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The State of Reproductive Organs of Young and Aged Male Rats at the Prolong Administration of Letrozole and after its Withdrawal

Alexander G. Reznikov[1,*], Olga V. Sachynska[1], Lyubov I. Polyakova[1], Oksana A. Faliush[1], Alexander V. Yanishevskiy[1]

[1]Department of Endocrinology of Reproduction and Adaptation, V.P. Komisarenko Institute of Endocrinology and Metabolism, National Academy of Medical Sciences of Ukraine, Kyiv< Ukraine

*Corresponding author: reznikov39@gmail.com

Abstract

Aim. Study of the reproductive organs at long-term administration of letrozole, the steroid aromatase inhibitor, followed by its withdrawal in young male rats and ones with age-related involution of reproductive organs.**Material and methods.** The experiments were carried out on Wistar rats with an initial age of 5 months and 15 months, which have been gavaged by letrozole every other day at a dose of 1 mg/kg b. w for 3 months and then 2 months after its discontinuation. The blood plasma testosterone and estradiol levels were measured by immunoassays. The spermatozoa concentrations in epididymal washes were determined. The testicles and accessory sexual glands were weighed, and morphology of gonads and ventral prostate have been studied. The results of the study were compared with those of control animals of corresponding age. **Results.**As the result of letrozole treatment, the ratio of testosterone and estradiol levels in blood plasma of aged rats increased. The spermatozoa

content in epididymis rose up by 28% at average. The histological study revealed functional activation of Leydig cells, a significant retardation of involutive changes of their number and morphology. Some Leydig cells demonstrated the signs of functional exhaustion. Letrozole exerted an increase of relative weights of the coagulation gland by an average of 40%, seminal vesicles by 31%, and ventral prostate by 33% compared with those of control animals. Two months after letrozolewithdrawal, there were no any signs of difference between letrozole-treated and control animals. In young rats, the effects of letrozole were almost not detectable. **Conclusions.**Letrozole administration to male rats with age-related involution of the reproductive system increases testosterone/estradiol ratio in blood plasma, the spermatozoa content in epididymises and the weights of androgen-dependent accessory sexual glands. This is accompanied by slowing down of age-related changes of the gonad and prostate gland morphology. Letrozole-induced reproductive effects are reversible.

Keywords: letrozole, reproductive organs, male, rats.

1. Introduction

One of the leading signs of aging of the male reproductive system is a gradual decrease in the production of testicular androgens, primarily testosterone. At the same time, the absolute or relative concentration of estrogen in the blood increases. The frequency of early age androgen deficiency in the men population, which is known as late-onset hypogonadism (LOH syndrome), varies from 2 % to 15 % [1].This pathological state is accompanied by sexual disorders, subfertility, psychological, somatic and neural disorders. It is considered a risk factor for the development of metabolic syndrome, cardiovascular diseases, diabetes, obesity, etc. [2-6], and even a fivefold increase in mortality compared with eugonadal men [7].

Obesity also leads to the above-mentioned negative consequences due to the fact that steroid aromatase of adipose tissue is capable of converting androgenic steroids into estrogens. Estrogen-dominant syndrome exacerbates androgen deficiency because estrogens reduce the secretion of pituitary luteinizing hormone and the level of free testosterone in the blood by increasing the concentration of sex hormone-binding globulin.

To date, testosterone replacement therapy remains the basic method for treatment of LOH syndrome. However, along with the positive effect, it can have unexpected consequences, namely, an increase in estrogen saturation of the body due to the metabolic transformation of exogenous testosterone to estradiol. This result was observed in men with hypogonadism without obesity [8, 9].

An alternative might be the use of pharmacological agents that inhibit the steroid aromatase, which is responsible for the formation of estrogens from aromatizable androgens. One of such drugs is letrozole, which is widely used in the treatment of breast cancer. It slows down the conversion of endogenous testosterone to estradiol-17β and androstenedione to estrone, resulting in reduction of the body's estrogen saturation. The high antiaromatase activity of letrozole was previously demonstrated by us in *in vivo* studies on female rats and *in vitro* on ovarian homogenates [10, 11].

Studies in healthy men have shown that the aromatase inhibitors, letrozole and fadrozole, can increase testosterone levels and decrease estradiol in the blood [12]. There are a few reports on increased testosterone levels and the therapeutic efficacy of letrozole in men with estrogen-dominant syndrome and concomitant excess of body fat [13, 14]. Therefore, the use of letrozole and other steroid aromatase inhibitors in LOH syndrome may be appropriate [15]. Restoration of spermatogenesis has been reported in some patients with oligo- or azoospermia under the influence of letrozole and anastrozole [16-19]. A significant increase in the ratio of testosterone to estradiol in the blood of men with subfertility, including LOH syndrome, was observed after the use of these drugs [19 -23].

Given the probable wide clinical use of letrozole and the lack of official indications for its use in men with estrogen-dominant syndrome, obesity, subfertility, LOH-syndrome, there is a need for experimental studies in male animals on its effect on the reproductive system. Data from the literature on this issue are extremely limited and contradictory [12, 24-26]. As our previous studies have shown, administration of letrozole to male rats for two weeks stimulates the prostate and other androgen-dependent accessory sex gland, mainly in aging animals [27].

The aim of this work was to study the condition of the reproductive system after long-term administration of letrozole followed by its withdrawal in young males and against the background of involutive changes in aging males in a comparative aspect.

2.Material and methods

The experiments were started on 5-month-old Wistar male rats with an initial body weight of 170-225 g and 15-month-old rats with an initial body weight of 220-320 g. According to the anatomical and physiological characteristics of laboratory white rats, 5 months of age is considered young, and 15-month – aging animals [28]. Based on the fact that in the age periodization of laboratory rats 1 day of life corresponds to 52 days (1.7 months) of human life [29], 5-month age of the rat corresponds to 22 years of man, and 15-month - 64 years.

The experiments were performed in compliance with the bioethical recommendations of the European Convention for the Protection of Vertebrate Animals Used for Scientific and Other Experimental Purposes (Strasbourg, 1986) and the recommendations of the I National Congress on Bioethics (Kyiv, 2001). Using randomization the rats were distributed to four groups of 20 animals each: 1 and 2 group - control and experimental young animals (average body weight 195 ± 3 g and 197 ± 3 g, respectively, P> 0. 05); groups 3 and 4 - control and experimental elderly animals (average body weight 273 ± 5 g and 266 ± 6 g, respectively, P> 0.05). The animals were kept in standard vivarium conditions (20-22 ^{0}C, relative humidity as 50-60%), on a standard diet and free access to drinking water. Letrozole (Letromara tablets, "Farmak", Ukraine) was introduced to experimental animals with gavage in the form of a suspension of tablet mass in Dorfman gel (0.9% sodium chloride solution containing 0.5% sodium carboxymethylcellulose, 0.4% tween-80 and 0.9% benzyl alcohol). Rats received letrozole once every 2 days at a dose of 1 mg / kg b. w. for 90 days. Individual doses were calculated according to the body weight at the time of the drug administration. Control animals were gavaged with the appropriate carrier.

Half of the animals in each group were decapitated under light ether anesthesia 24 h after the last administration of the drug, the other half - in 2 months after its withdrawal, taking into account the full cycle of spermatogenesis. The ventral lobe of the prostate (VP), the coagulating gland (CG, the anterior lobe of the prostate), the seminal vesicles (SV), the testes, and the epididymis were isolated and weighed. Fragments of the VP and testes were fixed in Bouin's fluid for histological examination. In dosed washes of the epididymis, the concentration of sperm was determined by counting in the Goryaev's chamber.

The levels of testosterone and estradiol in the blood plasma were assayed with ELISA Testosterone and ELISA Estradiol kits (DRG, Germany) and the reader Stat Fax (USA).

The data were statistically processed using Student's *t*-test. The difference between the studied measures in the experimental and control groups was considered significant at $p \leq 0.05$.

3.Results

Rats gained weight during the experiment and tolerated letrozole well. The dynamics of changes in body weight during the experiment was the same in experimental and control animals. Two aging control rats and three ones from letrozole-treated groups dropped out of the study by the time of completion of letrozole administration for various non-drug use reasons.

At the end of letrozole introduction, the mean plasma testosterone level and testosterone / estradiol ratio in the 18-month-old controls which were determined in December, were more than twice as low as that of the 8-month-old controls. This age difference was absent in late February, after two months of drug withdrawal. The levels of estradiol in control animals of different ages did not differ.

Letrozole treatment did not cause significant changes in the levels of each of the studied hormones in the plasma of both young and aging animals due to a wide variability. The testosterone / estradiol ratio in aging males increased by an average of 69%, which was very close to the level of significance.

In two months following letrozole withdrawal, the studied hormonal parameters, including testosterone / estradiol ratio, did not differ from the corresponding control values in both age groups (Fig. 1, 2).

Hormonal changes caused by letrozole are evidenced by changes in the content of sperm in the epididymis and the weights of accessory sexual glands, which is an integral indicator of androgen saturation, as well as by morphological features of activation of gonadal endocrinocytes. In young animals, after the introduction of letrozole, no changes in sperm content were observed, while in the elderly it increased from $(34.3 \pm 2.4) \times 10^6$ / ml to $(44.0 \pm 2.1) \times 10^6$ / ml of medium ($p < 0.05$) and did not differ from control in 2 months after drug withdrawal.

Histological examination of the gonads of young males receiving letrozole, compared with the control (Fig. 3a), showed moderately enlarged islets of interstitial Leydig cells with some activated endocrinocytes having a large nucleus and enlighten nucleolus (Fig. 3b). There were no activated cells in the control, probably due to seasonal depression of sexual activity (the experiment was completed in December). Typical age-related changes in the morphology of the gonads, namely hyperplasia and hypertrophy of Leydig cell, were observed in control aging animals (Fig. 3c) against the background of a significant decrease in testosterone

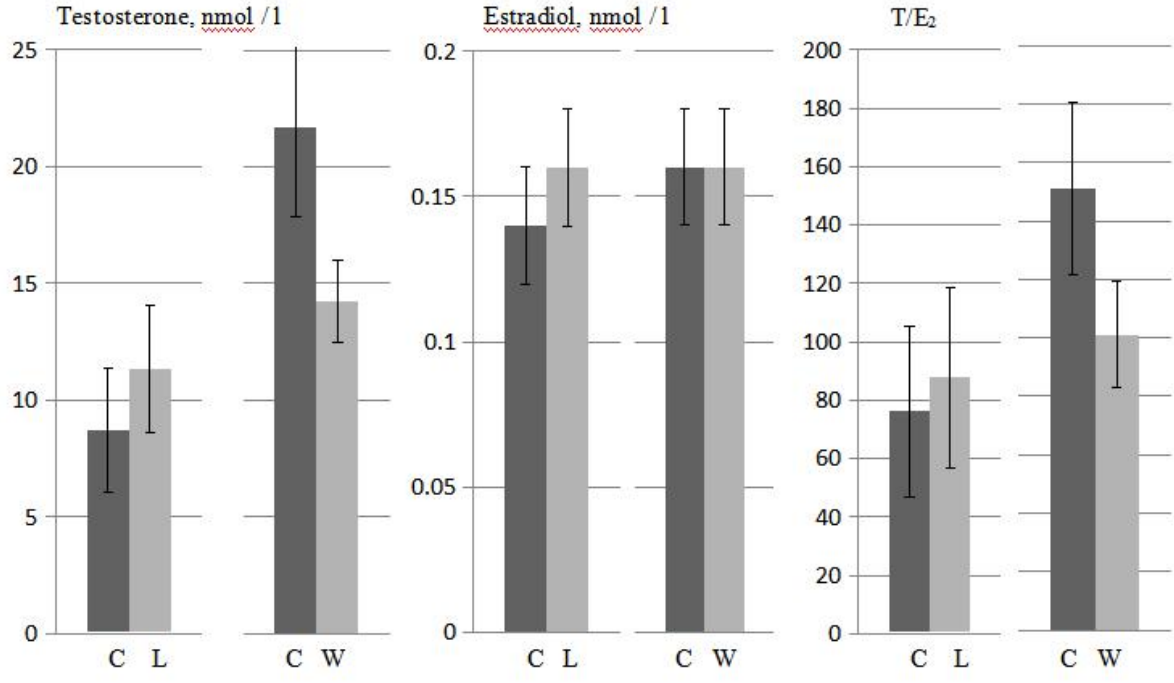

Figure. 1. The levels of sex hormones in the blood plasma of young male rats after administration of letrozole for 3 months. and in 2 months. after its withdrawal (M + m). C, control; L, letrozole; W, after withdrawal of L; T, testosterone; E2, estradiol.

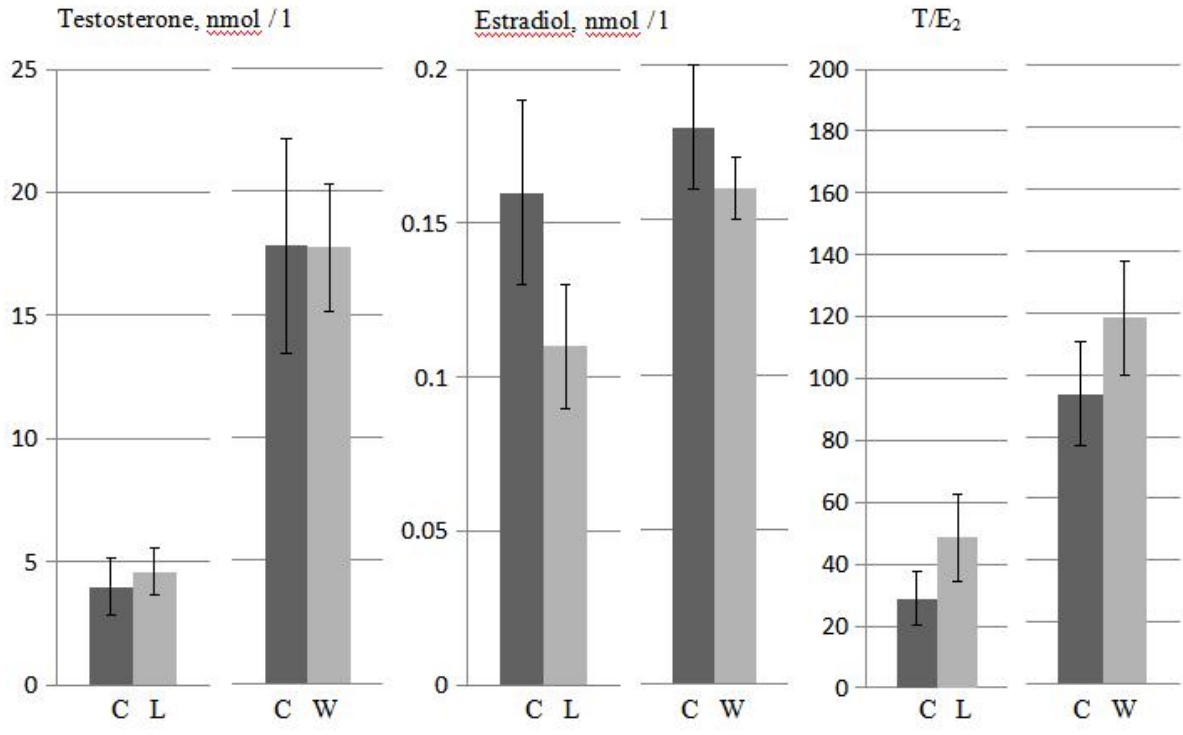

Figure. 2. The levels of sex hormones in the blood plasma of aging male rats after administration of letrozole for 3 months. and in 2 months. after its withdrawal (M + m). C, control; L, letrozole; W, after withdrawal of L; T, testosterone; E_2, estradiol.

production. In letrozole-treated aging rats, Leydig cells were similar in size and number of islets to those in control young animals. The cell composition of the islets differed in heterogeneity: along with activated cells that had normochromic nuclei and a large-volume cytoplasm vacuolated at the periphery, there were small cells with pyknotic nuclei and large endocrinocytes with shrunken hyperchromic nuclei (Fig. 3d). The latter probably acquired such a structure due to functional exhaustion.. In general, the state of endocrinocytes correlates with the results of hormonal tests. In 2 months after letrozole withdrawal (in late February) there was a seasonal activation of Leydig cells (Fig. 3e, f), and the difference compared with the corresponding control groups was not detected. The morphological structure of the spermatogenic layer of the seminal tubules in young and aging males did not differ from those in control animals both immediately after the introduction of letrozole and after 2 months after its withdrawal.

In control animals, the relative weights of CG, SP and epididymis were lower in aging males compared to young ones ($p < 0.05$), indicating age-related involution of androgen-dependent organs due to age-related testosterone deficiency. Under the influence of letrozole, these values in aging animals increased and reached normal indices in young males (Table 1).

The relative weight of CG increased by an average of 40%, SV - by 31% compared with control animals of the appropriate age. The increase in the VP weight was by 33% and approached the level of statistical significance. The total relative weight of the ventral and anterior lobes of the prostate increased from 115.4 ± 14.4 mg / 100 g b. w. to 156.1 ± 11.8 mg / 100 g b. w., *i.e.* 36% on the average (p <0,05).

In young animals, the organs studied did not change compared with the control group of the same age.

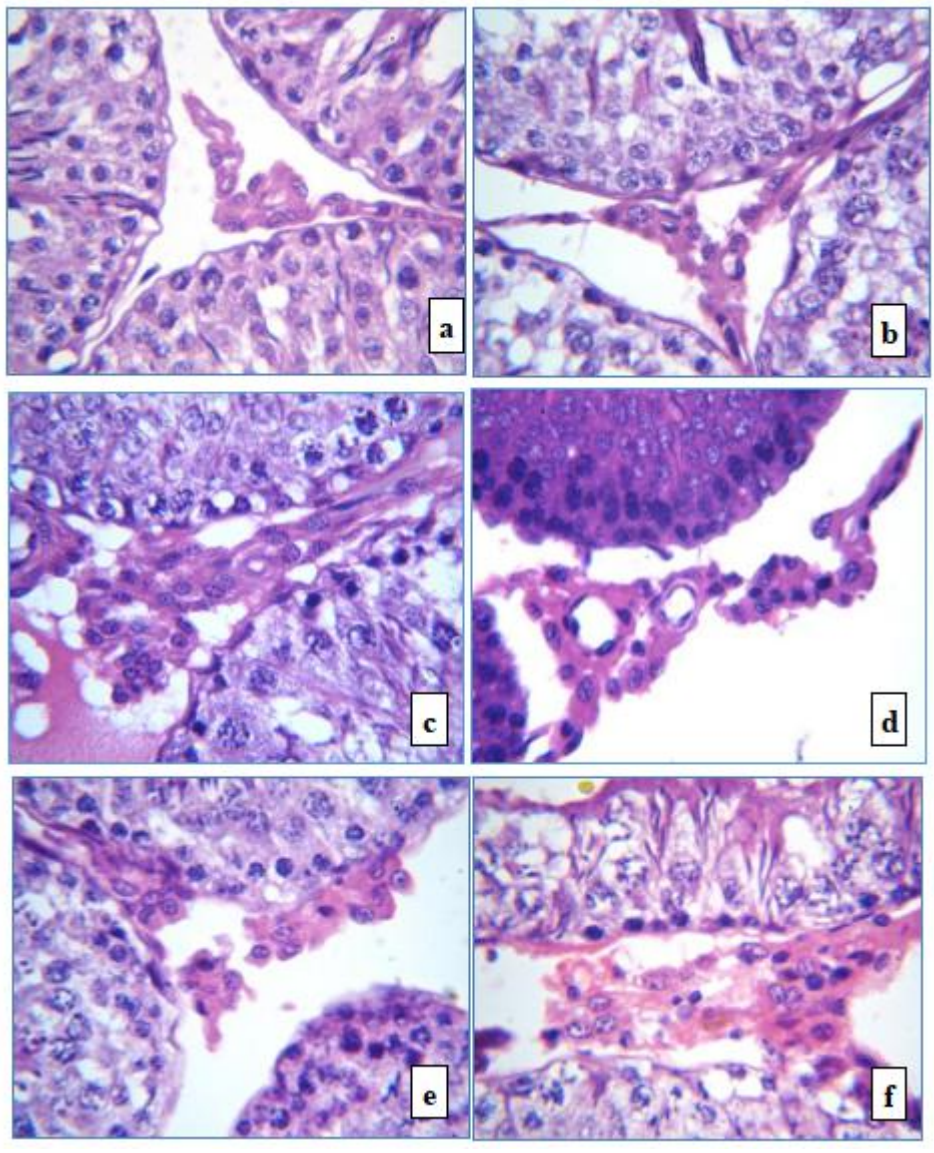

Figure 3. Microphotographs of rat testes: a, control young rat; b, letrozole treated young rats; c, control aging rat; d – letrozole treated aging rat; e, young rat in two months after letrozole withdrawal; f, aging rat in two months after letrozole withdrawal. Hematoxylin-eosin, × 400.

Table 1. Relative Weights of the Reproductive Organs (mg / 100 g b. w.) after Letrozole Treatment

Group of animals	*n*	VP	CG	SV	Epididymis	Testes
Young rats (8 months)						
Control	10	100,1 ± 10,4 (48–161)	55,2 ± 4,8 (28–78)	89,0 ± 4,3 (72–114)	356,7 ± 11,2 (310–428)	1158,0 ± 44,8 (990–1404)
Letrozole	10	108,0 ± 27,8 (59–136)	62,3 ± 2,0 (51–71)	90,9 ± 2,5 (78–103)	341,9 ± 7,9 (308–395)	1108,6 ± 39,1 (943–1320)
Aging rats (18 months)						
Control	8	74,8 ± 10,6 (26–114)	40,6± 4,3 (18–48)	68,6± 7,5 (43–102)	296,1± 20,1 (198–367)	1013,0 ± 52,6 (789–1254)
Letrozole	7	99,7 ± 9,2 (65–121) *p*>0,05	56,5 ± 4,0 (40–67) *p*<0,05	89,8 ± 3,7 (78–102) *P*<0,05	332,3 ± 16,3 (286–407) *p*>0,05	1114,3 ± 59,9 (947–1325) *p*>0,05

Notes: *n,* number of animals in each group; the data are presented as mean values and minimum and maximum figures (in the brackets). Statistical analysis by Wilcoxon-Mann-Whitney non-parametric *U*-criterion .*p* compared to controls.

In two months after discontinuation of the drug, the difference in the weights of reproductive organs between experimental and control rats disappeared, except for SV (Table 2).

Table 2. Relative Weights of the Reproductive Organs (mg / 100 g b. w.) after Letrozole Withdrawal

Group of animals	*n*	VP	CG	SV	Epididymis	Testes
Young rats (10 months)						
Control	10	110,9 ± 6,2 (86–137)	67,3 ± 2,4 (58–80)	85,0± 2,3 (74–95)	309,0 ± 9,1 (293–342)	962,7 ± 21,9 (990–1094)
Letrozole	10	121,7 ± 12,2 (87–219) p>0,05	67,0 ± 3,7 (48–79) p >0,05	93,8 ±3,8 (80–119) p >0,05	295,8 ±13,1 (197–336) p >0,05	884,2 ±53,2 (500–1075) p >0,05

Aging rats (20 months)						
Control	8	117,6 ± 10,1 (75–178)	71,4 ± 5,6 (48–111)	81,8 ± 2,9 (67–93)	302,2 ± 7,7 (262–341)	929,8 ± 26,9 (787–1026)
Letrozole	7	130,0 ± 12,1 (87–207) *p*>0,05	71,9 ± 4,8 (48–105) *p* >0,05	96,6 ±3,7 (81–117) *p*<0,01	290,4 ±9,2 (240–331) *p* >0,05	951,7 ±25,8 (803–1047) *p*>0,05

Notes: *n,* number of animals in each group; the data are presented as mean values and minimum and maximum figures (in the brackets). Statistical analysis by Wilcoxon-Mann-Whitney non-parametric *U*-criterion .*p* compared to controls.

Histological examination showed that in control young rats VP is composed of large acinuses, which are closely adjacent to each other and separated by thin layers of connective tissue (Fig. 4a, b). In the central part of the VP, majority of the acinuses are lined with cubic epithelium. The epithelium showed signs of high secretory activity. The nuclei of epithelial cells are spherical, well-contoured, often contain one, sometimes two nucleoli, a small amount of heterochromatin and are located in the basal part of the cells. Basophilic granularity was abundantly stained around the nuclei and in the apical part of the cell. The oxyphilic Golgi zone was clearly distinguished. Fine-grained secretion is present in the VP.

Following 3-months letrozole treatment of young animals, there were more the VP acinuses lined with a cylindrical epithelium in comparison with control group. Their size was also often larger than in control animals (Fig. 4c). Two types of changes were observed in the VP. On the one hand, most secretory cells demonstrated high secretory activity, as evidenced by increased vacuolation of their cytoplasm, expansion of the Golgi zone, the formation of convex cell tops (Fig. 4d). On the other hand, the signs of depletion and degeneration were found as light cytoplasm, hyperchromic and shrunken nuclei. Apoptotic bodies were also found in the epithelium. Such changes are likely to be the result of depletion due to excessive secretory activity induced by letrozole.

In contrast to the normal young males, in aging rats the VP had smaller acinuses, increased tortuous shape. "Amyloid" bodies were often found in the acinar cavity. The layers of connective tissue are much wider than in young animals. In some places, the epithelium formed two layers with hypochromic cytoplasm and lipofuscin granules in the cell (Fig. 5b). Most of the acinuses are lined with squamous epithelium, while in the end sections part of the

cylindrical epithelial cells is preserved (Fig. 5a). The connective tissue contained a considerable

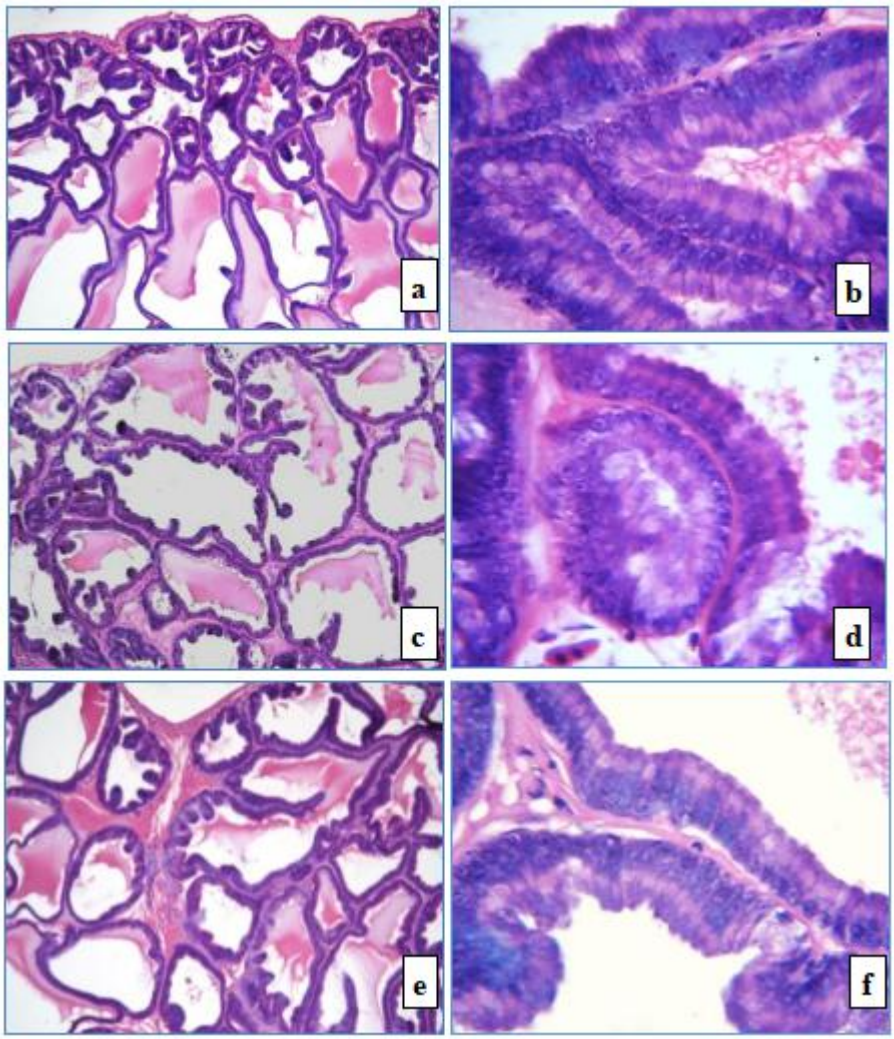

Figure 4. Microphotographs of the ventral lobe of the prostate of young rats: a, b, control; c, d - letrozole; e, f - in 2 months. After letrozole withdrawal. Hematoxylin-eosin, a, c, e – about × 40; b, d, f – about × 400.

number of cells and fibers. The nuclei of epithelial cells are often hyperchromic, preformed, has small nucleoli. Cytoplasm of all cells had very few basophilic granules, the Golgi zone was preserved.rarely, that indicates a very weak secretory activity of these cells. In letrozole-treated aging rats, the size of the acinuses increased and the tortuosity decreased. The amount of secretion granules grew up (Fig. 5c). Myoid cells were less noticeable, stretched. The layers of connective tissue are thin, they mainly consisted of amorphous matter. Large papillary outgrowths of high cylindrical epithelial cells of normal structure with normochromic spherical nuclei, clear nucleoli, abundant basophilic granularity, and a clear Golgi zone appeared in many terminal sections of the VP (Fig. 5d). Mitosis of epithelial cells

was found. In general, the described picture approached that of young males and indicated a partial "rejuvenation" of the histological structure of the gland and the activation of secretory processes in the epithelium with the use of letrozole.

In two months after discontinuation of letrozole, the VP of young rats was presented by large acinuses in accordance with the seasonal activation of the reproductive system (Fig. 4e). The acinuses were lined with a high cylindrical epithelium with many papillary outgrowths in the most secretory active end sections of the glands (Fig. 4f) and a cubic epithelium in the central zone. Histological appearance of the VP was almost the same as the corresponding control.

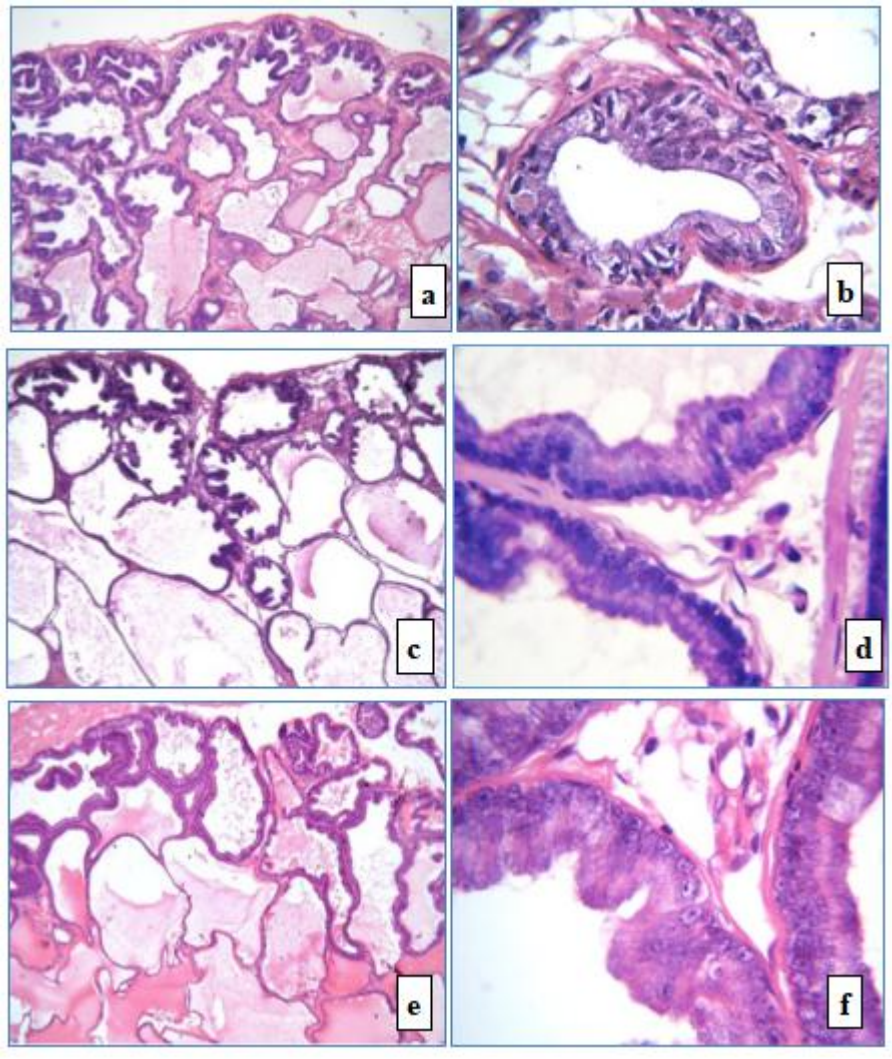

Figure 5.Microphotographs of the ventral lobe of the prostate of aging rats. Notes; see fig. 4.

In two months after withdrawal of letrozole, seasonal activation of the VP (Fig. 5e, f) and no difference with the corresponding control were observed in aging animals.

4.Discussion

Under physiological conditions, involutive changes in the reproductive system are the consequences of the natural aging process and one of its most sensitive indicators. The results obtained in the study on the difference in testosterone concentration and the ratio of testosterone and estradiol levels in the plasma of young and aging control rats, which were determined in December, show the aging of the reproductive system. It is worth noting that this difference was absent in 2 months after letrozole withdrawal, in late February, i.e. on the eve of spring, which is probably due to seasonal activation of the gonads. This also applies to the age difference in the histological structure of the testes, VP and the weights of the VP and accessory sex glands.

In animals of both age groups, no significant changes in the levels of each of the studied sex hormones in the blood plasma were found. This does not preclude a decrease in estradiol production due to inhibition of peripheral conversion of testosterone to estradiol or estrogen synthesis in the testes of rats, as in the case of aromatase inhibition by anastrozole [24]. The authors of this work showed that two-day administration of anastrozole to rats probably reduces the concentration of estradiol in the intercellular fluid of the gonads against the background of no changes in peripheral blood. In our work it was shown that the increase in the ratio of testosterone and estradiol in the blood plasma of aging animals under the influence of letrozole approached statistical significance limit.

Thus, in contrast to males, the sexual hormonal system of male rats is much less responsive to aromatase blockade. For example, only after 19 weeks of anastrozole [24] or 15 weeks of another inhibitor (vorozole), an increase in testosterone levels in male rats was observed since 12 months of age [30].

An important argument for the stimulating effect of three months letrozole treatment on the gonads and androgen-dependent reproductive organs is their weights and histological structure, especially in aging males. Therefore, long-term use of letrozole increases the androgen saturation of aging male rats, which stimulates the relevant organs. The changes in the reproductive organs caused by it are reversible.

Conclusions

1. Under conditions of oral administration of letrozole for 3 months at a dose of 1 mg / kg b.w. once every 2 days, the drug does not cause side effects in either young or aging male rats. In particular, it does not affect body weight, animal behavior, appearance, consumption of food and water.

2. The introduction of letrozole according to the above scheme leads to an increase in androgenic saturation of the body of aging male rats, against the background of age-related involution of the reproductive system.

3. According to all studied indicators, in 2 months after letrozole withdrawal there is a leveling of the difference compared with control animals.

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