

Altered Expression of *GDF9* and *BMP15* Genes in Granulosa Cells of Diminished Ovarian Reserve Patients: A Case-Control Study

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Abstract

Objective: Diminished ovarian reserve (DOR) is a challenging issue encountered during assisted reproductive technology. Growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) belong to the transforming growth factor-beta (TGF- β) superfamily which are essential for folliculogenesis. We aimed to the evaluation of the *GDF9* and *BMP15* expression in the granulosa cells (GCs) of DOR patients.

Materials and Methods: This case-control study included 14 women with DOR and 12 controls, who were between 28-40 years of age undergoing controlled ovarian stimulation with a gonadotropin releasing hormone (GnRH) antagonist protocol. DOR patients were selected by the Bologna criteria. The GCs were extracted from the aspirated follicular fluids and RNA isolated from this. The fold change of gene expressions was assessed by real-time polymerase chain reaction (PCR).

Results: *GDF9* expression in patients was 0.23 times lower than the control group, which was significant ($P < 0.0001$). *BMP15* expression in patients was 0.32 times lower than the control group, which was significant ($P < 0.0001$). The number of archived oocytes, MII, and two pronuclei (PN) embryos was higher in the control group and these differences were statistically significant ($P < 0.05$).

Conclusion: Given that *GDF9* and *BMP15* are specifically involved during follicular recruitment, we expect expression of these two genes in DOR patients which is greatly reduced by reducing follicular reserve.

Keywords: Bone Morphogenetic Protein 15, Growth Differentiation Factor 9, Ovarian Reserve

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Introduction

Diminished ovarian reserve (DOR) is one of the causes of infertility in women. Subfertility is considered to be related to DOR in older women (1). Approximately 10% of women seeking fertility treatments have DOR (2).

DOR is manifest heterogeneous disorder with various etiology such as infertility, genetics, autoimmune, idiopathic, and iatrogenic. Iatrogenic causes include ovarian surgery, radiation, and chemotherapy (2, 3). DOR is associated with infertility and poor ovarian response following controlled ovarian hyperstimulation. Delays in childbearing have led to an increase in a significant number of couples needing assisted reproductive technology (ART) due to older age. DOR has adverse effects on ART outcomes such as decreased ovarian response to gonadotropins, and quality of oocytes and embryos, implantation rates, and live birth rates (4, 5). Sub-fertile women are regularly screened for DOR before

starting therapy because of the relationship between DOR and poor *in vitro* fertilization (IVF) outcome (6). The Bologna Criteria, published in 2011, defines poor ovarian response as women having at least two of the following criteria: maternal age (≥ 39 years), poor ovarian reserve history (DOR; ≤ 3 oocytes), and abnormal ovarian reserve tests (antral follicle count [AFC] $< 5-7$ follicles or anti-Müllerian hormone [AMH] $< 0.5-1.1$ ng/ml) (7).

The functional unit of the ovary is the ovarian follicle (8). Development of the ovarian follicle requires coordination between somatic cell proliferation and differentiation with oocyte growth and its maturation (9). Granulosa cells (GCs) are one of the somatic cells of the follicular environment that are closely associated with the developing oocyte, ovulation, and fertilization (10). The ovarian function resulted from feedback of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and local ovarian factors

(11). In addition, the transforming growth factor-beta (TGF- β) superfamily is comprised of >35 proteins that have similar common structural motifs. These proteins include activin/inhibin, growth and differentiation factor (*GDF*), bone morphogenetic protein (*BMP*) subfamilies, and *AMH* (12). Among these, *GDF9* and *BMP15* play a critical role in ovarian function.

GDF9 and *BMP15* are crucial for folliculogenesis which involved in primary, secondary, and antral follicles except primordial follicles. However, the mRNAs level of *GDF9* and *BMP15* detected in all stages of folliculogenesis (13). Spontaneous mutations of either *GDF9* or *BMP15* affect fertility in females (14).

As with other members of the TGF- β superfamily, both *GDF9* and *BMP15* exert their biological functions by forming heteromeric complexes with types 1 and 2 receptors on the cell surface (13, 15).

GDF9 derived from the oocyte, is necessary for folliculogenesis, cumulus expansion, and GCs proliferation (15). *GDF9* knockout mice were infertile because of disruptions to folliculogenesis, (16). *GDF9* motivates the expression of the FSH receptor (*FSHR*), decreases expression of the LH receptor (*LHR*), prevents follicle atresia, and affects the GCs to steroidogenesis (17).

BMP15 with *GDF9* plays a critical role in folliculogenesis and fertility. In contrast to *GDF9*, *BMP15* knockout mice had subfertility because of defects in ovulation and early-stage embryonic development (18). *BMP15*, during the initial and final stages of folliculogenesis, has a positive role in follicular development (17).

BMP15 and *GDF9*, like other TGF β superfamily members, are translated as pre-proteins (19). *In vitro* studies show that both *BMP15* and *GDF9* can be expressed as linked homodimers or heterodimers (20, 21). Since studies about *GDF9* and *BMP15* focused on premature ovarian failure and comprehensive information in DOR is not available, we aimed to assess the fold of expression of *GDF9* and *BMP15* as important genes involved in folliculogenesis in the GCs of DOR patients.

Materials and Methods

This case-control study approved by the Reproductive Biomedicine Research Center Ethics Committee at Royan Institute (IR.ACECR.ROYAN.REC.1397.067). Participants were included patients admitted for infertility treatment at Royan Institute, Tehran, from 2017 to 2020. Twenty-six women (14 in the study and 12 in the control group), between 28-40 years of age undergoing IVF/intracytoplasmic sperm injection (ICSI) treatment were enrolled in this study. All patients gave their consent for collection and use of their discarded follicular fluid for research purposes.

Participants were divided into two groups, DOR and control (NOR). The Bologna criteria were used to

select DOR patients. Patients were considered to have DOR if they had serum AMH levels <1.1 ng/ml and/or an AFC <5, in conjunction with serum FSH levels >10 IU/l. The NOR participants were women of normal ovarian reserves with male factor infertility cause. All women had normal karyotype and those with fragile X mental retardation 1 (*FMR1*) gene permutation, polycystic ovary syndrome (PCOS), endometriosis as well as autoimmune disorders were excluded.

Ovarian stimulation protocols

The antagonist protocol is increasingly used in the management of women with a DOR who undergo ARTs. All DOR patients and the control group underwent pituitary down-regulation with a gonadotropin releasing hormone (GnRH) antagonist protocol. Patients received exogenous gonadotropins on the second or third day of menstruation while the leading follicle reached 13 mm in diameter, followed by a GnRH antagonist. Human chorionic gonadotropin (hCG) was prescribed when the follicles were dominant in terms of size. When follicles reached \geq 18 mm diameters, follicles puncture was performed and GCs from follicular fluid were collected. Following follicles puncture oocytes number, quality, and embryos grading were assessed (22).

Purification of granulosa cells from follicular fluid

The aspirated follicular fluid was centrifuged at 2000 g for 10 min, then 4 ml of salt taylor added to the pellet, and it was slowly layered on a 50% sill select gradient and centrifuged at 3000 g for 30 min. The GCs collected and removed using a sterile transfer pipette and placed into a 15 ml tube that contained 3 mL of cold DMEM/F-12 media supplemented with 1x penicillin/streptomycin and 10% fetal bovine serum, followed by centrifugation. Enzymatic digestion with hyaluronidase enzyme was performed to disperse the GCs (23).

Multinucleated giant cells were then washed in DMEM/F-12. The tube was centrifuged at 1500 rpm for 5 minutes at 21°C, and then 5 ml RBC lysing buffer (RLB) added to the pellets. The RLB solution consisted of ammonium chloride, potassium bicarbonate, and EDTA. The diluted solution was kept at room temperature for 2-5 minutes and centrifuged at 1500 rpm for 3 minutes at 21°C. Then, it was washed once with DMEM/F12. Cell counts and viability assessments were performed before the second centrifugation. The GCs were washed, centrifuged at 1500 rpm for 5 minutes, pelleted, and frozen at -80°C until RNA extraction (24, 25).

RNA extraction

RNeasy Mini-kit (Qiagen, Valencia, CA, USA, cat. no: 74004) was used according to the manufacturer's protocol to extract the RNA. The amount of RNA was measured by using a Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Nano Drop spectrophotometer).

cDNA synthesis

For cDNA synthesis, we used 30 ng total RNA according to the manufacturer's instructions in the QuantiTect Whole Transcriptome Kit (Qiagen, cat. no: 207045).

Quantitative real-time polymerase chain reaction

Polymerase chain reaction (PCR) targets were created from the template RNA using the manufacturer's protocols. Gene-specific primers were designed by using Perl Primer software. Then, the primers examined by Primer-BLAST to ensure that they were not linked to non-specific sites of the genome. The Primers used for real-time PCR are shown in the Table 1.

Table 1: Primer sets used for real-time RT-PCR

Gene	Primer sequence (5'-3')	Product size (bp)
<i>GAPDH</i>	F: AGAAGGCTGGGGCTCATTG	228
	R: TGATGGCATGGACTGTGGT	
<i>GDF9</i>	F: AGAAGTCACCTCTACAACACTG	132
	R: AACGGTAGTAATGCGATCCA	
<i>BMP15</i>	F: TGTGAACTCGTGCTTTTCATG	102
	R: CTCAATCAGGGGCAAAGTAGG	

mRNA quantification was performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a Step-One RT-PCR system (Applied

Biosystems, USA). Amplification of the genes of interest and *GAPDH* were performed in duplicate wells. *GAPDH* was used as the endogenous control for normalization.

Statistical analysis

Statistical calculations were performed using the IBM SPSS statistic 22 software (IBM, United states). The student's t test with a two-tailed distribution for equality of variances was used. $P < 0.05$ indicated statistical significance. Gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ algorithm to calculate the *GDF9* and *BMP15* mRNAs level relative to the level of *GAPDH*.

Results

Clinical characteristics

Table 2 demonstrates the demographic data and clinical characteristics of DOR and the NOR at baseline. There was no significant difference between the two groups in age and age at menarche.

The result of IVF revealed achieved oocytes, MII, two PN were higher in the NOR group compared to DOR patients these differences were statistically significant ($P < 0.05$, Table 2).

The expression profile for target genes in NOR and DOR groups granulosa cells

Quantitative expression of these genes in GCs of DOR patients and the control group was performed by RNA extraction and cDNA synthesis with primers designed for each gene. The *GAPDH* primer was used as the internal control for real-time PCR.

Table 2: Demographic characteristics, oocyte and embryo parameters between DOR and NOR groups

Criteria	Case (n=14)	Control (n=12)	P value*
Age (Y)	34.07 ± 3.91	34.25 ± 2.98	0.898
Age at menarche (Y)	13.71 ± 1.32	13.5 ± 0.797	0.63
Oocyte number	2.14 ± 0.66	7 ± 2.98	<0.00
MI	0.85 ± 0.37	1.62 ± 1.06	0.093
MII	1.42 ± 0.51	5.58 ± 2.46	<0.00
GV	1 ± 0	1.16 ± 0.408	0.51
2PN	1.41 ± 0.51	4.09 ± 2.73	0.003
Embryo quality			
Good (AB or B)	1.14 ± 0.37	3.82 ± 2.75	0.022
Fair (BC or C)	1.29 ± 0.48	1 ± 0	0.35
Poor (CD or D)	0	0	-

Data are presented as mean ± SD. *; Obtained by independent sample t test. Statistically significant level at 0.05. MI; Metaphase I, MII; Metaphase II, GV; Germinal vesicle, PN; Pronuclei, DOR; Diminished ovarian reserve, and NOR; Normal ovarian reserve.

The standard curve was used to evaluate the efficiency of primers and relative quantification. For normalization of the *GDF9* and *BMP15* genes and drawing a standard curve, was investigated in gene database sites such as NCBI and Gene Cards. The highest expression of these genes was found in testicular tissue. Therefore, standard curves were prepared by serial dilution to evaluate the efficiency of primers and real time PCR procedures.

GDF9 mRNA expressions in granulosa cells

All amplified RT-PCR products were at the expected size for *GDF9* and *GAPDH* (housekeeping) genes. *GDF9* expression in patients was 0.23 times lower than the control group, which was significant ($P < 0.0001$, Fig.1).

BMP15 mRNA expression in granulosa cells

All amplified RT-PCR products were at the expected size for *BMP15* and *GAPDH* (housekeeping) genes. *BMP15* expression in patients was 0.32 times lower than the control group, which was significant ($P < 0.0001$, Fig.1).

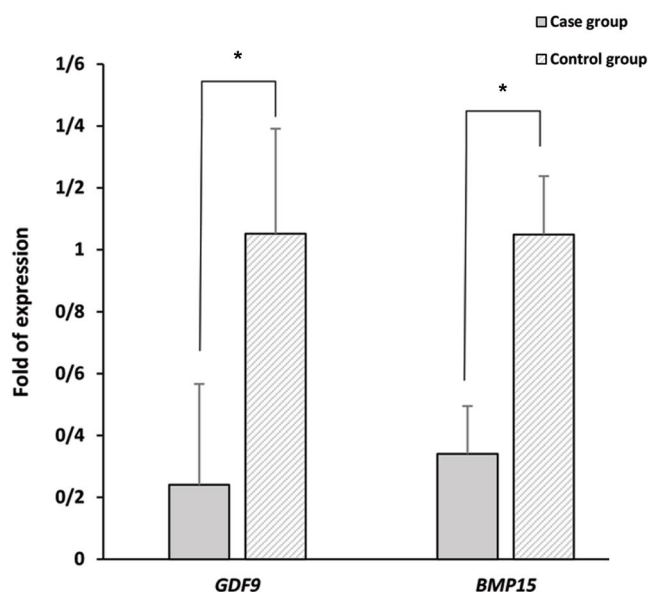


Fig.1: Comparison of *GDF9* and *BMP15* expressions between DOR and NOR groups. *. Statistical significant level at 0.05, DOR; Diminished ovarian reserve, and NOR; Normal ovarian reserve.

Comparison of elevated serum hormone levels

Although the serum level of AMH was significantly decreased in the case group, the mean serum level of FSH and LH on the third day of menstruation was significantly increased in cases in comparison with control participants ($P < 0.0001$, Fig.2). The serum thyroid-stimulating hormone (TSH) level was not different between the two groups ($P \geq 0.05$, Fig.2).

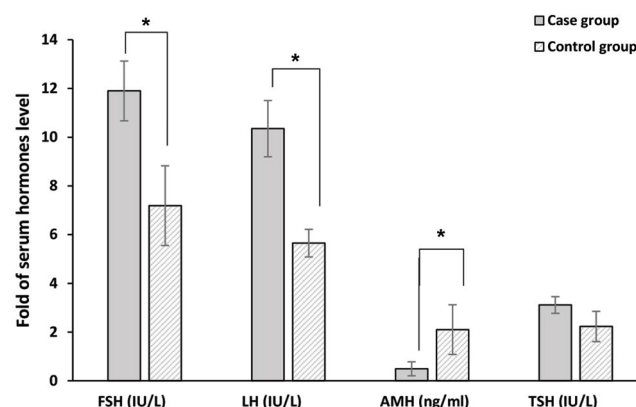


Fig.2: Mean serum hormones level between DOR and NOR groups. *. Statistical significant level at 0.05, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, AMH; Anti-Müllerian hormone, TSH; Thyroid-stimulating hormone, DOR; Diminished ovarian reserve, and NOR; Normal ovarian reserve.

Discussion

In our study, we assessed the expression of *GDF9* and *BMP15* in GCs of DOR patients with Bologna criteria and 28-40 years old. In comparison with male factor patients as a control group, we observed that the mean relative expressions of *GDF9* and *BMP15* were significantly lower in GCs of the infertile DOR patients compared with the control group.

Recent studies of genetic mutations in sheep, goats, and mice highlight the importance of oocyte-secreted factors in regulating ovarian follicular development and ovulation (13, 19, 26). Although some genetic causes of DOR are established, little is known about definitive gene mutations associated with DOR (2).

During the procedure of folliculogenesis, interplays between the oocyte and the somatic cells that surround it (GCs) are recognized that are the enduring effects of this interaction on the potential for the stages after fertilization for embryonic development (23).

The TGF- β superfamily, in particular *GDF9* and *BMP15*, oocyte-secreted growth factors play a critical role in ovarian organization and fertility. These factors are essential for the growth, development, and function of GCs according to the results from studies carried out on animals.

Knockout mouse technology has been used over the past decade to define the essential role of ovarian gene expression and the discovery of genetic interactions.

The distribution of the *GDF9*, *BMP15* mRNA, proteins, and BMP receptor mRNA, were evaluated in goat and bovine ovaries (13, 27, 28) to determine if these TGF- β members may play an important role in follicular development in goats (13).

In humans, there is a lack of expression level of these genes in DOR and only polymorphisms and mutations of *GDF9* are associated with DOR. In a study of Chinese

women, 3 out of 139 (2.2%) women with DOR had a specific mutation (p.R146C), whereas this mutation was not present in the control group (n=159) (15). In another study, *GDF9* was associated with DOR. A study compared 103 Chinese women with DOR to 123 age-matched women with normal ovarian reserve. The women were analyzed for three single nucleotide polymorphisms (SNPs) of *GDF9*. A higher prevalence of the GA/AA genotype was found in those with poor ovarian response (32%) during IVF cycles compared with those with the control group (19.5%) (29).

Recently, Gong et al. (30) had evaluated *GDF9* and *BMP15* expression in GCs of poor patients according to Bologna criteria. They subdivided patients via age including <35 years, 35-40 years, and >40 years. Also they observed the expression of these genes decreased with the age of poor patients especially after 40 years which related to lower oocyte quality and pregnancy outcome.

In the current study, the number of achieved oocytes, MII, and 2PN embryos were significantly lower in DOR patients. Since, these genes involved in folliculogenesis it seems lower expression of these genes involved in poor oocyte achievements in DOR patients. Because achieve oocyte from DOR patients is difficult analysis was performed in limited sample size and further studies with more samples is essential.

BMP15 and *GDF9* are oocyte-secreted factors that we assessed expression of these genes in the targeted tissue (granulosa cells) because of limited number of oocyte. A better suggestion is to check their receptor or target genes in GCs. In future, we collect enough samples and are going to study these receptor or target genes.

Conclusion

In the current study, we observed the significant reductions of *GDF9* and *BMP15* gene expressions in GCs of DOR patients compared to the control group. The decreased oocyte numbers and 2PN embryos in the DOR group might be secondary to the decrease in the expressions of the above genes. Based on the above findings and the information we have regarding the function of these two genes, it seems that there is a role for these paracrine factors in the folliculogenesis process as well as the interaction of GCs with oocytes which further studies with larger sample size is essential for its confirmation.

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Authors' Contributions

M.O.; Conceived and carried out experiments, literature

review, and manuscript drafting. A.M.M.; Administrative support, contributed to conception and design, and literature review. P.E.-Y.; Samples collection and draft revision. P.M., Z.Ch.; Contributed to the collection and assembly of data, draft revision. All authors read and approved the final version of the manuscript.

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