CLINICAL ASSISTED REPRODUCTION

The Effects of Gonadotropin-Releasing Hormone Agonist on Androstenedione Production and Follicular Development During Controlled Ovarian Hyperstimulation

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Submitted: January 30, 1998

Accepted: April 1, 1998

Purpose: We performed a prospective randomized study to assess the effects of a GnRH agonist (GnRH-a) on follicular development and steroidogenesis during controlled ovarian hyperstimulation (COH).

Methods: Patients undergoing in vitro fertilization (IVF) for tubal infertility received human menopausal gonadotropin (hMG) stimulation with or without the GnRH-a, buserelin, beginning in the midluteal phase of the prior cycle. We analyzed serum hormone levels, follicular development, and outcome of IVF.

Results: The mean number of retrieved oocytes was significantly greater, and the implantation rate per embryo was significantly higher, in the GnRH-a/hMG group ($n = 101$) than in the hMG-only group ($n = 97$). The concentration of androstenedione (A) and the A/estradiol ratio in the serum were significantly lower in the GnRH-a treatment group throughout the follicular phase.

Conclusions: The concomitant use of GnRH-a during COH prevents atretic change of the follicles and enhances follicular development by reducing androgen accumulation, resulting in a higher developmental competence of the oocytes.

KEY WORDS: androstenedione; controlled ovarian hyperstimulation; follicular atresia; gonadotropin-releasing hormone agonist; in vitro fertilization.

INTRODUCTION

Gonadotropin-releasing hormone agonist (GnRH-a) has recently been used during controlled ovarian hyperstimulation (COH) for in vitro fertilization and embryo transfer (IVF-ET). Although considerable evidence suggests that such a concomitant use of GnRH-a improves follicular recruitment and subsequent IVF outcome (1–4), the precise mechanism for that improvement remains unclear. The administration of GnRH-a during COH suppresses the basal levels of luteinizing hormone (LH), preventing premature LH surges and premature luteinization (1,4,5). A spontaneous LH surge is associated with poor oocyte maturation, poor oocyte recovery, and a poor fertilization rate (6). However, the role of a premature progesterone (P) elevation in oocyte maturation, embryogenesis, endometrial development, and subsequent pregnancy remains controversial (7–9).

Gonadotropins and estrogens are follicle survival factors that can suppress the apoptotic DNA fragmentation, an event that is suggested to underlie ovarian follicle atresia, whereas androgens are potential atretogenic factors (10,11). The intrafollicular steroid environment plays an important role in the maturation of the oocyte and its acquisition of developmental competence (12,13). The process of atresia is associated with a relative increase in the production of follicular androgen (12,14). The viability and the subsequent fate of oocytes are closely related to the levels of estrogen and androgen in follicular fluid.

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The purpose of this prospective, randomized study was to assess the effects of the concomitant use of GnRH-a during COH on follicular development and atresia in humans. Accordingly, we measured daily changes in the serum levels of gonadotropins and steroid hormones, including androstenedione (A), the major product of thecal steroidogenesis, in patients stimulated for IVF-ET with human menopausal gonadotropin (hMG) alone or combined with GnRH-a. We evaluated the differences in hormone levels, follicular development, and the subsequent outcome of IVF between the two stimulation regimens.

MATERIALS AND METHODS

Study Design

This study was conducted between October 1993 and December 1996 at the IVF unit of Tokyo Dental College, Ichikawa General Hospital. A total of 300 women, aged 25–42 years (mean, 33.6 years) was recruited into the IVF program and randomized to receive ovarian stimulation with hMG, in conjunction with ($n = 150$) or without ($n = 150$) the GnRH-a buserelin acetate (D-Ser[TBU]₆-des-Gly-NH₂ GnRH ethylamide, Suprecur; Hoechst Japan, Tokyo). Randomization was performed as follows: 300 sealed envelopes, each containing a card indicating the method to be used (150 for each method), were shuffled and one envelope was drawn before ovarian stimulation. Each patient was informed of the nature, scope, and possible consequences of the clinical trial and gave informed consent to participate. The study was approved by the institutional review board of our hospital. Subjects who were assigned to receive hMG only are referred to as group A, while those who were assigned to receive a combination of GnRH-a and hMG are referred to as group B.

Selection of Subjects

Criteria for inclusion were tubal infertility as the only indication for IVF, undergoing of their first IVF attempt at our institution, and normal ovulatory cycles (28 ± 4 days). Criteria for exclusion were a diagnosis of infertility that was caused by an endocrine abnormality, a male factor, or an immune factor.

A total of 102 patients was found to be ineligible for analysis of results for these reasons: (a) there was a failure to aspirate the medium and large follicles in 57 cases; (b) the semen specimen values fell below

the normal range (concentration, $<20 \times 10^6$ cells/ml; motility, $<50\%$; morphology, $<50\%$ normal forms) at the time of IVF in 28 cases; and (c) oocyte retrieval was canceled in 17 cases. Thus, results were analyzed in 198 subjects, 97 in group A and 101 in group B. The incidence of cancellation of oocyte retrieval in the two groups did not differ significantly: 10 patients (6.7%) in group A and 7 (4.7%) in group B.

IVF Procedure

Beginning on day 3 of the menstrual cycle, all patients received gonadotropin stimulation with 4 ampoules of hMG [75 IU of follicle-stimulating hormone (FSH) and 75 IU of LH per ampoule; Humegon; Japan Organon, Tokyo] administered intramuscularly daily for 2 days. Subsequent doses of hMG (2 or 3 ampoules daily) were individualized thereafter for 3–8 days according to serum estradiol (E_2) levels. In group B, these patients received buserelin acetate intranasally at a daily dose of 900 μ g, starting on the sixth day of the basal body temperature elevation of the preceding menstrual cycle.

Follicular development was monitored daily by ultrasonography and the measurement of serum E_2 . When the largest follicle was 18 mm or greater in the greatest diameter, human chorionic gonadotropin (hCG; Gonatropin; Teikokuzoki Co. Ltd., Tokyo) was administered intramuscularly at a dose of 10,000 IU. Buserelin administration was terminated on the day of hCG injection. Oocytes were retrieved 36 hr after hCG administration by transvaginal ultrasound-guided follicle aspiration during intravenous analgesia. Oocyte retrieval was canceled in those patients with a low E_2 response [E_2 , < 300 pg/ml (conversion factor to SI units, 3.671)] to hMG during follicular stimulation.

In vitro fertilization and transcervical ET were performed according to previously described standard techniques (15). Normal fertilization was confirmed microscopically by observing the presence of two polar bodies and two pronuclei 16–18 hr following insemination. After an overall incubation period of approximately 45 hr, the inseminated oocytes were examined for cleavage and a maximum of three embryos was transferred immediately. The luteal phase of each subject's cycle was supported with 25 mg of P in oil (Luteum, Teikokuzoki Co. Ltd., Tokyo) daily and 2500 IU of hCG every other day administered intramuscularly starting on the day after follicular aspiration. Only those clinical pregnancies, confirmed by ultrasound, were counted as such in the analyses of the results.

Blood samples were drawn each morning starting on day 3 of the menstrual cycle until the day of follicular aspiration. During the administration of hMG, blood samples were obtained approximately 2 hr before the administration of hMG. The serum was immediately separated by centrifugation and one portion of each daily aliquot of serum was used for rapid E₂ determination. The remainder was stored at -20°C for later analysis. Day -1 blood samples were obtained approximately 12 hr before the administration of hCG, while day +1 samples were collected 1 hr before oocyte retrieval.

Hormone Assays

Serum concentrations of E₂, P, and A were assayed with solid-phase ¹²⁵I-labeled steroid radioimmunoassay kits (Nippon DPC, Tokyo). Estradiol and P were measured without extraction, whereas A was measured after extraction with ethyl ether. The intraassay and interassay coefficients of variation (CVs) were 5.3 and 6.4% for E₂, 7.2 and 7.9% for P, and 5.5 and 8.8% for A, respectively. Serum levels of LH and FSH were determined by immunoradiometric assay using two monoclonal antibodies (SPAC-S LH, SPAC-S FSH; Daiichi Radioisotope Laboratory, Tokyo). The intra-assay and interassay CVs for LH were 2.5 and 4.2% and 1.5 and 2.9% for FSH.

Statistical Analysis

Results are reported as mean $\pm$ standard error (SE). Differences between the means of the two groups were evaluated with Student's two-tailed *t* test. Differences in the concentrations of LH, FSH, E₂, and P and in the values for A/E₂ were assessed by two-way analysis of variance, with the two independent variables being cycle day and treatment group. This analysis was followed by Scheffe's test to determine whether the two groups differed. Chi-square analysis was used to compare the implantation rate and the pregnancy rate (PR) in the two groups. A level of *P* less than 0.05 was defined as statistically significant.

RESULTS

The mean ages of the patients in the two treatment groups were similar (Table I). Although the average day of hCG administration did not differ significantly between the two groups, the mean number of hMG ampoules required by group B was significantly greater

Table I. Results of Ovarian Stimulation and Outcome of IVF in Patients Receiving hMG with or Without GnRH-a

	Group A (hMG only)	Group B (GnRH-a/hMG)
No. of cycles	97	101
Age (yr)	33.6 $\pm$ 0.4	33.6 $\pm$ 0.4
Day of hCG administration	9.7 $\pm$ 0.1	9.9 $\pm$ 0.1
hMG (No. of 75 IU ampoules) ^a	18.7 $\pm$ 0.4	21.9 $\pm$ 0.4
Cycles oocyte(s) obtained ^b	95 (97.9)	100 (99.0)
Transferred cycles ^b	89 (91.8)	94 (93.1)
No. of pregnancies	36	46
PR per retrieval (%)	37.1	45.5
PR per transfer (%)	40.4	48.9

^a Significantly different, *P* < 0.01.

^b Values in parentheses are percentages per retrieval.

than that of group A. The mean numbers of follicles aspirated and of oocytes retrieved, fertilized, and cleaved per cycle were significantly higher in group B than in group A (Table II). The mean numbers of follicles aspirated and oocytes retrieved per ampoule of hMG were also significantly higher in group B than in group A: 0.68 $\pm$ 0.05 vs. 0.51 $\pm$ 0.03 and 0.46 $\pm$ 0.03 vs. 0.30 $\pm$ 0.02, respectively. No significant differences in the fertilization rates or the cleavage rates were observed between the two groups. Since a maximum of three embryos was transferred, the mean number transferred in each group was comparable. Although the PRs did not differ significantly between the two groups, the implantation rate per embryo was significantly higher in group B than that in group A (Tables I and II).

The serum concentrations of LH measured during the follicular phase did not change substantially in

Table II. Outcome of IVF in Patients Receiving hMG with or Without GnRH-a

	Group A (hMG only)	Group B (GnRH-a/hMG)
No. of follicles aspirated ^a	9.1 $\pm$ 0.5	13.6 $\pm$ 0.9
No. of oocytes retrieved ^a	5.3 $\pm$ 0.3	9.4 $\pm$ 0.5
No. of oocytes fertilized ^a	3.9 $\pm$ 0.3	6.3 $\pm$ 0.4
Fertilization rate (%)	75.2 $\pm$ 2.7	68.3 $\pm$ 2.6
No. of oocytes cleaved ^a	3.1 $\pm$ 0.2	6.1 $\pm$ 0.4
Cleavage rate (%)	60.2 $\pm$ 3.0	65.0 $\pm$ 2.7
No. of embryos transferred	2.8 $\pm$ 0.2	2.8 $\pm$ 0.1
Implantation rate per embryo (%) ^b	18.1	25.8

^a Significantly different, *P* < 0.01.

^b Significantly different, *P* < 0.05.

group B, whereas LH gradually increased during the late follicular phase of group A (Fig. 1). Although the serum levels of LH measured during the early follicular phase were lower in group B than those in group A, these differences were not significant. The levels of LH measured during the late follicular phase (between day -3 and day +1) were significantly lower in group B than in group A. The serum concentrations of FSH did not differ significantly between the two groups throughout the days examined.

The serum concentrations of P did not differ significantly between the two treatment regimens (Fig. 2). We determined serum P value on the day of hCG administration (day -1) above or below which the PR was affected. The PRs were 43.0% (58/135), 41.5% (22/53), and 20.0% (2/10) when serum P levels on day -1 were 0.9 ng/ml or less (conversion factor to SI units, 3.180), between 0.9 and 1.9 ng/ml, and greater than 1.9 ng/ml, respectively. The elevated P levels (greater than 1.9 ng/ml) measured on the day of hCG administration, with which PR was found diminished, were observed in six cycles (6.2%) in group A and four (4.0%) in group B.

Levels of E_2 and A continuously rose in both groups during hMG stimulation, while the A-to- E_2 ratio

Fig. 1. Daily concentrations of serum LH and FSH in group A (○) and group B (●). The asterisks indicate statistically significant difference between the two groups. * $P < 0.05$; ** $P < 0.01$. hCG, hCG administration; OPU, oocyte pickup.

Fig. 2. Daily concentrations of serum E_2 and P in group A (○) and group B (●). hCG, hCG administration; OPU, oocyte pickup.

(A/ E_2) declined gradually (Figs. 2 and 3). The A/ E_2 values were obtained by dividing the A value by the E_2 value (both as nM) measured in the same sample. In contrast to the differing numbers of follicles found between the two groups, the serum concentrations of E_2 did not differ significantly. The levels of A were significantly lower and the values for A/ E_2 were significantly smaller in group B than those in group A throughout the days examined. No correlations were found between the serum concentrations of A and P or A and LH on the day of hCG administration (data not shown).

DISCUSSION

This prospective, randomized study was designed to compare follicular development, steroidogenesis, and IVF outcome in two groups of patients treated with hMG, one group with GnRH-a and the other without GnRH-a. The subjects were well defined, since the indication for IVF was tubal disease only and all patients were undergoing their first attempt at our institution. We showed that, in those patients receiving GnRH-a/hMG, the number of follicles aspirated and

Fig. 3. Daily concentrations of A and the A to E₂ ratio in the serum of group A (○) and group B (●). The asterisks indicate statistically significant difference between the two groups. ***P* < 0.01. hCG, hCG administration; OPU, oocyte pickup.

the number of oocytes retrieved were significantly greater, the serum concentrations of A were significantly lower, the A/E₂ ratios in the serum were significantly lower, and the implantation rate per embryo was significantly higher than those in patients receiving hMG without GnRH-a. Results suggest that the concomitant use of GnRH-a with hMG induces a viable follicular environment and enhances follicular development, resulting in more optimal oocyte and embryonic development.

Studies that investigated the use of GnRH-a during COH show beneficial effects of GnRH-a on the IVF outcome: a higher number of growing follicles (1); higher levels of serum E₂ (3); and an increased number of oocytes collected, fertilization rates, and PRs (2–4). In these studies, however, the patients with prior sub-optimal ovarian stimulation were selected (1,4), the indications for IVF treatment were variable (1,3,4) or the number of subjects was too small to make meaningful comparisons (1,2,4). Thus, the precise mechanisms for those beneficial effects of GnRH-a on the IVF outcome remains unclear.

The endocrinologic consequences of COH include frequent atresia of growing follicles and premature luteinization (1,4), which may be associated with spontaneous LH surges (5). The associated increase in the PR with the concomitant use of GnRH-a during COH suggests a beneficial effect of GnRH-a on oocyte quality by preventing premature luteinization of the follicles (1,4). However, the clinical significance of premature luteinization and the precise cutoff value for serum P that indicates its occurrence remain controversial (7–9). We found that the PR diminished when the serum P level on the day of hCG administration exceeded 1.9 ng/ml. However, the incidence of both the elevation of serum P level and the cancellation cycle did not differ significantly between the two stimulation regimens. It seems, therefore, reasonable to consider that any negative impact of premature luteinization on the outcome of IVF reflects factors other than the elevated P level itself.

Circulating levels of A progressively increase during the late follicular phase to reach peak levels at midcycle in spontaneous ovulatory cycles (16,17). The adrenal contribution to peripheral A is relatively constant throughout the menstrual cycle, and the midcycle increase in peripheral A is an ovarian origin (17). The present study demonstrated similarly elevated A levels in the hMG-stimulated patients with or without GnRH-a as those in the normal menstrual cycles. The serum E₂ and A levels are a result of steroids produced by large, well-vascularized preovulatory follicles and are unlikely to reflect steroid secretion in atretic follicles. During early follicular atresia, however, the steroid concentration profile of follicles may change from that of healthy follicles to that of atretic follicles before any morphological change is apparent (13,14). Therefore, the serum A levels may reflect the early atretic changes of developing follicles.

Sex steroids contribute significantly to the regulation of the apoptotic DNA fragmentation, which is suggested to be the underlying mechanism of follicle atresia, with estrogens preventing apoptosis and androgens antagonizing the effect of estrogens (10). An increased A/E₂ ratio in follicular fluid is commonly associated with the morphological features of atresia (12). We found that a greater number of follicles developed in the GnRH-a/hMG group. In addition, the serum concentration of A and the A/E₂ ratio in the serum were significantly lower, indicating a higher viability of the follicles in the GnRH-a/hMG group. These data suggest that the concomitant use of GnRH-a during COH may enhance follicular recruitment and reduce

the proportion of atretic follicles by decreasing the accumulation of androgen in the follicles.

Development of ovarian follicles is primarily under the control of gonadotropins. Recent study has demonstrated an inhibitory effect of FSH treatment in vivo on granulosa cell DNA fragmentation (11), confirming that FSH suppresses apoptotic cell death during atresia. In the present study, the mean number of hMG ampoules required by the GnRH-a/hMG group was significantly greater than that of the hMG-only group. However, the serum concentrations of FSH were similar in the two treatment regimens throughout the stimulated follicular phase. In addition, the number of follicles aspirated and the number of oocytes retrieved per ampoule of hMG were significantly higher in the GnRH-a/hMG group. It is unlikely that more oocytes were obtained in the GnRH-a/hMG group simply because more ampoules of hMG were given. The differences in the follicular development and the steroidogenesis between the two regimens cannot, therefore, be explained on the basis of FSH levels.

Androstenedione is the main precursor of follicular estrogen, being present in large quantities and selectively aromatized under the control of FSH. We found that in the GnRH-a group, the A/E₂ ratio was significantly lower, suggesting an increased conversion of androgen to estrogen. Gonadotropin-releasing hormone and its agonists directly modulate ovarian function (18,19). Recent data revealed that buserelin, at concentrations near those achieved in clinical use, can enhance the secretion of E₂ by cultured human granulosa-lutein cells when A is added to the culture media (19). Buserelin quickly reaches the ovarian follicular fluid after its intranasal administration at therapeutic doses (20). Because the serum levels of FSH were similar in the two treatment regimens, it is possible that GnRH-a itself, rather than gonadotropins, might directly stimulate aromatase activity through specific GnRH-binding sites in the granulosa cell layer (21). Whether GnRH-a is involved in ovarian steroidogenesis during COH remains speculative.

Because LH stimulates thecal androgen production, and because elevated androgen production is associated with follicular atresia (12), it is possible that the suppression of endogenous LH by GnRH-a may enhance the growth of follicles and induce a better follicular environment for oocyte maturation. We found that the serum concentration of A as well as the A/E₂ ratio were significantly lower throughout the stimulated follicular phase in the group administered GnRH-a. In contrast, the serum concentrations of LH in the early follicular phase were lower, but not signifi-

cantly so, and those in the late follicular phase were significantly lower, in the GnRH-a/hMG group than those in the hMG-only group. Although LH suppression cannot be achieved in the early follicular phase, the biological activity of LH in the serum is decreased during GnRH-a treatment (22). The ratio of bioactive LH to immunoreactive LH is significantly increased in the late follicular phase in the absence of GnRH-a (23). Thus, the reduction in bioactive LH throughout the follicular phase and the suppression of LH secretion in the late follicular phase may be responsible for the suppression of ovarian A production during treatment with GnRH-a.

Although similar numbers of normally appearing embryos were transferred in both groups of patients, the implantation rate per embryo was significantly higher in the GnRH-a treatment group than that in the hMG-only group. The serum concentrations of P and E₂ did not differ between the two groups, and the luteal phase supplementation protocol was similar in both. These findings suggest that the higher implantation rate observed with GnRH-a treatment may not have been due to endometrial receptivity but, rather, to the developmental competence of the embryos. However, the putative inhibitory effects of androgens on endometrial tissues (24) may suggest a direct adverse effect of androgens on endometrial receptivity. The possibility that the elevation in serum A may be directly responsible for the impaired implantation in the hMG-only group cannot be excluded.

In conclusion, this prospective study leads us to hypothesize that the concomitant administration of GnRH-a during COH prevents atretic changes of the follicles by reducing androgen accumulation, thus enhancing follicular development, leading to a higher developmental competence of the oocytes. The reduction in androgen accumulation may have resulted from the suppression of LH activity. Further studies are required to clarify the direct action of GnRH-a on the ovary.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid for Scientific Research 06671689 from the Ministry of Education, Tokyo.

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