Leptin Affects Pregnancy Outcome of In Vitro Fertilization and Steroidogenesis of Human Granulosa Cells

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Purpose: This study was to examine the serum leptin levels in the prediction of pregnancy outcomes in women receiving ovarian hyperstimulation. Effect of leptin on the steroidogenesis was evaluated for granulosa cell (GC) culture in vitro.

Method: Serum levels of leptin and estradiol were measured on Day 2, the time of hCG administration and oocyte retrieval in 50 women undergoing long-course GnRH agonist ovarian hyperstimulation. The production of estrogen and progesterone in granulosa cell culture were detected after administration of leptin at the absence or presence of FSH 1 mIU.

Results: Leptin levels at the time of hCG injection were significantly lower in the pregnant women than in those without pregnancy. Estradiol concentrations were not correlated with leptin levels during the time of hCG injection and oocyte retrieval. High leptin concentration suppressed both basal estradiol and progesterone productions in GC. Leptin in high doses suppressed estradiol production, but did not alter progesterone production of GC in the presence of FSH.

Conclusions: Serum leptin levels may be predictive of IVF pregnancy outcome with the effect on intraovarian progesterone/estradiol ratio during the follicular phase. Significantly low serum leptin levels were noted in the pregnant women than in the nonpregnant women.

KEY WORDS: Granulosa cells; leptin; pregnancy outcome; steroidogenesis.

INTRODUCTION

Leptin, a 16-kDa amino acid peptide protein hormone, is secreted exclusively by adipocytes, and acts on the neurons of the central nervous system to regulate energy balance and eating behavior (1). Since the discovery of leptin and leptin receptor in human follicles, granulosa cells, and cumulus cells (2,3), and leptin modulation of gonadotrophins and gonadotrophin-releasing hormone secretion

(4), attention has been increased on the role of leptin in reproductive physiology.

Leptin is an important signal of the onset of puberty (5–7). Recent evidence showed an increase of leptin concentration during ovarian hyperstimulation (8). Another study on leptin levels of sera and follicles in an IVF program indicated that serum leptin increased 22–198% during ovarian hyperstimulation, suggesting high leptin levels might reduce ovarian responsiveness to gonadotrophins (9). In rats, leptin suppressed the synergistic action of insulin-like growth factor-I on FSH-dependent estradiol-17 β secretion of ovarian granulosa cells (10). More recently, there was a report that lower leptin concentrations in follicular fluid were a marker of assisted reproduction treatment success in normal women (11). Also, leptin can be one of the markers of follicular hypoxia and

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suboptimal embryo development (12) since the mRNA and protein of leptin and OB-R mRNA were expressed in oocytes and embryos (13,14), and human placental trophoblasts (15,16). The effect of leptin on ovarian steroidogenesis is controversial. Leptin directly stimulates estradiol, but not progesterone production in GC culture (17). Another study showed that leptin had no effect on estradiol production, alone or in the presence of FSH, or on progesterone production in GC culture (18). Moreover, leptin suppression of progesterone production by human luteinized GC has also been reported (19). This prospective study was designed to examine whether the serum leptin levels during ovarian hyperstimulation in women receiving IVF affect pregnancy outcome and, if this is the case, what role leptin plays at the intraovarian level during oocyte maturation.

MATERIALS AND METHODS

Clinical Study

This study utilized a prospective designed protocol and was approved by the Ethical Committee of the hospital. We recruited 50 women (age ≤ 40 years) undergoing IVF from January 1, 1999, to December 31, 1999. Included criteria were tubal disease, male infertility, immunological infertility, and idiopathic infertility. Women who had polycystic ovarian disease and had abnormal ovarian reserve (FSH ≥ 10 IU/L, or estradiol ≥ 80 pg/mL on cycle Day 3) were excluded. Polycystic ovarian disease fulfilled the diagnostic criteria of characteristic ovarian morphology, with biochemical evidence of hyperandrogenemia (20). Only nonsmoking women were included in order to avoid the bias from the smoking effect on ovarian function (21,22). Short-term pituitary down-regulation was applied for ovarian hyperstimulation by the regimens of HMG/FSH (on Days 3, 4 and 5, and followed by HMG daily) and GnRH agonist (Suprecur^R nasal spray, 900 μ g per day from Day 1) until the day at least two follicles larger than 1.8 cm in diameter were found. Fasting serum samples were collected on Day 2, at the time of hCG injection (10,000 IU), and at the time of oocyte retrieval (34–36 h after hCG injection). All sera were stored at -20°C until radioimmunoassays for estradiol and leptin by commercial kits (Diagnostic Systems Laboratories, Texas and Linco, St Charles, MO, respectively) with the sensitivity of 20 pg/mL and 0.5 ng/mL, respectively. FSH levels on Day 2 were also

measured by radioimmunoassay (Diagnostic Systems Lab., Texas). The inter- and intraassay coefficients of variation in all assays were less than 12%. The end point of this study was a positive pregnancy test with an intrauterine gestational sac, confirmed by vaginal ultrasonography.

Granulosa Cells Culture

To investigate whether leptin acts at an intraovarian level to affect oocyte maturation and, in turn, influences the pregnancy outcome, pooled luteinized GC were obtained from the follicular aspiration via transvaginal ultrasound-guided oocyte retrieval from women during IVF. Briefly, only clear follicular fluids were pooled and centrifuged, at $\times 200g$ for 10 min at room temperature. The pellet was washed in serum-free HTF media and suspended again with 3 mL of HTF media. After adding 3 mL Ficoll solution, and centrifugation ($\times 300g$, 10 min, room temperature), GC were harvested from the middle layer of the tube. GC were then washed twice with 6 mL of HTF media and suspended in 5 mL of HTF media containing 0.1% collagenase. The suspension was shaken for 30 min at room temperature, followed by centrifugation and washed twice. Granulosa cells were then incubated in serum-free HTF media at 37°C for 24 h. The vitality of GC, showing over 80%, was examined by trypan blue before use. Granulosa cells were submitted to culture in 96-well plate, each well containing 5×10^4 GC in final 280 μ l HTF media. Three experiments were performed according to the reagent(s) added as follows: 1) various concentrations of FSH (0, 0.1, 1, 10, and 100 mIU/mL); 2) various concentrations of leptin (0, 1, 5, 10, 20, 40, 60, 80, and 100 ng/mL); and 3) various concentrations of leptin (0, 1, 5, 10, 20, 40, 60, 80, 100 ng/mL) at the presence of FSH (1 mIU/mL). Each test was composed of three separate experiments (i.e. three different patients), of which each ran with four wells for each treatment. After incubation at 37°C for 48 h, the media were harvested and stored at -20°C until radioimmunoassays for estradiol and progesterone.

Statistical Evaluation

Analyses of differences for significance within and between groups were performed in clinical study using the Student's *t* test, Mann-Whitney test, and repeated measures ANOVA followed by Duncan's test. Pearson correlation coefficients were used to test the

correlation between estradiol and leptin levels in the stimulated cycles. A $p < 0.05$ was considered statistically significant for all comparisons. Power analysis for comparing two means were detected with a two-sided test at a significance level of 0.05.

RESULTS

The clinical characteristics of 50 women treated with IVF are shown in Table I, divided into two groups according to the outcome: pregnancy ($n = 20$) and nonpregnancy ($n = 30$). The intrauterine pregnancy was confirmed by vaginal ultrasonography at 5–6 weeks of gestation, and the clinical pregnancy rate was 40%. There was no significant statistical difference in age, body mass index (BMI), and Day 2 serum FSH levels among the two groups. There was no significant statistical difference between the two groups in the mean numbers of follicles, oocytes retrieved, oocytes fertilized, embryos transferred, and total ampoules of gonadotrophins used.

In Table II, the serum leptin levels were lower in the pregnant group throughout the course of stimulation by two-way repeated measures analysis of variance ($F = 7.04$, $p = 0.01$). There was a significant interaction effect between pregnancy outcome and leptin concentration ($F = 3.63$, $p = 0.03$). Following it by means comparison with Duncan's test, serum leptin levels showed a significant difference ($p < 0.05$) at the time of hCG injection between the pregnant

Table I. Characteristics in Women With Pregnancy and Women Without Pregnancy Undergoing In Vitro Fertilization

Outcome	Pregnant	Nonpregnant	<i>p</i> value
No. of patients	20	30	
Age (years)	32.3 ± 4.5	30.7 ± 5.6	ns
BMI (kg/m ²)	21.2 ± 3.6	22.6 ± 4.9	ns
FSH level on Day 2 (U/mL)	5.9 ± 2.1	7.1 ± 3.1	ns
Ampoules of gonadotrophins	31.0 ± 2.0	33.5 ± 2.5	ns
Number of follicles (size >1.6 cm)	11.0 ± 6.7	10.9 ± 7.6	ns
Number of oocytes retrieved	12.9 ± 10.0	13.8 ± 10.1	ns
Number of oocytes fertilized	9.5 ± 8.0	11.2 ± 10.3	ns
Number of embryos transferred	3.4 ± 1.8	4.1 ± 1.3	ns

Note. Data are presented as mean ± SD. *p* value is calculated with Student's *t* test. BMI – body mass index. ns – not significantly different.

Table II. Fasting Serum Leptin and Estradiol Levels (mean ± SD) in Women With Pregnancy ($n = 20$) and Women Without Pregnancy ($n = 30$) Undergoing In Vitro Fertilization

	Outcome		<i>p</i> value
	Pregnant	Nonpregnant	
Leptin levels (ng/mL)			
Day 2	3.8 ± 1.4	5.3 ± 3.7	ns
Time of hCG injection	4.6 ± 1.7	8.6 ± 5.0 ^a	$p < 0.05$
Time of oocyte retrieval	6.3 ± 2.8 ^a	8.7 ± 6.1 ^a	ns
Estradiol levels (pg/mL)			
Day 2	38.2 ± 20.5	28.5 ± 11.0	ns
Time of hCG injection	3302.0 ± 2016.7	3288.5 ± 2351.1	ns
Time of oocyte retrieval	1819.1 ± 1289.5	2072.6 ± 1696.3	ns

Note. Comparing the means with Duncan's test reveals significantly decreased leptin concentration in the pregnant women at the time of hCG injection ($p < 0.05$). ns – not significantly different.

^a Indicating significant elevation of leptin levels compared with the levels on Day 2.

(4.6 ± 1.7 ng/mL) and nonpregnant (8.6 ± 5.0 ng/mL) women; power analysis documented was more than 95%. No statistical difference was found on Day 2 leptin between the pregnant (3.8 ± 1.4 ng/mL) and nonpregnant (5.3 ± 3.7 ng/mL) women; power analysis was 52.2%. There was no statistical difference between pregnant (6.3 ± 2.8 ng/mL) and nonpregnant (8.7 ± 6.1 ng/mL) groups in leptin concentration at the time of oocyte retrieval; power analysis was 77.4%.

In the nonpregnant group, significant differences in leptin concentration were found between Day 2 and the time of hCG injection (power analysis = 82.8%), and between Day 2 and the time of oocyte retrieval (power analysis = 74%). In the pregnant women, significant differences of leptin levels were only found between Day 2 and the time of oocyte retrieval (power analysis = 94.6%). The estradiol levels increased obviously from Day 2 to the time of hCG injection in both pregnant and nonpregnant groups; however, there was no statistical significance of estradiol levels at each time between the two groups (Table II).

The primary human luteinized GC culture in vitro displayed the effect of leptin on the steroidogenesis in the presence or absence of FSH. By repeated measures of ANOVA, FSH equal to or more than 1 mIU/mL stimulated significant estradiol and progesterone secretion (data not shown). High

Fig. 1. Effects of FSH and leptin on the steroidogenesis of luteinized granulosa cells culture. Effects of leptin on basal estradiol (a) and progesterone (b) productions. Effects of leptin on estradiol (c) and progesterone (d) productions at the presence of FSH (1 mIU/mL). Each bar means granulosa cells cultured from three different patients and each runs with 4 wells for each treatment. Data are expressed as mean $\pm$ SD. Repeated measures ANOVA were performed. *Indicating significantly different ($p < 0.05$) from the basal levels. **Denoting significantly different ($p < 0.05$) from the level marked by*. C – control. E₂ – estradiol.

leptin (40–100 ng/mL) concentration inhibited basal estradiol levels significantly in the absence of FSH (Fig. 1(a)). Leptin concentration (60–100 ng/mL) inhibited basal progesterone levels (Fig. 1(b)). In the presence of FSH (1 mIU/mL), leptin levels (40–100 ng/mL) significantly decreased estradiol secretion (Fig. 1(c)). However, leptin did not affect progesterone secretion (Fig. 1(d)). For analysis of the correlation of estradiol and leptin concentration during the time of hCG injection or oocyte retrieval, Pearson correlation coefficients were calculated. There was no significant correlation between estradiol levels and leptin levels after division into the three groups of total number of patients ($n = 50$), pregnant women ($n = 20$), and nonpregnant women ($n = 30$) (Fig. 2).

DISCUSSION

The increased serum leptin levels between baseline (Day 2) and maximal hyperstimulation (at the time of hCG injection) in the nonpregnancy group are consistent with the report of Butzow *et al.* (9). However, we have demonstrated that, during ovarian hyperstimulation, lower serum leptin concentrations at the time of hCG injection (LH surge) are significantly associated with successful pregnancy outcome, but they are not shown in the failed pregnancy group. The leptin level on Day 2 in the pregnant group is lower than the nonpregnant women, though it does not show statistical difference, although with the power of 52.2%, we cannot exclude a relationship. The likelihood of

Fig. 2. Pearson's correlation coefficients are calculated between serum estradiol and leptin concentration at the time of hCG (left panel) and oocyte retrieval (right panel). No significant correlations are found after dividing into the pregnant women ($n = 20$), nonpregnant women ($n = 30$), and total patients ($n = 50$).

serum leptin levels in the follicular phase in association with pregnancy outcome is attributed to the leptin effect on the quality of oocytes rather than on the quantity of oocytes since there are no statistical differences in the numbers of follicles and oocytes

retrieved between the two groups. In the pregnant women, the elevated leptin levels after hCG injection (at the time of oocyte retrieval) might imply the effect of leptin on the implantation potential or embryo development. This finding is comparable to the

previous report of a postovulatory increase in the serum leptin (3).

It is interesting to note how leptin acts on the intraovarian level to influence the oocytes quality. Recent study in an IVF program has shown that higher serum leptin levels in women, would be reflected by higher levels in follicular fluid (11). FSH receptors are not detected on GCs until the preantral stage (23). The follicular maturation is dependent on the amount of FSH available to the ovaries and the sensitivity of follicles to the gonadotrophins. The ovarian estradiol is produced by FSH-induced aromatization of androgen in the estrogenic microenvironment of GC. In antral follicular fluid, estrogen is the dominant steroid in the presence of FSH. Nevertheless, in the absence of FSH, androgens become dominant (24). On the other hand, LH is not normally detected in follicular fluid until midcycle. The positive correlation of leptin and LH pulsatility in circulation has been demonstrated in the normal menstrual cycle of women (25). Leptin levels in the follicular fluid of developing follicles are similar to the levels measured in serum of normal women and IVF women (11). That a higher leptin level presents in antral fluid at the midcycle might also reflect a higher LH level, resulting in the decrease of granulosa mitotic activity and increase of androgen level in follicle. The androgen/estrogen ratios increase, which are most likely to house an unhealthy oocyte (26). In normal cycles of women, mean estradiol concentration and progesterone/estradiol ratio are significantly lower in follicular fluid containing degenerating oocyte than those containing other oocyte stages (27). It has been demonstrated that leptin levels correlate negatively with follicular oxygen concentrations and a positive correlation with follicular PCO₂, affecting oocyte development and embryo quality (12). Our results demonstrate that leptin performs an inhibitory effect on both estradiol and progesterone productions of GC. However, in the presence of FSH, leptin in doses of more than 40 ng/mL exhibits an inhibitory effect on estradiol production. This effect is not seen in progesterone production. There is no doubt that inappropriately high leptin levels in follicles would affect the LH-leptin interaction in steroidogenesis. Our results indicate, at the time of hCG injection, a significantly higher leptin level presents in nonpregnant women than in women with pregnancy. It is conceivable that there is a higher level presented in preantral or antral follicular fluid in those women. Thus, the quality rather than quantity of oocytes is affected directly or indirectly during follicular development at the stage of

midcycle, a tendency which was observed in the present study.

How the mechanism of interaction between leptin and LH in follicle related to clinical pregnancy outcome is not clear. Notably, in successfully pregnant women, a significant increase of serum leptin at the time of oocyte retrieval suggests an important role for leptin at this critical time too. This leptin elevation does not occur in nonpregnant women after hCG injection. In normal menstrual cycle, serum leptin levels and their pulsatile patterns positively correlate with LH levels, especially at the time of the LH surge (28), suggesting leptin involvement in the regulation of hypothalamic-pituitary-gonadal axis. LH receptor can be detected only in theca cells and its concentrations are highest in the area closest to the basement membrane, separating the luteinized granulosa cells from the stroma. Acquisition and up-regulation of lipid uptake and storage pathways in luteinizing GC are known to be LH-dependent phenomena. Leptin is well known to be related to lipid deposit in adipocytes. Leptin has been reported to regulate LH secretion (29). It is reasonable to imply that leptin is involved in the lipid-rich vacuoles in the luteinized granulosa cells. Meanwhile, that the luteinized granulosa cells transform into corpus luteum also implies the possibility of leptin regulation in the ovarian steroidogenesis.

Leptin induces LH and FSH secretions from pituitary gland (30). Leptin (100 ng/mL) has also been shown to inhibit LH as well as IGF-I-stimulated estradiol production in human granulosa/thecal cells (2). Another study showed leptin (0.5–10 ng/mL) directly stimulates estradiol production in human luteinized GC (17). In bovine ovaries, leptin inhibits progesterone secretion (31). In human GC, leptin (0–100 ng/mL) inhibited hCG-stimulated progesterone production (19). Interestingly, there is evidence that leptin (0–100 ng/mL) had no effect on estradiol production alone or in the presence of FSH in GC, or on progesterone production (18). Our results show that leptin at dosages larger than 40 ng/mL inhibits both estradiol and progesterone production of human GC. Nevertheless, it still inhibits estradiol production but does not alter the progesterone production in the presence of FSH. It seems that the effects of leptin on the ovarian steroidogenesis are quite controversial. From the in vitro culture study, an inappropriate estradiol concentration or progesterone to estradiol ratio in follicle would result in impairment of oocyte quality during the follicular phase. Recently, Butzow *et al.* (9) have reported a similar observation

that during IVF a high relative leptin increase was associated with adiposity and a reduced ovarian response. Therefore, the likelihood to have an ongoing pregnancy after IVF treatment is less in the group of obese patients (32).

Leptin stimulates CRH gene expression; thus, lower leptin levels would lower CRH (33), since CRH can directly inhibit hypothalamic GnRH secretion (34), leading to lower gonadotrophin production. Although lower gonadotrophin levels can result in lower nuclear maturation to metaphase II of oocytes (35), the amount of gonadotrophin administration affects leptin levels neither in blood nor in follicular fluid (11). Meanwhile, leptin can directly impair the IGF-I-mediated augmentation of FSH-stimulated estradiol synthesis by granulosa cells (10,18). The influence of GnRH analogues on the regulation of leptin in IVF women is also unclear. It has been demonstrated that in hirsute women treated with GnRH analogues (a single 3.75 mg of triptorelin, I.M. injection), the responses of leptin, similar to LH and FSH, show a significant increase on Day 1 and a significant suppression on Day 21 (36). The present report confirms that serum leptin levels may be predictive of IVF pregnancy outcome, and that leptin can modulate in vitro steroidogenesis by granulosa cell culture. Several questions remain to be settled, for example, the expressions of mRNA of leptin and leptin receptors in human endometrium (37), embryos as well as placental trophoblasts have raised another issue for leptin study in human reproduction.

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