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Follicular fluid leptin as a marker for pregnancy outcomes in women in general subfertility population undergoing IVF/ICSI treatment

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SUMMARY

AUTHORS' CONTRIBUTION: (A) Study Design · **(B)** Data Collection . **(C)** Statistical Analysis · **(D)** Data Interpretation · **(E)** Manuscript Preparation · **(F)** Literature Search · **(G)** No Fund Collection

Background: Leptin is a hormone released mostly by adipocytes that regulates energy balance by boosting energy expenditure and limiting food intake, playing an important role in body weight regulation.

Aim of the Work: The purpose of this study was to investigate the relationship between follicular fluid leptin concentrations and IVF/ICSI success in the form of clinical pregnancy.

Patients and Methods: This prospective observational cohort study was carried out at Ain Shams University Maternity Hospital's Assisted Reproductive Technology (ART) unit from June 2020 to January 2022 on a total of 92 subfertile women, where follicular fluid was aspirated and collected from follicles after oocyte isolation to determine the leptin level.

Results: The current study revealed no statistically significant differences in pregnancy based on age, BMI, reasons of sub-infertility, or trial number. The findings of our study revealed that oocyte quality, Fertilization rate, Embryos number, Embryos quality, and Embryos transfer day were statistically substantially higher in positive clinical pregnant cases, with no significant difference in Oocytes retrieved and Embryos transferred number. In terms of Follicular Fluid Leptin, our findings demonstrated that it was statistically lower among positive clinical pregnant cases, with no statistically significant difference between causes of sub-infertility and trial number. Consequently, follicular fluid leptin had a statistically significant positive correlation with gonadotrophin dose, as well as a statistically significant negative correlation with fertilization rate, cleavage rate, embryo number, embryo quality, and embryo transfer day. Our results revealed that Follicular fluid leptin had significant moderate diagnostic performance with a cut-off value of ≤ 112.0 ng/mL with high Sensitivity, Negative Predictive value and Negative likelihood ratio in predicting pregnancy occurrence.

Conclusion: Leptin concentration in the follicular fluid may be an important predictive marker of a successful outcome of IVF treatment. However, combining leptin in serum and/or FF with other factors such as BMI, may act as useful and more informative marker to predict IVF outcome.

Keywords: Follicular Fluid Leptin; Pregnancy; General Subfertility Population; IVF/ICSI

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INTRODUCTION

Infertility has traditionally been regarded as one of the most serious and costly health issues in various societies. Several investigations have already been conducted to determine the causes of infertility; among these studies, a high leptin level has been identified as a key and effective factor [1].

Leptin has been proven to regulate energy homeostasis and food intake, as well as to have a variety of effects on female reproductive function [2].

Leptin is a hormone released mostly by adipocytes that regulates energy balance by boosting energy expenditure and limiting food intake, playing an important role in body weight regulation. It has distinct impacts on the reproductive axis, with stimulatory effects at the hypothalamo-pituitary level and inhibitory interactions at the gonads. Leptin is also critical for fetal growth and development, and it regulates placental processes such as implantation, placental angiogenesis, nutrition transport, and immunomodulation [3]. Leptin promotes the secretion of gonadotrophins and gonadotrophin-releasing hormone, however the episodic pulses of leptin and LH are controlled independently [4]. Other studies have shown the role of leptin in the onset of puberty and its connection with gonadotrophins and other hormones [5]. Leptin and its receptors are expressed in the blastocyst and endometrium, indicating that leptin plays an important role in the implantation process [6].

Leptin levels rise during the follicular phase and fall during the luteal phase of the menstrual cycle [7] demonstrating that estradiol increase has a disproportionate effect on leptin secretion [8]. Serum leptin levels rise during IVF cycles in response to exogenous follicle-stimulating hormone (FSH) injection [9].

Leptin membrane receptors have been found in the granulosa and theca cells of the ooytes. Leptin inhibits steroid production within these cells by inhibiting insulin-like growth factor I (IGFI) [10].

Leptin increases trophoblast cell proliferation and survival by an autocrine action and an anti-apoptotic effect. It has been observed that HCG has a beneficial influence on leptin gene expression in the placenta [2]. Leptin also plays a crucial role in the earliest stage of pregnancy by influencing placental cell proliferation, protein synthesis, invasion, and apoptosis [11].

In some studies, reduced follicular fluid and serum

leptin concentrations correlate with an increased pregnancy rate [12] and elevated levels of serum and follicular fluid leptin are associated with reduced pregnancy rates [13] Low levels of leptin in follicular fluids correspond to high embryo quality, high implantation rate, and positive pregnancy rate [13]. However, in the context of obesity, leptin resistance develops, and increased body fat levels are associated with high circulating levels [3].

Some researchers believe that leptin plays a dual role in reproduction regulation. They discovered that when leptin levels are lower than normal, the endocrine system, which regulates reproduction, fails, whereas when leptin levels are greater than normal, the ovary and fetus development function abnormally [1].

AIM OF THE WORK

The purpose of this study was to investigate the relationship between follicular fluid leptin concentrations and IVF/ICSI success in the form of clinical pregnancy.

PATIENTS AND METHODS

This Prospective Observational Cohort Study was conducted on a total of 92 sub-fertile women attending Assisted Reproductive Technology Unit (ART) clinics in Ain Shams university maternity hospitals for IVF/ICSI from June 2020 to January 2022, following ethical committee approval and informed consent from the patients prior to controlled hyperstimulation.

Inclusion criteria:

A. Women in reproductive age; 20 to 40 years.

B. Medically free.

C. BMI less than 30.

D. Sub-fertile women with the following causes of subfertility: Anovulatory, tubal factor, endometriosis, unexplained subfertility.

E. Sub-fertile women in different spectrum of expected response: Expected normo-responders (AFC 7-15 and AMH 1.2-3 ng/ml) [14].

Exclusion criteria:

A. Women younger than 20 or older than 40 years.

B. Women with any underlying complex medical disorders e.g. cardiovascular disease.

C. BMI more than 30.

D. Subfertile couples with severe male factor infertility: Severe oligozoospermia: sperm concentration fewer than 5 x 10*6/ml), severe asthenospermia: all sperms are immotile or only non-progressive motile, severe teratozoozpermia: absence of normal morphology, Azoospermia: no spermatozoa in the ejaculate [15].

E. Sub-fertile women with abnormal uterine cavity: endometrial polyps, submucous myoma, uterine malformations e.g septate uterus, intra-uterine Synechiae.

F. Women with Recurrent implantation failure: This term refers to women who have had three failed embryo transfer attempts with good quality embryos [16].

Sample size:

Assuming a proportion of success of IVF of 50%, a sample size of 92 cases achieved a power of 80% to detect an effect size of 0.8 using two-sided independent t-test with level of significance of 5%.

Study procedure:

All eligible patients undergoing IVF/ICSI at Ain Shams University Maternity Hospital's ART unit were subjected to:

- Complete medical history including age, previous obstetric history, menstrual history, infertility duration and previous assisted reproductive technique attempts details.
- **Physical examination:**

General examination: Weight, Height and BMI. BMI was calculated as weight (kg)/square of height (m2), vital data as Blood pressure, temperature and pulse, abnormal hair or fat distribution.

Abdominal examination: any abnormal hair distribution, scars of previous operations, abdominal masses or swelling.

Pelvic examination: any vulval, vaginal, cervical or uterine pathology or anomaly, any adenexal masses e.g ovarian masses, hydrosalpinx.

- **Ultrasound examination to determine:** Antral Follicular Count on cycle day 2-3, any ovarian cysts, hydrosalpinx, distorted uterine cavity by endometrial polyp or fibroids, any other uterine pathology e.g. adenomyosis.
- **Laboratory examination:** Basal FSH, LH, E2 done on cycle day 2-3, AMH, TSH, serum prolactin, semen analysis, viral markers for both couples, other routine pre ICSI laboratory investigations as CBC, serum creatinine, ALT, AST, Random Blood sugar, Blood group and Anti rubella IgG.
- **Hystro-salpingography:** to assess tubal patency and uterine cavity adequacy.
- Prior to controlled ovarian hyperstimulation, all women meeting the eligibility criteria must provide written informed consent. The long luteal phase protocol was employed for controlled ovarian hyperstimulation; 0.1 mg/day of gonadotropin-releasing hormone agonist Triptorelin was given subcutaneously every day from the midluteal phase of the menstrual cycle to the day of the HCG trigger. On cycle day 2, pituitary desensitization is

confirmed by: Lab criteria: serum estradiol levels of less than 50 pg/ml, LH levels of less than 5 IU/L, and serum progesterone levels of less than 1ng/ml Ultrasound criteria: Endometrial thickness of less than 6mm, follicular size of less than10mm, and no cysts larger than 15mm [17].

- Ovarian stimulation was initiated with highly purified urinary follitropine (Fostimone® 75 I.U. I.M. vial, IBSA-Switzerland). Depending on follicular growth, the daily dose of gonadotrophins was maintained on an individual basis.

When more than three follicles with diameters greater than 18 mm are present, and more than 50% of the expanding follicular cohort has a diameter greater than 14 mm, and serum E2 levels exceed 500 pg/ml. 10.000 IU of HCG was administered intramuscularly to the patient (Choriomon® 5000 I.U.I.M, IBSA -Switzerland).

Each patient's total dose of gonadotropins, number of days of ovarian stimulation, and serum E2 on the day of HCG trigger were all recorded.

Under general anesthesia, transvaginal oocyte retrieval was performed 34-36 hours following the HCG injection with ultrasound guidance [18]. For each patient, the number and quality of aspirated oocytes were documented.

Following oocyte isolation, bloodless aspirated follicular fluid was collected from follicles and centrifuged at 1000xg for 20 minutes at -4°C to remove debris, blood, and granulosa cells before being frozen at -20°C until analysis.

The investigation did not include follicular fluids contaminated with considerable amounts of blood cells. Leptin assay was conducted out as a single batch using ELISA (Human LEP (Leptin) ELISA kit, NTCO, Egypt).

The sandwich enzyme-linked immune-sorbent assay technology is used in this kit. Well plates were precoated with anti-LEP antibody. As detection antibodies, biotin conjugated Anti-LEP antibodies were utilized. Following that, the standards, test samples, and biotin conjugated detection antibody were added to the wells, and the wells were washed with wash buffer. Unbound conjugates were washed away with a wash buffer after HRP-streptavidin was introduced. The HRP enzymatic reaction was seen using TMB. TMB was catalyzed by HRP to yield a blue product that became yellow when an acidic stop solution was added. The yellow density was proportionate to the amount of LEP sample caught in the plate. The concentration of LEP was estimated by reading the O.D. absorbance at 450nm with a microplate reader.

Follicular leptin level was recorded for each patient.

Fertilization was evaluated 16-18 hours after oocyte injection. Normal fertilization was established by the existence of two obviously distinguishable pronuclei or the development of a second polar body [19]. Fertilization rate was recorded for each patient.Embryo quality was evaluated and recorded for each patient. Cleavage stage embryos was graded according to Nasiri, 2015 [20]. While blastocyst embryos were graded according to Gardner Blastocyst Grading Scale for embryo quality. The degree of expansion ranged from 1 (least expanded) to 6 (fully hatched).

Fresh embryos were transferred under ultrasound guidance on day 3 or day 5 of oocyte retrieval, depending on the number of available surviving embryos [21]. For luteal phase support, all patients received a daily dose of 400mg progesterone vaginal pessaries (Prontogest® 400 mg vaginal pessaries, Marcyrl Co., Egypt) for 14 days after embryo transfer till the end of the first trimester [22].

Pregnancy diagnosis was established using serum quantitative b-HCG two weeks after embryo transfer, followed by transvaginal ultrasound confirmation of intrauterine gestation sac 2 weeks later [23].

Statistical analysis

IBM SPSS statistics (Statistical Package for Social Sciences) software version 22.0, IBM Corp., Chicago, USA, 2013, and Microsoft Office Excel 2007 were used to code, tabulate, and statistically analyze the obtained data. The level of significance was set at P value 0.050, which indicates that the data is significant; otherwise, it is not.

RESULTS

Tab. 1. show that follicular fluid leptin levels were statistically lower in pregnant women. **Tab. 2.** shows that there was no statistically significant difference in follicular fluid leptin levels based on the cause of infertility or trial number.. **Tab. 3.** show that: FOLLICULAR fluid leptin had statistically significant positive correlation with dose of gonadotrophins, as well as statistically significant negative correlations with fertilization rate, cleavage rate, embryos number, embryos quality and Embryos transfer day. There was no statistically significant correlation with Age, BMI, stimulation days, Antral follicular count, Oocytes retrieved, Oocytes quality, Embryos transferred number and Hormonal profile. **Tab. 4.** Only had significant moderate diagnostic performance in predicting. **Tab. 5.** shows that: Follicular fluid leptin ≤ 112.0 ng/mL had high sensitivity, negative predictive value and negative likelihood ratio, but moderate other diagnostic characteristics.

DISCUSSION

This prospective observational cohort study was carried out at Ain Shams University Maternity Hospital's Assisted Reproductive Technology (ART) unit from June 2020 to January 2022 on a total of 92 subfertile women, where follicular fluid was aspirated and collected from follicles after oocyte isolation to determine the leptin level.

During this trial, 112 patients were evaluated for eligibility. Based on the inclusion criteria, 14 potential patients were eliminated from the study, while 6 patients declined to participate. Finally, the study relied on the information of 92 subfertile women.

In our study, 26 (28.3%) of the 92 cycles resulted in clinical pregnancies, with no cycles exhibiting clinical criteria of ovarian hyperstimulation syndrome.

Tab. 1. Comparison according to pregnancy regarding laboratory findings.

Lab	Pregnancy		^ p-value
	Positive (N=26)	Negative (N=66)	
FSH (IU/L)	6.9 ± 1.8	7.7 ± 3.0	0.227
LH (IU/L)	5.8 ± 2.5	6.2 ± 2.9	0.505
AMH (ng/mL)	2.3 ± 0.8	2.0 ± 0.9	0.195
Follicular fluid leptin (ng/mL)	100.3 ± 15.5	123.5 ± 17.8	**<0.001***

^ Independent t-test. *Significant

Tab. 2. Comparison according to cause of subinfertility and trial number regarding follicular fluid leptin (ng/mL).

Characteristics		N	Mean ± SD	p-value
Cause of sub infertility	Unexplained	70	116.0 ± 20.4	#0.705
	Tubal	20	119.8 ± 19.6	
	Endometriosis	2	122.1 ± 15.9	
Trial	First	67	117.5 ± 20.5	^ 0.693
	Repeated	25	115.6 ± 19.1	

^ Independent t-test. #ANOVA test

Tab. 3. Correlation between follicular fluid leptin and studied factors.

Variables	Follicular fluid leptin	
	r	p-value
Age	0.054	0.611
Body mass index	0.048	0.649
FSH	0.065	0.54
LH	0.055	0.603
AMH	-0.118	0.261
Antral follicular count	-0.126	0.232
Dose of gonadotrophins	0.255	**0.014***
Stimulation days	0.057	0.587
Oocytes retrieved	-0.18	0.087
Oocytes quality	-0.115	0.277
Fertilization rate	-0.264	**0.011***
Cleavage rate	-0.211	**0.043***
Embryos number	-0.307	**0.003***
Embryos quality	-0.398	**<0.001***
Embryos transferred number	-0.169	0.108
Embryos transfer day	-0.356	**0.001***

Pearson correlation. r: Correlation coefficient. *Significant

Tab. 4. Diagnostic performance of follicular fluid leptin in prediction of pregnancy occurrence

Factors	AUC	SE	p-value	95% CI	Cut point
Follicular fluid leptin	0.835	0.045	**<0.001***	0.746–0.924	≤112.0 ng/mL

AUC: Area under curve. SE: Standard error. CI: Confidence interval. *significant

Tab. 5. Diagnostic characteristics of follicular fluid leptin ≤ 112.0 ng/mL in prediction of pregnancy occurrence

Characters	Value	95% CI
Sensitivity	80.8%	60.6% – 93.4%
Specificity	77.3%	65.3% – 86.7%
Diagnostic accuracy (DA)	78.3%	68.4% – 86.2%
Youden's index	58.0%	39.8% – 76.3%
Positive Predictive value (PPV)	58.3%	40.8% – 74.5%
Negative Predictive value (NPV)	91.1%	80.4% – 97.0%
Positive likelihood ratio (LR+)	3.55	2.19 – 5.76
Negative likelihood ratio (LR-)	0.25	0.11 – 0.55
Diagnostic odds ratio (DOR)	14.28	4.60 – 44.32

CI: Confidence interval.

The current study found no statistically significant differences in pregnancy based on age, BMI, causes of subinfertility, or trial number (p values = 0.262, 0.185, 0.889, 0.314) respectively.

The findings of our study demonstrated that oocyte quality, fertilization rate, embryos number, embryos quality, and embryo transfer day were statistically substantially higher in positive clinical pregnant women (p value<0.05) with no significant difference as regard Oocytes retrieved and Embryos transferred number (p value=0.164, 0.056).

In terms of Follicular Fluid Leptin, our findings demonstrated that it was statistically lower among positive

clinical pregnant cases (p value<0.001), with no statistically significant difference in terms of causes of sub-infertility or trial number (p values = 0.705, 0.693) respectively.

As a result, follicular fluid leptin had a statistically significant positive association with gonadotrophin dose, as well as a statistically significant negative correlation with fertilization rate, cleavage rate, embryo number, embryo quality, and embryo transfer day.

These findings are consistent with the systematic review done by Al-Aqbi and his colleagues in 2022 [3] which revealed, similarly at day of OPU, that follicular fluid leptin levels were significantly lower in those who got pregnant group compared to those who did not (p value=0.006) and found no statistically significant difference as regard oocyte retrieval and embryo transfer number between both groups.

Elevated leptin levels have been linked with diminished ovarian stimulation response, follicular development, embryo quality, and pregnancy outcomes. These data imply that leptin influences embryo quality and may be a sensitive predictor of IVF success (Anifandis et al., 2005, Al-Aqbi et al., 2020) [3,13].

This is also consistent with prior research conducted by Barroso and his colleagues in 1999,24 who found that; the higher the follicular fluid leptin levels, the poorer the embryo quality in IVF cycles, implying that leptin, similar to vascular endothelial growth factor, is a marker of follicular hypoxia. Simultaneously, Butzow and his colleagues in 1999 discovered that a significant increase in leptin levels during controlled ovarian stimulation (COH) is associated with diminished ovarian stimulation response [9].

Similar findings were later reported by other researchers, including Anifandis and his colleagues in 2005 and Gürbüz and his colleagues in 2005 [13,25].

Again, our study results are in concordance with Mantzoros and his colleagues who performed a study in 2000 and were the first to report, that women who became pregnant during IVF cycles had considerably lower intrafollicular leptin concentrations than women who did not.12 Following that, other researchers showed that high serum or intrafollicular leptin levels are associated with reduced pregnancy rates in IVF cycles [26,27].

On the other hand, Ahmeid in 2017 conducted a trial on 54 infertile women who underwent their first ICSI cycles to investigate the relation between follicular fluid leptin levels and pregnancy rates in Iraqi women undergoing IVF/ICSI and discovered that 20.37% of them achieved pregnancy, while 79.63% did not, but importantly, follicular fluid leptin at the day of OPU showed no significant difference between women who got pregnant compared to those who did not [28].

Consistently with Ahmeid's study results, another research work conducted by Asimakopoulos and his colleagues in 2009, which enrolled 77 women undergoing IVF/ICSI cycles exclusively due to male factor infertility. They found that the fertilization rate was 24.6% (19/77) with no obvious symptoms of ovarian hyperstimulation in all cycles of IVF. In contrast to our findings, Asimakopoulos reported that total leptin concentrations in both serum and follicular fluid samples showed no significant differences between pregnant and non-pregnant groups. Asimakopoulos found also no significant difference in follicular leptin concentrations and fertilization rate, embryo number, and embryo quality [29].

This disagreement between our results and Asimakopoulos results and Ahmeid results could be explained by the fact that leptin concentrations can vary greatly depending on the technique of follicular fluid collection used. Each published study employed a different method of detecting leptin concentrations in blood and follicular fluid and not all assays, are equally sensitive or selective. It is generally understood that detecting the same cytokine in the same sample using different assays might yield different results. This is more noticeable in follicular fluid samples because not all assays have been validated for these types of samples.

Without a doubt, leptin is required for optimal reproductive function. It acts at various levels of the reproductive axis, and its effects appear to be dose-dependent [30]. It was found that high leptin levels can affect directly ovarian function, though, it was found that high leptin levels are unlikely to act centrally and affect the hypothalamus-pituitary axis because the saturable route of leptin across the blood-brain barrier prevents excess levels of leptin from reaching hypothalamic receptors [31].

It is also worth noting that the hypothalamus-pituitary axis is carefully controlled and inhibited during the downregulation that precedes IVF and embryo transfer. As a result, leptin's impacts on GnRH neurons and gonadotropic cells seems unlikely, although its direct actions on ovarian cells may be significant [32].

In terms of direct actions on ovarian cells, it is thought that high leptin concentrations block numerous growth factors and hormones stimulatory effects on gonadotropin-stimulated steroidogenesis in theca and granulosa cells. It is also found that low leptin levels either do not affect or even augment granulosa cell aromatase activity [32].

Our study tried hardly through a thorough detailed analysis, to determine which follicular fluid leptin levels are defined as high, and which as low.

Follicular fluid leptin had significant moderate diagnostic performance with a cut-off value of ≤ 112.0 ng/mL with high Sensitivity, Negative Predictive value, and Negative likelihood ratio in predicting pregnancy occurrence, according to our study results.

In this regard, the study population's selection may have had a substantial influence on the results, as leptin expression can be altered by factors such as BMI and inflammatory conditions [29].

This study comprised healthy, nonobese women who had ICSI cycles.

CONCLUSION

As evident from the current study, Follicular fluid leptin was significantly lower among positive clinical pregnant cases and, Follicular fluid leptin had statistically significant positive correlation with dose of gonadotrophins, as well as statistically significant negative correlations with fertilization rate, cleavage rate, embryos number, embryos quality and Embryos transfer day.

Consequently, Leptin concentrations in the follicular fluid may be important predictive markers of a successful outcome of IVF treatment.

However, combining leptin in serum and/or FF with other factors such as BMI may be a more helpful and informative marker for predicting IVF outcome.

CONFLICT OF INTEREST

The candidate declared that there was no conflict of interest, and the cost of the study was paid by the candidate (no funding third-party was available for this study).

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