Overt leptin response to controlled ovarian hyperstimulation negatively correlates with pregnancy outcome in *in vitro* fertilization-embryo transfer cycle

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Submission: 29.05.11
Review completed: 17.11.11
Accepted: 28.04.12

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/0974-1208.101021

ABSTRACT

CONTEXT: A critical body mass of adipose tissue is essential for the normal development of female reproductive functions. Leptin, an adipocyte-derived hormone encoded by the 'Ob' gene has been proposed as a peripheral signal indicating the adequacy of nutritional status for reproductive functions. It is reported as a direct regulator of gametogenic and steroidogenic potential of ovary. Though leptin is widely present in reproductive tissues, its relationship to reproductive hormones is still poorly understood. **AIMS:** Present investigation attempts to explore ovarian response to secretory profile of leptin and its impact on pregnancy outcome in women undergoing controlled ovarian hyperstimulation for *in vitro* fertilization and embryo transfer (IVF-ET). **SETTINGS AND DESIGN:** Patients enrolled for IVF-ET underwent pituitary-ovarian suppression by 'Long Protocol' GnRH-agonist downregulation followed by ovarian stimulation. **MATERIALS AND METHODS:** Sera were procured at different phases of IVF-ET for the assay of estradiol, progesterone, human chorionic gonadotropin, and for leptin. Ovarian follicular fluids were also assayed for leptin. Luteinized granulosa cells were cultured *in vitro* to evaluate their steroidogenic potential. **STATISTICAL ANALYSIS USED:** Statistical analyses were done by student's t-test, ANOVA, and Chi-square tests as applicable. All results were expressed as Mean ± SE. P values < 0.05 were considered significant. **RESULTS:** Positive correlation was observed between serum and ovarian follicular fluid leptin. A negative correlation was noted between the serum leptin levels and endometrial thickness. **CONCLUSIONS:** Elevated leptin response may exert adverse impacts on pregnancy success during IVF-ET possibly by modulating uterine receptivity.

KEY WORDS: Controlled ovarian hyperstimulation, endometrium, hypothalamo-pituitary-gonadal axis, *in vitro* fertilization and embryo transfer, leptin, pregnancy outcome

INTRODUCTION

Female reproductive function is exquisitely sensitive to the alteration in body's metabolic states. A critical body mass of adipose tissue is essential for the normal development of female reproductive functions.[1] The mechanistic link between body mass and reproductive functions, however, is not clearly elucidated.[2] Leptin, an adipocyte-derived hormone encoded by the 'Ob' gene has been proposed as the peripheral signal indicating the adequacy of nutritional status for reproductive functions.[3] It is important in regulating energy homeostasis, and by this virtue impacts the reproductive systems in diverse ways.[4] Leptin perhaps exerts direct regulatory action on ovarian folliculogenesis.[5] It is also reported to regulate ovarian function through its modulating effects on the hypothalamo-pituitary-ovarian axis (HPO axis) and contributing to the release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus and gonadotropins from pituitary.[6] Leptin concentrations showed fluctuations during menstrual cycle.[7] Moreover, leptin mRNA and its protein production have been documented in granulosa cells, oocytes, and early cleavage stage embryos.[8-12] However, though leptin is widely present in reproductive tissues, its relationship to reproductive hormones is still poorly understood. Controversial results have been reported during hormone replacement

therapy (HRT), oral contraceptive intake, and ovulatory disorders.[13] Controlled ovarian hyperstimulation (COH) for *in vitro* fertilization and embryo transfer (IVF-ET) involves ovarian suppression followed by stimulation and thus provide ample scope to adjudge the differential and dynamic alteration in the ovarian steroid hormone status. The present study is centered on the objective of evaluating the ovarian response to the circulating leptin level and its impact on pregnancy outcome in women undergoing COH for IVF-ET.

MATERIALS AND METHODS

Study subjects

Infertile patients (n = 18) aged between 24 and 36 years diagnosed with unexplained, tubal block or male factor infertility and recruited for IVF-ET[14,15] were selected for the proposed study. The investigations were performed with the approval from the institutional research ethics board. Women were at first assessed for their baseline endocrine profiles and leptin. All of them were ovulatory, euthyroid, and normoprolectinemic. None of the patients in this cohort had detectable pituitary or hypothalamic dysfunction.

Intervention

Ovarian stimulation

Women participating in the *in vitro* fertilization (IVF) programme underwent pituitary-ovarian suppression by standard 'Long Protocol' GnRH-agonist (GnRHa) administration (LA: leuprolide acetate: SUN Pharmaceutical Ind. Ltd.; 1 mg sc/day) commencing on day 21 of the preceding menstrual cycle. Ovarian stimulation was initiated with urofollitropin (Rec. FSH: Gonal F: Serono, Switzerland) and /or menotropin (hMG: Menopur: Ferring GmbH, Wittland 11, D24109, Kiel, Germany). Gonadotrophin stimulation was commenced only when complete pituitary–ovarian suppression has been achieved by prior administration of GnRH-a. The stimulated cycles were monitored by daily transvaginal ultrasonography (TVS) and intermittent assessment of serum E_2 levels. The daily dose of gonadotrophin was individualized according to an individual dose-response scheme. Stimulation was continued until serum E_2 levels reached ≥150 pg / dominant follicle with a diameter of ≥ 1.7 cm. Final maturation of oocytes was induced by injection of 10,000 IU of human chorionic gonadotrophin (hCG; Pregnyl; Organon: OSS, The Netherlands).[14,15] Stimulated cycles characterized by fewer than two dominant follicles and inconsistent E_2 levels were cancelled before hCG administration. The total numbers of antral follicles were counted and transvaginal ultrasound (TVS)-guided oocyte retrievals were performed 35/36 hours after hCG administration.

Collection of follicular fluid

Ovarian follicular fluids (FFs) were collected from preovulatory ovarian follicles of the individual patient who underwent ovarian stimulation with standard "long"- protocol GnRH-agonist downregulation followed by rFSH and hCG stimulation.[14,15] The FF was aspirated by transvaginal puncture using ultrasonic guidance. All visible follicles were aspirated separately. The yellow, clear FF samples obtained were first centrifuged for 10 minutes at 1500 rpm and then collected supernatants were stored at -40°C for leptin assay.

Granulosa cell culture

This assay is based on the capacity of hCG to stimulate luteinized granulosa cell (LGC) production of estrogen cultured *in vitro*. LGCs from the FF of individual patients were collected by centrifugation of follicular contents at 1500 rpm for 10 minutes and pellets were washed two times with HAM F-10. Percoll (50%) column centrifugation was done at 500 rpm for 30 minutes followed by the aspiration of granulosa cells from the interface for culture. All the reagents for this cell culture method were obtained from Sigma chemical Co., St. Louis, MO, USA. Cells were cultured in 15 mm 4-well culture plates (NUNC, Denmark) for 48 hours at 37°C in a humified, 95% air-5% CO_2 incubator (Forma Scientific). Each well contained 5000 ± 888 viable cells in 0.5 mL of Ham's F10 medium, supplemented with calcium lactate (120 mg/L), BSA (100 mg/L), penicillin (100 IU/mL), and streptomycin sulfate (100 μg/mL). The cells were grown in the presence of diethylstelbesterol (DES) (10^{-4}M), a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (MIX, 1 mg/mL), insulin (1 mg/mL), hCG (10-100 mIU/well), and androstenedione (10^{-3} M) as the substrate for aromatase. The conditioned media were assayed for Estrogen (E_2; pg/mL) by fully automated chemiluminescence assay (ACS: 180 from Chiron Diagnostics Corp., East Walpole, MA, USA) and a dose-response curve was plotted.

Measurement of endometrial thickness

USG measurement of endometrial thickness was performed.

Outcome measures

Samples of maternal serum were collected on the day of hCG administration, at the time of oocyte retrieval, and on day 16 post-transfer. Sera were procured after centrifugation and stored at –40°C for the assay of estradiol, progesterone, hCG, and for leptin. Depending on serum leptin levels on ovum pick-up day (OPU day), women were categorized under three leptin groups (low (<10 ng/mL), medium (10-30 ng/mL), and high (>30 ng/mL). Follicles were identically categorized into three groups based on FF leptin values. Moreover, based on morphology, oocytes were graded into high quality and dysmature oocytes and accordingly follicular aspirates were categorized under three groups: those yielding high quality oocytes, dysmature oocytes, or no oocyte. Stimulation outcome was assessed with respect to number of antral follicle produced, number of oocytes

retrieved, gradation of oocytes, fertilization, and cleavage rates. Embryo development was assessed on post-retrieval day 3 (three) on the basis of number and morphology of blastomeres. Embryos with ≥5 blastomeres of equal size with good to excellent morphology were graded as superior; those with <5 blastomeres with good to excellent morphology were categorized as good, and the rest were defined poor quality oocytes. Biochemical pregnancy was defined as the >30 IU/L hCG as measured on day 16 post-transfer. Implantation and clinical pregnancy was confirmed by visible gestation sac on ultrasound.

Biochemical assay

All serum samples were assayed for leptin with immunoradiometric assay (IRMA; REF-DSL-23100; Diagnostic Systems Laboratories Inc., 445 Medical Center Blvd, Webster, TX, 77598, USA, with assay sensitivity: 0.10 ng/mL; intra- and inter-assay coefficient of variation: 3.73% and 5.2%, respectively). Assay for estradiol (E_2) was done using enzyme-linked fluorescent assay (ELFA; VIDAS, Bio Merieux; Marcy I'Etoile, France; assay sensitivity: 9-3000 pg/mL; intra- and inter-assay coefficient of variation 4.12% and 6.32%, respectively). Samples were further assayed for progesterone with ELFA (assay sensitivity: 0.25-80 ng/mL; intra- and inter-assay coefficient of variation: 4.32% and 4.48%, respectively). Samples were evaluated for hCG by a two-site chemiluminescence sandwich immunoassay system (Bayer Diagnostics Corporation, Tarrytown, NY; assay sensitivity: 2.0-1000 mIU/mL). In addition, prolactin (assay sensitivity: 0.3-200 ng/mL; intra- and inter-assay coefficient of variation: 3.03% and 4.03%, respectively) and thyroid stimulating hormone (TSH) (assay sensitivity: 0.011-150 μIU/mL; intra- and inter-assay coefficient of variation: 3.59% and 4.30%, respectively) were measured in these samples by the two-site chemiluminescence sandwich immunoassay system. Furthermore, serum levels of leutinizing hormone (LH: assay sensitivity 0.07-200 μIU/mL; intra- and inter-assay coefficient of variation: 4.81% and 6.05%, respectively) and follicle-stimulating hormone (FSH: assay sensitivity 0.3-200 μIU/mL; intra- and inter-assay coefficient of variation: 1.9% and 4.6%, respectively) were measured using ACS: 180-automated two-site chemiluminescence sandwich system. Apart from this, serum testosterone was measured by radioimmunoassay (RIA), using a commercial kit from Immunotech (BP, 177-13276; Marseille, Cedex 9, France; REF-IM1119) with assay sensitivity 0.025 ng/mL and intra- and inter-assay coefficient of variation 14.8% and 15%, respectively. Measurement of androstenedione was carried out through IMMULITE 2000-competitive chemiluminescent enzyme immunoassay (assay sensitivity: 0.3-10 ng/mL; intra- and inter-assay coefficient of variation: 6.42% and 9.34%). Baseline insulin of all patients was measured with RIA using a commercial kit (Lot TKIN 1 939, 060630, Diagnostic Products Corporation, Los Angeles, CA, USA) with assay sensitivity 1.3 μIU/mL and intra- and inter-assay coefficient of variation 5.2% and 7.3%, respectively.

Statistical analysis

All results are expressed as Mean ± SE. Statistical analyses were done by Student's t-test, ANOVA, and Chi-square tests as applicable using PRISM Statistical Software Package (PRISM Version 4.03@1992-2005; GraphPad Software Inc.). *P* values < 0.05 were considered significant.

RESULTS

Table 1 represents the baseline endocrine profiles of the patients recruited for IVF-ET. Among the whole patient population, basal leptin concentrations correlated with BMI [Figure 1]. Individual leptin response and stimulation outcome have been presented in Table 2. The findings demonstrate that leptin concentrations are patient specific. Maternal serum leptin levels increased significantly from hCG-day to OPU day in ~80% of the patients [Table 2], while in the rest there were very negligible rise. In none of the patients who achieved pregnancy, leptin levels on OPU day increased over 50% of hCG day, and no patient with >60% leptin increase achieved pregnancy. Serum E_2 levels positively correlated with oocyte retrieval rate [Table 3]. A good positive correlation was observed between serum and FF leptin [Figure 2]. There were no differences in the number of retrieved oocytes among women having low, medium, or high FF leptin levels on OPU day [Table 3]. Oocyte retrieval rates were also comparable between the medium and higher serum leptin group. But in those with <10 ng/mL of serum leptin, the retrieval rate was poor [Table 3]. 30% reduction in hCG-stimulated E_2 production was evidenced when granulosa cells were collected from follicles yielding no oocytes [Figure 3]. FF leptin levels had no impact on oocyte maturity [Table 3]. Rates of fertilization, cleavage, and embryo development were comparable between the oocyte populations collected from low, medium, and high serum or FF leptin levels [Table 3]. A negative correlation was noted between the serum leptin levels and endometrial thickness [Table 3].

Table 1: Baseline characteristics of the study population

Study parameters	Patient population (*n* = 18)
Age (years)	28.28 ± 0.87
BMI (kg/m^2)	24.52 ± 0.73
LH (IU/L)	5.28 ± 0.35
FSH (IU/L)	5.79 ± 0.31
LH: FSH ratio	0.91 ± 0.07
Estradiol (pg/mL)	33.65 ± 2.90
Testosterone (ng/mL)	0.48 ± 0.12
Androstanedione (ng/mL)	1.47 ± 0.18
Fasting insulin (μIU/mL)	12.48 ± 4.16
Fasting leptin (ng/mL)	16.36 ± 1.12

Table 2: Leptin response between hCG and OPU day in women who achieved and did not achieve pregnancy following IVF-ET

Patient no.	Age (years)	Terminal E_2 (pg/mL)	Antral follicle no.	Leptin (ng/mL)		% Increase of leptin	Pregnancy (P/NP)
				hCG day	OPU day		
1	35	3780	12	32.0	39.2	22.5	P
2	23	3740	20	20.0	28.5	42.5	P
3	25	1480	11	29.0	49.2	69.65	NP
4	29	2460	16	25.0	52.2	108.8	NP
5	23	1660	12	22.0	41.7	89.54	NP
6	29	2100	09	21.0	23.5	11.90	P
7	30	1280	12	15.0	26.0	73.33	NP
8	36	223	03	22.0	42.8	94.54	NP
9	36	1740	15	16.0	35.2	120	NP
10	29	2540	15	19.0	27.8	46.31	P
11	33	740	06	14.0	15.7	12.14	P
12	25	2020	08	20.0	33.5	67.5	NP
13	35	3780	12	32.0	58.7	83.43	NP
14	25	1480	11	29.0	31.7	9.31	P
15	23	1660	12	32.0	56.7	77.18	NP
16	26	3780	27	19.5	25.6	31.28	P
17	27	740	06	33.0	60.1	82.12	NP
18	25	2490	19	26.0	43.4	66.92	NP

Pregnant (P) 38.88%; not pregnant (NP) 61.11%.

Table 3: Ovarian response and endometrial thickness under the influence of variable serum/FF leptin levels and peak E_2 following COH

	FF leptin (ng/mL)			Serum leptin (ng/mL)			Serum estradiol (pg/mL)		
	I	II	III	I	II	III	I	II	III
	<10	10-30	>30	<10	10-30	>30	<1000	1001-2000	>2000
No. of oocytes retrieved	9.3 ± 2.2	9.6 ± 2.6	8.9 ± 2.5	9.6 ± 2.8	10.1 ± 3.1	9.8 ± 3.1	3.7 ± 1.0^a	6.6 ± 1.7^b	10.6 ± 2.2^c
No. of oocytes fertilized	6.8 ± 1.6	7.2 ± 1.9	6.8 ± 0.2	6.5 ± 0.2	7.0 ± 0.2	7.5 ± 0.3	2.6 ± 0.2^a	4.7 ± 0.2^b	8.3 ± 0.3^c
Fertilization (% ± SEM)	73.4 ± 3.4	75.4 ± 3.1	76.8 ± 3.8	68.2 ± 2.8	70.1 ± 3.4	74.0 ± 4.2	70.0 ± 3.2	72.3 ± 3.3	78.2 ± 3.4
Cleavage rate (% ± SEM)	90.4 ± 3.8	95.2 ± 4.2	90.4 ± 3.8	86.3 ± 5.2	92.6 ± 4.3	92.3 ± 3.9	90.0 ± 3.2	93.4 ± 3.8	93.1 ± 3.6
Transferable embryo Grade I	58.8%	66.9%	60.7%	59.4%	64.6%	64.3%	65.1%	66.8%	68.4%
Grade II	31.6%	28.8%	29.2%	30.6%	28.2%	29.0%	23.2%	26.3%	23.3%
Endometrial thickness				10.4 ± 0.5^a	9.3 ± 0.6^a	6.9 ± 0.3^b	7.4 ± 0.3^a	9.5 ± 0.4^b	9.8 ± 0.4^b

Values with different superscripts in a row under individual group/subgroup differ significantly. a versus b, b versus c and a versus c: $P < 0.0001$

DISCUSSION

Reproductive potential in women undergoes adverse alteration following severe changes in nutritional status and energy availability in both directions. These adaptive changes are reversible when nutritional status is normalized.[16-18] Ovary is an ever-changing tissue and dynamic multi-compartmental organ which is under the chief regulatory control of hypothalamic pituitary principles. The hypothalamic-pituitary control over ovarian functions however is precisely governed by a plethora of external and internal principles including many of ovarian origin. Leptin has emerged as a potential regulator of many reproductive functions including gametogenic and steroidogenic potential of ovary. Leptin and leptin receptors are found in reproductive tissues. It is considered as a possible link between nutrition and reproduction.[19] Leptin receptors and mRNA have been identified in the HPO axis.[20] Furthermore, Leptin mRNA and protein production have been documented in granulosa cells, oocytes, and early cleavage stage embryos.[7-12] To elucidate the regulatory interaction between leptin and ovary, in this present investigation, leptin response was evaluated in women undergoing COH for IVF-ET. The major highlights of the present findings include an overall adverse impact of leptin on pregnancy outcome in IVF-ET. A rise in serum leptin level between hCG and OPU day leading to >60 ng/mL was associated with no term pregnancy. In 90% of these women who registered pregnancy wastage had elevated leptin levels. During COH, a positive correlation between serum and FF leptin concentrations was noted, but between the follicles leptin level variation was commonly observed. This signifies that follicles do not produce leptin and different follicles may differentially respond to a common

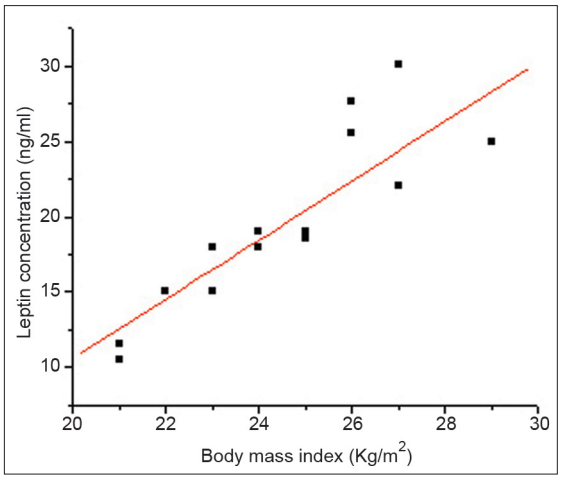

Figure 1: Correlation and regression line between serum leptin concentration and BMI in study population (r = 0.88148; *P* < 0.0001)

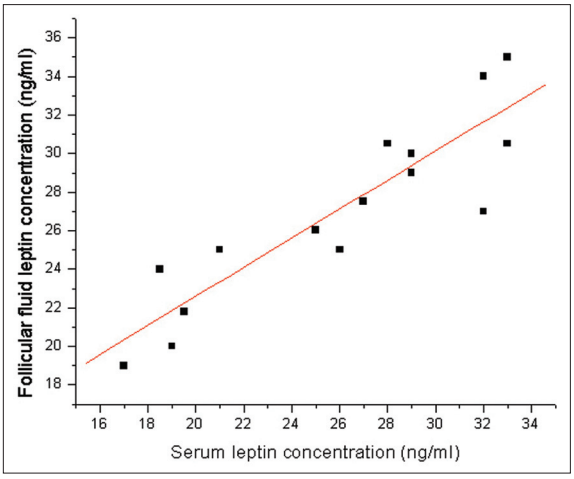

Figure 2: Correlation and regression line between serum leptin concentration and FF leptin concentration in study population (r = 0.89667; *P* < 0.0001)

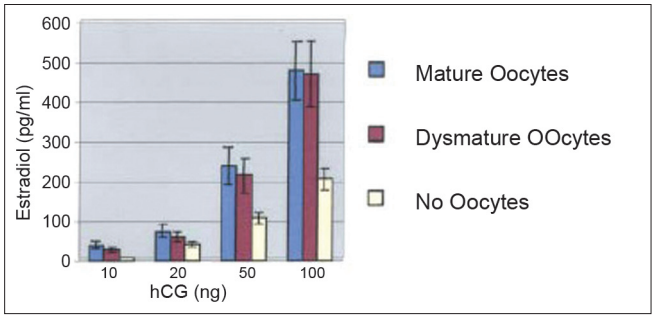

Figure 3: Granulosa cell competency to respond to dose-dependent increase of hCG-induced E_2 production; comparative study of E_2 production by LGCs collected from follicles harboring different grades of oocytes. There was a gradual rise in E_2 production in response to an increment in the hCG dose in all the three groups. Rates of increase in E_2 production was comparable between those collected from follicles producing mature or immature oocytes, but 30% reduction in hCG-stimulated E_2 production was evident when LGC were collected from follicles yielding no oocytes

systemic signal. Successful implantation and establishment of pregnancy is dependent on a synchronized interaction between the embryo and secretory endometrium.[21] Endometrial receptivity, therefore, is another important contributor to the success of pregnancy. Impaired uterine receptivity may be a possible factor involved in the high leptin level-associated pregnancy failure. Endometrial thickness is considered an indirect marker of endometrial potential to support pregnancy.[22] It was observed that women with higher leptin levels had significantly thinner endometrium. Moreover, expression of leptin mRNA in human secretory endometrium[23] suggests that endometrium may be capable of responding to leptin. An estrogen up-regulated 64.0 kDa uterine fluid protein is reported to correlate positively with endometrial thickness as well as its receptivity.[24] However, it remains to be elucidated whether leptin inhibits the 64.0 kDa protein and impedes with optimum endometrial bed preparation for the establishment and maintenance of pregnancy. On the other hand, quality of transferred embryo plays a crucial role in implantation and pregnancy success.[25] An additional role of leptin in embryo development is suggested by its presence within the pre-implantation embryo where the polarity of its distribution might imply a regulatory role in development.[26] We, however, observed no significant effects of leptin on pre-embryonic development as the rate of fertilization and production of good-quality embryos did not differ between the low and high serum and FF leptin groups. Reports[27] indicate that endometrial thickness bears positive correlation with follicular phase serum estradiol concentration. In order to rule out any masking effects of estradiol on leptin-associated endometrial thickness, we isolated three categories of patients who had peak estradiol level between 1800 and 2500 pg/mL and exhibited varied leptin responses: high, moderate, and low between post-hCG and OPU day. A significant difference was observed in the endometrial thickness between the three subgroups. The higher the leptin response, the thinner the endometrium. We, therefore, hypothesize that increased leptin response to COH may exert adverse impacts on pregnancy success by way of modulating uterine receptivity. This hypothesis gets further support by the observation of successful pregnancy in the cycles characterized by low pre-transfer leptin levels. In our patient population, eight patients had more than one transfer cycles (maximum three) with cryopreserved[28] embryos because of pregnancy failure in the stimulated cycle. In all transfer cycles, serum leptin was measured 4 days prior to transfer. It was observed that pregnancy was achieved in the cycles when leptin levels dropped significantly from that of the stimulated cycle. Apparently, a positive correlation was noted between leptin and estradiol, while progesterone exhibited no correlation. *In vitro* culture studies to evaluate the steroidogenic potential of LGCs collected from follicles with similar leptin milieu but producing mature, immature oocytes or no oocyte responded identically to hCG in the absence of leptin. But

a 30% reduction in hCG-induced estradiol production was evident in the presence of leptin when LGCs were collected from follicles producing no oocyte.

Taken together, these observations provide possible indication of attenuating effects of leptin on endometrial bed preparation that may be involved in pregnancy failure in women with elevated leptin response. The small population size limits the statistical power to judge the precise correlation; however, elevated leptin response in COH exhibits a trend to adversely impact endometrial bed preparation and consequent pregnancy outcome. The observations presented herein should be viewed as a prelude to what future holds.

ACKNOWLEDGMENT

This study was supported financially partly by University Grants Commission, New Delhi, India.

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How to cite this article: Chakrabarti J, Chatterjee R, Goswami S, Chakravarty B, Kabir SN. Overt leptin response to controlled ovarian hyperstimulation negatively correlates with pregnancy outcome in in vitro fertilization-embryo transfer cycle. J Hum Reprod Sci 2012;5:194-9.
Source of Support: Financial support: University Grants Commission, New Delhi, India Equipments and others: Institute of Reproductive Medicine Kolkata, India and Indian Institute of Chemical Biology, Kolkata, India No conflict of interests exist between the authors regarding the work plan, experiments, observations and inferences done from this study., **Conflict of Interest:** None declared.