Evaluation of Leptin in Sera and Follicular Fluids of Infertile Women Undergoing Intra-Cytoplasmic Sperm Injection and Their Effects on Pregnancy Outcome

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Abstract

Background	Leptin may serve as the critical link between the body's adipose tissue and the hypothalamo-pituitary axis, thus it is considered a possible link between nutrition and reproduction.
Objective	To evaluate leptin in the serum and follicular fluid and its effect on fertilization rate and pregnancy outcome in infertile female patients undergo intra-cytoplasmic sperm injection cycle.
Methods	Seventy four infertile women who agreed to participate in the study were selected randomly from those attending the Fertility Centre in Al-Sader Teaching Hospital, Holly Najaf. Hormonal analysis was done for serum and follicular fluid leptin hormone at the day of ovum pickup.
Result	Serum and follicular fluid leptin hormone levels were increased above its normal cutoff level according to the kit used. There was a relationship between the fertilization rate and leptin in serum and follicular fluid. Fertilization rate was significantly ($p = 0.049$) different depending on the serum leptin level while it has nothing to do with follicular fluid leptin hormone. On the other hand, follicular fluid leptin significantly ($p = 0.04$) affect pregnancy outcome.
Conclusion	Leptin provides possible impact on oocyte and/or embryo quality leading to impaired endometrial bed preparation that may be involved in pregnancy failure.
Keywords	Leptin, ferilization rate, pregnancy outcome.

List of abbreviation: BMI = body mass index, IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, OR = ovarian response, FF = follicular fluid, FR = fertilization rate, hCG = human chorionic gonadotrophin, FSH = follicle stimulating hormone, LH = luteinizing hormone, E2 = estradiol, PN = pronuclei, ET = embryo transfer, PR = pregnancy rate, ART = assisted reproductive technologies.

Introduction

Fertility is strongly dependent on the presence of a critical amount of total fat [1]. It is known that a drastic reduction in the size of the adipose reserve, as it happens in over trained athletes or in pathological situations, is associated with amenorrhea and infertility, and this association persists until the body mass index (BMI) returns to normal values [2]. This was classically interpreted as the result of the existence of a permissive signal produced by the adipose tissue: the "critical weight hypothesis," originated from the observation that the age at menarche is more closely related to body weight than to chronological age [3,4].

Despite its primary role in the regulation of body weight, it is becoming clear that leptin exerts widespread and unanticipated actions on other endocrine systems, linking the adipose tissue with the hypothalamus and exerting a regulatory role on many

neuroendocrine systems, such as the growth hormone axis, the thyroid hormone axis, and the reproductive axis [5,6]. Leptin receptors and leptin mRNA have been identified in the human hypothalamus and ovary, and leptin mRNA and protein production have been discovered in ovarian granulosa cells, oocytes, and early cleavage stage embryos [7,8].

During in vitro fertilization (IVF) /intra-cytoplasmic sperm injection (ICSI), a high relative leptin increase is associated with adiposity and a reduced ovarian response (OR) [9]. These observations support the possibility that high leptin concentrations might reduce ovarian responsiveness to gonadotropins. Leptin might explain in part why obese individuals require higher amounts of gonadotropins than lean subjects to achieve ovarian hyperstimulation; thus, leptin is considered a possible link between nutrition and reproduction [4].

Many studies have demonstrated adverse effects of leptin on IVF outcomes, including inhibition of ovarian follicular development and steroidogenesis [10,11]. However, some have reported no adverse effects [12,13]. Therefore, up to the present, the research community has failed to reach consensus on this issue.

The aim of our study is to study the level of serum and follicular fluid (FF) leptin hormone and its relation to fertilization rate (FR) and pregnancy outcome in infertile patients undergo ICSI cycle.

Methods

A hospital-based cohort study was conducted to determine the levels of leptin with FR in ICSI cycle. Seventy four infertile women agreed to participate in this research were selected randomly from those attending the Fertility Centre in Al-Sader Teaching Hospital, Al-Najaf Holly City during the period from March 2013 to November 2013. The study was approved by the Institute Review Board of the College of Medicine, Al-Nahrain University.

The mean age for infertile female patients was 31.41±5.45years. Infertility due to a female cause was present in 39 (52.7%) and to a male cause in 35 (47.3%) of the cases. Primary infertility was present in 57 (77%) whereas only 17 (23%) patients have secondary infertility. The duration of infertility for the entire patients group (8.11±3.94 years).

Those women with visible ovaries on ultrasound (model AMIB7, Canada), no uterine fibroid, uterine anomaly or ovarian cyst measuring ≥ 20 mm in diameter, negative screening tests for hepatitis B and C, as well as for human immune deficiency viruses, inability to achieve pregnancy in a period of ≥ 12 months despite regular unprotected intercourse, and no matter the cause of infertility was female or male factor were selected for the study.

All the participants were asked to come back on cycle day 2 for complete medical evaluation.

Treatment protocols and ovarian monitoring

Single injection of gonadotropin-releasing hormone (GnRH) agonist, Decapeptyl (Diphereline; 0.1 mg, Beaufond, Ispenpharma-France) was administered subcutaneously at CD 2; thereafter, results of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2) were monitored. If FSH serum level below 10 IU/ml, LH serum level below 8 IU/ml and E2 below 50 pg/ml, then the patient follow treatment by short protocol.

Fifty seven infertile patients enrolled in the short induction protocol. They are given 0.1 mg/day of Decapeptyl as a morning dose and purified 75 IU/ml FSH either as highly purified urinary FSH (Metrodin-HD serono, Switzerland) or recombinant DNA technology prepared agent Gonal-f (serono, Switzerland) as an evening dose subcutenously or intra-muscularlly. The dose of FSH in each regimen can be adjusted according to hormonal results, age and response of folliculogenesis.

Seventeen patients who have high FSH, LH or E2 level or with endometrial thickness more

than 10 mm at CD2 follow long type of induction protocol. They administered GnRH agonist, Decapeptyl CR (tiptorelin; Ferring, Germany 3.75 mg/ampule) subcutaneously at cycle day 21 of the menstrual cycle. Then FSH, LH and E2 level is checked 10-14 days after injection or after the cycle started; if FSH < 10 IU/ml and LH < 8 IU/ml, E2 level below 50 pg/ml and endometrial thickness < 5 mm, then Gonal-f or metrodin HP given daily for 6 days under strict indication.

The patients were monitored for follicular growth and endometrial thickness by serial transvaginal ultrasound and serum E2 from the 6th day of stimulation with gonadotropins.

Titration of FSH (upward or downward) is based on the response of folliculogenesis; when at least 2 dominant follicles of 17 mm in diameter in each ovary is ready, ovulation is triggered by hCG-pregnyl 5000-10,000 IU (Serono S.P.H, Italy) intramuscularly, to stimulate the women natural LH surge which stimulate the final growth and maturation of the oocyte. Thirty six hours following hCG trigger, follicular aspiration is done by transvaginal guidance.

Women who develop > 20 follicles measuring > 10 mm in diameter or having E2 level > 3000 pg/ml (ovarian hyper stimulation); in addition, those who develop at least 3 follicles measuring 18 mm following 14 days of FSH treatment, or E2 level < 200 pg/ml, (poor responder) were excluded from the study.

Under general or local anesthesia, ovum pick up was done through transvaginal aspiration usually timed 34-36 hours following hCG [pregnyl 5000-10,000 IU (Serono S.P.H, Italy)] and carried out via ultrasound guidance. Those patients eligible for ICSI cycle were scheduled for oocyte pick up after programmed ovulation induction.

Ten ml of FF samples were collected in plain tubes at the day of ovum pick up between 08:00-10:00 am. The FF first received by the embryologist for oocytes pick up and leaves the reminder fluid for further hormonal assay. At the sametime, 10 ml of venous blood were collected in plain tubes. Both samples left at least 15 minutes at room temperature before centrifugation at 3000 rpm (Rotofix 32A, Germany) for 10 minutes for measurement of leptin hormone (Beckman Coulter, Germany) by sensitive Enzyme Linked ImmunoSorbant Assay technique "ELISA" (BioTek, USA).

Once oocyte was received by the embryologist, denudation after identification, then examination of corona-complex.

After insemination, fertilized oocytes must be examined 16-20 hours for the presence of two round nuclear structures, the male and female pronuclei (PN). Pronuclei must be scored within the appropriate time span, before they merge and are no longer visible. This ensures only normal zygotes with two pronuclei (2PN's) are cultured for embryo transfer (ET). Usually, the laboratory will assign a grade for each embryo to identify the best quality embryos that are then selected for embryo transfer or cryopreservation.

The selection criteria or grading systems must be applied for all cleavage stages from day 2-4 to allow selection of the most viable embryos. Embryo development was evaluated approximately every 24 h. Slow dividing, non-dividing (arrested) or fragmenting embryos are selected against. Compacting embryos on day 3 that have closely apposed cell membranes are selected for. Standard morphological criteria used in evaluating embryo quality include the rate of division judged by the number of blastomeres, size, shape, symmetry, and cytoplasmic appearance of the blastomeres, and the presence of a nucleate cytoplasmic fragment. The quality of each embryo is assessed using the following grading system.

Grade I: Excellent-quality embryos with equally sized blastomeres without or with up to 10% a nucleate fragment.

Grade II: Embryos with 10-20% fragmentation considered good

Grade III: Fair-quality embryos with fragmentations between 20-50%.

GradeIV: Embryos with > 50% fragmentations and with unequally sized blastomeres were said to be poor-quality embryos.

The total number of pregnancies was detected by counting how many ladies have increasing positive serum hCG (more than 20 IU/ml) at least 15 days after ET. Clinical pregnancy was determined by observation of gestational sac with fetal heartbeats on transvaginal U/S 6-7 weeks of gestation. In general, pregnancy rate (PR) was defined as the number of pregnant ladies after ICSI divided by the whole number of patients under went ICSI cycles multiplied by 100.

Data Analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 18. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as mean and standard deviation 95% confidence interval. Pearson's correlation coefficient was used to study the relation between two continuous variables. A *P* value of ≤ 0.05 was considered as significant.

Results

Seventeen (23.0%) infertile patients had positive pregnancy test while 57 (77%) showed negative pregnancy test. Forty eight (64.9%) patients have a FR of ≥ 50 % and 26 (35.1%) have FR of < 50% while the mean FR was 0.61±0.30 (Table1).

Leptin level was ≥ 7.36 ng/ml in 79.1% and < 7.36 ng/ml in 20.9% of infertile patients.

Table 1 and fig. 1 illustrate the relationship between the FR and leptin in serum and FF. FR was significantly (p = 0.049) different depending on the serum leptin level while it has nothing to do with FF leptin hormone. On the other hand, FF leptin significantly (p = 0.04) affect pregnancy outcome.

Discussion

In our study, the effects of serum and FF leptin levels on pregnancy outcomes were evaluated in short protocols of assisted reproductive technologies (ART). Both serum and FF leptin hormone levels were increased. These findings were in harmony with the results of Al-Bderi [14] who demonstrated that serum and FF leptin hormone levels were significantly high in infertile women. On the other hand, FF leptin levels were lower than simultaneous serum leptin levels, which contradict the findings of Ergenoğlu et al [6]. Also we could not establish a relationship between serum leptin levels and pregnancy outcomes, similar to Gürbüz et al [15] and Ergenoğlu et al [6] but contrary to Yang and Huang [16].

Table 1. The differences in fertilization rate by serum and follicular fluid leptin hormones

Leptin hormone		No.	Fertilization Rate (Mean ± S.D)	t-test	*P* value
Serum	≥ 7.36 ng/ml*	9	0.44 ± 0.28	2.115	0.049*
	< 7.36 ng/ml	34	0.66 ± 0.31		
Follicular fluid	≥ 7.36 ng/ml	14	0.66 ± 0.35	0.651	0.518
	< 7.36 ng/ml	29	0.59 ± 0.30		
Leptin hormone		No.	Pregnancy outcome (Mean ± SD)	t-test	*P* value
Serum	Positive	11	16.14 ± 7.23	0.445	0.659
	Negative	32	14.75 ± 9.44		
Follicular Fluid	Positive	11	8.47 ± 3.24	2.122	0.040*
	Negative	32	13.45 ± 7.50		

* cutoff point of hormone according to kit used
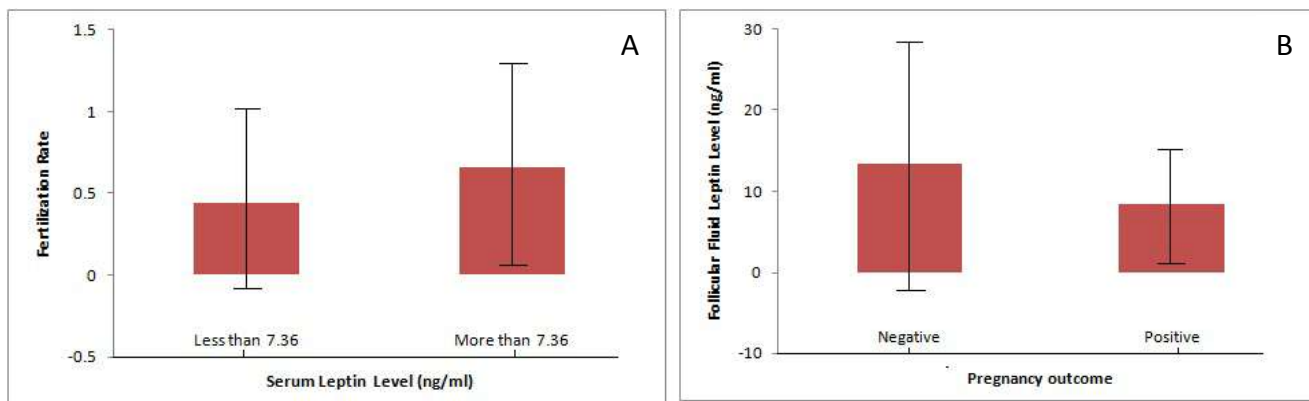
Fig. 1. (A) Relation of serum leptin level to fertilization rate and (B) follicular fluid leptin to pregnancy outcome

One of the major highlights of the present findings includes adverse impact of high FF leptin on pregnancy outcome. These patients who became pregnant from ICSI had lower mean FF concentrations of leptin than patients who did not become pregnant. This is probably due to that leptin can affect follicular development through a central (hypothalamic-pituitary) and end organ (ovary and endometrium) effects [17]. Our findings were also reported by Mantzoros et al [18]; Brannian et al [19] and Chakrabarti et al [1] but in contrast to the findings of Chang et al [20] who demonstrated that the FF leptin concentration is not significantly related to oocyte maturity and corresponding embryo development.

Additionally, Anifandis et al [11] considered the possible mechanisms by which leptin affects fertility and reduce pregnancy success are direct inhibitory action of high leptin levels on the ovaries that lead to ineffective follicular maturation, embryo quality and response. However, others failed to prove those associations [21,22].

As well, during IVF\ICSI, relatively high leptin is associated with adiposity, reduced OR, and low PR [3,4]. Some reports indicated that elevated serum and FF leptin levels may be used as predictive markers of ART failure [18]. In contrast, Agarwal et al [23] considered blood and FF leptin as non-suitable markers of oocyte maturation, embryo quality or ICSI outcome.

Taken together, these observations provide possible indication of the impact of elevated leptin on oocyte and/or embryo quality leading to impaired endometrial bed preparation that may be involved in pregnancy failure in women with elevated leptin response and consequent pregnancy outcome. The small population size limits the statistical power to judge the precise correlation.

In the present study, the effects of serum and FF leptin levels on pregnancy outcomes were evaluated in short protocols of assisted reproductive technologies (ART).

Both serum and FF leptin hormone levels were increased above its normal level. These findings were in harmony with the results of Al-Bderi [14] who demonstrated that serum and FF leptin hormone levels were significantly high in infertile women. On the other hand, FF leptin levels were lower than simultaneous serum leptin levels, which contradict the findings of Ergenoğlu et al [6]. Also, establish a relationship between serum leptin levels and pregnancy outcomes, similar to Gürbüz et al [15] and Ergenoğlu et al [6] but contrary to Yang and Huang [16].

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Author contribution
Dr. Farhood collects the data and follow the patients, Dr. Hamdan and Al-Salihi supervise the study and write the paper and revise it.

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