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ESTRADIOL AND CALCIUM AFFECT THE GROWTH HORMONE PRODUCING CELLS IN FEMALE MIDDLE-AGED RATS

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The effects of multiple doses of estradiol dipropionate (EDP) or calcium glucoheptonate (Ca) on the growth and function of pituitary somatotropes (GH cells) were studied. Female middle-aged rats were receiving i.p. EDP (0.625 mg i.p./kg b.w.), or Ca (11.4 mg/kg b.w) every day for two weeks. Blood samples were collected for hormone analyses and pituitaries dissected for histological and morphometric evaluation 24 h after the last injection. GH-producing cells were examined using the peroxidase-antiperoxidase (PAP) immuno-histochemical procedure. Both EDP- and Ca-treatment significantly decreased all morphometric parameters of GH cells ($p<0.05$) in comparison with the corresponding controls. Serum concentration of growth hormone (GH) in EDP- or Ca-treated groups was lower by 65% and 13% ($p<0.05$) respectively, comparing to the controls. The difference between all morphometric parameters of EDP- and Ca-treated rats was statistically significant ($p<0.05$) in relation to the controls. These findings suggest that multiple EDP, or Ca application affects (directly or indirectly) the control of growth and secretory activity of GH cells in middle-aged female rats.

Key words: estradiol, GH cells, growth hormone, middle aged, rat females.

INTRODUCTION

Middle-age (8-11 months old) and ageing (16-18 months old) are critical periods for the control of growth hormone (GH) and somatostatin gene expression in higher animals of both sexes (Wise and Camp 1984, Martinoli *et al.* 1991). Ageing is a biological process, that includes alterations of the humoral regulatory system in which the hypothalamo-pituitary axis plays an important role. Some of the hormones secreted from the anterior pituitary are related to the potency of survial during ageing (Ooka 1993). Age-related changes in hormonal secretion can also be secondary to physiological changes in circadian and seasonal rhythms, or in the frequency or height of hormonal pulses (Mooradian 1993). Plasma growth hormone (GH) secretion was found to be gradually

decreasing from maturity and middle-age (8-15 months of age) in rats of both sexes (Sonntag *et al*. 1982, Mobbs 1996). Takahashi (1992) reported an increase of somatostain release and a decrease in growth hormone-releasing hormone (GHRH), associated with a reduction of hypothalamic catecholamine activity in middle-aged rats. The amplitude of GH pulses was also reduced in middle-aged and old rats, but not the values or intervals between each pulse, which averaged about 6 min (Takahashi *et al*. 1987).

In the rat's pituitary *pars distalis* the nuclear concentration of estradiol was observed in all cell types including acidophils, basophils and chromophobes. More specifically, localization of estradiol is not confined to gonadotropes but occurs also with a decreasing intensity in somatotropic, lactotropic and thyreotropic cells (GH, PRL and TSH cells, respectively) (Keefer 1981). Friend *et al.* (1997) identified several estrogen mRNA isoforms in the rat's pituitary and characterized their regulation by gonadal steroids. Takahashi (1992) also showed that estrogens acted by decreasing the percentage of GH cells in rats of both sexes.

In addition to hormones, secretory processes in the anterior pituitary cells are controlled by regulatory molecules such as Ca^{2+} ions, vitamins, metabolites and growth factors (Perez *et al.* 1995). Calcium is an essential nutrient involved in most metabolic processes and calcium phosphate salts provide mechanical rigidity to the bones and teeth, where 99% of body's calcium resides (Nordin 1997). Thus, the consequences of calcium deficiency include not only osteoporosis, but also arteriosclerosis, hypertension, senile dementia and disturbances in cellular functions (Fujita 1985). Hence, calcium supplements that guarantee an adequate supply of this essential mineral in perimenopausal and postmenopausal women are widely applied (Avioli 1984).

The above facts prompted us to examine the characteristics of immunoreactive GH cells in the pituitary of middle-aged female rats multiple treated with estradiol dipropionate (EDP) or Ca-glucoheptonate. For this purpose histological, sterological and biochemical methods were employed.

MATERIAL AND METHODS

Animals. Wistar 14-month-old female rats, bred at the Institute for Biological Research, Belgrade, were used. Middle-aged female rats were i.p. receiving estradiol dipropionate (EDP; 0.625 mg i.p./kg b.w.), or calcium glucoheptonate (Ca; 11.4 mg/kg b. w) every day for two weeks. The animals were kept under a 12:12 h light-dark cycle, at 22±2 ^{o}C. They had free access to food (a product of Veterinarski zavod Subotica, Subotica, Serbia and Montenegro) and water. Cytological examinations of vaginal smears started at 13 months of age. The oestrous cycle of these females was irregular with long oestrus stages (persistent vaginal cornification) interspaced by one or two days of proestrus or diestrus.

Light microscopy and immunocytochemistry. Pituitary glands were excised, fixed in Bouin's solution for 48 h and embedded in paraffin. Tissue sections 5 μm thick were deparaffinized in xylol and alcohol. Pituitary hormones were localized by the peroxidase-antiperoxidase-complex (PAP) method of Sternberger *et al.*

(1970). Endogenous peroxidase activity was blocked by incubation in 9 mmol/L hydrogen peroxide in methanol for 30 min at room temperature. Before the application of specific primary antisera, non-specific background staining was minimized by incubating the sections with non-immune, porcine serum diluted with phosphate buffered saline, pH 7.4 (PBS) for 60 min. The sections were then overlaid with the appropriate dilutions of the specific primary antibodies (hGH-antisera, "Dako A/S", Glostrup, Denmark) for 24 h at 4 ^{o}C. After washing in PBS, the sections were incubated for another 60 min with the second antibody swine-antirabbit IgG for 45 min, rinsed again with PBS for 10 min and incubated with rabbit PAP serum for 45 min. The localization of antibodies was visualized by incubating the sections in Tris-HCl buffered saline (0.05 mol/L, pH 7.4) supplemented with 3,3-diaminobenzidine tetrachloride (DAB) (Serva, Heidelberg, Germany) and 9 mmol/L hydrogen peroxide. The slides were thoroughly washed under running tap water, counterstained with haematoxylin and mounted in Canada balsam ("Alkaliod", Skopje, FYR Macedonia). Control sections were incubated without primary antisera or by substituting non-immune rabbit serum for the primary antiserum.

Morphometry. Measurements were performed on the widest portion of the pituitary gland and immunocytochemically labelled GH cells were analyzed by the M_{42} test system (Weibel, 1979). For the calculations of cellular and nuclear volumes, the formula of Weibel (1979) was used.

Hormone assay. Serum concentrations of GH in the control and treated rats were measured by Delfia method (hGH-Delfia kits, LKB, Turky, Finland).

Statistical analyses. Biochemical and morphometric data obtained for each group were averaged and the standard deviation of the mean was calculated. A one-way analysis of variance (ANOVA), followed by the multiple range test of Duncan (Pharmacological Calculation System, 1986) was used for statistical comparisons of the differences between the groups. A probability value of 5% or less was considered statistically significant.

RESULTS

In female rats, treated with either EDP or Ca-glucoheptonate the body weight was significantly decreased ($p<0.05$) in comparison with the controls. However, the absolute and relative pituitary weights in each of the treated groups were significantly increased ($p<0.05$) in comparison with the corresponding controls (Table 1).

Immunocytochemically identified GH cells in the control rat's pituitaries were ovoid to pyramidal in shape, with a spherical centrally located nucleus. GH cells were usually situated along sinusoids (Fig. 1a). In either estradiol- or Ca-treated pituitaries, GH cells were smaller, irregularly shaped, with more intensely stained secretory granules (Figs. 1b, 1c) and dilated blood capillaries (Fig. 1b).

Morphometric studies revealed a significant decrease in the number and volume densities of perikarya (Fig. 2) of somatotropic cells in all treated rat females. Relative volume density of these cells was decreased by 86% and 21%, in EDP- and Ca-treated group, respectively, in comparison with the controls the

difference was statistically significant. An extremely striking difference in all morphometric parameters measured in the present study was observed in EDP-treated animals.

Table 1. The effect of multiple EDP or Ca-glucoheptonate treatment on body weight, absolute and relative pituitary weight of middle-aged rat females

Experimental group	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg/%)
Control	336 ± 7.0	17.3 ± 0.8	5.3 ± 0.3
EDP	307 ± 4.0* (–9%)	36.4 ± 5.4* (+110%)	11.8 ± 1.6* (+123%)
Ca	306 ± 1.0* (–9%)	30.5 ± 3.5* (+76%)	7.4 ± 0.8* (+40%)

The given values are for mean ±S.E.M.; *$p<0.05$

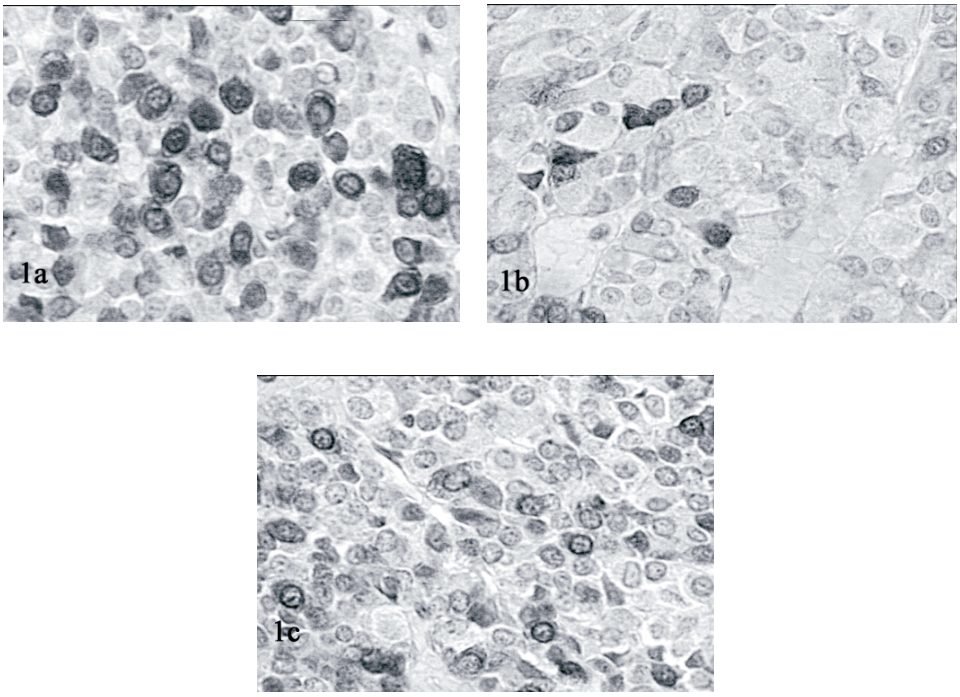

Figure 1. Immunohistochemically labelled GH cells (arrows) in: a) controls, b) EDP-treated rats and c) Ca^{2+}– treated rats. (PAP; X 1256)

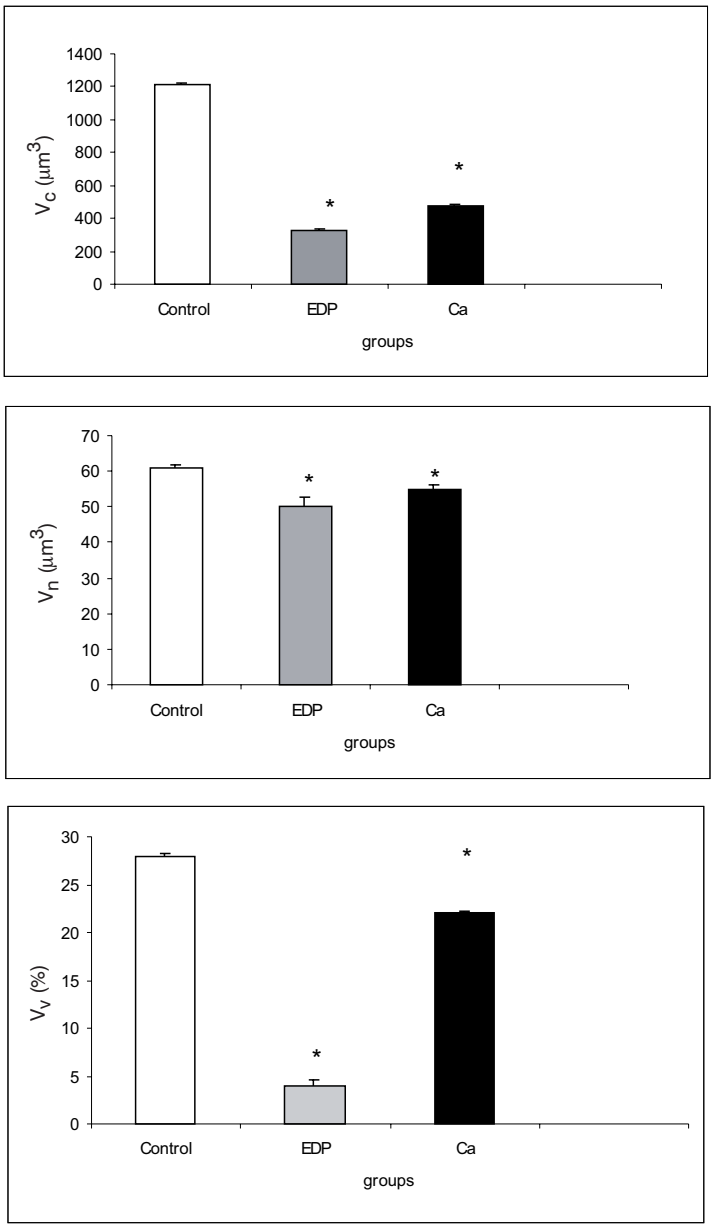

Figure 2. A) Cellular volume (V_c; μm^3) of the immunoreactive GH cells B) Nuclear volume (V_n; μm^3) of GH cells; C) Relative volume density (V_v; %) of GH cells expressed as percentages of total glandular tissue. The values are the means ± S.D. (n=5/group), $^a p<0.05$ *vs*. control

The serum concentration of GH was also significantly decreased in EDP- and Ca-treated rats (by 65% and 13%, respectively; $p<0.05$) in comparison with the controls (Fig. 3).

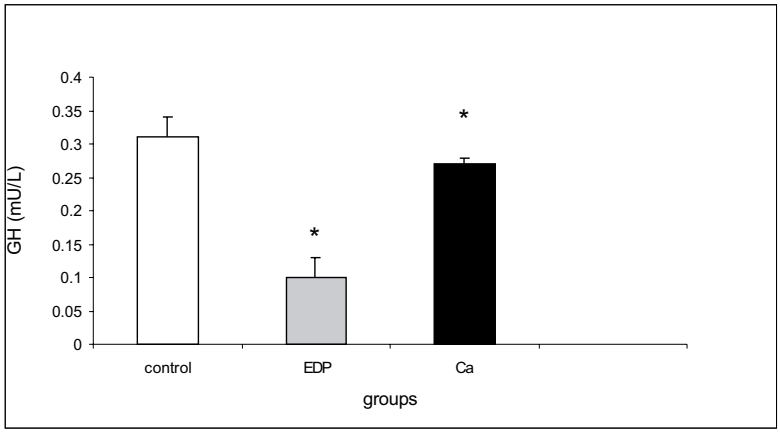

Figure 3. Serum concentration of growth hormone in Wistar female rats treated with EDP or Ca-glucoheptonate. Data are expressed as the means ±S.D. (n=5/group; *p<0.05 *vs*. control

DISCUSSION

Ageing is associated with a myriad of hormonal and anatomical changes of the endocrine glands, notably as a result of programmed cell death, autoimmune-mediated destruction of the gland, or neoplastic transformation of granular tissue (Mooradian, 1993). The mechanisms underlying these changes are variable. In a previous study, we have observed a reduction of pituitary TSH- (Sekulić *et al*. 1998) and gonadotrophic- (FSH and LH; Lovren *et al*. 1999) cell activity in middle-aged rats. In middle-aged rats the structure and function of C cells was also reduced.

The results obtained throughout the present study clearly demonstrate that multiple EDP doses given to middle-aged female rats resulted in an increase of absolute and relative pituitary weight. This could be explained by an increased number of chromophobe prolactin (PRL) cells (Pantić, 1980, 1995; Lovren and Sekulić 1994) and ACTH cells (Kostić *et al*., 2003). Takahashi and Kawashima (1987) observed a significant age-related increase of the DNA content in PRL cells in rats of both sexes, but this increase was more conspicuous in females than in males, suggesting a significant increase in the number of PRL cells, as well as in PRL secretion.

Results demonstrate that pituitary GH cells of middle-aged female rats responded to multiple treatment with either EDP or Ca-glucoheptonate. This is

supported by the values of measured morphometric parameters. Relative volume density of GH cells and volume of the perikarya were significantly lower in both treated groups in comparison with the corresponding controls. Average blood serum GH concentration was also decreased after multiple doses of either EDP or Ca-glucoheptonate.

An extremely striking difference in all measured parameters was observed in EDP-treated animals. As early as in 1982, Sontag *et al*. observed that the GH secretion in rats was declining with age. On the other hand, several authors examined the cause of a decreased pituitary GH content with ageing in rats (Takahashi 1992, Mobbs 1996). Serum GH levels in young female rats were found to be significantly higher than those of middle-aged animals (Takahashi *et al*. 1987). This decrease of serum GH level observed in middle-aged female rats was interpreted to be partly due to an increase in somatostatin release from the hypothalamus (Takahashi *et al*. 1987), a decrease in GHRH release, as well as a reduced pituitary responsiveness to GHRH stimulation (Pantić 1980). The amplitude of GH pulsatile release was also lower in middle-aged than in young rats (Takahashi *et al.*1987). Shibasaki *et al.* (1984) observed an age-related decrease in growth hormone (GH) responsiveness to growth hormone-releasing hormone (GHRH) and somewhat later, Abribat *et al*. (1991) reported a reduced pituitary adenylate cyclase response to altered GHRH binding sites of the pituitary gland. Morphologically classified growth hormone cell populations, changed in accordance with the GH secretory activity. Thus, it is highly probable that morphological heterogeneity of GH cells reflects functional heterogeneity and/or the maturation process of these cells (Kurosumi *et al*. 1986). Estrogens were shown to inhibit GH secretion and to reduce growth hormone-releasing hormone (GHRH) (Pantić 1980). Takahashi (1992) demonstrated that estrogen acted increasing the percentage of GH cells of Type II and Type III. Type II GH cells contain large and small secretory granules (100-150 nm in diameter), while cells of type III contain small secretory granules. Age-related decrease in the number of estrogen receptors of the pituitary (Mooradian 1993) or impaired nuclear binding to the receptors (Belisle *et al*. 1990) were shown to be associated with a decreased uterine responsiveness to estrogens.

In the present study, we observed that multiple treatment of middle-aged female rats with Ca resulted in a decrease of all measured morphometric parameters of GH cells, as well as of the blood serum GH level. Calcium is known as an essential nutrient involved in a number of metabolic processes and its phosphate salts provide mechanical rigidity to bones and teeth, where 99% of body's calcium occurs (Nordin 1997). Calcium also controls numerous cell functions in general, including pituitary cells. Zorec (1996) reported that a rise in cytosolic Ca^{2+} ions represents an important trigger for hormone secretion from pituitary cells. In our earlier studies we have shown that chronic treatment of middle-aged female rats acted reducing morphofunctional characteristics of gonadotrophic and TSH cells (Sekulić *et al.*, 1998; Lovren *et al*., 1999).

Based on results the presented results and the data of other authors, it can be concluded that multiple application of EDP and Ca to middle-aged female rats results in a suppression of morphofunctional characteristics of pituitary GH cells.

However, the observed decrease of immunocytochemical and morphometric parameters was much more pronounced in the EDP-treated group than in Ca-treated group of experimental animals.

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REFERENCES

1. *Abribat T, Deslauriers N, Brazeau P, Gaudreau P,* 1991, Alterations of pituitary growth hormone releasing factor binding sites in aging rats, *Endocrinol,* 128,1, 633-5.
2. *Avioli LV,* 1984, Calcium and osteoporosis, *Annu Rev Nutr,* 4, 471-91.
3. *Belisle S, Bellabarba D, Lehoux J,* 1990, Hypothalamic-pituitary axis during reproductive aging in mice, *Mech Aging Develop*, 52, 2-3, 207-17.
4. *Friend K, Resnick E, Ang L, Shupnik M,* 1997, Specific modulation of estrogens receptor mRNA isoforms in rat pituitary trought estrous cycle and in response to steroid hormones, *Mol Cell Endocrinol*, 131,2, 147-55.
5. *Fujita T,* 1985, Aging and calcium as an environmental factor, *J Nutr Sci Vitaminol (Tokyo),* Suppl: S15-19.
6. *Keefer DA*, 1981 Quantification of in vivo 3H-estrogen uptake by individual and pituitary cell types of male rat: a combined autoradiographic-immunocytochemical technique, *J Histochem Cytochem*, 25, 4,167-74.
7. *Kostić N, Nestorović N, Negić N, Milošević V,* 2003, The effect of estradiol on the morphofunctional characteristics of ACTH cells in middle-aged rats, International Proceedings Division, 1137-40
8. *Kurosumi K, Kovama T, Tosaka H*, 1986, Three types of growth hormone cells of the rat anterior pituitary revealed by immunoelectron microscopy using a colloidal gold antibody method, *Arch Histol Jpn*, 49,2, 227-42.
9. *Lovren M, Sekulić M,* 1994, Immunocytochemical and morphometric changes of gonadotrophs during development of oestrogen-sterilized female rats, *Yugoslav Med Biohem*, 13, 3-4, 13-8.
10. *Lovren M., Sekulić M., Milošević V., Mićić M., Radulović N,* 1999, Effects of estradiol and calcium on gonadotrophic cells in middle-aged female rats, *Histochem J*, 31, 1, 671-76.
11. *Martinoli MG, Ouellet J, Rheaume E, Pelletier G,* 1991, Growth hormone and somatostatin gene expression in adult and aging rats as measured by quantitative in situ hybridization. *Neuroendocrinol,* 54, 6, 607-15.
12. *Mobbs C,* 1996, Neuroendocrinology of aging. In: Schneider EL, Rowe JW, eds. Handbook of the Biology of Aging. San Diego: Academic Press, 234-82.
13. *Moordian AD,* 1993, Mechanisms of age-related endocrine alterations, *Drugs and Aging*, 3, 2, 81-146.
14. *Murai I, Ben-Jonathn N,* 1990, Acute stimulation of prolactin release by estradiol: mediation by the posterior pituitary, *Endocrinol,* 126, 6, 3179-84.
15. *Nordin BE,* 1997, Calcium and osteoporosis, Nutrition, 13, 2, 664-86.
16. *Ooka H,* 1993, Proliferation of Anterior pituitary cells in relation to aging and longevity in the rat, *Zool sci,* 10, 4, 385-92.

17. *Pantić V,* 1980, Adenohypophyseal cell specificities and gonadal steroids In: Jutz M, Mckerns KW, eds. Synthesis and Release of Adenohypophyseal Hormones, New York: Plenum Press, 335-62.
18. *Pantić V*, 1995, Biology of hypothalamic neurons and pituitary cells, *Int Rev Cytol*, 159,1, 1-112.
19. *Perez F, Rose J, Schwartz J*, 1995, Anterior pituitary cells: getting to know their neighbours, *Mol Cell Endocrinol*, 111, C1-C6.
20. *Sekulić M, Lovren M, Milošević V,* 1998, Immunoreactive TSH cells in the pituitary of female middle-aged rats after treatment with estradiol or calcium, *Acta Histochem,* 100, 4, 185-91.
21. *Sekulić M, Lovren M, Milošević V, Mićić M, Balint-Perić L,* 1998, Thyroid C cells of middle-aged rats treated with estradiol or calcium, *Histochem Cell Biol*, 109, 3, 237-62.
22. *Shibasaki T, Shizume K, Nikahara M, Masuda A, Jibiki K*, 1984, Age-related changes in plasma growth hormone response to growth hormone releasing factor in man, *J Clin Endocrinol Metab*, 58, 1, 212-4.
23. *Sonntag W, Forman L, Miki N, Trapp J, Gottschall P, Meites J,* 1982, L-dopa restores amplitude of growth hormone pulses in old male rats to the observed in young male rats, *Neuroendocrinol*, 34 3, 163-18.
24. *Sternberger LA, Hardy PHJ, Cuculius JJ, Meyer HG*, 1970, 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*, 18, 315-33.
25. *Takahashi S, Gottschall P, Quigley K, Goya R, Meites J,* 1987, Growth hormone secretory patterns in young, middle-aged and old female rats, *Neuroendocrinol*, 46 1, 137-42.
26. *Takahashi S, Kawashima S*, 1987, Proliferation of prolactin cells in rat: Effects of estrogen and bromocryptine, *Zool Sci*, 4, 2, 855-60.
27. *Takahashi S,* 1991, Immunocytochemical and immuno-electron-microscopical study of growth hormone cells in male and female rats of various ages, *Cell Tissue Res*, 266, 2, 275-84.
28. *Takahashi S,* 1992, Heterogeneity and development of somatotrophs and mammotrophs in the rat, *Zool Sci*, 9, 3, 901-24
29. *Wise MP, Camp P*, 1984, Changes in concentrations of estradiol nuclear receptors in the preoptic area, medial basal hypothalamus, amygdale and pituitary gland of middle-aged and cycling rats. *Endocrinol*, 114, 1, 92-8.
30. *Zorec R.* 1996, Calcium signaling and secretion in pituitary cells, TEM, 7,10, 384-8.
31. *Weibel ER*, 1979, Stereological methods, 1. Practical methods for biological morphometry, New York: Academic Press, 1-415.

DELOVANJE ESTRADIOLA I KALCIJUMA NA ĆELIJE HIPOFIZE KOJE LUČE HORMON RASTA U ACIKLIČNIH ŽENKI PACOVA

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SADRŽAJ

Ispitivani su efekti višekratnih doza estradiol dipropionata (EDP) ili kalcijum glukoheptonata (Ca) na rast i funkciju hipofiznih ćelija hormona rasta (GH) acikličnih ženki Wistar pacova. Aciklične ženke su svakodnevno tokom dve nedelje i.p. dobijale 0.625 mg EDP/kg t.m., ili 11.4 mg Ca/kg t.m.. Krv je sakuplja-

na radi određivanja koncentracije hormona rasta u serumu, a hipofize su pripremane za histološku i morfometrijsku analizu. GH ćelije su imunocitohemijski obeležavane metodom peroksidaza-antiperoksidaza (PAP). Morfometrijski parametri su bili značajno smanjeni ($p<0.05$) u životinja tretiranih EDP-om, ili kalcijumom u poređenju sa odgovarajućim kontrolama. Koncentracija hormona rasta u serumu bila je takođe značajno niža ($p<0.05$) u obe tretirane grupe (za 65% odnosno za 13%) u poređenju sa odgovarajućom kontrolom. Dobijeni rezultati ukazuju da višekratni tretman acikličnih ženki pacova EDP-om ili Ca-glukoheptonatom deluje inhibitorno na GH ćelije i da su i estrogeni i kalcijum uključeni na specifičan način u rast i sekretornu aktivnost GH ćelija hipofize.