Insulin-Like Factor 3 (INSL3) in Serum and Follicular Fluid: Realtion With Marker Of Ovarian Reserve

Maroua Khalaf Lateef [1], Muayad Sraibit Abbod [2], Mufeda Ali Jwad [3]

1 M.B.Ch.B, Iraqi Board in Gynecology and Obstetric
2 Assistant Professor, Clinical Reproductive Physiology Department, High Institute for Infertility Diagnosis and Assisted Reproductive Technology, Al-Nahrain University, Baghdad, Iraq.
3 Assistant Professor, Clinical Reproductive Physiology Department, High Institute for Infertility Diagnosis and Assisted Reproductive Technology, Al-Nahrain University, Baghdad, Iraq.:

Abstract

Background: Granulosa cells produce most ovarian function indicators in infertile women. FSH, estradiol, AMH, and inhibin B are these indicators. Female peptide hormone insulin-like factor 3 is novel. INSL3 was formerly considered a male hormone. It is produced by Leydig cells and is a biomarker of their performance.

Aim: to investigate how INSL3 levels may be linked to the number and quality of oocytes, fertilization rate, embryo quality, and the success of pregnancy in women undergoing fertility treatments.

Materials, and Method: In this cross-sectional study, 75 infertile couples were chosen in advance based on certain criteria at Al-Nahrain University's High Institute for Infertility Diagnosis and Assisted Reproductive Technologies from October 2022 to April 2023. For the ICSI cycle, the antagonist protocol was used to control how much the ovaries were stimulated. Based on the levels of INSL3 in the serum on the second day of the menstrual cycle and in the follicular fluid on the day of oocyte retrieval, a comparison was made with other markers of ovarian reserve (AFC, FSH, E, and AMH) and ICSI cycle outcomes, such as the number and quality of oocytes retrieved, the number and quality of embryos, the pregnancy rate, and other things. Researchers looked at the connection between INSL3 levels in serum and follicular fluid, I. A test called an Enzyme-Linked Immunosorbent Assay was used to find out how much INSL3 there was.

Results: The study involved 75 infertile Iraqi women undergoing intracytoplasmic sperm injection (ICSI), and 28.0% of them achieved pregnancy. INSL3 was detected in both serum and follicular fluids of all patients. Positive significant correlations were found between serum INSL3 levels and anti-mullerian hormone (AMH) and testosterone. Moreover, significant positive correlations were observed between serum INSL3 levels and antral follicle count,. However, there was no significant difference in follicular fluids INSL3 levels according to body mass indices.There was a positive but insignificant correlation between serum INSL3 and follicular fluids INSL3. The study did not find significant differences in serum and follicular fluids INSL3 levels between females with primary and secondary infertility. Serum INSL3 levels significantly differed among patients with different causes of infertility, but there was no association between follicular fluids INSL3 and causes of infertility.

In conclusion: The study provides compelling evidence for the significant role of INSL3 in female fertility and its association with the success of intracytoplasmic sperm injection (ICSI) in infertile Iraqi women. The presence of INSL3 in both serum and follicular fluids underscores its importance in the reproductive process, particularly in the context of assisted reproductive techniques like ICSI. Positive correlations between serum INSL3 levels and anti-mullerian hormone (AMH) and Antral follicle count AFC indicate its potential involvement in ovarian reserve and hormonal regulation, further highlighting its relevance to female reproductive health.

Keywords: Theca Cell Marker Insulin-Like Factor 3; ICSI; Follicular

Introduction:

Fertility is the ability to get pregnant and have children (Barbieri, 2019), and infertility affects millions of women of childbearing age all over the world. Infertility is defined by the World Health Organization (WHO) as "a disorder of the reproductive system that makes it impossible to get pregnant after 12 months or more of regular, unprotected sexual activity" (2020). This inability to get pregnant could be caused by sexual or non-sexual (unknown) factors (Elhussein et al., 2019). Reproductive problems and infertility are quite common, affecting 10-15% of couples worldwide (Yatsenko & Rajkovic, 2019). This has a negative effect on the quality of life and happiness of those who are affected. Some problems with the female reproductive system, like polycystic ovary syndrome, endometriosis, blocked fallopian tubes, and Asherman syndrome, can lead to infertility (Zhao et al., 2019). More than 186 million couples around the world have trouble getting pregnant. Most of these couples live in developing countries where they don't have access to good medical care. So, infertility is one of the most common health problems in the world. Infertility, on the other hand, can be treated and fixed (Allegra, 2017). Several studies have tried, in different ways, to predict the results of studies on infertility. With the development of assisted reproductive technology, early infertility prediction may be able to help doctors a lot more than just help patients with their physical, mental, and social problems. A lot of research is going on to find ways to find, analyze, and predict infertility early on. (The 202 of Koshy and Anuradha). Insulin-like factor 3 (INSL3) is a unique peptide hormone that is mostly made by Leydig cells in the testicles. It may be available to women. INSL3 is not like any other hormone in a woman's body. It shows the activity of theca cells in recruited antral follicles before the LH surge, so it may be a sign of follicle waves. A study from 2013 (Anand-Ivell, et al. 2013). Both the theca internal layer of antral follicles and the corpora lutea have mRNA for INSL3. A study shows that (Satchell et al., 2013). The goal of this study was to find out if there is a link between INSL3 levels and the number and quality of oocytes, the rate of fertilization, the quality of embryos, and whether or not a woman gets pregnant after fertility treatments.

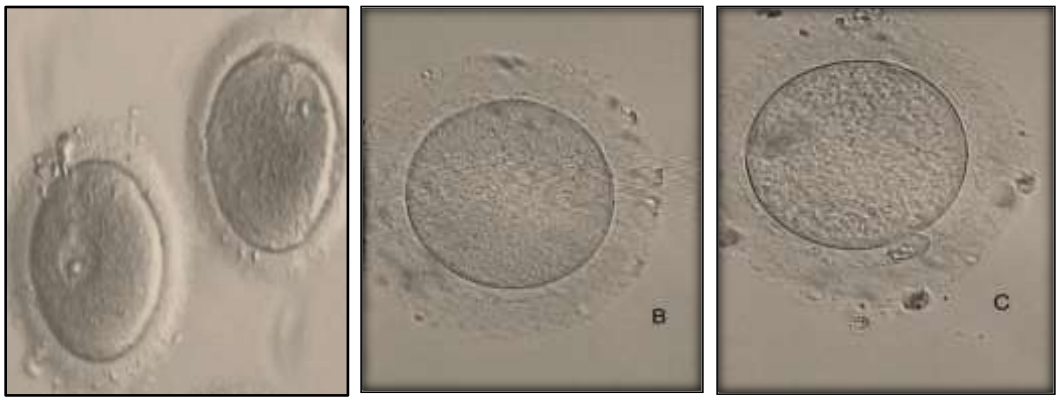
Materials and Methods

This prospective cross-sectional study was done at Al-Nahrain University's High Institute for Infertility Diagnosis and Assisted Reproductive Technologies from October 2022 to May 2023. Its goal was to find out what made 75 Iraqi couples who had intracytoplasmic sperm injection (ICSI)

treatment unable to have children. Before the ICSI program started, all of the women who were going to take part were given a thorough evaluation that included a medical history, a physical exam, an ultrasound, and an analysis of their hormones. The study looked at the relationship between serum Insulin-Like Factor 3 (INSL3) levels and things like the number of oocytes and embryos and whether or not a pregnancy was successful. In the study, infertile Iraqi women between the ages of 18 and 43 with both open and closed fallopian tubes took part. They were treated with ICSI using the GnRH antagonist protocol. Exclusion criteria included male partners with non-obstructive azoospermia or a testicular biopsy, uterine abnormalities, frozen embryos in cases of poor endometrium and ovarian hyperstimulation syndrome, and other medical conditions like thyroid dysfunction, diabetes, or heart, kidney, or liver disease. Before starting the ICSI cycle, each couple had a general history, physical and gynecological exams, hysterosalpingography or sonohystrosalpangography, general tests, hormone tests, and a baseline transvaginal ultrasound. Based on WHO reference values from 2010, seminal fluid tests were done on male partners. The GnRH antagonist protocol was used, and patients' levels of estradiol in their blood and ultrasounds of their genitalia were tracked. When three or more follicles with a diameter of 17 mm or more were present, ovulation took place. On the day that the oocytes were taken out, INSL3 levels were checked by taking follicular fluid. Embryo transfer happened about 3 days later, depending on how many and how good the embryos was.

The GnRH antagonist protocol was used in the study to treat infertile women with intracytoplasmic sperm injection (ICSI). Gonadotropin (recombinant FSH-Gonal F) injections were given based on ovarian reserve tests, BMI, and the results of the previous cycle. Cetrotide, which blocks GnRH, was also given. Recombinant chorionic gonadotropin (Ovitrelle®) was used to make a woman ovulate when an ultrasound showed three or more follicles with a diameter of 17 mm or more and E2>300 .

A transvaginal ultrasound-guided method was used to get the oocytes 34 to 36 hours after the hCG injection. Before ICSI, the cumulus and corona cells were taken out of the oocytes. MII oocytes were known to be mature because the germinal vesicle nucleus was gone and the polar body was there.



-A- **-B-** **-C-**

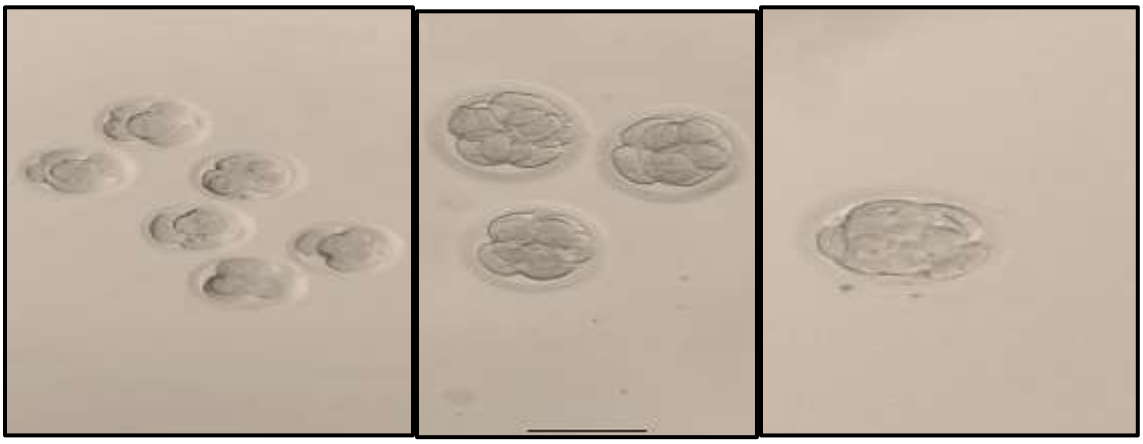
Figure 2: Morphology Markers characterizing the meiotic status of oocytes: A- Immature oocyte (GV) B- Immature oocyte(MI) C- Mature oocyte (MII), (Adapted from High Institute for Infertility Daignosis and Assisted Reproductive Technologies).

During the microinjection procedure for intracytoplasmic sperm injection (ICSI), a single spermatozoon was carefully selected based on its size and shape. The selected spermatozoon was immobilized by placing it in a droplet of PVP. Each egg and sperm were placed in separate droplets for the microinjection process. The micromanipulation was carried out using an inverted phase microscope with a heated stage. The injection pipette absorbed the immobilized sperm tail. The oocyte was held using a holding pipette, and the injection pipette was inserted at a specific angle to avoid interfering with the spindle. The sperm was then slowly injected into the oocyte's cytoplasm.

Figure 3: Intra-cytoplasmic Sperm injection (Adapted from High Institute for Infertility Daignosis and Assisted Reproductive Technologies).

The fertilization process after intracytoplasmic sperm injection (ICSI) was monitored in the study. After 14-16 hours, oocytes were checked for signs of fertilization, which were indicated by the presence of two separate pronuclei and a second polar body. The cleavage phase of the embryo was observed 25-27 hours after fertilization, and early embryo cleavage was assessed.

On the day after ICSI, the embryo cleavage was checked, and on day 3, the quality of the embryos was judged based on morphological criteria, including the number and shape of blastomeres and the rate of fragmentation. A grading system was used to assess embryo quality, with Grade A embryos considered the best, having equal-sized blastomeres without fragments or obvious morphological abnormalities.



-A- **-B-** **-C-**

Figure 4: Grade I embryos at different stages, A-Day 2 embryo, B-Day 3 embryos,C-Day 3 embryo,Early compaction , (Adapted from High Institute for Infertility Daignosis and Assisted Reproductive Technologies).

The study describes the procedures and techniques used during the intracytoplasmic sperm injection (ICSI) treatment and subsequent embryo transfer. Before the procedure, patients are positioned in lithotomy with a full bladder, and the vagina is washed with normal saline. Embryos are then transferred into the uterus using an intrauterine catheter guided by abdominal ultrasound.

Luteal support is provided to patients through intramuscular and vaginal progesterone supplementation. Patients receive progesterone injections every three days and vaginal suppositories until 14 days after embryo transfer, and if pregnancy occurs, the support continues until 10 weeks gestation.

The evaluation of INSL3 levels in both serum and follicular fluid is a crucial aspect of the study. Blood samples are taken from patients on the second day of the ovarian stimulation phase, and follicular fluid samples are collected on the day of oocyte retrieval using ultrasound guidance. The INSL3 levels are measured using an enzyme-linked immunosorbent assay (ELISA) technique, allowing for the quantification of INSL3 concentration in the samples.

Overall, the study highlights the specific steps involved in the ICSI procedure, luteal support, and the assessment of INSL3 levels to better understand its relationship with ICSI outcomes in infertile women.

Statistical Analysis:

The study utilized Statistical Package for Social Sciences (SPSS) version 23.0 and Microsoft Office 2010 for data analysis. Descriptive statistics, such as frequency, range, mean, and standard error, were employed to describe the data. To compare groups, the independent sample t-test and analysis of variance (ANOVA) were used. Pearson's correlation coefficient (r) was used to measure the relationship between continuous variables.

Results

Seventy five infertile females were enrolled in the present cross sectional study; the results were expressed in mean plus minus standard error of the mean. The mean patients' age was 29.88 ± 1.32 and the mean body mass indices were 26.37 ± 0.87. Twelve females (16.0 %) were normal weighted, 45 females (60.0%) were over weighted and 18 females (24.0 %) were obese as represented in table1.

Table2 demonstrated the baseline hormonal levels; accordingly mean LH level was 5.64 ± 0.29 mIU/ml, mean FSH was 4.88 ± 0.29 mIU/ml, mean AMH was 3.95± 0.22 ng/ml, mean basal E2 was 40.38 ± 0.93 pg/ml, mean E2 at day of HCG trigger was 1497± 94 pg/ml, mean testosterone was 0.33 ± 0.02 ng/ml, mean prolactin was 22.70 ± 2.50 ng/ml and mean TSH level was 2.78 ± 0.35 mIU/ml.

The baseline antral follicle count were presented in table3, according to the results; the mean antral follicle count was 19.60 ± 0.82,

Table 1: Characteristics of the patients in this study at the start

Parameters		Range	
Age (years)		20 - 42	29.88 ± 0.75
BMI (Kg/m²)		20.95 – 36.73	27.59 ± 0.44
Duration of infertility (years)		1 - 13	5.52 ± 0.35
Parameters		N. (%)	
Type of infertility	Primary	69 (92.0 %)	
	Secondary	6 (8.0 %)	
Cause of infertility	PCOS	25 (33.3 %)	
	Other female causes	19 (25.3%)	
	Male causes	18 (24.0 %)	
	Unexplained	13 (17.4 %)	
BMI ranking	Normal weight	12 (16.0%)	
	Over weight	45 (60.0%)	
	Obese	18 (24.0%)	

Table 2: Baseline hormone levels of people who took part in this study

Hormone	Range	Mean±SE
LH (mIU/ml)	0.5 – 12.82	5.64 ± 0.29
FSH (mIU/ml)	1.3 – 10.4	4.88 ± 0.29
AMH (ng/ml)	0.77 – 9.3	3.95 ± 0.22
Basal E2 (pg/ ml)	24 – 50	40.38 ± 0.93
E2 at day of trigger (pg/ ml)	790 – 2300	1497 ± 54
Testosterone (ng/ml)	0.1 – 0.97	0.35 ± 0.2
Prolactin (ng/ml)	2.35 – 50.0	22.70 ± 2.50
TSH (mIU/ml)	0.5 – 4.50	2.78 ± 0.20

FSH: Follicle stimulating hormone; LH : Luteinizing hormone;E2: Estradiol ;AMH: antimullerian hormone ;TSH: Thyroid stimulating hormone

Table 3: Baseline antral follicle count

ICSI outcome	Range	Mean±SE
Antral follicle count	5 -33	19.60 ± 0.82

Table 4: Baseline levels of serum and follicular fluids INSL3 levels

Parameters	Range	Mean ± SE
Serum insulin like-3 (INSL3)	34.40 – 98.80	71.31 ± 1.86
Follicular fluid insulin like-3	260.6 – 515.3	379.8 ± 5.22

SE: Standard error; INSL3: Insulin like factor 3

There was a significant negative correlation between serum INSL3 and the length of infertility (r=-0.327, p=0.004). There was also an insignificant negative correlation between serum INSL3 and the patient's age and body mass indices. On the other hand, there was no significant correlation between follicular fluids INSL3 and the patient's age, body mass indices, or length of infertility, as shown in table 5

Table 5: Correlations between serum and follicular fluid INSL3 and the patient's age, body mass index, and length of infertility

Parameters		Serum INSL3	Follicular fluids INSL3
Age	r	-0.018	-0.008
	p value	0.879 NS	0.949 NS
Body mass index	r	-0.278	0.101
	p value	0.118 NS	0.399 NS
Duration of infertility	r	**-0.327**	-0.05
	p value	**0.004 S**	0.670 NS
r; Pearson's correlation coefficient; NS: Not significant (p > 0.05); S: Significant (p< 0.05)			

There was a positive significant correlation between serum INSL3 with serum AMH (r= 0.578 & *p*= 0.001) and testosterone (r= 0.378 & *p*= 0.001); however there were no significant correlations between both serum and follicular fluids INSL3 with serum LH, FSH, prolactin, TSH, basal E2 and E2 at day of trigger as presented in table 6. There were significant positive correlations between serum INSL3 with antral follicle count as presented in table 7.

Table 6: Relationships between serum and follicular fluids INSL3 and the hormones of a patient

Hormones		Serum INSL3	Follicular fluids INSL3
LH	r	-0.003	0.172
	p value	0.979 NS	0.141 NS
FSH	r	-0.145	-0.050
	p value	0.215 NS	0.671 NS
AMH	r	**0.578**	0.084
	p value	**0.001 S**	0.471 NS
E2 basal	r	0.059	0.090
	p value	0.620 NS	0.453 NS
E2 \ trigger	r	0.339	0.050
	p value	0.090 NS	0.671 NS
Testosterone	r	**0.378**	0.107
	p value	**0.001 S**	0.362 NS
Prolactin	r	-0.027	-0.261
	p value	0.820 NS	0.183 NS

TSH	r	0.129	-0.021
	p value	0.137 NS	0.864 NS

Table 7: Correlation between serum and follicular fluids INSL3 and the number of antral follicles

ICSI parameters		Serum INSL3	Follicular fluids INSL3
Antral follicle count	r	**0.474**	0.090
	p value	**< 0.001 S**	0.445 NS

r: Pearson's correlation coefficient; NS: Not significant ($p > 0.05$); S: Significant ($p \leq 0.05$)

Discussion

Ovarian function is a "black box" in reproductive medicine since so little is known about it. "(Santoro, 2017)" Modern diagnostic procedures are often more accurate and better predict treatment results than pregnancy itself once DOR has been confirmed (Deadmond et al, 2022). About 10% of women have rapid fertility loss as they age, with severely diminished ovarian reserve by their early 30s and full infertility by age 35; those who are afflicted often show no symptoms and have no obvious risk factors associated with them. This is according to (Kyweluk, 2020). The peptide hormone insulin-like factor 3 (INSL3) was previously thought to be produced exclusively by male testicular Leydig cells (Ivell & Anand-Ivell, 2020). Its action has been shown to regulate the production of androgens in the theca and to stimulate the growth and function of antral follicles. Growth differentiation factor 9 (GDF9) expression in oocytes might be induced or oocyte maturation sped up as a result (Xue et al, 2014). Increased cell apoptosis and follicular atresia contribute to female infertility in Insl3-null mice. This suggests that INSL3 plays a critical role in the ovarian microenvironment throughout the early stages of follicular development and function (Zhu et al, 2020). Loss of INSL3 expression appears to be one of the early indications for follicular atresia, earlier than any loss of steroidogenic enzymes such cytochrome P450 side-chain cleavage (P450scc) or 3-hydroxysteroid dehydrogenase(3β-HSD) INSL3 expression has primarily been identified in developing antral follicle(Irving-Rodgers et al,2002) . In the current study, Correlations between serum levels of INSL3 with patient's clinical parameters namely age, body mass index (BMI), type and duration of infertility. The likelihood of pregnancy in women receiving assisted reproduction is

mostly determined by their age (Wang et al., 2018). Consistent with previous studies that reported no association between patient age and serum INSL3 (Gambineri et al., 2007), the current investigation observed no significant link between serum INSL3 and follicular fluids INSL3. The body mass index was also studied as a variable (BMI) Obesity has been associated to poorer fertility, yet the mechanisms behind this association are still poorly understood (Marinelli et al, 2022). Due to its effect on hypothalamic gonadotropin release, which reduces both follicle count and progesterone levels, obesity is a major risk factor for infertility in women (Gautam et al, 2023). Serum INSL3 was shown to have a weak inverse relationship with BMI in the current investigation; nevertheless, a different result was drawn from the same data by (Zhu et al, 2021). The success rate of ICSI may be affected by the duration of infertility, according to the findings of previous research. Conception rates dropped dramatically with increasing infertility duration (Bakhtiari et al., 2020), indicating a correlation between infertility duration and ICSI success. Serum INSL3 was significantly inversely related to infertility time in this research. In this work, we found that INSL3 levels are linked to indicators of ovarian reserve. Indicators like AMH, E2, FSH, and LH are mostly secreted by granulosa cells in follicles. Ovarian reserve is seldom evaluated based on the role of theca cells. According to (Zhu et al., 2021). In females, the ovary is the primary source of circulating INSL3 (Ivell et al 2013), during the follicular phase, INSL3 is expressed within the theca interna layer of antral follicles in the ovary (Xue et al, 2014). The significant correlation between INSL3 and AMH blood concentrations reported in this study might support the conception that those peptides may reveal impaired follicle maturation in the infertile women ,similar finding in which circulating level of INSL3 positively associated with AMH in women with normal ovarian reserve and those with premature ovarian insuffeciency (Zhu et al., 2021). Furthermore, in our analysis, no evidence that serum INSL3 was associated with serum FSH. Similarly, INSL3 and FSH in PCOD show no statistically significant relationship (Seyam & Hefzy, 2018). Despite this, a research by (Zhu et al, 2020) indicated that INSL3 levels were positively correlated with FSH in women with normal ovarian reserve and those with Pregnancy has profound physiological effects on the thyroid gland, and thyroid illness has a long history of being associated to female infertility (Mazzilli et al, 2023). Both serum INSL3 and serum TSH showed no significant associations in the present investigation. It has been hypothesized for a long time that hyperprolactinemia impairs fertility by causing anovulation or luteolysis due to an inhibition of LH production (Zhang et al, 2020). There was no link between serum INSL3 and prolactin levels. Women's post-menstrual surge in circulating INSL3 is

likely due to healthy antral follicle production that was recruited by the inter-cycle increase in FSH.(Anand-Ivell et al, 2013). In the current study There were significant positive correlations between serum INSL3 with antral follicle count ,similar result reached by (Satchell et al, 2013:Zhu et al, 2020).

In conclusion: The study provides compelling evidence for the significant role of INSL3 in female fertility and its association with the success of intracytoplasmic sperm injection (ICSI) in infertile Iraqi women. The presence of INSL3 in both serum and follicular fluids underscores its importance in the reproductive process, particularly in the context of assisted reproductive techniques like ICSI. Positive correlations between serum INSL3 levels and anti-mullerian hormone (AMH) and. Antral follicle count AFC indicate its potential involvement in ovarian reserve and hormonal regulation, further highlighting its relevance to female reproductive health.

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