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Multiple dexamethasone treatment affects morphometric parameters of gonadotrophic cells in adult female rats

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Abstract: Exposure to glucocorticoids leads to numerous changes in various biological systems including the reproductive system. The aim of the present work was to find out whether dexamethasone (Dx) treatment of adult female rats would influence the histological and morphometric characteristics of the pituitary gonadotrophic cells (luteinizing - LH cells and follicle stimulating - FSH cells). One group of female Wistar rats received Dx injections on three consecutive days in doses 1.0, 0.5 and 0.5 mg/kg b.w. respectively, while the control rats were treated with equivalent volumes of saline. Experimental and control animals were sacrificed 24 h and 72 h after the last injection. The peroxidase-antiperoxidase (PAP) immunocytochemical procedure was used to study the LH and FSH cells. The stereological and morphometric analyses showed that multiple Dx treatments of female rats significantly decreased the volume of LH cells and the volume of their nuclei 24 h and 72 h after the last Dx injection in comparison with control values. At 24 h after Dx treatment, the volume density of LH cells was significantly increased, but at 72 h differences between the experimental and control groups were insignificant. The increase in number of LH cells per unit area (mm^2) was significant at both timepoints (24 h and 72 h). Stereologic and morphometric characteristics of FSH cells was changed after Dx treatment in the same manner as that of LH cells, except for the volume density, where a significant increase was established 24 h and 72 h after the last Dx application. These results clearly demonstrate that 24 h and 72 h after the last of three Dx injections there were changes in the immunocytochemical and morphometric features of gonadotrophic cells. (www.cm-uj.krakow.pl/FHC)

Key words: Dexamethasone - Gonadotrophs - Immunocytochemistry - Morphometry - Rat, female

Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis by a variety of stressors is associated with changes in gonadotrophin secretion and disruption of ovarian cyclicity [7]. Glucocorticoids, the end product of the HPA axis have also been known to interrupt normal gonadotrophin secretion, acting at the level of the hypothalamus, pituitary gland and ovaries [26]. It is interesting that glucocorticoids affect the two gonadotrophins differently: plasma concentrations and pituitary content of follicle stimulating hormone (FSH) increased following exposure to stress or glucocorticoid administration [17, 26], whereas serum luteinizing hormone (LH) levels decreased but the pituitary content of LH was unchanged. The decline in plasma LH after elevation of glucocorticoid level blocks ovulation by preventing the preovulatory surges [15]. However, the increased synthesis and storage of FSH in the pituitary gland and the increase in plasma concentrations in this condition, may assist in the restoration of ovulatory cycles and reproductive functions after glucocorticoid levels fall [13].

It has been reported that glucocorticoids cause changes in plasma gonadotrophin levels and their pituitary content, and indirectly contribute to the inhibition of reproductive functions. In the present study, we investigated the effects of dexamethasone on immunocytochemical and morphometric characteristics of LH and FSH cells in adult female rats. We were also interested whether the intensity of any changes in the gonadotrophic cells alter in the period from 24 h to 72 h after multiple dexamethasone treatment.

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Materials and methods

Animals. Three-month-old virgin Wistar female rats were reared in the Institute for Biological Research in Belgrade, under standard conditions of controlled temperature (22°C), and a 12 h light/dark cycle. Food and water were freely available. The animals were divided into four groups. The experimental groups (Dx 24; n=8 and Dx 72; n=8) received subcutaneous injections of 1.0, 0.5 and 0.5 mg Dx (Dexamethasone phosphate - Krka, Novo Mesto, dissolved in

0.9% saline)/kg b.w. on three consecutive days, respectively, and were sacrificed at 10 a.m. by decapitation under ether anesthesia, 24 h or 72 h after the last injection. Control groups (C 24; n=8 and C 72; n=8) were injected with 0.9% saline in equal volumes as for the experimental groups, and were decapitated 24 h and 72 h after the last injection. Since the examined histological and morphometric parameters showed no significant differences between groups C 24 and C 72, all data were combined into one control group (C).

Light microscopy and immunocytochemistry. The pituitary glands were excised, fixed in Bouin's solution for 48 h and embedded in paraffin wax. Serial 5 μm thick tissue sections were deparaffinized in xylol and decreasing series of ethanol. Pituitary hormones were localized immunohistochemically using the peroxidase-anti-peroxidase complex (PAP) method of Sternberger [23]. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 15 min at room temperature, followed by a rinse in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 5 min. Before application of specific primary antibodies, nonspecific background staining was prevented by incubation for 45 min with nonimmune serum, *i.e.* normal porcine serum diluted 1:10 v/v in PBS; and then incubated with primary antibody for 45 min at room temperature. Antisera for βLH and βFSH (gifts from Dr A. F. Parlow, National Institute of Health, Bethesda, MD, USA) were diluted with PBS; 1:500 for βLH and 1:300 for βFSH. Subsequent to a 5 min rinse in PBS, the sections were incubated for 45 min with secondary antibody (1:500 v/v swine anti-rabbit IgG; DAKO A/S, Glostrup, Denmark), rinsed for 5 min in PBS, incubated for 45 min with rabbit antiperoxidase serum (1:100; DAKO A/S, Glostrup, Denmark), and again rinsed in PBS. The antigen-antibody complex was visualised by incubating the sections with 0.05% 3,3'-diaminobenzidine (DAB; Serva, Heidelberg, Germany) and 0.03% H_2O_2. The incubated sections were counterstained with haematoxylin. Control sections were incubated with normal porcine serum without primary antisera.

Morphometry. Immunocytochemically stained sections of pituitaries cut at three tissue levels of the *pars distalis* were used for morphometric examinations of anti-rat LH and anti-rat FSH reactive cells with visible nuclei. The cell volume of gonadotrophs (Vc), their volume densities (V_V) and the volume of the nucleus (Vn) were measured in 50 test areas per pituitary gland under a light microscope at the magnification of × 1000, on 5 μm thick sections, using the M_{42} multipurpose test system [27]. The volumes of LH- and FSH-positive cells and their nuclei were expressed in μm^3. Volume densities of gonadotrophic cells were expressed as percentages of total pituitary cells in mm^3. At the same time, the number of immunoreactive cells per unit area (mm^2) was analyzed in each section, by simple point counting.

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

Statistical analysis. Morphometric data obtained from each rat were averaged per experimental group and mean and standard deviations were calculated. One-way analysis of variance, followed by Duncan's multiple range test, was used for statistical comparison of the groups. A probability value of 5% or less was considered as statistically significant.

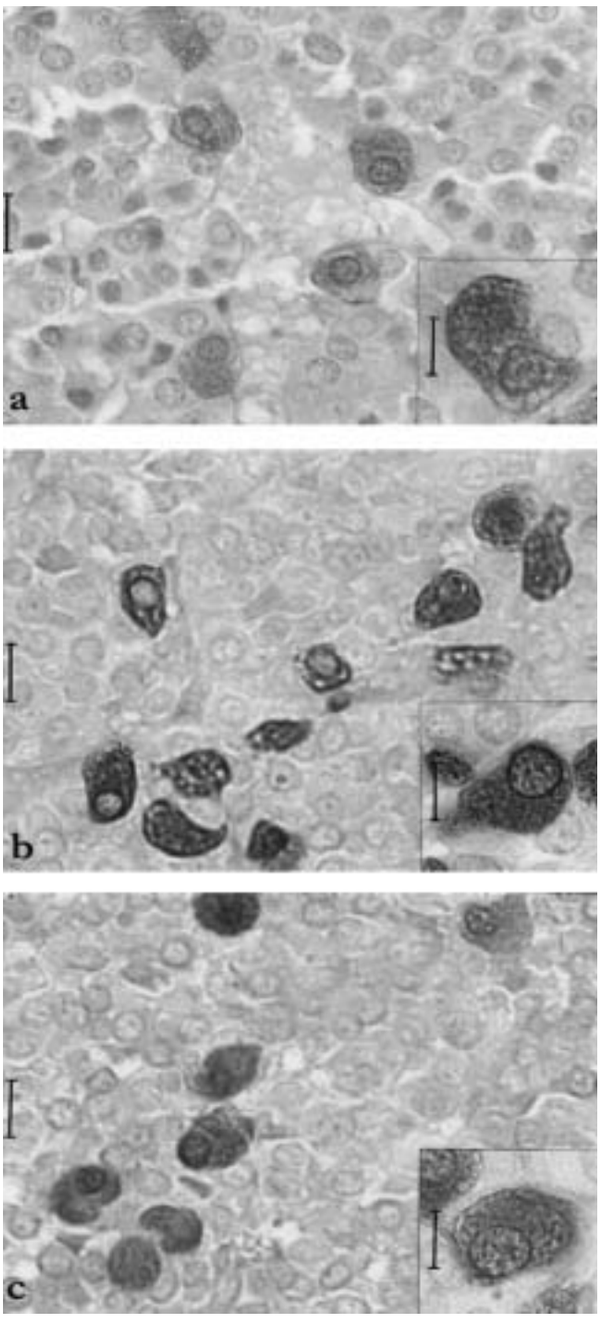

Fig. 1. Pars distalis of the adenohypophysis of control and Dx-treated female rats; **(a)** immunoreactive βLH cells in a control rat (bar=13 μm); **(b)** immunoreactive βLH cells 24 h after Dx treatment (bar=13 μm); **(c)** immunoreactive βLH cells 72 h after Dx treatment (bar=13 μm); insets (bar=8 μm).

Results

Histological analysis

The gonadotrophic cells of the control females were large, polygonal, oval or polyhedral in shape, with prominent, often eccentrically located nuclei. They showed strong immunopositivity for LH and FSH and were positioned throughout the pituitary *pars distalis* alone or in groups, often in close contact with blood capillaries (Figs. 1a, 2a).

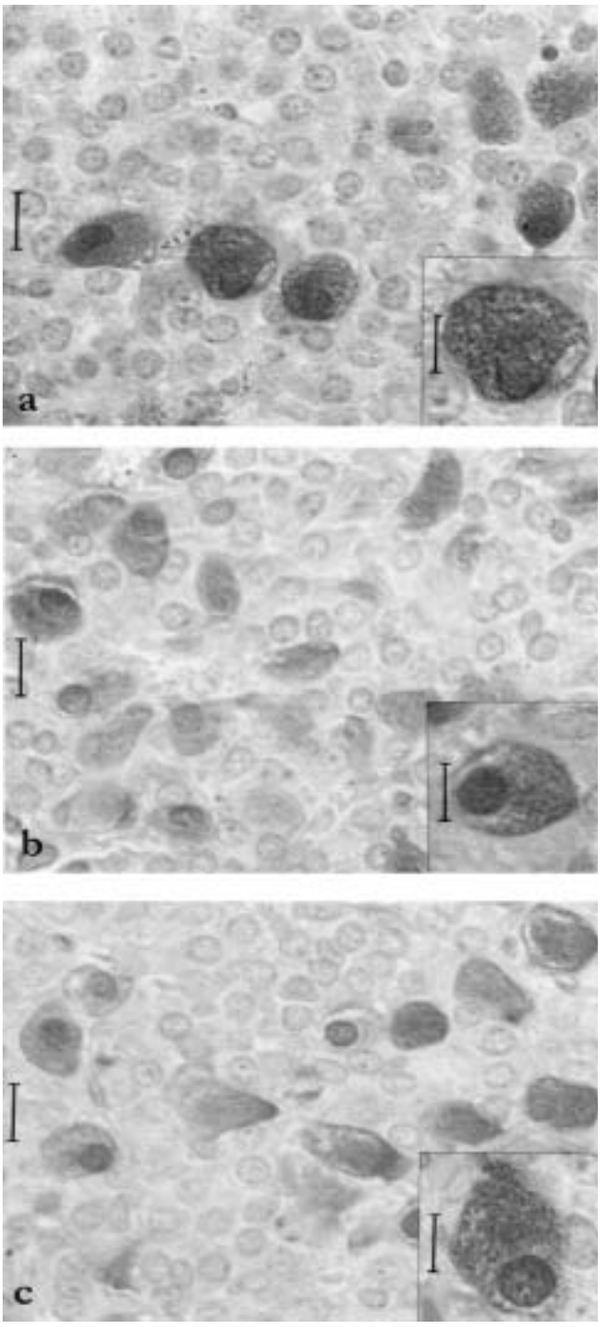

Fig. 2. Pars distalis of the adenohypophysis of control and Dx-treated female rats; **(a) i**mmunoreactive βFSH cells in a control rat (bar=13 μm); (**b)** immunoreactive βFSH cells 24 h after Dx treatment (bar=13 μm); (**c)** immunoreactive βFSH cells 72 h after Dx treatment (bar=13 μm); insets (bar=8 μm).

After Dx treatment, the LH cells were more intensely immunostained than in the controls, smaller in size but more numerous and with granulated cytoplasm (Figs. 1b,c). FSH cells after Dx treatment were weakly immunocytochemically stained, numerous and smaller than the FSH cells in control animals (Figs. 2b,c).

Morphometric analysis

LH cells. Stereological analysis showed that multiple Dx treatment led to significant changes in LH cells 24 h and 72 h after the last Dx administration. The volume of immunopositive LH cells was reduced at each timepoint by 31% ($p<0.05$) and 19% ($p<0.05$) respectively, compared with the control values (Fig. 3a). The nuclear volume of LH-positive cells was decreased by 57% ($p<0.05$) at 24 h, and by 52% ($p<0.05$) at 72 h in comparison with control females (Fig. 3b). The percentage of LH cells per unit volume of total pituitary gland tissue, *i.e.* the volume density was increased by 20% ($p<0.05$) in experimental group Dx 24, but insignificantly changed in Dx 72 group, (Fig. 3c). In the pituitaries of Dx-treated rats, the number of LH immunoreactive cells, per unit area (mm^2) was increased by 21% ($p<0.05$) at 24 h and by 8% ($p<0.05$) at 72 h, as compared with the controls (Table 1).

When the values for the two experimental groups were compared, the following results were obtained. The mean volume of LH-positive cells in group Dx 72 was by 17% larger ($p<0.05$), than that for group Dx 24 (Fig. 3a). The mean volume of the nuclei in LH-positive cells in group Dx 72 was also significantly increased (by 13%; $p<0.05$), in comparison with Dx 24 (Fig. 3b). The volume density and number per unit area of LH-positive cells were reduced by 18% (Fig. 3c) and 11% (Table 1), respectively, and the differences between the groups were again statistically significant ($p<0.05$). The LH cells 72 h after Dx treatment showed weaker immunostaining than LH cells 24 h after Dx treatment (Figs. 1b,c).

FSH cells. Multiple Dx treatment led to similar changes in the stereological and morphometric paramethers of FSH cells 24 h and 72 h after the last dose of Dx, as for LH cells. Thus, the volume of immunopositive FSH cells was decreased by 12% ($p<0.05$) at 24 h and by 9% ($p<0.05$) at 72 h (Fig. 3a), and the volume of their nuclei was also reduced at each timepoint by 26% ($p<0.05$) and by 19% ($p<0.05$), respectively, in comparison with the controls (Fig. 3b). The volume density of FSH-positive cells 24 h and 72 h after Dx treatment was increased by 12% ($p<0.05$) and by 9% ($p<0.05$), respectively (Fig. 3c). The number of FSH cells per unit area (mm^2) was increased by 39% ($p<0.05$) at 24 h and by 41% ($p<0.05$) at 72 h in comparison with the control rats (Table 1).

When the values for the Dx 24 and Dx 72 were compared, the following results were obtained. The mean volume of FSH-positive cells in group Dx 72 was insignificantly increased (by 4%), in comparison with Dx 24 (Fig. 3a). The mean volume of the nuclei in FSH-positive cells in group Dx 72 was significantly

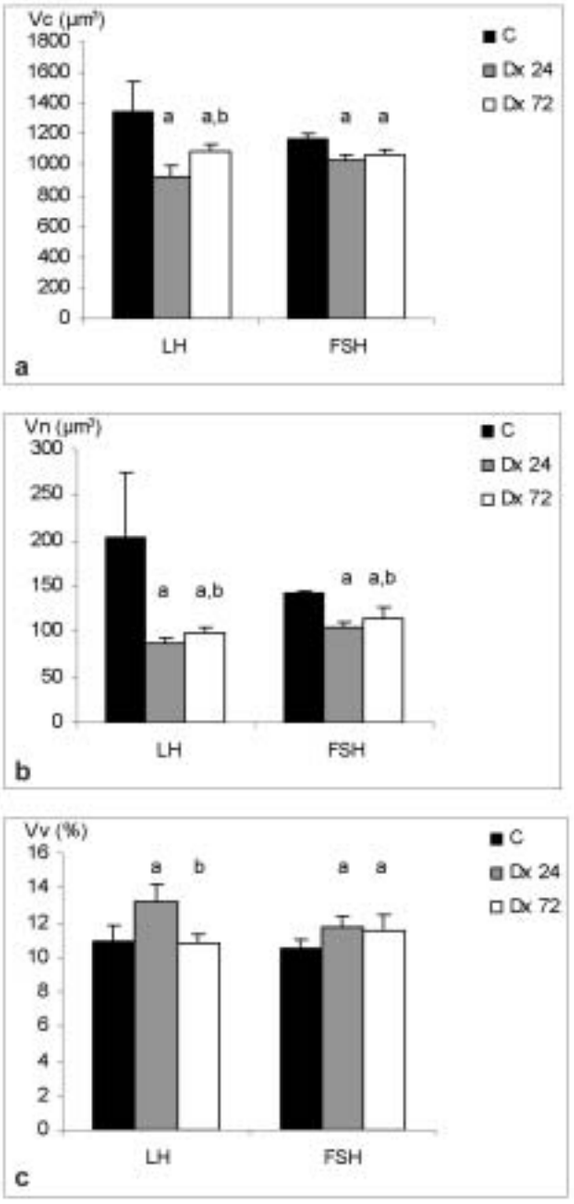

Fig. 3. (a) The cell volume (Vc) of LH- and FSH-immunopositive cells of control (C) and Dx-treated female rats 24 h (Dx 24) and 72 h (Dx 72) after the last Dx dose. **(b)** The nuclear volume (Vn) of LH- and FSH-immunopositive cells of control and Dx- treated female rats 24 h and 72 h after the last Dx dose. **(c)** Volume density (Vv) of immunoreactive LH and FSH cells expressed as a percentage of total adenohypophyseal cells volume in control and Dx-treated rats 24 h and 72 h after the last Dx dose. All values are means ±SD, n=8. [a]p<0.05 (C vs. Dx 24; C vs. Dx 72), [b]p<0.05 (Dx 24 vs. Dx 72).

increased (by 10%; p<0.05), (Fig. 3b), but the volume density and number per unit area of FSH-positive cells were not significantly changed (Fig. 3c, Table 1) in comparison with Dx 24. The FSH cells 72 h after Dx treatment were slightly more intensely immunostained than FSH cells 24 h after Dx treatment (Figs. 2b, c).

Table 1. The number (No) of immunoreactive LH and FSH cells per unit area (mm^2) in control and Dx-treated female rats 24 h and 72 h after the last Dx injection.

LH cells	C	Dx 24	Dx 72
No/mm^2	290±9	352±24	313±14
		(+21%)[a]	(+8%)[a], (-11%)[b]
FSH cells	C	Dx 24	Dx 72
No/mm^2	168±6	234±6	238±9
		(+39%)[a]	(+41%)[a]

Results are means ±SD (n=8). [a]p<0.05 (C vs. Dx 24; C vs. Dx 72), [b]p<0.05 (Dx 24 vs. Dx 72).

Discussion

Our results have shown that Dx treatment of female rats caused significant reductions of both cell and nuclear volume of LH and FSH cells, together with an increase in their volume density and number per unit area, 24 h and 72 h after the last Dx injection.

Dexamethasone is often used in medical practice to treat a wide variety of pathological states [12], as well as in studies of stress-induced effects of glucocorticoids. Its action is mediated through the activation of intracellular glucocorticoid receptors, which are expressed in almost all cell types [1].

It is possible that glucocorticoids have more than one site of action on LH cells, but the most important is the control of LH function at the level of the hypothalamus and pituitary gland [6].

Some of the effects of glucocorticoids on LH cells may be due to inhibition of gonadotrophin-releasing hormone (GnRH) secretion, the major controller of LH and FSH cell function [8]. The presence of glucocorticoid receptors (GR) in GnRH neurons [2] indicates that glucocorticoids act at the level of the hypothalamus. Glucocorticoids directly inhibit the expression of GnRH genes and GnRH secretion [5] associated with a decline in portal GnRH concentrations [19]. The effects of glucocorticoids on GnRH neurons are due at least partially to a decline in LH synthesis [10], with a consequent decrease in LH cell and nuclear volume, as seen in our experimental groups Dx 24 and Dx 72. It is also interesting that treatment of female rats with glucocorticoids in the absence of a GnRH signal, had no influence on α and β LH mRNA levels and LH synthesis [13]. It is possible that GnRH neurons are the key site of glucocorticoid action on the synthetic function of LH cells. However, the ability of glucocorticoids to inhibit LH secretion *in vitro* [3] suggests that they can directly affect LH cells through the glucocorticoid receptor, at the pituitary level [11]. Ringstrom *et al.* [17] observed that the main influence of glucocorticoids on LH cells seems to be blocking the release of LH, as manifested in our results

by intense immunocytochemical staining of LH cells after Dx treatment. The finding that glucocorticoids inhibit calcium ionophore-stimulated release of LH indicated that the release mechanism is disrupted [10]. Tohei and Kogo [26] also suggest that exposure to glucocorticoids generally suppresses serum LH levels, without affecting pituitary content of LH. It is obvious that the decrease in LH cell volume found in our study was accompanied by an increase in the number of LH cells. Some of the LH cells containing trace amounts of hormones could not be visualized by immunocytochemical staining in control females, but after Dx treatment they became visible and available for stereological and morphometric analyses. This could also contribute to the increase in the number of LH cells observed in experimental animals. Such compensatory effect contributes to the maintenance of LH content in the pituitary gland after exposure to glucocorticoids.

Glucocorticoids may reduce the pituitary response to GnRH [25] by acting directly or indirectly on gonadotrophic cells to inhibit their responsiveness to GnRH. Consistent with a direct action, the glucocorticoid receptor has been identified within gonadotrophs [11]. This receptor, however, has also been localized in folliculostellate cells within the pituitary [16]. Folliculostellate cells respond to glucocorticoids by synthesizing annexin-1, an inhibitory paracrine factor, with a receptor in gonadotrophic cells [22]. It is possible, therefore, that the inhibitory effects of glucocorticoids on the responsiveness to GnRH, as well as on LH release could be mediated indirectly via annexin-1 from folliculostellate cells [4, 9].

Glucocorticoid receptors are also present in the ovaries [21]. However, few data have shown the effects of dexamethasone on ovarian function *in vivo*. Tohei and Kogo [26] suggest that dexamethasone inhibits ovarian function and synthesis of inhibin and estradiol. Dexamethasone also reduces the ability of estrogen to stimulate LH secretion [10]. Thus, it is possible that under these circumstances, ovarian feedback effects have no important influence on LH secretion, but they are very important in the regulation of FSH secretion.

Decreases in FSH cell and nuclear volume, as well as increases in volume density and number per unit area were found in this study 24 h and 72 h after the last Dx dose. Decrease in cell volume and weak immunopositivity of FSH cells after Dx treatment could be a consequence of stimulated FSH release. It is interesting that glucocorticoids can stimulate FSH release by direct action on FSH cells [17] and by inhibition of the ovarian function [20] and inhibin secretion from ovarian granulosa cells [26]. Increase in the volume density and number of FSH-positive cells in the same experimental conditions are in accordance with the increase in tissue level of FSH reported by McAndrews *et al.* [13]. Ringstrom *et al.* [17] observed that exposure to glucocorticoids *in vivo* significantly elevated the pituitary content of FSH, mainly by increasing its synthesis or blocking intracellular degradation by direct action on the gonadotrophs and these effects were independent of endogenous GnRH.

Glucocorticoids can stimulate FSH synthesis by influencing FSH genes [18]. Thus, treatment with glucocorticoids increases pituitary FSH mRNA and pituitary content of FSH [13], suggesting that glucocorticoids affect FSH at the pituitary level directly rather than via the hypothalamus.

It is obvious that multiple dexamethasone treatment changed the dynamics of pituitary gonadotroph populations and caused an increase in volume density and number per unit area of FSH cells. It is well known that dexamethasone inhibits mitotic activity of pituitary cells [14], therefore the increase in FSH cell number could be a result of differentiation from undifferentiated pituitary cells or stem cells which are the major source of a newly formed pituitary cells under various physiological conditions [24]. Also, some of the FSH cells, as well as LH cells, became visible and available for stereological and morphometric analyses after Dx treatment, and that could contribute to the increase in number of these cells. Increases in volume density and number of FSH cells after Dx administration allow storage of more FSH in the pituitary gland.

Thus, our results showed that between 24 h and 72 h after the last dose of Dx there was an increase in LH cell and nuclear volume, together with a decrease in their volume density and number per unit area, *i.e.* the values for Dx 72 group approached those found in the control group. The intensity of immunostaining of LH cells in Dx 72 group was lower than in Dx 24 group. Changes in these parmeters indicate a gradual normalization of LH cell function with time after the treatment. Stereological and morphometric parameters, as well as intensity of immunostaining of FSH cells in Dx 72 group were only slightly changed in comparison with Dx 24 group. This indicates that normalization of FSH cell function after Dx treatment needs more time than normalization of LH cell function in the same experimental conditions.

Besides confirming that elevated levels of glucocorticoids cause significant changes in the stereologic and morphometric paramethers of LH and FSH cells, we have also shown that the changes after Dx treatment are reversible, as the effects decrease with time after treatment allowing for normalization of the gonadotroph function. These results are important in view of widespread dexamethasone use in clinical practice.

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