J Med Biochem 2012; 31 (1) DOI: 10.2478/v10011-011-0041-3

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 31: 34–39, 2012

Original paper
Originalni naučni rad

IMMUNOHISTOMORPHOMETRIC FEATURES OF ACTH CELLS IN JUVENILE RATS AFTER TREATMENT WITH ESTRADIOL OR HUMAN CHORIONIC GONADOTROPIN

IMUNOHISTOMORFOMETRIJSKE ODLIKE ACTH ĆELIJA U JUVENILNIH PACOVA NAKON TRETMANA ESTRADIOLOM ILI HUMANIM HORIONSKIM GONADOTROPINOM

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Summary: Estradiol and human chorionic gonadotropin (hCG) are very important in controlling the secretory activity of hormone producing cells in the female rat pituitary glands. The aim of the present study was to examine the morphometric parameters of immunohistochemically labeled ACTH cells in juvenile (16th day) female rat pituitaries after treatment with five doses of estradiol dipropionate (EDP) and two doses of hCG during the neonatal period of life. The controls were treated on the same schedule with an equivalent volume of vehicle. All animals were sacrificed 24 h after the last treatment. ACTH-producing cells were studied using the peroxidase-antiperoxidase immunohistochemical procedure. The absolute and relative pituitary weights were increased ($p<0.05$) only in the EDP treated group by 120.0% and by 121.1% respectively, in comparison with the controls. In this group, the volume of ACTH cells, volume of their nuclei and volume density were significantly decreased ($p<0.05$) by 6.4%, 33.3% and 46.2% respectively, compared to the corresponding controls. After treatment with hCG, there were no significant ($p>0.05$) changes neither in the volume of ACTH cells nor in the volume of their nuclei, in comparison with the controls. On the basis of the results obtained in our study, it can be concluded that EDP, injected into female rats during the neonatal period of life, has an inhibitory effect on the immunohistomorphometric parameters of ACTH cells, but such an effect is not clearly expressed after treatment with hCG.

Keywords: ACTH cells, EDP, hCG, female rats

Kratak sadržaj: Estradiol i humani horionski gonadotropin (hCG) veoma su važni u kontroli sekretorne aktivnosti ćelija hipofize koje proizvode hormone. Cilj ove studije je bio ispitati morfometrijske parametre imunohistohemijski obeleženih ACTH ćelija hipofize ženki pacova, u juvenilnom periodu (16. dan), nakon tretmana sa pet doza estradiol-dipropionata (EDP) ili dve doze humanog horionskog gonadotropina (hCG). Kontrole su bile tretirane na istovetan način sa odgovarajućom količinom rastvarača. Sve životinje su bile žrtvovane 24 h nakon poslednjeg tretmana. ACTH ćelije su bile imunohistohemijski obeležene metodom peroksidaza-antiperoksidaza (PAP). Dobijeni rezultati ukazuju na značajno povećanje ($p<0,05$) apsolutne i relativne mase hipofize za 120% i 121,1% kod ženki tretiranih sa EDP u odnosu na kontrolu. U ovoj grupi ženki pacova volumen ACTH ćelija i njihovih jedara, kao i njihova volumenska gustina su bili značajno smanjeni ($p<0,05$) za 6,4%, 33,3% i 46,2% u poređenju sa kontrolom. Nakon tretmana hCG-om, volumen ACTH ćelija, kao i volumen njihovih jedara, nisu bili značajno ($p>0,05$) promenjeni u odnosu na kontrolu. Na osnovu dobijenih rezultata možemo zaključiti da EDP, injeciran ženkama pacova tokom neonatalnog perioda života, ima inhibitorni efekat na imunohistomorfometrijske parametre ACTH ćelija, što nije tako jasno izraženo nakon tretmana hCG-om.

Ključne reči: ACTH ćelije, EDP, hCG, ženke pacova

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Introduction

The hypothalamic-pituitary-adrenal (HPA) axis represents an essential stress response system and a dynamic interface for multiple physiological systems. Biochemically interconnected, it generates rhythmic hormone secretion. The respective hormones, corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and principle glucocorticosteroids (CORT; i.e. cortisol in humans, corticosterone in rats) modulate their own secretion through feedforward and feedback mechanisms within the HPA axis and exert physiological effects in target organs outside the HPA axis (1). Corticotrophs (ACTH cells) are basophilic cells, which produce proopiomelanocortin (POMC) and its derivatives including ACTH, melanotropin (MSH) and lipotropin (LPH). These cells represent approximately 15% to 20% of adenohypophysial cells (2). ACTH release from the anterior pituitary is regulated by a variety of hormones and neuropeptides, through both cAMP-dependent and cAMP-independent mechanisms (3, 4).

It was shown that estrogen treatment decreases the level of POMC mRNA and lowers the ACTH response in stress-stimulated ovariectomized adult female rats (5). In contrast, estradiol treatment increased the ACTH level in middle-aged female rats (6). Schreihofer et al. (7) report that the estrogen receptor β (ER β) level is very low in rodent pituitaries, while, unlike follicle-stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) cells, less than 5% of ACTH cells express estrogen receptor α (ERα) in the human pituitary (8). Considering the low proportion of ER, it seems that the indirect mechanisms of estradiol action are considerable in ACTH cells.

Placental human chorionic gonadotropin (hCG) is a crucial hormone during pregnancy, maintaining the corpus luteum during early gestation. It is a heterodimeric glycoprotein, composed of 244 amino acids, with two subunits: α (alpha), identical to that of LH, FSH and thyroid-stimulating hormone (TSH); and β (beta), responsible for hormone-specific biological functions (9). Human chorionic gonadotropin interacts with the luteinizing hormone/choriogonadotropin (LHCG) receptor and promotes the maintenance of the corpus luteum during the beginning of pregnancy, causing it to secrete the hormone progesterone (10). Also, it was observed that hCG significantly increases the number of pituitary FSH, LH and TSH cells (11, 12).

The present study was focused on the histological and morphometric characteristics of immunopositive ACTH cells of juvenile female rats, neonatally treated with multiple doses of estradiol or with two doses of hCG.

Material and Methods

Wistar female rats, bred at the Institute for Biological Research, Belgrade, Serbia, were used in the experiment. The animals were kept under a 12:12 h light-dark cycle, at 22±2 °C. They had free access to food (a product of the Veterinary Institute Subotica, Subotica, Serbia) and water. Females were divided into three groups of five animals. The first group (EDP) received five intraperitoneal (i.p.) injections of estradiol dipropionate (EDP; 0.25 mg/kg b.w., ICN Galenika Pharmaceuticals, Belgrade, Serbia) every second day from the 4th to the 14th day after birth. After the treatment with EDP, the animals were sacrificed at the juvenile stage, when they were 17 days old. The second group of females (hCG) i.p. received two doses of pregnyl-gonadotrophinum chorionicum (hCG; 50 IU/kg b.w.; N.V. Oregon, Netherlands) on the 15th and 16th day of life. The control group was injected with an equivalent volume of the vehicle. All animals were sacrificed 24 h after the last treatment.

Pituitary glands were excised, weighed, fixed in Bouin's solution for 48 h and embedded in paraplast. The relative pituitary weights were calculated from the ratio of the measured pituitary weight and the body weight for each animal.

The experimental protocols were approved by the Animal Care Committee of the Institute for Biological Research (Belgrade, Serbia) in conformity with the recommendation provided in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123, Appendix A).

Immunohistochemical studies

ACTH-producing cells were identified by immunohistochemistry using the peroxidase-antiperoxidase method (PAP), described by Sternberger et al. (13). Endogenous peroxidase activity was first blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min. Reduction of non-specific background staining was achieved by incubation with normal porcine serum (DAKO A/S, Glostrup, Denmark), diluted in phosphate-buffered saline pH 7.4 (PBS; 1:10), for 45 min. Sections were then overlaid with commercially diluted primary antibodies (hACTH antiserum DAKO A/S, Glostrup, Denmark) for 24 h at 4 °C. This antibody strongly cross-reacts with rat ACTH (Starčević et al. (14); verified by Dr B. A. Yang of Dako Corp.). After washing in PBS for 5 min, sections were incubated for 60 min with a second antibody, swine anti-rabbit IgG (DAKO, Glostrup, Denmark; diluted 1:100 in PBS), rinsed again in PBS for 5 min and then incubated with rabbit PAP complex (DAKO A/S, Glostrup, Denmark; diluted 1:100 in PBS), for 45 min. Binding sites were visualized using 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany)

and 0.03% hydrogen peroxide in 0.2 mol/L TRIS-HCl buffer, pH 7.4. The sections were counterstained with hematoxylin and mounted in Canada balsam (Molar Chemicals KFT, Budapest, Hungary). For the control sections, the primary antibody was omitted and replaced by PBS, pH 7.4.

Morphometry

Rat pituitaries were serially cut to 5 μm thick sections. Two sections from the dorsal, three from the middle and two from the ventral part (seven sections, 20 μm apart in total) of the rat pituitary glands were analyzed. The point counting method was used at an overall magnification of x1000 (15). The M_{42} multipurpose test grid, inserted into the ocular of a Zeiss light microscope (Jena, Germany), was randomly positioned on the pituitary section at the beginning of counting. Counting was carried out on the following 50 test fields per section. Average values were calculated per pituitary i.e. per animal (7 sections, 350 test fields) and five pituitaries were analyzed per group. Cell volume (Vc; μm^3), volume of the nuclei (Vn; μm^3) and volume density (percentage of immunoreactive cells in μm^3, Vvc; %) were determined for ACTH-immunoreactive cells.

The following parameters were counted:

Pn – number of points hitting on the nuclei of immunohistochemically labelled cells inside the test field

Ptc – number of points hitting on the cytoplasm of immunohistochemically labelled cells inside the test field

Nn – number of the immunohistochemically labelled cell nuclei inside the test field.

The formula for calculating the nuclei volume was:

$$Vn = V_{Vn}/N_V,$$

and that for the cell volume calculation was:

$$Vc = 1/N_V, \text{ where}$$

V_{Vn} is – the volume density of ACTH cell nuclei and Nv – the numerical density of ACTH cells. Nuclei volume density (V_{Vn}) provides information about nuclei attendance in the estimated cells and is calculated as follows:

$$V_{Vn} = \Sigma Pn/\Sigma Ptc.$$

Since rat ACTH cells are mononuclear, Nv corresponds to the number of cells per cubic millimeter, according to the formula:

$$Nv = (k/\beta)\ (Na^{3/2}/V_{Vn}^{1/2}).$$

On the basis of earlier karyometric studies (16), the shape coefficient β for pituitary cells was estimated to be 1.382, k is a factor related to cell distribution according to their size (in the case of ACTH cells its value is 1) and NA is the number of cells in the plane of the pituitary tissue section. Na is calculated as follows:

$$N_A = \Sigma Nn/\Sigma Ptc \cdot a,$$

where a represents the rhombic area belonging every point of the test system and is calculated using the formula:

$$a = d^2\ 3^{1/2}/2, \text{ where}$$

d is – the test line length in the test system employed.

Volume density (V_{VC}) is calculated as the ratio of the sum of Pn and Ptc (Pn+Ptc) and the total number of points in the test system. Since the test system with 42 points was used and parameters were calculated using 50 test fields, the definite formula was:

$$V_{VC} = (Pn+Ptc)/\ 50 \cdot 42.$$

Statistical analysis

STATISTICA® version 5.0 (StatSoft, Inc) was used for all statistical analyses. Morphometric data obtained for the experimental groups were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range test (Pharmacological Calculation System, 1986) was used for *post hoc* comparisons between groups. A confidence level of $p<0.05$ was considered statistically significant. The data are presented as means ± SD.

Results and Discussion

The data summarizing body weights, absolute and relative pituitary weights of the female rats, treated with EDP or hCG, are presented in *Figure 1A-C*. Body weights in the EDP-treated group were not significantly different ($p>0.05$) compared to the corresponding controls, which was also observed in our earlier study, after estrogen treatment of rats during the neonatal period (17). hCG had no effects on the body weights in this study, and this is in accordance with Lijesen et al. (18) who reported that there is no scientific evidence for hCG efficacy in the treatment of obesity. Absolute pituitary weight was significantly increased ($p<0.05$) only in the EDP-treated animals by 120.0%, in comparison with the controls (*Figure 1B*). Relative pituitary weight was significantly increased ($p<0.05$) in the EDP-treated females by 121.1%, compared to the controls (*Figure 1C*). In middle-aged females, chronic EDP treatment was shown to increase absolute pituitary weight (5). Lovren et al. (10) and Sekulić et al. (12) have reported that EDP application, in the critical neonatal period, has stimulatory effects on the absolute pituitary weight and glycoprotein-producing cells morphometric parameters. Also, it was demonstrated that EDP treatment increases the

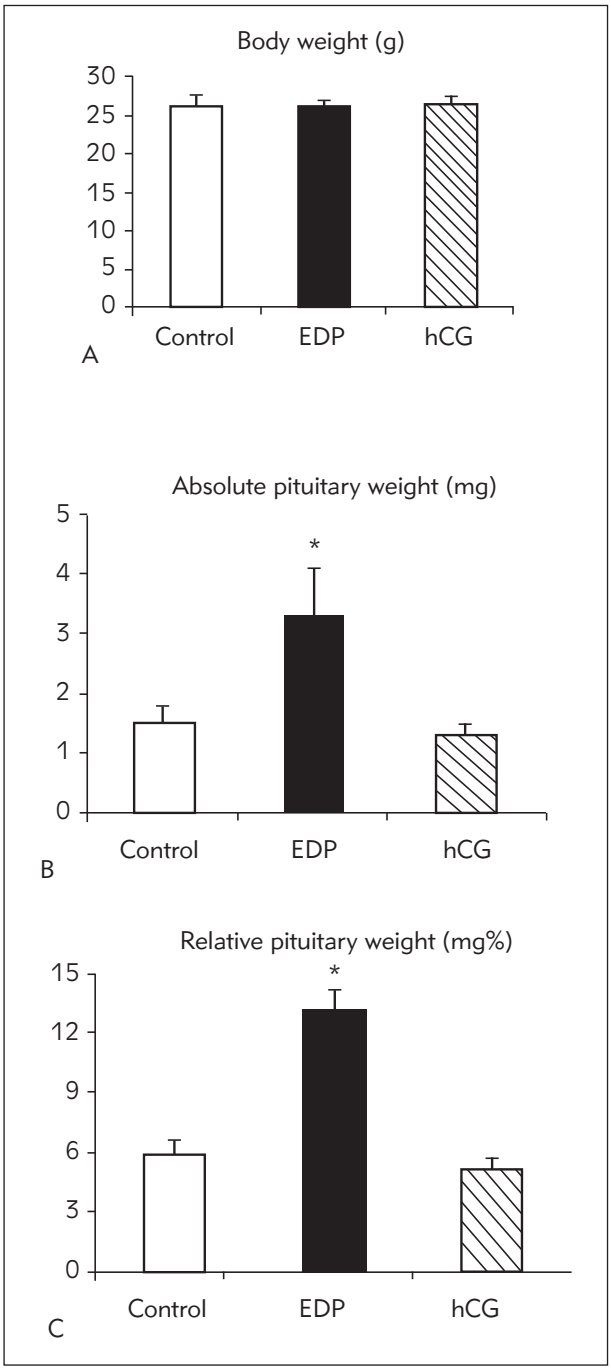

Figure 1. Body weight (g); B. absolute pituitary weight (mg); C. relative pituitary weight (mg%): in control, estradiol dipropionate (EDP) and human chorionic gonadotropin (hCG) treated female rats. All values are means ± standard deviation, n = 5 animals per group, *p<0.05 vs. control.

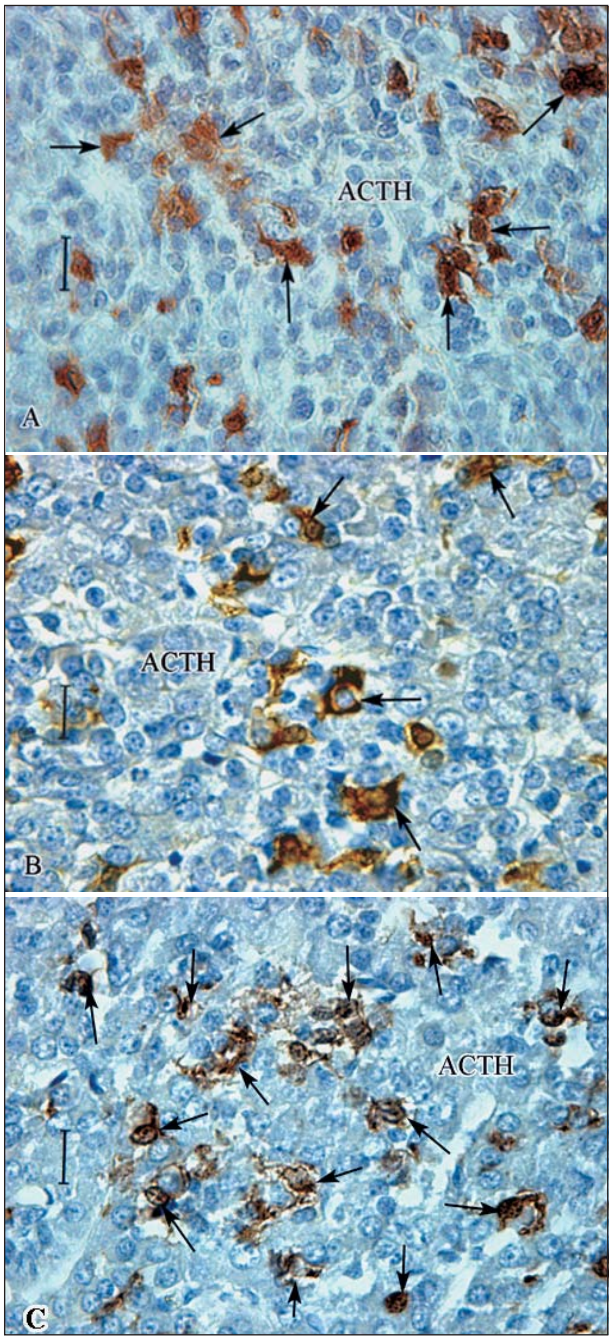

Figure 2. Immunopositive ACTH cells in the *pars distalis* of the pituitary gland from a control female rat; B. small ACTH cells, with intensely immunostained cytoplasm, in an estradiol dipropionate (EDP) treated female rat; C. less immunostained ACTH cells in a human chorionic gonadotropin (hCG) treated female rat. PAP, bar = 16 μm

number of chromophobes, PRL (19, 20) and LH cells (11), which confirms our results.

ACTH cells were positioned throughout the pituitary *pars distalis* and *pars intermedia*. The characteristic findings for the immunohistochemically labeled ACTH cells in control juvenile females considered their stellate shape with cytoplasmatic processes among neighboring (mostly somatotrophic) cells, and the cell localization between capillaries. The nuclei were often centrally located (*Figure 2A*). In females treated with EDP, neither the shape nor the localization of ACTH immunopositive cells were altered com-

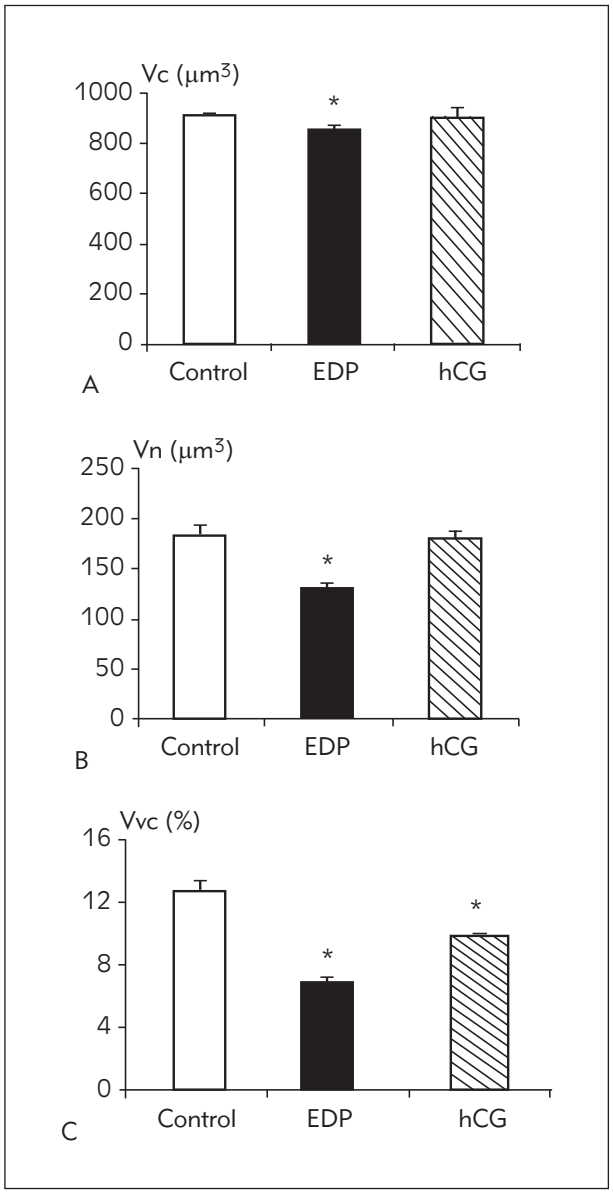

Figure 3. Cell volume (Vc; μm^3) of ACTH cells; B. volume of the nuclei (Vn; μm^3) of ACTH cells; C. volume density (V_{VC}; %) of ACTH cells expressed as a percentage of total gland tissue. All values are means ± standard deviation, n = 5 animals per group, *p<0.05 vs. control.

pared to the controls. These cells were smaller in size, with intensely immunostained cytoplasm (*Figure 2B*). Kostić et al. (6) previously demonstrated that after EDP treatment, ACTH cells in middle-aged female rats do not change their immunohistochemical appearance. In animals treated with hCG the shape and localization of ACTH cells were not changed, but the cytoplasm was less intensely immunostained than in the corresponding controls (*Figure 2C*).

Different studies have established a positive correlation between the morphological changes of ACTH cells and their functional state. The morphometric parameters measured in the present study, i.e. the volume of ACTH cells and the volume of their nuclei, as well as volume density are depicted in *Figure 3A-C*. In animals treated with EDP, the ACTH cell volume, volume of their nuclei and the volume density were significantly decreased ($p<0.05$) by 6.4%, 33.3% and 46.2% respectively, in comparison with the corresponding controls. It was reported that estrogen replacement lowers the POMC mRNA level and the ACTH response in ovariectomized rats (5), but some studies suggest the ACTH level rises after estradiol application (6). Furthermore, estradiol increases the presence of PRL (21) and LH cells (11). It was demonstrated that estrogen stimulates the hypothalamic production of somatostatin (22), which was shown to decrease the morphometric parameters of ACTH cells (14). Most likely, an indirect mechanism of estradiol action, involving somatostatin, is responsible for the changes in ACTH cells observed in our study. In animals treated with hCG the ACTH cell and nuclei volume were not changed ($p>0.05$), but the volume density was significantly decreased ($p<0.05$) by 23.1%, in comparison with the controls. Considering the previously observed hCG induced stimulation of pituitary FSH, LH and TSH cells (11, 12), some potential compensatory inhibition of ACTH cells, after hCG application in this study, seems possible.

In conclusion, our results indicate that neonatally injected EDP exerts a significant inhibitory effect on the immunohistomorphometric characteristics of ACTH cells in female rats, most likely due to indirect mechanisms. After hCG application, an inhibitory effect on the same characteristics of ACTH cells is not as clearly expressed.

Acknowledgments. Thanks are due to Mr. Hinko Sauter (Swiss Concept, Belgrade, Serbia) and Ms. Milana Milošević for the technical support. This work was supported by the Ministry for Education and Science of Serbia, Grant number 173009.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

References

1. Stratakis C, Chrousos G. Hypothalamic hormones. In: Conn PM, Melmed S, ed. Endocrinology: Basic and Clinical Principles. Totowa Humana Press, 1997: 185–209.
2. Horvath E, Kovacs K. Morphology of adenohypophysial cells and pituitary adenomas. In: Imura H, ed. The Pituitary Gland. New York Raven Press, 1994: 29–62.
3. Schmid A, Schoeffter P. Functional activity of the multiligand analog SOM230 at human recombinant somatostatin receptor subtypes supports its usefulness in neuroendocrine tumors. Neuroendocrinology 2004; 80: 47–50.
4. Tzoncheva A. Neuroendocrine tumors-laboratory diagnosis. Journal of Medical Biochemistry 2010; 29; 4: 254–264.
5. Redei E, Li L, Halasz I, Mcgivern RF, Aird F. Fast glucocorticoid feedback inhibition of ACTH secretion in the ovariectomized rat: effect of chronic estrogen and progesterone. Neuroendocrinology 1994; 60: 113–23.
6. Kostić N, Nestorović N, Negić N, Milošević V. The effect of estradiol on the morphofunctional characteristics of ACTH cells in middle-aged rats. Clinical Chemistry and Laboratory Medicine, International Proceedings Division, 2003; 1137-40.
7. Schreihofer D, Stoler M, Shupnik M. Differential expression and regulation of estrogen receptors (Ers) in rat pituitary and cell lines: estrogen decreases Erα protein and estrogen responsiveness. Endocrinology 2000; 141: 2174–83.
8. Friend K, Chiou Y, Lopes M, Laws E, Hughes K, Shupnik M. Estrogen receptor expression in human pituitary: correlation with immunohistochemistry in normal tissue, and immunohistochemistry and morphology in macroadenomas. J Clin Endocrinol Metab 1994; 78: 1497–504.
9. Pierce J, Parsons T. Glycoprotein hormones: structure and function. Ann Rev Biochem 1981; 50: 466–95.
10. Gregory J, Finlay J. Alpha-fetoprotein and beta-human chorionic gonadotropin: their clinical significance as tumour markers. Drugs 1999; 57: 463–7.
11. Lovren M, Sekulić M, Milošević V, Mičić M, Radulović N. Alterations of pituitary immunohistochemical and morphometric parameters in juvenile female rats following estradiol and hCG treatment. Jugoslov Med Biohem 1997; 16: 83–8.
12. Sekulić M, Šošić-Jurjević B, Filipović B, Manojlović-Stojanoski M, Milošević V. Immunoreactive TSH cells in juvenile and peripubertal rats after estradiol and human chronic gonadotropin treatment. Acta Histochem 2006; 108: 117–23.
13. Sternberger LA, Hardy PHJ, Cuculius JJ, Meyer HG. The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970; 18: 315–33.
14. Starčević V, Milošević V, Brkić B, Severs W. Effects of centrally applied somatostatin on pituitary adrenocorticotropes in female rats. Pharmacology 2000; 60: 203–7.
15. Weibel ER. Stereological Methods. 1. Practical Methods for Biological Morphometry. New York: Academic Press 1979: 1–415.
16. Malendowicz LK. Sex differences in adrenocortical structure and function. II. The effects of postpubertal gonadectomy and gonadal hormone replacement on the rat adrenal cortex evaluated by stereology at the light microscope level. Cell Tissue Res 1974; 151: 537–47.
17. Milošević V, Sekulić M, Lovren M, Nikolić D, Uščebrka G, Velkovski S. Somatotrops in female rats neonatally treated with estradiol: Stereological analysis. Folia Anat (supp. 1), 1999; 27: 20–1.
18. Lijesen GK, Theeuwen I, Assendelft WJ, Van Der Wal G. The effect of human chorionic gonadotropin (hCG) in the treatment of obesity by means of the Simeons therapy: a criteria-based meta-analysis. Br J Clin Pharmacol 1995; 40: 237–43.
19. Pantić V. Adenohypophyseal cell specificities and gonadal steroids In: Jutz M, Mckerns KW, eds. Synthesis and Release of Adenohypophyseal Hormones. New York: Plenum Press 1980: 335–62.
20. Pantić V. Biology of hypothalamic neurons and pituitary cells. Inter Rev Cytol 1995; 159: 1–112.
21. Van Bael A, Denef C. Evidence for a trophic action of the glicoprotein hormone – subunit in rat pituitary. J Neuroendocrinol 1996; 8: 99–102.
22. Frawley LS, Hoeffler JP. Hypothalamic peptides affect the ratios of GH and PRL cells: role of cell division. Peptides 1988; 9: 825–8.

Received: June 17, 2011
Accepted: July 22, 2011