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Morphological and enzymatic changes caused by a long-term treatment of female rats with a low dose of gonadoliberin agonist and antagonist

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: Long-term treatment with gonadoliberin analogs is used to block the hypothalamic-pituitary-gonadal axis. The use of these agents is generally considered to be safe; however, some observations suggest the possibility of adverse effects.

Material/Methods: We investigated whether a 3-months administration of a low dose (6 μg/kg b.w.) of dalarelin – a new agonist, and cetrorelix – a known antagonist of GnRH to female rats causes morphological changes in pituitary gland, ovaries, uterus and liver (HE and VG staining); effects on pituitary, hepatic and blood enzyme activities (histochemical and kinetic methods, respectively), and on the blood lipid profile (colorimetric methods); and to what extent these changes are reversible.

Results: Applying analogs effectively inhibited ovulation, affected the uterine endometrium and changed histological appearance of the liver (e.g., steatosis). They altered activities of marker enzymes of cellular respiration, gluconeogenesis and intracellular digestion in the liver and, partially in the pituitary gland, caused undesirable changes in the activities of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and creatine kinase, and a concentration of cholesterol HDL fraction and triglycerides in the blood. Both morphological and enzymatic effects were more evident after antagonist administration; changes in the blood lipid profile were more evident after agonist administration. In both analogs histological and enzymatic changes persisted a relatively long time after the discontinuation of the treatment.

Conclusions: The low dose of dalarelin and cetrorelix is sufficient to cause limited damage of hepatic cells and may modify the function of pituitary, ovaries, uterus and liver as well as other organs, even after discontinuation of the treatment.

key words: **GnRH analogs • Dalarelin • Cetrorelix • side-effects • long term treatment • low dose treatment**

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BACKGROUND

One of the main ways to control the hypothalamic-pituitary-gonadal axis is by way of a pulsatory release of the gonadoliberin (GnRH) by the neurons of hypothalamic nuclei [1,2]. In addition to the hypothalamus and gonadotrophs, both GnRH and its receptors (GnRHR) are synthesized in pituitary gonadotrophs and corticotrophs, in the testis, endometrium, placenta, and in the granulosa cells of the ovary [3–5]. Their expression has also been confirmed in organs that are not related to the hypothalamic-pituitary-gonadal axis, such as the liver, heart, skeletal muscles, kidneys and the cells of lymphatic system [6,7], and in a variety of cancers [8]. In physiological conditions, GnRH, due to its fast proteolytic degradation in the blood, is present only locally (mostly in pituitary portal system and brain), and does not reach distal organs. The content of GnRH in the hypothalamus is the highest (1300 pg); in the ovaries it is 7 pg, and in the hypophysis it is only 3 pg [9]. In extra-pituitary tissues, by binding with receptors, GnRH acts as a para- and autocrine regulator of intercellular communication, preventing uncontrolled cell growth and proliferation and it may somehow be involved in apoptosis or programmed cell death [10].

Disturbances in the secretion of hypothalamic GnRH affects the proper function of the hypothalamic-pituitary-gonadal axis. It has been observed that a high GnRH concentration lasting for a longer time leads to desensitization, or down-regulation, of the pituitary gland, and causes an inhibition of the axis due to prolonged excitation of GnRHR [11]. The mechanism of desensitization has been used in the treatment of hormone-dependent cancers, endometriosis, uterine leiomyomas, and precocious puberty [12].

Studies of potential physiologic signaling via GnRHR in extra-pituitary tissues are flawed by the sole use of GnRH analogs with long half-lives, often at pharmacologic levels. This is because natural gonadoliberin is not bound by blood proteins and is destroyed by proteases. In addition, its biological half-life is very short – only about 2-4 minutes [13]. Gonadoliberin analogs can be divided into 2 main groups: GnRH agonists, where amino acids at positions 6 and 10 are usually modified, and GnRH antagonists, where modified amino acids are usually located at positions 1, 2, 3, 6 and 10 [14,15]. Agonists, as well as antagonists, compete with endogenous GnRH for the receptor binding site; however, the mechanism of action of analogs differs. Agonists trigger the enhanced release of gonadotropins (flare-up effect), which is accompanied by a temporary increase in the concentration of steroid hormones such as estradiol, and then they inhibit secretion of gonadotropins by way of desensitization, which requires a long-lasting concentration of the agonist in the blood. After long-term treatment, an inhibition of sex hormone action at their target organs, as well as a decrease in their synthesis and secretion, was noted. The binding of antagonists to GnRHR does not trigger its excitation, but immediately inhibits secretion of LH and FSH, as well as steroid hormones [16].

Chemically modified GnRH analogs are more effective than natural gonadoliberin. They contain more hydrophobic aminoacids, which significantly increases their half-life and their affinity for the receptor. The half-life for analogs such as leuprorelin, triptorelin or buserelin ranges from minutes to hours [13,17]. The half-life for cetrorelix given to rats i.v. in a dose of 0.1 mg/kg b.w. was 1.7 h., and when delivered subcutaneously at a dose of 0.02 mg/kg b.w. it was 1.3 h. At a higher dose (0.5 mg/kg) the half-life extended to 80.7 h [18]. Analogs are more resistant to enzymatic degradation, and reach the systemic circulation. As a consequence, an inhibitory action of analogs on the release of the pituitary gonadotropins, and also their inhibition of the hypothalamic-pituitary-gonadal axis, lasts longer [14]. Analogs are metabolized in the liver to biologically inactive C-terminal metabolites [19].

Besides having chemical castration effects in the reproductive system, the agonistic and antagonistic GnRH analogs were shown to exert a direct effect on target cells in other organs through GnRHR coupling, which is why long-term treatment with GnRH analogs can affect both the morphology and function of many organs controlled by hormones. Davidson et al [20] demonstrated that activation of the GnRHR results in cell adhesion and cytoskeletal remodeling. Cytoskeletal remodeling is dependent on focal adhesion kinase I, c-Src, ERK and Rac, and is independent of the classic phospholipase C signaling pathway [21]. The collective data of Dong et al. [22] reveals that GnRH is capable of regulating cardiac contractile function via a GnRHR/PKA-dependent mechanism. If present in the human heart, dysfunction of such a system may play an important role in cardiac pathology observed in men treated with GnRH agonists for prostate cancer. The GnRH agonist leuprolide (100 μg/kg b.w.) seemingly caused a decline in the estradiol levels that contribute to the accompanying reduction in vascular permeability [23]. In clinical practice relatively high doses of analogs are used [24]. Known undesirable clinical effects of GnRH analogs include hot flashes, headaches, heart palpitations, atrophic changes of reproductive organs, and impotency in men, as well as changes in bone-mineral density (osteoporosis) and in lipidogram [25]. Some authors have observed rare but very serious negative effects such as pulmonary embolism and heart attack after prolonged use of analogs [13]. Furthermore, a significant, but transitional increase in body weight was observed in children during therapy with GnRH analogs as treatment of precocious puberty [24].

Dalarelin {(D-Ala[6], NHET10)-GnRH} is a new GnRH agonist, not yet introduced into clinical practice [26]. Adverse effects of dalarelin exposition have not been described in the literature. Cetrorelix {(Ac-D-Nal(2)[1], D-Phe(4Cl)[2], D-Pal(3)[3], D-Cit[6], D-Ala[10])-GnRH} is a known GnRH antagonist [27]. The aim of this study was to investigate whether a long-term administration of a low dose of dalarelin and cetrorelix: 1) causes morphological changes in pituitary gland, ovary, uterus and liver; 2) affects pituitary, hepatic and blood enzyme activities, and 3) to what extent these changes are reversible.

MATERIAL AND METHODS

Animals

This study was performed on sexually mature female Sprague-Dawley rats weighting 200–220 g. The animals were purchased from the Center for Experimental Medicine,

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Medical University of Silesia. During the experiment animals were housed in cages, within the parameters of standard conditions, a 12 hr/12 hr light cycle, and free access to feed and water. The animals were weighed each week to adjust the dose of hormones to a proper level. The study was accepted by the local Ethics Committee.

GnRH analogs

Dalarelin acetate (BAPEX, Ryga) – a gift from prof. F. Ryszka (Biochefa; Sosnowiec, Poland) – and cetrorelix acetate (ChemPep, Inc., USA) were used in the experiment. Their injection forms were prepared by Pharmaceutical Research & Production Factory Biochefa (Sosnowiec, Poland).

Experimental procedure

The animals were divided into 6 control groups (groups K) and 12 experimental groups, with 6 rats in each. Animals of experimental groups received subcutaneous injection of dalarelin (groups D) or cetrorelix (groups C) every morning at a dose of 6 µg/kg b.w. For the injection, both analogs were dissolved in 0.9% sodium chloride, and administered for: 1 month (group ID and IC), 2 months (group IID and IIC) and 3 months (group IIID and IIIC). The control rats received a placebo solution of 0.9% sodium chloride for 1 (IK), 2 (IIK) and 3 months (IIIK). After the specified 1, 2 or 3 months of analog or placebo administration to the appropriate groups, and a 1-week (groups IIIK+1, IIID+1, IIIC+1), 2-week (groups IIIK+2, IIID+2, IIIC+2) and 4-week (groups IIIK+4, IIID+4, IIIC+4) testing period for each group following the 3-month treatment duration, the animals were sectioned to excise the pituitary gland, ovaries, uterus, and liver and to draw a sample of blood. Liquid was removed from the uteri by making small incisions on uteri horns and drying on blotting-paper. The control rats, at the moment of decapitation, were in the proestrous phase of the reproductive cycle.

Morphological studies

Staining with hematoxylin and eosin

Excised pituitary glands, ovaries, uteri and sections of liver left lobe were fixed in 10% formaldehyde, dehydrated in increasing concentrations of ethanol-xylene series, and embedded in paraffin. The obtained paraffin blocks were cut into 4 µm slices, which were then deparaffinized and rehydrated by passing through a series of xylenes and alcohols, and stained with hematoxylin and eosin (H-E) by standard procedure.

Verhoeff-Van Gieson staining

To visualize the connective tissue fibers in the liver and uterus we used Verhoeff-van Gieson staining [28]. Paraffin sections were deparaffinized and stained with a solution containing hematoxylin, ferric chloride and Lugol solution for 20 minutes. The specimens were differentiated with 2% ferric chloride and then washed in 95% alcohol. In the next steps van Gieson solution (saturated picric acid, 1% acid fuchsine, and distilled water) was applied for 3-minute staining. Finally, the specimens were dehydrated in alcohols and xylene.

Histochemical examination

Immediately after excision, sections of hypophysis and liver were frozen with carbon dioxide and cut at –15°C into 5 µm slices on a CRYO-CUT microtome (American Optical Corp.). In such specimens the reactions for activities of the following marker enzymes were performed: succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), NADH-tetrazolium reductase (NADH-TR), Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase), acidic phosphatase (AcP) and, in the liver, also glucose-6-phosphatase (G6Pase) [29].

All morphological and histochemical stainings were evaluated microscopically (Nikon Eclipse E600 equipped with a Sony SSC-DC58AP camera). Histochemical changes were evaluated densitometrically using ImageProPlus software (Media Cybernetics, USA).

Biochemical analysis of the blood

Blood was drawn for analysis, and was allowed time to clot. The samples were centrifuged. The obtained serum was kept at –20°C until analyzed. The following biochemical parameters were measured using appropriate kits (BioMerieux): (1) enzyme activities: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK) – by kinetic methods, and (2) total cholesterol, HDL cholesterol, triglycerides (TG), glucose and total protein content – by colorimetric methods.

Statistical analysis

Statistical analysis of obtained data was performed with the one-way ANOVA. The Mann-Whitney U test and Kruskal-Wallis test were used if normal distributions could not be assumed. Differences were considered significant if P was less than 0.05. The values shown in Tables and Figures are expressed as mean ±SD.

RESULTS

Weight of the body and organs

Dalarelin and cetrorelix administered for 3 months at a dose of 6 µg/kg b.w. did not cause significant changes in the body weight of the experimental animals as compared to the control groups (Table 1). The liver weight in the control and experimental groups was also comparable. However, statistically significant changes were noted in the weight of ovaries (Table 1). In rats receiving dalarelin for 1, 2 and 3 months the weight of the ovaries approximately doubled the ovarian weight in the control groups (p<0.01). In days after the last injection of dalarelin the weight of ovaries gradually decreased, reaching the control level after 14 days. An opposite situation was noted in groups receiving cetrorelix. A low dose of this antagonist caused a 50% decrease in the weight of ovaries when compared to the control group, which persisted up to 7 days after the last injection (p<0.01). As in case of dalarelin, 14 days after the cetrorelix last injection, the weight of ovaries returned to those noted in control groups. Throughout the whole experiment the weight of uteri of rats receiving dalarelin was close to 40% of control (p<0.01). Even 30 days after the last injection of dalarelin, the weight of uteri was still lower (Table 1). All uteri

Table 1. The weight (g) of the body, ovaries, uterus and liver of female control and experimental rats during one (I), two (II) and three month (III) lasting administration of placebo, dalarelin and cetrorelix, as well as after one (III+1), two (III+2) and four (III+4) weeks after discontinuation of a treatment.

Group	Weight	I	II	III	III+1	III+2	III+4
Control	Body	254±14.5	254±9.2	272±13.1	278±13.5	282±12.8	287±10.3
	Ovary	0.10±0.01	0.09±0.01	0.08±0.01	0.07±0.01	0.08±0.02	0.09±0.02
	Uterus	0.61±0.05	0.62±0.07	0.54±0.1	0.50±0.06	0.57±0.21	0.70±0.25
	Liver	10.03±0.88	8.78±0.41	6.30±0.35	5.70±0.50	6.51±1.10	7.58±0.59
Dalarelin	Body	239±18.1	249±12.3	255±14.9	262±16.1	261±21	285±14.8
	Ovary	0.20±0.04**	0.22±0.02***	0.16±0.05***	0.09±0.01*	0.07±0.01	0.08±0.02
	Uterus	0.25±0.06***	0.26±0.02***	0.21±0.03**	0.47±0.16	0.37±0.05**	0.40±0.06*
	Liver	8.30±0.96	7.99±0.68	6.66±0.61	5.43±0.47	5.65±0.83	8.77±0.85
Cetrorelix	Body	243±16.5	269±5.6	267±11.7	279±11.2	280±11.1	292±8.8
	Ovary	0.06±0.01**	0.05±0.01**	0.04±0.01***	0.05±0.01**	0.07±0.01	0.08±0.01
	Uterus	0.55±0.07	0.57±0.16	0.52±0.1	0.49±0.1	0.56±0.15	0.51±0.12
	Liver	7.83±0.38	7.82±0.93	8.27±0.98	7.42±1.13	7.13±0.79	7.43±0.93

*, **, *** – The difference statistically significant as compared to appropriate control group for $p<0.05$, $p<0.01$ and $p<0.001$, respectively.
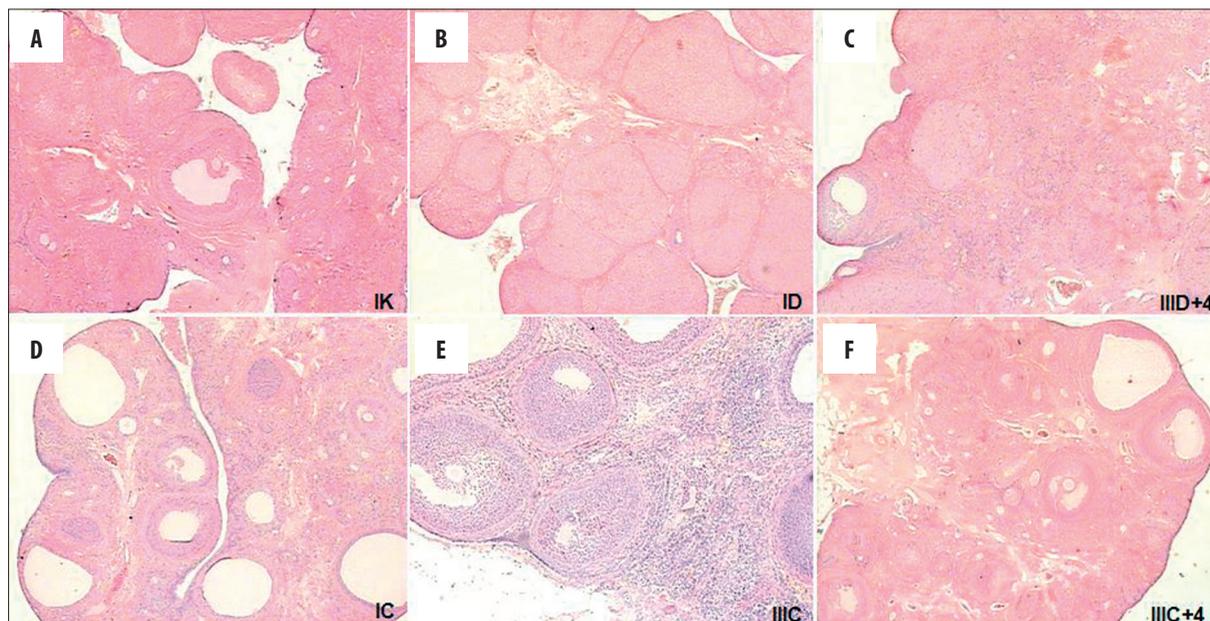

Figure 1. Different effects of dalarelin and cetrorelix on morphological appearance of rat ovary (HE staining). Sections taken from control rats (**A**), and: after one month of dalarelin treatment (**B**), four weeks after a discontinuation of the three months lasting dalarelin treatment (**C**); after one (**D**) and three (**E**) months of cetrorelix administration, and four weeks after its discontinuation (**F**). Magnification: 40×.

exposed to dalarelin were filled with liquid and swollen. We did not note significant changes in the weight of the uteri in the groups of rats given cetrorelix.

Morphological studies

Pituitary gland

Long-term exposure to dalarelin and cetrorelix did not cause significant changes such as increased proliferation, degenerative or vascular changes in the pituitary gland of studied rats.

Ovaries

Morphological images of rat ovaries from control groups were characteristic of the follicular phase with follicles at different stages of development – from primordial and growing, through maturing and fully mature (Figure 1A). There were few scarring corpora lutea and several atretic
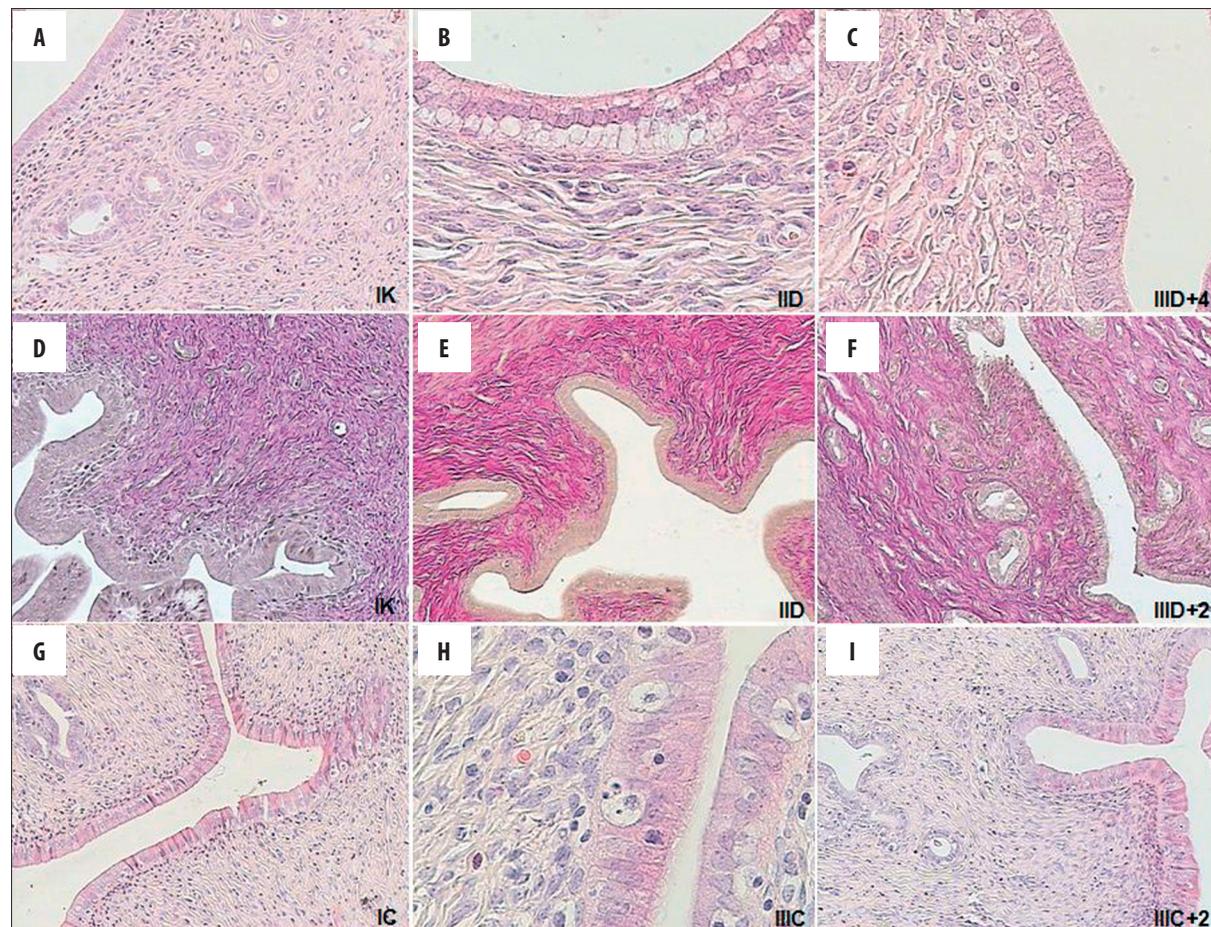
Figure 2. Effects of dalarelin and cetrorelix on morphological appearance of rat uterine endometrium. (**A–C**) HE staining; (**A**) control, 100×; (**B**) after two months of dalarelin injections (subnuclear and apical vacuolization in epithelial cells) and (**C**) four weeks after a discontinuation of the three months lasting dalarelin treatment, 200×; (**D–F**): VG staining, 100×; Collagen fibers are stained purple; (**D**) control; (**E**) two months of dalarelin treatment (signs of fibrosis), (**F**) two weeks after a discontinuation of the three months lasting dalarelin treatment; (**G–I**): HE staining; (**G, H**) After one and three months of cetrorelix administration (infiltration of granulocytes, apoptotic bodies), respectively; (I) two weeks after the cetrorelix treatment discontinuation; Magnification: (**G, I**) 100×, (**H**) 200×.

follicles. A well developed interstitial gland was prominent in the ovarian cortex. After 1 month of dalarelin treatment the number of growing and maturing follicles significantly diminished. Instead, we could see many well-developed corpora lutea containing lutein and paralutein cells (Figure 1B). Such a structure of the ovary was observed throughout the whole period of dalarelin treatment.

In contrast, as soon as 1 month after cetrorelix administration, the development of ovarian follicles was inhibited (Figure 1D). This was mostly noted at the stage of maturing follicles, which accumulated liquid – oocytes, if visible – and granulosa cells showed the signs of degeneration; theca interna and externa were significantly thickened. A number of follicles at early stages of development were atretic, and corpora lutea were absent. All the above changes persisted as long as cetrorelix was delivered (Figure 1E).

Histological changes described above persisted for a few weeks after the last injection of both analogs. Even 4 weeks after cessation of dalarelin treatment we observed enhanced atresia, scarring and luteolysis of corpora lutea, as well as reduced number of follicles at early stages of development

(Figure 1C). Discontinuation of cetrorelix injections resulted in enhanced, but expanded in time, atresia of large follicles that were stopped during maturation, increased number of follicles at different stages of development and appearance of just a few corpora lutea (Figure 1F).

Uterus

Uteri of rats in control groups were in the proliferative phase (Figure 2A). Both simple columnar epithelium lining the uterus, and glandular epithelium, showed a proliferative but not a secretory pattern. The functional layer of the endometrium was well-developed, contained few collagen fibers, numerous blood vessels, and straight glands. Below the epithelium an accumulation of mononuclear lymphoid cells, and deeper, in proximity of myometrium, eosinophils was documented.

In uteri of rats receiving dalarelin we observed features characteristic of early secretory phase: subnuclear (group ID) and apical (group IID) vacuolization in glandular epithelial cells (Figure 2B) and extension of endometrial glands. In group IIID the number of vacuolated cells diminished. The epithelial cells were densely packed and lower than in
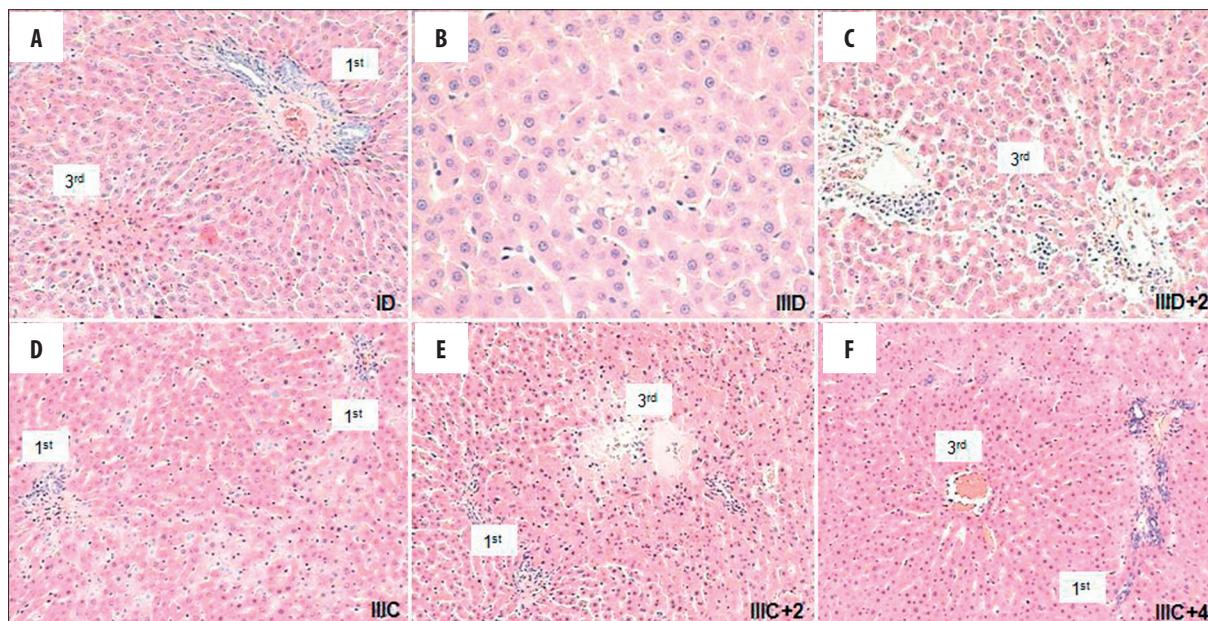
Figure 3. Effects of dalarelin and cetrorelix on morphological appearance of rat liver (HE staining). (**A–C**) rats treated with dalarelin for one (ID, focal necrosis) and three months (IIID, macrovesicular steatosis) and two weeks after the treatment discontinuation (IIID+2, increased number of mononuclear cells), respectively; (**D–F**) rats treated with cetrorelix for three months (IIIC, basophilic hepatocytes) and two (IIIC+2, signs of congestion, degenerating hepatocytes) and four weeks (IIIC+4) after the discontinuation, respectively. 1st and 3rd – zone 1 and 3 of liver acini, respectively. Magnification: (**A,C–F**) 100×; (**B**) 200×.

control rats. The lumen of glands and uterus contained acidophilic secretion. Moreover, the connective tissue component of endometrial stroma expanded in the functional layer (Figure 2D–F). Within the endometrium we observed features of fibrosis. The number of granulocytes significantly diminished. These changes were not accompanied by changes in the vascular bed. Four weeks after the cessation of treatment, the histological picture of the uterus resembled the adequate control appearance (Figure 2C).

Macroscopically, the uteri of rats receiving cetrorelix were larger than those from rats receiving dalarelin. Microscopically, we observed an expansion of endometrium containing many glands. The lining epithelium contained high, columnar, acidophilic cells with large pale nuclei; the glandular cells were much lower and more basophilic (Figure 2G). In fact, these cells resembled those of the epithelium during late proliferative phase. Some epithelial cells contained apoptotic bodies, which were especially numerous in groups IIC and IIIC (Figure 2H). Throughout the whole period of an analog delivery, the epithelial cells did not display secretory activity. There were numerous acidophils, both in deeper layers and just beneath the epithelium. Infiltration of granulocytes increased with a duration of the time of exposure. We did not observe the changes in the thickness of the muscularis proper. Morphological changes in uteri from rats receiving cetrorelix were stable and lasted for 4 weeks after analog treatment discontinuation (Figure 2I).

Liver

Injection of rats with GnRH analogs (dalarelin and cetrorelix) affected their liver morphology in a different way. In group ID (Figure 3A), we observed very gentle changes, such as different staining of individual hepatocytes and focal necrosis of cells (features characteristic for early necrosis). In the following months of exposure (groups IID and IIID) the number of dying cells diminished, and single hepatocytes appeared with macrovesicular steatosis (group IID) and finally, (group IIID) single hepatocytes with microvesicular steatosis could be noted (Figure 3B). Focally, there were groups of strongly acidophilic hepatocytes with pyknotic nuclei characteristic of apoptotic cells. No inflammatory reaction was seen in groups I–IIID.

One week after dalarelin treatment discontinuation, (group IIID+1) the morphology of the liver was comparable to that observed in groups IID and IIID. In groups IIID+2 and IIID+4 there was an appearance of congestion and increased number of mononuclear cells in the perivascular areas (Figure 3C).

In group IC (animals receiving cetrorelix for 1 month) we observed numerous basophilic hepatocytes around the central veins (zone 3 of liver acini) and single hepatocyte microvesicular steatosis, an increased number of mononuclear cells, and signs of organ congestion. The morphology of livers from groups IIC and IIIC was similar, but there was an increase in number of basophilic hepatocytes and mononuclear lymphoid cells and some level of congestion (Figure 3D). Additionally, as a new morphological feature observed after 3 months of analog delivery (group IIIC), we noted focal necrosis of groups of hepatocytes in the perivascular areas. After cetrorelix treatment discontinuation, the areas of basophilic hepatocytes became restricted to zone 1 of liver acini and the number of mononuclear cells decreased (group IIIC+4). In the meantime (groups IIIC+1 and IIIC+2), an appearance of extensive polymorphic areas of degenerated, swollen hepatocytes was observed (Figure 3E,F).

No pathological fibrotic changes were noted during the experiment, although in the groups receiving cetrorelix the
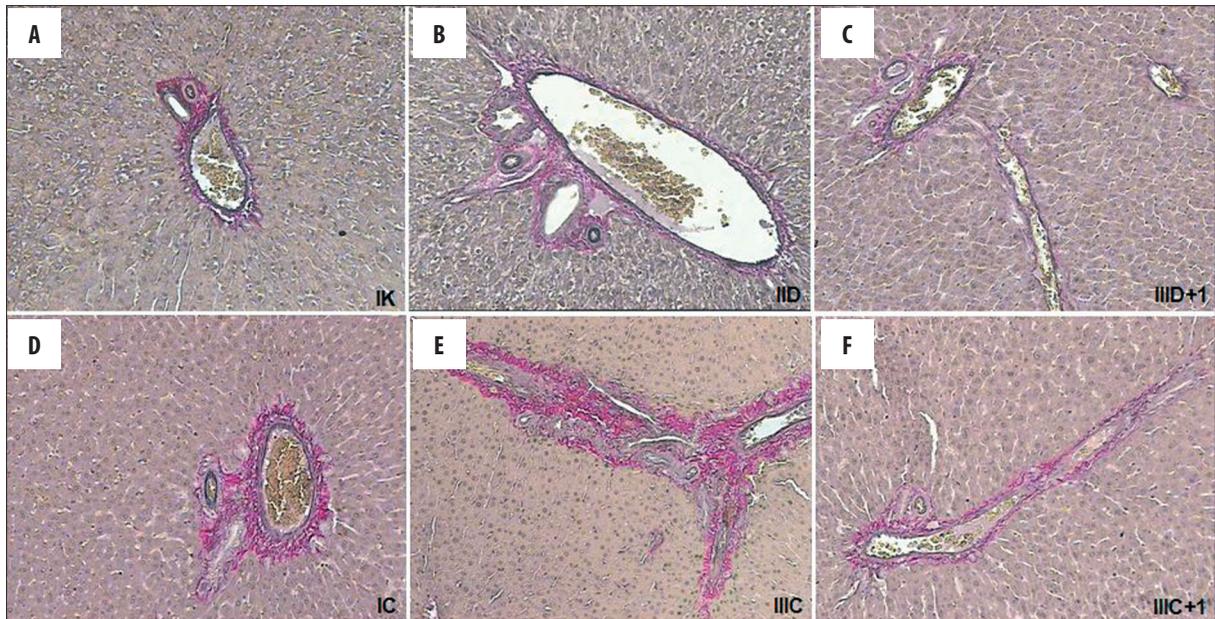
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Figure 4. Identification of connective tissue fibers in the liver of rats administered dalarelin and cetrorelix and after the treatment discontinuation (VG staining). Portal space and surrounding cells are shown. Collagen fibers are stained purple, elastic fibers are stained black. (**A**) control; (**B**) two months of dalarelin treatment; (**C**) one week after a discontinuation of the three months lasting dalarelin treatment; (**D**) one month and (**E**) three months of cetrorelix treatment; (**F**) one week after the cetrorelix treatment discontinuation; Magnification: 100×.

collagen content in some periportal areas was slightly higher than in the groups receiving dalarelin (Figure 4).

Histoenzymatic studies

Pituitary gland

Activity of marker enzymes in pituitary gland (SDH, NADH-TR, and Mg^{2+}-ATPase) did not change after long-term treatment with dalarelin (Table 2). Activity of LDH diminished by about 20% after 2 and 3 months of exposition and then, after discontinuation of analog, returned to control values (Figure 5, Table 2). Cetrorelix induced activity of AcP between the first and third months of treatment, up to 160% at the first month. It inhibited the activities of SDH on month 1 and NADH-TR after month 3 of the experiment. Changes in AcP and NADH-TR activity were reversible after 4 weeks of treatment discontinuation (Figure 5, Table 2).

Liver

Succinic dehydrogenase

Despite the initial decrease of granular-diffusive reaction reflecting lower SDH activity (group IC), from the second month of cetrorelix delivery up to 4 weeks after its discontinuation, we noted a statistically significant increase in the enzyme activity in zones 1 and 3 of liver acini. The effect of dalarelin was limited and the increase in SDH activity was noted mostly in the first month of treatment (Table 3, Figure 6).

NADH-tetrazolium dehydrogenase

Histochemical staining indicated that aside from a transient, small increase (p<0.05) in the enzyme activity after 1 month of dalarelin and 2 months of cetrorelix treatment,

there were no changes in NADH-TR activity in the liver (Table 3, Figure 6).

Lactate dehydrogenase

There was an increase of diffusive reaction after cetrorelix, indicating its inductory action on the activity of LDH in zones 1 and 3 of liver acini, intensifying with time of exposition from the second month of treatment, and persisting up to 4 weeks after analog discontinuation. Dalarelin delivery resulted in significant decrease in LDH activity on month 3 of treatment (group IIID), returning to control values 4 weeks after discontinuation (Table 3, Figure 6).

Glucose-6-phosphatase

After 2 to 3 months of dalarelin or cetrorelix treatment, we observed significant increase in G6Pase activity in hepatocytes of zones 1 and 3 of liver acini, manifested in increasing midgranular-diffusive reaction. It was especially evident (a 2-fold increase) after cetrorelix treatment in zone 1 of liver acini. This was a reason for differentiation of G6Pase activities between the zones of acini. G6Pase activity returned to control values soon after discontinuation of dalarelin administration. In the case of cetrorelix, increased G6Pase activity in zones 1 and 3 of liver acini was evident even 4 weeks after cessation of its injections (Table 3, Figure 6).

Mg^{2+}-dependent ATPase

The presence of a product of enzymatic reaction for ATPase was noted both in bile canaliculi and an epithelium of interlobular blood vessels and bile ducts. A transient, insignificant increase in ATPase activity was noted after 2 months of cetrorelix treatment and again after its discontinuation (Table 3; Figure 6).

Table 2. Densitometric evaluation of histochemical reactions for the activities of succinate dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR), lactate dehydrogenase (LDH), Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) and acidic phosphatase (AcP) in rat adenohypophysis, during one (I), two (II) and three month (III) lasting administration of placebo (K), dalarelin (D) and cetrorelix (C), as well as after one (III+1), two (III+2) and four (III+4) weeks after discontinuation of a treatment. All values are expressed as units of optical density.

Group	I	II	III	III+1	III+2	III+4
SDH						
K	100.1±3.7	89.0±8.3	89.2±8.9	94.1±12.3	90.4±15.1	101.5±11.7
D	92.4±13.2	90.0±7.0	81.2±10.5	74.8±4.3*	93.8±5.2	95.5±10.0
C	74.0±5.2*	92.3±5.8	79.4±2.6	89.9±5.0	80.7±4.0	92.0±4.9
NADH-TR						
K	53.0±9.9	66.9±10.3	87.0±4.6	82.1±6.0	84.4±4.4	85.3±8.6
D	69.6±9.3	75.2±7.1	81.5±10.6	75.3±6.0	87.1±6.2	80.1±5.5
C	65.3±3.9	64.2±6.0	57.7±11.3*	69.7±11.5*	70.11±3.8*	78.0±3.6
LDH						
K	105.0±8.7	118.7±15.5	106.4±6.7	110.3±1.5	111.1±8.6	109.8±12.1
D	100.0±3.3	93.4±4.5*	86.8±6.7*	116.0±8.9	103.7±10.3	109.0±10.9
C	110.4±7.3	108.6±11.3	100.4±5.6	115.3±3.6	118.6±2.9	93.0±15.1
Mg^{2+}-ATPase						
K	109.7±26.7	100.9±18.2	108.6±5.7	104.7±12.5	106.6±19.9	108.6±5.7
D	112.1±11.1	104.8±7.3	115.2±23.5	119.4±8.8	127.5±8.6	115.1±9.8
C	108.7±13.2	108.7±3.9	107.0±7.2	103.0±8.1	102.0±14.3	105.6±6.4
AcP						
K	88.5±14.0	84.8±10.2	114.0±5.4	99.0±13.4	109.1±4.3	110.9±4.4
D	105.2±12.1	103.0±9.9	117.7±18.0	118.8±5.7	115.5±8.3	112.5±3.1
C	123.3±10.0*	135.8±4.9*	135.2±1.7*	123.7±6.0*	133.8±5.0*	113.6±3.8

* – The difference statistically significant as compared to appropriate control group for $p < 0.05$.

Acidic phosphatase

There was a statistically significant decrease in AcP activity after the second and third months of dalarelin delivery, and after 3 months of cetrorelix delivery. There was a decrease of granular and granular-diffusive reaction in hepatocytes surrounding bile canaliculi and in Kupffer cells, both in zones 1 and 3 of liver acini. The observed decreases in AcP activity after 3 months of treatment were stable and persisted even 4 weeks after discontinuation of both analogs (Table 3, Figure 6).

Biochemical parameters of blood

Total protein and glucose content

Throughout the whole 4-month experiment, both dalarelin and cetrorelix did not cause any changes in the total protein and glucose content, as compared to control groups (Table 4).

Total cholesterol and HDL cholesterol

We noted a statistically significant decrease in the content of cholesterol and its HDL fraction in the blood drawn from rats' injected with dalarelin and those observed after the discontinuation of analog treatment, as compared to control groups. After the first and third month of treatment with dalarelin, the total cholesterol concentration diminished by 31% and after 2 months by 40%. On the 14th and 30th days after the last injection was given, the cholesterol concentration was 21–25% lower than in control groups, but the difference was not statistically significant. HDL concentration dropped to 50% after 1 month, by 35% after 3 months of treatment, and by 30% after the discontinuation of dalarelin, which was tested on the 14th and 30th days after cessation of injections. An opposite effect was observed in every cetrorelix treated group; a tendency to increase the concentration of cholesterol and HDL fraction was noted after cetrorelix treatment, although the changes were more pronounced for total cholesterol concentration. In case of cholesterol concentration, its decrease was observed after the cessation of treatment. HDL concentration returned to the control level 2 weeks after the discontinuation of exposition (Table 4).

Triglycerides

In rats receiving dalarelin for 2 months, TG blood concentration increased by 300% ($p < 0.05$) and after 3 months
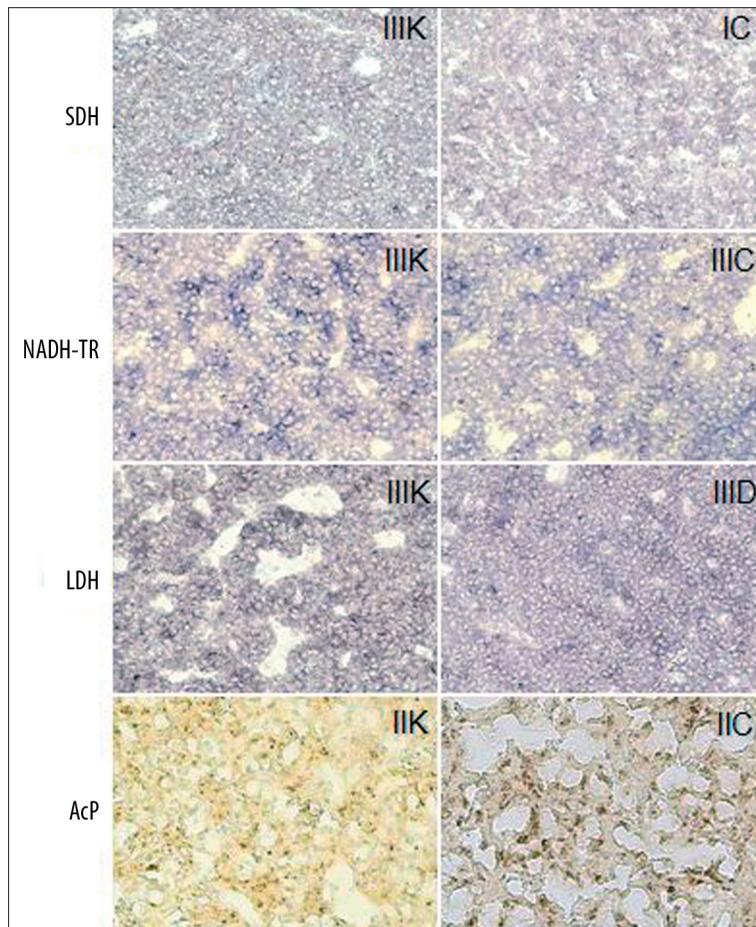
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Figure 5. Histochemical reactions for the activity of succinate dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR), lactate dehydrogenase (LDH), Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) and acidic phosphatase (AcP) in the rat adenohypophysis in control groups (IIK, IIIK), and dalarelin (IIID) or cetrorelix (IC, IIC, IIIC) treated rats. Magnification: IIC – 200×; others – 100×.

by 350% (p<0.001), as compared to controls. Seven and 14 days after the last dose, we observed decrease in TG concentration below the control values and then it normalized. In rats receiving cetrorelix, we observed a statistically significant increase in blood TG concentration up to 194% (p<0.001); however, only after 3 months of treatment. After the last dose we observed a temporary decrease and then normalization of TG blood concentration (Table 4).

Alanine and aspartate aminotransferases

After 1 month of treatment of rats with dalarelin, statistically significant increase in activities of ALT (by 27%) and AST (by 54%) was noted. However, after 2 and 3 months, activities of both enzymes dropped below the control values and returned to control values after the discontinuation of treatment. Cetrorelix caused statistically significant decrease in ALT activity after 1, 2 and 3 month of treatment by 37%, 30% and 59%, respectively. Cessation of cetrorelix injections caused a significant increase in ALT activity (p<0.01), followed by its decrease during the next 7 days, below the control values. In case of AST, cetrorelix injections significantly increased the enzyme activity (p<0.05) after 2 months of treatment and then significantly decreased (p<0.05) after 3 months of the study. Fourteen days after the last dose of the analog, activity of AST reached the control values (Table 5).

Lactate dehydrogenase

In groups receiving dalarelin we noted a statistically significant increase in LDH activity, which after 3 months exceeded by 3-fold the values of respective control groups (p<0.001). Seven days after the last injection we observed significant decrease in the activity (p<0.01) as compared to control, and then gradual normalization. Cetrorelix increased LDH activity after 2 and 3 months of injection; however, in lower degree as compared to rats receiving dalarelin. Soon after the last injection of cetrorelix, LDH activity returned to control values (Table 5).

Creatine kinase

After the first and second month of dalarelin administration, a decrease in CK activity was observed. However, after 3 months of treatment, we noted statistically significant increase in CK activity (p<0.01), as compared to control. After discontinuation of hormone injections we observed significant decrease in CK activity – by 16% and 36% after 7 and 14 days, respectively, and by 53% after 30 days, compared to control values. In groups of rats receiving cetrorelix, we noted temporary decrease in enzyme activity after 1 month of hormone injection. After discontinuation of cetrorelix injections, there was a significant increase in CK activity (p<0.001) as compared to control groups – 60% and 84% after 14 and 30 days, respectively (Table 5).
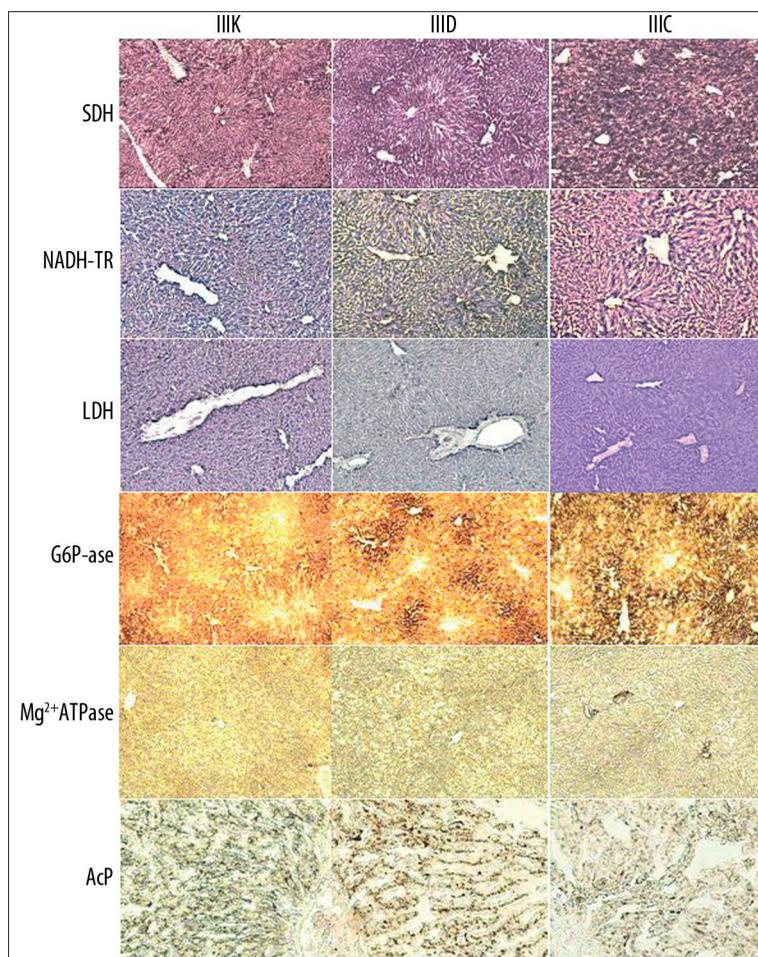
Figure 6. Histochemical reactions for the activity of succinate dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR), lactate dehydrogenase (LDH), glucose-6-phosphatase (G6Pase), Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) and acidic phosphatase (AcP) in the liver of rats from control group (IIIK), and rats treated three months with dalarelin (IIID) or cetrorelix (IIIC). Magnification: AcP – 200×; others – 100×.

DISCUSSION

The observations that GnRHR exist in the extrapituitary tissues are pertinent to the possibility that peripheral tissues, including tumors, may be direct targets for GnRH analogs. These agents may trigger signaling via local GnRHR that is different from potential physiologic endo- and/or paracrine signaling from GnRH/GnRHR activity [6,9,21]. Already low doses of analogs show significant desensitization effectiveness, indicating their high suppressive potential both on main and local hormonal axes. In female rats, cetrorelix at a dose of 2 µg/rat was sufficient to completely block ovulation. Lower doses significantly limited this process. Low doses of analogs did not cause evident adverse effects, and after extended treatment discontinuation hormonal function of gonads returned to normal [27]. In pharmacological as well as long-term toxicological studies on rats and dogs, cetrorelix did not cause systemic adverse effects. Delivered to rats i.v. at a dose of 1 and 4 mg/kg b.w. it did not interfere with respiratory and cardiovascular function, and at a dose of 1.5 mg/kg b.w. it did not cause edema related to increased histamine release. Differential changes in organs, adequate to produce pharmacodynamic effects, were not progressive or were morphologically and/or functionally reversible after treatment discontinuation. Direct organ toxicity in acute, subacute or chronic toxicological experiment, allergic or teratogenic properties, as well as negative effect on early embryonic development and implantation, were

not confirmed at clinical doses. Mutagenic tests were negative [27]. Nevertheless, other studies showed that GnRH agonists, at a dose of 1 µg/day, delivered for 10 days to immature female rats, may disturb the process of maturation, leading to an increase in the weight of ovaries and a decrease in the weight of the uterus. The above effects were not observed in rats treated with a lower dose of agonist – 0.05 µg/day [30]. Chronic treatment of mature female rats with agonists caused an interruption of the cycles, atrophy of the ovaries, and a decrease in the weight of the uterus – these changes were reversible after treatment discontinuation. Therefore, it was assumed that the effect of agonists on the morphology of ovaries depends on the age of the animal [30].

We did not note significant differences in the body weight of mature female rats receiving dalarelin or cetrorelix at doses of 6 µg/kg b.w. for 3 months. The liver weight was not affected. However, in rats receiving dalarelin, after 1 month of treatment, we observed a more than 100% increase in weight of the ovaries, accompanied by increase in number of corpora lutea and an approximately 60% decrease in weight of the uterus, accompanied by an accumulation of a large amount of fluid. The latest was, most probably, the consequence of tissue dehydration and enhanced secretory processes in epithelial cells, but they were not a result of changes in the vascular bed. Other authors [16] observed that shorter (12 days) treatment of rats with

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Table 3. Densitometric evaluation of histochemical reactions for the activities of succinate dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR), lactate dehydrogenase (LDH), glucose-6-phosphatase (G6Pase), Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) and acidic phosphatase (AcP) in 1st and 3rd zone of rat liver acini during one (I), two (II) and three month (III) lasting administration of placebo (K), dalarelin (D) and cetrorelix (C), as well as after one (III+1), two (III+2) and four (III+4) weeks after discontinuation of a treatment. All values are expressed as units of optical density.

Group	I		II		III		III+1		III+2		III+4	
Zone of acinus	1st	3rd	1st	3rd	1st	3rd	1st	3rd	1st	3rd	1st	3rd
SDH												
K	198.2±4.1	178.8±8.1	162.8±9.7	134.2±28.3	123.7±13.7	123.4±16.6					139.2±8.3	135.8±9.7
D	212.3±9.0*	205.2±12.0*	170.6±16.8	134.2±6.3	163.1±21.0*	131.6±20.5	116.2±14.0	118.9±15.6	149.9±31.9	136.2±15.7	151.1±7.1	135.8±10.4
C	174.0±8.4*	164.4±4.8*	165.5±2.7	163.3±4.3*	179.4±8.9*	173.3±4.5*	178.5±4.0*	178.9±8.1*	179.4±4.4*	173.1±3.6*	173.2±3.5*	173.1±5.8*
NADH-TR												
K	122.2±11.7	115.0±11.3	94.7±8.4	82.5±19.4	110.1±21.4	103.4±17.9					120.0±16.9	116.2±16.7
D	140.0±9.8*	141.8±11.2*	92.2±13.0	93.5±11.1	105.3±26.3	85.8±14.9	124.9±18.2	88.2±18.5	108.5±21.2	104.8±20.2	143.0±14.9	142.9±13.7
C	119.0±9.8	107.9±12.4	130.4±11.8*	122.7±9.2*	123.9±5.3	117.3±7.3	129.3±4.5	131.1±7.2	107.8±11.8	98.9±13.6	131.0±13.8	122.7±14.1
LDH												
K	177.7±13.0	167.8±11.5	132.8±9.5	126.0±9.1	95.3±15.3	93.8±14.4					119.5±17.4	115.3±17.6
D	169.4±20.7	159.7±29.9	135.7±11.9	131.9±9.6	46.3±14.6*	37.0±15.5*	62.1±9.7*	53.0±9.5*	58.8±13.0*	58.3±15.6*	91.3±22.0	86.2±17.4
C	143.5±17.9*	142.2±16.2*	166.0±6.3*	163.2±7.6*	155.1±4.3*	150.5±3.9*	148.1±6.3*	149.7±5.5*	181.8±5.0*	185.8±7.2*	177.8±8.4*	176.9±10.7*
G6Pase												
K	148.1±19.6	106.4±20.7	73.4±14	71.7±14.6	87.9±9.7	80.5±10.6					89.6±6.8	70.5±7.3
D	154.6±21.9	117.3±17.7	107.5±15.6*	81.1±14.6	102.4±4.7*	100.6±5.9*	89.3±10.6	83.7±9.8	101.4±11.8	65.1±6.4	86.1±11.8	60.6±6.6
C	141.4±14.6	99.9±12.4	146.3±9.5*	98.6±10.7*	160.5±10.4*	109.5±15.3*	155.2±3.8*	122.3±14.7*	159.3±4.9*	120.6±12.2*	164.6±8.3*	130.6±8.2*
Mg²⁺-ATPase												
K	147.7±24.4	149.3±26.7	137.3±15.5	137.7±17.7	148.5±7.4	142.8±14.2					138.1±17.1	132.6±19.1
D	136.0±6.7	129.0±9.6	130.0±11.0	132.0±12.0	134.6±6.8	138.1±8.2	140.6±2.7	130.8±11.0	162.0±8.8	165.4±5.3	152.0±15.7	156.0±15.3
C	142.5±4.9	151.0±4.5	147.2±4.9	154.3±3.8	139.7±8.9	149.6±5.3	152.7±5.1	162.1±13.5	154.4±3.6	156.9±7.7	157.8±13.1	162.4±14.6
AcP												
K	132.0±8.3	131.9±8.9	154.4±5.1	148.8±10.0	162.3±3.0	164.8±6.5					152.2±5.1	153.4±3.7
D	130.3±3.0	133.5±6.6	130.9±18.1*	132.7±17.0*	141.0±7.0*	143.9±3.3*	154.0±4.9*	153.2±5.9*	148.1±6.5	146.8±12.2	135.9±11.1*	127.3±8.0*
C	138.1±19.5	134.7±16.5	153.5±3.1	149.2±3.9	145.5±4.4*	146.6±7.2*	147.8±8.8	147.4±3.8*	148.4±5.8*	150.4±5.8*	139.1±5.9*	140.1±7.6*

* – The difference statistically significant as compared to appropriate control group for $p<0.05$. Values for groups III+1 and III+2 were compared with IIIK and IIIK+4 controls, respectively.

GnRH agonist triptorelin, at a dose of 10 μg/kg b.w., did not affect the weight of the ovaries and only slightly decreased the weight of the uterus. In groups of female rats receiving cetrorelix we noted 50% reduction in the weight of the ovaries, and the weight of the uterus was comparable to controls. Roth et al. [16] administered cetrorelix at a dose of 10 μg/kg b.w. and also noted a slight reduction in the weight of the ovaries and no changes in the weight of the uterus. The used dose was sufficient to cause a GnRHR-dependent decrease in FSH and LH concentration in the blood [31]. In the same 12 day period of injection, administration of the agonist (triptorelin) or the antagonist (cetrorelix), at a dose 10 times higher (100 μg/kg b.w.), resulted in a significant decrease in the weight of both ovaries

and uterus. In turn, in a study of acute and subacute toxicity of GnRH antagonist detirelix on rats and monkeys of both sexes, a significant reduction in body weight of male rats and monkeys was noted [32]. Also, an increase in the body weight of female rats and finally, a lack of significant changes in female monkeys were documented, in comparison to control groups. Three months of detirelix treatment resulted in a significant decrease in the weight of the ovaries and the uterus in rats and the weight of the uterus in monkeys as well. In male rats and monkeys, the analog used in a broad range of doses (0.4–8.0 mg/kg b.w.) caused a significant decrease in the weight of the testes, seminal vesicles, and the prostate gland, independently of the dose. The above results indicate that at low doses

Table 4. Biochemical parameters of the blood of female rats during one (I), two (II) and three month (III) lasting administration of placebo, dalarelin and cetrorelix, as well as after one (III+1), two (III+2) and four (III+4) weeks after discontinuation of a treatment.

Group	Parameter	I	II	III	III+1	III+2	III+4
Control	Protein (g/l)	69.1±4.4	63.6±4.8	65.4±4.3	68.2±3.2	67.8±4.7	67.4±6.3
	Glucose (mmol/l)	6.76±0.3	5.58±0.6	5.96±0.9	6.09±0.6	6.42±0.6	6.74±0.4
	Total Chol. (mmol/l)	1.53±0.1	1.67±0.4	1.97±0.2	2.04±0.4	2.23±0.3	2.42±0.2
	HDL-chol (mmol/l)	0.64±0.1	0.41±0.01	0.63±0.1	0.53±0.12	0.72±0.22	0.91±0.09
	TG (mmol/l)	0.93±0.3	0.80±0.3	0.98±0.2	1.36±0.3	1.34±0.3	1.3±0.4
Dalarelin	Protein (g/l)	62.63±3.1	67.97±6.9	64.72±2.2	66.91±4.2	66.78±3.0	68.78±4.8
	Glucose (mmol/l)	6.38±1.1	6.19±0.7	5.66±0.4	6.3±1.2	6.01±0.4	6.92±0.2
	Total Chol. (mmol/l)	1.06±0.19**	0.98±0.1*	1.36±0.27**	1.79±0.11	1.77±0.21	1.83±0.36
	HDL-chol (mmol/l)	0.32±0.05***	0.46±0.02	0.41±0.08**	0.66±0.11	0.48±0.06**	0.67±0.06**
	TG (mmol/l)	0.69±0.29	2.51±1.2*	3.56±1.28***	0.9±0.21	0.64±0.14***	1.68±0.46
Cetrorelix	Protein (g/l)	74.62±8.1	66.88±12.2	74.62±11.5	73.22±12.5	73.66±7.9	76.67±7.4
	Glucose (mmol/l)	5.23±0.53	5.68±1.42	5.76±0.64	6.75±1.34	6.72±0.67	6.17±1.37
	Total Chol. (mmol/l)	2.3±0.39**	2.19±0.24	2.58±0.48	1.58±0.14**	1.57±0.25*	1.59±0.22**
	HDL-chol (mmol/l)	0.59±0.04	1.13±0.36*	0.86±0.22	1.01±0.06***	0.73±0.18	0.96±0.16
	TG (mmol/l)	0.99±0.32	1.11±0.04	1.9±0.14***	0.88±0.24	1.42±0.15	1.09±0.29

*, **, *** – The difference statistically significant as compared to appropriate control group for p<0.05, p<0.01 and p<0.001, respectively.

Table 5. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) in the blood of female rats during one (I), two (II) and three month (III) lasting administration of placebo, dalarelin and cetrorelix, as well as after one (III+1), two (III+2) and four (III+4) weeks after discontinuation of a treatment.

Group	Enzyme (U/l)	I	II	III	III+1	III+2	III+4
Control	ALT	58.87±7.26	61.11±7.54	75.01±2.66	31.86±7.57	37.01±12.23	45.04±10.86
	AST	75.08±8.00	70.93±12.21	73.68±1.46	60.06±17.44	57.29±15.72	54.53±15.26
	LDH	3009±390	3112±103	2790±419	3412±515	3365±476	3318±488
	CK	933±49	778±169	580±153	515±142	595±154	515±142
Dalarelin	ALT	74.73±6.22*	50.28±11.87*	50.28±8.50*	28.37±10.98	29.33±12.67	39.46±10.81
	AST	115.93±10.66**	50.63±8.98*	52.03±13.54*	67.65±18.22	61.98±23.61	59.36±17.76
	LDH	4371±724*	4561±942**	8418±1577***	1295±724**	3305±1576	3309±926
	CK	753±123*	707±196	797±94	434±68*	381±31**	243±81**
Cetrorelix	ALT	36.82±15.56**	42.60±10.66*	30.31±5.50**	51.68±9.37**	34.18±15.11	26.76±10.94*
	AST	77.64±12.51	90.36±6.27*	52.52±9.09*	48.97±11.02*	61.98±16.98	54.82±13.44
	LDH	2936±642	4857±660**	4276±757*	3964±886	3141±305	3333±839
	CK	656±74**	748±90	718±120	684±157	954±66***	949±146***

*, **, *** – The difference statistically significant as compared to appropriate control group for p<0.05, p<0.01 and p<0.001, respectively.

of GnRH analogs the changes in the weight of rat ovaries and the uterus are dependent on the type of analog used and the duration of the exposure.

A reduced number of growing and maturing follicles and a large number of corpora lutea observed by us in the ovaries

after dalarelin treatment, indicates that a significant release of LH during first days of exposure (flare-up effect) would take place and would result in significant maturation of the follicles and their luteinization, as well as, indirectly, stimulation of secretory activity of epithelial cells lining the endometrium. Concerning the influence of GnRH antagonists on

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the morphology of ovaries, both our studies and data in the literature, describe a lack of corpora lutea, growth inhibition of maturing follicles, and their enhanced atresia [29]. The presence of atypical oocytes, inhibition of growth and maturation of ovarian follicles, the large number of apoptotic bodies in the epithelium and glands of the uterus after cetrorelix treatment, indicates that apoptotic processes were enhanced in cells of both organs. Recent studies confirmed that GnRH induces apoptosis of granulosa cells in rat ovaries [32]. The mechanism underlying the proapoptotic effect of GnRH analogs is not fully known and remains controversial in cancer studies. In some ovarian cancer cells, as well as in uterine leiomyomas, GnRH analogs may possess 2 counteracting activities (anti-proliferative vs. anti-apoptotic) that are mediated by 2 distinct signaling cascades, but are triggered by the same Gα protein. It is also known that GnRH analogs may directly up-regulate apoptosis in association with inhibition of cell growth by down-regulating proliferation in the cells [10,34,35]. The antiproliferative effect of GnRH analogs may be mediated, in part, through an interaction with the mitogenic signal transduction pathway of the EGF receptor in association with a decrease in EGF mRNA expression, thus inhibiting the autocrine/paracrine mitogenic activity of EGF and autophosphorylation of EGF receptors [34]. Several lines of evidence have suggested a role of the ERK1/2 signaling pathway. Depending on the cell context, GnRH analogs may attenuate the mitogenic action of growth factors, suppress the ERK cascade, inhibit phosphatidylinositol kinase activity, stimulate lysophosphatidic acid hydrolysis, as well as down-regulate telomerase reverse transcriptase, acidic ribosomal phosphoprotein, and prostate specific antigen expression [10]. The antiproliferative action of GnRH agonists may be also mediated by lysophosphatidic acid (LPA) hydrolyzing activity that is linked to GnRHR, preventing LPA-induced cell growth and survival. LPA mediates a wide range of proliferative and/or morphologic effects, including enhanced cell growth, decreased cell death, increased cancer cell invasion, and improved wound healing and regeneration [36]. Thus, the balance of these 2 activities (apoptosis and proliferation) may be a critical factor in controlling ovarian structure and function during analog administration and tumorigenesis.

To summarize our results, an antiovulatory effect of analogs was reached during the entire period of exposure, which confirms the efficiency of applied dose, although agonists and antagonists seemed to act by different mechanisms. The changes in the weight of ovaries persisted for 1 week after discontinuation of treatment and returned to control values after 2 weeks. Concurrent histological changes persisted much longer – even up to 4 weeks after the discontinuation of treatment. Enhanced processes of structural reconstruction, namely atresia, scaring and recruitment of follicles were seen. Histological changes caused by GnRH analogs in the uterus lasted for an equally long period of time. These effects, as well as the extended period of time required for recovery of the ovaries and uterus after analog treatment discontinuation, and an especially long time needed for new follicle growth and maturation, indicate that after treatment with both synthetics, long-term hormonal dysfunction could take place.

It is known that hypoestrogenism is a functional consequence of long-term treatment with GnRH analogs [37].

However, contradictory data concerning an increase or inhibition of steroidogenesis in vitro and in vivo indicate that different factors, such as type and dose of analog, mode of delivery, advancement of follicular development, type of ova, etc., may play key roles in this process [30]. The influence of GnRH analogs on the blood lipid profile is unequivocal as well. In women treated for 13 weeks with buserelin for uterine leiomyoma, an increase in total cholesterol concentration and a slight increase in LDL fraction were observed in the blood. Similar data were obtained for women receiving buserelin for 1 year due to endometriosis [38,39]. On the other hand, changes in blood lipid profile concerning HDL fraction, with the exclusion of total cholesterol and TG concentration, was shown in patients with endometriosis treated with goserelin for 6 months. Observed changes were positive, decreasing the risk of cardiological complications [40]. In studies done on groups of patients treated with triptorelin, neither changes in a concentration of total cholesterol and its' LDL and HDL fraction, nor changes in concentration of TG, were observed [41]. In our studies the changes in the lipid profile, mostly in total cholesterol concentration and its HDL fraction, were observed. These changes were dependent on the type of analog used, but were not dependent on the time of exposure. It seems that dalarelin, to a higher extent than cetrorelix, may disturb the lipid metabolism by diminishing the cholesterol HDL fraction and increasing TG concentration in the blood. Such changes in patients subjected to long-term therapy with GnRH analogs could increase the risk of cardiovascular complications, especially in patients who are predisposed to vascular disease, such as in atherosclerosis.

The impact of GnRH analog therapy on liver function and metabolism is largely unknown. It may be that changes observed in the blood lipid profile are caused by direct action of analogs on liver cells. In animal models the GnRH agonist histrelin has been safely administered to both rats and rabbits [42]. However, Chester et al. [32] investigated long-term toxicity of the GnRH antagonist detirelix and noted a significant dose-dependent increase in AST and ALT activity in blood of rats of both sexes. Clinical cases reported so far indicate that the incidence of GnRH analog-induced liver toxicity is very low, even during multi-drug therapy. It was observed that 99.6% of patients treated for prostate cancer with the antiandrogen flutamide and the GnRH agonist [D-Trp^6, des-Gly-NH$_2$^{10}] GnRH-ethylamide, experienced transient elevation in aminotransferase serum levels during the first 6 months of treatment [43]. Single patients exhibited AST and ALT activities or total serum bilirubin and alkaline phosphatase above upper normal limits, or they developed clinical manifestations of liver disease. The histopathologic findings showed a mixed pattern of cytotoxic and cholestatic changes. All clinical and biologic features of liver toxicity rapidly disappeared upon discontinuation of flutamide alone. In women with ovarian hyperandrogenism, randomly assigned to receive triptorelin and administered an oral triphasic contraceptive in combination with drugs effective for the treatment of hirsutism during a 9-month study period, none of the patients had abnormal liver function test results [44]. Gabbi et al. [45] reported a patient with prostate carcinoma who developed a nonalcoholic fatty liver disease (NAFLD) associated with a focal hepatic lesion, as well as a marked iatrogenic hypotestosteronemia and a metabolic syndrome, including insulin resistance, after 32-month

therapy with the GnRH agonist leuprorelin. The absence of any hepatic abnormalities before treatment supports a causal and secondary to androgen-deprivation role of leuprorelin in inducing metabolic derangement [45]. Freundl et al. [46], using leuprorelin in patients treated for endometriosis, detected only slight changes in ALT, AST and LDH activities in blood. In men with advanced prostate cancer who were treated with histrelin implant, the most common adverse effects were hot flashes, implant site reaction, renal impairment, testicular atrophy, nausea, fatigue, and gynecomastia [42]. Nevertheless, only 8.7% of these patients developed hepatic impairment, defined as occasional random anomalous laboratory values. In some clinical reports there were only a few cases of liver toxicity manifested by transiently elevated enzymes in a hepatocellular pattern – AST, ALT and alkaline phosphatase after the initiation of histrelin treatment [42]. The liver biopsy revealed marked necro-inflammatory injury with bridging necrosis, which favors a typical pattern for medication- or toxin-mediated hepatocellular damage.

In our experiment, temporal increase in the activities of ALT and AST were observed in blood of rats receiving a low dose of dalarelin. In animals receiving cetrorelix the ALT activity was diminished throughout the whole period of exposure. LDH activity increased significantly as a result of administration of both GnRH analogs; however, only during the exposure period. In contrast to other enzymes, changes in CK activity, which may reflect status of muscle cells, appeared as a consequence of discontinuation of analogs treatment, although the direction of these changes in both cases was opposite. Enhanced ALT, AST and LDH activities may suggest limited damage to hepatic cells. In the livers extracted from female rats receiving dalarelin, we noted temporary early signs of necrosis, steatosis and apoptotic changes in some hepatocytes. Predominant changes after cetrorelix administration included changes in hepatocytes stain, which could indicate enhanced metabolism, and vascular changes indicating liver congestion and increased number of inflammatory cells. Finally, after 3 months of exposure, a slight degeneration of hepatocytes became obvious, at first focal and then, after a treatment discontinuation, more wide-spread. Thus, in case of long-term treatment with both analogs we can expect less (after agonist) or more (after antagonist) enhanced morphological symptoms in the liver, even after discontinuation.

In parallel to those findings, we observed changes in hepatic enzyme activities, indicating that analogs affected the basic metabolic functions of hepatic cells. There was an increase in the activity of mitochondrial SDH, playing a key role in the Krebs cycle and indirectly with the generation of biological energy in the form of ATP. This increase was dependent on the type of analog and was much more intense and lasted longer (even after discontinuation) after cetrorelix administration. However, NADH-TR activity, which is routinely used in the qualitative assessment of respiratory complex I deficiency and measuring the complex I-catalyzed transfer of electrons between NADH and an artificial electron acceptor, nitroblue tetrazolium appeared to be less responsive to GnRH analogs. Observed changes in the activity were small and temporary. A potential complication is the fact that although the NADH-TR activity is widely used to indicate the mitochondrial respiratory complex

I activity, this activity is present also in the endoplasmic reticulum, notably that associated with several observations indicating that the contribution of the microsomal NADH-TR activity is relatively small [47]. Cetrorelix did not affect a ratio of SDH to NADH-TR activity between the zones of hepatic acini. We stated that administration of GnRH analogs, and especially the antagonist, may significantly stimulate oxygen-dependent production of energy in hepatocytes. This occurs more by stimulation of mitochondrial SDH activity than by respiratory complex I activity.

Observed enhancement in LDH activity after cetrorelix may indicate that in hepatocytes, where LDH isoforms involved in lactic acid metabolism dominate, demand for products of gluconeogenesis increases. It may be due to more intense mitochondrial activity and the increased need for glucose. Confirmation of such a scenario could be a significant elevation in G6Pase activity, an avid marker of gluconeogenesis, mostly in groups of animals exposed to cetrorelix and, to a lesser extent, to those exposed to dalarelin (in which LDH activity decreases). After both analogs the active transmembrane transport was not intensified, which was reflected by stable Mg^{2+}-ATPase activity during the exposure. An increase in activities of these marker enzymes of cellular respiration and gluconeogenesis was accompanied by the decrease in AcP activity – a lysosomal marker – in hepatocytes and Kupffer cells, indicating that intracellular digestion of endo- and exogenous substances diminished.

Morphological and histo-enzymatic changes in the pituitary gland of humans and animals subjected to GnRH analog treatment are poorly documented in the literature. However, in patients aged 60–78 years, receiving GnRH agonist goserelin therapy for a long time for metastasizing prostate carcinoma, clinical findings suggestive of proliferative histopathological pituitary changes (partial nodular hyperplasia) were observed [48]. In some cases, in the histopathological examination, the tissues consistent with a gonadotropinoma were recognized after being given a dose of leuprolide and goserelin [49,50]. In these cases hemorrhage within the gland was also observed, but there was no evidence of pituitary dysfunction in hormonal studies. These observations confirmed that GnRH agonists may cause adenomas in rat pituitary glands, as reported previously [48]. Our studies did not confirm histopathological changes in any part of the pituitary gland in rats treated for a long time with a low dose of the GnRH analogs dalarelin or cetrorelix. However, we observed functional changes in the adenohypophysis, indicating an inhibitory effect of analogs, mostly cetrorelix, on the energetic functions of pituitary cells and stimulatory effect of the antagonist on lysosomal activity in these cells. Direction of these changes, especially in the case of cetrorelix, is clearly opposite to that observed in liver cells and suggests the tissue-specific regulatory mechanism of enzyme activities by GnRH analogs.

CONCLUSIONS

In summary, we found that a dose of 6 μg/kg b.w. of the GnRH analogs dalarelin and cetrorelix can effectively inhibit ovulation in rats during 3-month exposure. The applied dose of analogs affected the morphology of the ovaries and uterus, changed histological appearance of the liver and altered some metabolic processes in the pituitary gland and

the liver (e.g., cellular respiration, gluconeogenesis and intracellular digestion). The influence of GnRH analogs was reflected by changes in the activities of ALT, AST, LDH and CK and biochemical parameters (cholesterol HDL fraction and TG concentration) of the blood, as well as changes in activities of marker enzymes. The activities of studied enzymes, both in the blood and the liver, as well as the range of morphological changes, indicate that although the perturbations induced by low doses of GnRH analogs are relatively small, they are sufficient to cause some functional consequences. Mechanisms and range of these changes was clearly dependent on the GnRH analog used – agonist or antagonist. Both morphological and enzymatic effects were more evident after antagonist administration; changes in the blood lipid profile were more evident after agonist administration. In case of both analogs, histological and enzymatic changes persisted long-term and they could modify the functions of pituitary, ovaries, uterus and liver, as well as other organs, even after discontinuation of the treatment.

List of abbreviations

AcP – Acidic phosphatase; **ALT** – Alanine aminotransferase; **AST** – Aspartate aminotransferase; **CK** – Creatine kinase; **FSH** – Follicle-stimulating hormone; **G6Pase** – Glucose-6-phosphatase; **GnRH** – Gonadoliberin; **GnRHR** – GnRH receptor; **LDH** – Lactate dehydrogenase; **LH** – Luteinizing hormone; **Mg^{2+}-ATPase** – Mg^{2+}-dependent ATPase; **NADH-TR** – NADH-tetrazolium reductase; **SDH** – Succinate dehydrogenase; **TG** – Triglycerides.

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