



Relation of serum and follicular level of GDF9 to oocyte quality, embryo quality, and pregnancy rate.

Zainab Hassan Hashim ¹, Lubna A. Al-Anbari ¹, Estabraq A. Al-Wasiti ²

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

²College of Medical, University of Al-Nahrain, Baghdad, Iraq

zainab.hassan@ierit.nahrainuniv.edu.iq

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ABSTRACT

It is well known that oocyte quality is essential in the determination of the developmental potential of the fertilized oocytes. The oocyte developmental potential decreases in all species with increasing age.

Use of serum and follicular fluid concentration of GDF9 as biomarkers of oocyte and embryo quality and their relation to pregnancy rate.

Eighty-eight women were included in this study are selected from those undergoing intra-cytoplasmic sperm injection.

Positive pregnancy was achieved by 14 women. The difference in mean serum and follicular GDF9 between pregnant and non-pregnant women was not significant. MI oocyte count was not significantly correlated to serum and follicular GDF9 ($p > 0.05$). MII oocyte count showed a non-significant correlation to serum and follicular GDF9 ($p > 0.05$). Grade 1 embryo count showed a non-significant correlation to serum and follicular GDF9. Grade 2 embryo count showed a non-significant correlation to serum and follicular GDF9. Also, grade 3 embryo count showed a non-significant correlation to serum and follicular GDF9.

The current study revealed that serum and follicular GDF9 could not be used as indicators for oocyte maturity, embryo quality, or pregnancy rate.

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KEYWORDS

GDF9, folliculogenesis, oocyte quality, ICSI

1. Introduction

Infertility is defined as the failure of a couple to get pregnancy after one year of unprotected and regular sexual intercourse. In about 85% of infertility cases, the cause is identifiable (Carson, S. A. et al., [2]). Ovulatory dysfunction, tubal disease, and male factor infertility are the most common causes of infertility. The other 15% of infertility cases have an unknown cause called (unexplained infertility) (Deshpande, P.S. et al. [3]).

Environmental factors and also lifestyle, such as obesity and smoking, can greatly adversely affect fertility (Song, Y., & Li, R., [4]). About 25% of infertility causes are due to ovulatory disorders, and polycystic ovary syndrome forms about 70% of these an-ovulatory disorders. Infertility can also be due to genetic causes, chronic medical diseases, and advanced age (Rakhimovna, K. D., et al., [5]). The key limiting factor in female fertility is the oocyte quality; the quality of the oocyte greatly affects early embryonic survival, also establishment with maintenance of

pregnancy, development of the fetus, and even causes some adult diseases (Adhikari, D. et al., [6]). Growth differentiation factor 9 (GDF9) has a unique feature within the Transforming growth factors- β super-family is that the expression of the protein is essentially restricted to the gametes (oocyte). GDF9 is expressed in the oocyte during folliculogenesis from the earliest stages (Sanfins, A. et al., [7]). It is expressed in high levels by the oocyte throughout folliculogenesis, so it could be regarded as a good indicator for oocyte quality, and measuring them in the serum, which is a rapid, non-invasive, and easy test, could give a great clue to female fertility (Da Broi, M. G. et al., [8]).

2. Patients, Materials and Methods

The prospective study was conducted in the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, from November 2020 to July 2021.

Subjects: The study involved eighty-

eight women who were selected from those who attended the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies.

Inclusion Criteria

- Couples undergoing IVF / ICSI protocols
- Women at age 18 to 47 years old.
- Infertility due to female factors: tubal blockage, unovulatory cycles, and mild-moderate cases of endometriosis that are diagnosed laproscopically.
- Couples with male factor Infertility
- Unexplained infertility

Exclusion Criteria

- All types of congenital anomalies of the reproductive system.
- Uncontrolled systemic and endocrine disorders.
- Women with a BMI of more than 30kg /m²

Methods and Study Design

A total of eighty-eight patients

undergoing the IVF/ICSI cycle were evaluated:

- Taking full obstetrical, medical, and surgical history with an assessment of weight and height to obtain (BMI).
- Examinations of the woman clinically and gynecologically to check for any abnormality.
- For male partners, the seminal fluid analysis was assessed according to WHO 2010.
- Doing an analysis of female hormones (LH, FSH, E2, Prolactin, Testosterone, and TSH) at the second day of the menstrual cycle.
- All women are enrolled to only one type of controlled ovarian hyperstimulation (COH) protocol, which is the Gonadotropin-releasing hormone antagonist protocol.
- Follow up of the patients by doing serial vaginal ultrasound and doing serum level of estradiol (E2) and then accordingly to the result; ovum pick up done.

- Oocyte retrieval is done with the guidance of trans-vaginal ultrasound after ovulation trigger with hCG about (35-36) hrs.
- At ova pick-up day, serum and follicular fluid samples were obtained from each woman for measurement of GDF9. Also, a serum sample was obtained from each post-menopausal women for measurement of GDF9.

The Antagonist Protocol

It involved the stimulation of the ovaries with gonadotropins since the second day of the menstrual cycle, followed by the administration of a GnRH antagonist (Cetrorelix acetate for injection 0.25 mg: Cetrotide®, Merk, Switzerland), using flexible method and given when the size of the largest follicles reach (13-14) mm. The initial dose of FSH was 75 - 300 IU daily, according to the patient's condition. With serial vaginal U/S for checking the number and size of ovarian follicles and for the endometrial thickness (ET), in addition, serum level of Estradiol (E2) was done. The serum level of (E2) Estradiol was

measured at the day of ovulation triggering by (hCG) administration.

Oocyte Grading:

The Oocyte at retrieval, could be either an immature oocyte, and this is called a Germinal vesicle (GV), in which the corona and cumulus cells are tightly packed around the oocyte, with the presence of a round structure inside it, is called the (germinal vesicle), the other immature oocyte is called Metaphase I (MI). MI oocyte is characterized by the absence of a polar body or a germinal vesicle, and it is an intermediate stage between the GV and MII (mature) stages. The mature oocyte is called Metaphase II (MII), which has a polar body.

ICSI Processes

The aspirated follicles were examined at the IVF laboratory in a petri dish immediately. Flushing was done, then kept for 1-2 hrs in the (37°C/ CO₂) incubator. All oocytes after that were subjected to denudation and grading in a Laminar Flow Cabinet. The mature eggs were selected by a specialized pipette. A single sperm was

held and immobilized by a very delicate, sharp, and hollow needle and then pick up of this sperm. After that, the sperm was inserted by the needle carefully through the eggshell into its cytoplasm, and then the eggs were kept in the CO₂ incubator and carefully monitor the result of cell division was by using Nikon ICSI Microscope.

Embryo Quality and Grading

Zygotes after insemination were observed after (18 - 20) hours to check for the presence of (2) pronuclei and after (25 - 29) hours to observe the presence of early cleavage, which is considered a sign of better implantation rates. The presence of 2 pronuclei at day one was regarded as a good prognostic sign. Then, at day two (43 – 45 hours after insemination) and day three (67 - 69 hours after insemination), the embryos were evaluated. Good quality embryos were considered when they were homogeneous, with normal kinetics (4) cells at day two and (7-9) cells at day 3, and containing <10% of cytoplasmic fragments. The embryos, at the third day, were classified as being with or

without compaction, which referred to all embryos that underwent the compaction process; the embryos could be at the beginning of compaction when the fusion of the membrane was visible, in this stage the counting of the number of cells is still possible, and those embryos with full compaction, in those embryos the distinguishing of cell boundaries was not possible.

Embryo Transfer: The dividing embryos were then replaced into the uterine cavity under pelvic ultrasound guidance and by an embryo transfer catheter. Pregnancy test, which was done 14 days after embryo transfer by doing B-hCG

3. Results

The Pregnancy Rate

The pregnancy rate in infertile women enrolled in the current study is shown in Figure (1). Positive pregnancy was achieved by 14 women, accounting for 19.0 %. Total number of patients was 88, and a number of cases were not included in counting pregnancy rate. This included five cases of

empty follicles, four cases of embryonic developmental arrest, six cases of failed fertilization, and one patient refuse embryo transfer. So, the number of cases that were included in counting pregnancy rate was 72 patients.

Characteristics of Infertile Women Enrolled in this Study

Characteristics of infertile women enrolled in this study are shown in Table 1. The mean age of all enrolled women was 32.25 ± 6.41 years, and the mean age of women with positive pregnancy was significantly lower than that of non-pregnant women (29.14 ± 4.54) years versus (32.76 ± 6.55) years, respectively ($p = 0.050$). The mean duration of infertility of all enrolled women was (7.89 ± 3.87) years, and the mean duration of infertility of pregnant women was lower than that of non-pregnant women (6.93 ± 3.08) years versus (8.05 ± 3.98) years; however, the difference did not reach statistical significance ($p = 0.319$). Out of all enrolled women, primary infertility was seen in 65 (74.0 %) women, whereas secondary

infertility was seen in 23 (26.0 %) women, and there was no significant difference in the frequency distribution of women according to the type of infertility with respect to pregnancy outcome ($p = 1.000$). The mean BMI for pregnant women was (26.71 ± 2.60), and for non-pregnant women (26.72 ± 3.01), there was no significant difference in the frequency distribution of women according to BMI with respect to pregnancy ($p = 0.958$) (Table 1).

Serum and Follicular Levels of GDF9 and their Relation to Pregnancy

At the day of ova pick up, growth differentiation factor 9 (*GDF9*) serum and follicular levels are shown in Table 2. There was no significant difference in mean serum GDF9 (192.00 ± 30.98) versus (202.34 ± 36.55) between pregnant and non-pregnant women. Also, there was no significant difference in mean follicular fluid GDF9 (146.43 ± 26.96) versus (155.36 ± 30.53) between pregnant and non-pregnant women (Table 2).

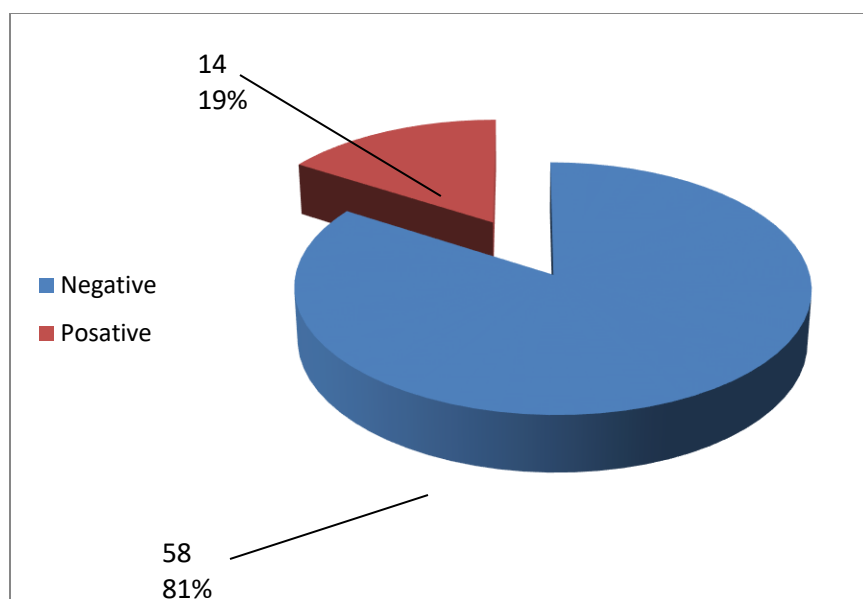


Figure 1: Pie chart showing the pregnancy rate of women undergoing ICSI

The Correlations of Serum and Follicular Fluid GDF9 to Oocyte Maturity

The correlations of serum and follicular fluid growth differentiation factor 9 (GDF9) that were measured at the day of ova pick up to oocyte maturity are shown in Table 3. MI oocyte count was not significantly correlated to serum (0.090) and follicular (-0.141) GDF9 ($p > 0.05$). MII oocyte count showed but showed a non-significant correlation to serum (0.009) and follicular (-0.103) GDF9 ($p > 0.05$) (Table 3).

The Correlations of Serum and Follicular Fluid GDF9 to Embryo Grading

The correlations of serum and follicular fluid growth differentiation factor 9 (GDF9) to embryo grading are shown in Table 4. Grade 1 embryo count showed non-significant correlation to serum (0.002) and follicular (0.004) GDF9. Grade 2 embryo count showed a non-significant correlation to serum (0.097) and follicular (-0.080) GDF9 ($p > 0.05$). Also, grade 3 embryo count showed a non-significant correlation to serum (0.092) and follicular (0.032) GDF9 ($p > 0.05$) (Table 4).

Table 1: Characteristics of infertile women enrolled in this study

Characteristic	Total <i>n</i> = 72	Positive pregnancy <i>n</i> = 14	Negative pregnancy <i>n</i> = 58	<i>P</i>
Age (years)				
Mean ±SD	32.25 ±6.41	29.14 ±4.54	32.76 ±6.55	0.050 I S
Range	20 -47	23 -40	20 -47	
Duration of Infertility (years)				
Mean ±SD	7.89 ±3.87	6.93 ±3.08	8.05 ±3.98	0.319 I NS
Range	1 -17	2 -12	1 -17	
Type of infertility				
Primary, <i>n</i> (%)	65 (74.0 %)	10 (71.4 %)	55 (74 %)	1.00Y NS
Secondary, <i>n</i> (%)	23 (26.0 %)	4 (28.6 %)	19 (26 %)	
BMI (Kg/m ²)				
Mean ± SD	26.72±2.81	26.71±2.60	26.72±3.01	0.958 I NS
Range	20.44 -30.75	21.46 -30.75	20.44 -30.70	

n: number of cases; **SD**: standard deviation; **I**: independent samples *t*-test; **Y**: Yates correction for continuity; **NS**: not significant at *p* > 0.05; **S**: significant at *p* ≤ 0.05

Table 2: GDF9 serum and follicular levels and their relation to pregnancy

Characteristic	Total <i>n</i> = 72	Positive pregnancy <i>n</i> = 14	Negative pregnancy <i>n</i> = 58	<i>P</i>
Serum GDF9				
Mean ±SD	200.89 ±35.86	192.00 ±30.98	202.34 ±36.55	0.320 I NS
Range	86 -308	151 -248	86 -308	
Follicular fluid GDF9				
Mean ±SD	154.11 ±30.09	146.43 ±26.96	155.36 ±30.53	0.305 I NS
Range	71 -251	108 -192	71 -251	

n: number of cases; **SD**: standard deviation; **I**: independent samples *t*-test; **NS**: not significant at $p > 0.05$;

Table 3: Correlations of serum and follicular fluid BMP15 and GDF9 to oocyte maturity

Characteristic	Correlation Index	Serum GDF9	Follicular fluid GDF9
Immature metaphase I (MI) oocytes	<i>R</i>	0.090	-0.141
	<i>P</i>	0.372	0.162
Mature metaphase II (MII) oocytes	<i>R</i>	0.009	-0.103
	<i>P</i>	0.932	0.309

Table 4: The correlations of serum and follicular fluid GDF9 to embryo grading

Characteristic	Correlation Index	Serum GDF9	Follicular fluid GDF9
Grade 1 embryo	<i>R</i>	0.002	0.004
	<i>P</i>	0.986	0.966
Grade 2 embryo	<i>R</i>	0.097	-0.080
	<i>P</i>	0.336	0.428
Grade 3 embryo	<i>R</i>	0.092	0.032
	<i>P</i>	0.162	0.091

4. Discussion

The main aim of this study was to find an easy, fast, not expensive, and available outpatient test to be an indicator for female fertility.

The serum biomarkers AMH, FSH, and E2 are used to predict female reproductive potential. These hormones are used to estimate growing follicles' number in the ovary and to estimate and predict the response of ovaries to stimulation by gonadotropin. These biomarkers provide only an indirect evaluation of oocyte function and yield no information about the quality of the oocyte because they are not derived from the oocyte itself (Victoria, M. [9]). GDF9 and BMP15 are known to be secreted only by the oocyte, essential for the process of folliculogenesis, quality of the oocyte, and female fertility, so these factors could be regarded as oocyte function biomarkers (Dayanir D. et al., 2019 [10]).

The Pregnancy Rate

Positive pregnancy was achieved by 14 women, accounting for 19.0 %. The rate was low when compared with other studies, like

a study done by De Geyter et al. (De Geyter et al. [11]) that found that the pregnancy rate was 28%, also other study done by Jassim WH. Found the pregnancy rate to be 25.4% (Jassim, W. H. et al. [12]). The pregnancy rate was low because there 4 cases of testicular biopsy and 2 cases of moderate endometriosis, and also there were 14 cases with ages above 40 years were included in this study. Furthermore, the SARS-CoV-2 (Covid-19) pandemic also might be one of the causes of the decrease in pregnancy rate. This is supported by study result done by Maya, W. D. C, et al., that found that germ cell destruction and testicular damage was clearly observed in patient with Covid 19 (Maya, W. D. C., et al. [13]) and other study showed that the testes that infected with SARS-CoV-2- showed extensive peritubular fibrosis, vascular congestion with extensive destruction of germ cell (Duarte-Neto, A. N., et al. [14]). Furthermore, SARS-CoV-2 could cause ovarian tissue damage and a decline in the function of the ovary and oocyte quality, causing female

infertility and may cause miscarriage (Duarte-Neto, A. N. et al. ^[14]).

Serum and Follicular Fluid Levels of GDF9 and their Relation to Pregnancy

There was no significant difference in mean serum GDF9 between pregnant and non-pregnant women. Also, there was no significant difference in mean follicular fluid GDF9 between pregnant and non-pregnant women, as shown in Table 2.

But, a study done by Li et al. on gene expression found that GDF9 mRNA expression levels were closely related to the outcomes of pregnancy (Li et al. ^[15]). Many factors might affect pregnancy rate other than oocyte quality, like malefactors, for example, abnormality in DNA as in sperm retrieved by testicular biopsy, also due to bad endometrial receptivity and psychological problems.

Correlations of Serum and Follicular Fluid GDF9 to Oocyte Maturity

The current study showed that MI oocyte count was not significantly correlated to serum or follicular fluid GDF9 ($p > 0.05$) Table 3. Furthermore, MII oocyte count

showed no significant correlation to serum or follicular GDF9. This result was not.

Agreed to a study done by Sanfins A et al. et al., which found that in the follicular fluid, higher levels of GDF-9 were significantly correlated with nuclear maturation of the oocyte (Sanfins, A, ^[7]).

The study result was not agreed with to study result done by Li et al. on gene expression, who found that the mRNA expression levels of GDF9 were closely related to the maturation of the oocyte, fertilization, and outcomes of the pregnancy (Li, Y., et al. ^[16]). Furthermore, a study done by (R. Romaguera stated that beneficial synergistic effects are exerted by OSFs on the maturation of the nucleus and cytoplasm, rapid energy utilization, and oxidative stress management (Chandra, V., & Sharma, G. T. ^[17]).

The oocyte-secreted factors, BMP15 and GDF9, are secreted by the oocyte in a primary follicle; these factors organize the granulosa and theca cells that surround the oocyte into the oocyte–cumulus–follicle complex. The granulosa at this time secretes

AMH, that has effects on the oocyte. Throughout the development of the follicle, this autocrine–paracrine dialogue between

the somatic cells and the oocyte proceed and is regarded as essential for establishing the fertilization potential and oocyte developmental competency (Michael, J. D. et al. ^[18]). The beneficial effect of OSFs were proved by Singh, A., who revealed that the spindle visualization rate is higher when the IVM medium was treated with GDF-9 (Singh, V. P., et al., ^[19]).

The Correlations of Serum and Follicular Fluid GDF9 to Embryo Grading

The correlations of serum and follicular fluid growth differentiation factor 9 (GDF9) to embryo grading are shown in Table 4. Grade 1 embryo count and grade 2 embryo count showed non- significant correlation to serum and follicular GDF9.

This result was not agreed with to study result done by Daneshjou, D. et al., that found that there is a positive correlation between the expression level of GDF9 and mRNA with the fertilization rate and grade I embryos (Daneshjou, D. et al., ^[20]). Also, other study

found that higher follicular fluid GDF-9 levels was correlated to embryo quality significantly (Sanfins, A. et al., ^[7]), also other

Study done by Kahraman, S et al. (2018) found that gene expression of GDF9 correlated with blastocyst quality (Kahraman, S et al., ^[21]).

Also, there were studies that support the role of GDF9 in enhancing embryo quality, like a study done by Li, J., Li C, et al., that found that the concentration of GDF9 in a culture medium is related to embryo quality and viability and had the potential to be regarded as a non-invasive biomarker for selection of good quality embryos (Li, J. Li C, et al., ^[22]), also Mohsenzadeh, M., et al., suggested that the GDF9 use in the IVM culture media enhance the rates of both formations of the embryos and blastocyst viability significantly (Mohsenzadeh, M. et al., ^[23]).

5. Conclusions

The GDF9 that is measured by specific Elisa Kit cannot be used as an indicator of oocyte quality, embryo quality, and pregnancy rate.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Clearance

The study was approved by the Ethical Approval Committee.

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