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Daidzein effects on ACTH cells: immunohistomorphometric and hormonal study in an animal model of the andropause

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Summary. Daidzein is a potential natural alternative to estradiol during therapy of some malignancies in men. Besides weak inhibition of tyrosine kinase activity, daidzein has a sizeable inhibitory effect on calcium channels. The aim of this study was to examine the effects of daidzein on the immunohistomorphometric features of pituitary adrenocorticotropes (ACTH cells) and circulating levels of ACTH and corticosterone, in comparison with estradiol, in an animal model of the andropause. Sixteen-month-old Wistar rats were divided into sham operated (SO), orchidectomized (Orx), estradiol treated orchidectomized (Orx+E) and daidzein treated orchidectomized (Orx+D) groups. Estradiol (0.625 mg/kg/day) and daidzein (30 mg/kg/day) were administered subcutaneously for three weeks, while the SO and Orx groups received the vehicle alone. ACTH cells were identified by the peroxidase-antiperoxidase (PAP) immunohistochemical procedure. Peripheral circulating concentrations of ACTH and corticosterone were measured by immunoassay. Orchidectomy reduced ($p<0.05$) the cell volume and volume density of adrenocorticotropes by 11% and 16%, respectively, in comparison to SO rats. In Orx+E rats, the volume density of ACTH cells decreased ($p<0.05$) by 25%, but the circulating level of ACTH increased ($p<0.05$) by 29%, compared to Orx rats. Daidzein treatment significantly decreased ($p<0.05$): volume density of ACTH cells, circulating ACTH and corticosterone by 24%, 48% and 33%, respectively, compared to the Orx group. In conclusion, this study revealed that daidzein negatively modulated the immunohistomorphometric features of ACTH cells and, unlike estradiol, decreased ACTH and corticosterone secretion, in an animal model of the andropause.

Key words: Daidzein, Estradiol, Stress, ACTH, Corticosterone

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Introduction

Ageing in males is associated with enhanced activity of the hypothalamic-pituitary-adrenal (HPA) axis, followed by stress-related psychiatric disorders, as well as osteoporosis, cardiovascular diseases and cancer (Hatzinger et al., 2000; Vance, 2003). Prostate cancer is a major cause of cancer-related death in elderly men. Besides radio- and chemo-therapy, the therapeutic approach in these patients includes androgen deprivation, *inter alia* orchidectomy and application of estrogen agonists (Tammela, 2004). However, a spectrum of unwanted side effects and an inferior quality of life accompanies androgen deprivation therapy, especially when using traditional estrogens (Daskivich and Oh, 2006).

Daidzein is a soybeans-derived isoflavone that has structural and functional similarity to 17ß-estradiol (Setchell, 1998), and when applied competes with endogenous estrogens for binding to estrogen receptors. Besides its mild estrogenic/antiestrogenic and antioxidant activities (Benassayag et al., 2002), daidzein exhibits weak inhibition of tyrosine kinase activity, but has almost the same inhibitory effect on L-type calcium channels as the structurally similar genistein (Yokoshiki et al., 1996).

Nutritional supplements containing isoflavones are widely used as alternative therapy for age related diseases. Data illustrating their effects on the HPA axis

and the response to stress are expanding. We have previously shown that subcutaneous administration of different doses of genistein modulated the immunohistomorphometric features of pituitary ACTH cells and decreased the circulating levels of both ACTH and corticosterone in an animal model of the andropause (Ajdžanović et al., 2009a; Milošević et al., 2009). Genistein also caused shunting of steroid metabolic pathways in the adrenals by favoring dehydroepiandrosterone (DHEA) and inhibiting corticosterone and aldosterone production in the same animal model (Ajdžanović et al., 2009b). Considering the slight but very important structural difference between daidzein and genistein, the weak inhibitory effect of daidzein on tyrosine kinase activity, as well as the absence of information about the influence of daidzein on ACTH morphofunctional features, additional insight into this field seemed necessary. Also, the parallel between daidzein and estradiol effects (potential contemporary and traditional therapeutic approach) on stress hormone secretion should be specifically investigated. In females, estradiol treatment was found to increase ACTH and corticosterone secretion (Suzuki et al., 2001; Kostić et al., 2003). Some evidence indicates that estrogen directly influences cellular activity and gene expression *via* estrogen receptors within the corticotrophin-releasing hormone (CRH) neurosecretory neurons (Simerly et al., 1990).

The precise aim of this study was to examine the effects of subcutaneous treatment with a therapeutic dose of daidzein on the immunohistomorphometric features of ACTH cells, as well as ACTH and corticosterone secretion, following the established approach (Doerge and Sheehan, 2002; Jefferson et al., 2007), and to create a parallel with the effects of estradiol in our animal model of the andropause.

Materials and methods

Animals and diets

The experiments involved 16-month-old male Wistar rats bred in the Institute for Biological Research, Belgrade, Serbia. They were housed two per cage, exposed to a 12-12 h light-dark cycle and kept at 22±2°C. Two weeks before the experiment, the rats started to eat a soy-free diet (according to Picherit et al., 2000) prepared in cooperation with the Department of Nutrition, School of Veterinary Medicine, Belgrade, Serbia, and INSHRA PKB, Belgrade, Serbia, with corn oil as the fat source.

The diet contained per 100 g: 20.3 g casein; 65 g carbohydrate (45 g cornstarch + 20 g sucrose); 5.2 g corn oil; 3.7 g fiber (crystalline cellulose); 1.5 g vitamin/mineral mix (Ca-phosphate deficient); 1.8 g dibasic calcium phosphate; 1g calcium carbonate; 1.5 g DL-methionine. Casein and crystalline cellulose originated from Alfa Aesar, Johnson Matthey Gmbh & Co.KG, Karlsruhe, Germany; carbohydrate, oil, vitamin/mineral mix, calcium carbonate and calcium phosphate were from INSHRA PKB, Belgrade, Serbia; and DL-methionine from Sigma Chemical Company, St. Louis, MO, USA. Food and water were available *ad libitum*.

Experimental design

Sham surgery and orchidectomy were performed under ketamine anesthesia (Ketamine hydrochloride; Richter Pharma, Wels, Austria; 15 mg/kg b.w.). Sham-operated (SO; n=8) and orchidectomized rats (n=24) were allowed to recover for 2 weeks. The orchidectomized rats were then divided into three groups of eight animals each. The first group was subcutaneously treated with estradiol-dipropionate (Galenika, Belgrade, Serbia; Orx+E) in a dose of 0.625 mg/kg b.w. every day except on Sundays for 3 weeks. The second orchidectomized group received daidzein (Nutraceutica, Monterenzio, Italy; Orx+D) at 30 mg/kg b.w. following the same regime. Daidzein was predissolved in a minimal volume of absolute ethanol (0.17 ml) and mixed with sterile olive oil (0.33 ml). The final volume injected was 0.5 ml *per* animal. The third orchidectomized group (Orx) and the SO group were given the same volume (0.5 ml) of vehicle alone. All animals were killed by decapitation 24 h after the last injection.

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

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.

ACTH-producing cells were identified by immunohistochemistry using the peroxidase-antiperoxidase method (PAP) as described by Sternberger et al. (1970). Endogenous peroxidase activity was first blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min at room temperature. 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., 2000; 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 0.2M 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 (Weibel, 1979). 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, V_{VC}; %) were determined for ACTH-immunoreactive cells.

The following parameters were counted:

Pn - number of points hitting on nuclei of immunohistochemically labeled cells inside the test field

Ptc - number of points hitting on cytoplasm of immunohistochemically labeled cells inside the test field

Nn - number of nuclei of immunohistochemically labeled cells inside the test field

The formula for calculating the nuclei volume was:

$Vn = V_{Vn} / N_V$,

and formula for the cell volume calculation was:

$Vc = 1/ N_V$, where

V_{Vn} is - the volume density of ACTH cell nuclei and N_V - 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, N_V corresponds to the number of cells per cubic millimetre, according to the formula:

$N_V = (k/ß) (N_A^{3/2} / V_{Vn}^{1/2})$

On the basis of earlier karyometric studies (Malendowicz, 1974), the shape coefficient ß 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 N_A is the number of cells in the plane of the pituitary tissue section. N_A is calculated as follows:

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

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

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

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

Biochemical analyses

Blood was collected from the trunk, and separated plasma and sera samples of all animals were stored at the same time at -70°C until assayed. Plasma levels of ACTH were determined without dilution by the IMMULITE method (DPC, Los Angeles, USA), in duplicate samples within a single assay, with an intra-assay coefficient of variation (CV) of 9.6%. The analytical sensitivity of this assay is 9 pg/ml. Serum corticosterone concentrations were determined without dilution by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate samples within a single assay, with an intra-assay CV of 8.0%. The sensitivity of this Corticosterone Immunoassay is typically less than 27 pg/ml.

Statistical analysis

STATISTICA® version 5.0 (StatSoft, Inc) was used for the statistical analysis. Morphometric and biochemical data obtained for the experimental groups were subjected to one-way analysis of variance (ANOVA) and Duncan's test. These tests were used for evaluation of the significance of differences between mean values of morphometric (ACTH: cell volume, volume of the nuclei and volume density) and biochemical (ACTH and corticosterone levels) parameters in the examined groups of rats.

Results

Body and pituitary weights

Data for body weight, absolute and relative pituitary weights are summarized in Table 1. A 10% decline ($p<0.05$) of mean body weight was observed in the Orx group in comparison to the SO group (before surgery they were around the same). Orchidectomy and subsequent estradiol treatment (Orx+E) decreased mean body weight by 16% ($p<0.05$) compared to that in the SO group. In daidzein treated orchidectomized rats (Orx+D) the value of mean body weight was similar to the corresponding in SO rats. The absolute pituitary weight in the Orx+E group increased ($p<0.05$) by 150%

and 156% in comparison to the SO and Orx groups, respectively. In the orchidectomized and daidzein treated group (Orx+D) the absolute pituitary weight was 8% greater ($p<0.05$) than in the Orx group. The relative pituitary weight increased by 15% ($p<0.05$) in the Orx group, in comparison with the SO group. Orx+E

Table 1. Body and pituitary weights in sham-operated (SO), orchidectomized (Orx), estradiol-treated orchidectomized (Orx+E) and daidzein-treated orchidectomized (Orx+D) middle-aged male rats.

Experimental group	Initial body weight (before surgery) (g)	Body weight before the treatment (g)	Body weight after the treatment (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg/100g body weight)
SO	680±30	654±8	650±29	17.0±1.7	2.20±0.14
Orx		639±63	586±35[a]	16.6±1.4	2.53±0.23[a]
Orx+E		639±63	548±32[a]	42.5±8[a,b]	7.75±0.59[a,b]
Orx+D		639±63	620±46[b]	18.0±1.4[b]	2.90±0.20[a,b]

Mean±SD; n=8; [a]: $p<0.05$ vs. sham-operated (SO) rats; [b]: $p<0.05$ vs. orchidectomized (Orx) rats

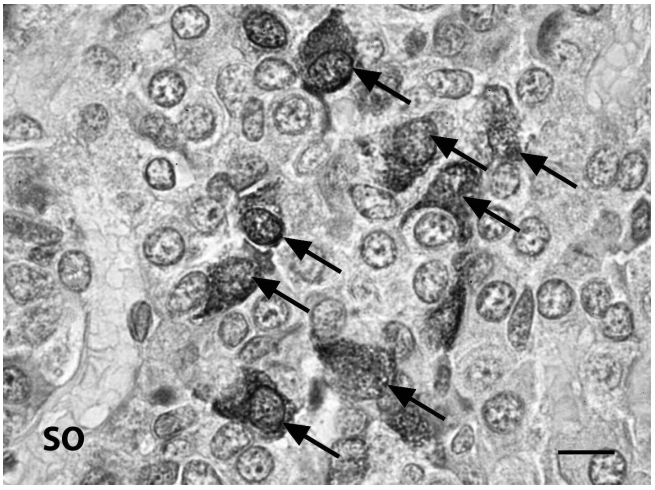

Fig. 1. Immunoreactive ACTH cells in the *pars distalis* of the pituitary gland from a sham-operated rat (SO), PAP. Bar: 8 μm.

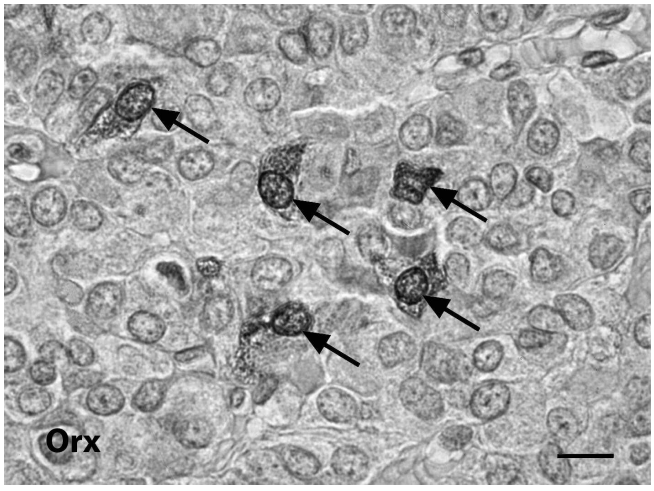

Fig. 2.. Less numerous ACTH cells from an orchidectomized rat (Orx), PAP. Bar: 8 μm.

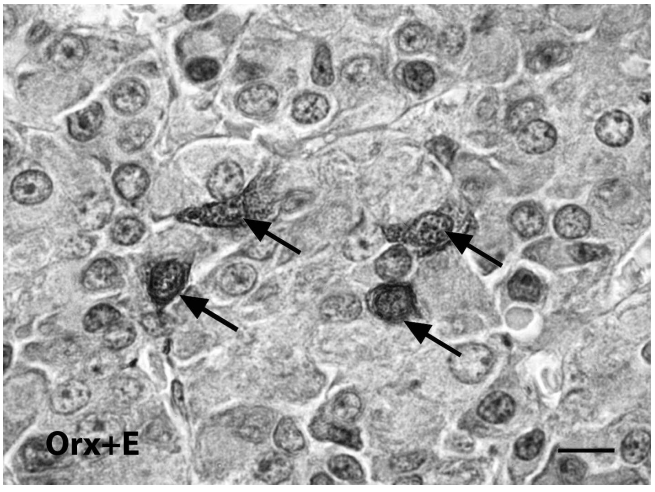

Fig. 3. Sparse ACTH cells in an orchidectomized and estradiol treated rat (Orx+E), PAP. Bar: 8 μm.

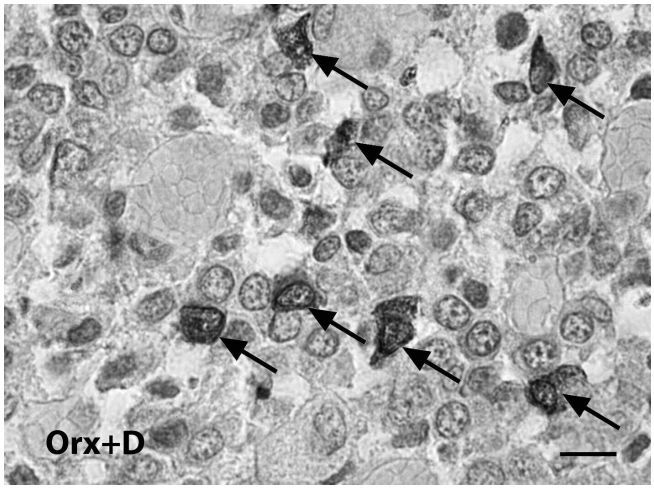

Fig. 4. Small dark ACTH cells in an orchidectomized and daidzein treated rat (Orx+D), PAP. Bar: 8 μm.

treatment elevated ($p<0.05$) the relative pituitary weight by 252% and 206% compared to values for the SO and Orx groups, respectively. In Orx+D rats, the relative pituitary weight was 32% and 15% greater ($p<0.05$) than in the SO and Orx groups, respectively.

Immunohistochemical findings

The ACTH-immunopositive cells were predominantly located in the central part of the pituitary *pars distalis*. In SO middle-aged males they were often present as small groups in close proximity to numerous capillaries. ACTH-immunopositivity was granular, uniformly distributed throughout the relatively small portion of cytoplasm surrounding the prominent nuclei. Corticotrophs were ellipsoid or polygonal in shape, often with expressed cytoplasmic projections (Fig. 1).

In comparison to the SO animals, ACTH-immunopositive cells in the Orx rats were less numerous, darker and variably shaped, although their location remained as in the SO group (Fig. 2).

Immunohistochemical analysis of the ACTH cells in Orx+E rats indicated some differences in comparison to the appropriate findings in the SO and Orx groups. Namely, the ACTH cells were sparse, round or elongated, with oval nuclei and leaned on the capillaries (Fig. 3).

In Orx+D rats, the ACTH cells appeared smaller, darker and were markedly irregular in shape compared to those in SO and Orx rats (Fig. 4).

Morphometric findings

Morphometric analysis revealed that the corticotrophs in the control SO group had a mean cell volume of 912 μm^3, a nuclear volume of 115 μm^3 and they occupied 20% of the pituitary volume (Fig. 5 a-c).

After orchidectomy, the cell volume and the relative volume density declined by about 11% and 16%, respectively ($p<0.05$; Fig. 5a,c) compared to the SO group.

In Orx+E rats the nuclear volume increased ($p<0.05$; Fig. 5b) by 11% and 12% in comparison to the SO and

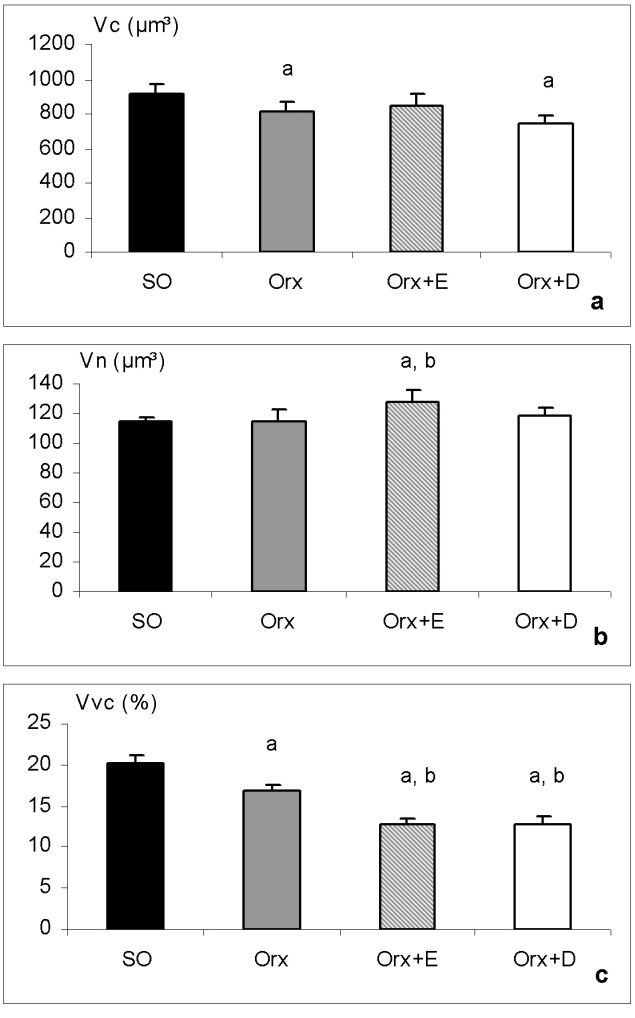

Fig. 5. a. Cellular volume (Vc; μm^3) of ACTH cells. **b.** Nuclear volume (Vn; μm^3) of ACTH cells. **c.** Relative volume density (V_{VC}; %) of ACTH cells expressed as percentages of total gland tissue. All values are means ± standard deviation, n=8 animals per group, [a]$p<0.05$ vs. sham-operated (SO), [b]$p<0.05$ vs. orchidectomized (Orx).

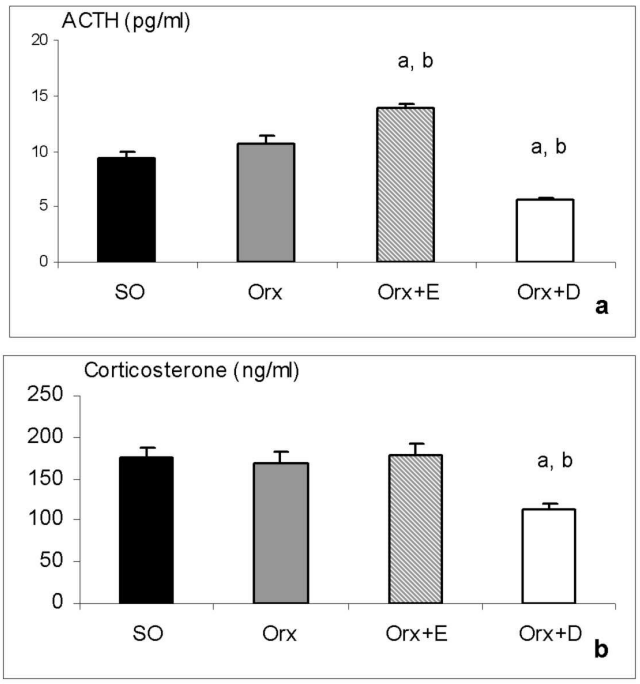

Fig. 6. a. Plasma ACTH concentration. **b.** Serum corticosterone concentration, in middle-aged male rats. The values are means ± standard deviation, n=8 animals per group, [a]$p<0.05$ vs. sham-operated (SO), [b]$p<0.05$ vs. orchidectomized (Orx).

Orx groups, respectively. After orchidectomy and estradiol treatment, the relative volume density was 37% and 25% ($p<0.05$) lower than in the SO and Orx groups, respectively (Fig. 5c).

Orchidectomy and daidzein treatment decreased ($p<0.05$) the cell volume by 18% in comparison to the SO group, while the relative volume density in these rats was 36% and 24% lower ($p<0.05$) than in the SO and Orx groups, respectively (Fig. 5a,c).

Plasma ACTH and serum corticosterone levels

The mean plasma levels of ACTH and serum levels of corticosterone are summarized in Fig. 6a,b. Orchidectomy did not affect these hormones, but in the Orx+E group plasma ACTH level was elevated by 47% and 29%, respectively ($p<0.05$) in comparison to the SO and Orx groups. Orchidectomy and subsequent daidzein treatment decreased ($p<0.05$) plasma ACTH by 40% and 48%, compared to the plasma level in the SO and Orx groups, respectively. Serum corticosterone concentration in Orx+D rats was 36% and 33% lower ($p<0.05$) than in the SO and Orx groups respectively (Fig. 6a,b).

Discussion

In parallel with the influence of estradiol, the effects of subcutaneous treatment with daidzein on immunohistomorphometric features of the pituitary ACTH cells and circulating ACTH and corticosterone levels were investigated in the andropause, mimicked using orchidectomized middle-aged rats.

Under our experimental conditions estradiol treatment further decreased the mean body weight of orchidectomized rats. This may be partly due to atrophy of skeletal muscles induced by testosterone deprivation (Antonio et al., 1999), but negative effects of estradiol application on body weight have also been observed (Kitay, 1963). As in our previous study with genistein (Ajdžanović et al., 2009a), the mean body weight of daidzein treated orchidectomized rats was similar to the value of sham-operated animals.

Orchidectomy and subsequent estradiol treatment markedly increased the absolute and relative pituitary weight compared to both control groups. Pituitary weight increase after estradiol treatment was observed long ago, and is the obvious consequence of prolactin (PRL) cell proliferation (Kitay, 1963). Thus, estradiol stimulates PRL synthesis and cell proliferation (Lieberman et al., 1978; Milošević et al., 2007). After orchidectomy and estradiol treatment, sparse, round or elongated ACTH cells leaned on the capillaries were depicted. The ACTH cell nuclei volume was significantly increased, but the relative volume density in orchidectomized and estradiol treated rats was lower than for both control groups. These changes were accompanied with a significant increase of plasma ACTH concentration with no alteration in corticosterone level, in comparison to the SO and Orx groups. Estrogens have been shown to lower POMC gene expression and the ACTH response to repeated stress stimulus in ovariectomized rats (Redei et al., 1994). In contrast, estradiol treatment increased the ACTH level in middle-aged female rats (Kostić et al., 2003). Also, it was observed that estradiol does not enhance ACTH stimulated corticosterone *in vitro* (Nowak et al., 1995). In our study, where the experimental animals suffered stressful stimuli (orchidectomy, daily treatment), the altered ACTH cell nuclear volume may indicate that estradiol affected POMC gene expression, but the decreased relative volume density accompanied by the transient ACTH rise suggests that the cells had already secreted their stored ACTH content. The nature of molecular events occuring in ACTH cells after estradiol treatment is not still clarified. The hypothesis, we postulated on the results obtained with the available methodology, needs verification by some advanced molecular and microscopy techniques.

Orchidectomy followed with daidzein treatment increased the relative pituitary weight in comparison to both SO and Orx groups. Similar results were observed earlier with the related isoflavone, genistein (Ajdžanović et al., 2009a; Milošević et al., 2009), and estrogenic effects on PRL cells explain the obtained rising trends after genistein and daidzein application. After orchidectomy and daidzein treatment inhibitory immunohistomorphometric and hormonal ACTH-corticosterone changes were observed. Immunohistochemistry indicated the presence of small, dark, irregularly shaped ACTH cells after daidzein treatment. Also, a low relative volume density of ACTH cells and reduced circulating ACTH and corticosterone levels were clearly evident in the Orx+D group when compared to both SO and Orx groups. In our previous ACTH-corticosterone study genistein treatment caused the same trend of changes in an animal model of the andropause (Ajdžanović et al., 2009a). The phenomenology of this was explained by genistein induced tyrosine kinase and 3ßHSD inhibition, and interruption of cytokine-CRH stimulated POMC gene expression (Akiyama et al., 1987; Katahira et al., 1998; Ohno et al., 2002). On the other hand, others found significantly increased serum PRL and LH levels in ovariectomized rats after the administration of the daidzein metabolite equol (Rachon et al., 2007). Considering the greater ER α presence in pituitary LH and PRL cells than in ACTH cells (Schreihofer et al., 2000), and the apparent daidzein/genistein effects on ACTH cells in our studies, it is obvious that ER-mediated as well as ER independent mechanisms of isoflavone action are operative in the pituitary. The proposed ER independent daidzein activity in the ACTH cells seems to be specific. In contrast to genistein, daidzein is a weak inhibitor of tyrosine kinase, but has almost the same inhibitory effect on L-type calcium channels (Yokoshiki et al. 1996). Pituitary POMC-derived hormones release in a calcium channel dependent manner is well known (Douglas and Taraskevich, 1978). Also, paracrine regulation of ACTH

secretion (Jia et al., 1992) and possible effects of daidzein on the paracrine factors should be considered. The concept of ACTH cells as autocrine/paracrine targets was postulated and a variety of factors regulating ACTH secretion have been identified (Denef, 2008). The inhibitory effects of soy isoflavones on IL-8 and follistatin have already been demonstrated *in vitro* (Handayani et al., 2006). Considering the protein binding/inactivating properties of isoflavones, some attention should be devoted to potential daidzein effects on paracrine loops in ACTH cells. It seems that, although weak, the tyrosine kinase inhibitory effect (interruption of POMC gene expression), blockade of hormone release and potential paracrine regulation effects lead to the morphofunctional status of ACTH cells we observed after daidzein treatment. The ACTH levels after daidzein application were low, opposite to rising tendencies in Orx+E rats. The reason for such a difference most likely involves secretion mechanisms. Similarly to genistein, daidzein acts as a potent inhibitor of 3ß-HSD (Ohno et al., 2002) and retains adrenocortical steroidogenesis. Together with attenuated ACTH release, blockade of peripheral steroidogenesis leads to the decreased circulating corticosterone level observed in our Orx+D group.

In conclusion, this study revealed that subcutaneous daidzein treatment negatively modulated the immunohistomorphometric features of ACTH cells and decreased ACTH and corticosterone secretion in our animal model of the andropause. Compared to estradiol, the traditional therapeutic option in some malignancies in men, the effects of daidzein were coherent and implied inhibition of ACTH, as well as corticosterone secretion.

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