The Association between Follicular Fluid Leptin, Insulin Resistance and ICSI Outcome in Women with Unexplained Infertility

Protocol of Thesis

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Introduction

Infertility has been always considered as one of the important, serious and costly health issues in different societies ***(Kamyabi andGholamalizade, 2015).***

Assisted reproduction is the scientific assistance provided to the infertile couples to achieve pregnancy by several assisted reproductive techniques that tend to overcome natural barriers to fertilization. Amongst these procedures, intracytoplasmic sperm injection which is a promising micro-manipulation technique, in which fertilization is accomplished by the injection of a sperm into a single egg ***(Rosen et al.,2010).***

In 25% of the infertile couples, etiology of infertility cannot be found. This unexplained infertility is described with normal semen counts together with optimal ovulatory functions, presence of patent tubes and a normal uterine cavity and failure of achieving pregnancy after at least 1year of unprotected intercourse***(Hart,2003).***

Patients with well-defined unexplained infertility may benefit from use of ICSI to fertilize all oocytes.The routine use of ICSI in couples with unexplained infertility offers significant benefit***(Lauren et al.,2013).***

Various studies have already been performed to discover the causes of infertility, among them high leptin level has been considered as one of the important and effective factors in several studies ***(Kamyabi and Gholamalizade,2015).***

Leptin is a hormone synthesized by white adipose tissue and is part ofthe adipokine family. It is involved in the regulation of food intake andenergy homeostasis by acting on the central nervous system (CNS)***(Catteau et al.,2015).***

The leptin gene, or ob(obesity) gene, was cloned and sequenced in1994. Mutations in this gene lead to morbid obesity and diabetes in humans and animals. Several leptin receptors have been located in many organs,and particularly in the brain. The serum leptin level is correlated with body fat mass quantity and distribution, providing a peripheral signal tothe CNS on the adequacy of nutritional status for reproductive function***(Catteauet al.,2015).***

Moreover, this hormone is involved in the control of many physiological functions, such as growth, metabolism and reproduction. In the case of female fertility, leptin is a key regulator and stimulator of the hypothalamic-pituitary-gonadal axis, through its direct action on the CNS leading to the regulation of gonadotrophin releasing hormone (GnRH) secretion in the hypothalamus ***(Quennell et al., 2009; Roa and Herbison, 2012).***

Leptin positively influences the reproductive system from the onset of puberty to pregnancy, establishing a close link between energy homeostasis and fertility. However, animal studies have clearly shown that excessive leptin secretion may have adverse effects on female fertility ***(Comninos et al.,2014).***

Although the physiological and pathophysiological consequences of this interconnection between leptin and ovarian physiology in humans are now extensively studied, particularly in assisted reproductive technology (ART) cycles, a small number of studies have tried to evaluate the interest for measuring the serum and/or follicular leptin levels (FFL)in infertile women undergoing IVF cycles, but have conflicting results ***(Anifandis et al., 2005; Gurbuz et al., 2005).***

Leptin receptors have been demonstrated in the ovarian granulosa and thecal cells, and within these cells leptin reduces steroid production by antagonizing insulin-like growth factor I(IGF-I) ***(Llaneza-Suarez et al.,2014).***

Leptin promotes the proliferation and survival of trophoblast cells by an autocrine action and an anti-apoptotic effect .The positive effect of HCG on leptin gene expression in placenta was recently reported ***(Catteau et al.,2015).***

This can be linked with the variation of serum leptin levels observed throughout pregnancy. Indeed, the serum leptin level progressively

increases during the first weeks of pregnancy to reach a maximum at the 28th week. Afterwards, the serum leptin level starts to decrease and reaches a minimum at delivery. These results suggest that leptin may not only play a key role between the endometrium and embryo at the time of implantation, but that it is also necessary for physiological placental function,finally allowing the maintenance of fetal development and normal pregnancy***(Stock et al., 1999).***

Some investigators have suggested that leptin might exert a double role in regulation of reproduction. They showed that when leptin level is lower than normal, it can exert a negative effect on endocrine system, regulating reproduction, while when leptin level is higher than normal, it negatively affects normal function of ovary and fetusdevelopment ***(Kamyabi and Gholamalizade, 2015).***

Aim of the Work

This study aims to investigate if follicular fluid leptin concentrations and insulin resistance are correlated with ICSI success.

Research Question:

In women with unexplained infertility undergoing ICSI, does high follicular fluid leptin concentration associate with insulin resistance and poor ICSI outcome?

Research hypothesis:

In women with unexplained infertility and undergoing ICSI, high follicular fluid leptin concentration may be associated with insulin resistance and poor ICSI outcome.

Patients and Methods

Design: A prospective observational (cohort) study.

Setting: This study will be conducted in assisted reproductive technology unit of Ain Shams University Hospital after approval of the research ethical committee.

Population: Thirty four women with unexplained infertility who will undergo ICSI will be enrolled in and a written informed consent will be obtained from all participants.

Sample size justifications:

Sample size was calculated using STATA® version 11 program, setting the type-1 error (α) at 0.05 and the power (1-β) at 0.8. Results from a previous study***(Llaneza-Suarez et al.,2014)*** showed that the mean FFL among live birth was 11.5 ± 4.6 while among non live birth it was 16.8±6.2 . Calculation according to these values produced a minimal sample size of 34 cases.

The formula used in calculating sample size:

$$\mathbf{n = (Z_{\alpha/2}+Z_{\beta})^2 * 2 * \sigma^2 / d^2},$$

where $Z_{\alpha/2}$ is the critical value of the Normal distribution at α/2 (e.g. for a confidence level of 95%, α is 0.05 and the critical value is 1.96), Z_{β} is the critical value of the Normal distribution at β (e.g. for a power of 80%, β is 0.2 and the critical value is 0.84), σ^2 is

the population variance, and d is the different you would like to detect.

Inclusion criteria:

- Women in reproductive period (age 18-38 years).
- Unexplained infertility
- Absence of any underlying complex disorders as diabetes,obesity and cardiovascular disease.

Exclusion criteria:

- Male factor infertility.
- Presence of an abnormal uterine cavity due to endometrial polyps, myomas, endometrial synechiae,septate uterus etc.
- Women with PCOS.
- Patient with poor ovarian reserve.
- Women with poor ovarian reserve.
- Patient with ovarian hyperstimulation.
- Poor responders to controlled ovarian stimulation.

<u>All the patients will be subjected to:</u>

- Full medical history including age, obstetric history, menstrual history, duration of infertility, number of previous assisted reproductive technique (ART) attempts.
- Physical examination (general including weight and height, abdominal and pelvic).

- BMI will be calculated as weight (kg)/square of height (m2) and categorized as low (<18.5 kg/m2), normal (18.5-24.99 kg/m2), or high (25 kg/m2).Women with high BMI values will further categorized as overweight (25.0-29.99 kg/m2) or obese (R30.0 kg/m2).
- The homeostasis-model assessment of insulin resistance (HOMA-IR) will be calculated by multiplying fasting insulin (μU/mL) and glucose (mg/dL) levels and then dividing this product by the constant (405).The patient will be considered to have normal insulin resistance if the score is below 3 and considered to have moderate insulin resistance if it is between 3 to 5 and sever insulin resistance if it is above 5.
- Investigations as LH, FSH &E2 levels on cycle day 3.
- Before beginning any of the controlled ovarian hyperstimulation protocols, a blood sample will be taken after an overnight fast and immediately sent to the hospital's chemistry laboratory for measuring Serum glucose and insulin.
- Controlled ovarian stimulation with long GnRH agonist (GnRH-a) down regulation protocol.Daily GnRH- administration of Triptorelin (Decapeptyl 0.1 mg/mL-Ipsen,S.p.A., Milan,Italy) will be started during the midluteal phase of the cycle before ovarian hyperstimulation till the day of human chorionic gonadotropin (HCG) administration.
- Ovarian hyperstimulation will be initiated once the serum estradiol (E2) level is ≤50 pg/mL.

- From day 2 of the menstrual cycle 150-300 IU of Human menopausal gonadotropin (HMG) (Fostimone, IBSA, Lugano, Switzerland) will be given intramuscularly.
- The dosages of gonadotropins will be individualized according to transvaginally ultrasonic monitoring of follicular growth.
- When at least 3 follicles ≥ 18 mm in diameter, the patient will be given 10,000 IU Human chorionic gonadotropin hormone (Profasi, Serono, Aubonne, Switzerland).
- Transvaginal oocyte retrieval will be performed with ultrasound guidance under general anesthesia 34- 36 hours after HCG injection.
- At the time of oocyte retrieval , bloodless aspirated follicular fluid will be collected from follicles , centrifuged for 20 minutes at 1000×g at 2 - 8°C to remove debris, and then stored at - 20C until total leptin determination will be performed as a single batch with the use of ELISA (Human LEP(Leptin) ELISA kit, NTCO, Egypt).
- This kit is based on sandwich enzyme-linked immune-sorbent assay technology. Anti LEP antibody will be pre-coated onto 34-well plates. And the biotin conjugated anti- LEP antibody will be used as detection antibodies. The standards, test samples and biotin conjugated detection antibody will be added to the wells subsequently, and wash with wash buffer. HRP-Streptavidin will be added and unbound conjugates will be washed away with wash buffer. TMB substrates will be usedto

visualize HRP enzymatic reaction. TMB will be catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the LEP amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of LEP can be calculated.

- In all cycles, after stripping the cumulus cells, ICSI will be performed with the sperm to mature metaphase II oocytes in all cycles.
- Fertilization will be assessed16-18 hours after injection of oocyte. Normal fertilization will be confirmed when 2 clearly distinct pronuclei are present or presence of 2^{nd} polar body.
- Embryo quality will be evaluated on the second day of development.
- A maximum of two fresh embryos will be transferred on day 5 oocyte retrieval under ultrasound guidance.
- For luteal phase support all patients will receive a daily dose of 400 mg vaginal pessaries of progesterone (Prontogest, Marcyrl, Egypt)for 14 days after embryo transfer.
- Pregnancy will be confirmed with the use of serum quantitative b-HCG and 3 weeks later by TVS verification of embryonic cardiac activity.

Outcome measure(s): The primary outcome will be biochemical pregnancy rate based on serum quantitative beta-hCG level at 9 days after ET. Secondary outcome will be clinical pregnancy rate

using trans-vaginal US examination at 7 weeks after embryo transfer to detect the presence of fetal sac and embryonic heart pulsations.

Fertilization rate (FR) is percentage of transformation of micro injected oocytes into two pronuclei ***(Jawed et al., 2016).***

Implantation rate (IR) is the number of gestational sacs observed on TVS divided by the number of embryos transferred ***(Kondapalli et al., 2012).***

Results:

All the collected data will be tabulated for further statistical analysis.

Statistical Analysis

The collected data will be revised, coded, tabulated and introduced to a PC using Statistical package for Social Science **(SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001).** Data will be presented as Mean and Standard deviation (±SD) for quantitative parametric data, and Median and Interquartile range for quantitative non parametric data.Frequency and percentage will be used for presenting qualitative data. Suitable analysis will be done according to the type of data obtained. **Student T** Test or **Mann Whitney test** will be used to analyze quantitative data while **chi square test and fisher exact test** will be used to analyze qualitative data.

- P- value: level of significance

  -P>0.05: Non significant (NS).

  -P< 0.05: Significant (S).

  -P<0.01: Highly significant (HS).

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