

Effect of Age on Serum and Follicular Fluid BMP15 and GDF9, The Oocyte Secreted Factors

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Abstract

Background: In female fertility, age is a most important factor. With increasing age, there will be decrease in both oocyte quantity and quality, and ovulatory dysfunction, is more common in younger female (1).

Aim of study: Evaluation of the effect of age on serum and follicular fluid BMP15 and GDF9, the oocyte secreted factors.

Subjects, Materials and Methods: One hundred and seventy six women were included in this study, eighty eight women (Group 1) are selected from those undergoing intra-cytoplasmic sperm injection, also eighty eight postmenopausal women (group 2).

Results: Age was not significantly correlated to serum or follicular fluid BMP15 and serum and follicular GDF9 ($p > 0.05$) in premenopausal group, but decreased significantly with age in postmenopausal group.

Conclusions: Very low level of serum BMP15 in women could be used as indicator of empty follicles and menopause.

Keywords: BMP15, GDF9, Folliculogenesis, Oocyte Quality, Oocyte Secreted Factors, IVF, ICSI.

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INTRODUCTION

Infertility: is defined as the inability, of a couple to have pregnancy after a period of one year, in those women under 35 years of age or after 6 months in those women above 35 years of age, in spite of regular (3 to 4 times / week), adequate and unprotected sexual intercourse (2). Fertility decreases with age, the female has her peak fertility between the ages of 18- 24 years, while, it begins to decrease after the age of (27) years and drops at a faster rate after the age of 35 years (1). Regarding ovarian reserve, typically the woman has (12 %) of her ovarian reserve at the age (30) and has only (3%) remain from her reserve at age 40 (3). Age is a limiting factor in female infertility and dysfunction of the ovulation occurred more commonly in younger than older female (1).

The key limiting factor in female fertility is the oocyte quality, and till now there is poor understanding of what factors that determine the oocyte quality or the mechanisms that governing it (4). The quality of oocyte greatly affects early embryonic survival, also establishment with

maintenance of pregnancy, development of the fetus, and even causes some adult diseases (5). Bone morphogenetic protein 15 (BMP15) and Growth differentiation factor 9(GDF9), have a unique feature, within the Transforming growth factors-b super-family is that the expression of the protein is essentially restricted to the gametes (oocyte). BMP15 and GDF9 are expressed in the oocyte during folliculogenesis, from the earliest stages (6). They are expressed in high levels by the oocyte throughout folliculogenesis, so they are could be regarded a good indicator for oocyte quality, and measuring them in the serum which is rapid, non- invasive and easy test could give a great clue to female fertility (7).

SUBJECTS, MATERIALS AND METHODS

A prospective study was conducted in the (High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University), from November 2020 to July 2021. One hundred and seventy six women were included in this study.

Subjects: The study involved eighty eight women who were

selected from those attended the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies (Group one), and eighty eight postmenopausal women that selected randomly from the general population (Group two).

Group one

Inclusion Criteria

- All couples undergoing IVF / ICSI protocols
- Women at any age from 18 to 47 years old.
- Infertility due to female factors: tubal blockage, un-ovulatory cycles, and mild to moderate cases of endometriosis that 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 BMI more than 30kg /m²

Group Two: Include any women in post- menopausal period (i.e at least one year amenorrhea) and their age more than 50 years.

METHODS AND STUDY DESIGN

- A total of eighty eight women enrolled in (IVF/ICSI) cycle were evaluated: Taking full obstetrical, medical, surgical history with 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 analysis of female hormones (LH, FSH, E2, Prolactin, Testosterone and TSH) at the second day of the menstrual cycle.
- All women for group one are enrolled to only one type of controlled ovarian hyperstimulation (COH) protocols which is 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 done with guidance of trans-vaginal ultrasound after ovulation trigger with HCG about (35-36) hrs.
- At the day of ova pick up, serum and follicular fluid samples were obtained from each woman

for measurement of GDF9 and BMP15, Also serum sample was obtained from each post - menopausal women for measurement of GDF9 and BMP15.

The Antagonist Protocol

It involved ovarian stimulation with gonadotropins since the second day of the menstrual cycle followed by the administration of a (GnRH) antagonist (Cetrorelix acetate) 0.25 mg by subcutaneous injection; 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 patient 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 day of ovulation triggering by (HCG) administration.

ICSI Processes

In the laboratory of IVF, examination of the aspirated follicles was done in a petri dish immediately. Flushing was done then kept 1-2hrs in (37°C/ CO₂) incubator. After that, denudation and grading of all oocytes was done in a Laminar Flow Cabinet. The mature eggs were selected by a specialized pipette, and by a very delicate, sharp and hollow needle which is used to held, immobilize and then pick up a single sperm. After that, the sperm was inserted by the needle carefully through egg shell into its cytoplasm, then the eggs were kept in the CO₂ incubator and carefully monitor the result of cell division, by using Nikon ICSI Microscope.

Embryo Transfer: The dividing embryos were then replaced gently into the uterine cavity with aid of vaginal ultrasound guidance and by specialized embryo transfer catheter.

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 patient was 88, 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 patient.

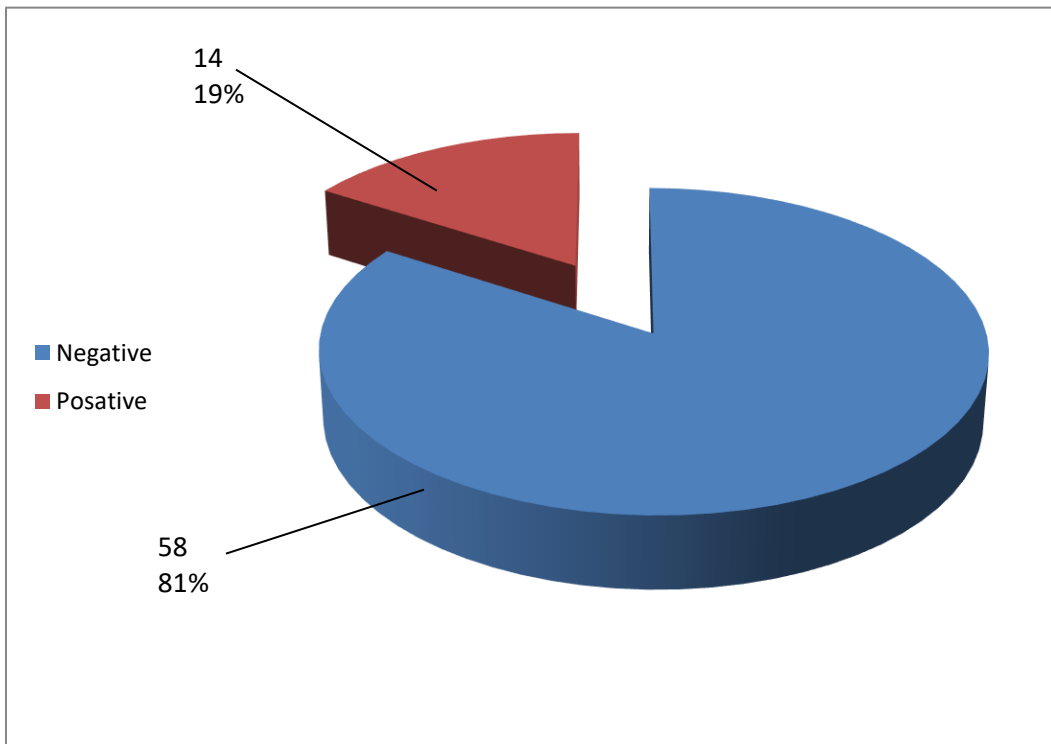


Figure 1: Pie chart showing pregnancy rate of women undergoing ICSI

Characteristics of Infertile Women Enrolled in this Study

Characteristics of infertile women that enrolled in the current study are shown in table 1. The mean age of all women enrolled in ICSI program 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; but 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 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: 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 \pm SD	32.25 ± 6.41	29.14 ± 4.54	32.76 ± 6.55	0.050 I
Range	20 -47	23 -40	20 -47	S
Duration of Infertility (years)				
Mean \pm SD	7.89 ± 3.87	6.93 ± 3.08	8.05 ± 3.98	0.319 I
Range	1 -17	2 -12	1 -17	NS
Type of infertility				
Primary, <i>n</i> (%)	65 (74.0 %)	10 (71.4 %)	55 (74 %)	1.00Y
Secondary, <i>n</i> (%)	23 (26.0 %)	4 (28.6 %)	19 (26 %)	NS
BMI (Kg/m ²)				
Mean \pm SD	26.72 ± 2.81	26.71 ± 2.60	26.72 ± 3.01	0.958 I
Range	20.44 -30.75	21.46 -30.75	20.44 -30.70	NS

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 $p \leq 0.05$

The Correlations of Serum and Follicular Fluid BMP15 and GDF9 to Age

The correlations of serum and follicular fluid bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) that measured at day of ova pick up to oocytes maturity are shown in table 2. Age was not significantly correlated to serum (-0.113) or follicular fluid BMP15 (-0.182) and serum (-0.018) and follicular (0.195) GDF9 ($p > 0.05$),

Table 2: Correlations of serum and follicular fluid BMP15 and GDF9 to age

Characteristic	Correlation Index	Serum GDF9	Serum BMP15	Follicular fluid GDF9	Follicular fluid BMP15
Age	R	-0.018	0.113	0.195	-0.182
	P	0.858	0.261	0.052	0.070

Correlations of Serum and Follicular Fluid BMP15 and GDF9 to Age Groups

By stratifying ages into three groups 20-29, 30-39 and 40-47,

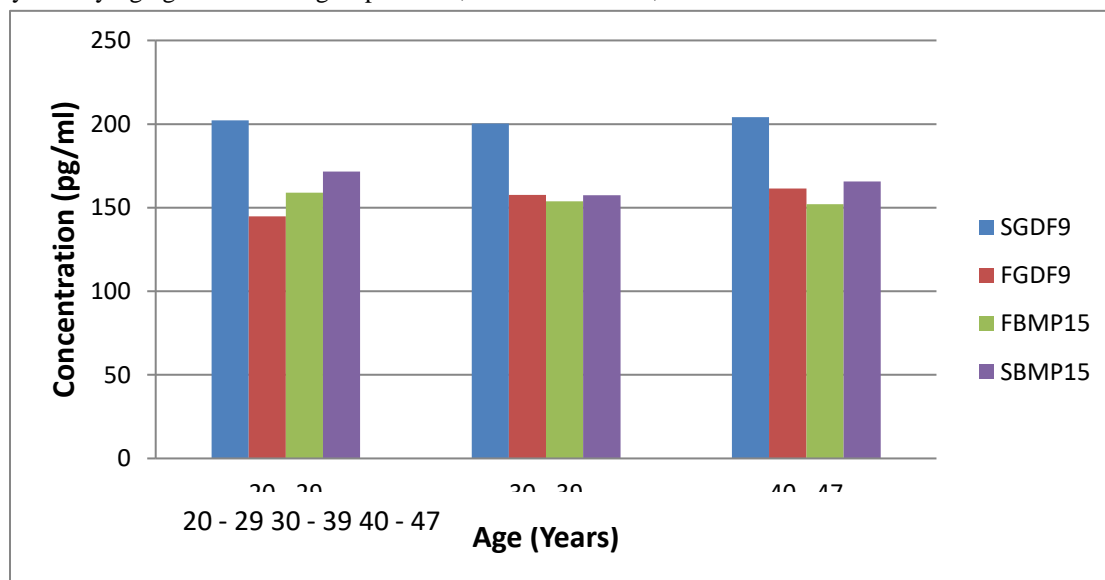


Figure 2: Mean level of serum and follicular fluid BMP15 and GDF9 in age groups

Correlation of Postmenopausal Group to Serum GDF9 and BMP15

In post-menopausal women serum GDF9 showed significant negative correlation to age (-0.27) ($p < 0.023$), serum

there was no significant correlation of ages of these stratified age groups to serum and follicular GDF9 and BMP15 ($p > 0.05$). 20-29 age group {SGDF9(0.190), FGDF9(-0.028), SBMP15(0.041), FBMP15(0.103)}, 30-39 age group {SGDF9(-0.184), FGDF9(0.054), SBMP15(0.044), FBMP15(-0.129)}, 40-47 age group {SGDF9(-0.100), FGDF9(-0.318), SBMP15(0.231), FBMP15(-0.043)}, as shown in table (3) and figure (2).

Table 3: Correlations of serum and follicular fluid BMP15 and GDF9 to age groups

Age Range	Correlation Index	Serum		Follicular fluid	
		GDF9	BMP15	GDF9	BMP15
20 - 29	R	0.190	0.041	-0.028	0.103
	P	0.280	0.820	0.880	0.560
30 - 39	R	-0.184	0.044	0.054	-0.129
	P	0.216	0.767	0.718	0.391
40 - 47	R	-0.100	0.231	-0.318	-0.043
	P	0.736	0.427	0.268	0.884

BMP15 showed highly significant negative correlation to age (-0.797) ($p < 0.001$), these result are shown in table 4.

Table 4: Correlation of age of postmenopausal group to serum GDF9 and BMP15

Characteristic	Correlation index	GDF9	BMP15
Age	R	-0.27	-0.797

	P	0.023*	< 0.001**
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*: Significant at $p \leq 0.05$; **: highly significant at $p \leq 0.01$

Comparison between Serum GDF9 and BMP15 in Premenopausal (Group 1) and Postmenopausal Women (Group 2).

In premenopausal women group 1 (G1) the mean value of serum GDF9 was (201.46), BMP15 (163.73), while mean value of postmenopausal group2 (G2) serum GDF9 was (179.75) and BMP15 (37.59), as shown in figure (3). Both of them showed highly significant difference between G1 and G2 ($P < 0.01$) Table 5.

Table 5: Comparison between Serum GDF9 and BMP15 in Premenopausal (group 1) and postmenopausal women (group 2).

Characteristic	Group 1 Mean± SD	Group 2 Mean± SD	P t- test
Serum GDF 9	201.46 ± 36.68	179.75± 50.04	0.0015 HS
Serum BMP 15	163.73 ± 45.22	37.59 ± 22.56	0.0001 HS

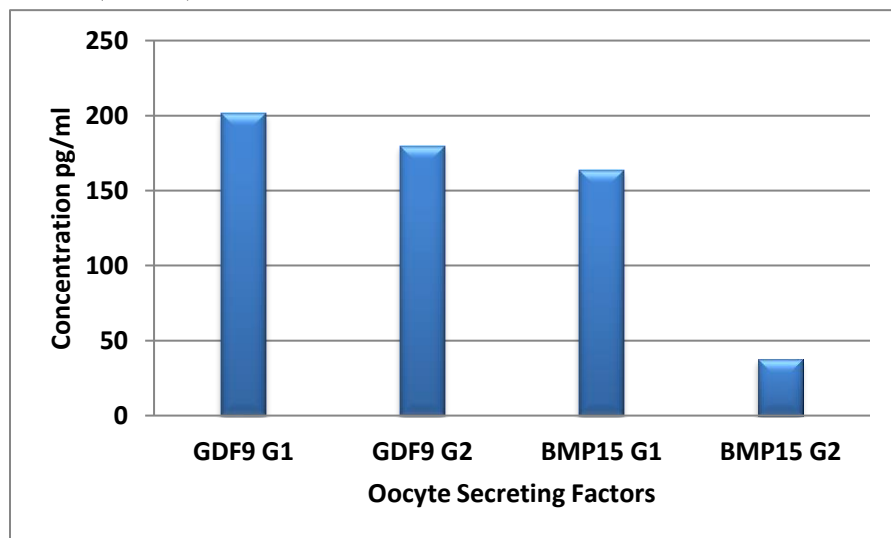


Figure 3: Mean level of Serum GDF9 and BMP15 in Premenopausal (group 1 /G1) and postmenopausal women (group 2/ G2).

Receiver Operating Characteristic (ROC) Curve Analysis to Find the Cutoff Value of GDF9 and BMP15 that Can Predict Menopause

Receiver operating characteristic (ROC) curve analysis was carried out to find the cutoff value of GDF9 and BMP15 that can predict menopause and the results are shown in

figure 4 and 5 and table 6. The cutoff value of GDF9 was ≤ 178 but with poor accuracy (67.1 %) since the area under curve (AUC) was less than 0.7. The cutoff value of BP15 was ≤ 70 and with excellent accuracy (96.1 %) since the area under curve (AUC) was more than 0.7.

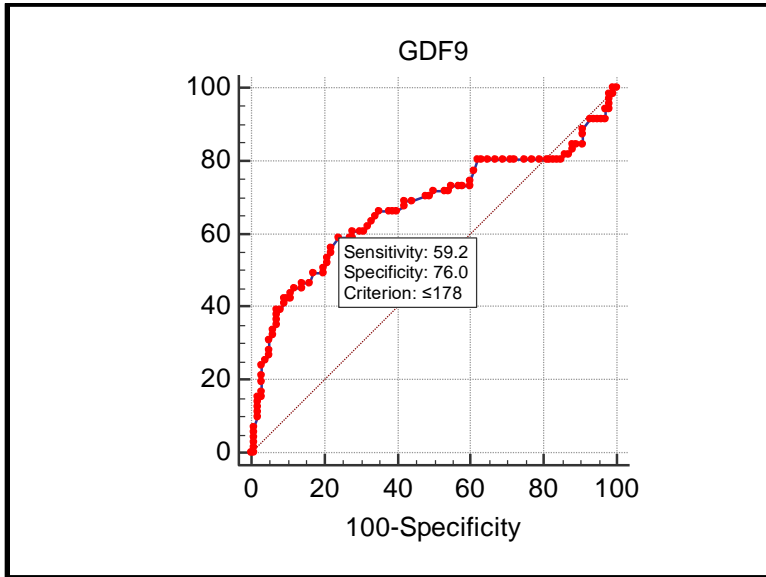


Figure 4: Receiver operating characteristic (ROC) curve analysis to find the cutoff value of GDF9 that can predict menopause

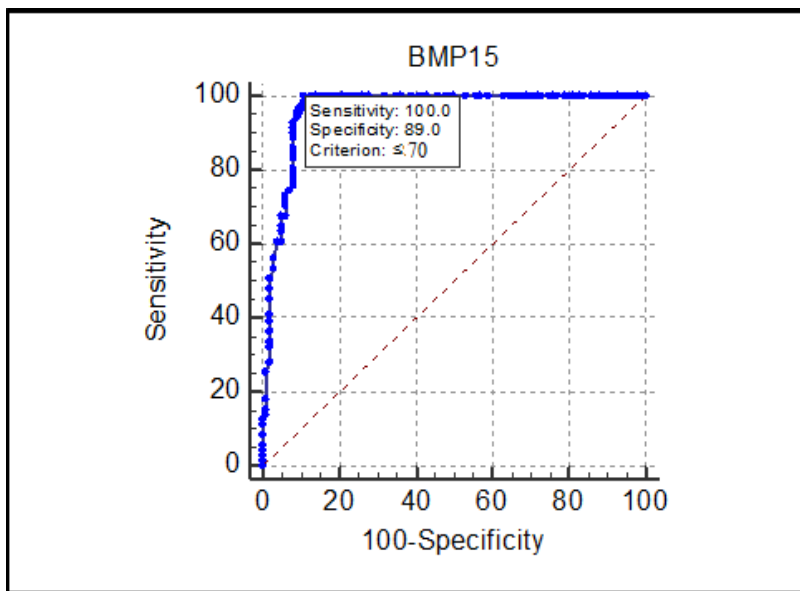


Figure 5: Receiver operating characteristic (ROC) curve analysis to find the cutoff value of BMP15 that can predict menopause

Table 6: The results of receiver operating characteristic (ROC) curve analysis to find the cutoff value of GDF9 and BMP15 that can predict menopause

Characteristic	GDF9	BMP15
Cutoff	≤ 178	≤ 70
AUC	0.671	0.961
95 % CI	0.595 to 0.741	0.921 to 0.985
p-value	<0.001***	<0.001***
Sensitivity %	59.2	100.0
Specificity %	76.0	89.0
Accuracy %	67.1	96.1

AUC: area under curve; CI: confidence interval; ***: significant at $p \leq 0.001$

Comparison of Serum and Follicular GDF9 and BMP15 in Women with Empty Follicles (Empty F.) and Women with non-Empty Follicles

Table (7) showed comparison of serum and follicular GDF9 and BMP15 in women with empty follicles (Empty F.) and women with non-Empty follicles (follicles containing

oocyte). Serum and follicular GDF9 showed non-significant difference ($p > 0.05$), while serum and follicular BMP15 showed highly significant difference between women with empty and non-empty follicles, ($p < 0.01$) as shown in figure (6).

Table (7): Comparison of serum and follicular GDF9 and BMP15 in women with empty follicles (Empty F.) and women with non-empty follicles

Characteristic	Non Empty (83) Mean \pm SD	Empty F. (5) Mean \pm SD	<i>P</i> <i>t-Test</i>
Serum GDF 9	201.46 \pm 36.68	190.00 \pm 7.58	0.49 NS
Serum BMP 15	163.73 \pm 45.22	41.00 \pm 9.19	0.0001 HS
Follicular GDF9	153.60 \pm 30.23	164.00 \pm 28.79	0.45NS
Follicular BMP15	155.63 \pm 30.46	39.60 \pm 6.75	0.0001 HS

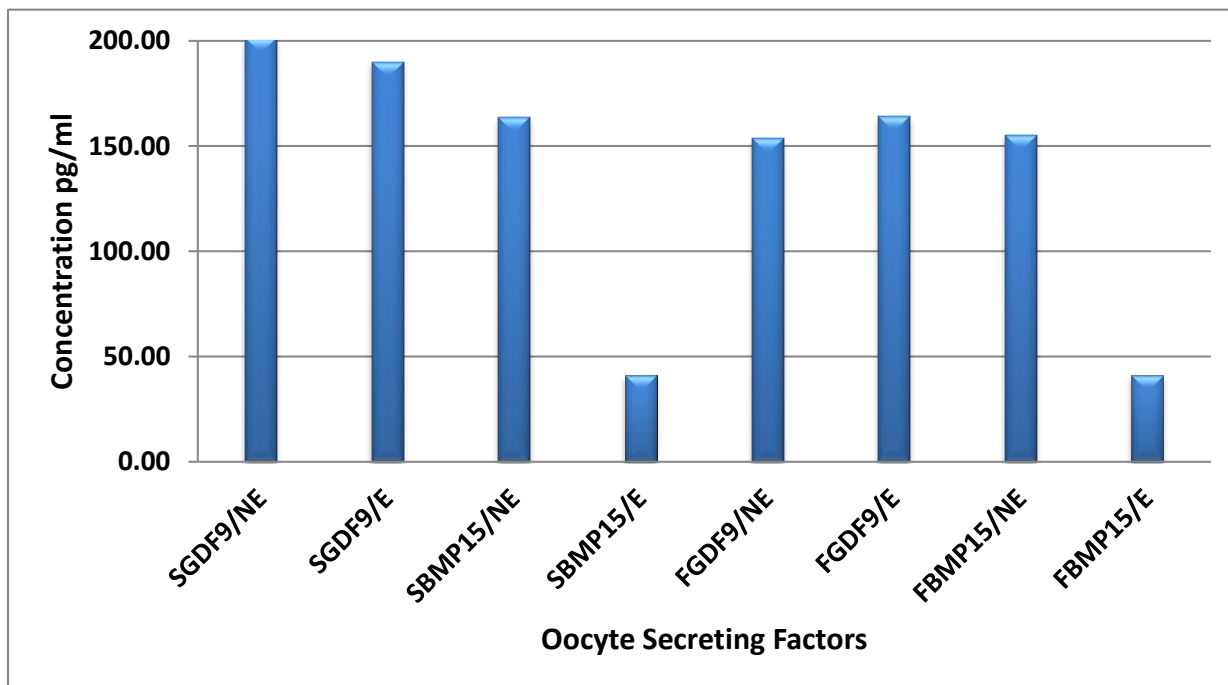


Figure (6): Mean level of serum and follicular GDF9 and BMP15 in women with Empty (E) and Non Empty (NE) follicles

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.

During ART, with increasing age, there is great decline in the oocyte quantity and quality (8). These consequences occur because with increasing age there will be increase in the incidence of aneuploidy, that caused by factors like disruption of spindle apparatus, mitochondrial damage and oxidative stress (9). The current study was done to study the possibility of using GDF9 and BMP15, the oocyte secreted

factors, as biomarkers for oocyte quality.

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. that found that pregnancy rate was 28% (10), also other study done by Jassim WH et al, found pregnancy rate to be 25.4% (11). The pregnancy rate was low because 4 cases of testicular biopsy, 2 cases of moderate endometriosis and also there were 14 case with age above 40 years were included in the this study, furthermore the SARS-CoV-2 (Covid 19)

pandemic also might be one of the causes of 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 (12), and the testes that infected with SARS-CoV-2- showed extensive peritubular fibrosis, vascular congestion with extensive destruction of germ cell (13). Furthermore, SARS-CoV-2 could cause ovarian tissue damage and decline in the function of the ovary and oocyte quality, causing female infertility and may cause miscarriage (13).

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.

Correlations of Serum and Follicular Fluid BMP15 and GDF9 to Age in premenopausal women

In the current study, age was not significantly correlated to serum or follicular fluid BMP15 and GDF9 in female that subjected to ICSI program ($p > 0.05$) table 2. This result correspond to that done by Riepsamen, A. H., et al. showed that BMP15 and GDF9 do not decrease greatly with age in female of reproductive age and no evidence of a strong correlation between serum (GDF9) or (BMP15) and age (14).

The oocytes developmental potential decreases in all species with increasing age, and this is called ovarian aging. In humans, female ovarian aging causes decline in the quantity and quality of oocytes. Ovarian aging starts at the age of thirty and continues until menopause. Today in the modern society, women postpone childbearing and the risk of infertility, abortion and congenital anomalies increased with increasing age accordingly. It's well known that the increase in age increases the incidence of human oocyte chromosomal aneuploidy, furthermore the older oocytes could show damage to the mitochondrial DNA, abnormality of the expression of mitochondrial gene and decline in the mitochondrial membrane potential (15).

But this result didn't correspond to that done by Park, M.J., et.al., that studied ovarian gene expression of GDF9 and BMP15 which stated that ovarian expressions of BMP15 and GDF9 decreased with age (16). It is well known that age affect the quality of oocyte and reproductive potential and was confirmed by Moghadam, et al., which stated that the increasing of maternal age is related directly to the fetal chromosomal abnormalities, and this fact confirms that the primary oocytes are more likely to have damage as they age (17). The results also disagreed with Gong, Y., et al., that stated that women with poor ovarian response, especially those over forty, BMP15 and GDF9 expression is decreased

along with increasing age (18).

Correlations of Serum and Follicular Fluid BMP15 and GDF9 to Age Groups

By stratifying ages into three groups (20-29), (30-39) and (40-47), there was no significant correlation of ages of these stratified age groups to serum and follicular GDF9 and BMP15 ($p > 0.05$), as shown in table (3) and figure (2). These results were surprising because GDF9 and BMP15 as known previously are secreted from the oocytes and with decreasing number of oocytes with increasing age these factors should be decreased, these results agreed with study result of Angelique Riepsamen, et al. (19).

Correlation of Postmenopausal Group to Serum GDF9 and BMP15

In post- menopausal women serum GDF9 showed significant negative correlation to age and serum BMP15 showed highly significant negative correlation to age, as shown in table 4. These results were agreed with study result of Riepsamen, A. H.,et al (19) that suggest that BMP15, but not GDF9, decreases with age., but the level of serum GDF9 still high in post- menopausal women and this result was supported by the same study that found out GDF9 in the serum of peri-menopausal and post - menopausal women, which is a surprising finding because of the low oocytes number remaining in the first group, and depletion of oocyte in the second group which signified that the GDF9 that detected in those women is mostly of non-oocyte origin, which is similar to pituitary secretion of human HCG. Other cell types may express those proteins and may contribute to the detection of them in peripheral serum, such as cumulus cells, granulosa cell, adrenal, or other tissues (14), also there is a possibility that GDF9 has number of subtypes, one or more of these subtypes originate from the ovary while the others originate from other sites in the body.

Riepsamen A.H.et al., Also showed that BMP15 concentrations were (4.5- and 4.6) folds lower in female > fifty five years of age as compared with female of (31 to 35) and (41 to 45) years of age, respectively (14).

These results indicate that the only source of BMP15 is the oocyte, while GDF9 has sources other than oocyte or could has subtypes one or more of them secreted from oocyte, and this explain the decrease in its concentration in postmenopausal women but its level remain high in comparison with the level of BMP15, as shown in table 4.

Comparison between Serum GDF9 and BMP15 in Premenopausal (Group 1) and Postmenopausal Women (Group 2).

In premenopausal women group 1 (G1) the mean value of serum GDF9 was (201.46), BMP15 (163.73), while mean

value of postmenopausal group2 (G2) serum GDF9 was (179.75) and BMP15 (37.59), as shown in figure (3), table 5. Despite of significant decrease in GDF9 in postmenopausal woman but it was still high and this confirm the non-oocyte origin, while the BMP15 reached very low concentrations, in some women reached nearly undetectable values, and this confirm that the BMP15 is secreted from the oocyte only.

Receiver Operating Characteristic (ROC) Curve Analysis to Find the Cutoff Value of GDF9 and BMP15 that Can Predict Menopause

In figures 4 and 5 and table 6, ROC curve was carried out and showed the cut off values with their sensitivity and specificity, for the limit of GDF9 and BMP12 serum concentration that showed the probability that women being in their menopause state and cannot have the ability to become pregnant. The cutoff value of GDF9 was ≤ 178 , the cutoff value of BMP15 was ≤ 70 .

Comparison of Serum and Follicular GDF9 and BMP15 in Women with Empty Follicles (Empty F.) and Women with non-Empty follicles

This study showed that serum and follicular GDF9 showed non-significant difference between women with empty and non - empty follicles, while serum and follicular BMP15 showed highly significant difference between women with empty and non-empty follicles, and BMP15 serum and follicular concentration reached very low level, as in table (7) and figure (6).

There was no previous study comparing these factors in empty and normal follicles. This results suggested that BMP15 secreted only from oocyte while GDF9 could be secreted from other sites in the body other than oocyte or could has sub-types that not discovered till now.

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