

# Luteinizing hormone response to gonadotrophin-releasing hormone in normal women undergoing ovulation induction with urinary or recombinant follicle stimulating hormone

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Oestradiol enhances pituitary sensitivity to gonadotrophin-releasing hormone (GnRH) in normal women, while in women undergoing ovulation induction the putative factor gonadotrophin surge attenuating factor (GnSAF) attenuates the response of luteinizing hormone (LH) to GnRH. To study the relationships between oestradiol and GnSAF during ovulation induction, 15 normally ovulating women were investigated in an untreated spontaneous cycle (control, first cycle), in a cycle treated with daily i.m. injections of 225 IU urinary follicle-stimulating hormone (FSH) (Metrodin HP<sup>®</sup>, uFSH cycle) and in a cycle treated with daily s.c. injections of 225 IU recombinant FSH (Gonal-F<sup>®</sup>, rFSH cycle). Treatment with FSH started on cycle day 2. The women during the second and third cycle were allocated to the two treatments in an alternate way. One woman who became pregnant during the first treatment cycle (rFSH) was excluded from the study. In all cycles, an i.v. injection of 10 µg GnRH was given to the women ( $n = 14$ ) daily from days 2–7 as well as from the day on which the leading follicle was 14 mm in diameter (day V) until mid-cycle ( $n = 7$ ). The response of LH to GnRH at 30 min ( $\Delta$ LH), representing pituitary sensitivity, was calculated. In the spontaneous (control) cycles,  $\Delta$ LH values increased significantly only during the late follicular phase, i.e. from day V to mid-cycle, at which time they were correlated significantly with serum oestradiol values ( $r = 0.554$ ,  $P < 0.01$ ). Initially during the early follicular phase in the uFSH and the rFSH cycles,  $\Delta$ LH values showed a significant decline which was not related to oestradiol (increased GnSAF bioactivity). Then,  $\Delta$ LH values increased significantly on cycle day 7 and further on day v with no change thereafter up to mid-cycle. On these two days,  $\Delta$ LH values were correlated significantly with serum oestradiol values ( $r = 0.587$  and  $r = 0.652$  respectively,  $P < 0.05$ ). During the pre-ovulatory period,  $\Delta$ LH values in the FSH cycles were significantly lower than in the spontaneous cycles. Significantly higher serum FSH values were achieved during treatment with uFSH than rFSH. However, serum values of oestradiol, immunoreactive inhibin, and  $\Delta$ LH as well as the number of follicles  $\geq 12$  mm in diameter did not differ significantly between the two FSH preparations.

These results suggest that in women undergoing ovulation induction with FSH, oestradiol enhances pituitary sensitivity to GnRH, while GnSAF exerts antagonistic effects. The rFSH used in this study (Gonal-F<sup>®</sup>) was at least as effective as the uFSH preparation (Metrodin-HP<sup>®</sup>) in inducing multiple follicular maturation in normally cycling women.

**Key words:** follicle-stimulating hormone/gonadotrophin-releasing hormone/gonadotrophin surge attenuating factor/luteinizing hormone/pituitary

## Introduction

Evidence has been provided that in women undergoing ovulation induction a putative ovarian factor named gonadotrophin surge attenuating factor (GnSAF) attenuates the endogenous luteinizing hormone (LH) surge through a significant reduction of LH response to gonadotrophin-releasing hormone (GnRH) (Messinis *et al.*, 1985; Messinis and Templeton, 1989, 1990a, 1991a). Studies in animals have also shown that the response of LH to GnRH during treatment with follicle-stimulating hormone (FSH) is significantly suppressed (Geiger *et al.*, 1980; Schenken and Hodgen, 1983; Sopolak and Hodgen, 1984; de Koning *et al.*, 1987). On the other hand, activity of GnSAF that is different from inhibin has been detected in human follicular fluid (Busbridge *et al.*, 1990; Fowler *et al.*, 1990; Knight *et al.*, 1990; Mroueh *et al.*, 1996).

Several studies in women have investigated in-vivo bioactivity of GnSAF during ovulation induction and the results have shown that FSH is a potent stimulus of GnSAF production both in the follicular and the luteal phase of the cycle (Messinis *et al.*, 1991, 1993a; b, 1994, 1996). In these studies, however, either FSH was given to the women for only a few days or when FSH was injected during the whole follicular phase, GnSAF bioactivity, estimated as the reduction of LH response to GnRH, was investigated only sporadically during the cycle. Therefore, the pattern of changes in LH response to GnRH during FSH treatment given for the whole follicular phase is not known. It has been suggested that in normal women, oestradiol enhances pituitary sensitivity to GnRH (Lasley *et al.*, 1975; Wang *et al.*, 1976; Quyyumi *et al.*, 1993), although during the normal menstrual cycle this is evident only towards the end of the follicular phase (Messinis *et al.*, 1994). On the other hand, in ovulation induction cycles GnSAF attenuates both the releasable pool (sensitivity) and the reserve pool of the pituitary gonadotrophs (Messinis and Templeton, 1991b). Although an antagonism between oestradiol and GnSAF has been postulated under these circumstances (Messinis and Templeton, 1991b; Messinis *et al.*, 1994), the relationships

between pituitary response to GnRH and oestradiol values during the whole period of ovulation induction with FSH have not been investigated. So far, all studies examining in-vivo bioactivity of GnSAF were performed during treatment of normal women with urinary gonadotrophins; it is not known whether preparations of recombinant FSH, recently available, can exert effects on GnSAF production similar to those of the urinary preparations.

The present study was undertaken to investigate LH response to GnRH during ovulation induction in women, in order to assess further the hypothesis that GnSAF plays a role in the secretion of gonadotrophins, and to compare the effects of a urinary and a recombinant preparation of FSH on the production of GnSAF.

## Materials and methods

### Patients

The study included 15 normally menstruating women, aged 24–35 years, with unexplained infertility who volunteered for the study and gave written informed consent. Approval for the study was obtained from the local ethical committee. Ovulation was confirmed in all women by serum progesterone measurement and ultrasound scans of the ovaries before admission to the study. Each woman was investigated during three menstrual cycles, i.e. an untreated spontaneous cycle (control, first cycle), a cycle treated with urinary FSH (uFSH cycle) and a cycle treated with recombinant FSH (rFSH cycle). Between treatment cycles the women were given at least a month's break. Urinary FSH (75 IU FSH per ampoule, Metrodin HP®; Serono, Faran, Athens, Greece) was injected i.m. at the daily dose of 225 IU. Recombinant FSH (75 IU per vial or ampoule, Gonal-F®; Serono, Geneva, Switzerland) was given s.c. at the daily dose of 225 IU. Treatment with urinary or recombinant FSH was allocated alternately, i.e. if one woman was started in the second cycle with the urinary preparation, the next woman was started in the same cycle with the recombinant preparation. Eventually, seven women received as the first treatment uFSH and seven women rFSH. One woman received only the rFSH preparation because she became pregnant during that treatment cycle and she was excluded from the study. Therefore the final comparison was made among the three cycles of the remaining 14 women. Treatment with urinary or recombinant FSH started on cycle day 2 and continued up to the day of human chorionic gonadotrophin (HCG) administration. The latter was given as a single i.m. injection of 5000 IU when the leading follicle reached the size of 18–20 mm. If on that day more than three follicles >16 mm in diameter were present, HCG injection was withheld and the women were advised to avoid intercourse.

In each woman, the response of LH to GnRH was investigated in all three cycles starting on cycle day 2 (at 0900 h and 2100 h) and then every day (0900 h) up to cycle day 7. In seven of the 14 women, randomly selected, the response of LH to GnRH was also investigated during the late follicular phase of all three cycles starting when the leading follicle reached the size of 14 mm and continuing up to the day of the endogenous LH surge in the spontaneous cycles or the injection of HCG in the FSH cycles. Each time, the dose of GnRH was 10 µg given as an i.v. injection. Blood samples in relation to the time of GnRH injection (time 0) were taken at –15, 0 and 30 min. The 30 min point was selected because at that time peak values of LH are achieved in the majority of women both during spontaneous and FSH treated cycles and this represents pituitary sensitivity (Wang *et al.*, 1976; Messinis and Templeton, 1991b). Further blood samples

were taken in the follicular phase of all cycles on the days on which a GnRH test was not performed and in the luteal phase 7 days after the detection of the LH surge or the HCG injection. The LH surge was detected in daily blood samples rapidly assayed from the day on which the dominant follicle was 16 mm in diameter. All blood samples were centrifuged at 1000 g for 15 min. and the supernatant was stored at –20°C until assayed.

### Hormone assays

Serum FSH and LH were measured using immunometric assays based on enhanced luminescence (Amerlite® FSH assay and Amerlite LH-30 assay respectively). Kits were purchased from Amersham International (Amersham, UK). The results are expressed as IU/l of standards calibrated against the WHO second IRP of human FSH (58/549) and the first IRP of human LH (68/40). Serum oestradiol was measured using a competitive immunoassay based on enhanced luminescence. Kits were purchased from Amersham (Amerlite® Estradiol-60 assay) and the results are expressed as pmol/l. Serum inhibin was measured using a solid-phase two-site immunometric assay (Inhibin-Easia®). Kits were purchased from Medgenix Diagnostics SA (Fleurus, Belgium) and contained a goat polyclonal antibody coated on the plastic wells and a mouse monoclonal antibody conjugated to peroxidase. Both antibodies recognize distinct epitopes on the α subunit of human inhibin. There was no cross-reaction with various growth factors, LH, FSH and activin at concentrations up to 100 ng/ml. The results are expressed as U/ml. The assay used for inhibin relates to the profile of inhibin A measured by a two-site enzyme immunoassay and does not give information about inhibin B (Groome *et al.*, 1996). The lower limit of detection for FSH, LH, oestradiol and inhibin were 0.5 IU/l, 0.12 IU/l, 50 pmol/l and 0.1 U/ml respectively, while inter- and intra-assay coefficients of variation were 7.5 and 6.0%, 9.0 and 6.9%, 9.3 and 8.5% and 8.6 and 2.9% respectively.

### Statistical analysis

Statistical analysis of the results was performed using one-way analysis of variance, unless stated otherwise. In order to achieve an approximately normal distribution of the data, hormonal values were log transformed before the statistical evaluation. However, the arithmetic means of the values are presented.

## Results

All 14 women displayed an endogenous LH surge during the spontaneous cycle. The mean ( $\pm$ SEM) cycle day on which the LH surge occurred was  $12.0 \pm 0.3$ . In the uFSH and the rFSH cycles, the mean ( $\pm$ SEM) day on which HCG was injected was  $10.7 \pm 0.2$  and  $10.4 \pm 0.2$  respectively, i.e. significantly earlier than the occurrence of the LH surge in the spontaneous cycles ( $P < 0.01$ ). In seven of the 28 FSH cycles (25.0%), an endogenous LH surge had already started before the administration of HCG (in three uFSH and in four rFSH cycles). In these FSH cycles, the first LH value (mean  $\pm$  SEM) indicating the onset of the LH surge ( $11.3 \pm 1.5$  IU/l) was significantly lower than that in the spontaneous cycles ( $21.4 \pm 1.8$ ,  $P < 0.001$ ). HCG was not injected in two women during the uFSH cycle and in two women during the rFSH cycle, as they were at a high risk of multiple pregnancy and the development of the ovarian hyperstimulation syndrome.

The response of LH to GnRH was assessed as the net increase of LH at 30 min ( $\Delta$ LH) above the basal value. The

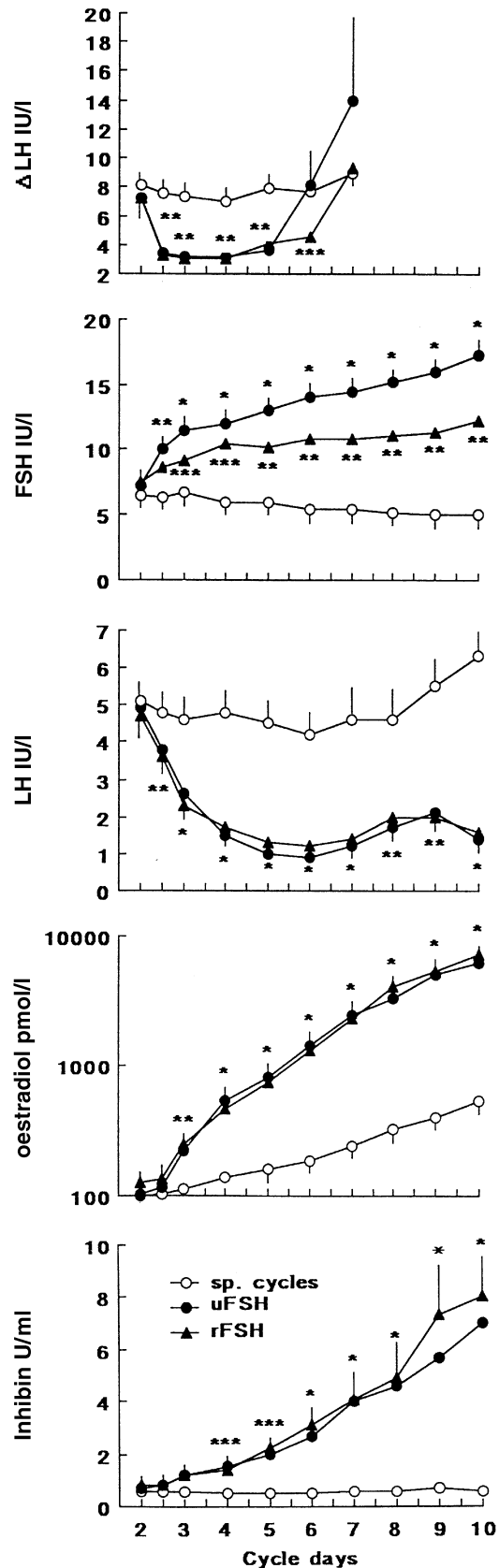
latter was calculated in each woman as the mean of the values at -15 and 0 min. Figure 1 shows  $\Delta$ LH, FSH, LH, oestradiol and immunoreactive (Ir) inhibin values during the follicular phase of the spontaneous and the FSH-treated cycles. Values of these hormones before the onset of the study on cycle day 2 did not differ significantly between the spontaneous and the two groups of FSH-treated cycles. Serum FSH concentrations increased significantly during treatment with FSH as compared with the spontaneous cycles, but the values were significantly higher ( $P < 0.01$ ) during treatment with the uFSH than the rFSH preparation (Figure 1).

$\Delta$ LH values in the spontaneous cycles did not change significantly from cycle days 2-7, although during the same period serum oestradiol values increased significantly ( $P < 0.01$ ). In contrast in the FSH cycles,  $\Delta$ LH values (mean  $\pm$  SEM) declined significantly 12 h from the first FSH injection (from  $7.2 \pm 0.6$  to  $3.4 \pm 1.0$  IU/l in the uFSH cycles and from  $7.2 \pm 1.3$  to  $3.3 \pm 0.6$  IU/l in the rFSH cycles,  $P < 0.01$ ). Then,  $\Delta$ LH values remained significantly lower in both groups of FSH cycles than in the spontaneous cycles up to days 6 or 7, increasing significantly thereafter to values similar to those in the spontaneous cycles (Figure 1).

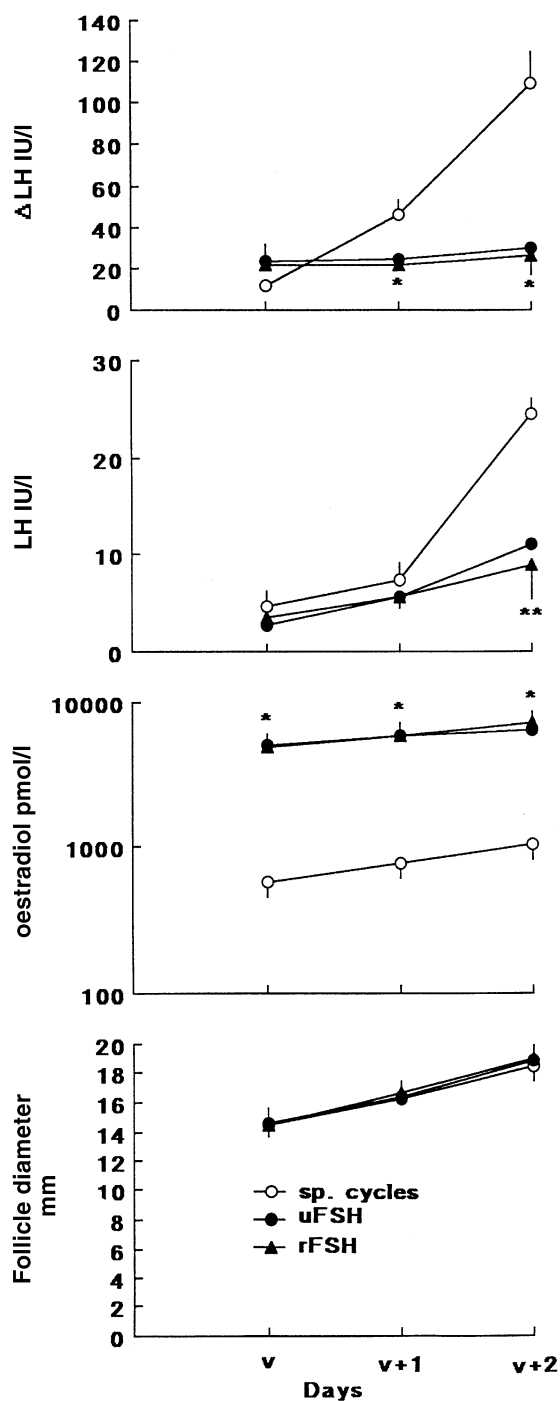
Basal LH values declined significantly in the FSH-treated cycles and remained significantly lower than in the spontaneous cycles, although there was a tendency for a slight but non-significant increase on cycle days 8 and 9 (Figure 1). Serum concentrations of oestradiol and Ir-inhibin increased significantly during treatment with uFSH or rFSH with no significant difference between the two groups of FSH cycles, and the values were significantly higher than in the spontaneous cycles. The increase of these two hormones, however, started 24 h after the first FSH injection. In the spontaneous cycles, oestradiol values increased significantly, while Ir-inhibin values remained unchanged (Figure 1).

Figure 2 shows  $\Delta$ LH, LH and oestradiol concentrations in late follicular phase, i.e. on the day the leading follicle reached the size of 14 mm in diameter, defined as day v, and the following 2 days in the three groups of cycles ( $n = 7$ ). In the FSH-treated cycles, day v occurred on average one day after day 7 ( $8.0 \pm 0.2$  days, mean  $\pm$  SEM), while in the spontaneous cycles on average 3 days after day 7 ( $10.4 \pm 0.3$  days). On day v,  $\Delta$ LH values were in the spontaneous cycles similar to those on cycle day 7, while in the uFSH and the rFSH-treated cycles significantly higher than on day 7 ( $P < 0.05$ ). Follicle size increased significantly from days v to v + 2 in all three groups of cycles (Figure 2).  $\Delta$ LH values (mean  $\pm$  SEM) in the spontaneous cycles increased significantly from day v ( $11.5 \pm 2.1$  IU/l) to days v + 1 ( $45.9 \pm 5.4$  IU/l,  $P < 0.001$ ) and v + 2 ( $109.4 \pm 15.1$  IU/l,  $P < 0.001$ ). At the same

time, serum oestradiol values (mean  $\pm$  SEM) also increased significantly from  $573 \pm 74$  pmol/l on day v to  $1035 \pm 87$  pmol/l on day v + 2 ( $P < 0.05$ ). In the uFSH- and the rFSH-

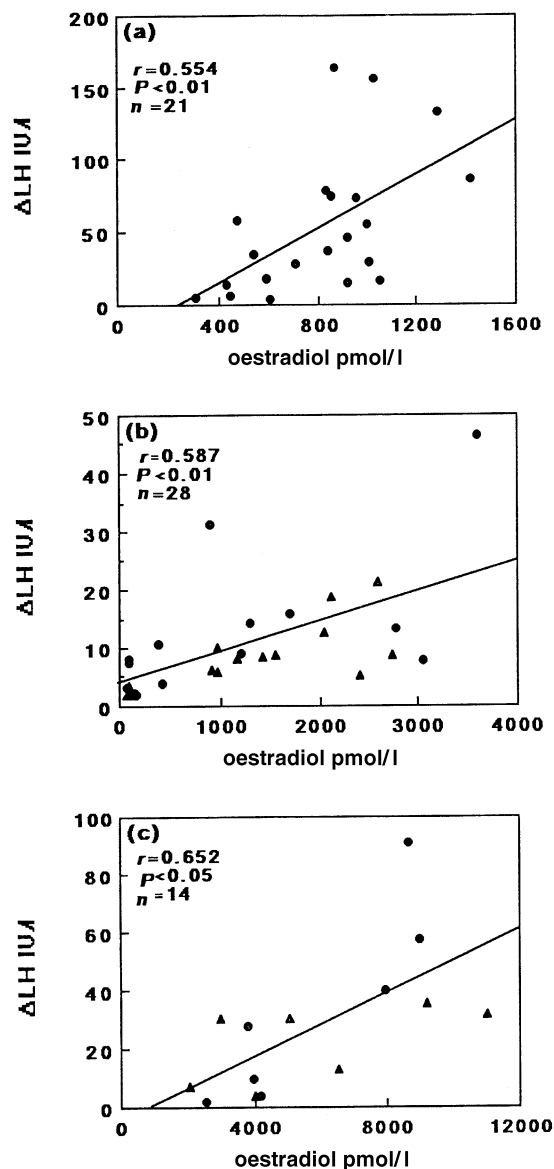


**Figure 1.** Serum values (mean  $\pm$  SEM) of  $\Delta$ LH [response at 30 min to 10  $\mu$ g gonadotrophin-releasing hormone (GnRH) i.v.], follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol and immunoreactive inhibin during the follicular phase of (○) untreated spontaneous cycles and cycles treated with (●) urinary FSH or (▲) recombinant FSH in 14 normally ovulating women. \*\*\* $P < 0.05$ , \*\* $P < 0.01$ , \* $P < 0.001$  (difference from spontaneous cycles).



**Figure 2.** Serum values (mean  $\pm$  SEM) of  $\Delta$ LH [response at 30 min to 10  $\mu$ g gonadotrophin-releasing hormone (GnRH) i.v.], luteinizing hormone (LH) and oestradiol on the day on which the leading follicle was 14 mm in diameter (day V) and the following two days during (○) untreated spontaneous cycles and cycles treated with (●) urinary follicle-stimulating hormone (FSH) or (▲) recombinant FSH in seven normally ovulating women. \*\* $P < 0.01$ , \* $P < 0.001$  (difference from spontaneous cycles).

treated cycles,  $\Delta$ LH values on day v ( $23.3 \pm 8.4$  and  $22.1 \pm 8.1$  IU/l respectively), although higher than in the spontaneous cycles, did not differ significantly from them and remained unchanged on days V + 1 and V + 2. On these two days,  $\Delta$ LH values were significantly lower in the FSH than in the spontaneous cycles with no significant difference between the



**Figure 3.** Relationship between  $\Delta$ LH values [response at 30 min to 10  $\mu$ g gonadotrophin-releasing hormone (GnRH) i.v.] and serum oestradiol concentrations (a) during the late follicular phase of untreated spontaneous cycles (days v, v + 1 and v + 2 of Figure 2), (b) on cycle day 7 and (c) on day v (defined as in Figure 2) of cycles treated with (●) urinary or (▲) recombinant follicle-stimulating hormone (FSH).

uFSH and the rFSH cycles (Figure 2). Serum oestradiol values in the two groups of FSH cycles were supraphysiological with further increase from days V to V + 2 (Figure 2). Due to the occurrence of an endogenous LH surge, basal LH values increased significantly from days V to V + 2 particularly in the spontaneous cycles and were on day V + 2 significantly lower in the two groups of FSH cycles than in the group of the spontaneous cycles (Figure 2). A significant positive correlation was found between individual  $\Delta$ LH and oestradiol values during the period from days V to V + 2 in the spontaneous cycles (Figure 3a,  $n = 21$ ) and on day V in the uFSH and the rFSH cycles combined (Figure 3c,  $n = 14$ ). A significant positive correlation was also found in the FSH

cycles between  $\Delta$ LH and oestradiol values on cycle day 7 (Figure 3b,  $n = 28$ ).

Multiple follicular development was induced during treatment with uFSH and rFSH. The numbers of medium sized follicles (12–15 mm) and of pre-ovulatory size ( $>15$  mm) on the day of HCG injection were similar in the uFSH ( $5.3 \pm 1.1$  and  $4.1 \pm 0.4$  respectively) and the rFSH cycles ( $4.3 \pm 0.5$  and  $3.7 \pm 0.4$  respectively). The size of the leading follicle (mean  $\pm$  SEM) on the day of the LH surge detection in the spontaneous cycles ( $18.9 \pm 0.2$  mm) did not differ significantly from that on the day of HCG injection in the uFSH ( $18.8 \pm 0.2$  mm) and the rFSH cycles ( $19.5 \pm 0.4$  mm). Ovulation of at least one follicle was confirmed by ultrasound and serum progesterone measurement in the 12 uFSH and the 12 rFSH cycles in which HCG was injected as well as in all spontaneous cycles. The duration of the luteal phase was significantly shorter in the uFSH ( $11.6 \pm 0.6$  days) and the rFSH cycles ( $11.4 \pm 0.5$  days) than in the spontaneous cycles ( $13.7 \pm 0.3$  days,  $P < 0.05$ ).

## Discussion

In the present study, a significant attenuation in LH response to GnRH was found during treatment with FSH. As in previous studies, this attenuation occurred before any significant increase in serum oestradiol or Ir-inhibin concentrations, thus confirming that the attenuation was related to GnSAF rather than to any other steroidal or non-steroidal ovarian substances (Messinis *et al.*, 1993a, 1994, 1996). One has to consider, however, that the immunoassay used for inhibin measurement does not evaluate dimeric inhibin A or B for which, as well as for activin A, specific two-site enzyme immunoassays have been developed (Groome *et al.*, 1996; Knight *et al.*, 1996).

In this study, changes in the response of LH to GnRH were investigated during almost the whole follicular phase of the FSH-treated cycles. The change in data presentation from a cycle day to a follicle size standardization was performed firstly for comparative purposes between FSH and spontaneous cycles, since treatment with FSH has been found to induce changes in follicular dynamics (Messinis and Templeton, 1990b), and secondly for patients' convenience, since  $\Delta$ LH response to GnRH in the natural cycle remains unchanged between cycle day 6 and 2 days prior to the mid-cycle LH surge (Messinis *et al.*, 1994) and this would reduce the blood sampling period in the spontaneous cycles.

The present study shows for the first time that the attenuation of LH response to GnRH, seen during the early follicular phase of the FSH-treated cycles, lasted for only 6 days, despite the continued treatment with FSH. Previous studies had investigated pituitary response to GnRH for a shorter period of time (Messinis *et al.*, 1991, 1993a, 1994, 1996). The increase in LH response to GnRH after day 6 and the significant positive correlation with supraphysiological oestradiol concentrations suggest that in the FSH cycles, oestradiol was able to overcome the attenuating activity of GnSAF and enhance pituitary sensitivity to GnRH around the mid-follicular phase. Nevertheless, the fact of no further increase in pituitary response to GnRH during the late follicular phase, despite the

continued rise in oestradiol concentrations, indicates that the ability of oestradiol to sensitize the pituitary to GnRH in the FSH cycles was limited by GnSAF, whose production therefore progressed throughout the treatment period. Thus, it is probable that in the FSH cycles GnSAF and oestradiol exerted antagonistic effects on LH response to GnRH. The possibility that oestradiol itself, when present at very high concentrations, is a limiting factor for the magnitude of LH response to GnRH, although not excluded, is not likely since in a previous study supraphysiological concentrations of oestradiol induced by the exogenous administration of oestrogen to women during the pre-ovulatory period of the normal menstrual cycle did not attenuate the amplitude of the endogenous LH surge (Messinis and Templeton, 1987).

An interesting finding in the present study is that, although on day 7 of the FSH-treated cycles the response of LH to GnRH was similar to that in the spontaneous cycles and serum oestradiol concentrations had already exceeded the pre-ovulatory threshold level for the positive feedback effect, an endogenous LH surge did not occur. This suggests that the factors which were responsible for the blockage of the endogenous LH surge in these cycles exerted their effects through mechanisms not involving the response of LH to GnRH. Whether this indicates that GnSAF affects the positive feedback effect of oestradiol through multiple mechanisms or that ovarian substances other than GnSAF are responsible for the blockage of the LH surge in stimulated cycles is at present unclear.

The physiological importance of the present findings is not clear. In this, as in a previous study (Messinis *et al.*, 1994), although serum oestradiol concentrations increased significantly from the early to mid-follicular phase of the spontaneous cycles, the sensitizing effect of oestradiol on the pituitary was evident only during the pre-ovulatory period, at which time a significant positive correlation between  $\Delta$ LH increase and oestradiol values was found. This, together with the present findings in the FSH stimulated cycles, supports the hypothesis that in the normal menstrual cycle the production of GnSAF is higher during the early and mid-follicular phase and lower in the late follicular phase (Messinis and Templeton, 1991a). In fact, an endogenous LH surge occurs during the early to mid-follicular phase of the normal cycle, whenever the oestradiol threshold level for the positive feedback effect is exceeded after the exogenous administration of oestradiol to women, but this surge is attenuated compared to the mid-cycle surge (Taylor *et al.*, 1995). It is possible, therefore, that the positive feedback effect of oestradiol on the pituitary has two components, i.e. one controlled by oestradiol, which triggers the endogenous LH surge, and another, controlled by GnSAF, which determines the amplitude of the surge.

The present study is the first in which normally menstruating women were treated with both the recombinant preparation of FSH, Gonal-F<sup>®</sup>, and the urinary preparation, Metrodin HP<sup>®</sup>, in two different cycles. Despite the significantly lower serum values of immunoreactive FSH during treatment with rFSH, the number of follicles as well as serum concentrations of oestradiol and Ir-inhibin were similar in the two treatments. This provides evidence that the preparation of rFSH used in

this study expressed a biopotency at least similar to that of the uFSH preparation. The comparison between the uFSH and the rFSH cycles demonstrates that the two treatments also exerted similar effects on pituitary response to GnRH. Certainly, an assessment of GnSAF bioactivity with the present in-vivo bioassay (LH response to GnRH) is rather broad and, therefore, a quantitative comparison of GnSAF bioactivity between the two treatments is difficult. So far, substances with GnSAF-like activity have been isolated from rat Sertoli cells condensed medium (Tio *et al.*, 1994) and porcine follicular fluid (Danforth and Cheng, 1995) and partially from human follicular fluid (Fowler *et al.*, 1995; Pappa *et al.*, 1995), but an immunoassay for GnSAF is not yet available.

In conclusion, the present results demonstrate for the first time that during the follicular phase of FSH stimulated cycles, the response of LH to GnRH shows a biphasic pattern, i.e. a decrease in the early follicular phase (predominant GnSAF effect), a recovery and further increase in mid-follicular phase (predominant oestradiol effect) and no further change in late follicular phase (GnSAF counterbalances the oestradiol effect). It is suggested that even in stimulated cycles, oestradiol enhances pituitary sensitivity to GnRH, while GnSAF exerts antagonistic effects. It is also suggested that the attenuation and the blockage of the endogenous LH surge in stimulated cycles are controlled by different mechanisms. Finally, the rFSH preparation used in this study (Gonal-F®) was at least as effective as the uFSH (Metrodin-HP®) in inducing multiple follicular maturation in normally cycling women.

## Acknowledgements

We wish to thank Ares Sero Group, Geneva, Switzerland, for the generous donation of Gonal-F®. We also thank Professor O. Tsolas, Director of the Department of Biological Chemistry, University of Ioannina for providing the laboratory facilities for the hormone assays.

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Received on February 6, 1998; accepted on June 17, 1998