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EFFECT OF ESTRADIOL OR CALCIUM TREATMENT ON MAMMOTROPHS OF FEMALE MIDDLE-AGED RATS

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The effects of estradiol dipropionate (EDP) or calcium glucoheptonate (Ca) on the morphology and stereology of the PRL cells in 14-month-old Wistar female rats were studied. The animals were treated daily with EDP in the dose of 0.625 mg/kg b.w. or calcium glucoheptonate (Ca; 11.4 mg/kg b.w.) for two weeks. The controls were injected with vehicle alone by the same schedule. Mammotrophs (PRL cells) were immunocytochemically localized by the PAP method. Blood PRL concentration was determined by Delfia procedure. In animals treated with EDP the volume of both, PRL cells and their nuclei, as well as the volume densities were significantly ($p<0.05$) increased by 17%, 9% and 38%, respectively, in comparison with the controls. In animals treated with Ca all morphometric parameters were insignificantly ($p>0.05$) decreased compared to control rats. Serum concentration of PRL was significantly increased ($p<0.05$) by 17% after estradiol treatment, but in Ca-treated females this parameter was insignificantly ($p>0.05$) changed by 2% compared to controls. Based on these results, it can be concluded that EDP expresses a strong stimulatory effect on the morphology and function of pituitary PRL cells.

Key words: PRL, mammotrophs, estradiol, calcium, middle-aged, female rats

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone that is synthesized and secreted from specialized cells of the anterior pituitary gland, lactotrophs or mammotrophs (Freeman *et al*., 2000). Chatelain *et al*. (1979) reported that mammotrophs (PRL cells) were detected on day 21 of gestation, but Watanabe and Haraguchi (1994) reported that PRL cells were first detected postnatally. The difference in the number and size of PRL cells in pituitary glands between adult rats may partially result from the sex difference in the mitotic activity of PRL cells during adult lifetime (Takahashi *et al*., 1984, 1992).

In rat's *pars distalis,* estradiol immunopositivity was observed in the nucleus of all cell types, including acidophils, basophils and chromophobes. According to Murai and Ben-Jonathan (1990) estradiol plays a crucial role in PRL release. Estrogen increased the percentage of Type I PRL cells, considered to be a mature type of PRL cells and decreased the percentage of immature Type II and III PRL cells (Takahashi and Kawashima, 1987).

The hypothalamo-pituitary system has a central importance in the regulation of biological mechanisms of aging. Some of the hormones secreted from the anterior pituitary, including PRL, are related to the potency of survival during the aging process (Ooka, 1993). Age-related changes in hormonal secretion can be secondary to physiological changes in the circadian and seasonal rhythm, or in the frequency or height of hormonal pulses (Mooradian, 1993). The percentage of PRL cells and PRL secretion significantly increased in female rats with age (Takahashi, 1992). On the contrary, reproductive decline in female rats begins in the same period, i.e. the function of gonadotrophic axis as well as the estrogen level were decreased during middle age (Lovren et al., 1999).

In pituitary cells secretory processes are controlled by regulatory molecules such as inorganic ions, Ca^{2+}, vitamins, metabolites and growth hormone (Perez *et al*., 1995). Calcium acts as an extracellular and intracellular messenger to regulate a diverse array of cellular functions (Zivadinovic *et al*., 2002).

Hormonal profiles during middle age are dramatically different from these found during the estrus cycle. Therefore, the aim of this study was to evaluate the morphometric and morphological characteristics of immunopositive PRL cells in middle-aged female rats after chronic estradiol dipropionate or calcium glucoheptonate treatment.

MATERIAL AND METHODS

Treatment of animals

Wistar 14-month-old female rats, bred at the Institute for Biological Research, Belgrade, were used. The animals were kept under a 12 : 12 h light-dark cycle, at 22±2 °C. They had free access to food (produced by Veterinarski zavod Subotica, Subotica, Serbia) and water. Middle-aged females were i.p. receiving estradiol dipropionate (EDP; 0.625 mg/kg b.w., ICN Galenika, Belgrade, Serbia), or i.m. calcium glucoheptonate (Ca; 11.4 mg/kg b.w. Novartis, Nypon, Switzerland) every day for two weeks. The control rats were injected with the corresponding vehicle by the same schedule. All animals, 5 rats per each group, were sacrificed in deep anesthesia 24 h after the last injection. Experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in "Guide for the Care and Use of Laboratory Animals" (1996 National Academy Press, Washington D.C.). Cytological examinations of vaginal smears started at 13 months of age. The estrous cycle of these females was irregular with long estrus 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. Serial 5 μm thick tissue sections were deparaffinized in xylol and serial alcohol. Three pituitary sections per animal, from dorsal, medial and ventral parts were prepared for immunocytochemical staining. 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 application of specific primary antisera, nonspecific background staining was minimized by incubation of the sections with non-immune, porcine serum diluted with phosphate buffered saline pH 7.4 (PBS) for 60 min. Sections were then overlaid with the appropriate dilutions of the specific primary antibodies-hPRL-antisera, ("Dako A/S", Glostrup, Denmark) at 4 °C for 48 h. After washing in PBS, 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. Antibody localization 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) and 9 mmol/L hydrogen peroxide. Slides were thoroughly washed under running tap water, counterstained with hematoxylin and mounted in Canada balsam ("Alkaloid", Skopje, Former Yugoslav Republic of Macedonia). Negative 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 PRL cells were analyzed by the M_{42} test system according to Weibel (1979). For the calculations of the cell and nuclear volumes the formula of Weibel (1979) was used. The mean value for each rat was computed from 50 fields from the three widest sections (n=150 measurements *per*/rat). Sections from the same pituitary level (ventral, medial and dorsal) of the control and experimental groups were compared.

Digital images were made on a DM RB Photomicroscope (Leica, Wetzlar, Germany) with a JVC TK 1280E Video Camera (Leica). For image acquisition Qwin program (Leica) was used.

Biochemical analyses

Serum concentrations of PRL in control and treated rats were measured by the Delfia method (hPRL-Delfia kits, LKB, Turkey, Finland).

Statistical analyses

Biochemical and morphometric data obtained from 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

between groups. A probability value of 5% or less was considered statistically significant.

RESULTS

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

Table 1. Effects of multiple EDP or Ca-glucoheptonate treatment on body weight, absolute and relative pituitary weights in middle-aged female rats

Experimental group	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (%)
Control	336 ± 19.8	17.3 ± 1.7	5.3 ± 0.6
EDP	307 ± 11.2* (-9%)	36.4 ± 3.9* (+110%)	11.8 ± 1.6* (+123%)
Ca	306 ± 2.8* (-9%)	30.5 ± 3.5* (+76%)	7.4 ± 0.8* (+40%)

The values are the means ± SD (n=5/group). * $p<0.05$ vs. controls.

Immunocytochemically labelled mammotrophs in the control pituitaries were polygonal, elongated in shape, with a spherical centrally located nucleus (Fig. 1A). Mammotrophs were sparse in the anterior-ventral portion of the gland. Large oval gonadotrophic cells usually surrounded PRL cells. In estradiol-treated female rats mammotrophs were elongated, irregularly shaped, with more intense black secretory granules (Fig. 1B). In the females treated with Ca the PRL cells were smaller and irregularly shaped, compared with the controls (Fig. 1C).

The morphometric parameters, *i.e.* the volume of the mammotrophs and their nuclei, as well as volume density of these cells after EDP or Ca treatment are shown in Table 2.

Volumes of PRL cells and their nuclei, as well as volume density of mammotrophs, were significantly ($p<0.05$) increased in EDP-treated rats by 17%, 9% and 38% respectively, in comparison with controls. In animals treated with Ca the volume of the mammotrophs, their nuclei and the volume density were not significantly ($p>0.05$) decreased compared to controls.

Serum concentration of PRL was significantly increased ($p<0.05$) by 17% after estradiol treatment, but in Ca-treated females serum PRL level was not significantly ($p>0.05$) changed in comparison with control rats (Fig. 2).

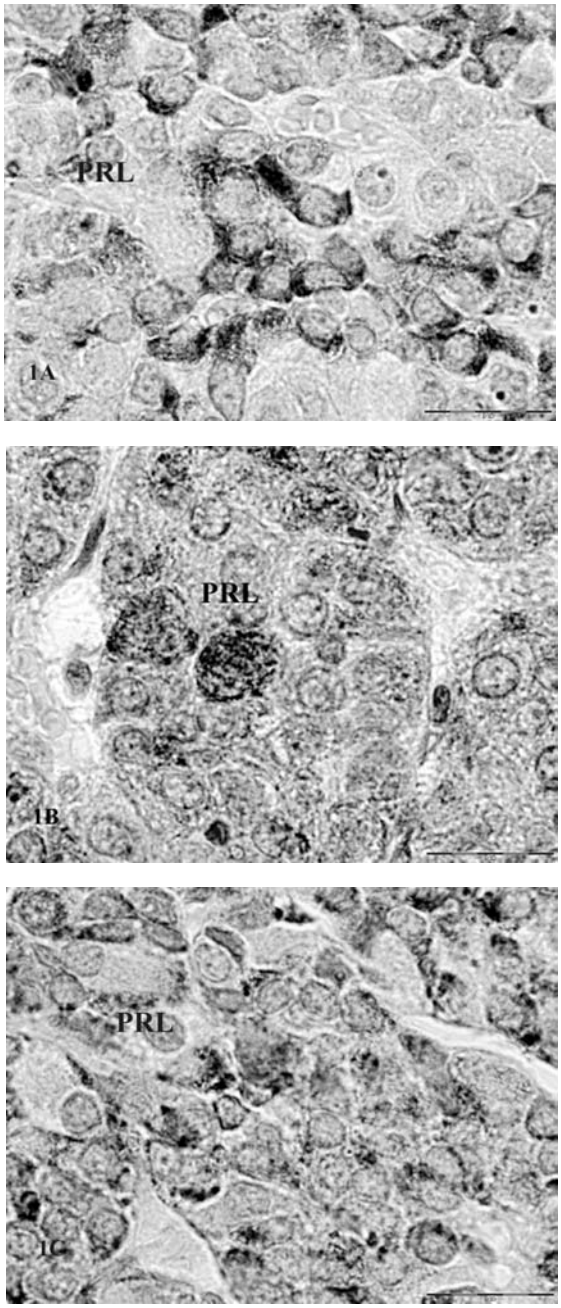

Figure 1. Immunocytochemically identified mammotrophs in: (A) control rats; (B) EDP, and (C) Ca-glucoheptonate treated rats. (Bar - 25 μm)

Table 2. Effects of multiple EDP or Ca-glucoheptonate treatment on morphometric features of the mammotrophs in middle-aged female rats

Experimental group	Volume of the mammotrophs (μm^3)	Volume nuclei of the mammotrophs (μm^3)	Volume density of the mammotrophs (%)
Control	1180 ± 14.1	124 ± 5.6	35.8 ± 0.8
EDP	1380 ± 0.1* (+17%)	135 ± 0.6* (+9%)	49.4 ± 2.1* (+38%)
Ca	1155 ± 63.6 (-2%)	116 ± 5.7 (-6%)	33.5 ± 2.12 (-6%)

The values are the means ± SD (n=5/group). * $p<0.05$ vs. controls.

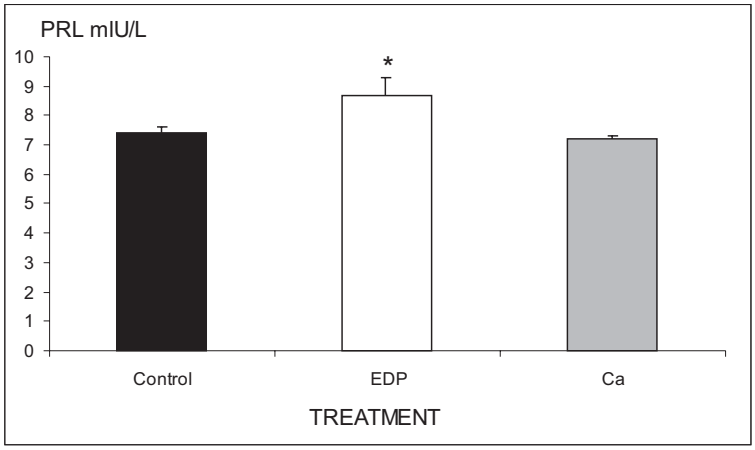

Figure 2. Serum concentration of PRL in middle-aged female rats treated with EDP or Ca-glucoheptonate. Data are expressed as mean values ± SD (n=5/group); *p<0.05 *vs*. control

DISCUSSION

Ageing is associated with a myriad of functional and anatomical changes of the endocrine glands, usually as a result of programmed cell death, autoimmune-mediated destruction of the gland, or neoplastic transformation of glandular tissue (Mooradian, 1993). The hypothalamic-pituitary-adrenal (HPA) axis is an autoregulating system with many modulatory mechanisms. Due to such regulation, the circulating levels of glucocorticoids are highly variable, according both to the spontaneous rhythmic fluctuations and to the responses towards stressful conditions (Ferrari *et al*., 2001). The mechanisms underlying these changes are variable. In previous studies, we have observed an activity reduction

of many pituitary cells, such as TSH- (Sekulić *et al*., 1998), gonadotrophic- (FSH and LH) (Lovren *et al*., 1999) and GH-cells (Milošević *et al*., 2005) in middle-aged rats. The structure and function of corresponding targets organs were also changed in middle-aged and aged rats (Sekulić and Lovren, 2000).

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 weights. This could be explained by an increased number of mammotrophs, chromophobes (Pantić, 1995) and adrenocorticotropic (ACTH) cells (Kostić *et al*., 2003) as demonstrated in this study.

Results presented here demonstrate that pituitary mammotrophs were larger in size, irregularly shaped, with more intensive immunostaining in estradiol-treated female rats then in controls. Volumes of mammotrophs and their nuclei, as well as volume density, were significantly increased in EDP-treated rats in comparison with controls. Serum concentration of PRL was also significantly increased by 17% after estradiol treatment. Takahashi and Kawashima (1987) observed a significant age-related increase of the DNA content in mammotrophs in rats of both sexes, but this increase was more conspicuous in the females than in males. A significant increase in the number of mammotrophs, as well as in PRL secretion was also observed by Takahashi *et al*. (1984) and Itoh *et al*. (2001) in middle-aged females. Takahashi and Miyatake (1991) reported that estrogen treatment significantly increased the mammotrophs volume density in male and female rats. Estrogen stimulated the proliferation of mammotrophs type I, and decreased percentages of type II and III cells (Takahashi, 1992). Type I PRL cells contained irregularly shaped, large secretory granules with a diameter of 300-700 nm; type II contained spherical granules with a diameter of 150-250 nm, while type III cells contained small round granules with a diameter of 100 nm (Ozawa and Kurosumi, 1989). Estrogen treatment for only 7 days may cause a significant proliferation of lactotrophs by 70%, and this proliferation was concurrent with higher serum and pituitary PRL levels (Mukdsi *et al*., 2004). The concentration of PRL in the blood of female rats increases with age (Ooka, 1993). Metka *et al*. (1994) reported that the estrogen-gestagen replacement therapy significantly increased PRL level in menopausal women.

In the present study, we observed that multiple treatment of middle-aged female rats with calcium resulted in a statistically insignificant decrease of all morphometric parameters of mammotrophs, as well as blood serum PRL level. In our earlier studies we have shown that chronic treatment of middle-aged female rats with calcium reduced morphometric and functional characteristics of gonadotrophic, TSH- and GH- cells (Sekulić *et al*., 1998; Lovren *et al*., 1999; Milošević *et al*., 2005). Calcium is known to be an essential nutrient involved in a number of metabolic processes and its phosphate salts provide mechanical rigidity to bones and teeth, where 99% of the 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 calcium ions represents an important trigger for hormone secretion from pituitary cells. Živadinović *et al.* (2002) indicate the mechanisms underlying the critical role of extracellular calcium on basal hormone secretion from anterior pituitary

somatotrophs and lactotrophs. The majority of basal GH and PRL secretion was an extracellular calcium dependent process, which is sensitive to blockage of voltage-gated calcium influx through L-type channels (Cota *et al*., 1990).

It can be concluded that the multiple application of EDP to middle-aged females results in increased morphological parameters of pituitary mammotrophs. However, in calcium treated animals the morphological characteristics of mammotrophs were not significantly changed.

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Milošević Verica *et al*.: Effect of estradiol or calcium treatment
on mammotrophs of female middle-aged rats

EFEKAT TRETMANA ESTRADIOLOM I KALCIJUMOM NA PROLAKTINSKE ĆELIJE U ACIKLIČNIH ŽENKI PACOVA

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SADRŽAJ

Ispitivani su efekti višekratnih doza estradiol dipropionata (EDP) ili kalcijum glukoheptonata (Ca) na morfološke i stereološke karakteristike mamotropnih (PRL) ćelija u acikličnih ženki pacova. Aciklične ženke su svakodnevno tokom dve nedelje dobijale 0,625 mg/kg tm EDP-a ili 11,4 mg/kg tm Ca. PRL ćelije su imunocitohemijski obeležavane PAP metodom. U životinja tretiranih sa EDP-om zapremina ćelija i njihovih jedara kao i volumenska gustina signifikantno ($p<0,05$) su bile povećane za 17%, 9% i 38% u poređenju sa kontrolama. U životinja tretiranih kalcijumom ispitivani morfometrijski parametri nisu bili značajno ($p>0,05$) promenjeni. Koncentracija PRL u serumu značajno ($p<0,05$) je bila povećana (za 17%) posle tretmana estradiolom dok tretman kalcijumom nije izazvao značajne promene (2% u odnosu na kontrole). Na osnovu dobijenih rezultata može se zaključiti da EDP ima snažan stimulatorni efekat na PRL ćelije u hipofizi.