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Relationship between BMP15 Gene Expression and FSH in a Sample of Iraqi Infertile Women with Polycystic Ovary Syndrome

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Abstract:The present study aimed to investigate the relationship between the FSH , LH and Prol level and BMP15 gene expression level in blood samples collected from 50 healthy fertile female as controls and 50 PCOS infertility female who subjected to detected the level of LH,FSH and Prol by ELISA test and the relative quantitative detection using RT-PCR technique. Specific primers designed for this purpose, the sequence of BMP15 retrieved from NCBI and designed by primer 3 software. The result data assessing the folding BMP15 gene expression in the polycystic ovary syndrome (PCOS) (0.507) , while the control group's was (1.000), PCOS group showed down regulation in the expression of BMP15 gene. Moreover, strong positive significant (r = 0.412) correlation observed between the levels of LH and FSH hormones, while the negative significant (r = - 0.338) correlation was observed between the FSH hormones level and the folding expression of BMP15 gene. In this study the FSH level considered an important criterion for infertility investigation because FSH is responsible for stimulates normal follicular growth and ovulation. The results provide that the expression of BMP15 was significantly decrease with increased in FSH hormone level, So the lack in BMP15 expression increase the level of follicle stimulating hormone leads to incomplete development at puberty and poor ovarian function.

Keywords: BMP15 gene,gene expression, Infertility, FSH ,PCOS .

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Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder that impacts many women of the reproductive age worldwide (1). It is estimated that approximately every 1 in 10 women face PCOS before menopause and struggle with its complications (2). Although the high ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) and increased frequency of gonadotropin-releasing hormone (GnRH) is known as the underlying causes of PCOS, the exact etiology and pathology have not been comprehensively well-known (3), (4). Evidence suggests the role of different external and internal factors, including environmental factors, genetic, and epigenetics. Ovarian hyperandrogenism, paracrine dysregulation of the follicle, menstrual dysfunction and insulin resistance development in ovaries are typical characteristics of PCOS(5),(6).Ovarian folliculogenesis is organized by the interaction of extra-ovarian and intra-ovarian factors that coordinate the processes of oocyte growth and follicular

development in the peri-ovulatory period(7). Primordial follicle growth is primarily influenced by paracrine and endocrine factors(8). Among the many extra-ovarian and intra-ovarian factors, the transforming growth factor beta (TGFB) superfamily in particular plays an important role in follicle growth. one of the most important related member of the TGFB superfamily is involved in the function and development of the ovary: is bone morphogenetic protein 15 (BMP15) (9). BMP15 play a critical role in follicle development, oocyte maturation, ovulation and embryo development (10).BMP15 functions are important in the regulation of normal follicular development and ovulation . BMP15 is mitogenic for somatic cells and is known to induce granulosa cell proliferation and affect the choice of the dominant follicle as well as the formation of follicular atresia (11).

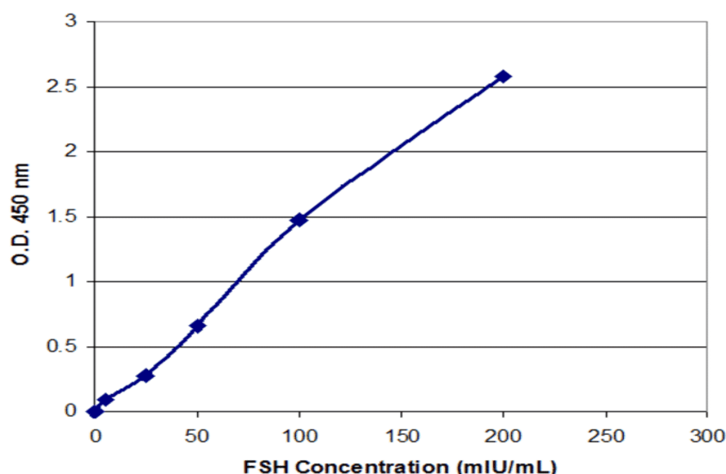
Materials and methods

Patients and controls

The study was designed to be futures study. The size of sample was 100 fertile and infertile women during their visit to at Al-Elwiya Educationl and Kadhimiya Teaching Hospital in Baghdad-Iraq, with average ages range between (25-45)years old. The selected 100 women were intentionally divided, in two group, first group: 50 infertile women with polycystic ovarian syndrome (PCOS), and second group: 50 healthy fertile women were also enrolled in this study, the PCOS specimens were selected according standardized diagnostic criteria (1).

Measurement of FSH hormone concentration

For the quantitative determination of human follicle stimulating hormone (FSH) concentration in serum, human FSH ELISA Kit. Ninety-six-well high-affinity binding microplates were having been precoated with a monoclonal antibody specific for FSH (12). The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. TMB substrate solution is added to each well. Only those wells that contain FSH and enzyme-conjugated antibody will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. In order to measure the concentration of FSH in the sample this Human FSH ELISA Kit includes a set of calibration standards (6 standards). The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of (O.D.) versus FSH concentration (mIU/mL). The concentration of FSH in the samples is then determined by comparing the O.D. of the samples to the standard curve, figure (1).



Figure(1): Standard Curve - Human Follicle Stimulating Hormone ELISA Kit

Measurement of luteinizing hormone concentration

For the quantitative determination of human Luteinizing hormone (LH) concentration in serum, human LH ELISA Kit. Ninety-six-well high-affinity binding microplates were having been precoated with a monoclonal antibody specific for LH. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, a TMB substrate solution is added to each well. Only those wells that contain LH and enzyme-conjugated antibody will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. In order to measure the concentration of LH in the sample this Human LH ELISA Kit includes a set of calibration standards (6 standards)(13). The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of (O.D.) versus LH concentration (mIU/mL). The concentration of LH in the samples is then determined by comparing the O.D. of the samples to the standard curve, figure (2).

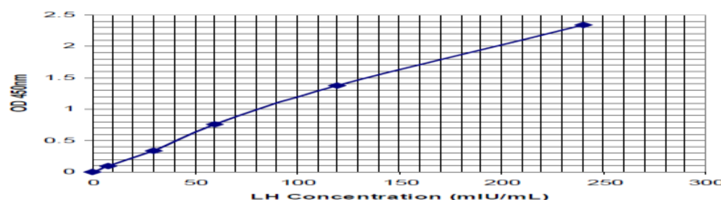


Figure (2): Standard Curve - human luteinizing Hormone ELISA Kit

Measurement of prolactin hormone concentration

The quantitative determination of human Prolactin concentrations in serum, human Prolactin ELISA Kit . Ninety-six-well high-affinity binding microplates were has been precoated with a monoclonal antibody specific for ProL. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, a TMB substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain Prolactin and enzyme-conjugated antibody will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm. In order to measure the concentration of Prolactin in the sample, this Human Prolactin ELISA Kit includes a set of calibration standards (6 standards)(14). The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of (O.D.) versus Prolactin concentration (ng/mL). The concentration of Prolactin in the samples is then determined by comparing the O.D. of the samples to the standard curve, figure (3).

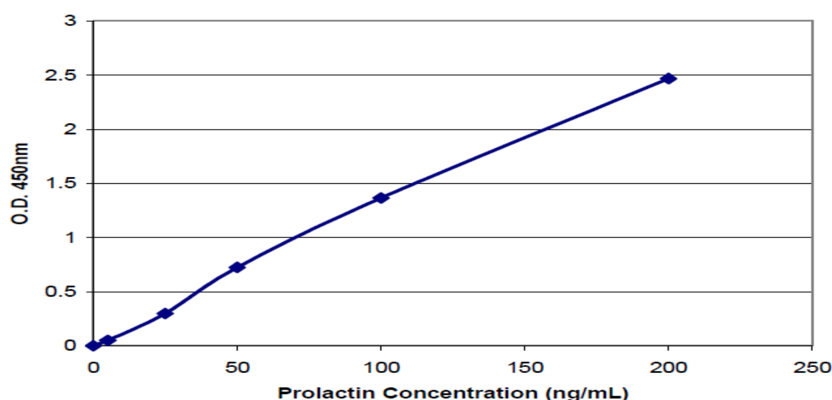


Figure (3): Standard Curve - prolactin Hormone ELISA Kit

Gene expression of BMP15

The expression of BMP15 gene which determine via the reverse transcription quantitative polymerase chain reaction (RTqPCR) method after isolation of total RNA. The reagent that a ready to use (TransZol Up Plus, TransGen, biotech) (Trizo,Trans;India) which used to isolate the total RNA, and the instructions of manufacture were followed. After that the cDNA reversely transcribed from the isolated RNA by using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit, total RNA was reverse-transcribed to complementary DNA (cDNA)(15). The procedure was carried out in a reaction volume of 20 µl according to the information of manufacturer's instructions. The total

RNA volume was (20µl) that which be reversely transcribed. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was carried out using TransStart® Top Green qPCR Super Mix and cDNA as a template (16). As showed in table (1) the forward and reverse primers(oligonucleotide) of BMP15 gene were designed. Also given the forward and reverse primers of the housekeeping gene GAPDH (reference gene: glyceraldehyde-3-phosphate dehydrogenase) (17).

Table (1): Primers used in the study

Primer	Sequence (5′→3′ direction)	primer size bp	Product size bp	Tm °C
BMP15 (Gene Expression)				
(Forward)	CTAGAAGAAT CCCCTGGCGA	**20**	**125**	**62**
(Reverse)	ATGGTGCGGT TCTCTCTAGG	20	125	62
***GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase**				
Forward	GAAATCCCAT CACCATCTTC CAGG	24	160	58
Reverse	GAGCCCCAGC CTTCTCCATG	20	160	

The mixture of reaction was adjusted to a final volume of 20 µl as suggested via the manufacturer, which included: 10µl of TransStart® Top Green qPCR Super Mix (2X), for 2-Step RT-qPCR , 1µl of each primer (10 mM), 2 µl cDNA, and 6µl Nuclease free water . The mix was transferred to a real time thermo cycler (MIC-4 Real-time PCR System, MX 3000P™ / Stratagen-eL/ USA), which was programmed for the following optimized cycles, Table (2).

Table (2): The thermal profile of GAPDH and BMP15 gene expression

Step	Temperature (°C)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	60	15	
Extension	72	20	
Dissociation	55 °C-95 °C		1

As $2^{-\Delta\Delta Ct}$ the gene expression was given, which represents the relative of the fold change. Therefore, the results were expressed as a fold change in the expression level of a target gene that was normalized to endogenous control (housekeeping gene) and relative to a calibrator, which is the target gene in control subjects (18).

Statistical analysis

Data where statistically analysis IBM SPSS Statistics 26 program was used to detect the effect of different factors on study parameters. One-way ANOVA and T-test was used to significantly compare between means. Data were appeared as mean ± standard deviation. Categorical variables were analyzed by Chi-square test used to significantly compare between percentage (0.05 and 0.01 probability). The difference in frequencies of genotypes and alleles between the patient and control group were analyzed by using the Chi-square test (19). Correlation analysis was carried out using Pearson's correlation and regression analysis using SPSS version 16.0.

Results and discussion

Assessment of fertility hormone

A total of women was investigated for infertility during this study and their FSH hormone, were assessed are shown in tables (3). Out of the 100 women assessed, 50 subjects with polycystic ovarian syndrome (PCOS) compared with 50 healthy married female (control) subjects. For the healthy (control) female, the hormone status of the FSH was (6.60± 0.62) (mIU/mL); while for polycystic ovarian syndrome (PCOS) women the hormone status of the FSH was (19.25 ± 0.05) (mIU/mL). Regarding the levels of FSH, the results showed that the FSH levels hormone, Figure (4) in married women with PCOS was highly significantly ($p<0.01$) when compared with healthy women (control).

Table (3): Comparison between two groups and FSH hormone

Group	FSH hormone mIU/mL
Control group	6.60± 0.62[b]
PCOS group	19.25 ± 0.05[a]
LSD value	2.341**
P-value	0.0001

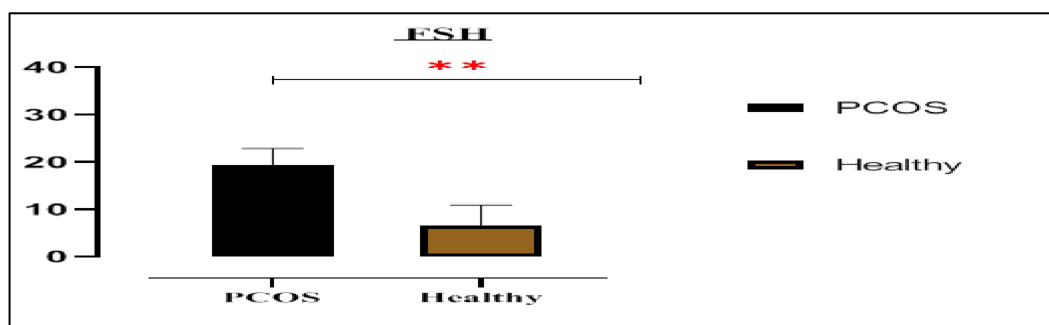


Figure (4): The comparison between difference groups and FSH hormone

Considering the FSH levels in married healthy women and in married women with PCOS, the results revealed a significant increase of this hormone. A similar result was reported by other researchers (20). which reported that Elevated FSH concentrations are a marker for decreased oocyte quality. While the

level LH hormone, were assessed are shown in tables (4). For the healthy (control) female, the hormone status of the LH was (4.38 ± 0.29) (mIU/mL); while for polycystic ovarian syndrome (PCOS) women the hormone status of the LH was (22.10± 1.19) (mIU/mL). Regarding the levels of LH, the results showed that the LH levels hormone, Figure (5) in married women with PCOS was highly significantly ($p<0.01$) when compared with healthy women (control).

Table (4): Comparison between difference groups and LH hormone

Group	LH hormone mIU/mL
Control group	$4.38^{b} \pm 0.29$
PCOS group	$22.10^{a} \pm 1.19$
LSD value	2.491**
P-value	0.0001

- Values are means ± standard deviation of means.
- Means in rows carrying similar small letters indicate a non-significant difference.
- Means in rows carrying different small letters indicate a significant difference
- ** Indicate to the highly significant ($p< 0.01$).

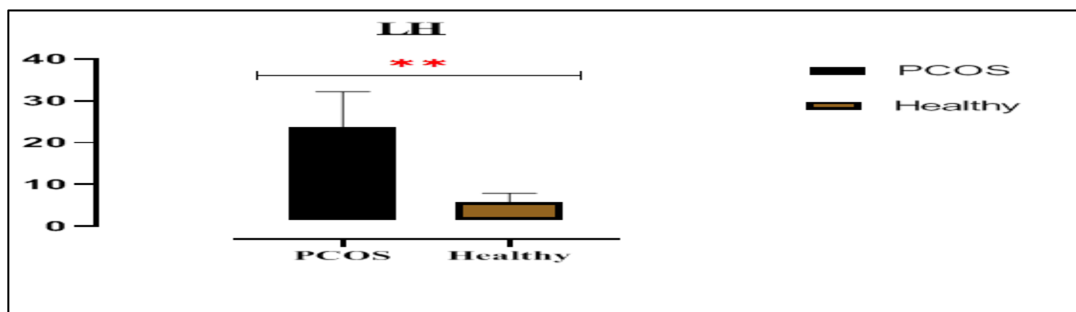


Figure (5): Comparison between difference groups and LH hormone

Abnormal patterns of gonadotropin secretion, including increased serum luteinizing hormone (LH) concentrations, have long been recognized as common characteristics of women with PCOS (21. Prolactin is a hormone secreted from anterior pituitary gland which has different functions throughout the body of the fertile females. The levels of Prolactin, were assessed are shown in table (5), for the healthy (control) female, the hormone status of the Prol was (12.83± 1.09) (mIU/mL); while for polycystic ovarian syndrome (PCOS) women the hormone status of the Prol was (16.35 ± 1.22) (mIU/mL). Regarding the levels of Prol, the results showed that the level of hormone, Figure (6) in married women with PCOs exhibit a mild elevation of serum prolactin level from normal.

Table (5): Comparison between difference groups and prolactin hormone

Group	prolactin hormone mIU/mL
Healthy group	12.83± 1.09[b]
PCOS group	16.35 ± 1.22[a]
LSD value	4.590**
P-value	0.0001

- Values are means ± standard deviation of means.
- Means in rows carrying similar small letters indicate a non-significant difference .
- Means in rows carrying different small letters indicate a significant difference
- ** Indicate to the highly significant (p< 0.01).

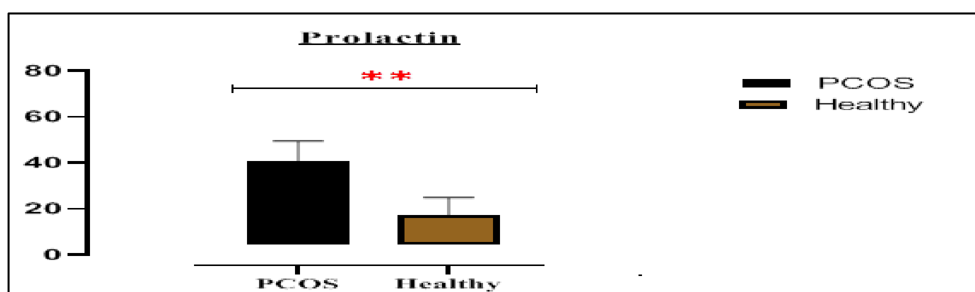


Figure (6): Comparison between difference groups and LH hormone

This result agreement with a previous study (22), which reported that married women with PCOs exhibit slightly elevation of serum prolactin level , but is more frequent in the females with PCOS than normal ovulatory females. Some was in agreement with this result and showed that serum prolactin level is higher than normal in PCOS patients (23) .This could be explained by the hypothesis that some points of the PCOS pathogenesis related to deficient hypothalamic dopaminergic activity which also responsible for elevated LH/FSH ratio and thyroid stimulating hormone (TSH) (24).

BMP15 gene expression

In table (6), The ΔCt mean±SD showed high significantly (p=0.0001**) difference in polycystic ovarian syndrome (PCOS)(6.312± 1.17) group when compared to corresponding in fertile control group(6.725± 0.18) . Assessing the $2^{-\Delta\Delta Ct}$ mean values in the polycystic ovarian syndrome (PCOS) (0.61) and fold of gene expression depending on $2^{-\Delta Ct}$ was (0.507) , while the control group's $2^{-\Delta\Delta Ct}$ mean value was (0.36) when the fold of gene expression depending on $2^{-\Delta Ct}$ was (1.000) , PCOS groups showed down regulation in the expression of BMP15 gene. These findings imply that the mean of $2^{-\Delta\Delta Ct}$ differed high significantly among these groups (p=0.002**).

Table (6): Folding expression level of BMP15 gene among PCOS infertile female and healthy fertile female (controls) samples

Study Groups	Mean ± SD of					
	BMP15 Ct value	ΔCT	ΔCTC	ΔΔCT	2-ΔΔ Ct	Fold of gene expression Depending on $2^{-\Delta Ct}$
Group 1: healthy	23.651± 1.03	6.725 ± 0.18	5.041± 0.83	1.683 ± 0.77	0.36± 0.19	1.000
Group 2: PCOS.	24.334± 1.11	6.312 ± 1.17	5.204± 0.67	1.101 ± 1.13	0.61± 0.47	0.507
P-value	0.0001**	0.0001**	0.0001 **	0.005 **	0.002 **	0.0001**

In the PCOS patients the down-regulation of BMP15 gene expression was observed, this results was in agreement with such findings, it has been reported that level of BMP15 was significantly decreased in female infertility. The authors also reported that the expression of BMP15 gene was lower in the female suffer from infertility factor, while rises in fertile control female (25). The BMP15 have been the aim of plentiful studies, which showed that it essential to folliculogenesis and also for oocyte maturation and ovulation (26). The present study highlights the importance of BMP15 on the ovarian function. Bone morphogenetic protein 15 (BMP15) is closely associated with reproduction and woman reproductive disease. As a multifunctional oocyte-specific secret factor, BMP15 controls female fertility and follicular development (27).In the female gonad, Transforming growth factor β (TGF-β) like BMP15 are produced physiologically and are involved in its normal function. Previous studies found that BMP15 played a critical role in follicular development and ovulation rate in mono-ovulatory mammalian species, especially in human, the BMP15 have regulatory role of female fertility (28). Lower level of BMP15 expression are associated with subfertility, damage to ovulation and even with ovarian failure in women (29). And therefore, this study conducted that the Iraqi women complaining from PCOS have down-regulation of BMP15 gene expression.

Pearson correlation analysis of PCOS patient group

To investigate the correlation between FSH and BMP15 gene expression, correlation coefficient analysis of (PCOS) patient group was done, the results showed in Table (7).

Table (7): The Pearson correlation coefficient analysis of (POCS) group

Parameters	LH	FSH	Prolactin	FOLD BMP15
LH	1	.412**	.069	-.247
FSH	.412**	1	.229	..338*
Prolactin	.069	.229	1	-.046
FOLD BMP15	-.247	..338*	-.046	1

The findings in table (7) shows a strong positive significant (r = 0.412) correlation observed between the levels of LH and FSH hormones in PCOS infertility women (30).
The negative significant (r = -0.338) correlation was observed between the FSH hormone level and the folding expression of BMP15 gene. Endocrinal disorder like hormones which regulate the oogenesis (e.g. FSH, LH, Testosterone) which contributed with the (TGF-β superfamily genes) like growth differentiation factors 9 (GDF9), BMP15, BMP6, to begin follicular growth leading to change these TGF-β superfamily genes levels. In this study the FSH level considered an important criterion for infertility investigation because FSH is responsible for stimulates normal follicular growth and ovulation and the un-oogenesis sometimes occurs concurrently with endocrine abnormalities (31). The results showed that the expression of BMP15 was significantly decrease with increased in FSH hormone level (32, BMP15 reduced FSH expression in human granulose cells through Smad and non-Smad pathways. This mechanism of FSH reduction by BMP15 may be utilized for controlling follicular growth. So, the rise of follicle stimulating hormone leads to incomplete development at puberty and poor ovarian function (ovarian failure). In this situation ovarian follicles do not grow properly and do not release an egg, thus leading to infertility (33,34).

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