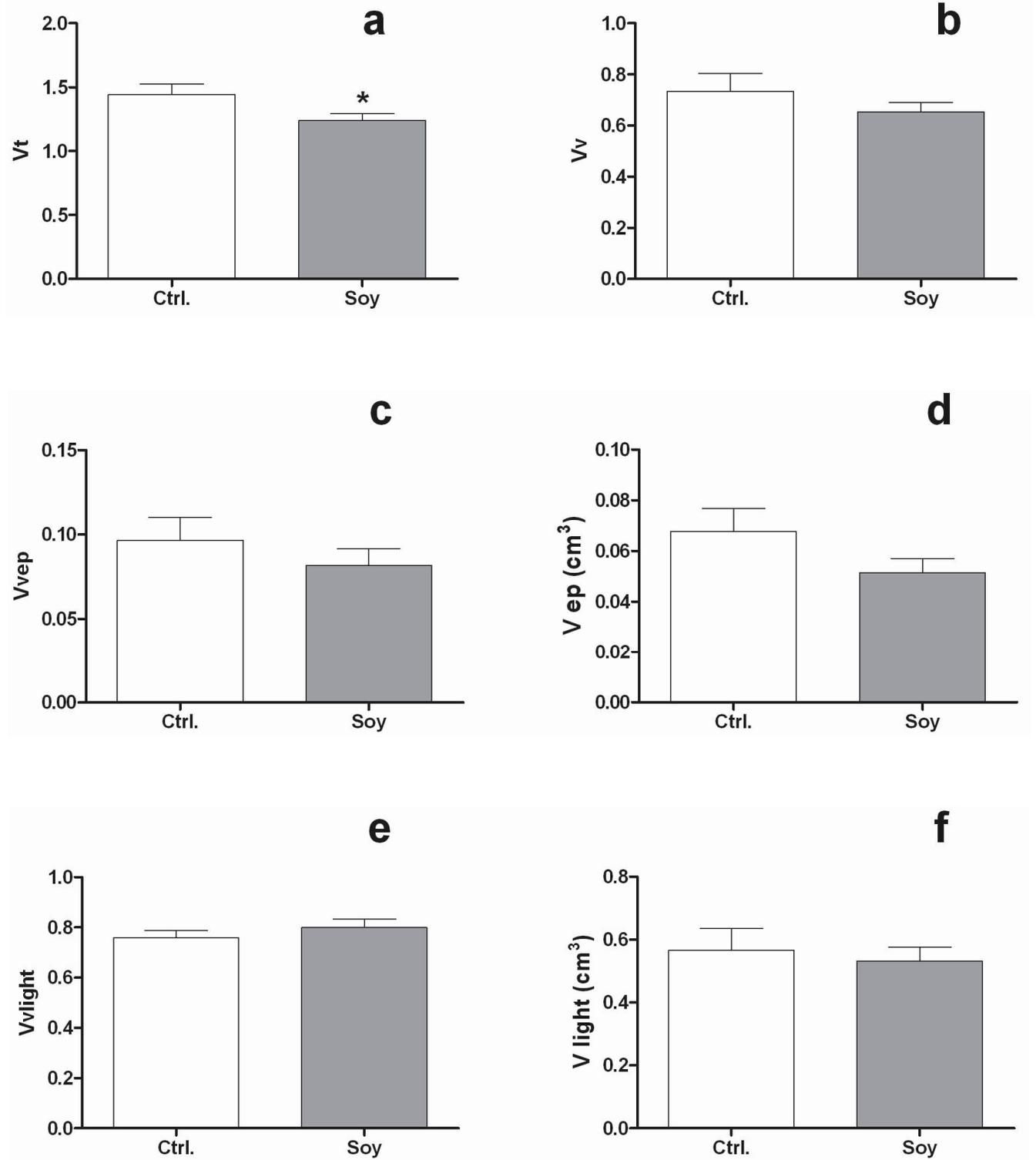


Fig. (3). Quantification of prostate volumes: Results are expressed as mean \pm SD. Asterisks indicate there are significant differences between groups ($p < 0.05$). **a)** Bar graph of total prostate volume in controls and in soy treated animals. **b)** Bar graph of the volume of the ventral prostate in the control group and in the soy treated group. **c)** Bar graph of the volume fraction of the ventral prostate epithelium in the control group and in soy treated group. **d)** Bar graph of total volume of the ventral prostate epithelium in the control group and in soy treated group expressed in cm³. **e)** Bar graph of the volume fraction of gland lumen in the ventral prostate of controls and animals treated with soy. **f)** Bar graph of total volume of gland lumen in the ventral prostate of controls and animals treated with soy expressed in cm³.

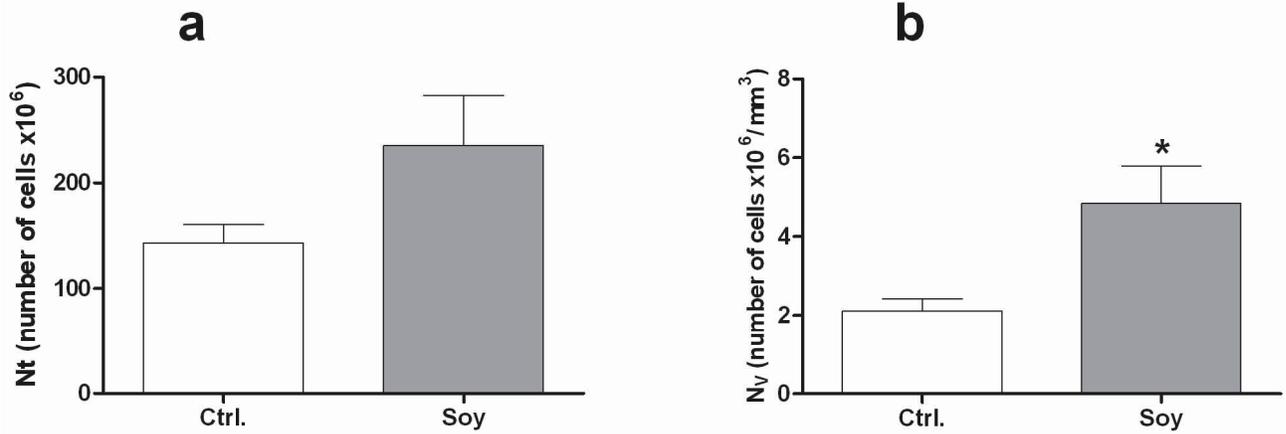


Fig. (4). Quantification of the numerical density and total number of cells in the prostate epithelium; Results are expressed as mean \pm SD. Asterisks indicate significant differences between groups ($p < 0.05$). **a)** Bar graph of total number of cells in the ventral prostate epithelium of the control group and the soy treated group. **b)** Bar graph of the numerical density of cells in the ventral prostate epithelium of the control group and the soy treated group.

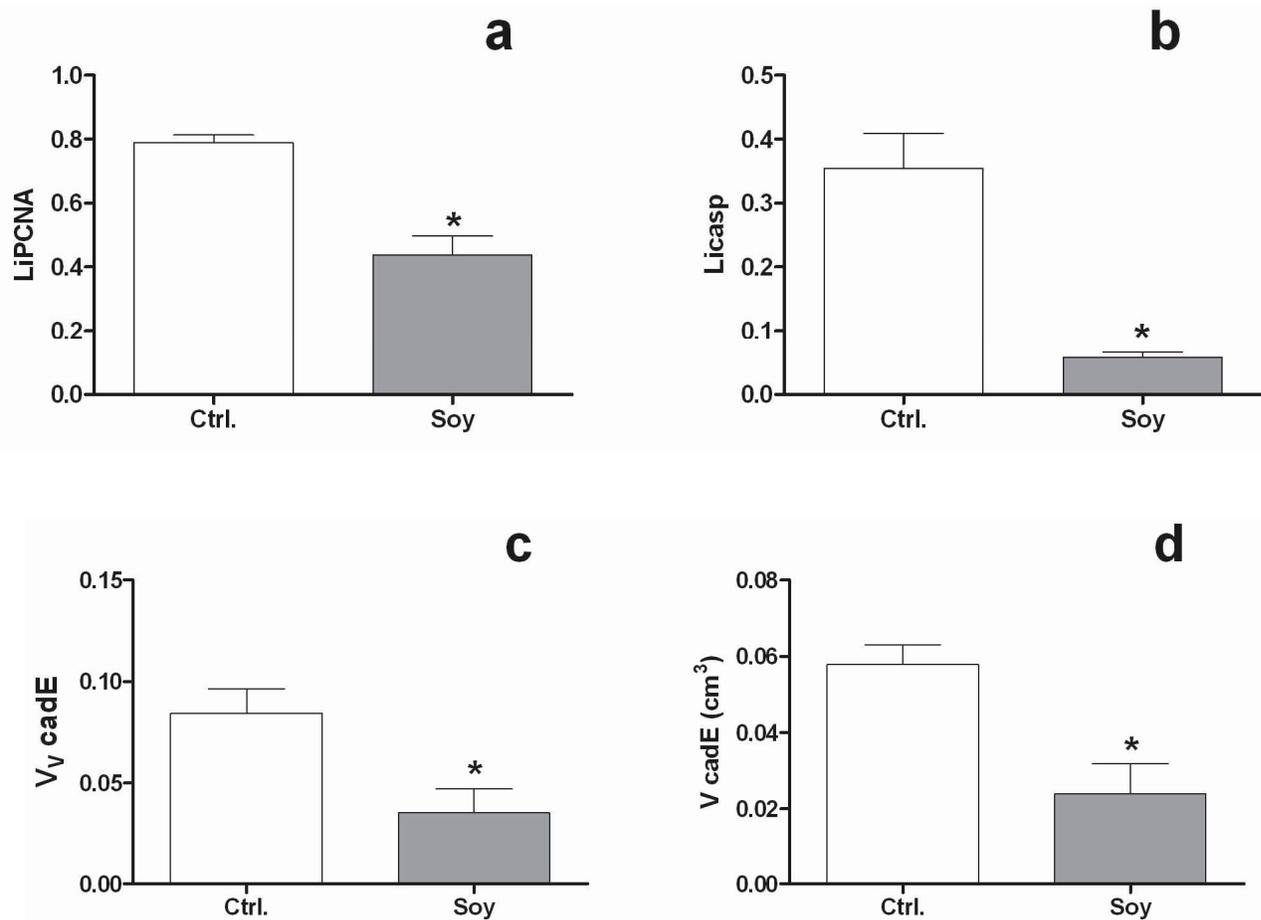


Fig. (5). Quantification of labeling indices and volumes of immunostaining; Results are expressed as mean \pm SD. Asterisks indicate significant differences between groups ($p < 0.05$). **a)** Bar graph of PCNA labeling index in the control group and in the treatment group. **b)** Bar graph of cleaved caspase 3 labeling index in the control group and in the soy treated group. **c)** Bar graph of the volume fraction of E-cadherin in the control group and in the soy treated group. **d)** Bar graph of the absolute volume of E-cadherin in the control group and in soy treated group.

Table 1. Summary of results expressed in figures 3-5.

Quantificated Feature	Significant Differences Between Groups p<(0.05)
Total prostate volume	Yes
Volume of the ventral prostate	No
Volume fraction of the ventral prostate epithelium	No
Total volume of the ventral prostate epithelium	No
Volume fraction of gland lumen in the ventral prostate	No
Total volume of gland lumen in the ventral prostate	No
Total number of cells in the ventral prostate epithelium	No
Numerical density of cells in the ventral prostate epithelium	Yes
PCNA labeling index	Yes
Cleaved caspase 3 labeling index	Yes
Volume fraction of E-cadherin	Yes
Absolute volume of E cadherin	Yes

In the present study some animals in the control group developed dysplastic lesions in the ventral prostate, beginning with a simple thickening of the epithelium and becoming a true epithelial dysplasia. This progression could be associated with aging, since it affected the untreated group. In animals fed with soy-enriched chow, the frequency of dysplasia is lower than in controls. This might be due to the estrogenic activity of soy that may have affected the development of prostate epithelium and then could protect against the development of dysplastic lesions.

Some studies show that prostatitis promotes changes in the epithelium that may evolve to a benign hyperplasia or even cancer [31, 32]. Prostatitis found in the control group is also a damage associated with aging [24]. The percentage of prostatitis in the soy treated group was smaller than in the control group, according to several studies [11, 33]. Kwon *et al.* [34] show that subcutaneous administration of isoflavones or orally administrated in pharmacologic doses (50-500 mg/kg/day), may cause prostatitis but confined to the lateral prostate. In this work we used doses within that range, but we did not study the lateral prostate with the exception of the gland volume, which may change due to prostatitis.

Loeb *et al.* [32] indicate that the volume of the prostate differs widely between individuals of advanced age. This is because it is common to develop hyperplasia with age, but it is also usual that the aging epithelium suffers an atrophic process as a result of a diminished testicular function. Therefore, we may find aged individuals that have their prostate volume increased and others in which it remains unchanged or even decreased.

In soy-treated animals, the epithelium of the prostate acini shows an atrophic process much more relevant than in controls. Cline *et al.* [35] described this effect of the soy and

associated it to its estrogenic activity. Quantitatively, there is a decrease in the volume of the epithelium but it is not significant. The volume of the gland lumen does not have much variation, neither does the ventral prostate volume. However, there is a significant change in the overall volume of the prostate, so the changes in the gland volume should have occurred mainly in the dorsolateral lobes of the prostate.

In this study the ventral lobes were chosen instead of the dorsolateral ones because there are many studies that have not found dysplasias in the dorsolateral lobes and others that show them in the ventral prostate [36-38].

The amount of epithelial cells per unit of epithelial volume was significantly increased in soy-treated rats. This increase in the relative number of cells is due to the atrophy of the epithelium and does not reflect a true increment of the total number of cells, because the absolute size of the epithelium in treated animals is quite similar to that observed in the control group. This insignificant change in the total number of cells could be related to the low expression of cleaved caspase 3 in soy-treated animals. The cleaved caspase 3 is one of the molecules involved in the activation of apoptosis, so in the group treated with soy this process is considerably less active than in controls. A lower rate of cellular death may turn out in a higher number of cells, although they are old aged, which may be associated to the atrophy observed in the epithelium. Having a higher number of cells in the same volume of epithelium, the numerical density is increased.

PCNA expression has been used in numerous studies as a marker of cell proliferation and it is even being considered to improve the accuracy in the diagnosis of prostate cancer [39, 40]. Abundant expression of PCNA was found in all animals in this study except in atrophic areas.

In soy-treated animals there were many atrophic areas, and it is in this group that we found a lower PCNA labeling index. This suggests that there is less cell proliferation by treating animals with soy, which can also be related to the atrophy observed in the epithelium.

Many works studying the effect of soy and its derivatives against cancer conclude that it is due to its estrogenic effects that the soy modulates the expression of certain genes, mostly related to cell proliferation. Many of these studies, using the PCNA labeling index, found that cell proliferation decreases when treating animals with soy or its derivatives [41, 42]. This agrees with our results.

There is evidence that the estrogenic effect of soy also affects genes that regulate the expression of E-cadherin [43, 44]. Soy causes atrophy in the epithelium. Therefore there is less contact surface between epithelial cells and the expression of E-cadherin may be reduced. Thus, although soy intake reduces the incidence of dysplastic lesions, it may increase the number of invasive tumors in animals that already have tumors [45].

The following conclusion can be drawn: a) Soy treatment induces atrophy in the epithelium, which is evidenced by an increased cell density without changes in the absolute number of cells. b) Soy intake over a long period may reduce the prostatic cell proliferation as it is shown in the PCNA labeling index. c) The apoptosis is also diminished through caspases way. d) The expression of E-cadherin is reduced probably due to the reduction of the epithelium thickness.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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