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Serum Follicular Fluid Growth Differentiation Factor 9 (GDF-9) and Bone Morphogenic Protein 15 (BMP-15) as Markers of Ovarian Reserve

Muhjah Falah Hassan[1,*] and Wasan Adnan Abdulhameed[2]

[1] *Department of Anatomy, Embryology and Histology, College of Medicine, Karbala University, Karbala, Iraq.*
[2] *Department of Embryology, High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad, Iraq.*

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ABSTRACT

Background: Growth differentiation factor (GDF-9) and bone morphogenic protein (BMP-15) (GDF-9 co-factor) are proteins that are released by oocytes and control key stages of follicular development and growth.
Objectives: To determine the ability of serum follicular fluid GDF-9 and BMP-15 in predicting ovarian reserve represented by their correlations with female age, traditional markers; antral follicle count (AFC) and anti-mullerian hormone (AMH).
Materials and methods: In this prospective cohort study, 114 infertile females from the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Baghdad, Iraq, were included. Their age was 18–43 years old and their body mass index (BMI) was 19–30 Kg/m^2. All were assessed on the second day of the menstrual cycle. Serum was taken to measure GDF-9 and BMP-15 using a special Kit by ELISA. Following oocyte retrieval, follicular fluid was collected for measuring GDF-9 and BMP-15.
Results: The serum and follicular fluid GDF-9 and BMP-15 showed a significant correlation with the age of the patient, while there was no such association with AMH. Significant positive correlations were found between serum and follicular fluid GDF-9 and AFC, while only follicular fluid GDF-9 exhibited a significant positive correlation with mature II (MII) oocytes number. While, significant positive correlations were found between follicular fluid BMP-15 and the total number of oocytes.
Conclusion: Serum follicular fluid GDF-9 and BMP-15 can be good predictors of ovarian reserve due to their significant negative correlations with age (oocyte quality) and significant positive correlations with AFC (quantity). Follicular fluid GDF-9, serum and follicular fluid BMP-15 can predict oocyte maturity.
Keywords: Ovarian Reserve Tests; Anti-Mullerian Hormone; Antral Follicle Count; Growth Differentiation Factor 9; Bone Morphogenic Protein 15.

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INTRODUCTION

Ovarian reserve is a term that represents the total number of follicles (primordial and growing follicles) within the ovarian follicular pool whose oocytes are arrested at the prophase stage of the 1st division of meiosis (primary oocytes), A clear or universal definition of ovarian reserve is still not present [1].

In females, folliculogenesis usually starts before birth. The total number of primordial follicles is about 2 million at the time of birth for a female fetus. There is a process of follicular depletion, so they decrease to approximately 400,000 at puberty [2]. Following puberty, follicular depletion continued, reaching about 40,000 at ages 25-30 year. Female ageing above 38 years tends to negatively affect not only the quantity but also the quality of the oocytes [3].

* Corresponding author: E-mail: doctor89muhjah@gmail.com

In order to assess these events, ovarian reserve tests were developed as a screening tests for the detection of diminished ovarian reserve (DOR) in an asymptomatic female who wishes to have a child as early as possible [4].

Anti-mullerian hormone (AMH) is secreted from granulosa cells of small antral follicles of a diameter of 4-6 mm, and when the size of antral follicles gets larger (up to 8 mm), its release declines until it is undetectable in pre-ovulatory follicles [5]. AMH regulates follicular progress from the primordial pool and controls the rate of follicular depletion [6]. AMH serum concentration slightly fluctuates during the menstrual cycle and is more likely to be stable within the cycle and from cycle to cycle. Serum AMH started to be used as an ovarian reserve test in 2002, as the concentration correlated positively with the number of antral follicles within the pool [7]. Until a few years ago, it was most commonly used in clinical practice [8]. Regardless of their ability to predict oocytes quantity, the predictive values of their quality are subpar and as too much research has shown, female age over 35 year decreases the quantity and adversely affects the quality of oocytes by affecting the meiotic spindles. Thus, female age is the best predictor of both oocyte quality and quantity [9].

Antral follicle count (AFC) is defined as the sum of the number of small antral follicles in both female ovaries during the 2nd to 4th days of the menstrual cycle. AFC is calculated using trans-vaginal ultrasound (TVUS) [10]. The follicular diameter that is used to define AFC is 2-8 mm [11]. Thus, reproductive specialists introduced it as an ovarian reserve test and started to be use it more commonly following the introduction of assisted reproduction [12]. Studies have been suggested that a significant positive correlation was found between AMH and AFC, and both have been recommended as ovarian reserve tests by American Society of Reproductive Medicine (ASRM) and European Society of Human Reproduction and Embryology (ESHRE). Together, both are negatively correlated with female age [13] and [14]. However, some confusion still exist about their abilities in determining oocyte quality [15]. Therefore, finding markers together with female age that have the ability to express oocyte quality is highly under researched.

Growth differentiation factor 9 (GDF-9) and bone morphogenic protein 15 (BMP-15) are oocyte-specific secreted growth factors belonging to the transforming growth factor (TGF-β) superfamily [16]. They seem to present throughout folliculogenesis and play an important role in folliculogenesis [17]. Both could be detected in the serum and follicular fluid and their levels were not affected by the day of the menstrual cycle and decreased with female age, especially above 40 years and in females with premature ovarian insufficiency [18].

BMP-15 and GDF-9 measurements in serum could have diagnostic usefulness in predicting female fertility potential and oocyte quality, as both are specifically secreted from primordial and primary follicles and their levels can give an idea about the number of stored follicles within the follicular pool [19]. Hence, this study aims to assess the ability of serum, follicular fluid, GDF-9, and BMP-15 to predict ovarian reserve and whether they can be used as ovarian reserve tests either alone or together with AMH and AFC.

MATERIALS AND METHODS

The study was conducted at the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad, Iraq throughout the period from September 2021 to January 2023. It is a prospective cohort, in which 114 sub-fertile couples were involved and all of them were subjected in the intracytoplasmic sperm injection (ICSI) program. Sample size was calculated according to the equation n= Z^2 p (1- p)/d^2 (n = sample size, Z = differential coefficient = 1.96, d = estimated error < 0.05 and p = prevalence of the problem (12%). n= (3.84×0.12×0.88)/0.0025. According to this equation, the sample size was equal to 162. The Local Medical Ethical Committee at Al-Nahrain University in Baghdad, Iraq where the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies were located approved the study. Informed consent was obtained from all infertile females to be included in the study.

The age of female partners ranged from 18-43 years old and their body mass index (BMI) ranged from 19-30 Kg/m^2. They visited the female Fertility Consultation Clinic at High Institute of Infertility Diagnosis and Assisted Reproductive Technologies seeking fertility treatment. Females were evaluated by medical and gynecological history, examination, anthropometric measures (weight, height, and BMI) and hormonal analysis (LH, FSH, and AMH), and TVUS for the assessment of ovarian reserve in the form of AFC which is the number of small follicles of 2-6 mm in diameter in both ovaries, ovarian pathology [ovarian cyst and polycystic ovarian (PCO) morphology], adnexal mass and endometrial thickness (ET) and endometrial pathology. These investigations were done on the second day of the menstrual cycle.

A 6 ml of venous blood was drawn from the ante-cubital fossa for assessment of GDF-9 and BMP-15 in the serum by ELISA using a GDF-9 and BMP-15 Kit (Elabscience, USA). Male partners were assessed by seminal fluid analysis according to WHO, 2010 [20].

Controlled ovarian stimulation was performed by the administration of different types of gonadptropins; human menopausal gonadotropin (HMG) in the form of in vitro fertilization-Menotropin (IVF-M),LG Chem Ltd, Korea 75-150 IU (75 IU FSH+75 IU LH) or recombinant FSH (r-FSH) in the form of Gonal–F, Merck KGaA Dermstadt, Germany 75-300 IU. When follicular recruitments is started and a good number of follicles reached a size of 14 mm, pituitary down-regulation was started by using a gonadotropin releasing hormone (GnRH) antagonist; Cetrotide Merck KGaA Dermstadt, Germany 0.25 mg 1×1 S.C (flexible protocol), ovulation trigger was done by the administration of 500 microgram of recombinant human chorionic gonadotropin (r-hCG); Ovitrelle, Merck Global, Germany S.C or GnRH agonist (Decapeptyl Ferring Pharmaceuticals, Switzerland 0.2 IU) S.C in hyper responder patients who are at risk of ovarian hyper stimulation syndrome (OHSS) when the leading follicles diameter ranged between 18–20 mm with an adequate E2 concentration in the serum > 1500 p/ml.

Inclusion criteria: females aged 18-43 years old, BMI 19-30 kg/m^2, a GnRH antagonist was used for pituitary down regulation, and those who accepted to participate in the study. While exclusion criteria: females aged less than 18 and more than 43 years old, BMI > 30 kg/m^2, pituitary down regulation with GnRH agonist, females with diminished ovarian reserve (hypogonadotropic hypogonadism, severe endometriosis, previous ovarian surgery, radiation or chemotherapy, premature ovarian failure), females who take oral contraceptives or GnRH agonist for 1 month before the start of ICSI program (as both affect ovarian reserve markers especially AMH) and those whom their male partners had severe oligo-asthenoterato-zo-spermia, azoospermia (frozen sperms obtained from

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the testes surgically) as poor sperm quality affects embryo quality following ICSI. Additionally, any participant who declined to participate in the current study was excluded.

Oocytes collection was performed under general anesthesia (GA) by follicular puncture guided by TVUS for 34-36 hours following the ovulation trigger. A 1 ml of follicular fluid was collected for measuring GDF-9 and BMP-15 using the same serum kit by ELISA. Microscopic assessment of oocytes maturity and quality was done by the embryologist according to the oocyte scoring system [21]. Luteal phase support using vaginal progesterone suppositories (Cyclogest 200 L.D. Collins & Co. Ltd. microgram × 3 per day and injectable progesterone intramuscularly (Primolute Depot Bayer AG Germany 250 micrograms every three days) was done starting from the evening of the day of oocyte pick up until the day of a positive chemical pregnancy test.

Nine females developed moderate OHSS (7.89%); they were included in the study; however, the resulting embryos of those females were frozen (freeze all) and fresh embryo transfer was cancelled.

Data were presented in an Excel, 2010 sheet and analyzed using the Statistical Package for the Social Sciences (SPSS), version 26. The number and percentage were used to express qualitative variables, while, quantitative variables were first evaluated for normality distribution using the Kolmogorov-Smirnov test. Then, normally distributed numeric variables were expressed as the mean and standard deviation. The association between any two categorical variables was evaluated by the Chi-square test. The difference in mean of numeric variables among more than two groups provided that these numeric variables were normally distributed, was evaluated by One way analysis of variance (ANOVA). In addition, ANOVA was followed by pos hoc least significant difference (LSD) test to evaluate individual differences in mean values between any two groups. The pearson correlation test was used to study bivariate correlations. The level of significance was considered at a P-value of less than 0.05.

RESULTS

The demographic characteristics of the patients enrolled in this study are shown in Table 1. The mean age of females was 32.37 ± 6.0 years, and the mean BMI was 23.47 ± 3.19 kg/m^2. The mean serum and follicular fluid GDF-9 were 124.14 ± 59.06 and 124.29 ± 54.51 pg/ml, respectively. While the mean serum and follicular fluid BMP-15 were 130.21 ± 53.73 and 132.74 ± 54.14 pg/ml, respectively.

The causes of infertility are illustrated in Table 2. The most common cause is unexplained sub-fertility (a couple fails to conceive after one year of regular, unprotected sexual intercourse when tests of ovarian function, tubal patency, and semen analysis are completely normal). While only four females are due to tubal obstruction.

Correlations of serum and follicular fluid GDF-9, and BMP-15 to demographic characteristics are shown in Table 3. Serum and follicular fluid GDF-9 and BMP-15 showed a significant (P-value < 0.05) and negative (r value is negative) correlation to age, but they were not significantly correlated to other demographic characteristic (P-value > 0.05).

Correlations of serum and follicular fluid GDF-9 and BMP-15 to serum hormonal levels are shown in Table 4. There was no significant correlation (P-value > 0.05).

Correlations of serum, follicular fluid GDF-9 and BMP-15 to oocyte characteristics are shown in Table 5. Serum GDF-9 showed a significant positive correlation to AFC. Follicular fluid GDF-9 showed a significant positive correlation to AFC and to MII. Serum BMP-15 showed a significant positive correlation to AFC and to MII. Follicular fluid BMP-15 showed a significant positive correlation to AFC, total oocyte count, and MII count.

Correlations of serum and follicular fluid GDF-9 and BMP-15 to stimulation characteristics are shown in Table 6. Serum and follicular fluid GDF-9 and BMP-15 were significantly and negatively correlated to the initial total but not total doses.

Table 1. The clinical variables of the females enrolled in the study.

Characteristic	N = 114, Mean ± SD
Age (years)	32.37 ± 6.0
Body mass index (kg/m^2)	23.47 ± 3.19
Duration of infertility (years)	7.68 ± 4.26
Type of infertility	
Primary, N (%)	90 (78.94%)
Secondary, N (%)	24 (21.05%)
AMH (pg/ml)	2.00 ± 1.85
FSH (miu/ml)	5.37 ± 1.98
LH (miu/ml)	4.61 ± 2.06
Serum GDF-9 (pg/ml)	124.14 ± 59.06
Follicular fluid GDF-9 (pg/ml)	124.29 ± 54.51
Serum BMP-15 (pg/ml)	130.21 ± 53.73
Follicular fluid BMP-15 (pg/ml)	132.74 ± 54.14

Table 2. Causes of infertility in the studied females.

Variables	Number	Percentage
Advanced age	14	12.28%
Male factor	19	16.66%
Male factor +advanced age	20	17.54%
Polycystic ovary syndrome	25	21.92%
Tubal factor	4	3.50%
Unexplained	32	28.07%
Total number	114	100%

DISCUSSION

Due to their potential to predict oocyte quality and quantity and their significant positive correlations to AFC, serum follicular fluid oocyte-secreted GDF-9 and BMP-15 can be used as ovarian reserve markers. The levels of both factors in the follicular fluid can be used to determine the quality (maturity) of the oocytes retrieved following stimulation. The novelty of our study is that previous researches studied serum concentrations of BMP15 and GDF9 during the in vitro fertilization (IVF) in women with reproductive pathologies and in women with PCOS and their relations to the number of produced oocytes [18, 19]. Besides, others studied the correlations of serum, follicular fluid GDF-9 and BMP-15 concentration and ICSI outcome represented by oocytes, embryos number, their qualities and chemical pregnancy rate [22]. However, no one studied their ability to predict ovarian reserve by studying their correlations with the ovarian reserve markers AFC and AMH.

The current study showed that serum follicular fluid GDF-9

Table 3. Correlations of serum and follicular fluid GDF-9 and BMP-15 to demographic characteristics*.

Characteristic	GDF-9 S		GDF-9 F		MP-15 S		BMP-15 F	
	r	p	r	p	r	p	r	p
Age (years)	-0.202	0.032	-0.211	0.024*	-0.194	0.038*	-0.197	0.035*
Body mass index (kg/m^2)	-0.163	0.083	-0.168	0.074	-0.164	0.081	-0.168	0.073
Type of infertility	-0.125	0.187	-0.134	0.155	-0.126	0.181	-0.128	0.176
Duration of infertility (years)	-0.126	0.182	-0.130	0.169	-0.114	0.229	-0.108	0.251
Cause of infertility	0.155	0.099	0.174	0.064	0.150	0.111	0.156	0.097

* significant at P-value < 0.05

Table 4. Correlations of serum and follicular fluid GDF-9 and BMP-15 to serum hormonal levels.

Characteristic	GDF-9 S		GDF-9 F		MP-15 S		BMP-15 F	
	r	p	r	p	r	p	r	p
AMH (pg/ml)	-0.124	0.190	-0.125	0.187	-0.125	0.187	-0.122	0.197
FSH (miu/ml)	0.160	0.088	0.161	0.087	0.173	0.065	0.174	0.065
LH (miu/ml)	-0.005	0.959	0.004	0.965	-0.006	0.951	0.007	0.944

Table 5. Correlations of serum and follicular fluid GDF-9 and BMP-15 to oocyte characteristics.*

Characteristic	GDF-9 S		GDF-9 F		MP-15 S		BMP-15 F	
	r	p	r	p	r	p	r	p
Antral follicle count	0.280	0.003**	0.290	0.002**	0.289	0.002**	0.293	0.002**
Total oocytes	0.169	0.072	0.176	0.061	0.175	0.062	0.185	0.048*
MII (mature) oocytes	0.182	0.053	0.189	0.044*	0.188	0.045*	0.196	0.036*

* significant at P-value < 0.05; ** significant at P-value ≤ 0.01.

Table 6. Correlations of serum and follicular fluid GDF-9 and BMP-15 to stimulation characteristics.*

Characteristic	GDF-9 S		GDF-9 F		MP-15 S		BMP-15 F	
	r	p	r	p	r	p	r	p
Initial total dose (iu)	-0.248	0.008**	-0.264	0.005**	-0.251	0.007**	-0.255	0.006**
Total dose (iu)	-0.070	0.459	-0.082	0.385	-0.076	0.424	-0.082	0.385

* significant at P-value < 0.05; ** significant at P-value ≤ 0.01.

and BMP-15 exhibited significant negative correlations to age, with no significant correlations to other demographic characteristics. A study by Ribeiro et al. demonstrated similar results that GDF-9 and BMP-15 concentrations in the follicular fluid significantly decreased with increasing female age [23]. Together, the expression of these oocyte secreted factors are decreased with advancing female age due to a decrease in the quality of the oocytes [24]. A study by Riepsamen et al. showed that serum and follicular fluid BMP-15 but not GDF-9 decreased with the age of the female [19]. Another study showed a significant positive correlation between GDF-9 and female age [25]. No correlation between BMP-15 and age in pre-menapousal women had also been exhibited [22].

Regarding the cycle day 2 hormones, the study showed that there were no significant correlations between serum and follicular fluid GDF-9 and BMP-15 and basal cycle day 2 hormones; AMH, LH and FSH. A study by Riepsamen et al. showed similar results, no correlation between serum GDF-9, BMP-15 and cycle day 2 AMH, FSH and LH [18, 19]. While, a recent study showed that serum level of BMP-15 exhibited no correlation with AMH and FSH and a significant positive correlation with LH [26]. Our result could be explained by the fact that both GDF-9 and BMP-15 are oocyte-specific secreted factors, released from the oocyte of the primordial and primary follicle stages, while AMH is released from the granulosa cells of pre- antral and antral follicle stage, and FSH and LH are released from the anterior pituitary [27, 28]. Regarding oocyte characteristics, the study showed that follicular fluid GDF-9 showed significant positive correlations to AFC and to MII oocyte count. Serum BMP-15 showed significant positive correlations with AFC and MII oocyte counts. Follicular fluid BMP-15 showed significant positive correlations to AFC, total oocyte count, and MII oocyte count. These results shared some similarities with the results of several

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studies, which exhibited that the serum and follicular fluid GDF-9 only, but not BMP-15 had significant positive correlation with the total number of oocytes within the follicular pool, AFC, and even ovarian volume [18, 19].

Regarding oocyte maturity, several studies were in agreement with the current study results, suggesting a significant positive correlation between GDF-9 and BMP-15 in the serum and follicular fluid with the number of mature oocytes and oocyte maturation rates [29, 30]. A study by Pantos et al. showed that the serum BMP-15 was significantly correlated with a total number of collected oocytes, MII oocytes, oocyte fertilization rate, and even embryo cleavage rate [31]. While, a study by Sumapradja et al. showed there are no correlations between the serum follicular fluid GDF-9 and the follicular fluid BMP-15 and the oocyte maturation and fertilization rates [28]. Regarding the dose of consumed gonadotropins, serum and follicular fluid GDF-9 and BMP-15 in the current study were significantly and negatively correlated to the initial total but not total doses. No study was found regarding the correlation between serum, follicular fluid GDF-9 and BMP-15 and the total and starting doses of gonadotropin during IVF. The results of the current study could be explained by the fact that when the serum and follicular fluid GDF-9 and BMP-15 increased, the initial dose of gonadotropin decreased. As shown previously, that the serum and follicular fluid of both factors were significantly correlated with AFC. This means that when the levels of both factors are high, AFC is high, and a high AFC usually needs a lower initial or starting dose of gonadotropins, therefore, this topic requires further research [32].

The single center and small sample size were the limitations of the current study.

CONCLUSION

It is important to discover a test of ovarian reserve that give an idea not about the quantity but the quality of oocytes. Serum GDF-9 and BMP-15 can be used for this purpose due to their significant negative correlations with age (oocyte quality) and significant positive correlations with AFC (quantity). While follicular fluid GDF-9, and serum and follicular fluid BMP-15 can predict oocyte maturity. However, we recommend further researches about the exact ability of GDF-9 and BMP-15 to predict oocyte quality depending on more invasive methods (genetic and chromosomal studies) for determining oocyte quality.

ETHICAL DECLARATIONS

Acknoweldgements

None.

Ethics Approval and Consent to Participate

The study was approved by the Ethical Committee of the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad, Iraq, as partial fulfillment of a degree of Doctor of Philosophy in infertility and clinical reproduction, November-17-2021, no.505. Informed consent was obtained from every participant.

Consent for Publication

Not applicable (no individual personal data included).

Availability of Data and Material

Data generated during this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contributions

All stated authors contributed significantly, directly, and intellectually to the work and consented it to be published.

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