Requirements for Human Chorionic Gonadotropin and Recombinant Human Luteinizing Hormone for Follicular Development and Maturation

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Purpose: *Our purpose was to evaluate the requirements for human chorionic gonadotropin (hCG) and recombinant luteinizing hormone (rec-LH) for follicular development and maturation in mice.*
Methods: *We carried out ovarian stimulation of immature mice. Output parameters were the preembryos created* in vivo *and frequency of blastocyst formation* in vitro.
Results: *hCG at 0 to 1 IU resulted in a dose-dependent recovery of preembryos (0 to 39.7 ± 4.3; mean ± SE) per mouse. hCG at 1 and 10 hCG gave similar results, whereas higher doses significantly reduced the number of preembryos. Potential for blastocyst formation was independent of hCG dose. hCG and rec-LH together exerted a synergistic effect on the recovery of preembryos.*
Conclusions: *Optimal follicular development required a combination of 20 IU follicle stimulating hormone and 1–10 IU hCG. The potency of hCG was higher than that of rec-LH, but a synergistic effect of rec-LH and hCG was observed. The results may be pertinent for the development of strategies for ovarian stimulation of women with low levels of endogenous LH.*

KEY WORDS: ovarian stimulation; oocyte maturation; human chorionic gonadotropin; recombinant luteinizing hormone; follicle stimulating hormone; preembryos; blastocysts.

INTRODUCTION

While follicle stimulating hormone (FSH) and luteinizing hormone (LH) are both required for normal follicular development and estrogen production in mammals, the precise contribution of LH in this process has been controversial (1,2).

In women, high circulating levels of LH have been shown to affect fertility negatively (3–5), and in connection with assisted reproduction, a great effort has been put into reducing levels of LH. Measures have concentrated mainly on the use of gonadotropin releasing hormone analogues (GnRHa) for pituitary down-regulation and the use of FSH preparations with reduced LH activity. These measures have successfully improved treatment outcome. However, with the introduction of highly purified FSH preparations and, especially, recombinant FSH preparations devoid of concomitant LH activity in connection with the use of a prolonged period of pituitary down-regulation, it became clear that the average circulation level of estradiol was reduced compared to that in patients who received LH-containing preparations (6,7). A reduced fertilization rate was also related to the use of pure FSH, suggesting a beneficial effect of LH on oocyte maturation. Although follicular development can be achieved by stimulation with pure FSH alone, studies in women with hypogonadal hypogonadism (8–10) showed reduced fertilization rates comparing FSH alone with preparations containing LH or human chorionic gonadotropin (hCG). Furthermore, no live births have been reported in hypogonadal women undergoing ovarian stimulation with pure FSH alone (11). In addition, new data seem to suggest that low levels of LH (i.e., <0.5 IU/L on stimulation day 8) results in a significantly increased pregnancy loss as compared to that in the >0.5 IU/L group (12). It was suggested that the low LH group lacked an optimal cytoplasmatic maturation of the oocyte due to a reduced estradiol load. About two-thirds of the patients in the study by Westergaard (12), which employed a standard long GnRHa protocol, occurred in the low-LH group, suggesting that the proportion of patients who may benefit

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from additional LH administration was not insignificant.

Taken together, the evidence suggests that levels of LH should be neither too high nor too low during the follicular phase of the menstrual cycle.

In the present study, we have used an animal model to titrate the level of hCG or rec-LH needed to achieve an optimal ovarian response and formation of preembryos in vivo. The model uses prepubertal immature mice with low levels of pituitary gonadotropins, thereby eliminating effects of endogenously derived gonadotropins. Using a fixed dose of FSH for ovarian stimulation, the mice simultaneously received different doses of hCG and/or rec-LH. After ovulation was induced with a bolus of hCG, the animals were mated, and the preembryos were recovered from the tubes. The capacity of the preembryos to develop into blastocysts in vitro was also used to assess follicular development and maturation in the various groups.

MATERIALS AND METHODS

Animals

B6D2-F_1 (C57Bl/6 × DBA/2J) immature female mice were randomly divided into groups of five animals. The mice were kept under 14 hr light/10 hr dark with free access to food and water. The mice were about 21 days old (weighing 15–17 g) when ovarian stimulation was initiated.

Stimulation Regime

On day 1 at 1400 hr each animal received a single dose of highly purified FSH (FSH-HP; Fertinorm HP; Serono Nordic, Denmark) and/or hCG (Physex; Leo, Copenhagen, Denmark; batch No. 9403E12E) and/or rec-LH (Serono, Geneva, Switzerland; batch No. 51703102) according to the planned stimulation protocol. The hormones were diluted in isotonic sodium chloride to a volume of 0.1 ml and administered intraperitonally (i.p.) as described previously (13).

In the first experiment mice were stimulated with 20 IU FSH-HP in combination with 0, 0.2, 0.4, 0.6, 0.8, 1.0, 10, 50, or 100 IU hCG or with 1.0 IU hCG alone. In the second experiment mice were stimulated with 20 IU FSH-HP in combination with 20 IU rec-LH or 0.2 IU hCG or 20 IU rec-LH plus 0.2 IU hCG. The doses of hCG and rec-LH were both chosen to be suboptimal to allow for evaluation of a possible additive/synergistic effect.

On day 3 at 0900 hr all mice received 2.5 IU hCG i.p. for ovulation induction. On day 3 at 1400 hr each female was placed with a male of proven fertility. Successful mating was defined as the presence of cleaved oocytes in the flushed tubes as described below.

Preembryo Recovery and Culture

On day 5 at 0900 hr the female mice were killed by cervical dislocation. The tubes from each mouse were removed and kept separate in Ham's F10 medium with 25 m*M* HEPES and L-glutamine (GIBCO, Paisley, Scotland) supplemented with 100 IU/ml penicillin (Leo) at room temperature. The preembryos were flushed from the tubes using a 34-gauge × 10-mm Luer square end needle (Coopers Needle Works, Birmingham, England). In the following, nonfertilized oocytes, fertilized noncleaved embryos and cleavage-stage embryos were collectively named preembryos. The flushed preembryos were subdivided into two groups: noncleaved one-cell and cleaved two-cell or >two-cell. The one-cell group contained both nonfertilized and fertilized oocytes. After flushing, the preembryos in each group were pooled and cultured in tubes (Falcon; Becton–Dickinson) (maximum, 100 preembryos per tube) for 72 hr at 37°C in 1 ml Earle's balanced salt solution (EBSS) (Flow Laboratories) supplemented with 100 IU/ml penicillin, 10.4 g/L $NaHCO_3$ (Merck, Darmstadt, Germany), 11 mg/L sodium pyruvate (Kock-Light Limited, Hoverhill, Suffolk, UK), and 0.5% (w/v) human serum albumin (Statens Serum Institute, Copenhagen, Denmark). After culture, the preembryos were subdivided into three groups: (i) blastocysts; (ii) less advanced developmental stages, e.g., the morula stages; and (iii) degenerated preembryos.

Histology

When the tubes were collected, ovaries from the group of animals receiving 20 IU FSH alone and 20 IU FSH plus 0.6 IU hCG were recovered, fixed, and processed for histology.

Statistics

The data were analyzed by ANOVA and Student's *t* test. Results are expressed as mean ± standard error of the mean (SE).

RESULTS

Experiment 1

Stimulation of FSH-HP in combination with hCG resulted in a dose dependent recovery of preembryos increasing from 0 to 1.0 IU hCG ($P < 0.001$) (Fig. 1). The recovery of 39.7 ± 4.3 preembryos per mouse when stimulating with 1 IU hCG and 20 IU FSH-HP was comparable to stimulation with 10 IU hCG and 20 IU FSH-HP (40.9 ± 8.3 preembryos) ($P > 0.10$). Increasing the concomitant dose of hCG to 50 and 100 IU reversed the picture and caused detrimental effects on the number of recovered preembryos, reducing the average to 12.3 ± 4.0 and 0.6 ± 0.1, respectively ($P < 0.001$ for both).

Increasing the stimulation dose of hCG up to 1 IU resulted in a dose dependent increase in the number of mice with preembryos present in the tubes ($P < 0.001$) as well as a dose-dependent increase in the number of preembryos recovered from each mouse ($P < 0.001$)(Fig. 2). Stimulation with 50 or 100 IU of hCG resulted in a decrease in the number of mice with preembryos and a decreased number of preembryos per mouse ($P < 0.01$).

Stimulation of mice with 20 IU FSH-HP alone resulted in a poor recovery of preembryos, only 0.2 ± 0.05 per mouse, all one-cell. As indicated in Fig. 3 preovulatory follicular development had taken place in these mice. However, the oocytes were still trapped within the follicles, showing that the vast majority of follicles was unable to respond to the bolus

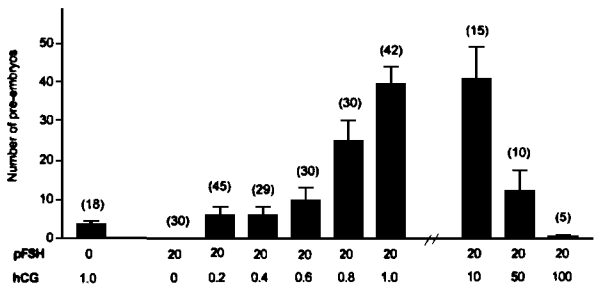

Fig. 1. Mean number of preembryos recovered by flushing the tubes of prepubertal immature mice stimulated with no or 20 IU of highly purified urinary FSH and different doses of hCG 2 days after mating. Data are mean ± SE (number of animals in parentheses). The 0-, 0.2-, and 0.4-IU hCG groups were not significantly different, whereas the number of preembryos in the 0.6-IU group was higher ($P < 0.05$). The number in the 0.8-IU hCG group was higher than that in the 0.6-IU hCG group ($P < 0.05$), and the number in the 1.0-IU hCG group was higher than that in the 0.8-IU hCG group ($P < 0.05$) but similar to that in the 10-IU hCG group ($P > 0.10$).

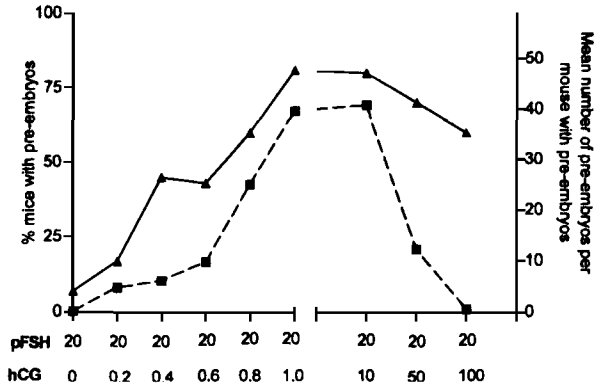

Fig. 2. Percentage of preembryos recovered from prepubertal immature mice stimulated with 20 IU of highly purified urinary FSH and different doses of hCG 2 days after mating. The solid line presents the percentage of mice with preembryos present in the tubes. The dotted line presents the mean number of preembryos per mouse with preembryos.

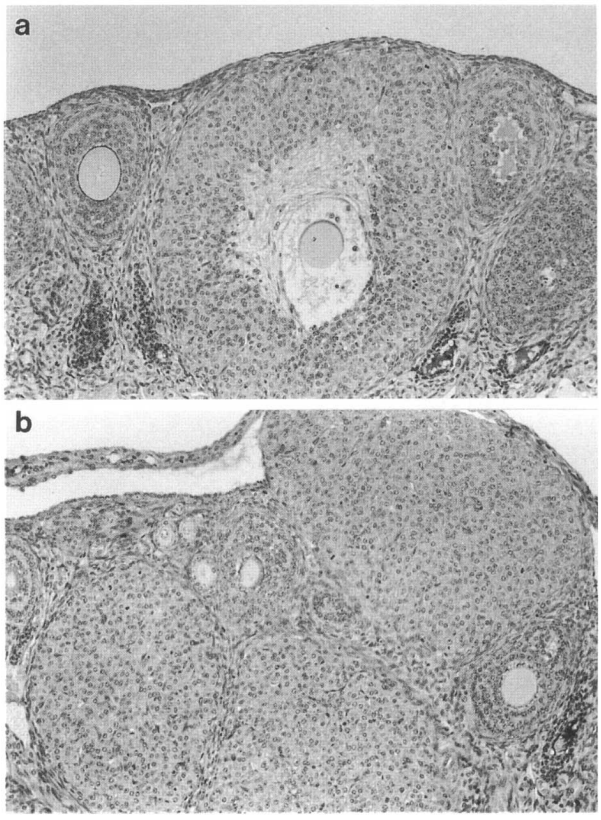

Fig. 3. (a) Histological sections of ovaries from mice stimulated with 20 IU FSH without concomitant hCG administration. Ovulation was induced with 2.5 IU hCG but the majority of follicles did not ovulate, resulting in luteinized unruptured follicles. (b) Ovaries that received 20 IU FSH and 0.6 IU hCG for ovarian stimulation. Ovulation and normal corpera lutea were achieved by administration of 2.5 IU hCG.

of hCG by undergoing ovulation. In contrast, the ovaries of mice stimulated with 20 IU FSH-HP plus 0.6 IU hCG show signs of ovulatory activity with early corpus luteum formation (Fig. 3b).

Stimulation with just 1 IU hCG and no concomitant FSH also results in a poor recovery of preembryos (3.7 ± 0.8 per mouse). In this group 85% of the preembryos had developed to at least the two-cell stage, whereas in all other groups more than 90% of the recovered preembryos had reached at least the two-cell stage.

The developmental competence of the derived cleavage-stage preembryos is shown in Fig. 4. The concomitant administered dose of hCG was without effect on the frequency by which the preembryos underwent blastocyst formation in vitro. On average, 77 ± 2.6% of the preembryos developed into blastocysts.

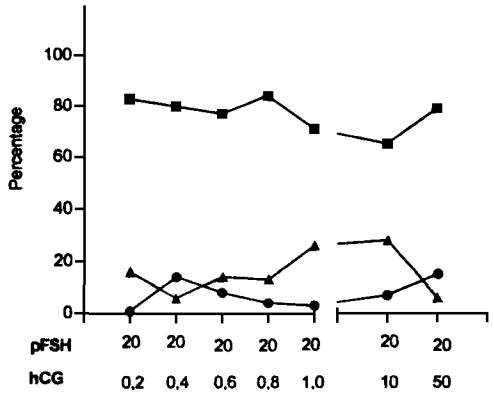

Fig. 4. The developmental capacity of recovered preembryos cultured for 3 days. The squares show the percentage of flushed preembryos developing into the blastocyst stage. The triangles show the percentage of flushed preembryos developing into less advanced stages and the circles show the percentage which was degenerated after 3 days of culture. The frequency at which the preembryos underwent blastocyst formation was similar among the groups.

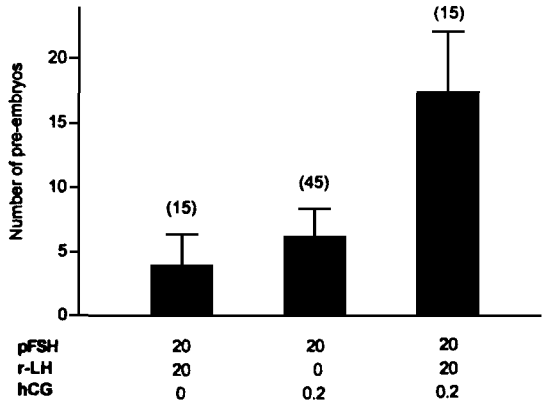

Fig. 5. Mean number of preembryos recovered by flushing the tubes 2 days after mating of ovarian-stimulated mice treated with 20 IU of highly purified urinary FSH with or without 20 IU of rec-LH and 0.2 IU of hCG. Data are mean ± SE (number of animals in parentheses).

Experiment 2

Stimulation of 20 IU FSH-HP plus 20 IU of rec-LH resulted in the recovery of 3.9 ± 2.5 preembryos per mouse with 83% in the cleavage stage (Fig. 5). Stimulation with 20 IU FSH-HP in combination with 0.2 IU hCG resulted in the recovery of 6.2 ± 2.2 preembryos per mouse, with 95% in the cleavage stage. However, when stimulation with 20 IU FSH-HP together with 0.2 IU hCG plus 20 IU rec-LH was performed, the recovery of 17.4 ± 4.7 preembryos per mouse, with 93% in the cleavage stage, was observed. This recovery was significantly higher than that for the additive effect of r-LH and hCG separately (P = 0.006). The frequency at which the cleavage stage preembryos developed into blastocysts was similar among the three groups (data not shown).

DISCUSSION

This study shows that both FSH and hCG/LH are needed to obtain follicular development and maturation that will allow for the formation of healthy preovulatory follicles containing oocytes capable of undergoing preembryo development in vivo. Optimal and maximal follicular stimulation and maturation are achieved with the administration of 1 to 10 IU hCG concomitant with 20 IU FSH-HP. On either side of this plateau of hCG, follicular development and maturation are severely compromised, resulting in the formation of a significantly lower number of preembryos. The immature mice used in this animal model have very low levels on endogenous gonadotropins. Therefore, the results define the relative contribution of FSH on one side and hCG on the other side for optimal follicular development and maturation. hCG activity accounting for as little as 5% of the FSH activity allowed for maximal recovery of preembryos, remaining constant while increasing the hCG activity to 50% of the FSH activity. hCG activity outside this range compromises the follicular response drastically.

The study by Mannaerts *et al.* (14) showed that pure FSH alone induced ovarian and aromatase activity in immature hypophysectiomized rats in a dose-dependent manner, while the plasma estradiol remained

unchanged. However, when pure FSH was supplemented with only 0.1 IU hCG plasma, estradiol started to rise, and 1 IU hCG caused a large augmentation of the aromatase activity. That study essentially proved the two-cell two-gonadotropin theory and, in connection with the present results, demonstrates that the production of estradiol and the production of viable preembryos are likely to be interrelated, reflecting correct guidance by the presence of critical amounts of FSH and LH-like activity.

In addition, this study parallels and confirms findings in women, in whom too high or too low levels of circulating LH seems to compromise reproductive performance. Women following ovarian stimulation with the long GnRHa protocol have low levels of endogenous gonadotropins during the period of ovarian stimulation (15). When ovarian stimulation in these women is performed with pure FSH preparations, they may experience levels of LH which are insufficient to allow for the formation of preembryos with an optimal pregnancy potential (6,12). This argument has also been applied to explain reproductive performance in women with hypogonadal hypogonadism (8,16). Extrapolating the results from the present animal model to women indicates women with low circulating levels of LH may benefit from the coadministration of hCG comprising at least 5% but not more than 50% of the FSH dose administered. However, actual studies in women are obviously needed to determine the necessary doses of hCG.

Interestingly, the results show that the developmental capacity of preembryos was independent of the dose of hCG used for ovarian stimulation, expressed by a similar frequency at which the preembryos develop into blastocysts in the various groups. Thereby this study confirms the recent findings in humans by Flemming *et al.* (17), who demonstrated that the formation of human blastocysts was independent of the concentration of circulating LH in the women from whom the oocytes originated. Furthermore, it points to a direct beneficial effect of hCG/LH on the maturation and development of the follicle-enclosed oocyte, maybe by improving its cytoplasmatic maturation and thereby its developmental capacity.

This study also confirms that ovarian stimulation with FSH-HP alone induces formation of preovulatory follicles (18). However, our study showed that the preovulatory follicles were unable to undergo ovulation when stimulated with ovulatory dose of hCG. This is in agreement with findings in hypogonadotropic patients (9) but in contrast with another study (15), which found that more than 90% of mice ovulated after stimulation with FSH-HP and ovulation induction with hCG. Compared to the latter study, we used only half the amount of hCG for ovulation induction (i.e., 2.5 vs 5 IU hCG), and the induction of a reduced number of follicular LH receptors when ovarian stimulation is performed with FSH alone compared to a combination of FSH/LH may explain the reduced ovulation rate in our study (19).

Previous studies in the animal model as applied in this study used human menopausal gonadotropin (hMG) to optimize the ovulatory response and the number of derived preembryos (13). hMG contains equal amounts of FSH and LH-like activity, and using a dose of 20 IU FSH and 20 IU LH-like activity, an optimal recovery of about 40 preembryos per mouse was obtained, as observed in this study. The LH-like activity of hMG preparations is composed of LH and hCG, the immunoreactivity of hCG amounting for as much as 25% of the total LH-like activity (20). Therefore, the hCG content of the hMG preparations is, by itself, enough to cause an optimal response in our animal model. This may suggest that the hCG content of hMG preparations is the important LH-active component in vivo, as this study also suggests, since the rec-LH were far less potent than the hCG. A likely explanation for this observation may be the fact that LH is much less glycosylated than hCG, resulting in a prolonged half-life of hCG compared to LH. However, it is interesting to note the synergistic effect of rec-LH and small amounts of hCG on the number of preembryos recovered. This may suggest that hCG has a function of its own in follicular development and maturation. Actually, small amounts of hCG are secreted in a pulsatile manner during the follicular phase of the human menstrual cycle (21). Maybe hMG preparations, unintentionally, contain a very suitable combination of LH-like activity consisting of mainly LH and small amounts of hCG, causing optimal follicular maturation. We are now trying to approach this question using our animal model.

In conclusion, this study shows that both FSH and LH-like activity were mandatory for the formation of preembryos in vivo in immature mice previously not exposed to circulating levels of gonadotropins. The maximal number of in vivo derived preembryos resulted from stimulation with a combination of hCG and FSH, in which hCG comprised from 5 to 50% of the FSH activity. In this model, human rec-LH was far less active than hCG, however, a synergistic effect between rec-LH and hCG was observed. The observed effects parallel those in women and suggest that valuable information may be generated in this animal

model with regard to the development of new gonadotropin preparations and the management of women with low circulating levels of LH.

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