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Sexual dimorphism in the effect of concomitant progesterone administration on changes caused by long-term estrogen treatment in pituitary hormone immunoreactivities of rats

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Since in clinical practice long-term estrogen (E) treatment is frequently applied, our aim was to study the effect of concomitant progesterone (P) administration on changes caused by long-term estrogen treatment in the secretion of LH, FSH, PRL and GH.

Material/Methods: Diethylstilbestrol (DES), P or both in silastic capsules were implanted under the skin of prepubertal Sprague-Dawley male and female rats. Animals survived for two or five months. We have also studied whether the changed hormone secretion caused by DES can return to normal level 1 or 2 months after removing DES capsule.

Results: 1.) The males more rapidly responded than females with decreasing basal LH release upon treatments. The basal FSH release was decreased only in males. The effect of DES persisted in males; however, in females basal LH and FSH levels were upregulated after removal of DES capsule. 2.) The basal GH levels were low in each group. The body weight and length were depressed by DES in both genders and P little blunted this effect. The body weight and length in males remained low after removal of DES capsule, in females it was nearly similar to intact rats. 3.) There was no sexual dimorphism in the effect of steroids on PRL secretion. In both genders DES extremly enhanced the PRL levels, P prevented the effect of DES. PRL levels returned to intact value after removal of DES influence. 4.) Removal of DES capsule reversed the changes in the immunohistochemical appearance of hormone immunoreactivities.

Conclusions: There was sexual dimorphism in the change of basal gonadotropic hormone and GH secretion but not of PRL upon DES and DES+P treatments. P was basically protective and this role may be mediated by P receptors locally in the pituitary gland.

key words: **anterior pituitary • immunohistochemistry • RIA • sexual dimorphism**

Abbreviations: **ACTH** – adrenocorticotropic hormone; **DES** – diethylstilbestrol; **E** – estrogen; **EB** – estradiol benzoate; **ecap** – empty capsule; **ER** – estrogen receptor; **E2** – estradiol; β FSH folliculus stimulating hormone; **GH** – growth hormone; **GHRH** – growth hormone releasing hormone; **GnRH** – gonadotrop hormone releasing hormone; **ir** – immunoreactive; **IR** – immunoreactivity; **LH** – luteinizing hormone; **NET** – norethisterone enanthate (synthetic progesterone); **P** – progesterone; **PR** – progesterone receptor; **PRL** – prolactin; **TSH** – thyrotropic hormone; **VIP** – vasoactive intestinal polypeptide

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Background

It is well known that sexual steroids, estrogen (E) and progesterone (P), among other factors regulate the secretion of pituitary tropic hormones directly acting on pituitary cells and through the central nervous system by a feed-back mechanism. It was demonstrated two decades ago that diethylstilbestrol (DES) induced appearance of prolactin (PRL)-secreting tumors [1].

Estrogen and progesterone receptors (ERs and PRs) were identified in various pituitary hormone secreting cells and in various hypothalamic and extrahypothalamic structures. Both ER and PR have two isoforms. ERα was identified by Koike and his co-workers [2], ERβ was cloned by Kuiper and his co-workers [3]. In a recent publication it was found that ERα is localized in the largest number in lactotropes, and in decreasing number in somatotropes, thyrotropes, gonadotropes. The number of ERβ was much lower and it was identified in somatotropes, lactotropes and gonadotropes. Immunoreactivity of both receptors was present in the nucleus, rough endoplasmic reticulum, secretory granules and in the free cytosol. The intracellular localization was dependent on the stage of estrous cycle [4]. In another study it was shown that ERβ was expressed by folliculus stimulating hormone (FSH) cells [5]. A and B isoforms of PR were demonstrated in the anterior lobe using RT-PCR technique [6] and immunohistochemistry [7]. The predominant form is the A-isoform (PRA). In rat PR immunostaining was seen in the nuclei of gonadotropes. Both ERs and PRs were also shown in the central nervous system including hypothalamic structures [8–11].

Besides classical hormones, the anterior pituitary also produces many other substances such as neurotransmitters, neuropeptides, and growth factors [12]. The classical pituitary hormones act far from the site of production and reach their target tissues through the general circulation. The small peptide molecules act in auto- and paracrine manners [13,14].

The effect of E and P on rat pituitary tropic hormon levels was investigated in *in vivo* and *in vitro* models [7,15–18]. In our previous experiment [19] long-term E treatment (implantation of DES capsule under the skin of neck) depressed the number of luteinizing hormone (LH) and thyroid stimulating hormone (TSH) cells, it enhanced the number of cells (the cells formed prolactinoma), it enhanced the size of growth hormone (GH) cells and it did not influence the number of adrenocorticotrop (ACTH) cells. DES extremly enhanced the number of vasoactive intestinal polypeptide (VIP) cells and induced appearance of VIP-oma. It was also demonstrated that E enhanced the VIP expression in the anterior pituitary by six times as compared with intact rats [20].

With the use of haemolytic plaque assay it was demonstrated that a chronic E treatment increased the percentage of PRL secreting cells and the amount of secreted PRL; however, there was a decrease in both the percentage of GH secreting cells and the amount of secreted GH. Following the haemolytic plaque assay *in situ* hybridization revealed that estradiol β (E2) increased the PRL mRNA while it decreased the GH mRNA [16]. This research group did not find any colocalization between GH and PRL expression in control male rat pituitary cell culture; however, in E2 treated culture about 10% of PRL secreting cells contained GH mRNA as well. It was also demonstrated that the effect of E in the pituitary is mediated through ERα [21].

The data in the literature concerning the effect of P on pituitary hormone secretion is controversial and not so abundant. The protective effect of P on E inducing undesired changes was observed about twenty-five years ago. It was demonstrated that P was able to reduce the size of E induced mammary tumor [22]. The protective role of P is also used to prevent preterm birth when being applied as vaginal suppository [23]. Genazzani and his co-workers [24] analysed how P and various synthetic progestins modulate the effect of E on hypothalamic gonadotropin-releasing hormone (GnRH) and on pituitary LH and PRL concentrations in ovariectomized rats. Two-week steroid treatments were applied. It was found that P and norethisterone enanthate (NET, synthetic progestin) reversed the depression of hypothalamic GnRH induced by the estradiol benzoate (EB) and elevated its level. Both P and NET blocked the EB induced increase of pituitary LH, and the plasma LH levels remained low. Progestins alone did not influence the PRL levels but reversed the EB induced increase.

In clinical practice sexual steroid treatment is a very common medicament. Steroid hormone contraception was suggested about seventy years ago [25]. It is well known that administration of E, P or the combination of E and P prevents the LH surge and the ovulation [26–29]. Sexual steroids are also applied to synchronize the ovarian cyclicity, to maintain the endometrium during pregnancy [30,31]. E is even recently used as replacement therapy after surgical removal of ovaries or in menopause as well [32–34]. In men E is used to treat prostatic cancer [35].

The aim of our present work was to further clarify the way how the concomitant P administration affected the changes in the anterior pituitary caused by long-term estrogen treatment. Long-term steroid treatments in experimental animals, similar to those applied in the human practice was rarely found in the literature. We decided to imitate the long-term clinical treatments. Two- and five-month survivals after implantation of hormones were chosen, both are long time in the life of rats. The animals were three and six-month-old when they were sacrificed. Three-month-old rat is a relatively young adult and the six-month-old rats are old. The continuous hormone influence was provided by the implantation of DES and P containing capsules. At the end of experimental periods we have examined LH, FSH, PRL and GH immunoreactivities in the anterior pituitary gland and basal LH, FSH, PRL and GH plasma levels in rats of both sexes. We have also examined whether removal of DES capsule two months after implantation can reverse the DES induced changes one or two months following the removal.

Material and Methods

Animals

Sprague-Dawley female and male rats were used for our experiments. They were kept in a light (light on at 5 h and off at 19 h) and temperature (22±2°C) controlled vivarium and fed with standard lab chow and water ad libitum. The treatment of the animals was in accordance with the rules of the „European convention for the protection of vertebrate animals used for experimental and other scientific

purposes", Strasbourg, 1986. Our protocol was approved by the Department of Animal Health Care, Budapest. Permission number: 37/1999. When the animals were 25 day-old they received empty capsule (ecap), DES, DES + P or just P containing silastic capsule implanted under the skin of the neck in the subcutaneous tissue.

Preparation of the hormone containing capsules

Silastic capsules (id 1.55 mm, od 3.13 mm, length 10 mm) (Dow Corning Corporation, Midland, MI) were filled with DES (Sigma, St. Louis, Mo) or P (Sigma) and the ends of the capsules were sealed by Szilorfix (Finomvegyszer Szövetkezet, Budapest, Hungary), a silicon based glue. A day after drying, the capsules were used for implantation. The steroids can pass through the wall of the capsule and can exert its effect through the general circulation [36].

Experimental protocol

The animals were grouped as follows.

Experiment 1.
1. intact (negative control) rats,
2. ecap (sham operated rats receiving ecap sealed with glue),
3. DES implanted rats,
4. DES+P implanted rats,
5. P implanted rats.

85 female and 79 male rats were included in this experiment. The survival time after implantation was 2 months.

Experiment 2.
Similar groups were included in this experiment (56 females and 56 males) as described in Experiment 1; however, the survival time was 5 months.

Experiment 3.
From 35 DES implanted animals (18 females and 17 males) the DES capsules were removed. Half of these animals was sacrificed 1 month after the removal and the other half was sacrificed 2 months after removal. Age matched controls were also included in all the groups (17 females and 16 males). The data obtained in these groups were compared with those of DES implanted rats and their age matched controls of Experiment 1.

In female rats the opening of the vaginal membrane was recorded. Then vaginal smear was taken daily for two weeks and for another two weeks before sacrificing. Females were handled in this way. The male rats were transferred from their cage to another and back every day for two weeks before sacrificing (handling) to blunt the stress effect.

At the end of the experimental period the body weight (BW) and body and tail length (B+TL) were measured and the animals were sacrificed by decapitation between 9 am and 11 am. The trunk blood was collected, its coagulation was prevented by EDTA (ethylene diamine tetraacetic acide sodium salt, Serva, Heidelberg, Germany). Plasma was used to determine hormone levels. Endocrine organs were weighted. Anterior pituitaries were immersed in Bouin solution (75% saturated picric acid, 25% formaldehyde, 5% acetic acid [Reanal, Budapest, Hungary]). Two days later tissue samples were washed in 0.1 M potassium-phosphate buffer (KPB) and put in ascending sucrose solution (10–20–30%) for cryoprotection. One day later the pituitaries were embedded in cryomatrix (Shandon, Pittsburg, PA) and 20 µm thick sections were cut on cryostat (Cryotome, Thermo Shandon, Pittsburg, PA) in 4 parallel series. The sections were stained for LH, FSH, GH and PRL immunoreactivities.

Radioimmunoassay (RIA)

The trunk blood was centrifugated at 4°C. Plasma was stored at –20°C until determination of pituitary hormones. Iodination was carried out by Chloramine-T method. Separation of the free and bound antigen was made by double antibody method. All hormone kits were obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow. Hormone levels were expressed in ng/ml plasma. The mean of two parallel determinations for each animal was subjected to one way analysis of variance (ANOVA) followed by a Student's *t* test.

Immunohistochemistry

Three animals of each group were anesthetized by ketamine-hydrochloride (75 mg/100g bw) (Sigma, St. Louis, MO) and perfused with 4% paraformaldehyde (Merck, Darmstadt, Germany) in potassium phosphate buffer (KPB) (pH 7.4, 0.1mol). The components were purchased from Sigma (St. Louis, MO). Pituitaries were removed and postfixed overnight. All the pituitaries (fixed in Bouin solution or PFA) were used for immunohistochemistry. After washing in KPB, the pituitaries were immersed in ascending sucrose solution (10–20–30%), then frozen on dry ice. The pituitary sections were rinsed in potassium phosphate buffer (KPB), pH 7.4. The tropic hormones were visualized by indirect fluorescence technique. The following primary antisera were used in our experiment: LH, FSH, GH (dilution 1:500) were raised in guinea pig and obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow. PRL antiserum (dilution 1:500) was raised in rabbit by Nagy and characterized by Demaria et al. [37]. Fluorescence labeled secondary antibodies were the following: rabbit anti-guinea pig IgG conjugated to FITC (DAKO A/S, Glostrup, Denmark), goat anti-rabbit IgG conjugated to Cy3 (DAKO A/S, Glostrup, Denmark).

Selected sections were double stained for PRL and GH immunoreactivities using fluorescence technique. First PRL staining was carried out using a monoclonal antibody (raised and characterized by Scammell [38] and TRITC conjugated secondary antimouse antibody (DAKO A/S, Glostrup, Denmark). Then the GH staining was done.

Results

Vaginal smears

In intact rats the vaginal membrane opened on 31.36±0.80 day of postnatal life. In all animals implanted with capsule (independently of their hormone content) the vaginal membrane opened earlier. In DES treated rats it happened much earlier than in intact animals (27.50±0.31, p<0.0001), although in animals implanted with ecap, DES+P or P alone the opening was also significantly shifted earlier than in intact rats, 28.36±0.47 (p<0.003) 28.06±0.34 (p<0.0005) and 29.09±0.51

Table 1. Body weight (BW) and body and tail length (B+TL) of female and male rats.

Group of animals	Female		Group of animals	Male	
	BW	B+TL		BW	B+TL
2 month survival					
intact (19)	244.1±4.19	38.50±0.25	intact (22)	394.8±12.42	44.11±0.28
ecap (12)	254.5±13.93	39.04±0.39	ecap (11)	418.8±10.14	44.30±0.51
DES (19)	144.9±4.25***	33.36±0.46***	DES (19)	174.2±5.59***	33.82±0.34***
DES+P (20)	158.8±5.25***	32.54±0.43***	DES+P (13)	179.8± 6.27***	33.98±0.33***
P (15)	250.7±6.71	37.89±0.50	P (14)	412.0±9.23	43.92±0.40
5 month survival					
intact (10)	311.3±7.93	41.68±0.58	intact (9)	540.7±15.67	47.45±0.46
ecap (12)	291.9±4.86	41.33±0.96	ecap (8)	497.3±11.20	46.28±1.20
DES (11)	143.7±4.37***	33.09±0.67***	DES (14)	178.6±3.17***	32.27±2.58***
DES+P (11)	239.4±21.06**	36.80±1.55**	DES+P (12)	352.9±52.60**	41.06±1.75**
P (12)	285.5±4.36**	40.50±0.51	P (13)	453.3±30.81*	47.01±0.34
DES removal					
intact (19)	244.1±4.19	38.50±0.25	intact (22)	394.9±12.42	44.11±0.28
DES (19)	144.9±4.25***	33.36±0.46***	DES (19)	174.2±5.59***	33.82±0.34***
intact (8)	225.9±7.46	39.03±0.29	intact (9)	422.6±19.43	46.33±0.66
DES 1 m (9)	200.1±14.41	36.91±0.29***	DES 1 m (8)	275.5±14.85***	40.88±1.14***
intact (9)	273.8±6.94	40.17±0.38	intact (7)	511.6±18.80	46.87±0.49
DES 2 m (9)	247.7±6.10*	38.52±0.82	DES 2 m (9)	391.6±14.89***	43.32±0.58***

* $p<0.01$; ** $p<0.001$; *** $p<0.0001$; 1 m – one month survival after removal of DES capsule; 2 m – two month survival after removal of DES capsule; the numbers in brackets indicate the number of animals in each experimental group.

($p<0.03$) day of postnatal life, respectively. Upon DES treatment in the vaginal smear persistent estrus was observed. DES+P did not interrupt the cyclicity but it was irregular and metestrus predominated, ecap and P alone had no effect.

Effect of treatments on the body weight and body length

Table 1 shows BW and B+TL in various groups of female and male rats 2 and 5 months after implantation. In DES and DES+P implanted animals BW and B+TL were significantly lower than in control rats 2 months after implantation; however, 5 months after implantation P moderated the effect of DES. P alone slightly decreased BW only in the case of 5-month treatment. When we removed the DES capsule BW and B+TL could not return to intact levels, but the difference was not significant in B+TL of females.

Effect of treatments on the weight of the endocrine organs

Table 2 shows the weight of the anterior pituitary and the ovaries in female rats. The weight of anterior pituitaries increased upon DES and DES+P treatments in the case of 2-month survival, but in the case of 5-month treatment P reversed the weight gain. When the DES capsule was removed the weight of pituitaries returned to control level. DES and DES+P extremly decreased the weight of ovaries after 2-month steroid treatment. In the case of 5-month survival P prevented the effect of DES and P alone slightly decreased the weight of ovaries. When DES capsule was removed the weight of ovaries returned to control levels.

Table 3 shows the weight of the anterior pituitary, testes and seminal vesicles of male rats. Similarly to female rats the weight of anterior pituitaries increased upon DES and DES+P treatment in the case of 2-month survival time, but in the case of 5-month survival P reversed the weight gain. DES and DES+P treatments extremly decreased the weight of testes and seminal vesicles, P alone enhanced those in the animals having 2-, but not 5-month survival time. Removal of DES capsule partially, but not completely, restored the weight of testes and seminal vesicles.

Effect of treatments on the classical pituitary hormone immunoreactivities

Radioimmunoassay

The basal LH level in female rats was depressed by DES treatment (Figure 1A,B), but it become significant only five months after implantation. Progesterone alone had no

Table 2. Weight of anterior pituitary and ovaries of female rats.

Group of animals	Anterior pituitary (mg)	Ovaries (mg)
	2 month survival	
intact (19)	10.8±0.24	100.1±5.19
ecap (12)	11.0±0.46	95.2±3.37
DES (19)	16.9±1.56***	56.2±7.61***
DES+P (20)	14.8±1.10**	44.3±6.02***
P (15)	10.0±0.41	94.8±2.36
	5 month survival	
intact (10)	13.3±0.49	132.2±13.11
ecap (12)	12.3±0.41	123.6±8.45
DES (11)	27.9±4.73**	33.0±6.92***
DES+P (11)	12.6±0.84	115.0±13.86
P (12)	12.3±0.68	99.5±4.00*
	DES removal	
intact (19)	10.8±0.24	100.1±5.19
DES (19)	16.9±1.56***	56.2±7.61***
intact (8)	11.4±0.46	100.7±7.54
DES 1 m (9)	12.6±0.75	102.1±6.29
intact (9)	13.8±0.80	96.4±3.45
DES 2 m (9)	12.2±0.54	94.7±4.66

* $p<0.01$; ** $p<0.001$; *** $p<0.0001$; 1 m – one month survival after removal of DES capsule, 2 m – two month after removal of DES capsule, the numbers in brackets indicate the number of animals in each experimental group.

significant effect. One month after removal of DES capsule LH level further decreased and it was significantly lower than in controls; however, two months after removal it was even higher than in age-matched controls (Figure 1C). In male rats DES and DES+P significantly decreased the LH levels already two months after implantation (Figure 1D). The lower levels persisted five months after implantation in DES+P treated rats (Figure 1E). After the removal of DES capsule the LH level remained lower than in controls for a month, and later it was similar to the control values (Figure 1F).

The basal FSH level in female rats was not significantly influenced in groups of 2- or 5-month survival (Figure 2A,B); but 2 months after the removal of DES capsule FSH was significantly higher than in the age-matched control rats (Figure 2C). In male rats both DES and DES+P treatment depressed the FSH levels two months after implantation (Figure 2D) but five months after the implantation the FSH levels were similar in each group (Figure 2E), likewise the females. After the removal of DES capsule the FSH levels remained lower and did not return to control values (Figure 2F).

The basal PRL levels showed very similar pattern in both female and male rats. DES extremly enhanced the PRL levels (Figure 3A,B and D,E). Two months after implantation P blunted the effect of DES (Figure 3A, D) and five months after implantation P completely reversed the effect of DES (Figure 3B, E). P alone had no effect on the PRL levels (Figure 3A,B and D,E). Removal of DES capsule gradually restored the PRL levels (Figure 3C,F).

The basal GH levels were very contradictory (Figure 4A–F). In females DES and DES+P treatment decreased the level (Figure 4A,B); however, it was significant only after 2-month treatment (Figure 4A). P alone did not influence the GH level 2 months after implantation (Figure 4A) but it decreased after 5 months (Figure 4B). When the DES capsule was removed GH levels reached the control value in one month and by the end of 2-month survival after removal and it was even higher than in controls (this elevation was not statistically significant) (Figure 4C). In male rats we did not find significant difference in any groups (Figure 4D,F).

Immunohistochemistry

The quantitative measurements obtained with RIA were confirmed with the qualitative observations provided by immunohistochemistry.

Distribution of immunoreactive cells in intact rats

In the anterior lobe of intact rats the distribution of tropic hormone immunoreactive cells is very characteristic (Figure 5A–H). The density of LH and FSH immunoreactive cells is much higher in the gonadotropic zone that is in the anterior pole of the anterior pituitary (Figure 5A–D). PRL is evenly distributed all over the anterior lobe (Figure 5E,F). GH immunoreactive cells are almost absent in the anterior pole of intact rats. Everywhere else they are evenly distributed (Figure 5G,H).

Distribution of immunoreactive cells in steroid treated rats

Steroid treatments differently influenced the immunoreactivity of the four hormone producing cells.

The number of LH and FSH cells decreased in both female and male rats. The effect of DES was more pronounced five than two months after DES implantation. Figure 5I and J show LH immunoreactive cells in the anterior pituitary of DES treated female rats after 2- and 5-month treatments, respectively. When P was implanted together with DES, the effect of DES was nearly prevented by P (Figure 5K,L). It seemed that only the density of gonadotropes in the rostral pole of the anterior lobe was lower than in intact rats. The appearance of LH and FSH immunoreactivities in male rats was very similar to that of females (not shown). P alone did not influence the LH and FSH immunoreactivities in the anterior lobe of both sexes (not shown).

DES enhanced the number and the size of PRL cells in both female and male rats (Figure 5M–O and Q, male). In intact rats these cells are cup-shaped (Figure 5N). In DES treated rats the cells are large, ovoid or rounded, and their diameter is nearly double than that of intact rats already after two-month steroid influence (Figure 5O). Figure 5P shows PRL cells from a male animal with DES+P treatment (2-month survival). In these animals

Table 3. Weight of anterior pituitary, testes and seminal vesicles of male rats.

Group of animals	Anterior pituitary (mg)	Testes (mg)	Seminal vesicle (mg)
2 month survival			
intact (22)	9.5±0.47	2651±275.9	1008±84.57
ecap (11)	10.0±0.49	3247±112.8	1367±95.55*
DES (19)	19.7±1.36***	284.6±23.14***	190.5±8.96***
DES+P (13)	16.3±0.72***	352.0±26.33***	171.8±9.82***
P (14)	10.0±0.35	3244±167.3*	1362±66.80**
5 month survival			
intact (9)	10.1±0.99	3357±112.9	1869±261.7
ecap (8)	11.1±0.58	3249±151.8	1864±123.1
DES (14)	33.2±5.33**	145.3±26.30***	243.6±27.97***
DES+P (12)	14.0±1.62	2125±536.5*	1305±266.7
P (13)	10.9±0.81	3603±82.70	1740±120.6
DES removal			
intact (22)	9.5±0.47	2651±275.9	1008±84.57
DES (19)	19.7±1.36***	284.6±23.14***	190.5±8.96***
intact (9)	11.5±0.43	3600±178.7	1356±165.1
DES 1 m (8)	10.6±0.42	2411±198.2***	766.7±92.80**
intact (7)	11.7±0.61	4014±128.0	1886±295.5
DES 2 m (9)	12.6±0.68	3370±222.6*	1490±237.3

* $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$; 1 m – one month survival after removal of DES capsule, 2 m – two month survival after removal of DES capsule, the numbers in brackets indicate the number of animals in each experimental group.

the size of the cells were only moderately enhanced compared to control rats and they lost their cup-shaped appearance. When the animals were treated with DES for 5 months prolactinomas developed. Figure 5Q shows a well developed prolactinoma. P prevented the effect of DES on PRL. The immunostaining was similar to control rats (Figure 5R). P alone did not affect the PRL immunostaining (not shown).

GH cells did not show a pronounced change either in male or female rats upon 2-month DES treatment but the distribution of these cells was very characteristic in animals with 5-month DES treatment. GH cells were completely missing in areas resembling prolactinomas (Figure 5S). Double labeling immunohistochemistry confirmed this observation (Figure 5T). In DES+P treated rats GH immunostaining was very similar to intact rats. The only difference was observed in the distribution of GH cells. They could be also observed in the anterior pole of the anterior pituitary gland (Figure 5U), this is the region where they are missing in intact rats.

Implantation of ecap did not alter the immunostaining of pituitary tropic hormone producing cells.

Effect of the removal of DES capsule on the distribution of immunoreactive cells

The removal of DES capsule from the animals restored the density and distribution of all the four hormon producing cells already in one month. One or 2 months after the removal of DES capsule the appearance of immunostaining was similar to those of intact rats (Figure 5V–X).

Discussion

In the present experiment we tried to imitate long-term steroid treatments which are frequently used in clinical practice. It is well known that continuous E or combined E + P administration prevents the ovulation. The modern contraceptive medication is based on these observations [26,39–41]. Nowdays high percent of girls around puberty uses contraceptive hormones [42], in some cases the steroids are used as medication of acne vulgaris in this early age [43]. In men E preparations (DES) are usually used in adult age to treat prostate cancer [35,44]. E is also used to ameliorate the postmenopausal syndromes and osteoporosis [34].

Our experiments provide new informations concerning the effect of long-term E and concomitant P treatments on the

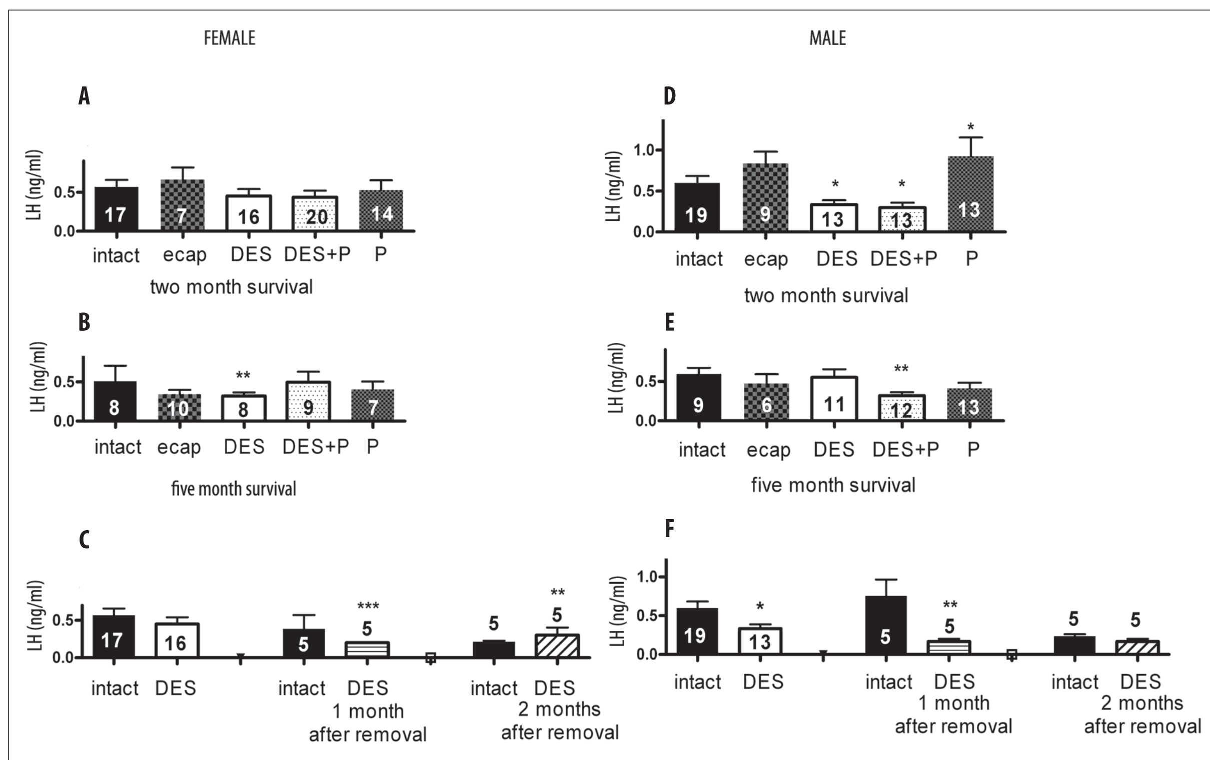

Figure 1. LH plasma levels in various experimental groups. (**A**) shows LH levels in female rats with two-month survival after the implantation of capsules, (**B**) with five month survival, and (**C**) shows LH in the animals two months after the implantation of DES capsule, and one and two months after the removal of DES capsule. (**D–F**) show LH data in male rats of similar experimental groups as in females. Numbers in the columns or above them indicate the number of animals included in the given group. Abbreviations: DES – diethylstilbestrol; ecap – empty capsule; P – progesterone.

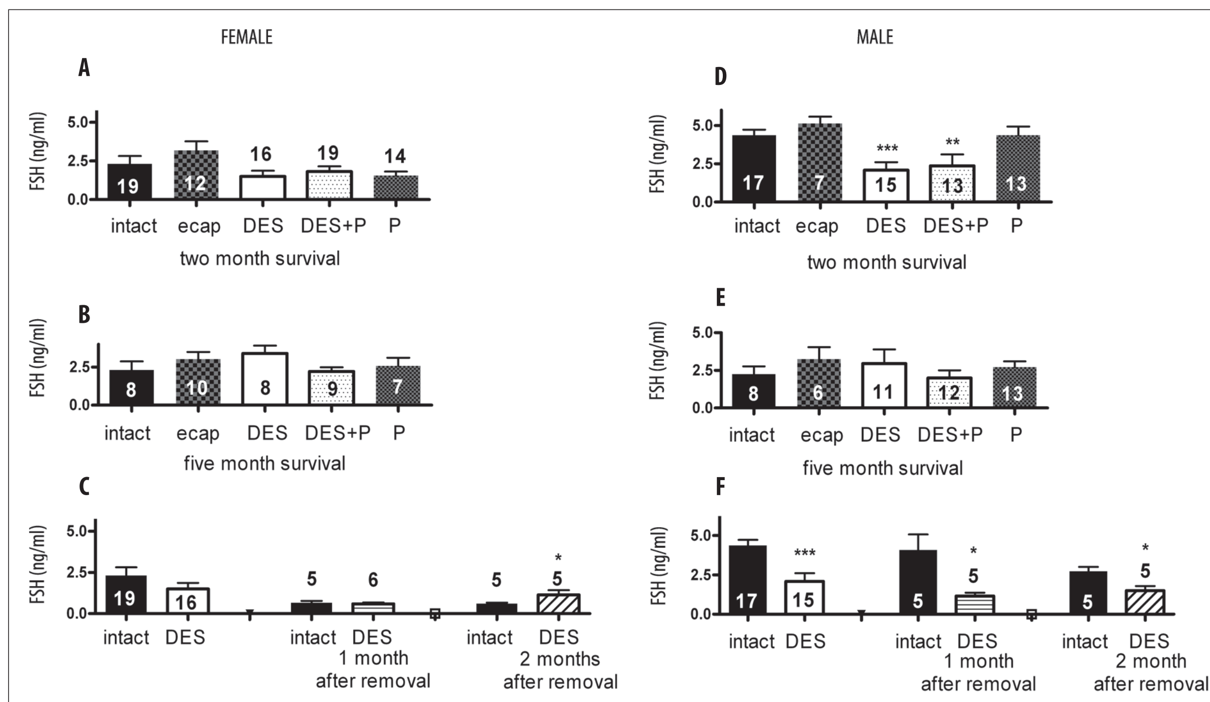

Figure 2. FSH plasma levels in various experimental groups. (**A**) shows FSH levels in female rats with two-month survival after the implantation of capsules, (**B**) with five-month survival, and (**C**) shows FSH in the animals two months after the implantation of DES capsule, and one and two months after the removal of DES capsule. (**D–F**) show FSH data in male rats of similar experimental groups as in females. Numbers in the columns or above them indicate the number of animals included in the given group. Abbreviations: DES – diethylstilbestrol; ecap – empty capsule; P – progesterone.

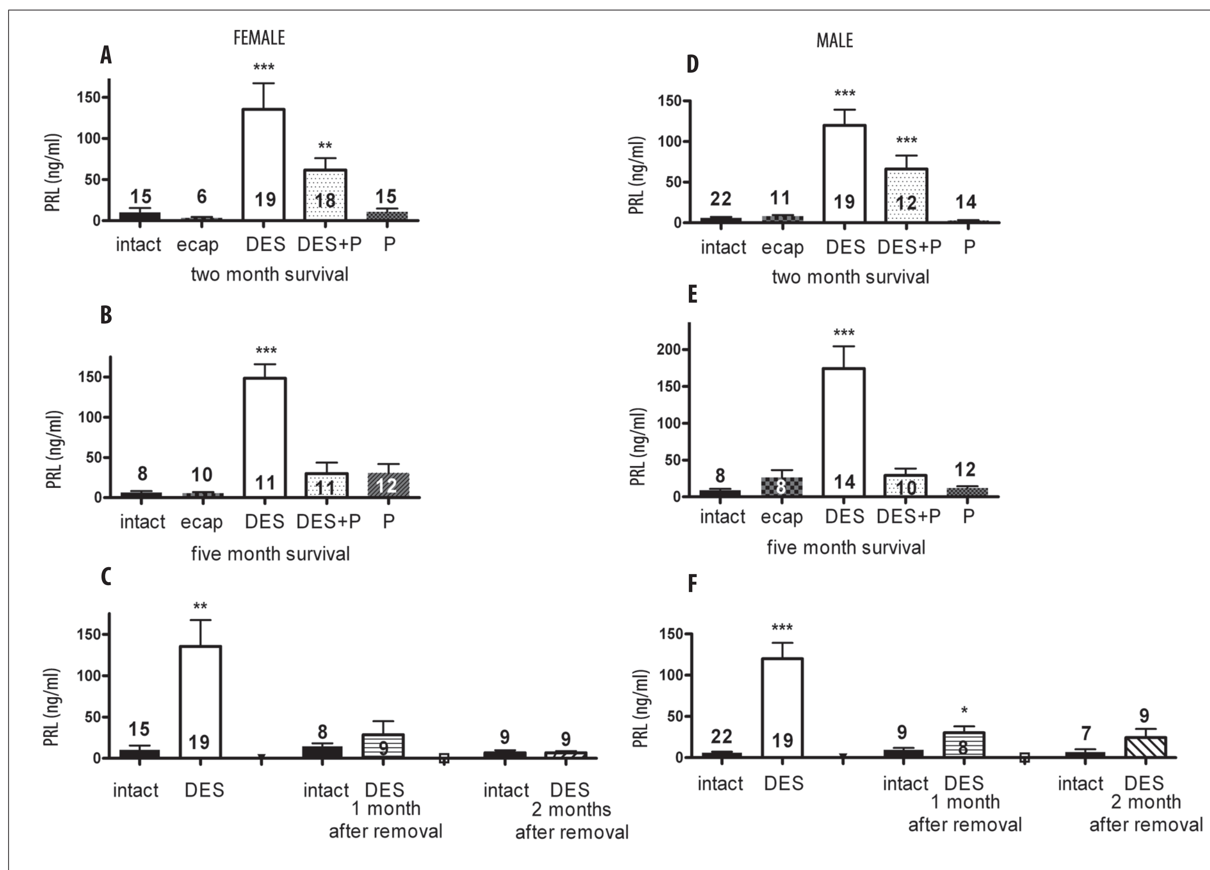

Figure 3. PRL plasma levels in various experimental groups. (**A**) shows PRL levels in female rats with two-month survival after the implantation of capsules, (**B**) with five-month survival, and (**C**) shows PRL in the animals two months after the implantation of DES capsule, and one and two months after the removal of DES capsule. (**D–F**) show PRL data in male rats of similar experimental groups as in females. Numbers in the columns or above them indicate the number of animals included in the given group. Abbreviations: DES – diethylstilbestrol; ecap – empty capsule; P – progesterone.

changes caused by E, and the consequence of the removal of E capsule after two months of its implantation on pituitary LH, FSH, PRL and GH synthesis and release.

New data are as follows:

1. There is sexual dimorphism in the change of basal LH and FSH release upon DES and combined DES+P treatments. The male rats more rapidly responded than female rats with decreasing basal LH release upon E and E+P treatments. The effect of P was ambiguous. It was protective in five-month treated females but not in males. The basal FSH release was decreased only in male rats and later it was recovered. Neither LH, FSH nor the weight of testes and seminal vesicles restored in male rats after the removal of DES capsule. However, the LH level and the weight of seminal vesicles were not significantly lower than in intact rats. It means that the effect of DES persisted in male. However, in females both basal LH and FSH levels were rather upregulated and the low ovarian weight returned to the control level.
2. There was no sexual dimorphism in the effect of long-term steroid treatments on PRL secretion. In both female and male rats the PRL release similarly enhanced and it was partially prevented after two month and completely after 5-month survival by concomitant P treatment. Removal of DES influence completely restored the PRL release by the end of further 2-month survival. There was a well definied parallel change between the PRL levels and weight of anterior pituitary that was extremly enhanced in DES treated rats and then the enlargement of pituitaries was completely prevented by concomitant P treatment in the case of 5-month survival.
3. A mild sexual dimorphism was also observed in GH level. It was significantly lower only in DES and DES+P treated female, not in male rats with two-month survival. The BW and B+TL were very much depressed by DES treatment in both female and male rats and P little blunted but not prevented this effect. The BW and B+TL in male rats remained low after the removal of DES capsule; however, in females it was nearly similar to intact rats.
4. P alone had no significant effect on the parameters studied; however, the concomitant P treatment with DES was usually protective.
5. Removal of DES capsule reversed the changes in the immunohistochemical appearance of LH, FSH, PRL and GH immunoreactivities.

It is well known that GnRH and LH are released in a cyclic pattern in females but not in male rats. The neural LH release apparatus was demonstrated at the first time by Everett and Sawyer [45,46]. A sexual dimorphism of prepubertal rats in GnRH and LH responses to steroid treatment was also

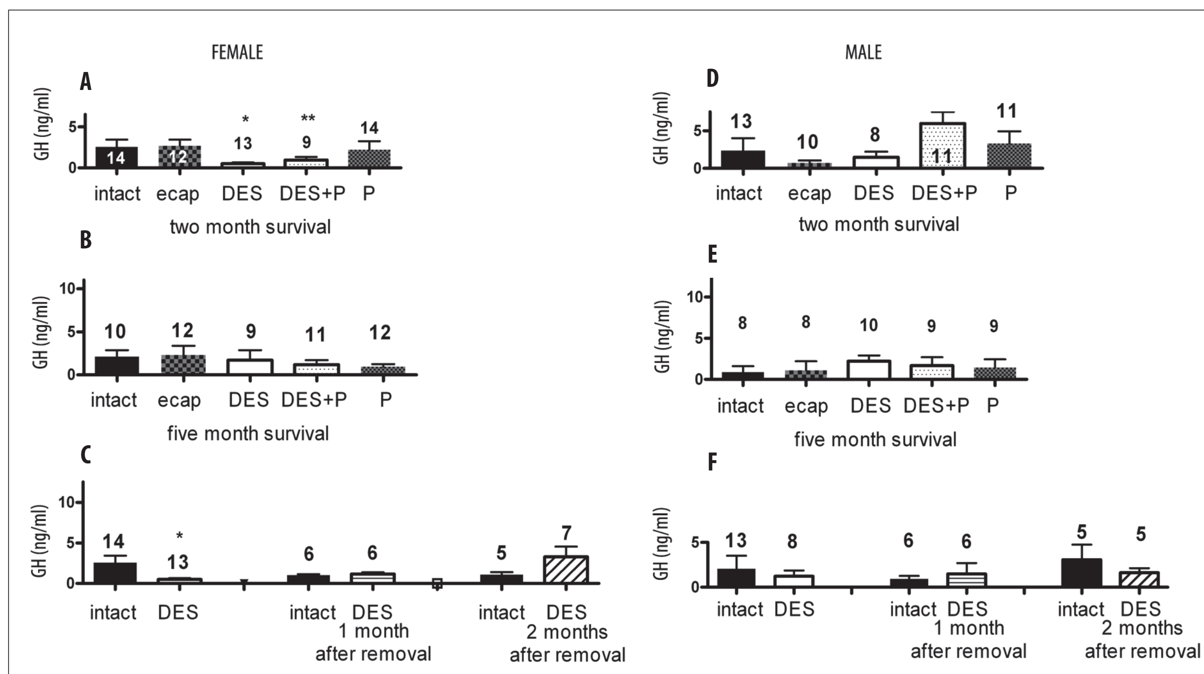

Figure 4. GH plasma levels in various experimental groups. (**A**) shows GH levels in female rats with two-month survival after the implantation of capsules, (**B**) with five-month survival, and (**C**) shows GH in the animals two months after the implantation of DES capsule, and one and two months after the removal of DES capsule. (**D–F**) show GH data in male rats of similar experimental groups as in females. Numbers in the columns or above them indicate the number of animals included in the given group. Abbreviations: DES – diethylstilbestrol; ecap – empty capsule; P – progesterone.

demonstrated in acute experiments. Dluzen and Ramirez [47] showed that E treated prebubertal females displayed a significant change in GnRH concentrations in the preoptic-suprachiasmatic area upon P treatment; however, males did not show any change. Sexual dimorphism was also observed in the gonadotropin α-subunit promoter activity to GnRH [48]. In the present study we have found sexual dimorphism in the response of basal LH level to long-term DES and P treatments as well.

In our model we did not find any sexual dimorphism in the effect of long-term steroid treatments on PRL secretion and on the weight of pituitaries; however, in other models some sexual dimorphism in the basal level of PRL was observed. It was published earlier that around the time of puberty females had higher PRL mRNA level than males [49], and the weight and PRL level of anterior pituitary in prepubertal females is significantly higher in females than in males [50]. In ovariectomized rats P implanted in silicone tubes subcutaneously induced nocturnal PRL surge, similar P sensitive central mechanism was not observed in adult male rats [51].

A sexual dimorphism was found in the plasma GH pattern and regulation as well. In the above-mentioned experiment it was also demonstrated that around the puberty the males had higher level of GH mRNA than females [49]. Neonatal masculinization happens in neonatal males which determines the secretory pattern of GH. The masculine type (high infrequent GH pulses with low plasma GH levels in between) promotes growth more effectively than the feminine type (an intermediate, rather constant level of plasma GH) [52]. In our model long-term DES treatment similarly depressed the body growth in both male and female rats indicating that the effect of DES is not mediated through GH. There are data which show that chondrocytes in the epiphyseal plate express ERs [53]. And in this relation the effect of E does not depend on a central mechanism.

P alone slightly depressed the body weight of both sexes in the case of five-month treatment. There were no other significant change. In the literature we did not find as long combined treatment as ours. That is why difficult to draw paralell to others and our experiments.

More data are available about the protective role of P when it is administered with E. The models are very different from what we used. Genazzani and his co-workers [24] studied the role of P and various synthetic progestins in the modulation of the effect of E on hypothalamic GnRH and on pituitary LH and PRL concentrations in ovariectomized rats. And they used only two-week steroid treatment. It was found that P and norethisterone enanthate (NET, synthetic progestin) reversed EB induced hypothalamic GnRH depression, that is elevated its level. Both P and NET blocked the EB induced increase of pituitary LH, and plasma LH levels remained low. Progestins alone did not influence the PRL levels but reversed the EB induced increase. Cho and his co-workers [54] also demonstrated that P suppressed E-enhanced PRL mRNA level.

In our previous experiment it was found that the DES treatment changed not only the number and distribution of tropic hormone producing cells, but the distribution of S-100 immunoreactive folliculostellate cells (FSCs) as well. Inside the prolactinomas FSCs were scattered but they surrounded the prolactinomas forming a demarcation line around them

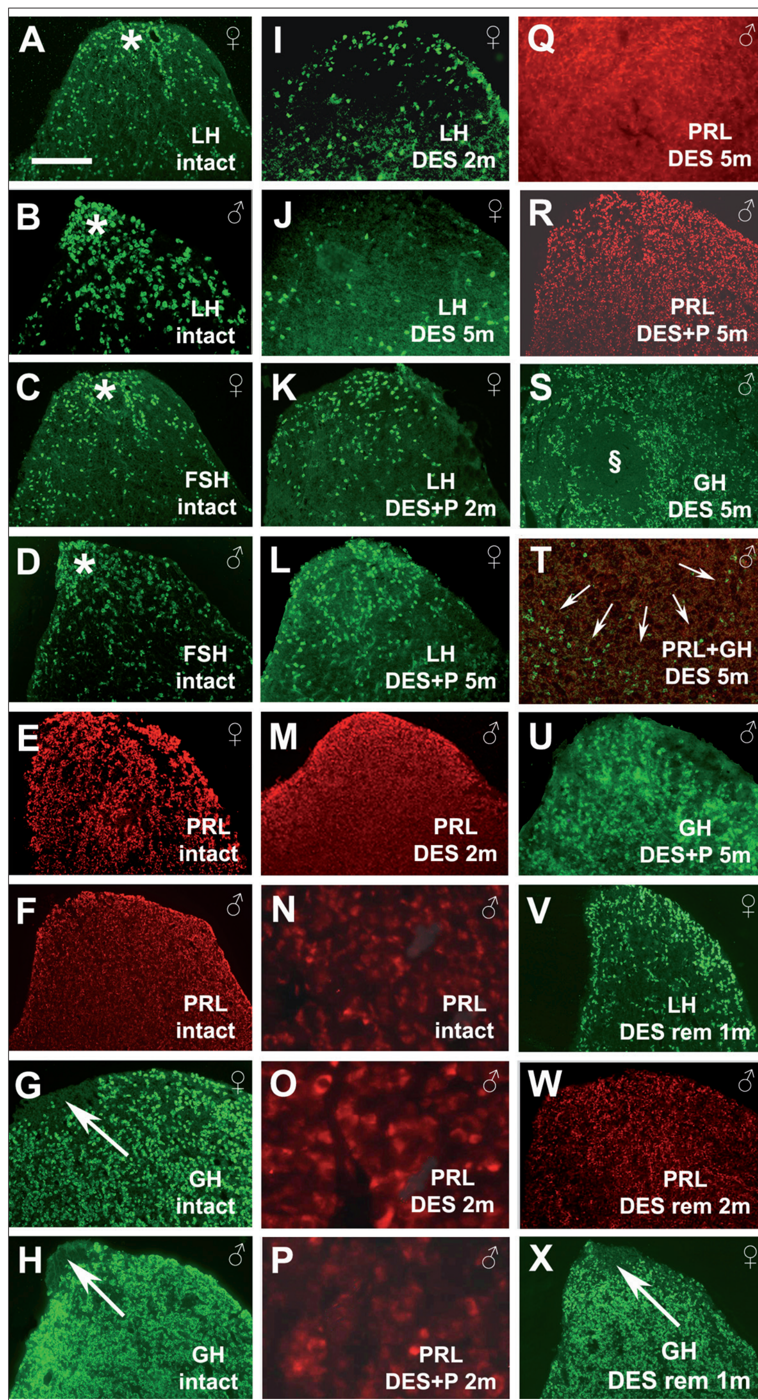

Figure 5. Microphotographs showing anterior pituitary sections stained for LH, FSH, PRL and GH immunoreactivities using indirect immunofluorescence method. (**A–H**) derived from intact rats, (**I–X**) from different experimental groups. In the gonadotropic zone of both female and male rats the density of LH and FSH cells is higher than in the other part of the anterior lobe indicated by asterisk (**A–D**). PRL cells are evenly distributed in intact rats (**E,F**). GH cells are completely missing from the gonadotropic zone (indicated by arrow) of both female (**G**) and male (**H**) rats, anywhere else they are also evenly distributed. In rats with 2-month survival after DES implantation the number of LH cells much lower than in intact rats (**I**). The decrease of LH cells is more pronounced 5 months after implantation (**J**). Implantation of P together with DES prevented the serious decrease in the number of LH cells (**K,L**). DES implantation for 2 months increased the size and number of PRL cells (**M**). (**N**) shows cup shaped PRL cells in an intact male rat, (**O**) shows hypertrophized ovoid and rounded PRL cells in a DES treated rat with 2-month survival, (**P**) shows medium sized PRL cells from a DES+P treated male rat. DES implantation in animals with 5-month survival induced appearance of adenomatous areas composed of PRL cells. One of these prolactinomas is shown in (**Q**). The histological appearance of PRL immunostaining is very similar to intact rats. Concomitant P implantation with DES for 5 months prevented the hypertrophy of PRL cells (**R**). (**S**) shows GH staining in a male rat with 5-month DES influence. GH cells are missing from a region resembling prolactinoma (§). Double labeling (**T**) for GH and PRL shows a prolactinoma (outlined by arrows). GH cells are missing from this adenomatous region. (**U**) shows GH staining in a male rat with DES+P implantation. GH cells show similar distribution than in intact rats; however, they are also present in the gonadotropic zone. Removal of DES capsule restored the appearance of immunostaining for LH (**V**), PRL (**W**) and GH (**X**). Abbreviations: 2 m – 2-month survival; 5 m – 5-month survival; rem 1 m – 1-month survival after removal of DES capsule; rem 2 m – 2-month survival after removal of DES capsule. Scale: 750 µm in (**A–M**) and (**Q–X**), and 100 µm in (**N–P**).

[55]. Inside the prolactinomas there were only a few GH cells. When DES was implanted with P the changes, characteristic for DES treatment, could not develop. Concomitant P influence prevented the morphological changes in the anterior pituitary. The distribution of FSCs and GH cells was very similar to that of controls.

In this present experiment in male rats DES permanently injured the gonadotropic functions. After the removal of DES capsule LH and FSH levels, the weight of testis and seminal vesicle remained lower than in controls. Interestingly, in females there was no permanent depression in basal LH and FSH levels and ovarian weight. Rather, both LH and FSH were upregulated. In the literature there is no animal

experiment in which so long steroid treatment was applied and plasma hormone levels were measured. In human medication sexual steroids are used for months to syncronize the ovarian cyclicity, functional dysmenorrhea, premenstrual syndrome, endometriosis and to limit the adult height of girls [56,57].

The question arises how E induces and P prevents the changes caused by a long-term E treatment. As it was mentioned in the introduction ERs and PRs are present not only in the hypothalamus but in the anterior pituitary as well. Steroids can act locally through these receptors on pituitary cells. In a recent publication it was found that ERα is localized in the largest number in lactotropes, and in decreasing number in somatotropes, thyrotropes, gonadotropes. The number of ERβ was much lower and it was identified in somatotropes, lactotropes and gonadotropes [4]. PRA immunoreactivity was seen in the nuclei of gonadotropes [7]. It is also possible that both E and P act through the hypothalamus as well. The distribution of these receptors was recently demonstrated by several authors. Merchenthaler and his co-workers [11] very precisely mapped the distribution of both ERα and ERβ in the hypothalamus. It was found that the density of ERα is very high in the arcuate nucleus and periventricular area and the density of ERβ is high in the paraventricular nucleus and the medial preoptic area where ERβ immunoreactivity colocalize with LHRH immunoreactivity [58]. In the arcuate nucleus colocalization between ERα and GHRH immunoreactivity but not ERα and somatostatin was published by Shimizu et al. [59]. PR containing neurons were demonstrated in the ventrolateral and in the medial part of the arcuate nucleus [10].

The mechanism how the E stimulates the cell proliferation in the anterior pituitary was thoroughly studied [60,61]. It was found that E through ERα induces production of TGF-β3 from lactotropes and this stimulates the bFGF production of FSCs which has a proliferative effect on lactotropes. The mechanism how the concomitant P treatment prevents the E induced proliferation is not fully explored. Calderon and his co-workers [62] used ovariectomized immature rats for analysis of action of P in response of anterior pituitary and hypothalamus to estradiol exposure. E induced the nuclear accumulation of ERs and the appearance of PRs reaching a peak at 12 hours, then declined to a plateau near the control level. If P was administered to the animals at the peak of PRs, subseqent nuclear accumulation of ER caused by estradiol injection was suppressed. This was observed only in the anterior pituitary, not in the hypothalamus. They concluded that P affected the response to estradiol in the pituitary gland by a well definied temporal pattern and using a same protocol it had no effect in the hypothalamus.

Conclusions

It seems that there is a cross-talk between ERs and PRs. In our protocol we ensured a consistently higher blood level of both DES and P than in controls by the implanted silastic capsules for 5 months. How the ER and PR levels changed during this long period is not known. And there is no data in the literature describing the level of these receptors in such a long-term experiment. There are several possibilities to explain the protective effect of P. 1) PRα is present in gonadotropes. P binding may stimulate these cells to influence the E binding to lactotropes and to prevent the production of TGF-β3. 2) ERs in lactotropes may have P responsive elements and P can bind directly to these receptors to prevent the production of TGF-β3. 3) The effect of P through the hypothalamus is not excluded. But the above-mentioned experiment indicates that the P acts on the pituitary locally rather than through the hypothalamus.

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