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Molecular and morphometric analysis of the rat ventral prostate injected with leptin

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ABSTRACT

The aim of this study was to evaluate the effect of leptin administration on the ventral prostate lobe of adult rat. Twenty adult male rats were divided into 2 groups: L-animals were daily injected with 50 μL of leptin (8 μg/100 g BW, subcutaneous) for four days and C-animals received the same volume of saline solution. Lipid profile and testosterone serum levels were evaluated. The prostate ventral lobe was processed for histomorphometric analysis. Gene expression of aromatase, androgen, leptin and estrogen receptors isoforms was evaluated by real-time PCR. Cell proliferation was evaluated by PCNA immunohistochemistry. Data were expressed as mean ± standard error and analyzed by student's *t*-test. Serum levels of cholesterol (C = 39.7 ± 4.2;L = 55.2 ± 4.2, mg/dL; $P<0.02$) increased and testosterone (C = 1.6 ± 0.43;L = 0.6 ± 0.15, ng/dL; $P<0.03$) decreased in L group. The histomorphometric analysis showed a reduction in cell density (C = 8868 ± 242; L = 8211 ± 210, mm^2; $P<0.04$), in total (C = 0.24 ± 0.026; L = 0.10 ± 0.009, mm^2; $P<0.001$) and in the internal acini areas (C = 0.16 ± 0.009; L = 0.08 ± 0.006, mm^2; $P<0.0002$). On the other hand, there was an increase in the epithelial height (C = 17.3 ± 0.3; L = 22.8 ± 0.2, μm; $P<0.0001$) and in the number of acini (C = 7.0 ± 0.2; L = 8.7 ± 0.1, mm^2; $P<0.0002$). The histomorphometric analyses together with PCNA immunohistochemistry results suggest that leptin increases cell proliferation. In relation to the gene expression, leptin treatment increased the expression of all genes, but ER-α, in more than 200 times compared to the expression in C group. In conclusion, in this paper we showed that leptin has a direct effect on the prostate gland of adult rats leading to an increase in proliferation and in the gene expression of aromatase, androgen, leptin and estrogen receptors isoforms that are important for the physiology of the prostate gland.

1. Introduction

The prostate is a sexual gland that produces important substances for the potency of sperm to fertilize eggs within the female reproductive tract. In order to accomplish this task, the prostate gland is finely regulated by neural and hormonal mechanisms, and possesses a complex histological organization. The function of the prostate is under complex endocrine control. Androgens have a strong impact on the prostate in adult subjects in terms of the maintenance of its morphology and secretory activity, with the ventral prostate being the main area that responds to androgen stimulation [1,2]. Prostate responses to androgens are mediated by the wide distribution of androgen receptors (AR) in epithelial cells, smooth muscle, and stroma [3]. Testosterone also requires conversion to dihydrotestosterone (DHT) in the prostate gland for full activity [4] and is metabolized to estrogens locally within the prostate via the aromatase enzyme [5].

Although the prostate is an androgen-dependent tissue, its physiology and pathology are also influenced by estrogens. Estrogens have significant direct and indirect effects on prostate gland development and homeostasis and have been long suspected in playing a role in the etiology of prostatic diseases [6]. Accordingly, estrogen receptors (ER) α and ß are expressed in the prostatic stroma and epithelium [7]. ER-α mediates a number of adverse effects upon the prostate, specifically aberrant proliferation, inflammation and malignancy. The induction of inflammation by ER-α may also promote the development of malignancy, as well as stimulate aromatase expression, which is altered in malignancy and may be driven by inflammatory cytokines, stimulating a cycle of activity. ER-β subsequently exerts a number of beneficial and protective effects upon the prostate, which may be anti-proliferative, anti-inflammatory, and anti-carcinogenic. Although currently unknown, the anti-inflammatory actions of ER-β may potentially also result in decreased aromatase expression, and, therefore, reduced local estrogens [5].

Leptin, a hormone discovered in the last decade, is 167-aminoacids product of ob gene expression [8] and although it is predominantly synthesized by adipocytes, the expression of the hormone has been detected in several other tissues including prostate [9]. To date, six splice variants of leptin receptors have been described. Among them the long isoform, OB-Rb, and the short one, OB-Ra, are the most relevant [10]. Despite leptin receptors are found in the hypothalamus, where it is involved in the regulation of feeding behavior

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[11], it is also expressed in several peripheral tissues such as prostate [9].

The leptin is considered a mitogenic, anti-apoptotic [12] and angiogenic mediator [13], because it activates many genes involved in cellular proliferation and angiogenesis regulation [14]. Data derived from leptin-deficient animals or humans have highlighted the importance of leptin in reproductive function and have suggested direct effects of leptin at the pituitary level to control reproduction [15].

The importance of the study arises from the fact that obesity seems to be a risk factor for the development of prostate tumours and increased leptin levels observed in obesity have been pointed as potential mediators which may promote prostate cancer progression and increase the risk of aggressive cancer. There is also multiple biological links between obesity and PCa including higher estradiol, insulin, insulin-like growth factor-1 (IGF-1), leptin and lower free testosterone, all of which may promote more aggressive cancers [16].

Nevertheless, the association between leptin and prostate cancer is a controversial issue since the results obtained by different studies differ. But most previous studies designed to address this question have been performed using cancer cell lines [17–19], and the effect of leptin on normal prostate cells was unknown.

The aim of this study was to evaluate whether leptin administration in adult rats promotes changes on the morphology of the prostate ventral lobe, the gene expression levels of leptin receptors, aromatase, and androgen and estrogen receptors that could be related to prostate cancer.

2. Methods

2.1. Experimental design

The project was developed on the Molecular and Cell Biology Laboratory on the Urogenital Research Unit. The handling and use of the animals followed the principles described in Marques et al 2009 [20]. Animal experimental protocols were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro CEUA/036/2010. Twenty male adult Wistar rats with 180 days of age were kept in a room with controlled temperature (18 °C ± 1 °C) and dark–light cycle (7:00–19:00 h). The animals were divided into two groups of 10 rats each: Leptin (L) - each animal was injected with 50 μL of rat recombinant leptin in a dose previously described in the literature [21–24] (8 μg/100 g BW, subcutaneous, PeproTech, cat # 400–21); Control (C) – each animal was injected with the same volume of saline solution. All the injections were given daily for four days, after the last injection all animals were killed with an overdose of thiopental. Blood samples were collected by cardiac puncture, processed and the serum stored at −20 °C for subsequent hormonal assay with specific radioimmunoassay and evaluation of lipid profile. The prostate ventral lobe was excised, weighed and divided into two fragments. One fragment was immediately stored on liquid nitrogen for Real Time PCR analysis, while the second one was fixed in 10% formalin, processed and included in paraffin for further histomorphometric analysis. Body weight and food intake were evaluated from the beginning until the end of the experiment.

2.2. Radioimmunoassay (RIA)

Testosterone serum concentrations were determined by specific RIA for the hormone (INC Pharmaceuticals, INC, CA, U.S.A). The variation coefficient within and between trials was 4.6 and 7.5% respectively, with RIA sensitivity 0.04 ng/mL.

2.3. Lipid profile

Lipid profile was measured using Bioclin commercial kit for triglycerides (K055), total cholesterol (K083) and HDL cholesterol (K071).

2.4. Real Time PCR

Total ribonucleic acid was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All RNA samples were rid of contaminating DNA by using DNA-free reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One μg of RNA was reversed transcripted with Superscript III (Invitrogen). The Real Time PCR amplification was performed using 2 μL of cDNA, specific primers for each gene and SyBR Green reagent (Invitrogen), in a final volume of 20 μL. The reactions were performed in triplicate. The primer sequences used are described in Table 1. Moreover, β-actin was used as internal control.

2.5. Histomorphometric analysis

Prostate samples were fixed in 10% buffered formalin, processed and embedded in paraffin. Sections of 5 μm were made and stained with Picro-Sirius Red for histomorphometric analysis of epithelium height and cell density with magnification of 1000×. The height epithelium acinus was calculated by measuring the distance between the basal pole and the apical pole of the acini epithelium cells. Ten linear measurements were made in 5 distinct fields randomly distributed throughout the acini in a total of 250 final measurements, expressed as μm. Cell density was measured by the proportion of acinar epithelium cell's nucleus numbers over an area selected and expressed as mm^2.

Internal and total acinar area analysis was performed in a microscopic magnification of 100×, where 10 acini per animal was selected and measured in a total of 50 acini in each group. The number of acinus was measured in a microscopic magnification of 100× by the proportion of acinus numbers over an area selected and expressed as mm^2. The Image_J 1.41 program (NIH, Bethesda, USA) was used for all histomorphometric analysis.

2.6. Histochemistry

For histochemistry analysis five deparaffined sections of each group were hydrated, treated with buffer TRIS–EDTA (pH 9.0) overnight at 60 °C for antigen retrieval, and then treated with 3% hydrogen peroxide solution in methanol for 10 min to block endogenous peroxidase activity. These steps were followed by washing the sections in PBS and

Table 1
Sequence of primer used for amplification in the Real Time PCR reaction.

Gene	Sequence
β actin	5' - ctgtccctgtatcgctctggtc - 3'
	5' - tgaggtagtccgtccggtccc - 3'
Aromatase	5' - tggagtgtgcatctgccat - 3'
	5' - tgctgtgtgtcttcagggag - 3'
Androgen Receptor (AR)	5' - ggcaaaggcactgaagagac - 3'
	5' - cccagagctacctgcttcac - 3'
Short isoform of Leptin receptor (Ob-Ra)	5' - taccaacctcccaacagtcc - 3'
	5' - agcatatgccccaactgaac - 3'
Long isoform of Leptin receptor (Ob-Rb)	5' - ctgaagaaaatcacggggaa - 3'
	5' - tgaacagacagtgagctggg - 3'
Estrogen Receptor β (ER-β1)	5' - gaagctgaaccacccaatgt - 3'
	5' - caatcatgtgcaccagttcc - 3'
Estrogen Receptor β (ER-β2)	5' - aggtgctaatggtgggactg - 3'
	5' - gccagtgagggtcttctgag - 3'
Estrogen Receptor α (ER-α)	5' - tccggcacatgagtaacaaa - 3'
	5' - tgaagacgatgagcatccag - 3'

subsequently incubating 10 min at room temperature with 10% goat serum to block unspecific binding. The sections were then incubated for 2 h at 37 °C with Mouse anti-PCNA - Proliferating Cell Nuclear Antigen (Invitrogen, CA, USA, cat#180110), diluted 1:200 in PBS with 1% BSA. Sections were then washed in PBS and incubated at room temperature for 20 min with biotinylated secondary antibody followed by incubation at room temperature for 10 min with streptavidin–peroxidase conjugate (Histostain-Plus Kit, Invitrogen, CA, USA). Sections were washed in PBS, then revealed with liquid diaminobenzidine (Histostain-Plus Kit, Invitrogen, CA, USA), and then counterstained with hematoxylin. The negative controls were processed by replacing the primary antibody with PBS and no indication of staining was observed. Benign prostate human was used as positive control.

2.7. Statistical analysis

Data were presented as mean ± standard error. The Kolmogorov-Smirnov one sample tests (K-S) was used to assess the normality of the distributions of each of the variables. Statistical significance was determined by Student *t*-test and the level of significance considered was $P<0.05$.

3. Results

Food consumption, body and prostate weights are shown in Fig. 1. The leptin treatment led to a significant reduction in food consumption (Fig. 1A) and no alteration in the body weight (Fig. 1B). In relation to food consumption, there was a significant ($P<0.0001$) reduction in the L group at days 2 and 3 compared to day 1. The food consumption was also significantly ($P<0.0001$) reduced in the L group at days 2 and 3 compared to the C group at the same days. The C group did not show any alteration in the food consumption during the experimental time. The leptin treatment led to a significant ($P<0.03$) increase in both absolute (Fig. 1C) and relative (Fig. 1D) prostate weights.

Lipid profile and testosterone serum concentrations are shown in Fig. 2. There was no significant alteration on triglycerides (Fig. 2A) and HDL (Fig. 2B) levels after leptin treatment, however, cholesterol increased significantly (Fig. 2C, $P<0.02$). Testosterone serum concentration presented a significant (Fig. 2D, $P<0.03$) decrease in the leptin treated group compared to the control one.

Fig. 3 shows the histomorphometric alterations. Leptin treatment led to a significant reduction in cell density (Fig. 3A, $P<0.04$), in the total (Fig. 3B, $P<0.002$) and internal area of the acini (Fig. 3C, $P<0.03$). On the other hand, there was a significant increase in the epithelial height (Fig. 3D, $P<0.0001$) and in the number of acini (Fig. 3E, $P<0.0002$). The photomicrograph which represents the above data is shown in Fig. 4 (Fig. 4A, B, C, D). The immunohistochemistry for PCNA showed that there is an increase in the number of stained cells in the leptin group, revealing an increase in the proliferation rate in this group (Fig. 4E, F).

In relation to the gene expression, the leptin treatment resulted in a significant increase in the expression of all genes evaluated in this study but ER-α which increment was not significant (Fig. 5). The difference in the gene expressions between the groups was about 200 times higher in L compared to C group.

4. Discussion

Obesity, a rapidly growing worldwide epidemic, is linked to development of numerous types of cancer. Also PCa is the third most common cancer in men globally [16]. Leptin has been considered an important link between nutrition and reproduction [11] but only recently the association between both PCa and obesity has been better investigated.

As obesity is associated with altered levels of numerous hormones such as testosterone, estradiol and leptin [16], in this paper we aimed to evaluated the gene expression of their receptors in the prostate gland besides the gland morphology.

As expected, leptin administration was able to reduce food intake although there was no alteration in body weight. Probably the time of leptin administration was not enough to induce a significant body weight loss. The significant reduction in the food intake from the beginning to the end of the experiment in the leptin treatment group suggests that the effect of leptin increases with continuous administration. Although there was no alteration in the body weight, the prostate weight was increased by the leptin treatment. As the

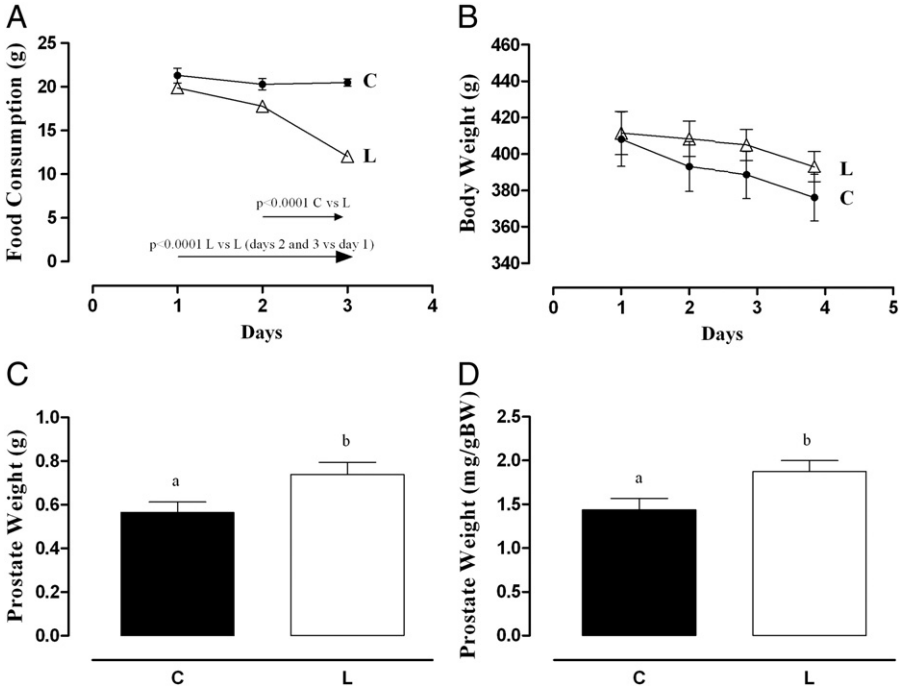

Fig. 1. Food consumption (A), body weight (B), prostate absolute weight (C) and prostate relative weight (D) of adult rats injected with saline solution, control group (C) or injected with 50 μL of rat recombinant leptin, leptin treated group (L). Values are given as mean ± SEM of 10 animals per group. Different letters mean statistical significance.

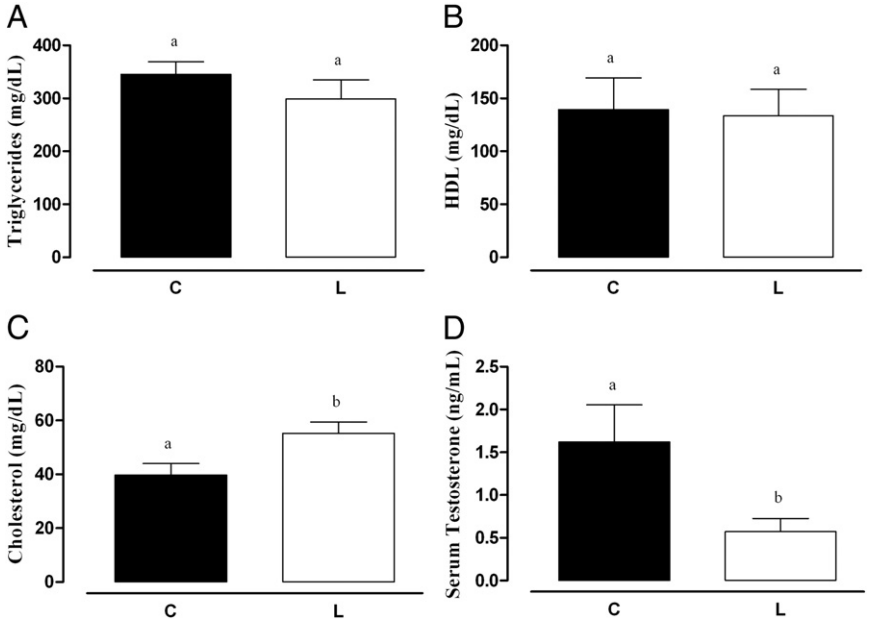

Fig. 2. Serum evaluation of triglycerides (A), HDL (B), cholesterol (C) and testosterone (D) of adult rats injected with saline solution, control group (C) or injected with 50 μL of rat recombinant leptin, leptin treated group (L). Values are given as mean ± SEM of 10 animals per group. Different letters mean statistical significance.

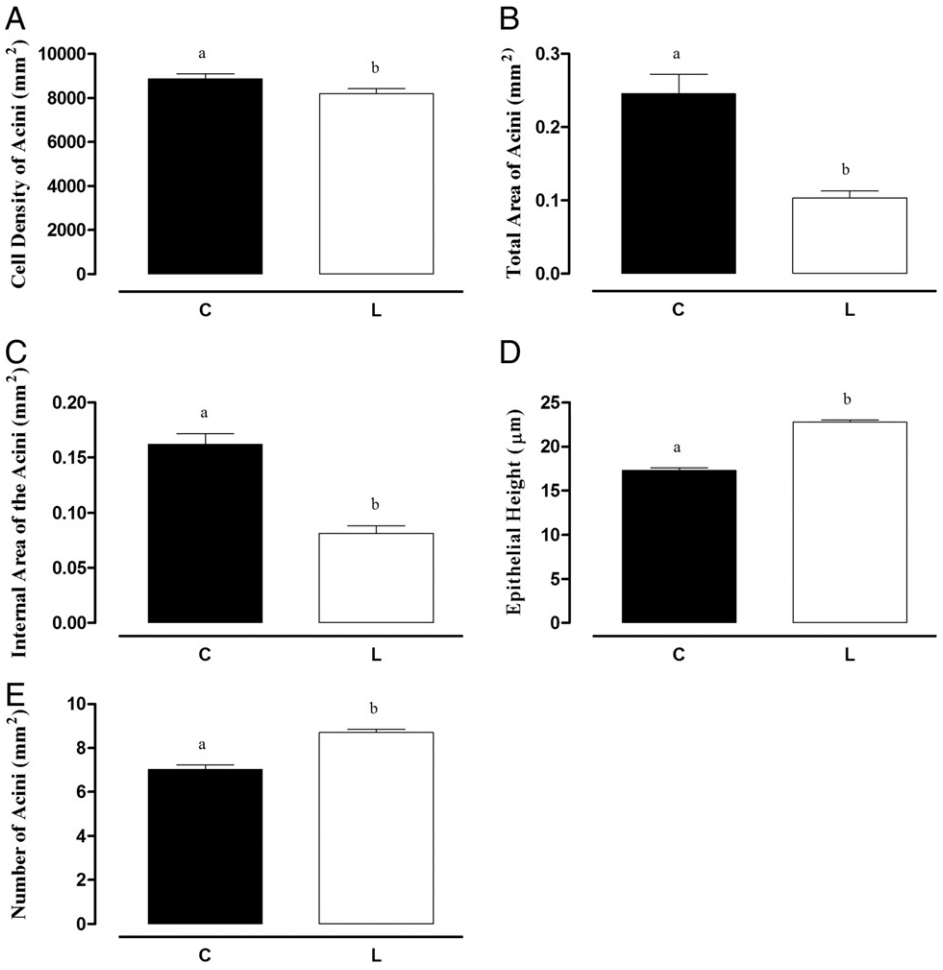

Fig. 3. Morphometric analysis of the ventral prostate lobe of adult rats injected with saline solution, control group (C) or injected with 50 μL of rat recombinant leptin, leptin treated group (L). Cell density (A), total acini area (B), lumen area (C), epithelial height (D), and number of acini (E). Values are given as mean ± SEM of 10 animals per group. Different letters mean statistical significance.

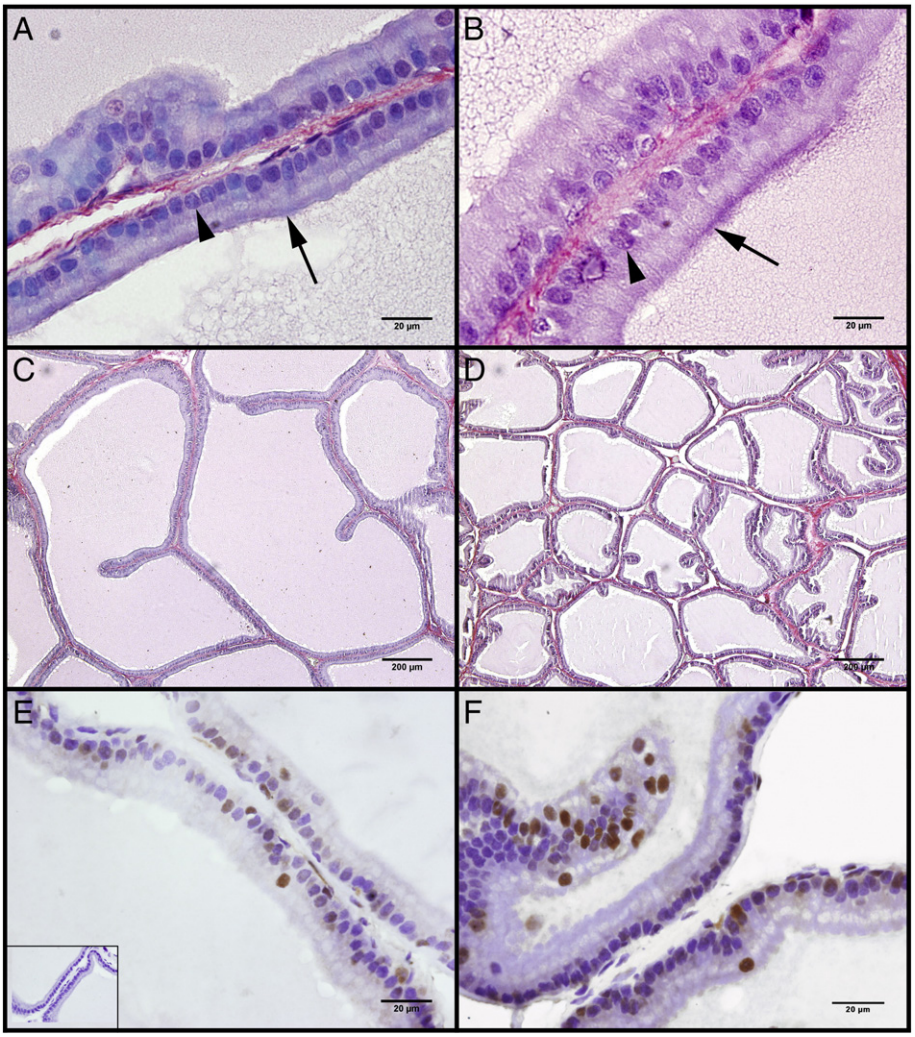

Fig. 4. Photomicrograph of the histomorphometric alterations of ventral prostate lobe of adult rats injected with saline solution, control group (C) or injected with 50 µL of rat recombinant leptin treated group (L). Arrows show the height of the epithelium and arrowheads epithelial nuclei in the control group (A) and leptin group (B). The size and number of acini of both groups are seen in control group (C) and leptin group (D). Immunohistochemistry for PCNA in ventral prostate lobe of adult rats injected with saline solution (E) or injected with 50 µL of rat recombinant leptin (F). Note the increase in the number of stained acinar epithelial cell nuclei in the leptin treated group. (Insert — negative control).

prostate gland express leptin receptors we can hypothesize that the increase in the tissue weight was a result of a direct action of the hormone.

It is known that leptin has proliferative and anti apoptotic effects in normal ovary tissue [25]. The increase in the acini number and in the epithelium height that occurred after the leptin treatment is consistent with proliferative and active acini and could also have contributed to the higher prostate weight in these animals. The histochemistry results for PCNA corroborate the proliferative effects of leptin. The reduction in cell density, total acini and internal areas of the acini could be due to the increment in the number of acini.

Leptin treatment led to an increase in the expression of several genes in the ventral prostate lobe of adult rat. We could not find any previous data showing that leptin treatment in vivo could up regulate the expression of Ob-Ra and Ob-Rb isoforms, aromatase, AR, ER-α, ER-β1 and ER-β2 in the prostate tissue. These results are very similar to our recent publication using an in vitro tissue culture system of ventral prostate [26]. The only difference was that the ex vivo exposure to leptin resulted in decreased ER-ß while the results presented here show an increase in ER-ß expression. We do not have enough data to explain this difference but we cannot forget that in an ex vivo model there is loss of several regulatory factors that in vivo could have interfered with the main regulation of the gland.

It is known that obese men present high serum levels of leptin and estradiol [27]. We can hypothesize that an increase in the expression of both Ob-Rs and ERs could probably mediate the estradiol and leptin effects since they have been linked to PCa [28,29]. In vitro leptin stimulates growth of androgen-independent, but not androgen-sensitive, PCa cell line [17].

Testosterone is also known by influencing growth, proliferation and physiology of the prostate gland [4]. In obese men testosterone serum levels are generally low [30]. Several studies found that, among PCa patients, decreased serum testosterone levels were associated with more aggressive disease, worse prognostic and worse treatment response [31,32].

All of the recent large studies suggest adult obesity is associated with decreased risk of low-grade disease, but increased risk of high-grade, advanced disease. The ultimate explanation for this apparent paradox is likely multifactorial and explains another link between obesity and PCa mortality.

As testosterone serum levels were decreased by leptin treatment, as previously showed by the literature [33,34], we can conclude that the increase in prostate weight, proliferation and gene expression was indeed due a direct effect of leptin treatment.

Several studies support a direct or central effect of leptin on peripheral aspects of lipid metabolism [35–37]. These studies have

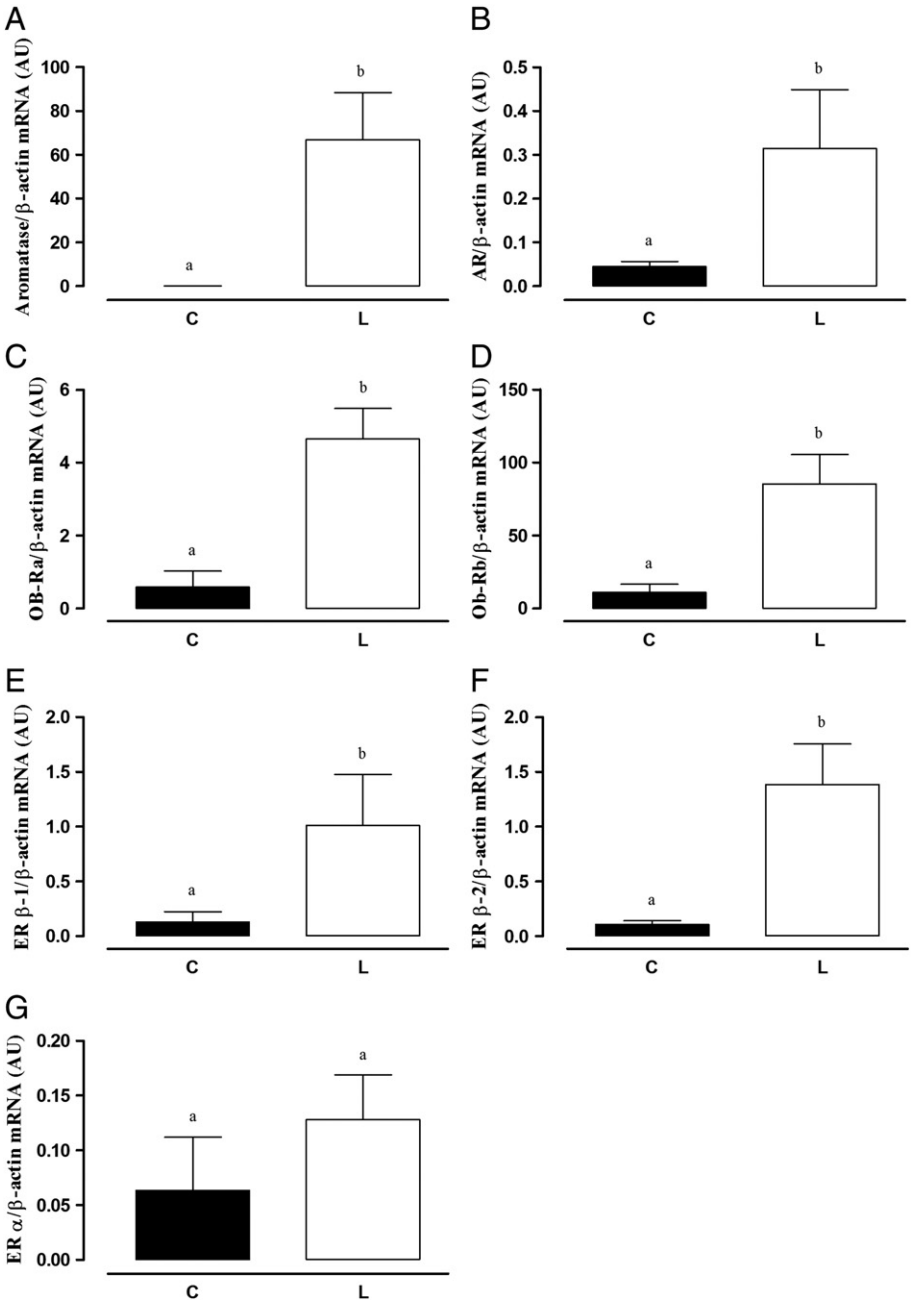

Fig. 5. Gene expression of aromatase (A), AR (B), OB-Ra (C), OB-Rb (D), ER-β1 (E), ER-β2 (F) and ER-α (G) evaluated by Real Time PCR and normalized by β-actin expression in the ventral prostate lobe of adult rats. Control group (C) or of rat leptin treated with group (L). Values are given as mean ± SEM of 10 animals per group. Different letters mean statistical significance.

used different ex vivo and in vitro models making it difficult to place the data within a proper physiological context and to compare with our present results. Despite the difference among our model and the others, we have shown that in vivo leptin treatment affects lipid metabolism by increasing serum cholesterol with no significative change in triglycerides and HDL levels.

In conclusion, in this paper we showed that leptin has a direct effect on the prostate gland of adult rats leading to an increase in proliferation and in the gene expression of aromatase, androgen, leptin and estrogen receptors isoforms that are important for the physiology of the prostate gland.

Conflict of interest

None of the authors have a conflict of interest.

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