

# EFECTOS DEL LETROZOL EN LA PRÓSTATA VENTRAL DE MURINOS

*Letrozole effect in murine ventral prostate*

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## RESUMEN

La glándula prostática es regulada por hormonas esteroides, sus complejas interacciones están en un delicado balance regulando el crecimiento y desarrollo de la glándula. Interesantemente, los cambios en las concentraciones de hormonas esteroides durante la edad senil afectan el equilibrio hormonal y contribuyen con la evolución de los cambios patológicos de esta. En este trabajo se ha analizado el efecto del letrozol, un inhibidor de la enzima aromatasa, sobre la estructura de la próstata ventral de animales intactos y de animales castrados. Los resultados demuestran alteraciones en la fisiología de la glándula después del tratamiento con letrozol: las concentraciones séricas de testosterona, el peso de la glándula y los índices de proliferación de las células epiteliales y luminales aumentaron. Las concentraciones de estrógeno no fueron alteradas dramáticamente, en contraste fue observado el aumento de las concentraciones de las gonadotrofinas en los animales castrados. La castración no alteró el índice de proliferación de las células basales. En los animales tratados con letrozol fue observada la reorganización de los diferentes compartimentos como el epitelio y estroma. Además, una disminución de la expresión del receptor de andrógeno fue observada en los animales después de 21 días del tratamiento con letrozol. Estos resultados fueron confirmados con inmunocitoquímica. Los anteriores resultados revelan aspectos nuevos de la relación entre el receptor de andrógeno y el metabolismo del estrógeno en la glándula prostática,

demostrando que alteraciones en las concentraciones hormonales durante un corto periodo de tiempo pueden inducir alteraciones significativas en el equilibrio de la glándula prostática.

## PALABRAS CLAVE

Próstata, Receptor de Andrógeno, Letrozol, Proliferación Celular

## ABSTRACT

The prostate gland is regulated by steroid hormones and complex interactions based on a subtle balance between androgen and estrogen (E2) regulate prostatic development and physiology. Interestingly, the changes in steroid hormone levels at old ages affect the hormonal milieu and contribute to the evolution of the pathological changes of the gland. We have analyzed the effects of letrozole, an aromatase inhibitor, on the structure in the ventral prostate of control and castrated adult rats. The results demonstrated alterations in prostate physiology after letrozole treatment. Serum levels of testosterone, prostate weight and proliferative index in luminal and basal cells were increased. Estrogen serum levels were not altered dramatically, in contrast to slight increase in gonadotrophin hormones seen in the castrated animals. Castration did not alter the proliferation index of basal cells. Reorganization

of tissue compartments was seen with significant increase in letrozole treated animals. A decrease in androgen receptor expression was seen 21-days after the beginning of treatment with letrozole. These results were confirmed by immunohistochemistry. These results reveal new aspects in the relationship between androgen receptor and steroid metabolism in the prostate gland, demonstrating that alteration in hormone levels during a short time period induces significant alterations in prostate homeostasis.

## KEY WORDS

prostate, androgen receptor, letrozole, cell proliferation

## INTRODUCTION

Prostate development is primarily influenced by androgens; testosterone (T) and its active metabolites elicit regulatory responses of urogenital sinus (UGS) from epithelial bud induction up to the total differentiation and functional maturity. However, prostate is also an estrogen-targeted organ and the expression of the estrogens receptors,  $\alpha$  and  $\beta$ , in prostate gland together with the presence of enzymes involved in estrogen metabolism suggest important role of this hormone in differentiation and metabolism of prostate gland<sup>1,2,3</sup>.

The biosynthesis of estrogens occurs via metabolism of an androgenic substrate and the enzyme involved in the irreversible transformation of androgen in estrogens is a complex known as aromatase, which is present in the endoplasmic reticulum of numerous tissues<sup>4</sup>, including the prostate gland, suggesting a direct intraprostatic effect of estrogen<sup>5</sup>. The aromatase complex is composed of two proteins; the NADPH-cytochrome p450 reductase and a cytochrome p450 aromatase, which contains the heme and the steroid binding pocket<sup>6</sup>. In humans, the p450arom is the product of a single Cyp19 gene, belonging to the cytochrome p450 family<sup>7</sup>.

The aromatase-modulated transgenic mice, such as the aromatase-overexpressing (AROM+) mouse and the aromatase-knockout mouse (ArKO) allowed the determination of the effects of estrogen/androgen unbalance in male reproductive functions<sup>4,8</sup>. From these effects, it is remarkable the prostate atrophy in AROM+ mice and prostate enlargement, elevated peripheral and intraprostatic androgen levels and increased androgen receptor expression in ArKO mice<sup>9</sup>.

Other important alterations are age-dependent changes in the hormonal balance, marked by a decrease in serum testosterone to estradiol ratio, when coupled with alterations in the concentrations of steroid receptors, may contribute to the evolution of pathological changes observed in benign prostatic hyperplasia (BPH) and carcinoma of prostate gland among older men<sup>10</sup>.

As shown in other reports, androgen receptor is expressed along the entire prostatic ducts in all three lobes from adult rats where it elicits the different responses after T and DHT exposure<sup>11, 12</sup>. Many studies are focused in the altered pattern of expression after exogenous compounds. For example, the normal developmental of the rat prostate lobes is affected by exposure of the neonatal animals to estrogens resulted in an altered pattern<sup>13</sup>. The purpose of this study was to investigate the effect of letrozole, an aromatase inhibitor, on androgen receptor expression in the ventral prostate structure and androgen receptor expression of the non-castrated and castrated rats, aiming at identifying processes that affect AR levels and AR function, and that might thereby influence proliferation and viability of androgen dependent cells.

## MATERIALS AND METHODS

### TREATMENTS

A total of 40 male Wistar rats (12 weeks old) were housed in standard plastic cages, maintained under controlled conditions (lights on 0700-1900h, temperature 20-24 oC) and allowed to water and rat chow ad libitum. The rats were divided into ten different groups: intact rats (Control), non-castrated rats treated with letrozole during seven days (LET 7), castrated letrozole-treated rats (CASLET) and castrated (CAS) with no further treatment. Analyses were performed 7, 14 and 21 days after treatments. Treated rats received 1 mg/kg/day of aromatase inhibitor, letrozole, dissolved in corn oil by oral gavage<sup>14</sup>. This dose has been previously demonstrated to produce a significant decrease in the concentration of estrogen in the serum of female rats<sup>15</sup>. Control rats received the same volume of the vehicle. Animals were killed by cervical dislocation. The prostates were removed, and ventral prostate (VP) were dissected. For immunohistochemical studies, VPs were fixed in 4% paraformaldehyde and embedding in paraffin. The experiments were carried out according to the Guide for Care and Use of Laboratory Animals and were approved by the Committee for Ethics in Animal Experimentation.

## **CHEMICALS AND ANTIBODIES**

Letrozole was purchased from Novartis (Basilea, Swiss), corn oil and protease inhibitor cocktail, were obtained from Sigma Chemical Co. (St Louis, MO, USA). Monoclonal mouse anti-Ki67 antibody was purchased from Dako (Carpentaria, CA, USA). Anti-AR rabbit polyclonal anti-AR (N-20) primary antibody and luminal reagent were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and The EasyPath ABCComplex/HRP kit from Novocastra Laboratories (Newcastle upon Tyne, UK).

## **HORMONE MEASUREMENT**

Plasma levels of testosterone (T), estradiol (E2), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were estimated by RIA. Blood samples were obtained by cardiac puncture immediately before death. The plasma was separated by centrifugation and stored at -20 C for subsequent hormone assays. T and E2, concentrations were measured in serum samples using Coat-a-count kits (Diagnostic Products, LA, CA, USA). Serum samples were assayed in duplicate.

Plasma LH and FSH were measured by double-antibody radioimmunoassay using specific kits provided from the National Hormone and Peptide Program (NIH / NIDDK, USA). All samples in the same experiment were measured in the same assay. The lowest detectable amount of LHRP3 standard was 0.05 ng/ml and the intra-assay coefficient of variation was 4%. The lowest detectable amount of FSHRP2 standard was 0.2 ng/ml and the intra-assay coefficient of variation was 3.2 %.

## **HISTOLOGY**

The VP was immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. Samples were then washed, partially dehydrated and embedded in Leica historesin. Two micrometer sections were obtained and stained with hematoxylin and eosin <sup>16</sup>.

## **IMMUNOHISTOCHEMICAL LOCALIZATION OF ANDROGEN RECEPTOR**

Paraffin-embedded sections (6  $\mu$ m) of VP were dewaxed and rehydrated in an ethanol graded series. Antigens were retrieved by boiling in 10 mM citrate buffer (pH 6.0) 3 times of 5 min. The cooled sections were incubated in 1% H<sub>2</sub>O<sub>2</sub> for 15 min to quench endogenous peroxidase.

To block nonspecific binding, sections were incubated in BSA 3% for 1h at room temperature. Sections were then incubated with anti-AR (1:100) or anti-Ki67 (1:100) in 1% BSA at 4°C. Negative controls were incubated with 1% BSA without primary antibody. The ABC kit was used to visualize the signal, according to manufacturer's manual. The sections were incubated in appropriated secondary antibody solution for 30 min followed by washing for 10 min with PBS and incubation in Avidin and Biotinylated HRP solution for 30 min. After washing in PBS, sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride substrate, lightly counterstained with methyl green, dehydrated through ethanol series and xylene, and mounted.

## **COUNTING OF PROLIFERATING CELLS**

To quantify and compare proliferation rates between VP of control and treatments groups, dividing cells were assessed by counting Ki67-positive and Ki67-negative (methyl green-stained) nuclei at 400 x magnification. A total of three immuno-stained sections per treatment were used. Separated counts were made for the epithelial and stromal compartments of the gland. Approximately 2,000 cells were counted for each treatment. The number of positive cells was expressed as the percentage of Ki67-positive cells.

## **STATISTICAL ANALYSIS**

Body weights, tissue weights, hormone serum levels (T, E2, LH and FSH), stereological data, cell proliferation and cell death index, were analyzed for statistical significance by analysis of variance (ANOVA). Comparisons among individual treatments conditions within each time period were further examined by Tukey's test for multiple comparisons using MINITAB® Release 14.20. Results were considered to be statistically significant when  $p < 0.05$ .

## **RESULTS**

### **BODY AND ORGAN WEIGHTS**

Body, prostate, and relative testis were not affected by letrozole treatment (Table 1).

After seven days, castration led the prostate to a 70% regression. After 14 and 21 days of castration the regression was 80%. The letrozole administration in castrated rats did not alter this effect.

**Table 1.** Body, prostate, relative prostate, and testis weight in grams, of adult rats after treatment with different drugs (values are given as means  $\pm$  SEM n=4).

TREATMENT	DAY	BODY WEIGHT (G)	PROSTATE (MG)	RELATIVE PROSTATIC WEIGHT	TESTIS (G)
CONTROL		336.75 $\pm$ 16.66	305.08 $\pm$ 30.67	0.090 $\pm$ 0.00	3.73 $\pm$ 0.07
LETROZOLE	7	327.50 $\pm$ 13.67	340.68 $\pm$ 21.61*	0.104 $\pm$ 0.00	3.59 $\pm$ 0.13
	14	314.75 $\pm$ 7.97	293.20 $\pm$ 32.83	0.093 $\pm$ 0.00	3.36 $\pm$ 0.14
	21	329.75 $\pm$ 25.41	330.73 $\pm$ 21.24	0.101 $\pm$ 0.00	3.36 $\pm$ 0.11
CASTRATED	7	317.25 $\pm$ 6.40	87.05 $\pm$ 18.51*	0.027 $\pm$ 0.00*	—
	14	330 $\pm$ 21.09	33.58 $\pm$ 5.52*	0.010 $\pm$ 0.00*	—
	21	326.50 $\pm$ 19.77	34.53 $\pm$ 8.19*	0.010 $\pm$ 0.00*	—
CASTRATED PLUS LETROZOLE	7	340.50 $\pm$ 25.51	74.73 $\pm$ 15.56*	0.022 $\pm$ 0.00*	—
	14	312 $\pm$ 12.33	38.23 $\pm$ 13.69*	0.012 $\pm$ 0.00*	—
	21	331 $\pm$ 20.30	32.43 $\pm$ 8.83*	0.009 $\pm$ 0.00*	—

\* =  $P < 0.05$ , as compared to the control

### SERUM HORMONES CONCENTRATIONS

Testosterone levels were altered after treatment with letrozole during the 14 day of the experiment (Table 2). At seven and 21 days of treatment the intact rats did not present significant alterations when compared with the control. In castrated animals

(treated or not with letrozole) T levels were below to detection level. LH and FSH levels in control group were  $3.8 \pm 0.7$  and  $8.7 \pm 1.6$ , respectively. Letrozole treatment did not affect these values. However there was a tendency in castrated animals for increased gonadotrophin levels. Serum estrogen levels were stable in all treatments.

**Table 2.** Serum Testosterone, Estradiol, Luteinizing hormone and Follicle stimulating hormone levels of adult rats after treatment with different drugs (values are given as means  $\pm$  SEM n=4).

TREATMENT	DAY	TESTOSTERONE (NG/DL)	ESTRADIOL (PG/ML)	LH (NG/ML)	FSH (NG/ML)
CONTROL		308.8 $\pm$ 29.35	16.64 $\pm$ 1.73	3.8 $\pm$ 0.68	8.65 $\pm$ 1.64
LETROZOLE	7	364.78 $\pm$ 15.80	23.45 $\pm$ 1.12	5.15 $\pm$ 0.71	10.9 $\pm$ 0.89
	14	625 $\pm$ 72.50	19.84 $\pm$ 2.10	5.73 $\pm$ 0.99	16.63 $\pm$ 2.09
	21	359 $\pm$ 55	24.65 $\pm$ 0.31	7.3 $\pm$ 0.58	9.53 $\pm$ 0.66
CASTRATED	7	—	20.69 $\pm$ 1.73	6.5 $\pm$ 0.72	24.18 $\pm$ 2.41
	14	—	23.93 $\pm$ 0.68	10 $\pm$ 0.43*	22.50 $\pm$ 0.31
	21	—	21.69 $\pm$ 4.08	12 $\pm$ 0.60*	53.50 $\pm$ 2.10*
CASTRATED PLUS LETROZOLE	7	—	10.74 $\pm$ 0.22	10.8 $\pm$ 0.20*	38.8 $\pm$ 7.60*
	14	—	12.04 $\pm$ 1.18	6.6 $\pm$ 0.56	46.30 $\pm$ 2.79*
	21	—	17.64 $\pm$ 0.02	10.25 $\pm$ 0.92*	40.90 $\pm$ 9.85*

\* =  $P < 0.05$ , as compared to the control

### HISTOLOGICAL ANALYSES

Examination of ducts in rat ventral prostate of adult rats (91-day-old) revealed a simple columnar epithelium, lined with tall columnar secretory epithelial cells (Fig. 1A)

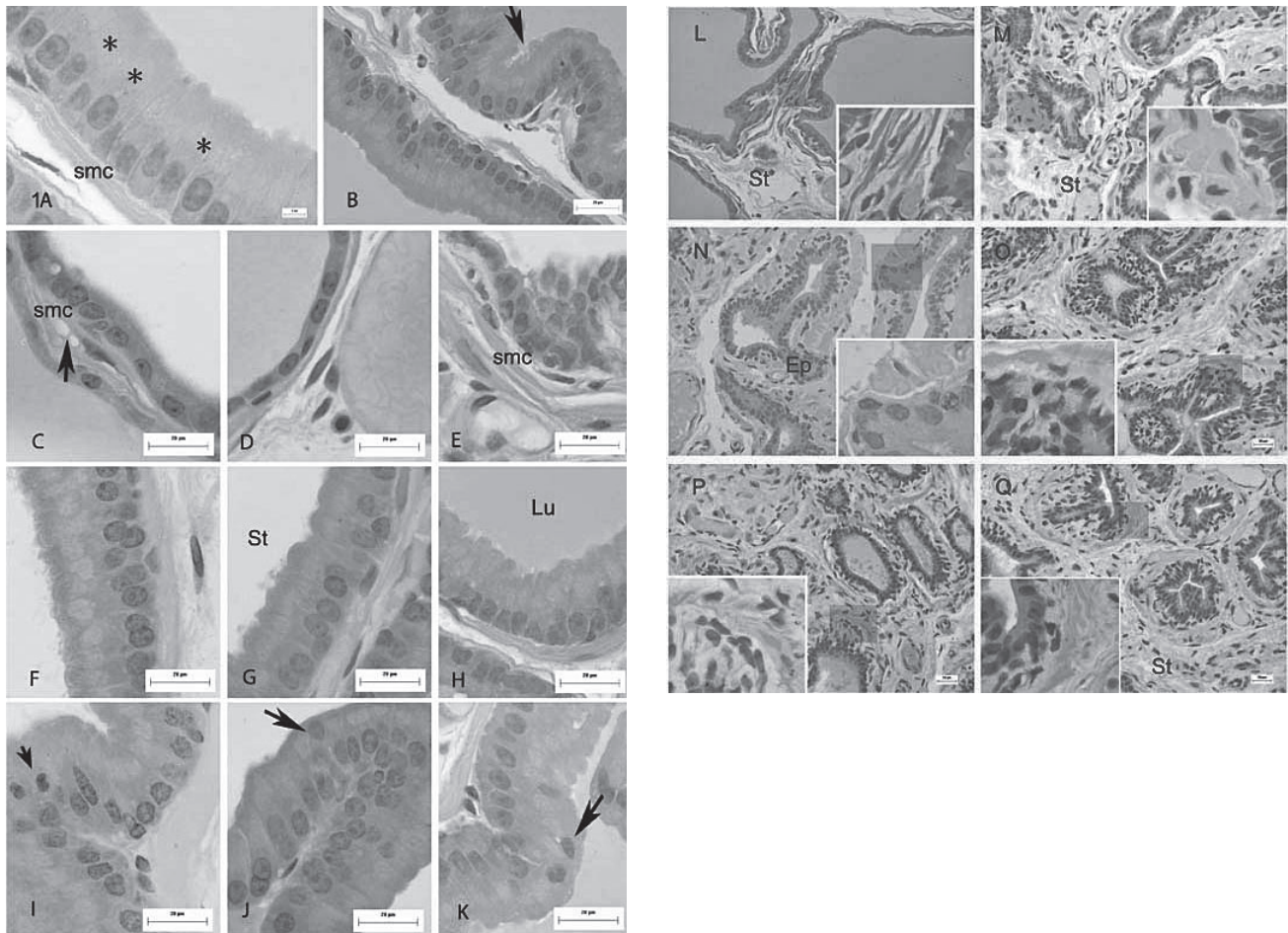
The characteristic infolding of the distal region is also present (Fig. 1B). After 7 (Fig. 1C), 14 (Fig. 1D) and 21 (Fig. 1E) days of beginning of treatment the proximal ductal region did not show differences when compared to the control. The proximal region of letrozole-treated animals in 7th day presented small vesicles in the layers

of stromal cells (Fig. 1C), and proximal regions of the 21st day presents a multilayered sheet of smooth muscle cells. However in absolute terms, a decrease of the smooth muscle cell was detected in the 7th and 21st but not in 14th day of treatment. Intermediate ductal regions (Figs. 1F-H) did not show significant alterations between treatments. Distal ductal regions presented pronounced mitotic activity (Figs. 1I-K), particularly in the 7th day after treatment absolute values of epithelium and lumen were increased and, after 21st days, there was a stimulatory effect only in the luminal compartment.



After seven days of castration, the prostate remodeled dramatically losing its characteristic morphology. The epithelial regression is evident and stromal cells became disorganized. In castrated rats (**Fig. 1L**) and castrated-letrozole treated rats (**Fig. 1M**) different patterns of smooth muscle cells were seen. After 14 days of castration the frequency of rudimentary

ducts increase in the prostatic area and the stromal cells are more evident (**Fig. 1N**). Letrozole-treated rats conserved this pattern (**Fig. 1O**). After 21 days of castration the acini were totally atrophic and their frequency was increased. (**Figs. 1P and Q**). No alteration was seen in the smooth muscle cell compartment.



**Figure 1.** The morphology of control, letrozole-treated, castrated and castrated letrozole-treated rats was examined by H&E staining after seven days of treatment with letrozole, and then collected after 7, 14 and 21 days of the beginning of treatment. A and B, control animals showing the active columnar epithelium and the characteristic infolding of the distal region (arrow).

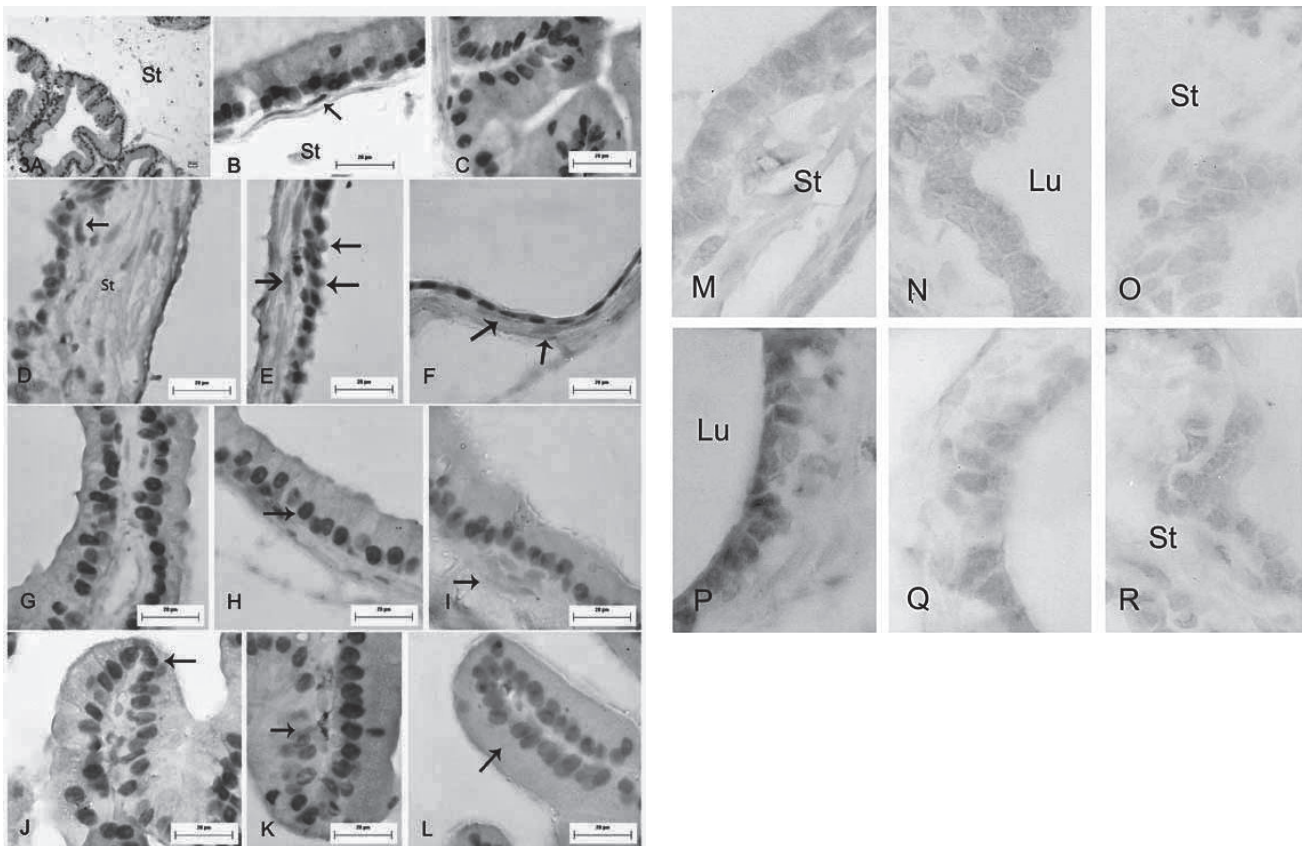
Proximal, intermediate and distal region of letrozole-treated animals after 7, (C, F and I), 14 (D, G and I) and 21 (E, H and K) days of the experiment. The proximal regions of the treatments show different characteristics in the stromal compartment (arrow). Intermediate regions conserved the same morphological organization and distal regions present intense proliferate activity (arrows)

Castrated animals after seven days (L) and treated with letrozole (M) present an epithelial regression. It was also notable different patterns in the stromal cells pattern (inset). After 14 days of castration the atrophied acini, in detail apoptotic figure (N) the treatment with letrozole present disorganization in an epithelial and stromal compartments (O). 21 days after castration the acini are reduced and the stroma is a predominant compartment (P) treatment with letrozole did not alter this pattern (Q). Apparently the letrozole treatment in castrated rats results in a dense cellular stromal phenotype. Lu = lumen; smc = smooth muscle cell, St = stroma.

### IMMUNOLocalIZATION OF ANDROGEN RECEPTOR IN VENTRAL PROSTATE LOBES

In control rats the total epithelial cells were AR-positive (Fig. 2A) and staining was also present in some stromal cells (Fig. 2B). The distal ductal region showed AR- positive cells with different intensity staining (Fig. 2C). In letrozole-treated groups, in the 7th (Figs. 2D, G and J) and 14th day (Figs. 2E, H, and K) the AR-staining in proximal, intermediate and distal regions of the prostate was not modified.

The main alteration in letrozole treated animals was found in 21-day group that showed a decrease in a staining in the three regions of ventral prostate. In the proximal region there was a decrease in staining in epithelial and stromal cells (Fig. 2F). In the intermediate and distal regions AR-staining was decreased (Fig. 2I) in the epithelial cells (Fig. 2L). Castration had a strong effect on AR-staining, which was completely abolished. Letrozole application did not alter this pattern (Fig. 2B).



**Figure 2.** Histological sections of the rat ventral prostate immunocytochemically stained for AR. In the control 91 day-old rats (2A-C) most of the epithelial cells within the acini were AR (+) (arrows). It was detected a presence of the stromal cells AR- positive (arrow).

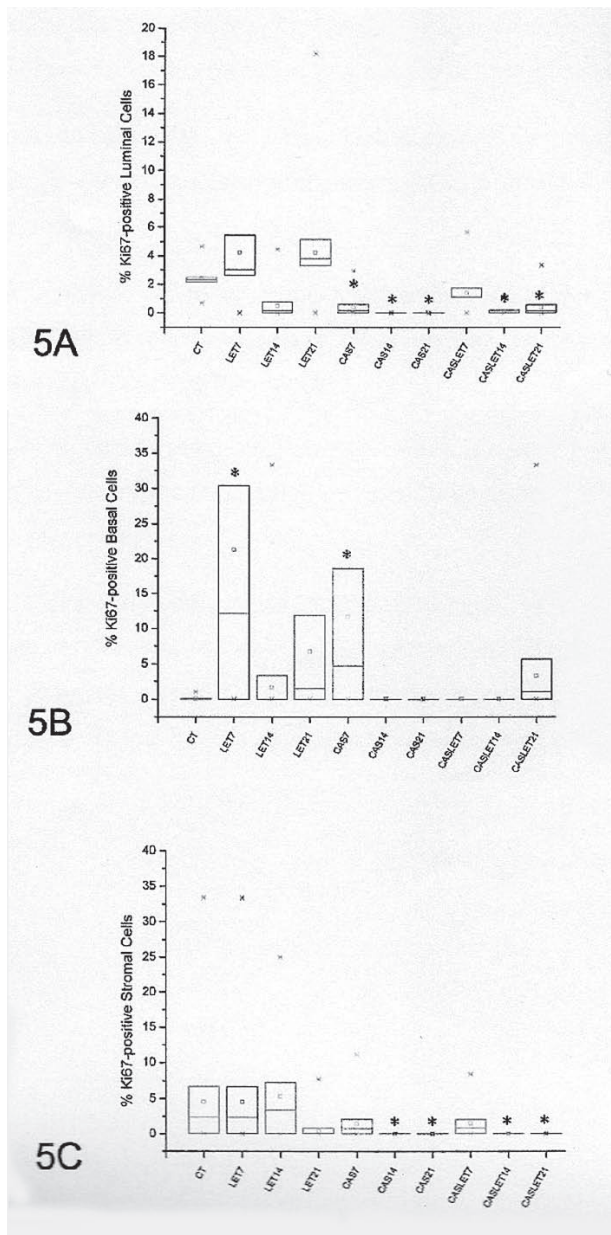
In the animals treated for seven days with letrozole the epithelium showed a strong nuclear staining in the proximal, intermediate and distal region (D, G, and J) respectively. Fourteen days after treatment a intense staining was seen in proximal region (E), while intermediate (H) and distal regions (K) conserve the staining. 21 days (F, I and L), the intense of the nuclear staining was fainter (arrows) staining decrease in the different regions.

Immunocytochemical localization of AR in the prostate of castrate rats. The epithelial cells showed only a weak staining. Neither nucleus nor cytoplasm was stained seven days after castration (M). In castrated letrozole-treated rats after seven days the nucleus and cytoplasm were weakly labeled (P). Fourteen (N) and twenty-one days (O) after castration, the epithelial cells were atrophic and the stroma was more evident. Labeling was abolished. Treatment with letrozole did not alter this pattern (Q and R). (Figs. 2B, 2D, 2F, 2H, 2L (925x)). Lu = lumen; smc = smooth muscle cell, st=Stroma  
**KI67 POSITIVE CELL**



## COUNTS

To assess the proliferative status of the basal and luminal compartments, the expression of the Ki67 antigen was measured. After seven days of treatment an increase in the proliferation index in basal cells of letrozole-treated animals was observed (**Figure 3**).



**Figure 3.** Box-plot representations of the median percentage of Ki67 positively stained cell in luminal (A and B) and stromal (C) compartments. The experimental groups are sequentially represented according to the different treatments. The plots correspond to counts of 20 separate fields of view from each experimental group. CT=Control; LET= Letrozole-treated animals; CAST= Castrated animals; CASTLET=Castrated letrozole treated animals

Fourteen and 21-days after treatment, the proliferative index was similar to that of control animals. After seven days of castration the proliferative index changed from 2.46% in luminal cells to 0.40%. However these values were not significant. At the 14th and 21st day not proliferative activity was detected in the epithelial compartment. Ki67 staining was detected after seven days of castration in basal cells. Treatment with letrozole in castrated rats did not alter this pattern. The prostate of castrated and castrated-letrozole treated animals were Ki67 negative.

## DISCUSSION

Multiple examples of physiological interplay between androgens and estrogens led us to hypothesize that there may be a direct interaction between the androgen receptor and estrogen receptors. In breast cancer for example, tumors containing both receptors (AR and ER) presented higher levels of endogenous steroid hormones than tumors showing either receptor alone<sup>17</sup>.

In prostatic tissue, androgen receptor (AR) synthesis is regulated by androgens. Therefore, it might serve as an effective molecular marker of androgen action in this tissue. In this study we showed that a deregulation of hormonal environment alters the normal expression of AR and interfere with the kinetics of cell proliferation.

Previous reports showed a relationship between AR and aromatase inhibitors. A breast cancer cell (MCF-7) was sensitive to aromatase inhibitors and blockade of the AR inhibited the antiproliferative effect of letrozole. These results suggest that aromatase inhibitors might exert their antiproliferative effect not only by reducing the intracellular production of estrogens but also by unmasking the inhibitory effect of androgens acting via the AR<sup>18</sup>. In the present study letrozole did not cause an inhibiting effect on the proliferating cells. On the contrary, a slight increase of proliferating cells was present which led us to hypothesize that an inhibition of the conversion of T to E2 by letrozole resulted in a significant increase in serum T levels as well as in alterations in the pattern of AR location, thus demonstrating that its expression is dependent on the hormonal environment and probably control cell proliferation. This inhibition of estrogen synthesis by letrozole was effective and the hormonal quantifications showed larger levels of testosterone

serum levels after treatment. The general tendency of an increased epithelial and luminal compartments and a decrease of the stromal compartment indicate a reorganization of the gland after disruption of the normal hormonal environment, besides an increase in proliferation index of the basal cells.

Variation in normal AR synthesis among the different treatment groups was noted, with the highest levels seen in the VP of control rats and the expression of the AR-staining shows a quick effect in AR-labeling in animals treated after 14 and 21 days.

The possible pathways activated after letrozole treatment was the increase of testosterone levels, after aromatase inhibition that stimulates the cell proliferation and produces the growth in the tissue compartments of the gland. Cell proliferation is also stimulated by the loss of the physiological ligands of estrogen receptors, as demonstrated by Weihua and co-workers<sup>19</sup> Androgen receptor down regulation likely results from the disruption of the hormonal milieu.

We conclude that letrozole down-regulates AR in the rat ventral prostate and alters the serum levels of androgens. This model represents a valuable model for analyzing the direct and indirect effects of unbalanced estrogen and androgen action.

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