Intrafollicular and Circulating Concentrations of Leptin Do Not Predict the Outcome in IVF-ICSI Cycles

Byron Asimakopoulos, PhD, Frank Köster, PhD, Ricardo Felberbaum, MD, PhD, Grigorios Tripsiannis, PhD, Gamze Sinem Caglar, MD, PhD, Nikos Nikolettos, MD, PhD, Safaa Al-Hasani, MDV, PhD, and Klaus Diedrich, MD, PhD

Leptin is involved not only in the regulation of food intake but also in other functions including reproduction. Because leptin has been demonstrated to influence ovarian steroidogenesis directly and leptin levels vary during the menstrual cycle and in stimulated cycles, we tested the hypothesis that serum or intrafollicular concentrations of leptin would correlate with reproductive outcomes in intracytoplasmic sperm injection cycles. Serum and follicular fluid samples were collected from 77 women undergoing ovarian stimulation, intracytoplasmic sperm injection and embryo transfer due to male factor infertility. The concentrations of total leptin, both in serum and in pooled follicular fluid samples, did not correlate with the number of oocytes, the fertilization rate or the embryo quality. Additionally, leptin concentrations did not differ between cycles that resulted in pregnancy and those that failed. These results raise objections to the prognostic value of leptin for the outcome of in vitro fertilization/intracytoplasmic sperm injection cycles.

KEY WORDS: Leptin, fertilization, embryo quality, pregnancy, ICSI.

INTRODUCTION

Leptin, the 16-kd product of the *LEP* gene located in 7q31.3, is mainly produced in adipose tissue and is considered to play an important role in appetite control, fat metabolism, and consequently in the regulation of body weight.[1,2] The levels of circulating leptin strongly correlate with body mass index (BMI).[1,2] *LEP* gene is also expressed in skeletal muscle, stomach, endometrium, and placenta whereas its expression in ovarian tissues is still in doubt.[1-3] The effects of leptin are mediated by receptors on the extracellular membrane with a single transmembrane domain. Leptin receptors are encoded by the *LEPR* gene found in 1q31 chromosome and belong to gp130 family receptors. Five leptin receptors have been described as the result of alternative splicing: Ob-Ra,Ob-Rb,Ob-Rc,Ob-Rd, and Ob-Re.Ob-Rb is considered to be the main functional receptor expressed in hypothalamus and in other sites as well. Ob-Re is a soluble receptor regulating the bioactivity of leptin. It seems that at least one isoform of leptin receptors is expressed in almost every tissue of the human body.[1-3]

Soon after its discovery, leptin was linked to reproductive functions. It was shown that it participates in the development of the reproductive system during puberty as well as in the reproductive functions during adulthood, acting as a satiety signal that influences the hypothalamus–pituitary–gonadal axis.[1-4] It seems that leptin, by binding on hypothalamic leptin receptors, modulates the secretion of gonadotropin-releasing hormone (GnRH)

From the Laboratory of Physiology, School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece (BA, GT, NN); Department of Gynecology and Obstetrics, University Clinic of Schleswig-Holstein, Campus Lübeck, Lübeck, Germany (FK, GSC, SA-H, KD); and Department of Obstetrics/Gynecology, Clinic of Kempten, Academic Teaching Hospital of the University of Ulm, Germany (RF).

Address correspondence to: Byron Asimakopoulos, PhD, Laboratory of Physiology, School of Medicine, Democritus University of Thrace, Dragana, 68100 Alexandroupolis, Greece. E-mail: basima@med.duth.gr.

Reproductive Sciences Vol. 16 No. 1 January 2009 113-119
DOI. 10.1177/1933719108324139

by hypothalamic neurons, thus influencing the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by gonadotrope pituitary cells. Leptin modulates further the release of FSH and LH by acting on gonadotrope cells, which also express functional leptin receptors.

The effects of leptin in ovarian function have gained a special attention. Several lines of evidence indicate that the ovary is a target organ for leptin; ovarian granulosa and theca cells[5] as well as the oocytes[6] express high-affinity receptors for leptin. Many studies have indicated that leptin has modulatory effects on ovarian steroidogenesis while at the same time sex hormones influence leptin expression.[5,7-10] It is also well documented that leptin levels show a considerable variation throughout the human menstrual cycle, with higher levels during the luteal phase.[11,12] The above findings suggest that leptin may both indirectly and directly influence follicular growth, oocyte development as well as implantation. This notion along with the knowledge that overweight and obese women have usually a poor outcome in in vitro fertilization (IVF) cycles[13] has fuelled the investigation on the prognostic value of leptin for the outcome of IVF cycles.

During the last years, many studies aimed to find an association between leptin levels either in serum or in follicular fluids (FFs) and the outcome of IVF cycles. However, these studies have failed to reach consensus.[10,14-28]

In the present study, the concentrations of leptin in both blood and FFs were investigated as possible prognostic markers for the outcome in intracytoplasmic sperm injection (ICSI) cycles. The relationships of leptin concentrations with those of other hormones were also investigated. The women who participated in the study had a normal reproductive system and followed controlled ovarian stimulation (COH), ICSI, and embryo transfer exclusively due to male factor infertility.

MATERIALS AND METHODS

Participants, COH, ICSI, and Embryo Transfers

The study was carried out in the Department of Gynecology and Obstetrics, University Clinic of Schleswig-Holstein in Campus Lübeck. The study population included 77 women without any apparent abnormality of their reproductive system as defined by their medical history, the clinical examination, and common hormonal tests. They participated in ICSI cycles exclusively due to male factor infertility. Their age was 32.8 $\pm$ 3.7 years (range = 22-41). Low responders as well as women with basal FSH $\geq$10 mIU/mL were excluded. The study was approved by the Internal Review Board. Only 1 cycle from each patient was included in the study.

Controlled ovarian stimulation was performed according to the multidose GnRH-antagonist protocol (''Lübeck antagonist protocol'') as described elsewhere.[29,30] Briefly, the pituitary suppression was achieved with cetrorelix (Cetrotide, Serono International SA, Geneva, Switzerland) 0.25 mg per day from cycle day 6 onward until ovulation induction. The ovarian stimulation was achieved with recombinant FSH (recFSH; Gonal-F, Serono International S.A., Geneva, Switzerland), which was individually adjusted according to serum estradiol (E2) levels and ovarian sonography. Final oocyte maturation was induced by injection of 10 000 IU human chorionic gonadotropin (hCG; Pregnyl; N.V. Organon, Oss, Holand) when at least 3 follicles reached a diameter of 17 mm and the serum E2 concentration increased to 300 to 500 pg/mL per follicle. Transvaginal oocyte aspiration with ultrasound guidance was performed 36 hours later.

The preparation of oocytes, ICSI, and culture of fertilized oocytes were performed as previously described.[21] Only fresh semen samples obtained by masturbation after 3 to 4 days of abstinence were used.

Embryo transfers were performed 48 hours after oocyte aspiration under ultrasound guidance. Up to 3 cleaving embryos were transferred, according to the German Embryo Protection Law. Before embryo transfers, according to the degree of fragmentation and the regularity of blastomeres, each embryo was graded as follows: G1 (very good quality), G2 (good quality), and G3 (bad quality). Each grade was assigned a value: G1 = 3, G2 = 2, and G3 = 1. The score for each embryo was the product of grade value multiplied by the number of blastomeres. The cumulative embryo score (CES) was the sum of the embryo scores of the transferred embryos.[31]

The luteal phase was supported daily with 600 mg natural progesterone (P) administrated vaginally (Utrogestan; Besins-Iscovesco, Paris, France). Pregnancies were defined by the presence of positive fetal heart beats. In these cases, the administration of P was continued up to week 12 of gestation.

Sampling and Measurements

On the day of hCG, peripheral blood samples were taken from each woman, sera were extracted and aliquots stored at −75°C.

During oocyte aspiration, FF samples from follicles containing oocytes were collected in separate tubes. Follicular fluids contaminated with flushing fluid or with obvious presence of blood were not collected. After denudation of oocytes, those tubes which contained fluids coming from follicles with metaphase II (MII) oocytes were pooled together whereas others were discarded. Pooled FFs were mildly centrifuged and the supernatants were stored at −75°C in aliquots for further analysis.

In serum samples, total leptin, E2, P, LH, and testosterone, whereas in FF samples, total leptin, E2, and P, were measured.

In serum samples, E2, P, LH, and testosterone were measured with Elecsys immunoanalyzer (Roche Diagnostics, Mannheim, Germany) having the following intra-assay and inter-assay variations (AV): <5% and <10% for E2, <3% and <5% for P, <5% and <7% for testosterone. All the other measurements were made with commercially available enzyme immunoassay kits as follows. E2: DSL-10-4300 Active Estradiol EIA (DSL, Webster, Tex), intra-AV: 3.3% to 4.8%, inter-AV: 6.5% to 8.2%, minimum detectable dose (MDD): 7 pg/mL, P: DE220 (R&D Systems Inc, Minneapolis, Minn), intra-AV: 4.9% to 7.6%, inter-AV: 2.7% to 8.3%, MDD: 8.57 pg/mL. Leptin: Quantikine DLP00 (R&D Systems Inc, Minneapolis, Minn), intra-AV: 3% to 3.3%, inter-AV: 3.5% to 5.4%, and MDD: 7.8 pg/mL.

As the above kits are validated for serum samples but not for FFs, before running FF samples, we checked the recovery rate of at least 3 spiked FF samples and the linearity of the results after multiple dilutions. The results were satisfactory, with recovery rates ranging from 80% to 130%. All samples were measured twice, according to manufacturers' instructions. In cases of very high or low results, the measurements were repeated with appropriate dilution.

Statistical Analysis

The cycles that resulted in pregnancy are referred to as group A and the cycles that failed to result in pregnancy are referred to as group B. The normality of all studied parameters was checked with the Shapiro-Wilks' *W* test and an additional evaluation of kurtosis and skewness. The statistical analysis included descriptive statistics for both studied groups. The comparisons of clinical data as well as hormonal and cytokine concentrations between the groups were performed either with the *t* test, or with 2 nonparametric tests: Mann-Whitney*U* test and Kolmogorov-Smirnov. Correlations were evaluated with the Spearman Rank test. The 2-tailed significant level was set at $P < .05$. Statistical analysis was performed using the software STATISTICA 6.0 (StatSoft Inc., Tulsa, Okla). All values are presented as mean ± standard deviation.

RESULTS

Among the 77 cycles, 19 resulted in clinical pregnancies. There were no cycles with clinical symptoms of ovarian hyperstimulation syndrome. The clinical characteristics as well as the measurements of the cycles that resulted in pregnancy (group A) and those that failed (group B) are presented in Table 1.

The 2 groups were similar in terms of age, BMI, and the quality of transferred embryos. Group A received lower amount of gonadotropins, had a slightly lower number of retrieved oocytes, and a slightly higher fertilization rate, in comparison to group B, though these differences were not statistically significant. Group A had also lower peak serum E2 concentrations than group B but not at a statistically significant level.

There were no significant differences between the 2 groups regarding the concentrations of total leptin and the studied hormones in both serum samples and pooled FFs.

The correlations among the studied parameters are presented in Tables 2 and 3. The amount of administrated recFSH showed positive correlations with intrafollicular P concentrations. Serum and intrafollicular leptin correlated positively and strongly with BMI. The number of oocytes retrieved, the fertilization rate as well as the CES did not correlate neither with serum nor with intrafollicular leptin. The number of oocytes retrieved correlated positively with peak E2 serum and serum P concentrations. The CES of the transferred embryos had a weak positive correlation with Peak E2 serum concentrations. Intrafollicular leptin correlated positively with intrafollicular P concentrations.

DISCUSSION

During the last decade, many studies have aimed to explore the association of leptin levels with the outcome of IVF cycles in terms of ovarian response to COH, oocyte quality, embryo quality, and the achievement of pregnancy. However, their results have been contradictory.

Barroso et al[32] reported that leptin FF levels correlate negatively with embryo quality in IVF cycles, suggesting that leptin, together with vascular endothelial growth factor, is a marker of follicular hypoxia. At the same time,

Table 1. Clinical Characteristics, Hormonal and Cytokines Concentrations of the 2 Studied Groups[a]

Parameter	Group A (n = 19)	Group B (n = 58)	*P*
Age	31.7 ± 3.6 (29.9-34)	33.2 ± 3.8 (32.2-34.2)	.914[b]
BMI	24.9 ± 4.6 (22.7-27)	23.7 ± 3.9 (22.6-24.7)	.272[c]
recFSH (IU)	1803.9 ± 593.4 (1517.9-2090)	2081.1 ± 765.9 (1877.9-2284.3)	.425[c]
Number of retrieved MII oocytes	8.7 ± 4.5 (6.6-11)	10.1 ± 5.2 (8.7-11.4)	.512[b]
Fertilization rate (%)	62 ± 20.5 (52.1-72)	55.1 ± 25.2 (48.4-61.8)	.365[c]
Number of embryos transferred	2.263 ± 0.653 (1.9-3)	2.421 ± 0.706 (2.2-2.6)	.346[c]
CES	21.4 ± 8.9 (17.1-26)	21.4 ± 10.3 (18.7-24.1)	.523[b]
Serum E2 (pg/mL)	1748.9 ± 1425.1 (1062.1-2436)	2117.4 ± 1035.6 (1837.4-2397.4)	.083[c]
Serum P (ng/mL)	0.8 ± 0.3 (0.6-1)	0.9 ± 0.4 (0.7-1)	.375[c]
Serum testosterone (ng/mL)	0.6 ± 0.5 (0.3-1)	0.5 ± 0.2 (0.4-0.6)	.675[c]
Serum LH (ng/mL)	1.9 ± 2.3 (0.8-3)	1.8 ± 1.6 (1.4-2.3)	.767[c]
Serum leptin (pg/mL)	20903 ± 21790.5 (8835.8-32970)	15979.2 ± 10174.5 (12885.8-19072.5)	.911[c]
FF-E2 (pg/mL)	834162.6 ± 400702.9 (628140.2-1040185)	744213.9 ± 414388.1 (632189.1-856238.6)	.453[c]
FF-P (ng/mL)	23553.3 ± 5262.2 (17019.4-30087)	20582.5 ± 10262.9 (16522.6-24642.4)	.22[c]
FF-leptin (pg/mL)	27700.3 ± 23681.6 (15524.3-39876)	24921.1 ± 19490 (19601.4-30240.9)	.857[c]

Abbreviations: BMI, body mass index; CES, cumulative embryo score; E2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MII, metaphase II; P, progesterone; recFSH, recombinant FSH.

[a] Group A: cycles resulted in pregnancy; Group B: cycles that did not result in pregnancy. Serum concentrations were measured at the day of hCG. The values are given as mean ± standard deviation; confidence intervals 95% are given in parenthesis.

[b] *P* estimated by *t* test.

[c] *P* estimated by Mann-Whitney*U* test.

Table 2. Correlations Among the Clinical Characteristics of the ICSI Cycles and the Serum Concentrations of Hormones and Cytokines at the Day of hCG[a]

	Serum E2	Serum P	Serum Testosterone	Serum LH	Serum Leptin
Age	−0.058	−0.136	−0.364[b]	0.172	−0.249
BMI	−0.179	−0.169	0.101	0.061	0.491[b]
recFSH	−0.02	−0.04	−0.28	0.002	0.077
Number of retrieved MII oocytes	0.497[b]	0.322[b]	0.184	−0.268[b]	−0.007
Fertilization rate	−0.109	−0.032	−0.079	0.051	−0.087
CES	0.291[b]	0.112	0.075	−0.036	0.117
Serum E2		0.572[b]	0.572[b]	0.151	−0.117
Serum P			0.522[b]	0.059	−0.2
Serum testosterone				0.493[b]	0.221
Serum LH					0.105
Serum leptin					

Abbreviations: BMI, body mass index; CES, cumulative embryo score; E2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; LH, luteinizing hormone; MII, metaphase II; P, progesterone; recFSH, recombinant FSH.

[a] Spearman *R* is given.

[b] $P < .05$.

Bützow et al[14] found that a considerable increase in leptin levels during COH is related with reduced ovarian response. Later on, similar findings were reported by other investigators.[22,23,26]

Mantzoros et al[15] were the first who reported that women who became pregnant during IVF or gamete intrafallopian transfer (GIFT) cycles had significantly lower intrafollicular leptin concentrations than women who failed to become pregnant. After that, several investigators reported that either serum or intrafollicular high levels of leptin are associated with lower pregnancy rates in IVF cycles.[10,16,20-23]

The present study opposes the notion that circulating or intrafollicular concentrations of leptin can serve as a

Table 3. Correlations Among the Clinical Characteristics of the Studied ICSI Cycles and the Concentrations of Hormones and Cytokines in Follicular Fluid Samples[a]

	FF-E2	FF-P	FF-Leptin
Age	0.16	−0.066	−0.3[b]
BMI	0.078	0.482[b]	0.584[b]
recFSH	0.012	0.402[b]	0.058
Number of retrieved MII oocytes	−0.181	−0.027	0.027
Fertilization rate	0.141	−0.021	0.019
CES	0.131	0.201	0.058
FF-E2		0.04	−0.025
FF-P			0.38[b]
FF leptin			

Abbreviations: BMI, body mass index; CES, cumulative embryo score; E2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; MII, metaphase II; P, progesterone; recFSH, recombinant FSH.

[a] Spearman R is given.

[b] $P < .05$.

prognostic factor for the outcome in ICSI cycles. According to our present results, neither circulating nor intrafollicular leptin concentrations were associated with the number of oocytes retrieved, the fertilization rate, the CES, and the achievement of pregnancy. These results are consistent with several previous studies.[17-19,24-27]

Indisputably, leptin is mandatory for the normal reproductive function. It acts at multiple levels of the reproductive axis, and it seems that its effects depend on its concentrations; high levels of leptin exert inhibitory direct actions on gonadal functions.[3] The hypothalamus–pituitary axis probably is not influenced by high circulating levels of leptin because the saturable system of leptin through the blood–brain barrier prevents excess concentrations of leptin binding to hypothalamic receptors.[33] It is also worth noting that during COH that precedes IVF and embryo transfer, the hypothalamus–pituitary axis is carefully controlled and ceased. Therefore, the actions of leptin on GnRH neurons and gonadotrope cells are probably meaningless, whereas its direct actions on ovarian cells might be considerable.

Regarding the direct actions on ovarian cells, a large body of evidence indicates that leptin at high concentrations antagonizes the augmenting effect of several growth factors and hormones on gonadotropin-stimulated steroidogenesis in both ovarian theca and granulosa cells, whereas low concentrations of leptin either do not influence or even stimulate the aromatase activity of granulosa cells.[5,7-10]

The question is which are the critical levels of leptin to affect the ovarian functions? In other words, which levels are ''high'' and which levels are ''low''?

In this respect, the selection of the study population might have influenced the results to a large extent as the expression of leptin may be influenced by factors as BMI and inflammatory conditions. This study included healthy, nonobese women who participated in ICSI cycles exclusively due to male factor infertility.

Moreover, the methodology of measurements also has an impact on the results. Different methods of measuring leptin concentrations in blood and FFs have been used in each published study. However, not all assays have comparable sensitivity or specificity. It is well known that measuring a cytokine in the same sample by different assays can bring about different results. This is more obvious in FF samples because not all assays are validated for this kind of samples. This methological problem obscures the definition of the ''high'' and ''low'' concentrations of leptin. By measuring the same samples with different assay kits, different ''high'' and ''low'' concentrations of leptin may arise.

Last but not least, not all the studies have followed the same methodology of FFs collection. A number of studies have measured leptin concentrations in individual follicles whereas others in pooled FFs. The first method is very useful when the main aim is to investigate the maturation status and the fertilization potential of individual oocytes as well as the development of the embryos that come from these oocytes. Pooled FFs are used to negate the variation in leptin concentrations in individual follicles as well as to be consistent with ''pooled embryo transfers,'' thus measurements in pooled FFs are supposed to be more reliable when the main aim is to investigate the ovarian response (in terms of the total number of MII oocytes retrieved), the CES of transferred embryos, and the pregnancy outcome. It is obvious that the concentrations of leptin might be very different according to the method of FF collection used.

Furthermore, it is not always clearly stated from what kind of follicles the fluids are coming from. Mature follicles containing MII oocytes have a different cytokine and hormonal profile than small follicles. We believe that FFs derived from follicles of different status of maturation give confusing and unreliable results. In this study, we measured leptin and hormone levels in pooled FFs coming from mature follicles containing MII oocytes.

For all the above reasons, the comparison of the results from different studies exploring the role of leptin on the outcome of IVF cycles is not always easy and the evaluation of the prognostic value of leptin levels becomes difficult. The results and conclusions of the relevant studies, including the present one, have to be considered only as indicative.

In our opinion, although high levels of leptin seem to disrupt the function of ovarian cells and compromise the quality and the fertilizability of the oocytes, the usefulness of leptin concentrations, either circulating or intrafollicular, as a prognostic marker for the outcome in IVF/ICSI cycles is not granted. It is not certain that leptin levels can always predict the fertilization outcome, the embryo development, or the pregnancy outcome in IVF/ICSI cycles. Furthermore, because follicular maturation, fertilization, and implantation are complex processes where a plethora of factors play a role, we can postulate that it is rather impossible for one of them, namely leptin, to be established as a reliable prognostic factor.

ACKNOWLEDGMENTS

The study was partially supported by DAAD and the Greek Scholarships Foundation (IKY) through the program IKYDA. The authors thank Dr Steven Waczek for revision of the English text.

REFERENCES

1. Mantzoros CS, Moschos SJ. Leptin: in search of role(s) in human physiology and pathophysiology. *Clin Endocrinol*. 1998;49:551-567.
2. Mantzoros CS. The role of leptin in human obesity and disease: a review of current evidence. *Ann Int Med*. 1999;130:671-680.
3. Caprio M, Fabbrini E, Isidori AM, Aversa A, Fabbri A. Leptin in reproduction. *Trends Endocrinol Metab*. 2001;12:65-72.
4. Goumenou AG, Matalliotakis IM, Koumentakis GE, Panidis DK. The role of leptin in fertility. *Eur J Obstet Gynecol Reprod Biol*. 2003;106:118-124.
5. Karlsson C, Lindell K, Svensson E, et al. Expression of functional leptin receptors in the human ovary. *J Clin Endocrinol Metab*. 1997;82:4144-4148.
6. Antczak M, Van Blerkom J. Oocyte influences on early development: the regulation proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod*. 1997;3:1067-1086.
7. Agarwal SK, Vogel K, Weitsman SR, Magoffin DA. Leptin antagonizes the insulin-like growth factor-I augmentation of steroidogenesis in granulosa and theca cells of the human ovary. *J Clin Endocrinol Metab*. 1999;84:1072-1076.
8. Brannian JD, Zhao Y, McElroy M. Leptin, inhibits gonadotrophin-stimulated granulosa cell progesterone production by antagonizing insulin action. *Hum Reprod*. 1999;14: 1445-1448.
9. Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H. Leptin directly stimulates aromatase activity in human luteinized granulosa cells. *Mol Hum Reprod*. 1999;5:708-713.
10. Tsai EM, Yang CH, Chen SC, et al. Leptin affects pregnancy outcome of in vitro fertilization and steroidogenesis of human granulosa cells. *J Assist Reprod Genet*. 2002;19:169-176.
11. Ludwig M, Klein HH, Diedrich K, Ortmann O. Serum leptin concentrations throughout the menstrual cycle. *Arch Gynecol Obstet*. 2000;263:99-101.
12. Geisthövel F, Jochmann N, Widjaja A, Horn R, Brabant G. Serum pattern of circulating free leptin, bound leptin and soluble leptin receptor in the physiological menstrual cycle. *Fertil Steril*. 2004;81:398-402.
13. Maheshwari A, Stofberg L, Bhattacharya S. Effect of overweight and obesity on assisted reproductive technology—a systematic review. *Hum Reprod Update*. 2007;13:433-444.
14. Bützow TL, Moilanen JM, Lehtovirta M, et al. Serum and follicular fluid leptin during in vitro fertilization: relationship among leptin increase, body fat mass, and reduced ovarian response. *J Clin Endocrinol Metab*. 1999;84:3135-3139.
15. Mantzoros CS, Cramer DW, Liberman RF, Barbieri RL. Predictive value of serum and follicular fluid leptin concentrations during assisted reproductive cycles in normal women and in women with polycystic ovarian syndrome. *Hum Reprod*. 2000;15:539-544.
16. Brannian JD, Schmidt SM, Kreger DO, Hansen KA. Baseline non fasting serum leptin concentration to body mass index ratio is predictive of IVF outcomes. *Hum Reprod*. 2001;16: 1819-1826.
17. Dorn C, Reinsberg J, Kupka M, van der Ven H, Schild RL. Leptin, VEGF, IGF-1 and IGFBP-3 concentrations in serum and follicular fluid of women undergoing in vitro fertilization. *Arch Gynecol Obstet*. 2003;268:187-193.
18. Welt CK, Schneyer AL, Heist K, Mantzoros CS. Leptin and soluble leptin receptor in follicular fluid. *J Assist Reprod Gen*. 2003;20:495-501.
19. Chen R, Fisch B, Ben-Haroush A, Kaplan B, Hod M, Orvieto R. Serum and follicular fluid leptin levels in patients undergoing controlled ovarian hyperstimulation for in vitro fertilization cycle. *Clin Exp Obstet Gynecol*. 2004;31: 103-106.
20. Nikolettos N, Asimakopoulos B, Nicolettos N, Efthimiadou A, Mourvati E, Demirel C. Evaluation of leptin, interleukin-1β, tumor necrosis factor-α and vascular endothelial growth factor in serum and follicular fluids of women undergoing controlled ovarian hyperstimualtion as prognostic markers of ICSI outcome. *In Vivo*. 2004;18:667-674.
21. Asimakopoulos B, Nikolettos N, Papachristou DN, Simopoulou M, Al-Hasani S, Diedrich K. Follicular fluid levels of vascular endothelial growth factor and leptin are associated with pregnancy outcome of normal women participating in intracytoplasmic sperm injection cycles. *Physiol Res*. 2005;54:263-270.

22. Anifandis G, Koutselini E, Louridas K, et al. Estradiol and leptin as conditional prognostic IVF markers. *Reproduction*. 2005;129:531-534.
23. Anifandis G, Koutselini E, Stefanidis I, et al. Serum and follicular fluid leptin levels are correlated with human embryo quality. *Reproduction*. 2005;130:917-921.
24. Robins JC, Srivastava R, Mershon JL, Thomas MA. Association of leptin with poor ovarian stimulation during in in vitro fertilization. *J Reprod Med*. 2005;50:356-360.
25. Wunder DM, Kretschmer R, Bersinger NA. Concentrations of leptin and C-reactive protein in serum and follicular fluid during assisted reproductive cycles. *Hum Reprod*. 2005;20: 1266-1271.
26. Gürbüz B, Yalti S, Ficicioglu C, Tasdemir S. The relation of serum and follicular fluid leptin and ovarian steroid levels in response to induction of ovulation in in vitro fertilization cycles. *Eur J Obstet Gynecol Reprod Biol*. 2005;118:214-218.
27. Asimakopoulos B, Abu-Hassan D, Metzen E, Al-Hasani S, Diedrich K, Nikolettos N. The levels of steroid hormones and cytokines in individual follicles are not associated with the fertilization outcome after intracytoplasmic sperm injection. *Fertil Steril*. 2008;90:60-64.
28. Hill MJ, Uyehara CFT, Hashiro GM, Frattarelli JL. The utility of serum leptin and follicular fluid leptin, estradiol, and progesterone levels during an in vitro fertilization cycle. *J Assist Reprod Genet*. 2007;24:183-188.
29. Diedrich K, Felberbaum R. New approaches to ovarian stimulation. *Hum Reprod*. 1998;13:1s-13s.
30. Asimakopoulos B, Nikolettos N, Al-Hasani S, Diedrich K. An evaluation of the multiple dose protocol of cetrorelix in single ovary women. *In Vivo*. 2003;17:89-92.
31. Asimakopoulos B, Nikolettos N, Al-Hasani S. Outcome of cryopreserved pronuclear oocytes obtained after ovarian stimulation with either HMG or recFSH and the GnRH-antagonist cetroerelix. *Reprod BioMed Online*. 2002;5:52s-56s.
32. Barroso G, Barrionuevo M, Rao P, et al. Vascular endothelial growth factor, nitric oxide and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. *Fertil Steril*. 1999;72:1024-1026.
33. Wong M-L, Licinio J, Yildiz BO, et al. Simultaneous and continuous 24-hour plasma and cerebrospinal fluid leptin measurements: dissociation of concentrations in central and peripheral compartments. *J Clin Endocrinol Metab*. 2004;89: 258-265.