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Genistein affects the morphology of pituitary ACTH cells and decreases circulating levels of ACTH and corticosterone in middle-aged male rats

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ABSTRACT

The soybean phytoestrogen, genistein, is increasingly consumed as an alternative therapeutic for age-related diseases, namely cardiovascular conditions, cancer and osteoporosis. However, despite the beneficial effects on health, concern has been raised that this isoflavone also acts as an endocrine-disrupting chemical. The aim of this study was to examine the effects of genistein on immunohistomorphometric features of pituitary adrenocorticotropic cells (ACTH) and blood concentrations of ACTH and corticosterone in orchidectomized middle-aged male rats. Sixteen-month-old Wistar rats were divided into sham-operated (**SO**), orchidectomized (**Orx**) and genistein-treated orchidectomized (**Orx+G**) groups. Genistein (30mg/kg/day) was administered subcutaneously for three weeks, while the control groups received the vehicle alone. ACTH cells were identified by the peroxidase-antiperoxidase (PAP) immunohistochemical procedure. Circulating concentrations of ACTH and corticosterone were measured by immunoassay. Orchidectomy reduced ($p<0.05$) the cell volume and the relative volume of ACTH cells in comparison to **SO** rats. Genistein treatment further decreased ($p<0.05$) these morphometric parameters and reduced ($p<0.05$) circulating ACTH and corticosterone concentrations by more than 20% in comparison to both **Orx** and **SO** rats. In conclusion, genistein modulated the immunohistomorphometric features of ACTH cells and decreased blood ACTH and corticosterone levels, which supports evidence that this isoflavone affects the hypothalamic-pituitary-adrenal axis and suppresses glucocorticoid hormone secretion.

Key terms: ACTH cells, corticosterone, genistein, middle-age, orchidectomy, rats

INTRODUCTION

Andropause is an age-related partial decline of serum testosterone that is usually accompanied by decreases of other hormones, namely dehydroepiandrosterone, growth hormone, thyroid hormones and melatonin (Lamberts et al., 1997). During this period of life the frequency of cardiovascular diseases, cancer, osteoporosis, as well as stress-related psychiatric disorders, depression, irritability and sleep disturbance, increases (Vance, 2003). Augmented activity of the hypothalamic-pituitary-adrenal (HPA) axis is associated with a high incidence of stress-related psychiatric disorders with advancing age (Hatzinger et al., 2000).

The soybean isoflavone, genistein, is structurally similar to estradiol 17β (Price and Fenwick, 1985; Setchell, 1998). It competes with a lower potency for binding to endogenous estrogen receptors (ERs) and exerts significantly higher affinity for ER β than for ER α (Kuiper et al., 1997). In addition to this mild estrogenic/antiestrogenic effect, genistein has strong antioxidative potency (Benassayag et al.,

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2002) and inhibited activity of the tyrosine kinase enzyme family (Akiyama et al., 1987). These mechanisms certainly contribute to its putative beneficial effects in the prevention and treatment of cancer, cardiovascular and other age-related diseases (Ramos, 2007). Combining genistein with radiation is a potentially important novel strategy for the treatment of prostate cancer (Raffoul et al., 2006). Consumption of high doses of purified isoflavones from soybean in the form of nutritional supplements has become very popular among the Western population in recent years. However, there is a growing concern that phytohormones also act as endocrine disruptive chemicals, which interfere with the function of different endocrine systems (Brevini et al. 2005; Caserta et al. 2008; Phillips and Tanphaichitr, 2008).

Estrogen treatment has been shown to decrease the level of proopiomelanocortin (POMC) mRNA, a precursor of the ACTH molecule, and lowered the ACTH response in stress-stimulated ovariectomized adult female rats (Redei et al., 1994). Studies concerning the potential effects of the phytoestrogen, genistein, on the function of the HPA axis are rather limited. Based on the findings that some cytokines, namely IL-1, IL-2 and IL-6, are important stimulators of pituitary ACTH secretion (Bateman et al., 1989; Besedowsky and del Ray, 1996), *in vitro* studies (Katahira et al., 1998) demonstrated a suppressive effect of genistein on cytokine-mediated stimulation of expression of the POMC gene. However, chronic treatment of weanling rats with genistein at 40mg/kg significantly reduced serum corticosterone concentration and elevated ACTH level by a feedback mechanism (Ohno et al., 2003).

Considering the available literature data the authors assumed that ACTH cell morphology can change after genistein treatment (hypothesis behind the aim) and their idea was to investigate how it can change, *i.e.* to make a certain step in this field, with available methodology. The precise aim of our study was to examine the effects of subcutaneous treatment with therapeutic doses of the soybean isoflavone, genistein, on immunohistomorphometric features of ACTH cells, ACTH hormone secretion and consequently glucocorticoid hormone synthesis in orchidectomized middle-aged male rats, which is an animal model of the andropause.

MATERIALS AND METHODS

Animals and diets

The experiments were performed on 16-month-old male Wistar rats. They were bred in the Institute for Biological Research, Belgrade, Serbia, housed two per cage, exposed to a 12-12 h light-dark cycle and kept at 22 ± 2ºC. 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 a 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 crystal cellulose originated from Alfa Aesar, Johnson Matthey Gmbh & Co.KG, Karlsruhe, Germany; carbohydrate, oil, vitamin/mineral mix, calcium carbonate, calcium phosphate 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 anaesthesia (Ketamine hydrochloride; Richter Pharma, Wels, Austria; 15 mg/kg b.w.). Sham-operated (**SO**; n=8) and orchidectomized rats were allowed to recover for 2 weeks. After recovery, the orchidectomized rats were divided into two groups of eight animals each. One group was

subcutaneously treated with genistein (Nutraceutica, Monterenzio, Italy; **Orx+G**) in a dose of 30 mg/kg b.w. every day except on Sundays for 3 weeks. Genistein was predissolved in a minimal volume of absolute ethanol (0.17 ml) and mixed with sterile olive oil (0.33 ml). The final volume of mixture injected was 0.5 ml *per* animal. The other orchidectomized group (**Orx**) and the **SO** group were given the same volume (0.5 ml) of vehicle alone. All animals were killed by decapitation 24h 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 Guide for the Care and Use of Laboratory Animals (1996, National Academy Press, Washington D.C.).

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; described by Sternberger et al., 1970). 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 45min. Sections were then overlaid with commercially diluted primary antibodies (hACTH antiserum DAKO A/S, Glostrup, Denmark) for 24h 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 45min. Binding sites were visualised 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 5μm thick sections. Two sections from the dorsal, three from the middle and two from the ventral part (totally seven sections, 200μm apart) of the rat pituitary glands were analyzed. The point counting method was used at total magnification of x1000 (Weibel, 1979). 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 analysed *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 labelled cells inside the test field, Ptc-number of points hitting on cytoplasm of immunohistochemically labelled cells inside the test field, Nn-number of immunohistochemically labelled cell nuclei inside the test field, Formula for the nuclei volume calculation was:

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

and formula for the cell volume calculation was:

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

V_{Vn} - volume density of ACTH cell nuclei and N_V - numerical density of ACTH cells. Nuclei volume density (V_{Vn}) provides information about nuclei attendance in estimated cells and is calculated using the formula:

$$V_{Vn} = \sum Pn / \sum Ptc$$

Since rat ACTH cells are mononuclear, the N_V corresponded to the number of cells per cubic milimeter, according to the formula:

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

On the basis of earlier karyometric studies (Malendowicz, 1974), the shape coefficient β for pituitary cells was estimated to be 1.382, k is factor related to cell distribution according to their size (in case of ACTH cells its value is 1) and N_A is number of cells in the plane of pituitary tissue section. N_A is calculated using the formula:

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

where a represents the rhomb area that belongs to every point of the test system and is calculated using the formula:

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

d, the test line length in the used test system.

Volume density (V_{VC}) is calculated as a ratio of Pn and Ptc sum (Pn+Ptc) and total number of points in test system. Considering the fact that 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 diluting the plasma, by the IMMULITE method (DPC, Los Angeles, USA), in duplicate samples within a single assay, with an intra-assay CV of 9.6%. Analytical sensitivity of this assay is 9 pg/ml. Serum corticosterone concentrations were determined without diluting the sera, 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 analyses

Morphometric and biochemical data obtained for the experimental groups were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range test was used for *post hoc* comparisons between groups. The confidence level of $p<0.05$ was considered statistically significant. The data are presented as means ± SD.

RESULTS

Body and pituitary weights

Data for body weight, absolute and relative pituitary weights are summarized in Table I. A 10% decrease ($p<0.05$) 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 genistein treatment did not affect the absolute pituitary weight of the animals. The relative pituitary weight increased by 15% ($p<0.05$) for the **Orx** group and 34% ($p<0.05$) for the **Orx+G** group in comparison with **SO** group.

Immunohistochemical findings

The ACTH-immunopositive cells were mostly 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 cytoplasmatic projections (Fig 1).

In comparison to the **SO** controls, the ACTH-immunopositive cells in **Orx** rats were less numerous, darker and smaller, although their location and shape remained as in the controls (Fig 2). After genistein treatment, ACTH cells appeared smaller and darker than in both **Orx** and **SO** rats. Their shape was highly irregular (Fig 3).

Morphometric findings

Morphometric analyses showed that the corticotrophs in the control **SO** group had a mean cell volume of 912.1 μm^3, a nuclear volume of 115.3 μm^3 and they occupied 20 % of the pituitary volume (Fig 4a-c).

After orchidectomy, the cell volume and the relative cell volume (relative volume density) were significantly decreased by about 11% and 16%, respectively ($p<0.05$; Fig 4 a,c) compared to the **SO** group.

In **Orx+G** rats the cell volume of pituitary corticotrophic cells was smaller than in the **Orx** group by about 16% ($p<0.05$; Fig 4a) and the relative volume density had decreased by 19% ($p<0.05$; Fig 4c). Compared to the **SO** group, Vc had decreased by about 25% ($p<0.05$; Fig 4a) and V_{VC} by about 32% ($p<0.05$; Fig 4c).

Plasma ACTH and serum corticosterone levels

The mean plasma levels of ACTH and serum levels of corticosterone are summarized in Figure 5 a-b. Orchidectomy did not affect these hormones, but in the **Orx+G** group both ACTH and corticosterone concentrations were reduced by about 23% and 31%, respectively ($p<0.05$; Fig 5 a,b) in comparison with the vehicle-treated **Orx** group and the **SO** group (by about 12% and 34%, respectively; $p<0.05$; Fig 5 a,b).

DISCUSSION

The effects of subcutaneous treatment with genistein on the morphology and function of pituitary ACTH cells and circulating corticosterone levels were studied in the andropause, mimicked by using orchidectomized middle-aged male rats.

Orchidectomy was also performed to avoid any confounding effects on the HPA axis of endogenous sex steroids, of which the testes are the major source. This is an established approach for examining the potential effects of sex hormone-like compounds on hormonal homeostasis. The dose of genistein employed (30 mg/kg b.w.) was chosen to mimic human exposure to elevated concentrations of isoflavones when nutritional supplements are used for therapeutic purposes (Doerge and Sheehan, 2002). Some new data (Jefferson et al., 2007) suggest that subcutaneous treatment is legitimate, considering that totality of the injected genistein enters the circulation (about 80 % is absorbed into blood when orally applied), so biological effects caused by either oral or subcutaneous application are very similar.

TABLE I

Body and pituitary weights in sham-operated (**SO**), orchidectomized (**Orx**) and genistein-treated orchidectomized (**Orx+G**) middle-aged male rats

Experimental group	Initial body weight (before surgery)(g)	Body weight before the treatment (g)	Body weigh after the treatment (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg/100g body weight)
SO		654 ± 8	650 ± 29	17.0 ± 1.7	2.20 ± 0.14
Orx	680 ± 30	639± 63	586 ± 35[a]	16.6 ± 1.4	2.53 ± 0.23[a]
Orx+G		639±63	632 ± 57	19.0± 1	2.96 ± 0.20[a]

Mean ± SD, n=8
[a]$p<0.05$ vs. sham operated (**SO**) rats

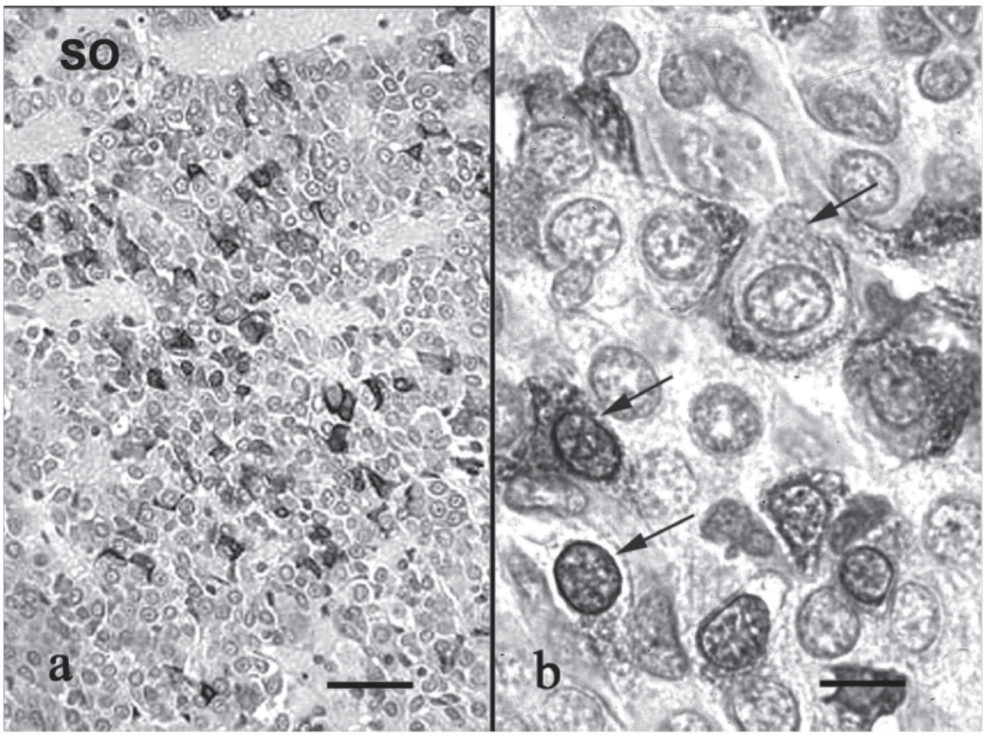

Figure 1a: Immunoreactive ACTH cells in the pars distalis of the pituitary gland from a control sham operated rat (**SO**), PAP, bar=40 μm, **1b.** higher magnification, bar=8 μm

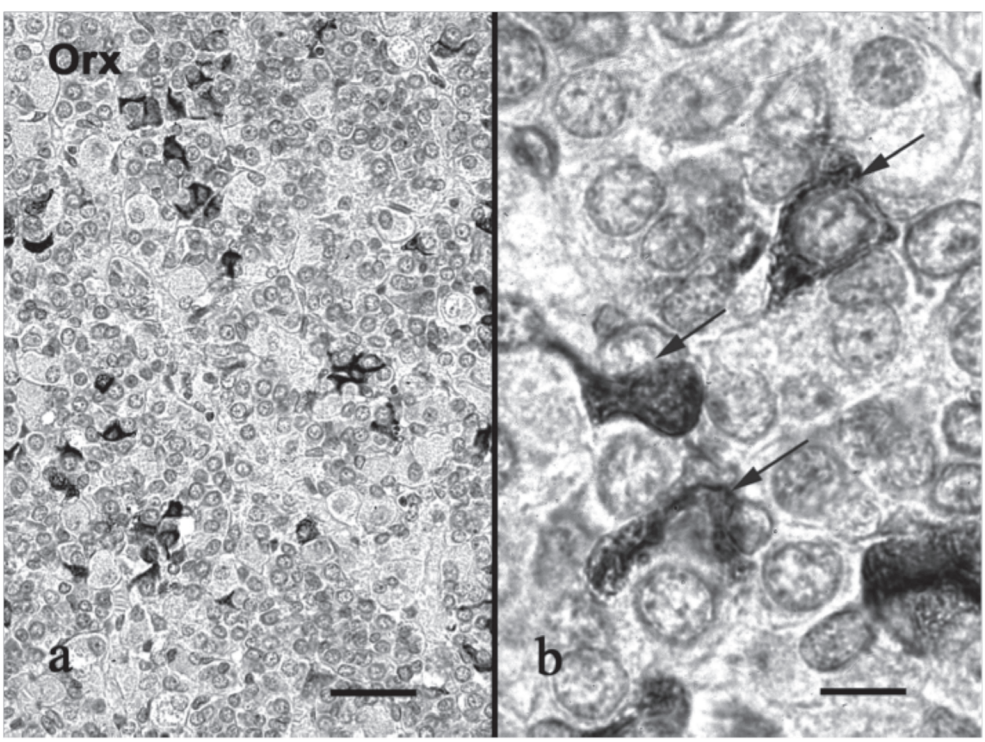

Figure 2a: Less numerous and smaller ACTH cells from an orchidectomized rats (**Orx**), PAP, bar=40μm, **2b.** higher magnification, bar 8=μm

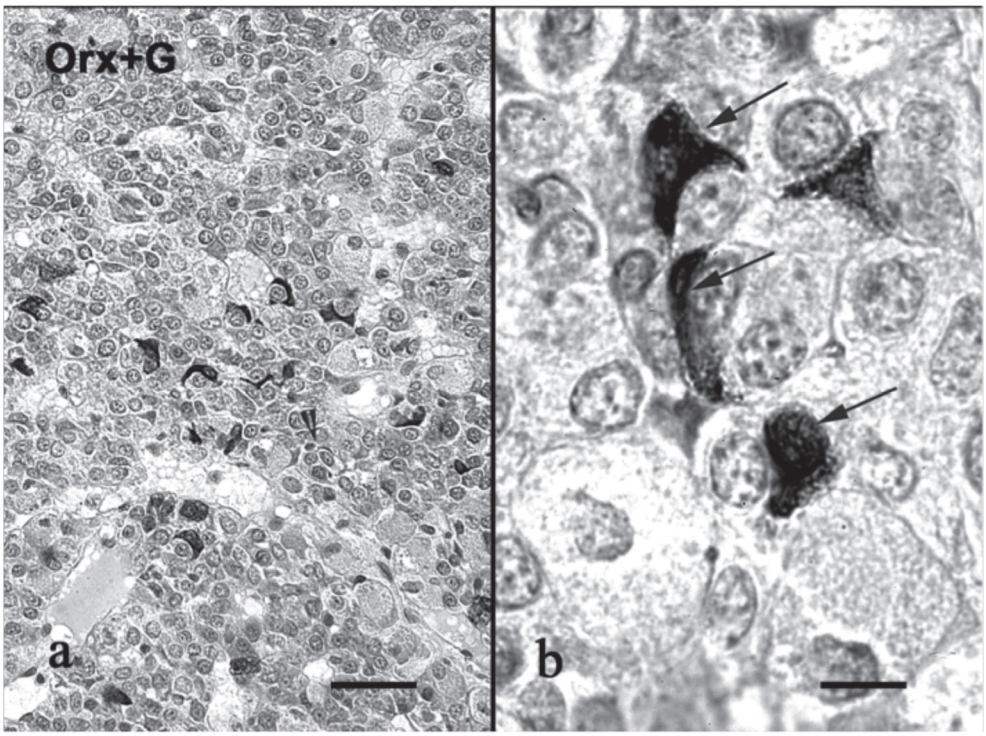

Figure 3a: Small, sparse ACTH cells in an orchidectomized and genistein treated rats (**Orx+G**), PAP, bar=40μm, **3b.** higher magnification, bar=8 μm

To that end, using an injection strategy, we have operated with the form of soybean isoflavone available after digestive transformation (orally consumed soybean based therapeuticals contain genistin being hydrolyzed to nonconjugated active form genistein, due to digestive enzymes). The injection strategy was, also, used with a view to easily control the applied dose.

Under our experimental conditions, a significant decrease of mean body weight was detected five weeks after orchidectomy. This is in accordance with previous reports (Malendowicz, 1976) and may be due to atrophy of skeletal muscles induced by testosterone deprivation (Antonio et al., 1999). Subcutaneous genistein treatment of orchidectomized rats did not affect body weight in our animals. The reported effects of genistein on body weight in males are contradictory, as decreased (McClain et al., 2006), increased (Penza et al., 2006) or, as in our study, no effect on body weight (Faqi et al., 2004) has been recorded, probably due to different treatment protocols and experimental conditions.

Orchidectomy and subsequent genistein treatment increased the relative pituitary weight when compared to **SO** rats. At least for **Orx** rats this is probably the consequence of decreased body weight, since no significant changes in absolute pituitary weights were detected. However, the detected increase of relative pituitary weight was higher in the **Orx+G** group and may be partly the result of an estrogenic effect of genistein on pituitary estrogen-responsive cells, namely prolactin cells. Consistent with these results was the trend towards increased relative pituitary weight in both male and female pups whose dams had received high doses of genistein (Delclos et al., 2001). On the other hand, it was shown that genistein facilitated the development of an estrogen responsive pituitary tumor cell line transplanted into rats (Fujimoto and Honda, 2003). Further examinations of pituitary prolactin cells in our experimental model are needed to confirm this hypothesis.

The immunohistochemical and morphometric analyses revealed a decreased volume and the relative volume density of ACTH cells without an accompanying decrease in plasma level of ACTH after orchidectomy of middle-aged rats. It was reported that orchidectomy causes persistent CRH release (Bingaman et al., 1994). The data of the cell number and total volume of the ACTH cells should help to brighten depict the morphofunctional changes after orchidectomy. On the other hand, 13% of plurihormonal cells, which contained both ACTH and gonadotropic hormones, were detected within the population of rat pituitary corticotropes (Childs et al., 1982). Therefore, it is possible to postulate the hypothesis (which, naturally, needs verification by using some advanced microscopy techniques), that the transdifferentiation of the multi-hormonal ACTH cells to gonadotropes, caused by orchidectomy, provokes the changes in the population of pituitary corticotropes.

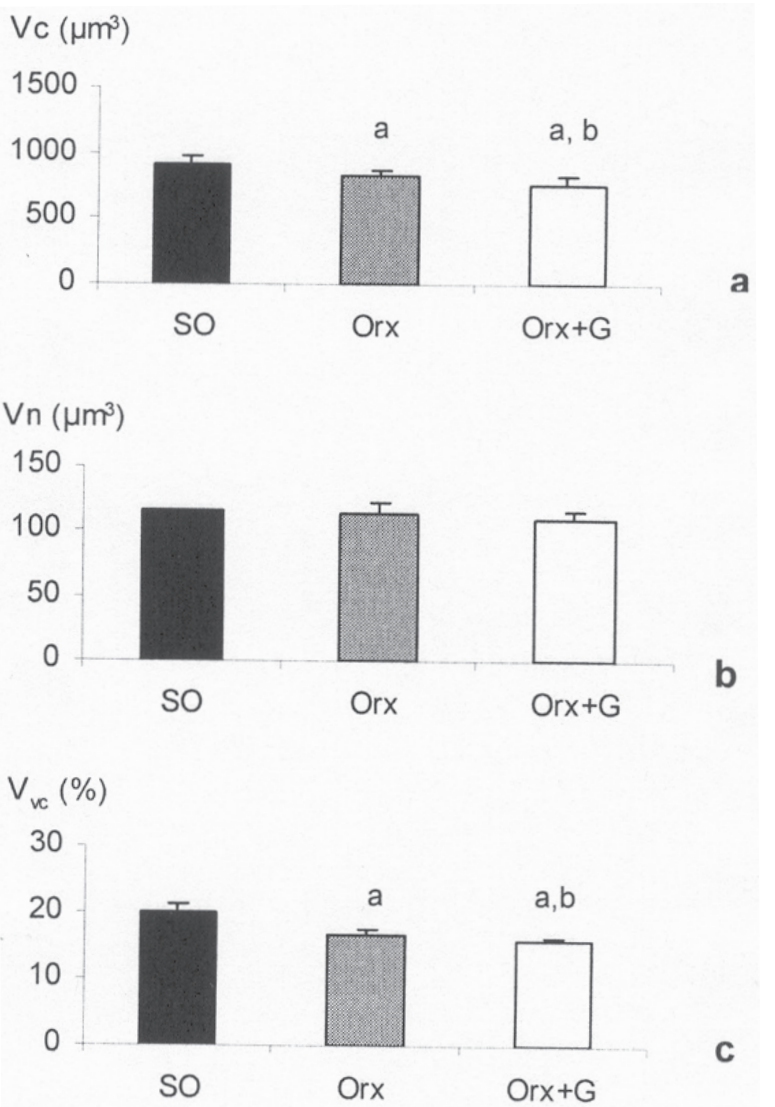

Figure 4a: Cellular volume (Vc; μm³) of ACTH cells; **b**. nuclear volume (Vn; μm³) of ACTH cells; **c**. relative volume density (V_{VC}; %) of ACTH cells expressed as percentages of total gland tissue; All values are the means ± standard deviation, n=8 animals per group, [a]p<0.05 vs. sham operated (**SO**), [b]p<0.05 vs. orchidectomized (**Orx**)

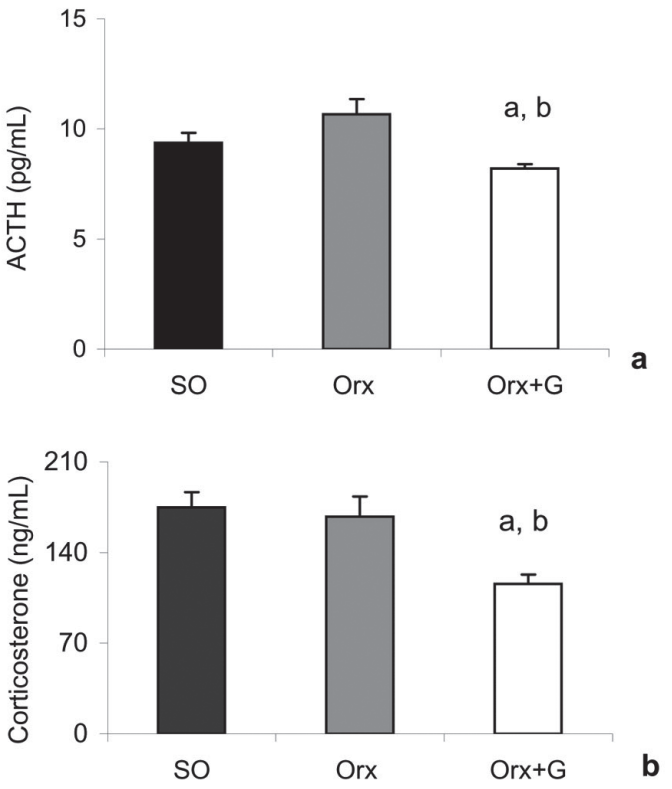

Figure 5a: Plasma concentration of ACTH; **b.** serum concentration of corticosterone, in middle aged male rats. The values are the means ± standard deviation, n=8 animals per group, [a]p<0.05 vs. sham operated (**SO**), [b]p<0.05 vs. orchidectomized (**Orx**).

In this study we demonstrated that high doses of genistein inhibited the HPA axis. Decreased immunohistomorphometric features of pituitary ACTH cells and reduced circulating ACTH and corticosterone levels were clearly evident in the **Orx+G** group when compared to both the **Orx** and **SO** groups. It was reported that estrogen replacement lowered the POMC mRNA level and the ACTH response to repeated stressful stimuli in ovariectomized rats (Redei et al., 1994). Besides the estrogenic mechanism, genistein may also reduce the level of ACTH through inhibition of tyrosine kinase phosphorylation cascades (Katahira et al., 1998). Various cytokines are generated during stress and regulate the HPA axis. Thus, IL-1 was shown to increase POMC mRNA concentrations *in vivo* (Harbuz et al., 1992) and *in vitro* in a primary culture of rat anterior pituitary cells (Suda et al., 1989); IL-2 stimulated ACTH secretion and/or POMC expression *in vivo* (Harbuz and Lightman, 1989; Naito et al., 1989; Hanisch et al., 1994) and *in vitro* (Karanth and McCann, 1991); IL-6 stimulated ACTH secretion at the level of both the hypothalamus and pituitary (Matta et al., 1992). As phosphorylation cascades represent the central signaling pathway for several cytokines and since they operate in ACTH cells, genistein, a broad spectrum inhibitor of protein tyrosine kinase and several other kinases, was proposed to act on ACTH cells by this mechanism (Katahira et al., 1998). Considering that our experimental animals suffered stressful stimuli (orchidectomy, subcutaneous treatment), we expect that effects of genistein were investigated under the conditions of increased cytokine generation, so decreased plasma ACTH after genistein treatment may be a result of indicated mechanism, as well. Considering that the controls were on equal stressful terms

(levels of ACTH were around 10 pg/ml) the genistein action is distinctive.

It seems logical to assume that, under our experimental conditions, genistein treatment reduced serum corticosterone through central effects, by lowering pituitary ACTH release. However, a direct action of genistein on the adrenal cortex cannot be disregarded. Geńistein was shown to suppress cortisol secretion in the human adrenocortiḉal cell line, H925, and the porcine adrenocortical cells (Mesiano et al., 1999; Kaminska et al., 2007). It was shown that subcutaneous administration of genistein at 3nmol/100g significantly lowered the blood corticosterone level in ovariectomized rats unrelated to its estrogen-like activity (Malendowicz et al., 2006). On the other hand, it was reported that genistein *in vitro* strongly inhibited human adrenocortical 3β-hydroxysteroid dehydrogenase (Ohno et al., 2002), similar to estrogen action (Rao and Chinoy, 1986), and reduced the activity of 21-hydroxylase (P450c21), the latter finding also confirmed (Mesiano et al., 1999).

After continuous administration of genistein to weanling rats, expansion of cells at *zona fasciculata* and *zona reticularis* of the adrenal cortex was reported, accompanied by decreased corticosterone and elevated ACTH concentrations in blood (Ohno et al., 2003). Contrary to our conclusions, the authors considered the increase in serum ACTH concentration to be the result of a feedback effect of genistein-induced decline in adrenal production of corticosterone. It is well-known that in developing rodents there is a short period (from 4th to 14th postnatal day) when central regulation of the adrenal response to stress is either minimal or non-existent, a stress hyporesponsive period. Therefore, the discrepancy between these results and ours are probably due to the different age of the experimental animals.

In conclusion, this study showed that subcutaneous genistein administration modulated immunohistomorphometric features of pituitary ACTH cells and altered the HPA axis by decreasing both ACTH and corticosterone levels in orchidectomized middle-aged rats.

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