

GnRH Antagonist-induced Down-regulation of the mRNA Expression of Pituitary Receptors: Comparisons with GnRH Agonist Effects

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Abstract. In order to compare the mechanism for the down regulation of the mRNA expression of pituitary receptors induced by GnRH antagonist (GnRHant) to that by GnRH agonist (GnRHa), we examined the effects of GnRHant (Cetrorelix, 333 µg/kg/day), GnRHa (leuprolide depot, 333 µg/kg), and GnRHant combined with GnRHa on LH response to exogenous GnRH, pituitary LH content, LHβ subunit mRNA, and GnRH receptor (GnRH-R) mRNA levels at 2, 5, 24, 72 hours, and 7 days after the treatment in ovariectomized rats. GnRHant significantly decreased serum LH, the LH response of the pituitary to exogenous GnRH, and the pituitary LH content compared to the control treatment, though GnRHa significantly increased serum LH. GnRHant with GnRHa significantly diminished the GnRHa-induced flare-up phenomenon. GnRHant significantly decreased LHβ mRNA and GnRH-R mRNA levels, but the magnitude of the decrease in these mRNA levels by GnRHant was significantly less than those by GnRHa until 72 hours following treatment. Prolonged treatment of GnRHant caused a marked inhibition of LHβ mRNA and GnRH-R mRNA expression, similar to that caused by GnRHa. Combination treatment with GnRHa and GnRHant was demonstrated to decrease LHβ mRNA and GnRH-R mRNA levels as much as GnRHa alone and GnRHant alone over 7 days of the treatment. The present study showed differences between GnRHant and GnRHa treatment in the reduction of GnRH-R mRNA levels up to 72 hours after the treatment, and indicated that the suppression of GnRH-R mRNA by GnRHant was the maximal by GnRHa 7 days after the treatment because more profound suppression was not observed upon additional treatment with GnRHa. The findings in the present study support the hypothesis that the mechanism by which GnRHant leads to down-regulation of the mRNA expression of pituitary receptors is similar to that of GnRHa.

Key words: GnRH antagonist, GnRH agonist, GnRH-R mRNA, LHβ subunit mRNA, Female rat

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GnRH is a decapeptide that plays an important role in the regulation of LH and FSH secretion from the anterior pituitary. The synthesis and release of these two pituitary glycoproteins are controlled by the complex interaction of multiple factors, including the frequency and

amplitude of pulsatile GnRH secretions. For example, at low concentrations, a pulsatile GnRH treatment causes up-regulation of the GnRH receptor (GnRH-R), whereas at high concentrations, a continuous GnRH treatment reduces the number of GnRH-R, and results in the desensitization of the pituitary gonadotropins [1]. In addition, administration of GnRH agonist (GnRHa) induces a transient rise in gonadotropin secretions, known as a flare-up phenomenon [2], and thereafter gonadotropin secretions decline steadily. This flare-up phenomenon is problematic for patients

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receiving GnRHa treatment because of its side effects such as a worsening of estrogen dependent diseases.

In contrast to GnRHa, since GnRH antagonist (GnRHant) inhibits gonadotropin secretions without exhibiting a flare-up phenomenon, this ability of GnRHant to induce hypogonadotropic hypogonadism led to its use in clinical situations for the treatment of estrogen-dependent diseases, and its introduction into protocols for assisted reproductive technologies [3]. Although GnRHant treatment is potentially useful for reducing gonadotropin secretions since it does not exhibit the flare-up phenomenon, chronic GnRHant administration remains problematic at the moment. This is because GnRHant causes local reactions such as erythema and itching [3]. In addition a suitable sustained delivery system for GnRHant, such as GnRHant depot, is not currently available. Instead, a combination therapy of GnRHa with GnRHant may be another choice for clinical use in estrogen-dependent diseases such as endometriosis and uterine leiomyoma at present. However, comparison between GnRHant and GnRHa on the mechanism of down-regulation of the mRNA expression of pituitary receptors is not clearly understood, and the interactions between GnRHa and GnRHant on gonadotropin secretions have not yet been established.

In order to compare the mechanism for the down-regulation of the mRNA expression of pituitary receptors induced by GnRHant to GnRHa, we therefore examined the effects of GnRHant, GnRHa, and GnRHant with GnRHa treatment on LH response to exogenous GnRH, pituitary LH content, LH β subunit mRNA and GnRH-R mRNA levels in ovariectomized rats.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (obtained at 7 weeks of age from Charles River, Yokohama, Japan) were maintained at a constant temperature of 24–26°C under controlled lighting conditions (lights on from 0500 to 1900 h) with food and water available *ad libitum*. Adult female rats were ovariectomized at the age of 8 weeks, and were used 4–9 weeks later. All animal housing and surgical procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Yokohama City University School of Medicine.

Experimental protocols

Groups of 5–10 rats were subcutaneously injected with 333 μ g/kg/day of GnRHant (250 μ g/ml, Cetrorelix acetate, Asta Medica, AG), 333 μ g/kg of GnRHa depot (940 μ g/ml, Leuprolide acetate, Takeda Chemical Ind., Ltd. Osaka, Japan), or GnRHant together with GnRHa. The effects of GnRHant, GnRHa and GnRHant with GnRHa were examined on serum LH, serum FSH, LH response to exogenous GnRH, pituitary LH content, LH β subunit mRNA and GnRH-R mRNA levels at 2, 5, 24, 72 hours and 7 days after the treatment, as shown in the figure of experimental protocols (Fig. 1). Control rats were injected with vehicle only. Cetrorelix acetate was dissolved in 0.3 N acetic acid solution and Leuprolide acetate was dissolved in 5% mannitol solution.

The rats were sacrificed by decapitation and trunk blood was collected. Serum was stored at –20°C until assayed. For RNA extraction, pituitaries were harvested under sterile conditions, and were immediately frozen in liquid nitrogen and stored at –70°C. To assess

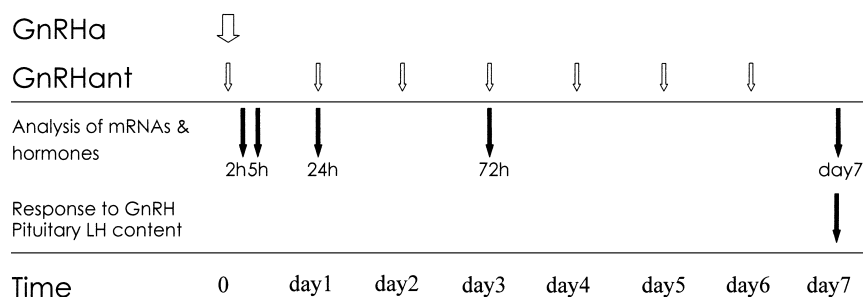


Fig. 1. The figure of experimental protocols

the pituitary response to exogenous GnRH following seven days of treatment, blood samples were obtained by heart puncture under light ether anesthesia before and 30 minutes after subcutaneous injection of 1 µg native GnRH (Lutamin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). For determination of pituitary LH content, pituitary glands were homogenized in distilled water and the supernatants were stored at -20°C.

Northern blot analysis

Total RNA was extracted from rat pituitaries using the RNeasy Mini kit (QIAGEN Inc., Tokyo, Japan). Levels of LHβ subunit mRNA were determined by Northern blotting with the probe for LHβ-subunit (5'-GCCGGCACAGATGCTGGTGGTGAA-3') which was end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase as described previously [2]. Levels of GnRH-R mRNA were determined by Northern blotting with ³²P-labeled GnRH-R single-stranded DNA probes as described previously [4]. The sequences of primers are as follows:

N-primer:

5'-GCCAAAATCATCTTTGCTCTCACG-3'

C-primer:

5'-GAATGCGACTGTCATCTTTAGCGT-3'

Radioimmunoassay and statistical analysis

Concentrations of LH and FSH in blood and LH in pituitary extracts were measured by double antibody radioimmunoassay with materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and generously donated by Dr. K. Wakabayashi (Gunma University, Maebashi, Japan). The reference standard was NIDDK rat LH-RP-3 for LH and NIDDK rat FSH-RP-3 for FSH, but the amounts of LH and FSH are expressed in terms of NIH LH-S1 and NIH FSH-S1, respectively. All data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's PLSD post-hoc test, and significance was attained at $p < 0.05$.

Results

Effects of GnRHant, GnRHa, and GnRHant together with GnRHa on serum LH and FSH

As shown in Fig. 2 and 3, significant differences in serum LH and FSH among the 4 groups at each time point were observed by ANOVA ($p < 0.01$). Compared to controls, GnRHa treatment significantly increased serum LH from 2 to 5 hours after treatment (*Post Hoc*, $p < 0.01$), and significantly decreased it by 7 days after treatment (*Post Hoc*, $p < 0.01$). GnRHant treatment, on the other hand, significantly decreased serum LH from

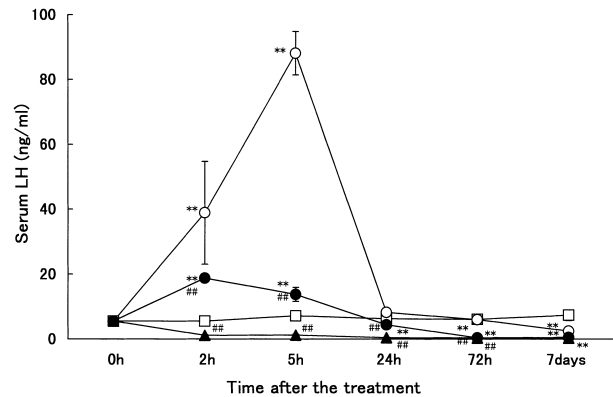


Fig. 2. Changes of serum LH after GnRH analog treatment in ovariectomized rats. (mean \pm SEM, $n = 5$) ##: $p < 0.01$ vs GnRHa; **: $p < 0.01$ vs controls. Ovariectomized female rats were treated with GnRHant (▲), GnRHa (○), GnRHant together with GnRHa (●), or vehicle only as the control (□).

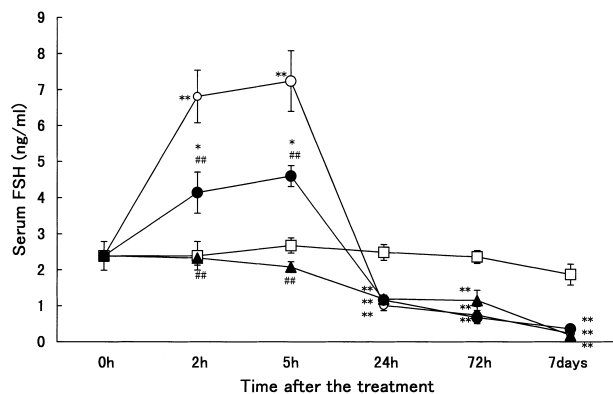


Fig. 3. Changes of serum FSH after GnRH analog treatment in ovariectomized female rats. (mean \pm SEM, $n = 5$) ##: $p < 0.01$ vs GnRHa; *: $p < 0.05$, **: $p < 0.01$ vs controls. Ovariectomized female rats were treated with GnRHant (▲), GnRHa (○), GnRHant together with GnRHa (●), or vehicle only as the control (□).

24 hours to 7 days after treatment (*Post Hoc*, $p < 0.01$) without showing any increase in serum LH as observed with GnRHa. GnRHant treatment together with GnRHa significantly inhibited the GnRHa-induced transient increase in serum LH from 2 to 5 hours (*Post Hoc*, $p < 0.01$), but did not prevent the GnRHa-induced decrease in serum LH observed at 7 days (Fig. 2).

GnRHant treatment significantly decreased serum FSH from 24 hours to 7 days compared to the control (*Post Hoc*, $p < 0.01$), without showing any increase in serum FSH as was evident in GnRHa treatment. In addition, GnRHant together with GnRHa diminished the GnRHa-induced transient increase in serum FSH (*Post Hoc*, $p < 0.01$), but did not prevent the GnRHa-induced decrease in FSH observed at 7 days (Fig. 3).

Effects of GnRHant, GnRHa, and GnRHant together with GnRHa on LH responses to GnRH and LH content of the pituitary

In the concentrations and net increases of serum LH at 30 minutes after GnRH administration, a significant difference among the 4 groups was observed by ANOVA ($p < 0.01$). After seven days' treatment, the concentrations and net increases in serum LH at 30 minutes following GnRH injection were significantly decreased (*Post Hoc*, $p < 0.01$) in both GnRHant- and GnRHa-treated rats compared to controls. In addition, GnRHant treatment together with GnRHa did not affect this inhibition of the LH response, indicating

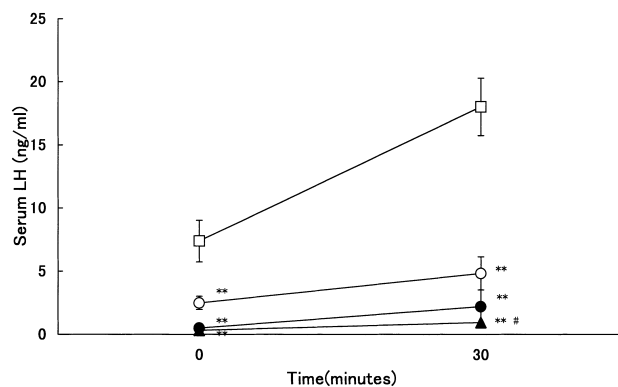


Fig. 4. Responses of serum LH to GnRH (1 μ g i.v.) 7 days after GnRH analog treatment in ovariectomized rats. (mean \pm SEM, $n = 9$) #: $p < 0.05$ vs GnRHa; **: $p < 0.01$ vs controls

Ovariectomized female rats were treated with GnRHant (▲), GnRHa (○), GnRHant together with GnRHa (●), or vehicle only as the control (□).

that GnRHant treatment did not affect GnRHa-induced desensitization of the pituitary to GnRH (Fig. 4).

A significant difference in the LH content of the pituitary was observed among the 4 groups by ANOVA ($p < 0.05$). After seven days' treatment, GnRHant treatment (*Post Hoc*, $p < 0.05$) as well as GnRHa treatment (*Post Hoc*, $p < 0.01$) significantly decreased the LH content of the pituitary compared to control, and GnRHant together with GnRHa did not prevent the GnRHa-induced decrease in LH content (Fig. 5).

Effects of GnRHant, GnRHa, and GnRHant together with GnRHa on LH β mRNA and GnRH-R mRNA

The levels of LH β mRNA and GnRH-R mRNA were examined 7 days after the treatment. Significant differences in LH β mRNA and GnRH-R mRNA levels among the 4 groups at each time point were observed by ANOVA ($p < 0.01$). GnRHant treatment significantly decreased LH β mRNA levels from 2 hours to 7 days after the treatment (*Post Hoc*, $p < 0.01$), as did GnRHa treatment (*Post Hoc*, $p < 0.01$), but the magnitude of the inhibitory effect of GnRHant on LH β mRNA levels was significantly less than that of GnRHa treatment from 2 to 5 hours after the treatment (*Post Hoc*, $p < 0.05$). GnRHant treatment together with GnRHa significantly diminished the GnRHa-induced inhibition of LH β mRNA levels from 2 (*Post Hoc*, $p < 0.01$) to 72 hours (*Post Hoc*, $p < 0.05$), but did not reduce it at 7 days after the treatment (Fig. 6).

GnRHant treatment significantly decreased GnRH-R mRNA levels from 2 hours to 7 days compared to control (*Post Hoc*, $p < 0.01$). As with the changes in LH β mRNA levels, the magnitude of the inhibitory effect of

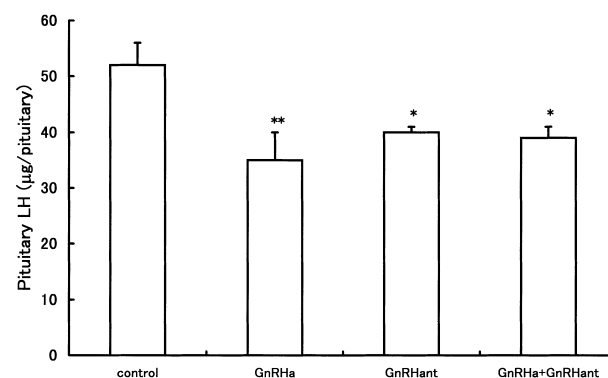


Fig. 5. Pituitary LH content 7 days after GnRH analog treatment in ovariectomized female rats. (mean \pm SEM, $n = 6$). *: $p < 0.05$, **: $p < 0.01$ vs controls

GnRHant on GnRH-R mRNA levels was significantly less than that of GnRHa treatment from 2 (*Post Hoc*, $p < 0.01$) to 72 hours (*Post Hoc*, $p < 0.05$). GnRHant treatment together with GnRHa diminished the GnRHa-induced inhibition of GnRH-R mRNA levels from 2 (*Post Hoc*, $p < 0.05$) to 24 hours (*Post Hoc*, $p < 0.05$), but the GnRHa-induced decrease of GnRH-R mRNA levels was not prevented by combined GnRHant treatment with GnRHa at 7 days after treatment (Fig. 7).

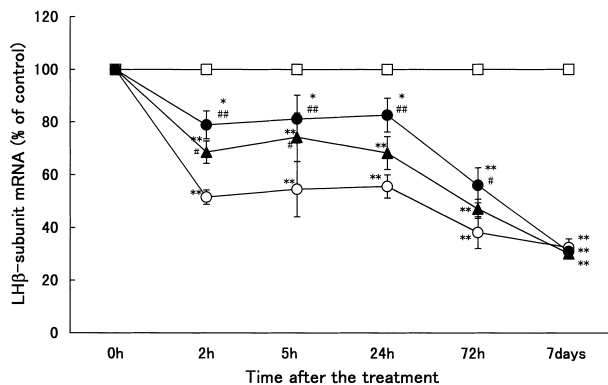


Fig. 6. Changes of LH β mRNA levels after GnRH analog treatment (mean \pm SEM, $n = 5$). #: $p < 0.05$, ##: $p < 0.01$ vs GnRHa; *: $p < 0.05$, **: $p < 0.01$ vs controls. Ovariectomized female rats were treated with GnRHant (▲), GnRHa (○), GnRHant together with GnRHa (●), or vehicle only as the control (□). The levels of LH β mRNA are expressed as a percentage of the control values.

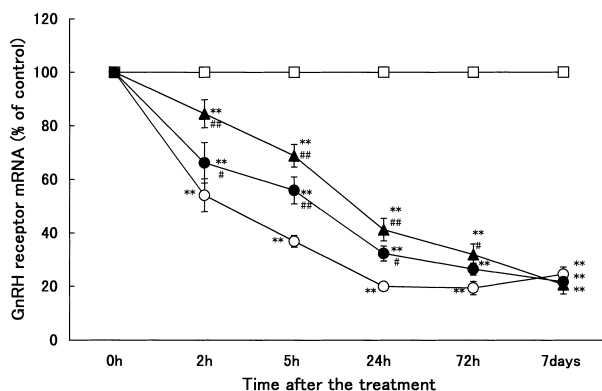


Fig. 7. Changes of GnRH-R mRNA levels after GnRH analog treatment in ovariectomized rats. (mean \pm SEM, $n = 5$). #: $p < 0.05$, ##: $p < 0.01$ vs GnRHa; **: $p < 0.01$ vs controls. Ovariectomized female rats were treated with GnRHant (▲), GnRHa (○), GnRHant together with GnRHa (●), or vehicle only as the control (□). The levels of GnRH-R mRNA are expressed as a percentage of the control values.

Discussion

In a clinical study, the dose of GnRHant used was 10–30 times as much as the dose of GnRHa [3], therefore, 333 $\mu\text{g/kg}$ BW/day of GnRHant and 333 $\mu\text{g/kg}$ BW/4 weeks of GnRHa depot were used in the present study. This dose of GnRHant is similar to that reportedly used by Pinski *et al.* [5] and Kovacs *et al.* [6]. Changes in LH β mRNA during GnRHa treatment almost coincided with those described in our previous reports on immature female rats [2, 7] and castrated male rats [8], and the decrease in LH β mRNA levels during prolonged GnRHant treatment was also similar to that reported previously [9]. LH β gene expression is suggested to be primarily regulated by a direct effect of GnRH [9].

There are several reports concerning changes in pituitary GnRH-R mRNA during GnRHant treatment. Regarding GnRHant treatment alone, the result of the present study is in accord with these reports. However, the previous studies investigated changes in GnRH-R mRNA levels only at one [5, 10] or two [6] time points after GnRHant treatment, and did not examine effects of GnRHant treatment combined with GnRHa. To clarify the differences between GnRHant and GnRHa on the reduction of GnRH-R mRNA concentration, we therefore examined the effects of GnRHant treatment on GnRH-R mRNA at multiple time points after the treatment, and compared GnRHant treatment with GnRHa treatment as well as GnRHa treatment in combination with GnRHant.

We found that the magnitude of the decrease in GnRH-R mRNA levels induced by GnRHant treatment was less than that of GnRHa treatment from 2 to 72 hours following the treatment, though prolonged administration of GnRHant caused a marked inhibition of GnRH-R mRNA expression, similar to that caused by GnRHa.

The reduction of GnRH-R expression by GnRHa involves the internalization and degradation of the agonist-occupied receptor [11], and GnRH-R mRNA decreased after the administration of long-acting GnRHa [12]. GnRHant treatment has been demonstrated to produce a considerable down-regulation of GnRH-R with prolonged treatment [13]. Not merely the occupancy of GnRH binding sites by GnRHant but also a time-dependent decrease in the concentration of GnRH-R has been demonstrated, using an *in vitro* method to disassociate Cetrorelix from its receptors

with chaotropic agents [14]. GnRHant did not influence the mRNA expression of the GnRH-Rs in the superfusion system [6], and the down-regulatory effect of GnRHant seems to result from a competitive inhibition of the stimulatory effect of endogenous GnRH.

The apparently opposing impact of GnRHant on GnRHa-induced down-regulation of pituitary receptors raises interesting questions regarding the potential effects of concurrent administration. Regarding the impact of GnRHant on the GnRHa-induced flare-up phenomenon, GnRHant partially inhibited the initial flare-up caused by GnRHa treatment in this study, and Pinski *et al.* [15] described that pretreatment with GnRHant (500 or 1000 µg/rat) 1 hour before GnRHa (50 µg/rat) in male rats could completely prevent the elevation of LH. However, GnRHant did not diminish GnRHa-induced gonadotropin desensitization during prolonged treatment in the present study. Illions *et al.* [16] demonstrated that the presence of GnRHant reduced the extent of gonadotropin desensitization 8 days after the treatment. These data are not consistent with our present findings. However, in their study, the dose of GnRHa used was quite low, which was equivalent to approximately one fiftieth the normal dose given to humans on a weight-adjusted basis. Moreover, GnRHa was administered to animals pretreated for 1 week with GnRHant. These differences in the experimental protocol may explain the differing effects of GnRHant on GnRHa-induced desensitization between Illions' work and our study.

Currently, no data are available regarding the impact of concomitant GnRHant treatment on the GnRHa-induced reduction of GnRH-R mRNA levels. In the present study, GnRHant treatment partially diminished the GnRHa-induced suppression of GnRH-R mRNA for a short period after the treatment. During long-term

treatment, combined treatment with GnRHa and GnRHant was demonstrated to decrease GnRH-R mRNA as much as either GnRHa or GnRHant alone. These findings indicate that the suppression of GnRH-R mRNA by GnRHant is the maximal inhibition by GnRH analogues during long-term treatment because more profound suppression was not observed upon additional treatment with GnRHa. These present findings support the hypothesis that the mechanism by which GnRHant leads to down-regulation of the mRNA expression of pituitary receptors is similar to that of GnRHa, though the internalization and degradation of bound antagonist-receptor complex needs further investigation to confirm whether the mode of action of GnRHant varies in some respects from that of GnRHa.

GnRHants are thought to act on the same receptor sites as GnRH [17]. The GnRH-R might be gradually saturated by prolonged treatment, becoming effective in time, because of the high binding affinity of GnRHant [6]. Our data demonstrate that the two GnRH analogs, GnRHa and GnRHant, exert differing actions on the GnRH-R mRNA levels in the pituitary only during short-term treatment. GnRHant is useful for inhibiting serum gonadotropin, and combined or sequential treatment with GnRHa is a possible alternative therapy until a long-acting GnRHant is available for clinical use. The present data therefore provide useful basic information for these treatments.

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