Morphological and morphometric changes and epithelial apoptosis are induced in rat epididymis by long-term letrozole administration

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ABSTRACT

The epididymis is an organ that plays a key role in sperm maturation. The aim of this study was to examine the association between the chronic treatment of mature male rats with letrozole and morphological evaluation and morphometric values of epididymis as well as changes in the number of apoptotic cells in epididymal epithelium. Adult rats were treated with letrozole for 6 months and the epididymis weight, morphology, morphometric values and the number of apoptotic cells in the epithelium were examined. Long-term aromatase inhibition resulted in presence of intraepithelial clear vacuoles, hyperplasia of clear cells and a hyperplastic alteration in the epithelium known as a cribriform change. Moreover, changes in diameters of the epididymal duct and the epididymal lumen and changes in the epididymal epithelium height were observed. The number of apoptotic epithelial cells was increased in letrozole-treated group. It can be indicated that chronic treatment with letrozole can affect morphology, morphometric values and apoptosis in the epididymis of adult male rats. Observed changes are similar to that observed in the aging processes and may also be important for patients treated with aromatase inhibitors.

Key words: Estrogen; aromatase; letrozole; epididymis; morphology; apoptosis.

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Introduction

The epididymis is an organ that plays a key role in sperm maturation. It is in this organ that spermatozoa develop their main abilities - forward motility and egg fertilizing.[1] The epididymis is derived from the Wolffian duct[2] and irregularities in the development and functioning of this organ may be the cause of male infertility.[3] The ductus epididymis is an elongated and convoluted duct connecting efferent ductules to the vas deferens. In most species, including humans and rodents, the epididymis is divided into four anatomical regions: the initial segment, head (caput), body (corpus) and tail (cauda),[4] which are additionally subdivided by connective tissue into separate intraregional segments.[5] Ten (10) such segments were identified in the epididymis of the mouse and 19 in the rat.[6] The epididymal duct consists of one epithelial layer with several basic cell types - pseudostratified columnar epithelium with stereocilia.[5] Principal cells, the most abundant type of cells in the entire epididymis epithelium, are responsible for the production of a large number of proteins actively secreted into the luminal space, as well as endocytosis of proteins already present there. Basal cells, located primarily in the initial and intermediate regions, are considered to regulate the principal cell electrolyte and water transport. Apical cells are involved in the transport of electrolytes and regulation of pH in the epididymal fluid. The function of narrow cells, such as apical cells, is associated with the regulation of transport between the lumen of the duct and the epithelial cells in the initial and intermediate regions. Narrow cells also have high lysosomal activity and are involved in sperm protection processes. Clear cells are present only in the caput, corpus, and cauda regions and are responsible for the absorption of proteins from the epididymal lumen, as well as the contents of the cytoplasmic droplet released by spermatozoa. Halo cells are present throughout the epididymal epithelium and have been described as lymphocytes or monocytes.[7,8] The epithelial layer is surrounded by connective tissue and smooth muscle cells. The number of muscle cells increases towards the distal part of the ductus epididymis.[9]

The proper functioning of the epididymal duct is regulated not only by androgens, testosterone (T) and dihydrotestosterone (DHT), but also by estrogens.[5,10] The conversion of androgens into estrogens is catalysed by the microsomal, enzymatic complex of cytochrome P450 aromatase (P450arom), encoded by the CYP19A1 gene. One place in the male reproductive system where estrogen synthesis occurs is the epididymal duct.[5,11,12] In humans, aromatase activity is noticed in the cytoplasm of the basal and principal cells in the proximal part of ductus epididymis.[13] Estrogens exert their biological effect through specific estrogen receptors, ESR1 and ESR2.[11] In mammals, ESR2 is distributed along the entire length of the ductus epididymis, while the distribution of ESR1 is species-dependent.[11,14,15]

Aromatase inhibitors (AIs) are used as a potential treatment for a number of male reproductive disorders. Letrozole, a third generation non-steroidal aromatase inhibitor,[16] has been used in men with hypospermatogenesis,[17,18] in men with non-obstructive azoospermia[19] and in men with severe oligospermia.[20] Normal serum hormone levels and semen parameters were observed in hypogonadotropic hypogonadic obese men following letrozole treatment.[21] The benefits of letrozole have been demonstrated in hormone-sensitive male breast cancer[22] and in prepubertal boys to improve final growth.[23] Letrozole is used in the present study to lower circulating estrogen levels.

The aim of this study was thus to examine the association between chronic treatment of mature male rats with letrozole and 1) morphological evaluation of the epididymis; 2) morphometric values of the epididymis; and 3) changes in the number of apoptotic cells in the epididymal epithelium. The morphometric analysis included: (i) the diameter of the epididymal head and tail; (ii) the diameter of the lumen of the epididymal head and tail; and (iii) the height of the epithelium in the head and tail of the epididymis.

Material and Methods

Animals and study design

Sexually mature 3-month-old male Wistar rats were maintained under standard (12L:12D) lighting and nutrition. The animals were randomly divided into control and experimental groups (6 rats per group). Rats in the experimental group received *per os* letrozole (Femara; Novartis Pharma, Nuremberg, Germany) - a non-steroidal inhibitor of cytochrome P450 aromatase, at a dose 1 mg/kg b.w./day for 6 months, as previously described in detail.[24] The letrozole was given to each experimental rat once per day (in the morning) in the form of a small pellet formed from letrozole powder and pressed into a piece of bread. The animals willingly ate the pellets from the hand of the person performing the experiment. The rats in the control group received a pellet without letrozole. At the end of the experimental treatment, the animals were sacrificed under thiopental anesthesia (120 mg/kg b.w., i.p., Biochemie GmbH, Vienna, Austria); the epididymis was resected and weighed. Two subdivisions were prepared, namely the cranial and the caudal parts (Figure 1), which were then fixed in Bouin's fluid and embedded in paraffin, from which a series of sections (3-5 μm) were prepared.

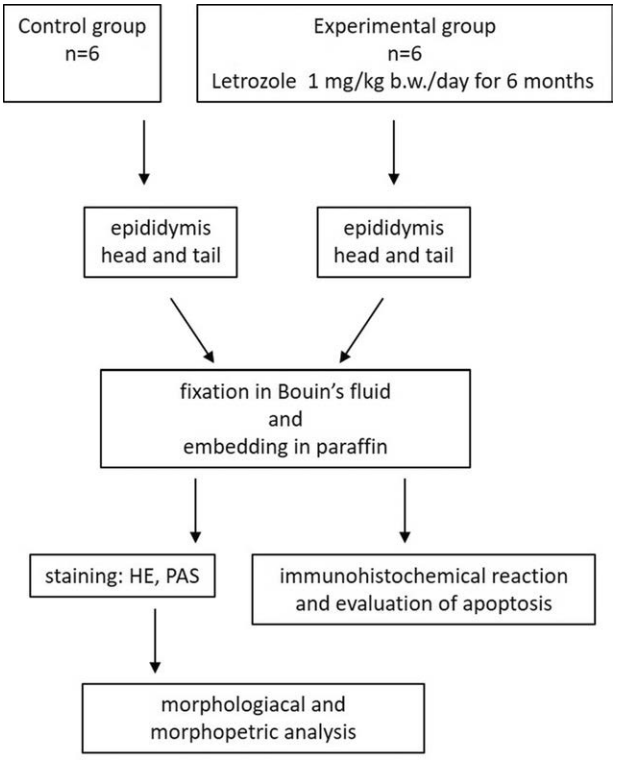

Figure 1. Schematic diagram of the experimental design.

Histological staining and morphometric analysis

For the morphological and morphometrical analyses, dewaxed and hydrated sections were stained with a nuclear stain, Mayer's hematoxylin, which consists of a dye (hematoxylin) and a binding agent (an aluminum salt), in a solution for 10 min. After rinsing in tap water for 10 min and distilled water for 5 min, the slides were stained with an aqueous solution of eosin for 10 min. Following the eosin stain, the slides passed through several changes of alcohol, then were rinsed in several baths of xylene.

Additionally, to identify the clear cells, the slides were stained with PAS Periodic Acid from a Schiff Hotchkiss Mc Manus kit (code 04-130802, Bio-Optica, Milan, Italy). In accordance with manufacturer's staining protocol, the slides were incubated with periodic acid solution for 10 min, washed in distilled water, incubated with Schiff reagent Hotchkiss McManus for 20 min, washed in distilled water, incubated with potassium methabisulphite solution for 2 min and fixative solution for 2 min, washed in distilled water, incubated with Mayer's Hemalum for 3 min and washed in tap water for 5 min. Following the PAS stain, the slides were passed through several changes of alcohol, then were rinsed in several baths of xylene. The slides were evaluated using a light microscope and LAS V4.4 software (Leica DM5000B, Germany). Morphometric measurements were made at a magnification ×20. Measurements (50 on average for each sample) were made on cross-sections of the head and tail duct of the ductus epididymis most similar to a circle. The diameter of whole cross section of the ductus epididymis, the lumen of the ductus epididymis, and the thickness of the epithelium lining the ductus were rated as the differences between the diameter of the entire cross-section of the ductus and the diameter of its lumen.[25] The epididymal diameter and lumen measurements were taken at the narrowest point between two parallel tangents to the outer and inner edges of the epididymal wall (Figure 2).

Immunohistochemical reaction and evaluation of apoptosis.

To identify apoptotic cells in the epididymal epithelium mouse anti-caspase-3 (1:300; cat.no.: sc-56053; Santa Cruz Biotechnology, Santa Cruz, CA, USA) monoclonal antibody was used. In order to expose epitopes, the deparaffinized sections were microwaved in a citrate buffer (pH 6.0). Once cooled and washed with PBS, the preparations were incubated for 60 min at room temperature with the primary antibody. Next, the slides were washed and incubated with a secondary antibody from an EnVision+System-HRr kit (code K4010, DakoCytomation, Glostrup, Denmark) for 30 min followed by incubation with avidin-biotin-peroxidase complex (DAKO) and development with diaminobenzidine chromogen for 5 min, in accordance with manufacturer's staining protocol. The sections were washed in distilled water and counterstained with hematoxylin. Positive staining was defined by visual identification of brown DAB pigmentation under a light microscope (Leica DM5000B, Germany). As a negative control, some specimens were processed in the absence of the primary antibody.

In order to calculate the degree of apoptosis, 500 epithelial cells were counted in randomly selected fields and the numbers of positive cells was used to calculate the apoptosis index.[26,27]

Statistical analysis

The results were analyzed statistically using Statistica 6.1 software. The arithmetical means and standard deviations (±SD) were determined for each of the studied parameters. The distribution of results for the individual variables was obtained using Shapiro-Wilk *W*-tests. As most of the distributions deviated from a normal Gaussian distribution, nonparametric Mann-Whitney U-tests were used for further analysis to assess the differences between the studied groups.

Results

Epididymis mass

The mean epididymal mass of the letrozole treated male rats was lower (0.658±0.042g) than in the control rats (0.78±0.127g). This difference was not statistically significant ($p<0.07$; Table 1).

Morphological study

The whole epididymal duct was lined by pseudostratified columnar epithelium in the control and letrozole-treated groups (Figure 3 A-I). We observed a small amount of connective tissue and circular smooth muscle cells around the epithelium. The layer of smooth muscle cells was thicker in the caudal part. The epididymis of the control rats presented a normal morphology (Figure 3 A,B). The chronic treatment with letrozole resulted in the morphological changes in the rat epididymal duct. Some sections showed intraepithelial clear vacuoles of varying sizes in the epididymis head (Figure 3 C,G) and tail (Figure 3D). Additionally, hyperplasia of clear cells were observed in the both parts of the epididymis following the hematoxylin-eosin (HE) staining (Figure 3 E,F), as well as signs of PAS reaction (Figure 3 G,H). Finally, a hyperplastic alteration in the epithelium known as a cribriform change was observed in the caudal region of the epididymis (Figure 3I) in the letrozole treated rats.

Morphometric study

A statistically significant increase in the diameter of the epididymal tail duct was observed in the experimental animals compared to the control animals, as well as a significant increase in the diameter of the lumen of the epididymal tail duct was found in the experimental group compared to the control group (Table 1).

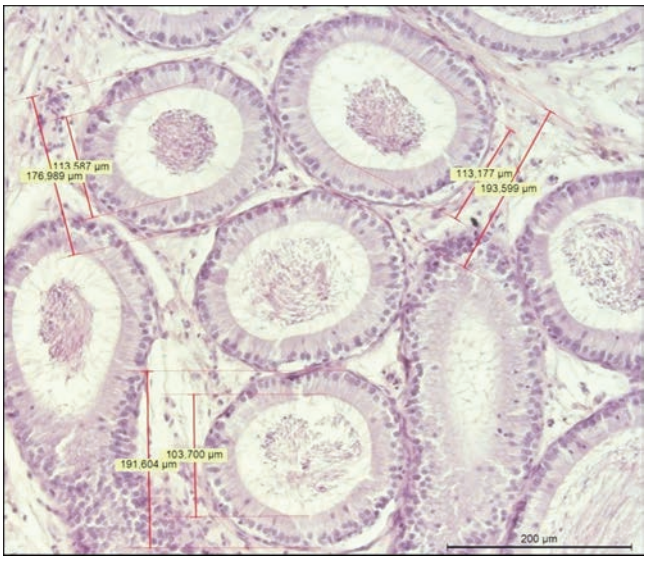

Figure 2. Figure showing the method of morphometric measurements. Sections through the caput epididymis of a control rat. HE staining. Objective magnification: x20.

A statistically significant increase in the epididymal epithelium height in the head and a statistically significant decrease in the epididymal epithelium height in the tail were found in the experimental group compared to the control group (Table 1).

A slight decrease in the diameter of the epididymal duct head was observed in the experimental animals compared to the control animals, but this was not statistically significant. There was a slight decrease in the diameter of the lumen of the epididymal duct head in the experimental group compared to the control group, but this was also not statistically significant.

Evaluation of apoptosis

The caspase-3-immunopositive product were localized in the nuclei of the epithelial cells lining the head and the tail of the epididymis (Figure 4). A much higher number of caspase-3-immunopositive cells were observed in the letrozole-treated rats (Figure 4 B,C,E,F) than in the control groups (Figure 4 A,D) in both regions of the epididymal duct (Figure 5).

Discussion

The epididymis is an androgen-dependent organ. As the use of testosterone as a hormone replacement therapy following castration does not restore the proper morphology of the epithelial cells in the initial segment of the epididymis,[28] a significant role in proper epididymal homeostasis has been suspected for estrogens.[29] Results present in this study show changes in epididymal morphology and morphometric values in experimental male rat, as well as changes in the number of apoptotic epithelial cells, suggesting the role of estrogen in these parts of the epididymis. Using the same rats that were used in this study, we found that letrozole significantly decreased the level of circulating estradiol in the adult male rats by an average of 43%.[24]

We found letrozole was damaging the structure of the ductus epididymis. This could be the underlying reason for the obvious decrease in epididymal weights. Although the epididymal weight loss was not statistically significant, we observed a 16% loss. In another study, decreased (20%-30%) epididymis weight was observed in adult rats treated with exemestane, an irreversible steroidal inhibitor of cytochrome-P450 aromatase.[30]

To focus attention on the morphology of the epithelial cells we investigated the tissue after HE and PAS staining. The 6-month-long treatment with letrozole resulted in morphological changes within the epididymal epithelium of the male rats. We observed large clear vacuoles between epithelial cells, especially in the caudal region of the epididymis. According to Serre and Robaire,[31] vacuolization, polymorphism of lysosomes, spermiophagy, and activation of the immune system, are the signs of aging found to be

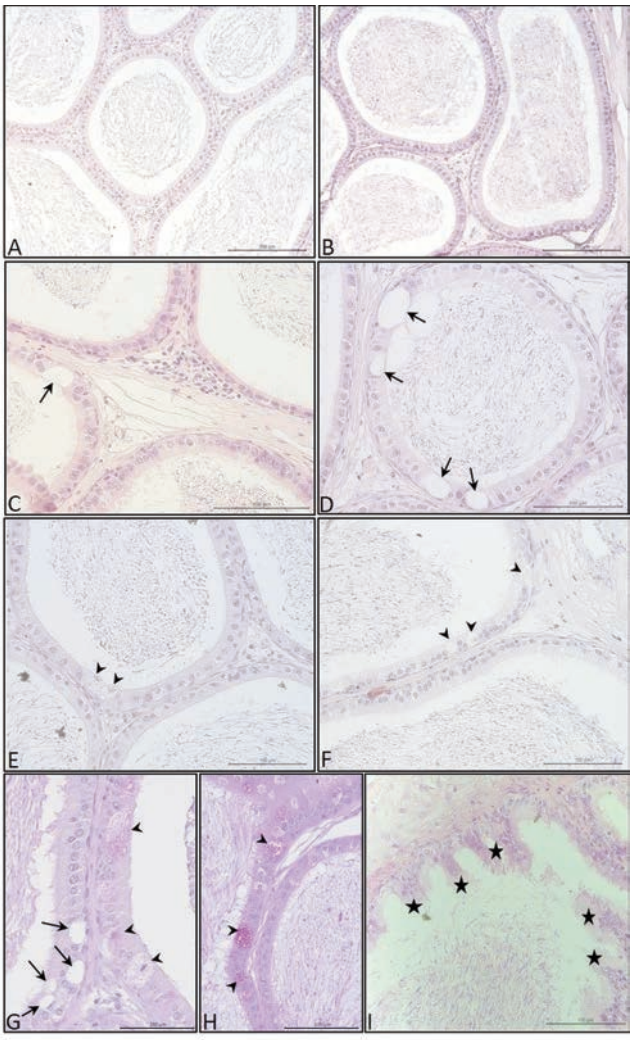

Figure 3. Histological examination of the caput and cauda epididymis. Normal aspect of the caput epididymis (A) and cauda epididymis (B) from the control group. Changes observed in the letrozole treated group in the caput epididymis (C,E,G) and cauda epididymis (D,F,H,I). Arrows, intraepithelial vacuoles; arrows head, hyperplasia of clear cells; stars, cribriform change. A-F, I) H&E staining; G,H) PAS staining. Objective magnification: A,B) x20; C-I) x40.

Table 1. Epididymal mass and morphometrical values in the head and tail of the epididymis of control and letrozole-treated male rats.

	Control (C) n=6	Letrozole (L) n=6
Epididymal mass (g): p<0.07	0.78±0.127	0.658±0.042
The diameter of the epididymal duct (µm): **p<0.01		
head	274.07±77.91	259.86±35.66
tail	335.17±72.82	352.20±82.55**
The diameter of the epididymal lumen (µm): ***p<0.001		
head	205.80±78.78	76.37±40.32
tail	279.77±67.13	302.09±79.94***
The height of the epithelium of epididymal duct (µm): **p<0.01		
head	68.27±15.90	83.48±19.11***
tail	55.40±16.54	50.11±13.85**

specific to the epididymis. Analogous changes, similar to those observed in the aging processes, were noticed in the rat testes in our previous study.[24] Therefore, considering both - the testis and epididymis, it can be concluded that the long-term estrogen deficiency produced histomorphological changes similar to those observed in the aging processes. Hess *et al*.[32] demonstrated significant abnormality of the epithelial cells within the whole epididymal duct, the accumulation of vacuoles, and PAS-positive granules in the epithelial cells, in studies with mice with targeted disruption of estrogen receptors.[33] Because it is known that epithelial cells of the epididymis are involved in multiple functions, including production of a large number of proteins, regulation of electrolyte and water transport, as well as sperm protection processes,[7,8] any alternation in their morphology can be found as a reason of disruption of the epididymal luminal environment.[33] Moreover, any abnormalities in the normal morphology of the epithelium may alter the epithelial cell-cell interactions mediated by adhering junctions, gap junctions, and tight junctions, which are necessary for cell adhesion and the formation of the blood-epididymal barrier.[34]

Another significant change observed by us was hyperplasia of clear cells. The enlargement of clear cells is caused by an increase in the number of lysosomes in their cytoplasm involved in endocytosis. This in turn is due to the increased accumulation of debris in the lumen.[35] In our experience, hyperplasia could again be associated with the previously described changes in the testes,[24] where we observed intense sloughing of premature germ cells of the seminiferous epithelium into the tubular lumen. So, it is very possible that in the present study, clear cell hyperplasia was more evident as there was likely increased endocytosis of undifferentiated sperm cells.

The appearance of cribriform change may be associated with both the aging processes and the alternations taking place in the testes.[35] Therefore, as in the case of the presence of a vacuole, foldings of the hyperplastic epithelium found by us in the present work in the letrozole-treated group can be combined with the aging processes.

Taking into account the notable histological alterations in the epididymis described above, it can be assumed that they may be secondary to the letrozole effects in the testis. We reported in our previous works significant changes following six-month-long letrozole treatment. The presence of sloughed premature germ cells in the lumen of seminiferous tubules was noticed first. The appearance of vacuoles and multinucleated giant cells within the seminiferous epithelium were also observed. These changes concerned not only the seminiferous epithelium, but also the wall of the seminiferous tubule. We noticed irregularities and infoldings of the tubular basement membrane and deep invaginations of the lamina propria with myoid cells. Finally, the changes also concerned the interstitial tissue, where the appearance of cells with changes characteristic of the aging processes was observed.[24] It is known that testicular factors contribute to the processes taking place in the epididymis.[36]

In the present study, increased diameters of the epididymal duct and the epididymal lumen in the caudal region were observed. We noticed an increase in the epididymal epithelium thickness in the head and a reduction in the epididymal epithelium thickness in the tail. According to Hess *et al*.[32] the tubular diameters of the efferent ductules and initial segment of the epididymis were dilated in estrogen receptor-α knockout experimental mice (α ERKO). We assume that the changes in epithelial thickness and epididymal

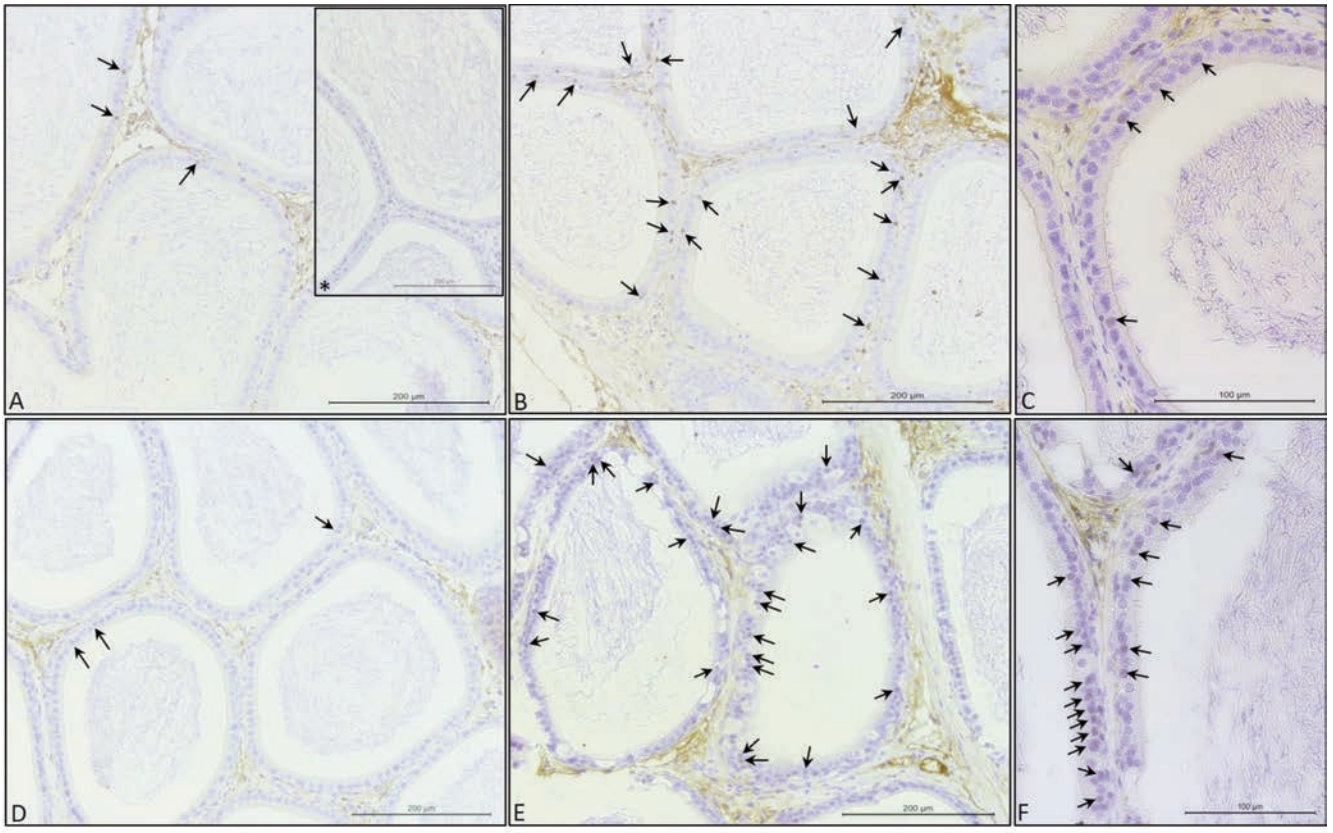

Figure 4. Immunolocalization of caspase-3 in the caput (A) and cauda (B) of the control group and caput (C) and cauda (D) of the letrozole-treated group. Arrows show caspase-3-immunopositive cells. Objective magnification: x20.

tubule diameter may be a consequence of the disorganization and degeneration of epithelial cells lining the epididymal duct described above. Moreover, it can also be assumed that any disturbances in hormonal balance effect morphometric changes, as similar results were observed by Marchlewicz *et al*.[25] in rats treated with soya isoflavones.

The results herein revealed a statistically significant increase in the number of apoptotic epithelial cells along the epididymis of the letrozole-treated group. After log-term treatment with letrozole an increase in the number of caspase-3-immunopositive cells was observed in the caput and cauda. These data indicate that cell death could be activated by the action of letrozole. Chronical treatment of adults rats with letrozole increased the apoptosis of cells in the seminiferous tubules, as was also presented in our earlier study.[37] Estrogens, synthesized by Leydig cells and germ cells, are present in low concentrations in the blood, but can be high in the semen.[29,38] Without testicular luminal fluid factors, many cells in the epididymis undergo apoptosis beginning in the initial segment and moving over several days to the cauda epididymis. It has been postulated that a factor or factors originating from the testis is/are responsible for maintaining the integrity and survival of spermatozoa (sperm) in the epididymal duct.[39,40]

In conclusion, this study shows significant effect of estrogen depletion on the epididymal duct. The results suggest a correlation between estrogen imbalance and the morphology of the epididymis, indicating that estrogens can act as regulators of some specific epididymal functions. It is known that epididymal epithelial cells *in vitro* synthesize androgens and mRNA for cytochrome P450 aromatase in the cultured epididymal epithelial cells of the rat, as well as them having the ability to aromatize the synthesized androgen to 17 beta-estradiol.[12] However, the effect of long-term letrozole treatment on the epididymis may not just be due to the direct effect of the aromatase inhibitor, but may also be due to indirect effects secondary to the changes taking place in the testis. The results of this study may be a signal for men who use aromatase inhibitors. The morphological and morphometric changes that we have observed are similar to those observed in the aging processes, which seems to be crucial for male patients treated with aromatase inhibitors.

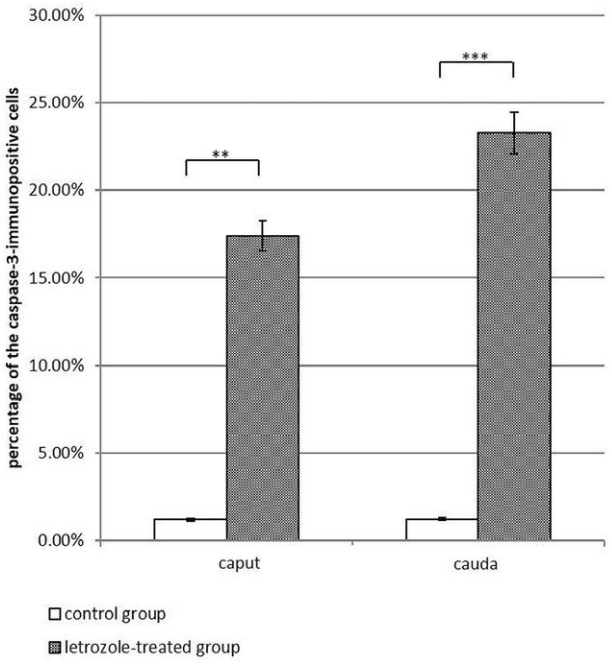

Figure 5. Comparison of the percentage of caspase-3-immunopositive cells in the caput and cauda epididymis of the control and letrozole-treated groups. **p≤0.01; ***p≤0.001 (Man-Whitney U test, n=6).

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