Effect of long-term treatment with low doses of the LHRH antagonist Cetrorelix on pituitary receptors for LHRH and gonadal axis in male and female rats

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Our previous studies showed that treatment of female rats with large doses of Cetrorelix, an antagonist of luteinizing hormone-releasing hormone (LHRH), reduces levels of serum LH, estradiol, progesterone, and the concentration of pituitary LHRH receptors (LHRH-Rs) and their mRNA expression. Serum LH and testosterone levels and pituitary LHRH-R in male rats are also decreased by high doses of Cetrorelix. This approach can be used for therapy of sex hormone-dependent cancers. However, in conditions where an incomplete hormone deprivation is indicated, lower doses of Cetrorelix may suffice. Thus, we investigated the effect of a 30-day treatment with a low-dose depot formulation of Cetrorelix (20–24 μ g per kg per day) on the pituitary-gonadal axis of male and female rats. In both sexes, lower serum LH levels were observed on day 4 after administration. In males, LH returned to control levels by day 10, whereas in females, a rebound LH elevation occurred. Testosterone levels in male rats were decreased up to day 20, but on day 30, the values were similar to controls. In females, serum estradiol was reduced on day 4; however, by day 10 it returned to normal. Progesterone levels were diminished through the entire period. Female rats showed diestrous smears during the first week of treatment and prolonged estrous periods thereafter. The weights of testes and ovaries were significantly lower, but not the weights of prostate, seminal vesicles, and uterus. Pituitary LHRH-R mRNA and LHRH-R protein levels were not significantly different from the controls. Thus, the treatment with low doses of Cetrorelix did not seriously impair gonadal functions. The results suggest that Cetrorelix in low doses induces only a partial pituitary-gonadal inhibition and might be indicated for treatment of endometriosis, leiomyomas, and benign prostatic hyperplasia.

luteinizing hormone-releasing hormone analog | luteinizing hormone-releasing hormone receptor

Luteinizing hormone (LH)-releasing hormone (LHRH) exerts its effect on pituitary gonadotropes through the high-affinity G protein-coupled LHRH receptors (LHRH-Rs) and stimulates the release of LH and follicle-stimulating hormone, which in turn regulate reproductive functions and sex steroid secretion (1–7). The responses to LHRH vary under different conditions and depend on the mode of application and the doses delivered to gonadotrope cells. Prolonged exposure to high doses of LHRH or its agonists leads to an inhibition of pituitary responses and the suppression of serum LH, follicle-stimulating hormone, and sex steroid levels (8–10). This effect is the result of the down-regulation of pituitary LHRH-Rs and inhibition of LHRH-R gene expression by mechanisms that have not been fully elucidated (11, 12). Chronic administration of LHRH agonists can produce reversible medical castration and is used clinically for treatment of sex hormone-dependent cancers and various gynecologic conditions (2, 6, 7, 10).

However, in clinical situations, in which an immediate suppression of gonadotropins is desired, LHRH agonists have the disadvantage of producing an initial stimulatory effect on hormone secretion. Therefore, the use of LHRH antagonists, which

cause a prompt and dose-related inhibition of LH and follicle-stimulating hormone release by competitive blockade of the LHRH-Rs is much more advantageous (6, 10, 13, 14). Cetrorelix is one of the most advanced antagonist produced to date and has been shown to be safe and effective in inhibiting LH and sex steroid secretion in many animal species and in various clinical settings in patients. Clinical trials in patients suffering from advanced carcinoma of the prostate, benign prostatic hyperplasia (BPH), and leiomyomas have already shown the efficacy of this modality of treatment with LHRH antagonists (7, 10, 13, 15–21). Cetrorelix and other LHRH antagonists are also successfully used in assisted reproductive technology. Thus, Cetrorelix is used in various protocols for *in vitro* fertilization and embryo transfer for blocking endogenous LH surges in women, before ovulation is induced with gonadotropins (10, 18). Cetrorelix is now available in long-acting pamoate formulations. Nevertheless, a complete suppression of sex steroids may not be necessary in indications such as uterine fibroma, endometriosis, and BPH. Additional investigations are required to elaborate new approaches and to determine the dose regimens of Cetrorelix for treatment of these conditions (22). In our previous study (23), we have shown that large doses of the LHRH agonist Decapeptyl and the antagonist Cetrorelix both greatly reduced the sex steroid concentrations and the weights of the reproductive organs, demonstrating an efficient blockade of pituitary-gonadal axis. However, Decapeptyl microcapsules elevated serum LH in female rats, but decreased LH levels in male rats, and the changes in LHRH-R mRNA expression did not parallel those of LHRH-R protein. On the other hand, the antagonist Cetrorelix reduced serum LH in both sexes and diminished LHRH-R mRNA and LHRH-R protein. The inhibitory effect of Cetrorelix on serum LH, LHRH-R mRNA, and LHRH-R protein in the pituitaries, was greater than that of Decapeptyl. In two other studies (24, 25), large doses of Cetrorelix lowered serum LH and reduced mRNA for pituitary LHRH-R in intact female or ovariectomized rats. Other investigations from our laboratory demonstrated that serum LH and testosterone levels and pituitary LHRH-Rs in male rats were strongly decreased by high doses of Cetrorelix (26–28). Because of the fact that in endometriosis, leiomyomas, BPH, and other conditions, only a partial inhibition of pituitary-gonadal axis may be necessary to obtain the desired clinical effect, the aim of the present study was to evaluate the long term effects of low doses of the depot formulation of Cetrorelix on LHRH-R mRNA and LHRH-R

Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone; LHRH-R, LHRH receptor; BPH, benign prostatic hyperplasia.

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protein, serum LH, sex steroid levels, and other endocrine parameters in male and female rats.

Materials and Methods

Peptides. Depot formulation of the LHRH antagonist Cetrorelix (Ac-D-Nal (2)¹, D-Phe(4Cl)², D-Pal (3)³, D-Cit⁶, D-Ala-¹⁰)LHRH (19) was supplied by Zentaris as Cetrorelix pamoate (D20762) and contained Cetrorelix peptide base and pamoic acid in a molar ratio of 2:1 and mannitol suspended in distilled water. The concentration of Cetrorelix pamoate in the suspension administered was 0.75 mg/ml.

Animals. Adult female and male Sprague–Dawley rats (Charles River Breeding Laboratories) were used, weighing 230–280 g at the start of the experiments. The animals were allowed standard rat diet and tap water ad libitum and were maintained under controlled conditions (24°C, 12-h light/12-h dark schedule). Vaginal smears were monitored daily and only rats showing two consecutive estrous cycles were used in the experiment.

In Vivo Experiments. A group of 11 male rats were treated with the suspension of Cetrorelix pamoate (0.71 mg/kg, 0.2 mg per rat) intramuscularly, whereas 13 female rats received similar injections at a dose of 0.625 mg/kg (0.15 mg per rat). The rats were randomly cycling at the start of the experiment. These doses, not considering the weight gain of the rats during the experiment, were estimated to release 23.7 and 20.8 µg/kg, respectively, daily for 30 days. Control female rats ($n = 44$) and male rats ($n = 10$) received vehicle only. Vaginal smears of female rats were controlled daily. Blood samples were taken from the jugular vein under isoflurane anesthesia before the experiment was started, and on days 4, 10, 20 (treated groups only), and 30 (or before decapitation). From the control females, 13 rats were randomly chosen for the blood sampling on days 0, 4, and 10. Serum was separated by centrifugation and stored at –20°C until assayed for LH, testosterone, or estrogen and progesterone. Treated and control males and the treated females were all killed in the morning of day 30 of the experiment, immediately after the last blood samples were collected. The control female rats were killed in four groups (in proestrus, estrus, diestrus I and II, $n = 11$ in each group) in the morning of the day closest to day 30. The testes, seminal vesicles, and prostate of male rats and the ovaries and uterus from females were removed and were weighed. Anterior pituitaries (five in each group) were homogenized in TRI Reagent (Sigma) and stored at –70°C for LHRH-R mRNA determination. The remaining pituitaries were frozen on dry ice and kept at –70°C for LHRH-R protein analysis.

RIA. Concentration of LH in the serum was determined by an RIA, using materials provided by A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, Torrance, CA): rLH-RP-3 (AFP-7187B), rLH-I-10 (AFP11536B), and anti-rLH-RIA-11 (AFP C697071P). Estradiol, progesterone, and testosterone were determined by using DSL-4800, DSL-3400, and DSL-4100 commercial kits (Diagnostic Systems Laboratories, Webster, TX).

Total RNA Extraction and RT-PCR Analysis. Total RNA was extracted from the pituitaries and the ovaries with the TRI Reagent following the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA and was then amplified by using the reagents and protocol of the GeneAmp RNA PCR core kit (Perkin–Elmer). Reverse transcription reaction and the PCR amplification were performed as described (23, 24) with the GeneAmp PCR System 2400 (Perkin–Elmer). The number of cycles was within the exponential range of PCR amplification, which in the pituitary was 18–19 cycles and 23–24 cycles for the β -actin and the LHRH-R, respectively. In the

ovary, the optimal cycle number was 15 for the β -actin and 20 for the LHRH-R, respectively. PCR products were separated by agarose gel electrophoresis, were stained with ethidium bromide, and were visualized under UV light. Bands of PCR-amplified products were analyzed semiquantitatively by using a Kodak DC290 zoom digital camera with an EDAS 290 imaging system (Kodak). The levels of rat LHRH-R mRNA products were normalized versus values of mRNA for rat β -actin and were expressed as percentage of the vehicle-treated controls.

Immunodetection of LHRH-Rs. Membrane fraction was prepared from rat pituitaries by the method described (23, 29). Membrane proteins (25 µg) were solubilized in 20 mM Tris-HCl buffer (pH 8.0) containing 10% (vol/vol) glycerol, 1% (wt/vol) SDS, 1 mM EDTA, and 1 mM DTT, and heated for 10 min. Proteins were resolved by SDS/10% PAGE and then were transferred to nitrocellulose sheets (Hoefer). The nitrocellulose sheets were soaked in Tris-buffered saline (10 mM Tris-HCl, pH 7.5/50 mM NaCl) containing 0.1% Tween 20. Excess protein-binding sites were saturated with (10 mM Tris-HCl, pH 7.5/50 mM NaCl) containing 0.1% Tween 20 containing 5% nonfat dried milk. The blotted membranes were incubated for 1 h at room temperature with goat polyclonal human LHRH-R antisera (Santa Cruz Biotechnology) diluted 1:1,000 in the (10 mM Tris-HCl, pH 7.5/50 mM NaCl) containing 0.1% Tween 20. The immunoreactive proteins were developed with peroxidase-conjugated anti-goat IgG antibodies (Santa Cruz Biotechnology) and were visualized by a chemiluminescence detection system (Pierce). The bands were analyzed with the imaging densitometer specified above and the relative protein levels were quantified.

Analysis of Data. Result are expressed as mean $\pm$ SEM. For statistical analysis of data, the computer software SIGMASTAT (Jandel, San Rafael, CA) was used. *In vivo* results were subjected to one-way ANOVA, followed by Bonferroni *t* test, and $P < 0.05$ was accepted as statistically significant.

Results

Effects of Low Doses of Cetrorelix Pamoate in Male Rats. The mean body weight of male rats treated with Cetrorelix at the start of the experiment was 280 ± 3.4 g, increasing to 426.4 ± 9.9 g on day 30, which was not different from the weight of the controls. As the rats received 0.71 mg/kg Cetrorelix pamoate on day 0 of the experiment, the estimated daily release of Cetrorelix was reduced from 23.7 µg per kg per day on day 1 to 15.7 µg per kg per day on day 30.

In the group treated with Cetrorelix, serum LH levels were decreased significantly by $>50\%$ on day 4 ($P < 0.01$), but returned to control levels on the subsequent blood sampling on day 10 and were not significantly different from the controls at later time points (Fig. 1*a*). Serum testosterone was reduced to 27% of the control value on day 4 ($P < 0.01$) and remained at this low level at least until day 20 of the experiment. However, on day 30, the serum testosterone concentrations returned to the control values (Fig. 1*b*). In the Cetrorelix-treated group, the weights of the prostate and seminal vesicles were smaller than those of the controls, but only the weight of the testes was significantly decreased ($P < 0.05$, Fig. 1*c*).

At the end of the experiment, the mRNA expression for pituitary LHRH-R in the group treated with Cetrorelix was not different from that in the control group (Fig. 2*a* and *c*). The LHRH-R protein levels in the Cetrorelix group were elevated by $>30\%$, as compared with the controls, but this difference was not significant (Fig. 2*b* and *d*).

Effects of Low Doses of Cetrorelix Pamoate in Female Rats. The mean body weight of the Cetrorelix-treated female rats increased from the initial 237.8 ± 4.9 g to 272.7 ± 7.3 g on day 30, and a similar

Fig. 1. Effects of low doses of Cetorelix pamoate on serum hormone levels in male rats during the treatment and on sex organ weights at the termination of the experiment. Shown are serum LH (a), serum testosterone (b), and sex organ weights (c) at the termination of the experiment. *, $P < 0.05$; **, $P < 0.01$ versus control.

rise occurred in the controls. As the rats received 0.625 mg/kg Cetorelix pamoate on day 0 of the experiment, this weight gain reduced the estimated daily release of Cetorelix from 20.8 μ g per kg per day on day 1 to 18.3 μ g per kg per day on day 30.

Vaginal smears were regularly followed during the 30-day period. The control rats all had regular cycles. Treated female rats that were in proestrus on the day of the Cetorelix injection went into estrus the next day, but thereafter had a prolonged diestrus. The rats in estrus, diestrus I, or II phase, all had a prolonged (4–6 days) diestrus phase after the injection. From all smears taken between days 1 and 5, only 6.2% were estrous smears. After day 6, there was a change in the vaginal smears: estrous smears dominated in most rats treated with Cetorelix. Between days 6 and 30, 75.5% of the smears examined showed estrus or occasionally proestrus (Table 1).

Depot Cetorelix significantly decreased LH levels on days 4 and 10, as compared with untreated controls on the same days ($P < 0.01$). On day 20, there was a substantial rebound-like increase in LH levels in most rats treated with Cetorelix with high individual variations. The LH levels on day 30 were above the average of the combined control values (calculated from the data of all control rats in four stages of the estrus cycle); nevertheless, the difference was not significant (Fig. 3a and a1). Estradiol levels in the group given Cetorelix were significantly reduced ($P < 0.01$) by 30% on day 4 of the experiment, but returned to control values after day 10 (Fig. 3b and b1). Serum progesterone in the rats that received Cetorelix were diminished

Fig. 2. Effects of low doses of Cetorelix pamoate on pituitary LHRH-R mRNA expression and LHRH-R protein levels in male rats after 30 days of treatment. (a) RT-PCR products of the pituitary LHRH-R mRNA and β -actin mRNA. M, 100-bp DNA molecular weight marker; lanes 1–4, Cetorelix pamoate; lanes 5–8, control. (b) Immunodetection of LHRH-R protein. Lanes 1–3, control; and lanes 4–6, Cetorelix. (c) Densitometric analyses of LHRH-R mRNA levels. (d) LHRH-R protein levels after 30 days. NS, not significant.

by >50% on day 4 and remained at this significantly lower level ($P < 0.01$) through the entire experiment (Fig. 3c and c1).

The mean weight of the uterus in female rats treated with Cetorelix was not different from the average uterine weight of control rats. The weights of ovaries of rats that received Cetorelix were decreased by >30%, as compared with those of the controls ($P < 0.01$, Fig. 3d).

The mRNA expression for pituitary LHRH-R of female rats treated with Cetorelix pamoate did not differ from the values in intact females (Fig. 4a and d). LHRH-R protein levels showed marked differences at different stages of the estrous cycle, being the lowest at estrus and the highest on the morning of proestrus (Fig. 4b and e). The content of the pituitary LHRH-R protein of the Cetorelix-treated rats was comparable with the values found in untreated rats at estrus (Fig. 4c and e). mRNA for ovarian LHRH-R was found to be significantly higher in the Cetorelix-treated rats than in the diestrus-I and estrus controls ($P < 0.01$), and this elevation was close to significance versus the diestrus II group ($P = 0.07$) and proestrus group ($P = 0.056$, Fig. 5).

Discussion

Previous studies and our former results (2, 7, 15, 16, 23–28) demonstrated that large doses of Cetorelix such as 0.3–0.5 mg per kg per day down-regulate pituitary LHRH-R in male and female rats. Such a down-regulation could take place in a clinical setting in patients with prostate cancers treated with large doses of the antagonists (2, 7, 15, 16). A down-regulation of pituitary LHRH-Rs may have occurred also in male volunteers treated with large doses (10 mg/day) of Cetorelix for 5 days (30).

Table 1. Vaginal smears of female rats treated with Cetrorelix pamoate during the 30-day period of the experiment

	Diestrus I and II: 93.8%						Proestrus and estrus: 75.5%																		
	Day 0	Day 4					Day 10					Day 20					Day 30								
I	—	—*	—*	—*	—*	—*	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
II	2	—	—	—*	—*	—*	—*	1	2	2	—	2	2	2	2	2	2	2	2	—	—	1	2	2	—
III	—	—	—*	—*	—*	—*	—*	2	2	—	2	2	—	1	2	—	—	Died							
IV	—	—*	—*	—*	—*	—*	2	2	—	—	1	2	—	2	—	2	—	2	2	2	2	2	2	2	—
V	1	2	—	—	—*	—*	—*	1	2	2	—	2	2	2	2	2	—	2	2	—	—	2	2	2	—
VI	1	2	—	—	—*	—*	1	2	2	—	—	2	2	—	1	2	—	—	2	2	—	—	2	2	2
VII	2	—	—	—*	—*	—*	—*	2	2	—	—	—	2	2	1	2	2	2	2	2	2	1	2	—	
VIII	1	2	—	—	—*	—*	—*	1	2	—	—	—	2	—	—	2	—	2	2	2	2	2	2	2	2
IX	2	—	—	—*	—*	—*	1	2	2	—	—	1	2	—	1	2	2	2	2	2	—	2	2	2	2
X	—	—	—*	—*	—*	—*	—*	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	—	
XI	1	2	—	—	—*	—*	2	2	2	2	2	2	2	1	2	2	2	2	2	—	2	—	2	2	2
XII	—	—	—*	—*	—*	—*	—*	—*	1	2	2	—	—	2	2	2	2	2	2	2	2	1	2	2	2
XIII	2	—	—	—*	—*	—*	1	2	2	—	2	2	2	2	—	2	2	2	2	2	2	2	2	2	2

Day 0: day of treatment. Smears on days 1–5 showed in 93.8% diestrus I or II, whereas 75.5% of the smears were estrus- and occasionally proestrus-like between days 6–30. 1, proestrus; 2, estrus; —, diestrus-I; —*, diestrus-II.

*Days when the extra diestrus smears were observed.

However, for indications in which the purpose of the therapy with Cetrorelix is a temporary blocking of the pituitary LH surges, such as in the case of *in vitro* fertilization and embryo transfer, smaller doses of Cetrorelix and other antagonists are applied (18). An incomplete inhibition of the pituitary gonadal axis and partial decrease in sex steroid levels might be also indicated in BPH, endometriosis and leiomyomas, and other conditions (10, 15, 16, 22, 31–33). Thus, in BPH patients it would be important to improve urinary symptoms without a suppression of testosterone to castration levels and other related side effects (2, 7, 10, 16, 34).

The lower dose of Cetrorelix (20–24 μ g per kg per day) used in this study appears to meet these goals. In male rats, testosterone levels were decreased by 50–60% up to day 20 of the study. However, castrations levels, which would involve a >90% decrease, were not induced. LH levels were minimally and not significantly reduced, except for day 4, during a period of probably more intense release of the active compound from the depot formulation. The reduction in the weights of the reproductive organs was also minimal. In addition, we found no substantial differences in the LHRH-R mRNA and protein levels in the Cetrorelix-treated groups versus the controls at the end of the experiment. This finding suggests that even if there was a decrease in the LHRH-R expression at some time during the treatment, such a reduction would be likely only transient. Although the depot formulation of Cetrorelix pamoate releases the active compound for $\approx$ 30 days, there is most probably a higher release in the first few days after administration (35).

When experiments are carried out in female rats, especially with agents influencing the reproductive system, it is always difficult to decide which phase of the estrous cycle should be used as control. For the blood sampling on days 0, 4, and 10, we used random cycling rats, but at the end of the 1-month period, four distinct groups of rats were killed at different phases of the cycle, between 9 and 10 a.m. This result also means that the pronounced changes which occur in the afternoon of proestrus could not be measured and detected in our experiments. However, with four groups of control rats, we could make a better evaluation of the results in the Cetrorelix-treated rats.

The effects of the uneven release of Cetrorelix pamoate from the depot preparation could be observed also in female rats. After the initial administration of Cetrorelix, the vaginal smears of all rats showed a prolonged diestrus II phase and then changed

to mostly estrous smears in the second week. These changes were likely induced by the higher release of Cetrorelix in the initial period, because it is known that high doses of LHRH antagonists cause constant diestrus smears (23), whereas low doses that are still sufficient to prevent ovulation are followed by prolonged estrous periods (36). These estrous periods are not equivalent to the true estrous phase and they are rather comparable with the anovulatory cycles in women. Similarly, the non-estrous-like smears during the last 20 days of the study were not real diestrus I or II smears, because they were not accompanied by elevated progesterone levels. The lack of ovulation is also supported by the permanent reduction of progesterone levels, and by the fact that on termination of the experiment, we did not see corpora lutea in the ovaries.

On day 4, probably because of the initial higher release of Cetrorelix, serum LH and estrogen levels were decreased. Thereafter, estrogen levels were not different from those in the random cycling rats, but the LH concentrations showed an interesting rebound effect, the serum levels of LH being much higher than those in the controls on day 20. The high SEM of these values is most likely due to the fact that the rebound peak did not reach its maximum at the same time in all of the rats. This rebound could be the consequence of the decreasing level of Cetrorelix concentrations in blood. An appropriate, but relatively low dose of an LHRH antagonist was shown to prevent LH release and cause accumulation of LH in the pituitary (36). When the effective dose of Cetrorelix is even lower, it can no longer prevent the release of LH from the pituitary, and the accumulated LH is liberated into the circulation.

mRNA levels for pituitary LHRH-R in rats treated with Cetrorelix did not show any differences versus the values found in various phases of the cycle. LHRH-R protein levels were similar to those in control rats in estrus, being significantly lower than the concentrations in control rats in diestrus II or the morning of proestrus.

Interestingly, mRNA for the ovarian LHRH-R was found to be significantly elevated. During a regular cycle, ovarian LHRH-Rs may be activated by locally produced LHRH (37), because the hypothalamic peptide is not present in the peripheral circulation in concentrations sufficient to influence ovarian receptors. The ovarian LHRH-Rs show cyclic changes (38, 39) that were also detected in our study. It was similarly demonstrated that the highest concentrations of LHRH-R mRNA are

Fig. 3. Effect of low doses of Cetrorelix pamoate on serum hormone levels in female rats during the treatment and weights of the uterus and ovary at the termination of the experiment. (a) Serum LH concentrations in the Cetrorelix pamoate-treated group and control group in random cycles. (a1) Serum LH in control female rats at different stages of the estrous cycle on day 30. (b) Serum estradiol levels in the Cetrorelix pamoate-treated group and control group in random cycles. (b1) Serum estradiol in control female rats at different stages of the estrous cycle on day 30. (c) Serum progesterone levels in the Cetrorelix pamoate-treated group and control group in random cycles. (c1) Serum progesterone in control female rats at different stages of estrous cycle on day 30. (d) Weights of uterus and ovaries on day 30. Wide bars show weights of these organs in the Cetrorelix pamoate-treated group and control group in random cycles, narrow bars show weights of uterus and ovary, at different stages of estrous cycle on day 30. **, $P < 0.01$ versus control; DI, diestrus-I; DII, diestrus-II; P, proestrus; E, estrus.

found in large follicles and atretic follicles (38, 39). In this article, the ovaries were polyfollicular, most probably many being atretic and there were no corpora lutea. This finding could logically explain the rise in ovarian LHRH-R mRNA.

Fig. 4. Effects of low doses of Cetrorelix pamoate on pituitary LHRH-R mRNA expression and LHRH-R protein levels in female rats after 30 days of treatment. (a) RT-PCR products of the pituitary LHRH-R mRNA and β -actin mRNA. M, 100-bp DNA molecular weight marker; lanes 1–3, proestrus; lanes 4–6, estrus; lanes 7–9, diestrus-I; lanes 10–12, diestrus-II, and lanes 13–15, Cetrorelix pamoate. (b) Immunodetection of LHRH-R in different stages of the estrous cycle. Lanes 1–3, estrus; lanes 4–6, diestrus-I; lanes 7–9, diestrus-II; and lanes 10–12, proestrus. (c) Immunodetection of LHRH-R. Lanes 1–3, rats in estrus; and lanes 4–6, Cetrorelix pamoate. (d) Densitometric analyses of LHRH-R mRNA levels after 30 days. The wide filled bar shows the treated group. Narrow bars show data of control rats at different stages of the estrous cycle. NS, not significant versus control estrus and diestrus-I value; **, $P < 0.01$ versus control proestrus and diestrus-II value. DI, diestrus-I; DII, diestrus-II; P, proestrus; E, estrus.

A high dose of Cetrorelix and other antagonists, even when administered as depot formulations, might produce multiple side effects such as impotence in male patients and amenorrhea and hot flushes in women. Such regimens of LHRH antagonists, which involve a profound deprivation of sex steroids, are suitable for therapy of malignant neoplasms. However, for treatment of BPH and benign gynecologic disorders, a low-dose treatment with a depot formulation of LHRH antagonists might produce the desired clinical result without major endocrine side effects. Under these conditions, the clinical goals would be achieved with a minimal effect on the reproductive endocrine system and fast restoration of gonadal functions after cessation of the treatment.

In conclusion, our results indicate that low doses of Cetrorelix, released from once administered depot pamoate formulation, caused only a temporary inhibition of serum LH and sex steroid concentration in male and female rats and did not markedly impair gonadal function or alter pituitary LHRH-R mRNA and

Fig. 5. Effects of low doses of Cetrorelix pamoate on LHRH-R mRNA expression in the ovary in female rats after 30 days of treatment. (a) RT-PCR products of the ovarian LHRH-R mRNA and β -actin mRNA. Lanes 1–4, proestrus; lanes 5–8, estrus; lanes 9–12, diestrus-I; lanes 13–16, diestrus-II, and lanes 17–20, Cetrorelix pamoate. (b) Densitometric analyses LHRH-R mRNA levels after 30 days. The wide filled bar shows the treated group. Narrow bars show the mRNA for ovarian LHRH-R of control rats at different stages of the estrous cycle. **, $P < 0.01$ versus Cetrorelix group; DI, diestrus-I; DII, diestrus-II; P, proestrus; E, estrus.

LHRH-R protein levels. Our findings suggest that in a clinical setting, low doses of Cetrorelix and other LHRH antagonists, released from depot preparations would induce only a partial inhibition of pituitary-gonadal axis without a marked down-regulation of pituitary LHRH-Rs. This transient suppression of pituitary-gonadal function by low doses of Cetrorelix could

provide suitable alternate approaches to the treatment of BPH, endometriosis, and leiomyomas.

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