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Histological and morphofunctional parameters of the hypothalamic–pituitary–adrenal system are sensitive to daidzein treatment in the adult rat

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ABSTRACT

The isoflavone, daidzein is a biologically active, plant-derived compound that interacts with estrogen receptors. Data from previous studies have suggested that daidzein exerts beneficial effects in many diseases; however, as an endocrine disrupter, it may also alter the functioning of the endocrine system. Data regarding the effect of daidzein on the morphofunctional and histological parameters of the hypothalamic–pituitary–adrenal (HPA) system is still lacking. Therefore, using the newCAST stereological software, we investigated the effects of chronic (21 days) daidzein treatment on corticotropin-releasing hormone (CRH) neurons within the hypothalamus and corticotropes (ACTH cells) in the pituitary, while image analysis was employed to-examine the intensity of fluorescence of CRH in the median eminence (ME) and adrenocorticotropin hormone in the pituitary in adult orchidectomized (Ovx) rats. Circulating ACTH and corticosterone levels were also analyzed. This study showed that daidzein treatment decreased the volume density of CRH neurons within the paraventricular nucleus as well as CRH immunofluorescence in the ME. The total number of ACTH cells was decreased, while ACTH cell volume and the intensity of ACTH fluorescence were increased following daidzein treatment. Both ACTH and corticosterone blood levels were increased after daidzein administration. The results of performed experiments clearly demonstrate that volume density of CRH neurons; total number and volume of ACTH cells, as well as stress hormones levels are vulnerable to the effects of daidzein.

1. Introduction

Activation of the hypothalamic–pituitary–adrenal (HPA) system is considered to be a characteristic response to physical or psychological stress, the purpose of which is to maintain equilibrium between organisms and their environment (de Kloet et al., 2005). Stress-related inputs are integrated in the paraventricular nucleus (PVN) of the hypothalamus to induce the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which stimulate adrenocorticotropic hormone (ACTH) secretion from the pituitary, subsequently inducing glucocorticoids production by the adrenals. The HPA system activation is terminated by a negative feedback action of glucocorticoids: elevated levels of glucocorticoids decrease the synthesis of AVP, CRH and ACTH in a direct and indirect manner through the pituitary, hypothalamus and hippocampus (Whitnall, 1993). The activity of the HPA system shows pronounced sex-related differences (glucocorticoid levels are thus higher in females than in males) and estradiol is believed to play a causal modulatory role (Handa et al., 1994). Inter alia, estradiol may directly enhance CRH gene transcription in the hypothalamus via binding to estrogen-responsive elements on the CRH gene (Vamvakopoulos and Chrousos, 1993).

Phytoestrogens are estrogens that present in some plant. They are structurally and functionally similar to estrogens (Patisaul and Jefferson, 2010). Daidzein, one of the major isoflavones in soybeans, works by binding to estrogen receptors (ER) (Kuiper et al., 1998). Previous studies have revealed that daidzein exerts beneficial effects such as: improvement of preovulatory follicles development (Liu and Zhang, 2008), anti-cancer (Messina et al., 1994) and neuroprotective effects (Zhang et al., 2002), has antioxidative properties (Dwiecki et al., 2009), and that it effects could be dose-dependant (Benassayag et al., 2002). On the contrary, in view of estrogen's essential role in growth, differentiation and homeostasis, isoflavones may be classified as endocrine disruptors since they alter the normal functioning of the endocrine system (Henley and Korach, 2010).

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And yet, few experiments have been conducted to assess the daidzein effects on the HPA system and, to our knowledge, none have examined the effects of daidzein on stereological parameters at the hypothalamic–PVN and pituitary–ACTH levels by using the newCAST stereological system in adult rats. This study was undertaken to provide additional insight into the actions of daidzein by analyzing its effects on the volume of PVN, volume density of CRH neurons, and the volume density, volume and total number of ACTH cells in adult rats, considering the importance of the stereology approach as essential in the discovery of new concepts in cell biology. In addition, the blood concentrations of ACTH and corticosterone were also determined.

2. Material and methods

All animal procedures complied with the EEC Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research, 'Sinisa Stankovi' University of Belgrade, Serbia.

The experiments were performed on adult, 2-month-old male Wistar rats, kept in the facilities of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Under standard environmental conditions (a 12 h light\dark cycle, 22 ± 2° C), the animals were kept in groups of two per cage. Before entering the experiment, all the animals were fed the standard chow diet for laboratory rats (Veterinary Institute Subotica, Serbia). Two weeks prior to the start of the experiment the rats were fed a soy-free diet prepared in cooperation with the Department of Food, School of Veterinary Medicine, Belgrade, Serbia, and INSHRA PKB, Belgrade, Serbia, according to Picherit et al. (2000). Food and water were available ad libitum.

Under anesthesia (ketamine hydrochloride 15 mg/kg b.w.), the animals were orchidectomized (Orx) or sham-operated (So). Given that sex steroids act on the whole HPA system, by orchidectomy we want to minimize steroids influence and separate the effects of sex steroid and daidzein. The first group of Orx rats was subcutaneously injected with daidzein (D) (Nutraceutica) (30 mg/kg b.w.) every day at 9 AM, for 3 weeks, two weeks after surgery. The applied dose of daidzein (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). Daidzein was dissolved in a minimal volume of absolute ethanol (0.1 ml) and then mixed with sterile olive oil (0.2 ml). The So and the second Orx group received the same volume of absolute ethanol in sterile olive oil and served as controls. The injection strategy is equivalent to oral consummation in terms of absorption rate (Jefferson et al., 2007) and provides an easy control of the applied dose. All rats were euthanized by decapitation under low ether anesthesia (ether ad narcosis Ph. Iug. III., Lek, Ljubljana, Slovenia) 24 h after the last injection. The low ether anesthetic provides relief from pain and tension (we used this anesthetic for 2 min), and doesn't affect ACTH and corticosterone hormone levels (de Haan et al., 2002).

2.1. Tissue preparation

The hypothalamus and pituitary glands were excised, fixed in Bouin's solution and dehydrated in increasing concentrations of ethanol and xylene. After embedding in Histowax (Histolab Product AB, Göteborg, Sweden), coronal serial sections of the hypothalamus (5 µm thick) and a serial section of the pituitary (3 µm thick) were obtained with a rotary microtome (RM 2125RT Leica, Glostrup, Denmark). Sakura Tissue-Tek Accu-Edge Low-Profile microtome blades for extremely thin sectioning were used. We used cresyl violet acetate solution to identify nuclear structures within the hypothalamus.

2.2. Immunohistochemistry

Hypothalamic CRH-containing neurons in the PVN and CRH immunoreactivity in the median eminence (ME) were determined using immunofluorescence. After dewaxing, hydration and rinsing in 0.01 M phosphate-buffered saline (PBS; pH 7.6 for 10 min), the sections were exposed to microwaves (700 W) in 0.05 M citrate-buffered (pH 6.0; for 2 × 10 min) for antigen retrieval. Subsequently, the sections were washed in PBS (3 × 10 min). To block nonspecific staining, the section were preincubated in normal donkey serum (1:10) for 30 min, and then incubated overnight with rabbit anti-CRH (1:500 in PBS; ab8901-100 Abcam). After washing in PBS, the sections were incubated with donkey anti-rabbit Alexa fluor 488 IgG (1:200; Invitrogen) for 2 h, then washed in PBS and mounted in mowiol. Antibody specificity has been evaluated by using blocking peptides (Trifunović et al., 2012) and the negative control and the results ware complete loss of immunoreactivity within PVN. Precisely, the specificity of CRH immunostaining was confirm by co-incubation with 5-fold excess of blocking peptide, while the sections were treated in the same way as described above: the antibody was neutralized by incubation with the blocking peptide; the antibody that was bound to the blocking peptide was no longer available to bind to the epitope present in the protein (Fig. 1). The sections were examined and photographed using a Zeiss Axiovert fluorescence microscope, equipped with a camera and EC Plan-Apochromat.

Pituitary ACTH was localized using the peroxidase-antiperoxidase method. Antiserum to rat ACTH (NIDDK-anti-r ACTH-IC; 1:200) was obtained from Dr. A.F. Parlow, National Hormone Peptide Program, Harbor-UCLA Medical Centre, Carson, CA, USA. The specificity of the antisera was assessed by the National Institute of Diabetes and Digestive and Kidney disease (NIDDK). Endogenous peroxidase activity was blocked by incubation in a hydrogen peroxide solution in methanol. After the blocking procedure and incubation with normal swine serum (1:10; Dako, Glostrup, Denmark) for 1 h, the sections were overlaid with the appropriate dilution of ACTH primary antibodies for 24 h at room temperature. After washing in PBS, the sections were incubated for another 1 h with the secondary antibody (polyclonal swine-anti-rabbit; Dako, Glostrup, Denmark) and again rinsed with PBS. 0.05% 3,3-diaminobenzidine tetrachloride liquid substrate chromogen system was used for antibody localization. Control sections were incubated with rabbit non-immune serum at the same concentration as the primary antibody (in omission of the primary antibody). This resulted in the complete loss of immunoreactivity in the pituitary gland sections (Fig. 1).

Also, pituitary ACTH cells were determined using immunofluorescence. After dewaxing, hydration and rinsing in PBS, the sections were preincubated in normal donkey serum (1:10) for 30 min and then incubated in ACTH primary antibody for 24 h at room temperature. After washing in PBS, the sections were incubated with donkey anti-rabbit Alexa fluor 488 IgG (1:200; Invitrogen) for 2 h, then washed in PBS and coverslipped with mowiol.

2.3. Image analysis

For the evaluation of CRH protein content within ME, measurements of the relative intensity of fluorescent signal (RIF) were performed with Image J, as described previously (Jensen, 2013). Using five immunostained sections from different areas of ME (rostral, three medial and caudal sections) for each animal the RIF for CRH within ME were analyzed. The formula: RIF = Integrated Density – (selected CRH positive area X Mean fluorescence of background readings) was used. The sections were examined and photographed using a Zeiss Axiovert fluorescence microscope, equipped with a camera and EC Plan-Apochromat. So, RIF for CRH content was measured in five sections per animal, followed by calculating the average value per animals, and finally the average per group.

ACTH cell Image analysis: Images were obtained using a confocal

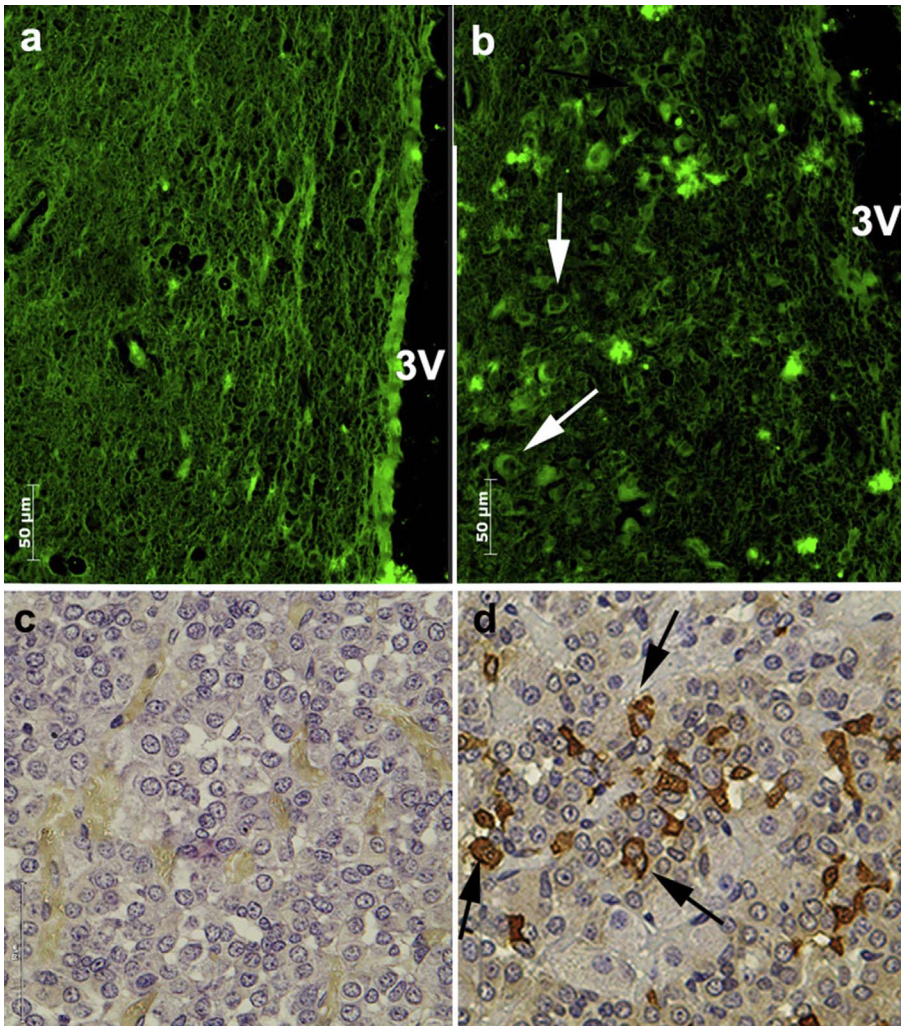

Fig. 1. Negative controls for: CRH and ACTH immune-staining in the paraventricular nucleus and the pituitary gland, respectively of adult rat. Photomicrographs a and b represent negative controls for CRH and ACTH immune-staining, photomicrographs b and d represent controls for CRH and ACTH immune-staining. Scale bar = 50 µm, white arrows indicate CRH neurons within PVN (a, b); Scale bar = 55 µm, black arrows indicate ACTH cells within pituitary.

laser scanning microscope Leica TCS SP5 II Basic (Leica Microsystems CMS GmbH; Germany). An Ar-ion 488 nm laser was used for excitation of green fluorescence. Imaging was done with a 40× or 63× objectives. Analysis of confocal microscopy images was performed using the Quantify option in LAS AF Lite software (Leica Microsystems CMS GmbH; Germany). Relative intensity of fluorescence (RIF) was calculated according to Waters and Swedlow (2007). Intensity of fluorescence was measured on 100–150 ACTH cells per animal, in which the nucleus was apparent.

2.4. Stereological measurements

Using a workstation controlled by a newCAST stereological software package (VIS – Visiopharm Integrator System, Denmark) all stereological analyses were carried out.

The volume of PVN (every 10th section from each tissue block was analyzed; interval between the section was 50 µm), pituitary volume and volume density of ACTH cells (every 20th section from each tissue block was analyzed; interval between the section was 60 µm) were estimated using Cavalieri's principle, according to the method described in detail by Trifunović et al. (2012).

The percentages of CRH neurons were obtained using photomicrographs of three fluorescently labeled sections from three levels (rostral-bregma-1,6 mm; medial-bregma-1,8 mm; caudal-bregma-2,0 mm; interval between the section approximately 0,2 mm) of the PVN for each animal. Photomicrographs for each animal were imported into the VIS program. The percentages of immunolabeled CRH neurons were calculated by counting the points hitting the CRH-ir neurons and dividing the number with the number of points hitting the PVN area x 100.

A fractionator/physical dissector design with two levels of sampling was used to estimate the total number of ACTH cells from all examined groups according to the method described in detail (Manojlović-Stojanoski et al., 2010). Sampling was systematically uniform from a random start and the sampling distance between the first sections in consecutive section pairs was 20 sections. Sections designated as section pairs were firstly captured into a super image. One section in the pair was designated the reference section and the other the 'look up' section. Then, the analysis was performed in both directions and this doubles the first sampling fraction from 1/20th to 1/10th (sampling fraction 1 (f1) = 1/10 = 0.1). We analyzed 1% of the selected tissue and sampling fraction 2 for ACTH cell number was f2 = 0.01.

As the mean volume of a single ACTH cell is equivalent to the total volume occupied by ACTH cells divided by their number, the size of a single ACTH cell can be calculated (de Lima et al., 2007).

2.5. Blood ACTH, corticosterone and testosterone assays

Blood was collected from the trunk in two types of glass tubes (anticoagulant-treated glass tubes, EDTA for ACTH analyses and tubes without anticoagulant for corticosterone analyses). The samples were stored at −70 °C until assayed for hormone concentrations in duplicate within single assays. IMMULITE method (DPC, Los Angeles, USA) with an intra-assay CV of 9.6%. was used for determination of ACTH

concentration. The corticosterone concentration was determined by immunoassay (R&D Systems Inc., Minneapolis, USA) with an intra-assay CV of 8.0%. Serum testosterone concentrations were determined without dilution, using a competitive immunoenzymatic colorimetric method (EIAgen Testosterone Kit, Adaltis Italia S.p.A., Bologna, Italy), in duplicate samples within a single assay, with an intra-assay CV of 6.2%.

2.6. Statistical analysis

All the results are expressed as means for six (stereological data) or 10 animals (hormonal level data) per group ± SD. Data were tested for normality of distribution by the Kolmogorov–Smirnov test, whereas homogeneity of variances was evaluated by Levene's test. Duncan's multiple range test was used for post-hoc comparisons between the groups. A confidence level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body mass and testosterone level

Data of the body mass and the testosterone level are presented in Table 1. Body mass and the testosterone level were significantly ($p < 0.05$) decreased after orchidectomy in comparison with So group. The significant ($p < 0.05$) changes of body mass and testosterone level in Orx + D group in relation to So and Orx rats, were observed.

3.2. Hypothalamus

The PVN was identified according to a rat brain atlas (Paxinos and Watson, 2004). The main references used to locate the PVN were the presence of the fornix and the 3rd ventricle. In adult rats, the PVN begins at 1.5 mm and extends to 2.1 mm posterior to the bregma level. The mean volumes of PVN were larger ($p < 0.05$) in the Orx (0.19 ± 0.01 mm^3; or 55%) and Orx + D (0.17 ± 0.02 mm^3; or 42%) compared to the So group (0.12 ± 0.01 mm^3) (Fig. 2B).

The majority of the CRH-immunopositive perikarya were located in the medial parvicellular subdivision of the PVN, which is in accordance with previous works (Kiss et al., 1991; Lennard et al., 1993; Simmons and Swanson 2009). Histological analysis showed higher CRH-ir in the Orx group compared to both So and Orx + D groups (Fig. 2A). The percentage of CRH-ir in the Orx group (26.78 ± 2.1%) was higher ($p < 0.05$) by 23% than in the So group (22.43 ± 2.3%), while the same parameter in the Orx + D group (23.8 ± 1.3%) was reduced in comparison to the Orx group (Fig. 2C).

CRH neurons with axons are projecting to hypophyseal portal capillaries in the external zone of the ME what is in accordance with previous studies (Makara et al., 1979; Alonso et al., 1986; Aguilera and Liu, 2012)

The stronger CRH staining intensity in the ME were detected in the Orx group when compared to the So and Orx + D groups. The ImageJ analysis showed higher ($p < 0.05$) relative intensity of fluorescence by 103% in the ME of the Orx group when compared to the So. Also, RIF in ME of the Orx group showed higher values ($p < 0.05$) by 41% when compared to the Orx + D groups. (Fig. 3)

Table 1
XXX.

Group	Body mass (g)	Serum level of testosterone (ng/mL)
So	397.1 ± 26.1	1.570 ± 0.050
Orx	330.4 ± 32.3*	0.034 ± 0.010*
Orx + D	319 ± 27.8*	0.11 ± 0.009*,**

All values are the means ± SD, n = 8 animals per group.
* $p < 0.05$ vs. So.
** $p < 0.05$ vs. Orx.

3.3. Pituitary

The ACTH immunoreactive cells were irregularly shaped and located alone or in groups between the capillaries in the pituitary pars distalis, in all experimental rats. Histological analysis revealed more or less the same number of ACTH cells in all experimental groups. In the Orx + D group the volume of ACTH cells was greater in comparison to the other two control groups (Fig. 4A).

The significant decrease ($p < 0.05$; 15%) of ACTH cells fluorescent intensity were detected in the Orx group compared to the So group. Following daidzein treatment, the same parameter increased by 46% and 72% in comparison to the So and Orx groups, respectively (Fig. 4B).

The volume of pituitary and the volume density of ACTH cells in the group Orx + D were not different from the respective values in the So and Orx rats. The total number of ACTH cells was decreased ($p < 0.05$) by 21% and 27% in the Orx + D group compared to the So and Orx rats, respectively. The cell volume of ACTH cells was 27% and 34% greater ($p < 0.05$) in the Orx + D group than in the So and Orx rats, respectively (Fig. 5).

3.4. Hormonal analysis

The mean plasma ACTH concentration in the Orx + D group was higher ($p < 0.05$) by 39% and 117% in comparison to the Orx and So groups, respectively. Also, the same parameter was 56% higher ($p < 0.05$) in the Orx group than in the So group (Fig. 6A). The mean serum corticosterone concentration in the Orx + D group was higher ($p < 0.05$) by 57% and 40% in comparison to the So and Orx groups, respectively (Fig. 6B).

4. Discussion

There is growing public concern that exposure to phytoestrogens may have deleterious effects on endocrine homeostasis. The purpose of the present study was to determine whether exposure of adult rats to the phytoestrogen daidzein influences histological, stereological and biochemical parameters in the HPA system. In this study, we used the orchidectomized rat as an appropriate model system for testing the effects of hormone-like substances.

Substantial evidence suggests that reproductive steroids modulate the response to stress. In our previous publication, we described in detail the stimulatory activity of orchidectomy on stereological and biochemical parameters at the hypothalamic and pituitary levels within the HPA system (Trifunović et al., 2012).

Following daidzein treatment, the qualitative histological as well as the stereological results showed decreased percentages of CRH neurons and CRH content within the ME. Considering the ERs distribution in the brain, more precisely in the parvocellular and magnocellular regions in the PVN (Simerly et al., 1990), and the ability of daidzein to cross the blood–brain barrier (Ma et al., 2010), a direct influence of daidzein within the CNS is viable. The reduced number of CRH neurons obtained in this study is opposed to the phytoestrogens-stimulated neurons proliferation observed earlier (Pan et al., 2012), and the disagreement could be explained (*inter alia*) by an indirect action of daidzein. Moreover, our results have revealed increased circulating corticosterone levels following daidzein exposure. It is well known that glucocorticoids serve a vital function in the negative feedback inhibition of their own secretion, so fluctuations in the corticosterone level may trigger changes at the hypothalamic level. Previous studies have indicated that glucocorticoids bind their membrane receptors on PVN neurons, eliciting an intracellular cascade that mobilizes the synthesis of endocannabinoids which inhibit glutamate release, reducing the neural activity of PVN neurons (Malcher-Lopes et al., 2003).

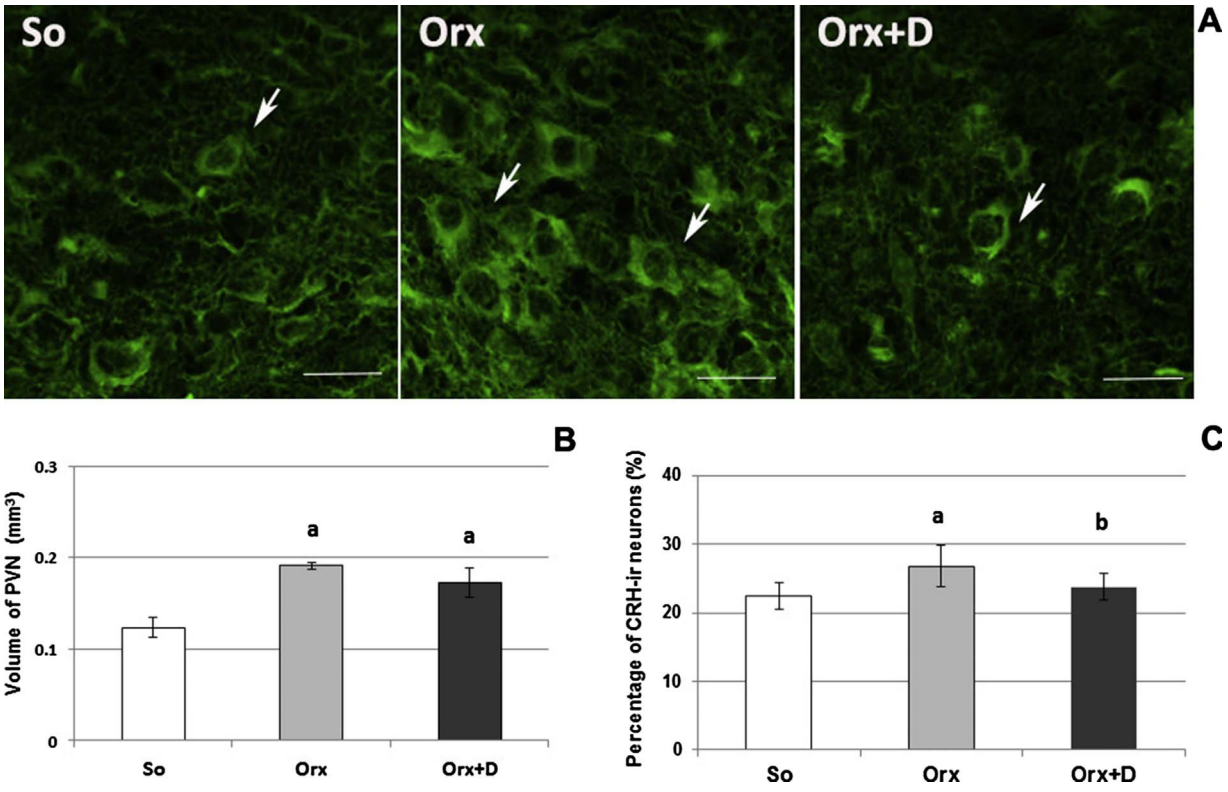

Fig. 2. CRH immuno-fluorescent stained neurons in the paraventricular nucleus-PVN (A); Volume of the PVN (B); percentage of CRH immunoreactive neurons (C) of sham-operated (So), orchidectomized (Orx) orchidectomized and daidzein treated (Orx + D) adult rats. Scale bar = 50 μm, white arrows indicate CRH neurons within PVN. The values are means ± standard deviation, n = 6 animals per group; a $p < 0.05$ vs. So, b $p < 0.05$ vs.

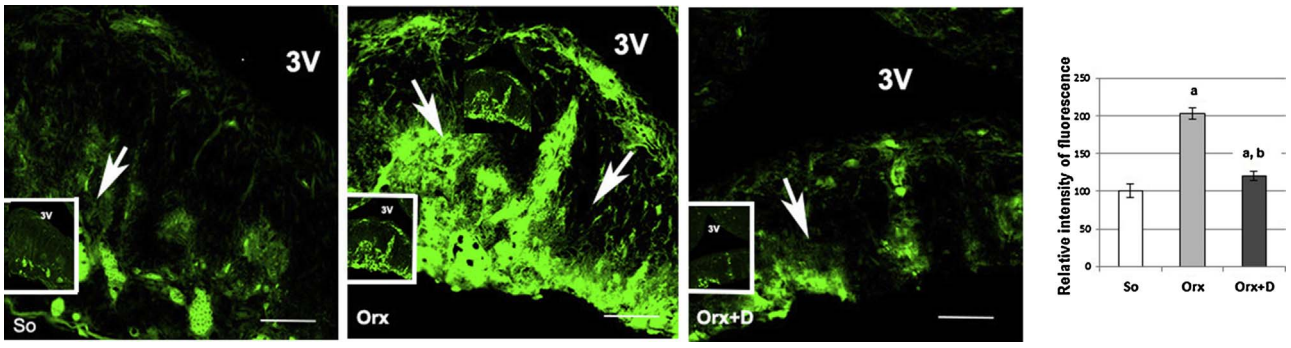

Fig. 3. CRH immunoreactivity and the relative intensity of CRH fluorescence in the median eminence in sham-operated (So), orchidectomized (Orx), orchidectomized and daidzein treated (Orx + D) adult rats; 3V- third ventricle; white arrows indicate CRH protein content within ME; scale bar 50 μm. The values are means ± standard deviation, n = 6 animals per group; a $p < 0.05$ vs. So, b $p < 0.05$ vs. Orx.

Furthermore, daidzein may have a direct effect on adrenals considering the moderate to high ERα expression in the gland (Kuiper et al., 1997). Also, lower levels of endogenous sex steroids, provoked by orchidectomy, might potentiate daidzein-induced corticosterone secretion, considering the competitive binding between the isoflavones and endogenous sex steroids (Tham, 2002). Earlier studies have revealed an increase (Hartley et al., 2003) or decrease (Caceres et al., 2014) in corticosterone secretion following daidzein exposure, and variances may be due to different animal ages and/or different experimental approaches. It seems logical to assume, considering the results from this study (the reduction of CRH activity), that at the hypothalamic level dominates the indirect influence of daidzein by the glucocorticoid negative feedback.

Daidzein treatment provoked a decrease in ACTH cell number, and increases in ACTH cell volume, intensity of ACTH fluorescence and ACTH circulating level. Daidzein may act on the pituitary directly, considering the ER distribution in all anterior and intermediate lobe cell types (Mitchner et al., 1998), or over the hypothalamus and adrenal. It seems, however, that lactotrophs (LH cell) are the major target for estrogen-like substances within the pituitary due to their abundance (40%) (Chen, 1987) and the high ER expression within LH cells (Mitchner et al., 1998) and reduced number of ACTH cells might be the result of pituitary plasticity, namely to poly-hormonal corticotropes (Childs, 1991). Also, the reduced CRH neuron activity might potentate the decrease of number of ACTH cells. On the other hand the direct action of daidzein, through ERs, could be cause for the increased production of ACTH (increased ACTH content and circulating ACTH hormone level). Apparently, ACTH cells represent the medium of complex, both direct and indirect, action of daidzein with the increase of ACTH levels as a resulting effect.

In summary, quantitative results from our stereological analysis plus biochemical data indicate that effects of daidzein are different at the level of the hypothalamus, pituitary and adrenals (under this experimental condition). Separately observing, each of these organs contain

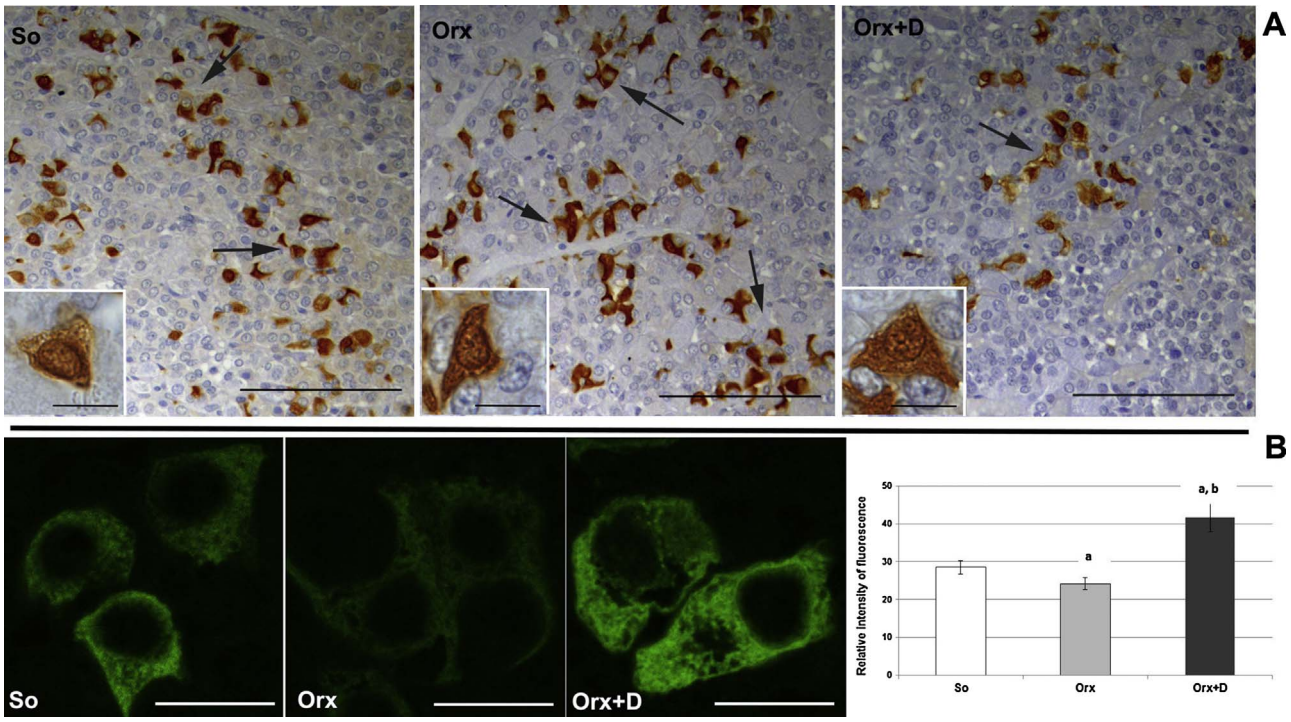

Fig. 4. Immunoreactive corticotropes (ACTH cells; A) and the relative intensity of ACTH fluorescence (B) in the pituitary gland in sham-operated (So), orchidectomized (Orx) and orchidectomized-daidzein treated (Orx + D) adult male rats. Scale bar 100 μm, insets scale bar 10 μm; the values are means ± standard deviation, n = 6 animals per group; a $p < 0.05$ vs. So, b $p < 0.05$ vs. Orx.

the ER and could be the direct target for daidzein. On the other hand, keeping in mind that it is an *in vivo* study, these organs should be observed within the totality of the HPA system with all the possible interactions. Our findings suggest that the morphofunctional parameters of stress system are sensitive to daidzein, in a view of: the increased ACTH and corticosterone levels, the decreased CRH activity and number of ACTH cell and increased ACTH cell activity. Accordingly, the daidzein action on HPA system appears to be some complex summation of direct influences and the indirect feedback mechanisms.

Conflict of Interests

The authors and manufacturers disclose no actual potential conflict of interests.

Acknowledgement

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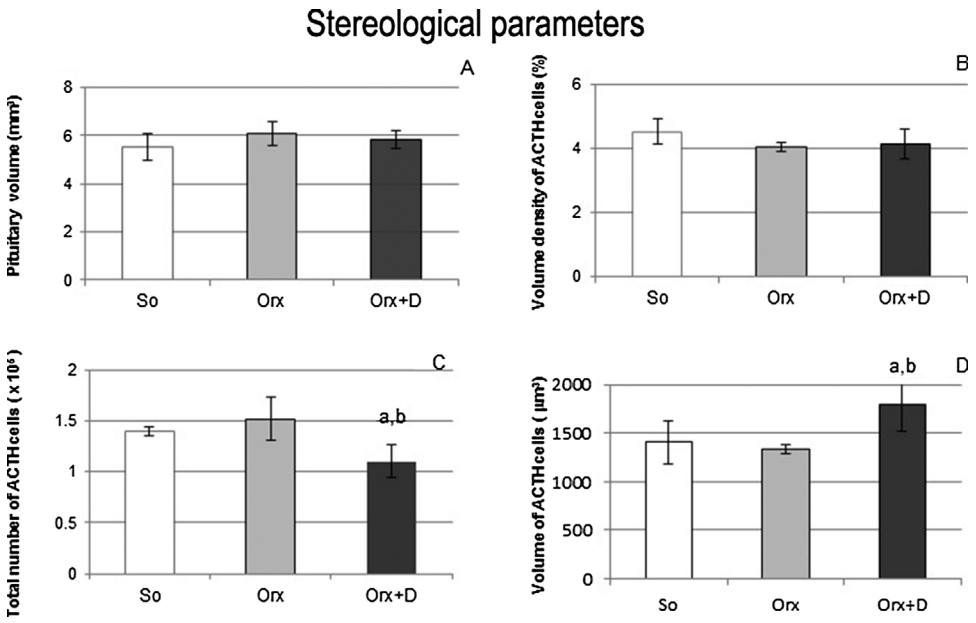

Fig. 5. Stereological parameters for pituitary corticotropes in sham-operated (So), orchidectomized (Orx), orchidectomized, orchidectomized and daidzein treated (Orx + D) adult rats: pituitary volume (A); volume density of corticotropes (ACTH cells); Total number of ACTH cells (C); Volume of ACTH cells (D). The values are means ± standard deviation, n = 6 animals per group; a $p < 0.05$ vs. So, b $p < 0.05$ vs. Orx.

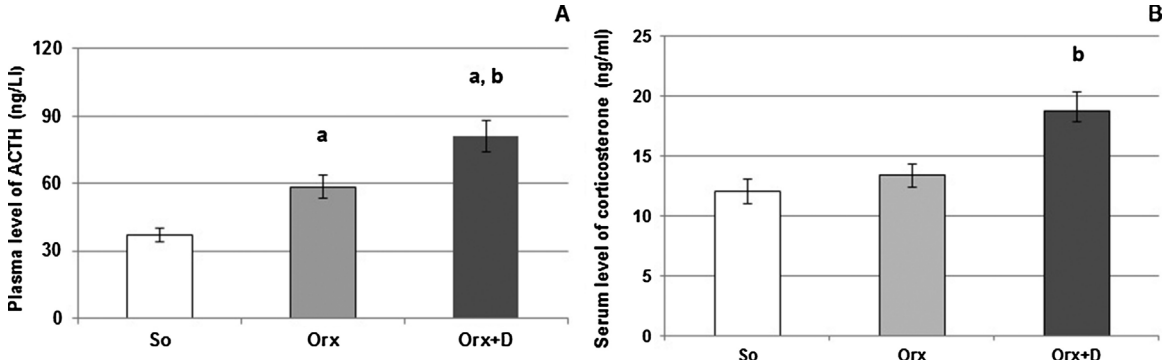

Fig. 6. Plasma ACTH concentration (A) and serum corticosterone concentration (B) in sham-operated (So), orchidectomized (Orx) and orchidectomized-daidzein treated (Orx + D) adult male rats. The values are means ± standard deviation, n = 10 animals per group; a $p < 0.05$ vs. So, b $p < 0.05$ vs. Orx.

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