Arch Biol Sci. 2016;68(4):821-828 DOI:10.2298/ABS151201070N

SEX STEROID APPLICATION REVERSES CHANGES IN RAT CASTRATION CELLS: UNBIASED STEREOLOGICAL ANALYSIS

Nataša M. Nestorović*, Svetlana L. Trifunović, Ivana M. Jarić, Milica N. Manojlović-Stojanoski, Nataša M. Ristić, Branko R. Filipović, Branka T. Šošić-Jurjević and Verica Lj. Milošević

Department of Cytology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

***Corresponding authors**: rnata@ibiss.bg.ac.rs

Received: December 1, 2015; **Revised**: February 17, 2016; **Accepted**: February 18, 2016; **Published online**: August 17, 2016

Abstract: The aim of the present study was to examine the morphometric characteristics of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) immunoreactive cells in the pituitaries of orchidectomized (Orx) and Orx testosterone- or estradiol-treated rats. Adult male Orx Wistar rats, 2 weeks post operation, received estradiol dipropionate (E) or testosterone propionate (T) for 3 weeks. Both controls, sham-operated (So) and Orx rats, were injected with solvent, in the same regime. Changes in the volume of *pars distalis*, the volumes of individual FSH- and LH-labeled cells, their numerical density and number were determined by unbiased design-based stereology. The FSH and LH intracellular content was estimated by relative intensity of fluorescence (RIF). We observed that Orx caused hyperstimulation of gonadotropic cells. Their volume, volume density, number, numerical density and intracellular hormone content significantly increased in comparison to So controls. Compared to Orx controls, T caused a significant decrease in the volume and volume density of gonadotropic cells and immunoreactive FSH and LH content in their cytoplasm. The volume of the *pars distalis*, the numerical density and number of gonadotropic cells were not changed as compared to Orx controls. Estradiol treatment caused a significant increase in the volume of the *pars distalis*, decreases in cell volume, volume and numerical density of gonadotropic cells, and FSH and LH intracellular content in comparison to Orx controls. The number of FSH-labeled cells increased. In conclusion, both T and E reversed all of the examined parameters of gonadotropic cells of Orx rats to the level of So controls, except in number.

Key words: estradiol; gonadotropic cells; orchidectomy; stereology; testosterone

INTRODUCTION

Gonadotropes are cells in the anterior pituitary (*pars distalis* of the adenohypophysis) that produce two gonadotropic hormones to regulate the ovaries and testes [1]. Gonadotropins include luteinizing hormone (LH), which is important in regulating ovulation and luteinization of the ovarian follicles in the female. It regulates Leydig cells in the testes of the male. Another gonadotropin is follicle-stimulating hormone (FSH), which regulates the development of follicles in the female and sperm production in the male. Immunocytochemical studies have shown that most gonadotropes are bihormonal, i.e. they store both FSH and LH. The percentage of bihormonal and monohormonal cells varies significantly with the physiological state of the animal, such as castration or estrous cycle [2].

Healthy reproduction requires the precise regulation of FSH and LH. This regulation consists of a number of feedforward and feedback signals. It is widely accepted that in male mammals the levels of circulating LH and FSH are largely determined by the stimulatory effects of the gonadotropin-releasing hormone (GnRH) and activins, and the inhibitory effects of steroids and inhibin [3]. Testicular androgens have a main negative feedback effect, which has been demonstrated in early studies where castration resulted in a significant increase in circulating LH and FSH and was subsequently prevented by the administration of physiological doses of testosterone [4]. Further studies have demonstrated that the feedback effects of testosterone are mediated at the level of the hypothalamus and the pituitary. However, estradiol can also act on the hypothalamus or pituitary via a feedback-regulating mechanism. The specific role of each sex steroid

in the regulation of gonadotropin negative feedback is still not completely explained.

Estrogen [5] and androgen [6] receptors have been found on the pituitary gonadotropic cells of rats, but their exact role and significance is not yet clear. Recently, O'Hara et al. [7] reported that androgen receptor signaling is dispensable in the mouse male pituitary for testosterone-dependent regulation of LH secretion, since blood levels of testosterone and gonadotropins were unchanged in mice lacking the pituitary androgen receptor. On the other hand, Lindzey et al. [8] reported that androgen receptor signaling pathways are important in regulating the hypothalamic GnRH content, and hence the observed blood levels of gonadotropins in male mice.

The effects of both estrogens and androgens and the role of their respective receptors in the regulation of gonadotropin synthesis and secretion have been mostly examined in the animal model of castrated rodents. Castration causes hyperstimulation of gonadotropes and promotes their bihormonal state [9]. These cells are called castration or signet ring cells, and are prominent in the *pars distalis* of castrated males [10]. Ultrastructurally, they contain an extremely dilated and rough ER, a well-developed Golgi complex and numerous secretory granules. Estradiol and testosterone both bring about the recovery of castration cells and suppression of circulating LH, while differently regulating the expression of granins, proteins colocalized with gonadotropins in secretory granules [11].

The classic stereological and morphometric approach has been used to characterize changes caused by castration [9,12]. However, to the best of our knowledge, stereological features of castration gonadotropes after they have been exposed to the influence of sex steroids have not examined. Bearing in mind the different effects of male and female sex steroids on the population of gonadotropic cells, the aim of this study was: (i) to estimate the changes in the morphology of gonadotropic cells following castration using an unbiased, design-based stereological approach; (ii) to analyze the recovery potential of testosterone and estradiol treatments; (iii) to evaluate the intracellular content of immunofluorescently labeled gonadotropic hormones in castration cells and after sex steroid treatments, and (iv) to compare the effects of testosterone and estradiol on all the examined parameters.

MATERIALS AND METHODS

Animals and experimental design

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade. All surgeries were performed under anesthesia, and all efforts were made to minimize the suffering of the animals.

The experiments were performed on adult 2-month-old male Wistar rats that were bred in the facilities of the institute. The animals were housed under standard environmental conditions (a 12 h light/dark cycle, 22±2C). Standard food pellets and water were provided *ad libitum*. The experimental animals were bilaterally orchidectomized (Orx) or sham-operated (So) under ketamine anesthesia (ketamine hydrochloride 15 mg/kg bw; Richter Pharma, Wels, Austria). Two weeks after surgery, the Orx rats were divided into three groups, each consisting of 6 animals. They were subcutaneously injected with 5 mg/kg bw testosterone propionate (T) (Fluka Chemie AG, Buchs, Switzerland) or 0.625 mg/kg bw of estradiol dipropionate (E) (Galenika, Belgrade, Serbia; Orx+E) every day for three weeks. The control Orx and So groups received equivalent volumes of sterile olive oil and served as controls.

The used dose of E was a high pharmacological dose previously shown to elicit a strong estrogenic effect on the somatotropic and hypothalamo-pituitary-adrenal axis in the experimental model of young castrated rats [13,14]. This dose is the same as used in the experiments of Nolan and Levy [15], who examined the trophic effects of estradiol on the mitotic activity of pituitary cells following gonadectomy, and is equivalent to about 1.3 mg per week per rat. T at a dose of 5 mg/kg bw shows osteoprotective effects in orchidectomized mice and rats [16,17], and in our experimental model raised the concentration of testosterone ten-fold in comparison to So rats (data not shown).

Tissue preparation and immunohistochemistry

The pituitary glands were excised, fixed in Bouin's solution for 48 h and dehydrated in increasing concentrations of ethanol and xylene. After embedding in HISTOWAX (Histolab Product AB, Göteborg, Sweden), serial sections of 3-μm-thick pituitary slices were placed on silica-coated glass slides (SuperFrost Plus, Prohosp, Denmark) and immunohistochemically stained. Series of sections cut through three tissue levels (dorsal, middle and ventral portions) of the *pars distalis* were used for immunohistochemical and immunofluorescence localization of βFSH- and βLH-containing cells. Sections were analyzed with a light (Olympus BX-51) and confocal laser scanning microscope (Leica TCS SP5 II Basic, Leica Microsystems CMS GmbH; Germany). After rehydration, the sections were stained immunohistochemically. Gonadotropic cells were visualized by the peroxidase enzymatic method or immunofluorescently, as previously described [18,19]. In both cases, anti-rat βFSH (1:300 v/v) and anti-rat βLH polyclonal antibodies (1:500 v/v) served as primary antibodies. Antisera to rat βFSH (NIDDK-anti-rBetaFSH-IC1, Lot# AFP-7798 1289P) and βLH (NIDDK-anti-rBetaLH-IC Lot# AFPC697071P) were obtained from Dr. A. F. Parlow, National Hormone Peptide Program (NHPP), Harbor-UCLA Medical Centre, Carson, CA, USA. After washing in PBS, the sections were incubated with secondary antibodies: either polyclonal swine-anti-rabbit IgG/HRP (Dako A/S, Glostrup, Denmark), or Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes, Inc., USA, 1:200). For light microscopy, antibody localization was visualized using a 0.05% DAB liquid substrate chromogen system (Dako A/S, Glostrup, Denmark). The sections were thoroughly washed under running tap water and counterstained with hematoxylin.

For the evaluation of the intracellular FSH and LH protein (hormonal) contents, an Ar-ion 488-nm laser was used for excitation of fluorescence. Analysis of confocal microscopy images was performed using the Quantify option in LAS AF Lite software (Leica Application Suite Advanced Fluorescence Lite/1.7.0 build 1240, Leica Microsystems CMS GmbH; Germany). Relative intensity of fluorescence (RIF) in the cytoplasm of pituitary gonadotropes was evaluated according to previously described procedures [14,19].

STEREOLOGICAL MEASUREMENTS

All stereological analyses were carried out using a workstation comprised of a microscope (Olympus BX-51) equipped with a microcator, a motorized stage and a CCD video camera. The whole system was controlled by the newCAST stereological software package (VIS-Visiopharm Integrator System, version 3.2.7.0; Visiopharm; Denmark). The main objectives were planachromatic 4, 40 and 100 × lenses. Control of stage movements and interactive test grids and unbiased dissector frames were provided by the newCAST software package.

The volume of *pars distalis* (V), which served as a reference volume, was estimated using Cavalieri's principle [20]. As every 20th section from each tissue block was analyzed, the mean distance between two consecutively studied sections was 60 μm.

Section pairs from the dorsal, middle and ventral portion of the pituitary *pars distalis* were immunohistochemically stained and used for estimation of the FSH and LH numerical densities (N_V). The numerical density, or the number of cells per volume unit, was estimated using a physical dissector design. Nuclei of gonadotropic cells were designated as the reference points, and the cells were counted according to the counting rules: (i) if their nuclei appeared within the unbiased counting frame in the reference section; (ii) if they did not touch forbidden lines of the frame or its extensions, and (iii) if they did not appear on the look-up section. The numerical density (N_V) is the number of counted cells (Q^-) contained in an analyzed tissue volume ($V_{analyzed}$). The volume of the analyzed tissue is estimated as the product of the number of counting frames (ΣPi), the area of the counting frame ($a=2500^2$) and the dissector height (h), which was equal to section thickness (3 μm). These relations are presented in the following formula:

$$N_V = \frac{Q^-}{V_{analyzed}} = \frac{Q^-}{\sum_{i=1}^{n} Pi \times a \times h}$$

The number of gonadotropic cells *per pars distalis* is the product of N_V and the volume of the *pars distalis*.

Cells that were counted using a physical dissector were sampled for cell volume estimation by a planar rotator tool as previously described in detail [21]; 150-200 FSH- and LH-immunoreactive nucleated cells were counted per animal (objective magnification was 100x). The volume density (V_V) of gonadotropic cells was calculated as the ratio of the number of points hitting FSH- or LH-immunoreactive cells and the number of points hitting the reference space. This parameter was estimated on the same sections that served for N_V and cell volume estimation.

Statistical analysis

All results were expressed as means for six animals per group±standard deviation (SD). The data were tested for normality of distribution by the Kolmogorov-Smirnov test. One-way analysis of variance followed by the Newman-Keuls test were used for comparison of differences between the groups. A probability value of 5% or less was considered as statistically significant.

RESULTS

Histological analysis

Gonadotropic cells in pituitaries of So males (Figs. 1a and b) are oval to polyhedral in shape, with often eccentrically positioned nuclei. They are mostly regularly distributed throughout the *pars distalis* and often can be seen in close contact with blood capillaries. After Orx, gonadotropic cells were hypertrophic, strongly staining immunohistochemically and often vacuolated (Figs. 1c and 1d). After the treatment with T, the appearance of the gonadotropic cells was similar to those in the pituitaries of So males. In comparison to Orx gonadotropic cells, they were smaller in size without cytoplasmic vacuolation (Figs. 1e and 1f). Treatment with E changed the histological appearance of gonadotropic cells. They were significantly smaller in size compared to cells in the pituitaries of Orx males and mostly polyhedral in shape (Figs. 1g and 1h).

Stereological analysis

The volume of the *pars distalis* (V) was not changed in Orx males compared to So males (Fig. 2a). However,

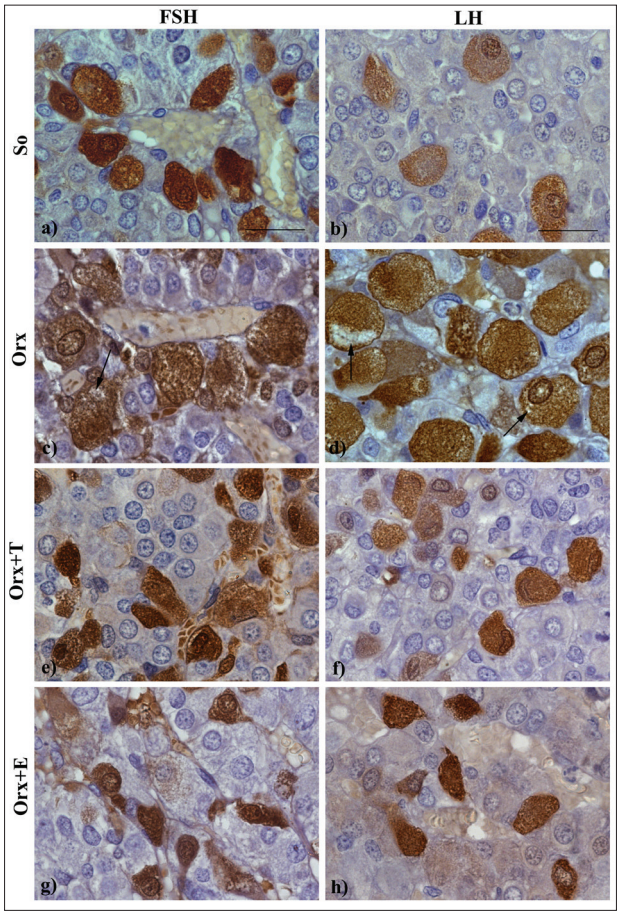

Fig. 1. Immunohistochemical staining of gonadotropes in the pituitaries of male rats. Representative micrographs of FSH (a, c, e, g) and LH (b, d, f, h) cells in pituitaries of So (a, b), Orx (c, d), and Orx rats treated with T (Orx+T; e, f) and E (Orx+E; g, h). In the pituitaries of Orx rats, gonadotropic cells are hyperstimulated (c, d), i.e. larger in size and with a vesiculated cytoplasm (arrows). After treatment with either T (e, f) or E (g, h), the FSH and LH cells were smaller, and a vesiculated cytoplasm was no longer present.

the volume of gonadotropic cells (Vc) significantly increased, by 81.4% in FSH- and by 49.3% in LH-immunolabeled cells after Orx (Fig. 2b). This was followed by 3- and 4-fold increases in volume density (V_V) of FSH- and LH-immunoreactive cells, respectively (Fig. 2c). Orchidectomy also caused an increase in the number (No) of gonadotropic cells (by 52.9% in FSH-labeled and by 89.6% in LH-labeled cells) (Fig. 2d), and their numerical density (N_V; by 43.2% in FSH-labeled and by 75.8% in LH-labeled cells) (Fig. 2e).

Compared to the Orx values, treatment with E increased the volume of the *pars distalis* (V) by 93.2% ($p<0.05$), while the treatment with T did not have a similar effect (Fig. 2a). However, the volume of go-

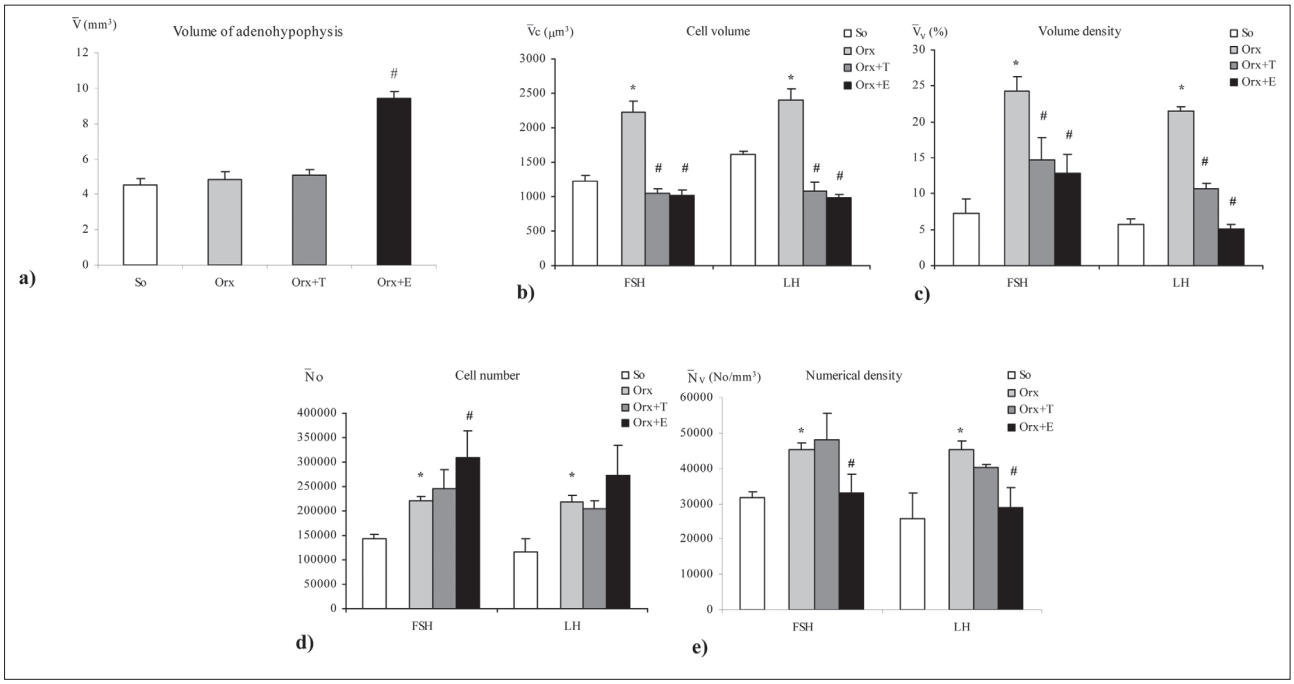

Fig. 2. Stereological parameters of gonadotropes in the pituitaries of male rats. **A** – volume of the *pars distalis* (V; mm^3); **b** – cell volume (Vc; μm^3); **c** – volume density (V_V; %); **d** – cell number (No); **e** – numerical density (N_V; mm^{-3}) of FSH- and LH-immunoreactive cells in the pituitaries of sham-operated (So), orchidectomized (Orx), and orchidectomized rats treated either with T (Orx+T) or E (Orx+E). All values are provided as the mean±SD; n = 6. *p<0.05 Orx *vs*. So; #p<0.05 Orx treatment *vs*. Orx control.

nadotropic cells (Vc) was decreased to the same extent (p<0.05) in both treated groups (Fig. 2b).

In the pituitaries of Orx+T males, the volumes of FSH- and LH-immunoreactive cells were decreased by 52.8% and 55.5%, respectively, as compared to the Orx group. E decreased the volume (Vc) of FSH- and LH-labeled cells by 54.7% and 58.8%, respectively (Fig. 2b). The volume densities (V_V) of gonadotropic cells were also significantly decreased, but to a greater extent after the treatment with E. Namely, E caused a decrease in V_V of FSH- and LH-immunoreactive cells by 47.4% and 76.1%%, respectively, while T caused decreases by 32.9% and 50.11%.

The total number (No) and numerical density (N_V) of gonadotropic cells did not change after the treatment with T, whereas in the pituitaries of Orx+E males, the total number of FSH-reactive cells was increased (p<0.05). The number (No) of LH-labeled cells was increased as compared to the Orx group, though statistical significance was not observed (Fig. 2d). On the other hand, the numerical density (N_V) of FSH- and LH-immunoreactive cells was significantly decreased in Orx+E males (Fig. 2e).

Immunofluorescence analysis

Gonadotropic cells of So males possessed a strong immunofluorescence signal of labeled hormones localized in the cytoplasm. After Orx, gonadotropic cells had more pronounced FSHβ (by 23.1%) and LHβ (by 45.3%) immunofluorescent (IF) signals as compared to the So group (Figs. 3a and 3b). The treatment of Orx males with T lowered the IF signal in both FSH- and LH-reactive cells, and the relative intensity of fluorescence (RIF) was significantly decreased by 39.8% and 41.8%, respectively, as compared to Orx animals (Figs. 3a and 3b). Estradiol also decreased RIF in both FSH- and LH-labeled cells by 29.5% and 30.1%, respectively (Figs. 3a and 3b).

DISCUSSION

Gonadectomy in rodents is a widely used experimental model for examining the regulation of FSH and LH synthesis and release. The population of gonadotropic cells undergoes a dramatic functional and morphological modification resulting from the removal of

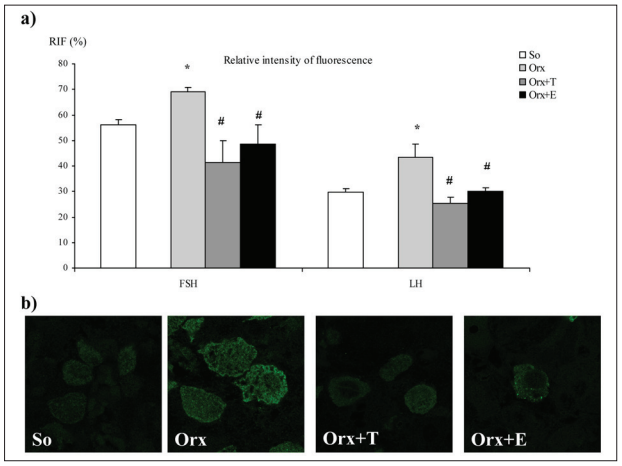

Fig. 3. Immunofluorescence of gonadotropic cells in the pituitaries of male rats. **A** – the relative intensity of fluorescence (RIF) of FSH and LH cells in the pituitary *pars distalis* of sham-operated (So), orchidectomized (Orx), and orchidectomized rats treated either with T (Orx+T) or E (Orx+E). All values are provided as the mean±SD; n = 6. *p<0.05 Orx *vs*. So; # p<0.05 Orx treatment *vs*. Orx control. **B** – representative micrographs of gonadotropic cells; immunofluorescence for βLH in the pituitary *pars distalis* of sham-operated (So), orchidectomized (Orx), and orchidectomized rats treated with T (Orx+T) or E (Orx+E).

the negative feedback exerted by gonadal hormones, both at the hypothalamus and at the pituitary. In this study we show that the chronic application of T and E recovered the morphological features of castration gonadotropes and the intracellular content of both gonadotropic hormones. The only parameter that did not return to the level of the So controls was the number of FSH- and LH-labeled cells.

Five weeks post Orx, the gonadotropes were hypertrophied and enlarged, with prominent vesiculation of the cytoplasm caused by the extremely dilated rough ER and a well-developed Golgi complex [11]. Inoue and Kurosumi [22] reported a 6.5-fold increase in LH-reactive gonadotropic cell volume three months following Orx. In our study, five weeks post Orx, the observed increases in cellular volumes of FSH- or LH-reactive cells were by about 81% and 49%, respectively. This discrepancy could be explained as due to the use of different techniques of measurement and sampling. Using the planar rotator, we measured the volume of unbiasedly sampled individual gonadotropic cells throughout the *pars distalis*. The increased volume of gonadotropic cells caused an increase in their overall percentage within the *pars distalis,* i.e. the volume density. Using classical stereological methods on dissociated pituitary cells, Childs et al. [9] documented that this increase was dramatic 24 h after castration, and that it gradually increased during the first month [12]. In our study, the increase in volume density of FSH- and LH-labeled cells was estimated to be two-fold. We observed a significantly larger number of gonadotropic cells/mm^3 in the *pars distalis*, i.e. an increase in their numerical density after Orx. The increase of this parameter was due to the increase in the number of gonadotropic cells since the reference volume (*pars distalis*) remained unchanged. The expansion of the gonadotropic population after Orx was recently observed as an increase in the number of GnRH-responding cells in mice [23-25]. Intracellular storage of FSH and LH in castration cells was increased, which was previously shown at the ultrastructural level [11].

It was shown that hypertrophy of gonadotropes after Orx is caused by increased stimulation by GnRH, and that it can be prevented by injection of anti-GnRH antibody [26]. The addition of GnRH into the pituitary culture medium results in hypertrophic gonadotropes similar to those observed following gonadectomy [27]. Estradiol and dihydrotestosterone (DHT), a nonaromatizable androgen, both provide a recovery of the GnRH content in Orx male mice, which suggests that androgen and estrogen signaling pathways can regulate the GnRH content in castrated males [8]. However, sex steroids can act on gonadotropic cells directly, since expression of androgen and estrogen receptors was found in gonadotropes of rats [5,6]. Whether they act at the hypothalamic or pituitary levels or both, T and E effectively suppress serum FSH and LH concentrations [8].

In our study, T and E both brought about the recovery of hyperstimulated gonadotropes. Histologically, this was observed as an absence of typical castration cells in the *pars distalis* of treated males. Stereologically, the volume and volume densities of FSH- and LH-labeled gonadotropes were significantly reduced. Intracellular βFSH and βLH fluorescence was decreased to the level observed in So controls. The differences between the sex steroid treatments were detected in the volume of the *pars distalis*, the number of gonadotropic cells and their numerical density. E caused an almost two-fold increase in the volume of the *pars distalis.* E stimulates the prolifera-

tion of pituitary cells [28], which was confirmed in prolactin [29] and adult stem cells [30], resulting in an increase in the volume of the *pars distalis*. T on the other hand had no effect on this parameter. The number of gonadotropic cells after treatment with T remained at the same level as in the *pars distalis* of Orx males, whereas after treatment with E it increased. A statistically significant difference was observed only in FSH-labeled cells; however, an increasing trend was also detected in LH-reactive cells. The numerical density, i.e. the number of cells per unit volume of reference space, is the quotient of the number of gonadotropic cells and the volume of *pars distalis*. Consequently, this parameter was unchanged after the treatment with T and decreased after treatment with E. The reason for this is the enlarged volume of the *pars distalis* after the administration of E. It should be noted that the expansion of the *pars distalis* volume was caused mostly by the multiplication of prolactin cells, and that it was not accompanied by the multiplication of gonadotropic cells to the same extent.

The literature data about the effects of E and T on the number of gonadotropic cells after castration are scarce, although the presented findings are in agreement with the expansion of gonadotropic cells after Orx. Inoue et al. [24] reported on a decrease in gonadotropic cells with mitotic figures after a week's treatment with T one week post Orx. On the other hand, it has been shown that aromatizable androgens, such as T that was used in our study, exert a transient stimulatory effect on pituitary mitotic activity [28]. However, the FSH- and LH-labeled cells observed in our study could have originated and increased not only as a consequence of mitotic division, but also as the result of the differentiation of immature cells into gonadotropes and the transdifferentiation of mature pituitary cells from another lineage into gonadotropes [31]. Childs [32] suggested that a population of multihormonal/multipotent cells exists in the pituitary in order to provide support for all of the requirements of the gonadotropins at mid-cycle in females. Growth hormone cells are possible candidates, since the co-expression of GnRH and growth hormone releasing hormone (GHRH) receptors has been documented. The expression of GnRH receptors is stimulated by E and possibly activin [32]. In our study, the high doses of E applied to males could have triggered transdifferentiation and sustained the high number of gonadotropic cells.

In conclusion, we have demonstrated that the exposure of Orx rats to T or E causes the recovery of castration gonadotropic cells in terms of their volume, volume density and specific hormone content. The number of gonadotropic cells was the only parameter that did not return to the control level and in which the difference between the effect of estradiol and testosterone treatment was observed.

Acknowledgments: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. 173009. The authors are grateful to Ms. Sonja Jeremić (Kefo d.o.o., Belgrade, Serbia) for her technical support.

Authors' contributions: NN, ST contributed equally to this work. NN, ST and VM contributed to the experimental design. IJ, MM-S and NR contributed in the stereological counting. BŠ-J and BF conducted the part of the experiment with the animals. NN drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest disclosure: The authors disclose no actual potential conflict of interest.

REFERENCES

1. Greep RO. The gonadotrophins and their releasing factors. J Reprod Fertil. 1973;20(0):1-9.
2. Childs GV. Division of labor among gonadotropes. Vitam Horm. 1995;50:215-86.
3. Vale W, Bilezikjian LM, Rivier C. Reproductive and other roles of inhibins and activins. In: Knobil E, Neill JD, editors. The Physiology of Reproduction Vol 1. New York: Raven Press; 1994. p. 1861-78.
4. Ramirez V, McCann S. Inhibitory effect of testosterone on luteinizing hormone secretion in immature and adult rats. Endocrinology. 1965;76(3):412-7.
5. Mitchner NA, Garlick C, Ben-Jonathan N. Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. Endocrinology. 1998;139(9):3976-83.
6. Okada Y, Fujii Y, Moore JP Jr, Winters SJ. Androgen receptors in gonadotrophs in pituitary cultures from adult male monkeys and rats. Endocrinology. 2003;144(1):267-73.
7. O'Hara L, Curley M, Tedim Ferreira M, Cruickshanks L, Milne L, Smith LB. Pituitary androgen receptor signalling regulates prolactin but not gonadotrophins in the male mouse. PLoS One. 2015;10(3):e0121657.
8. Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS. Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone

content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. Endocrinology. 1998;139(10):4092-101.
9. Childs GV, Ellison DG, Lorenzen JR, Collins TJ, Schwartz NB. Immunocytochemical studies of gonadotropin storage in developing castration cells. Endocrinology. 1982;111(4):1318-28.
10. Farquhar MG, Rinehart JF. Electron microscopic studies of the anterior pituitary gland of castrate rats. Endocrinology 1954;54(5):516-41.
11. Watanabe T, Banno T, Jeziorowski T, Ohsawa Y, Waguri S, Grube D, Uchiyama Y. Effects of sex steroids on secretory granule formation in gonadotropes of castrated male rats with respect to granin expression. Endocrinology. 1998;139(6):2765-73.
12. Ibrahim SN, Moussa SM, Childs GV. Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. Endocrinology. 1986;119(2):629-37.
13. Trifunović S, Manojlović-Stojanoski M, Ajdzanović V, Nestorović N, Ristić N, Medigović I, Milošević V. Genistein stimulates the hypothalamo-pituitary-adrenal axis in adult rats: morphological and hormonal study. Histol Histopathol. 2012;27(5):627-40.
14. Trifunović S, Manojlović-Stojanoski M, Ajdžanović V, Nestorović N, Ristić N, Medigović I, Milošević V. Effects of genistein on stereological and hormonal characteristics of the pituitary somatotrophs in rats. Endocrine. 2014;47(3):869-77.
15. Nolan LA, Levy A. The trophic effects of oestrogen on male rat anterior pituitary lactotrophs. J Neuroendocrinol. 2009;21(5):457-64.
16. Vandenput L, Ederveen AG, Erben RG, Stahr K, Swinnen JV, Van Herck E, Verstuyf A, Boonen S, Bouillon R, Vanderschueren D. Testosterone prevents orchidectomy-induced bone loss in estrogen receptor-alpha knockout mice. Biochem Biophys Res Commun. 2001;285(1):70-6.
17. Filipović B, Sošić-Jurjević B, Ajdžanović V, Pantelić J, Nestorović N, Milošević V, Sekulić M. The effects of sex steroids on thyroid C cells and trabecular bone structure in the rat model of male osteoporosis. J Anat. 2013;222(3):313-20.
18. Nestorović N, Manojlović-Stojanoski M, Ristić N, Sekulić M, Šosić-Jurjević B, Filipović B, Milosević V. Somatostatin-14 influences pituitary-ovarian axis in peripubertal rats. Histochem Cell Biol. 2008;130(4):699-708.
19. Medigović IM, Živanović JB, Ajdžanović VZ, Nikolić-Kokić AL, Stanković SD, Trifunović SL, Milošević VL, Nestorović NM. Effects of soy phytoestrogens on pituitary-ovarian function in middle-aged female rats. Endocrine. 2015;50(3):764-76.
20. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc. 1987;147(Pt 3):229-63
21. Manojlović-Stojanoski M, Nestorović N, Ristić N, Trifunović S, Filipović B, Sošić-Jurjević B, Sekulić M. Unbiased stereological estimation of the rat fetal pituitary volume and of the total number and volume of TSH cells after maternal dexamethasone application. Microsc Res Tech. 2010;73(12):1077-85.
22. Inoue K, Kurosumi K. Mode of proliferation of gonadotrophic cells of the anterior pituitary after castration--immunocytochemical and autoradiographic studies. Arch Histol Jpn. 1981 Mar;44(1):71-85.
23. Durán-Pastén ML, Fiordelisio-Coll T, Hernández-Cruz A. Castration-induced modifications of GnRH-elicited [Ca2+](i) signaling patterns in male mouse pituitary gonadotrophs in situ: studies in the acute pituitary slice preparation. Biol Reprod. 2013;88(2):38.
24. Inoue K, Tanaka S, Kurosumi K. Mitotic activity of gonadotropes in the anterior pituitary of the castrated male rat. Cell Tissue Res. 1985;240(2):271-6.
25. Sakuma S, Shirasawa N, Yoshimura F. A histometrical study of immunohistochemically identified mitotic adenohypophyseal cells in immature and mature castrated rats. J Endocrinol. 1984;100(3):323-8.
26. Arimura A, Shino M, de la Cruz KG, Rennels EG, Schally AV. Effect of active and passive immunization with luteinizing hormone-releasing hormone on serum luteinizing hormone and follicle-stimulating hormone levels and the ultrastructure of the pituitary gonadotrophs in castrated male rats. Endocrinology 1976;99:291-303.
27. Shiino M. Morphological changes of pituitary gonadotrophs and thyrotrophs following treatment with LH-RH or TRH in vitro. Cell Tissue Res. 1979;202:399-406.
28. Nolan LA, Levy A. The effects of testosterone and oestrogen on gonadectomised and intact male rat anterior pituitary mitotic and apoptotic activity. J Endocrinol. 2006;188(3):387-96.
29. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiol Rev. 2000;80(4):1523-631.
30. Rizzoti K., Akiyama H., Lovell-Badge R. Mobilized adult pituitary stem cells contribute to endocrine regeneration in response to physiological demand. Cell Stem Cell. 2013;13(4):419-2.
31. Nuñez L, Villalobos C, Senovilla L, García-Sancho J. Multifunctional cells of mouse anterior pituitary reveal a striking sexual dimorphism. J Physiol. 2003;15(549):835-43.
32. Childs GV. Development of gonadotropes may involve cyclic transdifferentiation of growth hormone cells. Arch Physiol Biochem. 2002;110(1-2):42-9.