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

Maroua K. Lateef [1], Muayad S. Abbod [1], Mufeda A. Jwad [1]

[1] **High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al Nahrain University, Baghdad, Iraq.**

marrwakhalaf@gmail.com

ABSTRACT

Granulosa cells produce most ovarian function indicators in infertile women like estradiol, AMH, and inhibin B. Female peptide hormone insulin-like factor 3 is produced by theca cell. Signaling of insulin-like factor 3 from theca cell stimulates antral follicle development and theca androgen production. To investigate how INSL3 levels may be a theca cell marker of ovarian reserve and the association with current markers of ovarian reserve, including AFC, FSH, E2, and AMH, in infertile women undertaking assisted reproduction. In this cross-sectional study, 75 Iraqi infertile couples were chosen at Al-Nahrain University's High Institute for Infertility Diagnosis and Assisted Reproductive Technologies from October 2022 to April 2023 for the ICSI cycle. Based on the levels of INSL3 in the serum on the day 2 of the menstrual cycle and on the day of retrieving oocyte in the follicular fluid. A comparison was made with current ovarian reserve markers (AFC, FSH, E2, and AMH) .From 75 infertile women undergoing intracytoplasmic sperm injection (ICSI), 21 of them achieved pregnancy. Positive Significant relations were found between serum INSL3 levels and anti-mullerian hormone (AMH and antral follicle count. there were no significant relationships between follicular fluids INSL3 with serum LH, FSH and E2 . 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). 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.

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KEYWORD

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

1. Introduction

Infertility is defined as a disorder of the reproductive system that makes it impossible to get pregnant after one year or more of regular sexual activity without protection (WHO, 2020 [1]). The inability to get pregnant could be caused by sexual or non-sexual (unknown) factors (Elhussein et al., 2019 [2]). Reproductive problems and infertility are quite common, affecting 10-15% of couples worldwide (Yatsenko & Rajkovic, 2019 [3]). 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 (Koshy and Anuradha, 2020 [4]). Insulin-like factor 3 (INSL3) is a unique peptide hormone that is mostly produced by Leydig cells in the testicles. In women, INSL3 is produced by the theca interna cells of the antral follicles of the ovary (Esteban-Lopez & Agoulnik, 2021 [5]). In healthy women, INSL3 is first detectable through the late stages of puberty (Hagen et al., 2015 [6]); its levels alter in a phasic way during the menstrual cycle, which is quite similar to what has been discovered by ultrasonography and becomes undetectable by the time of menopause (Ivell & Anand-Ivell, 2018 [7]) .

Insulin-like factor 3 (INSL3) shows the activity of theca cells in recruited antral follicles before the LH surge, so it may be a sign of follicle waves (Anand-Ivell et al., 2013 [8]). Development of preantral follicles in vitro, which was stimulated by INSL3, strongly shows that paracrine regulatory mechanisms play a role in folliculogenesis. Both the theca internal layer of antral follicles and the corpora lutea have mRNA for

INSL3 (Satchell et al., 2013 [9]). The reduction of INSL3 expression seems to be one of the early indicators for follicular atresia, as indicated by a comprehensive immunohistochemistry examination of the bovine ovary (Ivell & Anand-Ivell, 2018 [7]). Its action has been shown to regulate the androgens expression in the theca and to stimulate the growing and function of antral follicles. Growth differentiation factor 9 (GDF9) expression in oocytes might be induced or oocyte development sped up as a result (Xue et al., 2014 [10]). Increased cell apoptosis and follicular atresia contribute to female infertility in Insl3-null mice. This recommends that INSL3 may play a critical part in the ovarian microenvironment throughout the early stages of follicular development and function (Zhu et al, 2020 [11]). The goal of the current study was to determine if there is a link between INSL3 levels association with current markers of ovarian reserve, including AFC, FSH, E, and AMH, in infertile women undertaking assisted reproduction.

2. Patients and Methods

This prospective cross-sectional study conducted at Al-Nahrain University's High Institute for Infertility Diagnosis and Assisted Reproductive Technologies from October 2022 to May 2023 aimed to understand the factors contributing to infertility in 75 Iraqi couples who underwent intracytoplasmic sperm injection (ICSI) treatment. Before initiating the ICSI program, all the women participants received a thorough evaluation, including a medical history, a physical exam, baseline transvaginal ultrasound, hormonal analysis, hysterosalpingography or sonohystrosalpingography, and general tests. The study assessed the correlation between serum Insulin-Like Factor 3 (INSL3) levels and the current markers

of ovarian reserve, including AFC, FSH, E2, and AMH, in infertile women between the ages of 20 and 42. 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. Based on WHO reference values from 2010, seminal fluid tests were done for male partners. Early follicular phase serum (day two of the menstrual cycle) was collected to measure the level of basal INSL3. The GnRH antagonist protocol was used; gonadotropin (recombinant FSH-Gonal F) injections were given based on ovarian reserve tests, BMI, and the results of the previous cycle if present. Cetrotide, the GnRH antagonist, was also given, and patients were monitored with serial transvaginal ultrasound and serum levels of estradiol. Ovulation triggered by recombinant chorionic gonadotropin (Ovitrelle®) in this protocol when three or more follicles with a diameter of 17 mm or more were present and E2> 300. Oocyte retrieval was performed 34-36 after hCG injection using a transvaginal ultrasound-guided technique; at the time of the oocyte retrieval, follicular fluid was collected to measure the INSL3 level . Before ICSI, the oocytes were denuded to remove the cumulus and corona cells. Mature MII oocytes were identified based on the absence of the germinal vesicle nucleus and the presence of the polar body, as shown in Figure 1. 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 existence of two distinct 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 depend on morphological criteria, including the count 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, as shown in (Figure). During the embryo transfer, 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.

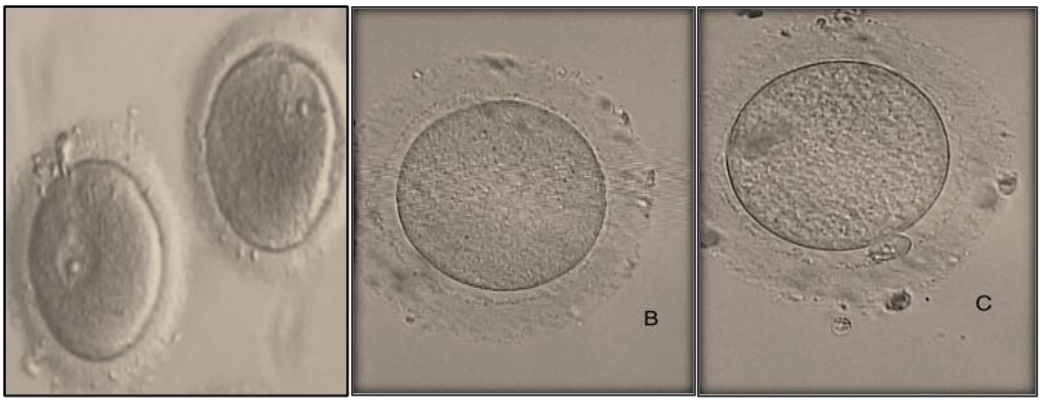
Luteal support is provided to patients through intramuscular and vaginal progesterone supplementation. Patients receive progesterone injections every three days and vaginal suppositories until 14 days after embryo transfer, and if pregnancy occurs, the support. Continues until ten weeks gestation.

The evaluation of INSL3 in serum and in the follicular fluid is a crucial aspect of the study. Measurement of INSL3 levels was done using a test called an enzyme-linked immunosorbent assay (ELISA) technique, allowing for 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:

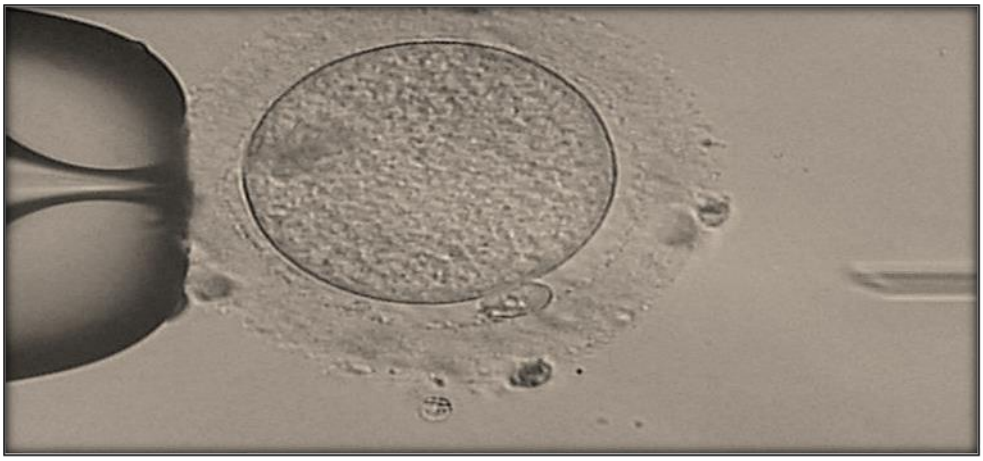
The study utilized Statistical Package for Social Sciences (SPSS) version 23.0 and Microsoft Office 2010 for data analysis. The data was explained using descriptive statistics, including frequency, range, mean, and standard error. The independent sample t-test and analysis of variance (ANOVA) were used to compare the groups. The correlation between continuous

variables was evaluated using Pearson's correlation coefficient (r).

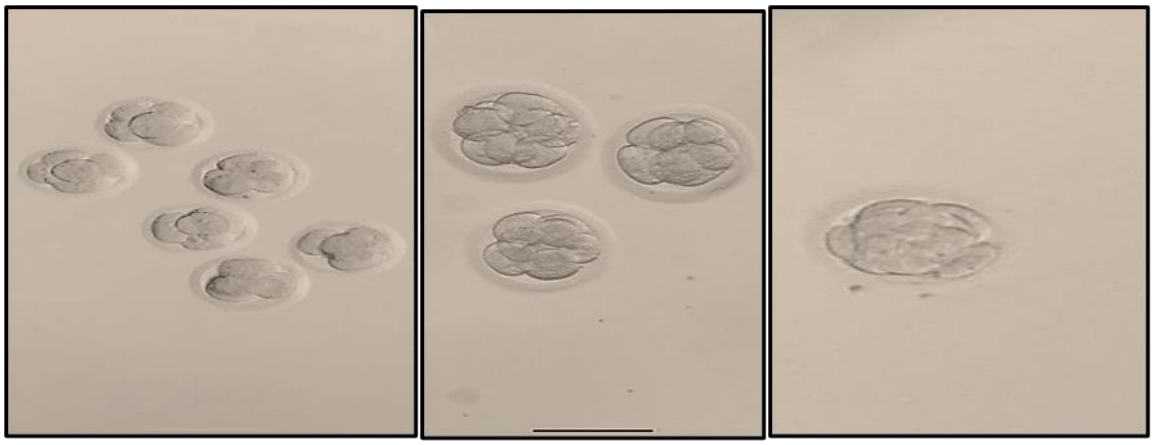


A B C

(Figure 1): Morphology Markers Illustrating the oocytes' meiotic status: A- Immature oocyte (GV) B- Immature oocyte (MI) C- Mature oocyte (MII)



(Figure 2): Intra-cytoplasmic Sperm injection.



A B C

(Figure 3): Grade I embryos at different stages, **A**-Day 2 embryo, -Day 3 embryos, **C**-Day 3 embryo, early compaction

3. Results

Seventy-five infertile females were enrolled in this cross-sectional study; the results were stated 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 overweighted, and 18 females (24.0 %) were obese, as represented in Table 1.

Table 2 demonstrated the baseline hormonal levels; accordingly, the mean LH level was 5.64 ± 0.29 mIU/ml, the 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 trigger day 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 as represented in table 2. The baseline antral follicle count was

presented in Table 3; according to the results, the mean antral follicle count was 19.60 ± 0.82. Serum INSL3 levels was (71.31 ± 1.86) and follicular fluids level was (379.8 ± 5.22), as demonstrated in Table 4. A significant negative association concerning 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 with the age of patients, body mass indices, or length of infertility, as shown in Table 5. A positive significant relation between serum INSL3 with serum AMH (r= 0.578 & p= 0.001) and testosterone (r= 0.378 & p= 0.001) was recognized; nevertheless, no significant correlations were identified between both serum and follicular fluids INSL3 with serum LH, FSH, prolactin, TSH, basal E2, and E2 at the day of the trigger as presented in table 6. There were significant positive correlations involving serum INSL3 with antral follicle count, as presented in Table 7.

Table *(1):* Characteristics of the patients in this study

Parameters		**Range**	**Mean ± SE**
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	19 (25.3%)	
	Male causes	18 (24.0 %)	
	Unexplained	13 (17.4 %)	
BMI ranking	Normal weight	12 (16.0%)	
	Overweight	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) (pg/ml)	34.40 – 98.80	71.31 ± 1.86
Follicular fluid insulin like-3 (INSL3) (pg/ml)	260.6 – 515.3	379.8 ± 5.22

SE: Standard error; INSL3: Insulin-like factor 3

4. Discussion

The likelihood of pregnancy in women receiving assisted reproduction is mostly determined by their age (Wang et al., 2018 [12]). The present study showed an insignificant negative association between the patient's age and serum INSL3, in line with earlier research (Gambineri et al. 2007 [13]) that likewise revealed no relationship

between the patient's age and serum INSL3. In contrast with the cross-sectional control population study, which found that INSL3 decreases significantly with aging (Anand-Ivell et al., 2013 [8]) while women undergo ART with normal ORT only displayed a negative correlation with age (Zhu et al., 2020 [11]) .

The body mass index (BMI) was also studied as a variable; obesity has been associated to poorer fertility, yet the mechanisms behind this association are still poorly understood (Marinelli et al., 2022 [14]). Due to its effect on hypothalamic gonadotropin release, which reduces both follicle count and progesterone levels, an increase in weight is a major risk factor for infertility in women (Gautam et al., 2023 [15]). Serum INSL3 was shown to have an insignificant negative relationship with BMI in the current research; (Zhu et al., 2021 [11]) and (Gambineri et al., 2007 [13]) made similar conclusions. Both found that levels of serum INSL3 we're not related to BMI. 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 [16]), indicating a correlation between infertility duration and ICSI success. In this research, serum INSL3 was significantly inversely related to infertility time. In this work, INSL3 levels' relation to other ovarian reserve indicators was studied. Indicators like AMH, E2, FSH, and LH are typically secreted by granulosa cells in follicles. Ovarian reserve is seldom evaluated based on the role of theca cells (Zhu et al., 2021 [11]). In females, INSL3 is produced inside the theca interna layer of antral follicles in the ovary (Xue et al., 2014 [10]). The significant positive association between INSL3 and AMH concentrations in the blood described in this study might confirm the conception that those peptides may reveal follicle

maturation impairment in infertile females. Similar finding in which circulating level of INSL3 is positively associated with AMH in females with normal ovarian reserve and those with premature ovarian insufficiency (Zhu et al., 2021 [11]). The same was for the infertility study by (Anand-Ivell et al., 2013 [8]) study of infertile attending infertility clinics regarding AMH; there was a highly significant correlation with INSL3 since the pool of recruited mid to large antral follicles (represented by circulating INSL3) would be dependent on the ovarian reserve (represented by circulating AMH), this likely explains the correlation observed .

It could be suggested that the increased INSL3 per se may up-regulate AMH production, although, at present, receptors for INSL3 in the granulosa cells have not yet been described (Pelusi et al., 2013 [17]). In contrast, for the prepubertal girl's serum, INSL3 was not correlated with AMH (Hagen et al., 2015 [6])

However, in this analysis, there is no evidence that serum INSL3 was associated with serum FSH. Similarly, INSL3 and FSH in PCOD show no statistically significant relationship (Seyam & Hefzy, 2018 [18]). Despite this, research by (Zhu et al., 2021 [11]) indicated that INSL3 levels were positively correlated with FSH in women. 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 (Ivell et al., 2013 [8]). In the current study, There were significant positive correlations between serum INSL3 with antral follicle count, with similar results reached by (Satchell et al., 2013 [8]) and (Zhu et al., 2021 [11]). Regarding the ovarian follicle number, the same result was reached (Gambineri et al., 2007 [13]). This result may reflect the connection of serum INL3 and total theca cell mass, which increases with a rise in the number of follicles in the ovary.

Furthermore, evidence has shown that the INSL3 level positively correlates with the Aral follicle count (Seyam & Hefzy, 2018 [18]) .

The current study discovered that the INSL3 content in follicular fluid was significantly higher than in serum, which represents direct secretion from theca cells, and that there were no significant correlations with antral follicle count or other marker of ovarian reserve.

There was an insignificant positive correlation between serum INSL3 and follicular fluid INSL3 that can reflect the number of follicles that produce INSL3 from theca cells. This result can be due to the LH spike during ovulation that is induced by the injection of 500-µg recombinant chorionic gonadotropin α (Ovitrelle®) subcutaneously, that negatively affects INSL3 production.

Similar results were shown in the study of (Zue et al., 2021 [11]). INSL3 content in follicular fluid was much higher than in serum and also consistent with the previous measurements in macaques (Henna et al., 2010 [19]) and in cows, the levels of INSL3 in follicular fluid were remarkably high, an approximately sixfold increase over the levels found in serum (Dai et al., 2017 [20]).

6- Conclusion

The study provides compelling proof for the significant effect 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 related to the context of techniques of assisted reproduction 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 of its relevance to female reproductive health is recommended.

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FAuthor Contribution

Maroua K. Lateef performed the study, Muayad S. Abbod and, Mufeda A. Jwad supervised the work .

Conflict of Interest

The authors declare no conflict of interest .

Ethical Clearance

The study was approved by the Ethical Approval Committee.

Financial Disclosure

There is no financial disclosure.

Ethical Clearance

The study was approved by the Ethical Approval Committee

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