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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Decreased GDF9 and BMP15 in follicle fluid and granulosa cells and outcomes of IVF-ET among young patients with low prognosis

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Abstract

Purpose To analyze the level of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) in follicle fluid (FF) and granulosa cells (GCs) derived from young patients with low prognosis for *in vitro* fertilization and embryo transfer (IVF-ET) treatment.

Methods A prospective cohort study was carried out by enrolling 52 young patients with low prognosis according to the POSEIDON classification group 3 (low prognosis group) and 51 young patients with normal ovarian reserve (control group). The concentration of the GDF9 and BMP15 proteins in FF was determined by enzyme-linked immunosorbent assay. The mRNA level of the *GDF9* and *BMP15* in the GCs was measured by quantitative real-time PCR.

Results The concentration of GDF9 (1026.72 ± 159.12 pg/mL *vs.* 1298.06 ± 185.41 pg/mL) and BMP15 (685.23 ± 143.91 pg/mL *vs.* 794.37 ± 81.79 pg/mL) in FF and the mRNA level of *GDF9* and *BMP15* in the GCs and the live birth rate per treatment cycle started (30.77% *vs.* 50.98%) and oocytes retrieved (4.25 ± 1.91 *vs.*12.04 ± 4.24) were significantly lower, whereas the canceled cycle rate was significantly higher (9.62% *vs.* 0) in the low prognosis group compared with the control group ($P < 0.05$). The expression of GDF9 and BMP15 in the ovary was positively correlated with live birth ($P < 0.05$).

Conclusion The expression of GDF9 and BMP15 in the ovary was decreased in young patients with low prognosis accompanied by a poorer outcome of IVF-ET treatment.

Trial registration ChiCTR1800016107 (Chinese Clinical Trial Registry), May 11, 2018. (http://www.chictr.org.cn/edit.aspx?pid=27216&htm=4).

Keywords GDF9 · BMP15 · Low prognosis · *In vitro* fertilization · Young women

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Introduction

Poor ovarian response (POR) poses a big challenge for *in vitro* fertilization and embryo transfer (IVF-ET) treatment. Poor ovarian responders have fewer oocytes and cleaved embryos, lower pregnancy rate, and a higher risk of miscarriage and canceled cycles [1–3]. The incidence of POR has ranged from 9 to 24% among various IVF centers [4]. The causes of POR have included advanced maternal age, ovarian and pelvic surgeries, autoimmune disorders, cigarette smoking, chemotherapy, and radiotherapy [5]. The physiopathology of POR is complex, which include follicular loss by atresia or apoptosis, endocrine disorders of the follicle, genetic mutations, chromosomal aberrations, and mitochondrial dysfunction of the oocytes [6–8].

Oocyte-secreted factors (OSFs), including the transforming growth factor β (TGF-β) superfamily, participate in the control of the function of granulosa cells (GCs) [9]. The TGF-β superfamily plays an essential role in the regulation of ovarian functions [10]. Growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*, also known as *GDF9B*), which respectively map to 5q31.1 and Xp11.22, are two important members of the TGF-β superfamily [11] and are mainly secreted by oocytes and can be detected in serum, follicle fluid (FF), and GCs [9, 12–14]. Both *GDF9* and *BMP15* play important roles in follicular development including preantral follicle recruitment, cumulus expansion, oocyte maturation, and ovulation [9, 10]. Previous studies have shown that mutation, decreased expression, and abnormal structure of the *GDF9* and *BMP15* genes may predispose to follicle atresia, multiple ovulation, and early exhaustion of the ovarian reserve [10, 15]. These pathological changes are associated with multiple pregnancy, premature ovarian insufficiency (POI), and female infertility [7, 10, 15]. Furthermore, the expression of the *GDF9* and *BMP15* genes has been associated with oocyte maturation, fertilization, embryo quality, and outcome of IVF treatment [12]. Some researchers have proposed that they may be used as biomarkers for predicting the potential of oocyte development [10, 12].

The Bologna criteria for the diagnosis of POR grouped together women who have differed significantly in biologic characteristics [1]. Therefore, in 2016, the POSEIDON (Patient-Oriented Strategies Encompassing Individualized Oocyte Number) classification has adopted a more specific new definition for "low prognosis" in place of POR [16]. The POSEIDON classification has divided patients into four groups with different degrees of low prognosis: Group 1, patients < 35 years with normal pre-stimulation ovarian reserve parameters (antral follicle count (AFC) ≥ 5, anti-Müllerian hormone (AMH) ≥ 1.2 ng/mL) and with an unexpected poor or suboptimal ovarian response; Group 2, patients ≥ 35 years with normal pre-stimulation ovarian reserve parameters and with an unexpected poor or suboptimal ovarian response; Group 3, patients <35 years with low ovarian reserve pre-stimulation parameters (AFC < 5 and/or AMH < 1.2 ng/mL); and Group 4: patients ≥ 35 years with low ovarian reserve pre-stimulation parameters [16].

For group 3, the prognosis is relatively good compared with groups 2 and 4 [16, 17]. We have previously found that the expression of GDF9 and BMP15 in the FF and GCs from young poor responders (similar to POSEIDON group 3) was significantly higher compared with older poor responders (age ≥ 35) and was associated with higher pregnancy rate [18]. However, few studies exist of the *GDF9* and *BMP15* in such patients and GDF9 and BMP15 in the ovary during IVF cycles in comparison with young controls have not been evaluated [7, 15, 19]. In view of their significant role in folliculogenesis, to determine their expression may be valuable for revealing the pathogenesis of poor ovarian response and formulating treatment protocols for such patients. Oocytes are supported and nourished by an intimate cross-talk with surrounding GCs, and the composition of FF may reflect the metabolic and endocrine status of the oocyte and facilitate interrogation of the biological process in the oocytes [20]. Therefore, this study was designed to analyze the IVF outcomes and the expression of GDF9 and BMP15 in the FF and GC of young patients with low prognosis.

Materials and methods

Ethics statements

The prospective cohort study was registered with the Chinese Clinical Trial Registry Center (Registration No. ChiCTR1800016107) and approved by Sichuan Provincial Women's and Children's Hospital (No. 20170724). Written informed consents have been obtained from all participants. All procedures have complied with the Ethical Standards for Human Experimentation of the Helsinki Declaration (2013 revision).

The primary outcome was the expression of *GDF9* and *BMP15* in FF and GCs. The secondary outcomes were treatment outcomes including oocyte and embryo quality, implantation rate, ongoing pregnancy rate, and live birth rate (LBR). Calculation of the sample size was based on the ongoing pregnancy rate in our center. The ongoing pregnancy rate per treatment cycle started of young poor

ovarian responders and age-matched tubal infertility patients were approximately 26% and 53%, respectively. As a result, 51 patients were assigned to each group, with α type error = 0.05 and β type error = 0.2 (power of statistical test = 80%), and a dropout rate of 6%.

Study population

Young patients with low prognosis diagnosed according to the criteria of POSEIDON classification group 3 (low prognosis group, $n = 52$) [16] and tubal infertility (control group, $n = 51$) in the Reproductive Medicine Centre were enrolled in the study from January 2019 to June 2021. The patients were 23 ~ 34 years old and underwent IVF-ET treatment. Patients were excluded should they meet any of the following criteria: (1) congenital uterine malformation, endometriosis, polycystic ovarian syndrome (PCOS), ovarian surgery and chemotherapy, and intrauterine adhesion; (2) systemic lupus erythematosus and/or sicca syndrome; (3) uncontrolled endocrinopathy such as diabetes, hyperthyroidism, hypothyroidism, and hyperprolactinemia; (4) basal follicle stimulating hormone (bFSH) ≥ 15 IU/L; (5) underwent IVF-ET treatment within three months; (6) regular cigarette smoking and/or alcohol consumption (five times per week within three months or discontinuous for at least one year); and (7) all intracytoplasmic sperm injection (ICSI) cycles, including male factor infertility to avoid the influence of sperm and the different fertilization protocol on the quality and development potential of embryos. In addition to the above criteria, patients with poor ovarian reserve (AFC < 5 and/or AMH < 1.2 ng/mL) were excluded from the control group. Medical history was taken from all participants, including regularity of menstrual cycle, duration of infertility and pre-treatment protocol. Abnormal menstruation patterns included oligomenorrhea (defined as menstrual cycles lasting more than 35 days or a menstrual pattern of fewer than eight periods per year), amenorrhea (defined as absence of menstruation for more than 6 months or absence of menstruation over three periods according to the woman's original menstrual cycle), and abnormal uterine bleeding (defined as bleeding from the uterine corpus with abnormal volume, regularity, and/or timing in non-pregnant women) [21]. Height and body weight without shoes and heavy clothing were measured. Body mass index (BMI) was calculated as body weight divided by the square of height (kg/m^2). On day 2 ~ 3 of menstruation or progestin induced withdrawal bleeding, blood samples were collected from elbow veins after an overnight fast. Antral follicles were counted by transvaginal ultrasonography.

Regimen of controlled ovarian stimulation (COS) and IVF

All patients underwent a GnRH antagonist protocol for controlled ovarian stimulation (COS). Human rFSH (Gonal-F, Merck-Serono KGaA., Darmstadt, Germany; Puregon®, Merck Sharp & Dohme, USA) was started from day 2 of the menstrual cycle, and the dosage was adjusted according to the follicular growth. Ganirelix (Merck Sharp & Dohme, USA) was administered when follicle diameter was > 13 ~ 14 mm. 250 μg of recombinant human chorionic gonadotrophin (rHCG) (Merck Sharp & Dohme, USA) was injected as the trigger when the leading follicle diameter reached ≥ 18 mm, or at least two follicles have reached a mean diameter of ≥ 17 mm. Oocytes were retrieved under transvaginal ultrasound guidance 36 h later without follicle flushing.

After 4 to 6 hours of conventional fertilization, mature oocytes with the first polar body were defined as being at the metaphase II (MII) stage, and 17 to 18 hours later, oocytes with 2 pronuclei were defined as normally fertilized. Cleaved embryos on day 3 were evaluated according to the Istanbul criteria [22]. Embryos of grade A ~ C were considered transferable, and higher-quality embryos were categorized as grades A or B [22]. Only one day 3 embryo was transferred should there be only one transferable embryo (grade A), or if the patient wanted to reduce the risk of twins. Otherwise, two embryos were transferred. Starting on the day after oocyte retrieval, progesterone oil (Zhejiang Xianju Pharmaceutical Co. Ltd. Taizhou, China) was injected 60 mg/d as luteal phase support. Ovarian hyperstimulation syndrome (OHSS) was diagnosed by following the proposal of Navot et al. [23].

The reasons for the cancellation of oocyte retrieval included follicular growth failure (10 days after COS, leading follicle diameter < 10 mm) and premature ovulation. Patients with abnormal endometrium thickness (< 7 mm or > 15 mm) or progesterone level (> 2.0 ng/mL) on the trigger day, or OHSS underwent frozen-thawed embryo transfer one month later. All other patients underwent fresh embryo transfer. Only the first embryo-transfer cycle was included in this study.

The rates of maturation or fertilization referred to the number of mature oocytes or normally fertilized oocytes divided by the number of all retrieved oocytes, respectively. The rate of cleaved embryo or higher-quality embryo referred to the number of grade A ~ C embryos or A ~ B embryos on day 3 divided by the number of normally fertilized oocytes. The definition of clinical pregnancy, and the rates of implantation and multiple pregnancy were calculated as reported previously [24]. Ongoing pregnancy was defined as detection of fetal heartbeat at 12 weeks gestation

[25]. The miscarriage rate was calculated as the number of miscarriage cycles before 12 weeks of pregnancy divided by the number of clinical pregnancy cycles. Live birth was defined as a live fetus born after 24 weeks of gestation. Patients were followed up until delivery.

Experimental procedures

Measurement of endocrine parameters

Estrogen, progesterone (P), total testosterone (TT), prolactin, luteinizing hormone (LH), FSH, and insulin levels were measured on an electrochemiluminescence immunoassay platform (Roche Diagnostics GmbH, Mannheim, Germany). Plasma glucose was measured by using the hexokinase method (Beijing Strong Biotechnologies, Inc., Beijing, China). AMH was measured with an enzyme-linked immunosorbent assay kit (Guangzhou Kangrun Biotech, Co. Ltd, Guangdong, China). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5. The intra- and inter-assay coefficients for the above variation values were set as < 5% and 10%, respectively.

Follicular fluid and primary granulosa cell isolation

FF and GCs from each patient were collected and considered as one sample. Briefly, the FF was collected from all follicles with a diameter of ≥ 16 mm measured on the day of oocyte retrieval without flushing and immediately centrifuged at 700 × *g* for 5 min at room temperature. The supernatant was stored at – 80 °C for the detection of GDF9 and BMP15 with ELISA. The precipitate was suspended in 2 mL FF and gently layered into 3 mL of 50% lymphocyte separation medium (Beijing Solarbio Science and Technology Corporation, Beijing, China). Red blood cells and debris were removed by centrifugation at 700 × *g* for 10 min. GCs layered at the interface of the gradient were collected and washed with 5 mL of phosphate buffer solution (PBS) (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, Jiangsu, China). Residual red blood cells were removed with a red blood cell lysis buffer (Solarbio Science and Technology Corporation, Beijing, China) and washed with 5 mL of PBS (Nanjing KeyGen Biotech. Co. Ltd., Jiangsu, China). The GCs were stored at – 80 °C immediately for future use.

Detection of GDF9 and BMP15 in the FF with ELISA

The concentrations of the GDF9 and BMP15 in FF were respectively determined by using Human GDF9 and Human BMP15 ELISA Kits (Elabscience Biotechnology Co. Ltd., Wuhan, Hubei, China). The specificity of the two ELISA kits have been described in our previous paper [18]. The supernatant of the FF samples for each patient was thawed and pooled together, and 100 µL was added to each well in duplicate according to the instructions from the manufacturer without concentrating or diluting. The absorbance value was measured at 450 nm with a Perlong DNM-9602G microplate spectrophotometer (Perlong New Technology Co. Ltd., Beijing, China). The sensitivity of the assays was 9.38 pg/mL.

Determination of GDF9 and BMP15 expression in the GCs by quantitative real-time polymerase chain reaction

A RNAprep Pure Micro kit (Tiangen Biotech Co. Ltd., Beijing, China) was used to isolate total RNA from the GCs after rapid thawing, and the quality of RNA was examined at 260 nm/280 nm by Nanodrop-2000 (Thermo Fisher Scientific, Waltham, Massachusetts, American). Synthesis of cDNA from total RNA was accomplished with a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan). By using a TB Green™ Premix Ex Taq™ II kit (TaKaRa), cDNA was amplified through quantitative real-time PCR (qRT-PCR) in triplicate. The specificity of the amplification was checked by melting curve analysis. The mRNA level of the target genes was calculated by using the $2^{-\Delta CT}$ method and expressed as a fold change relative to that of the internal control (glyceraldehyde-3-phosphate-dehydrogenase, *GAPDH*). All experiments were repeated for three times. The sequences of primers for *GDF9*, *BMP15*, and *GAPDH* were listed in Table 1.

Statistical analysis

All data were analyzed by using SPSS 20.0 software (SPSS Inc., Chicago IL, USA). The Kolmogorov–Smirnov test was used to assess for normality of data distribution. Continuous variables with normal distribution were

Table 1 Sequences of qRT-PCR primers

Gene	Primer (5′ → 3′)	Annealing temperature (°C)
GDF9	F: TGGAGCATCCTTCAGCAC R: GCAGCCTCTTCTCCCACA	57.2
BMP15	F: TTTACCGCCATCATCTCCAA R: TTTCCAAGCGTTAGACATCA	53.4
GAPDH	F: ACGGATTTGGTCGTATTGGG R: CGCTCCTGGAAGATGGTGAT	57.4

expressed as mean ± standard deviation (SD) and compared with independent-samples *t* test. Categorical data were compared using Chi-squared test. In young patients with low prognosis, Pearson's correlation was applied to analyze the correlation between GDF9 and BMP15 with AMH level, and Spearman rank correlation was applied to analyze the correlation between GDF9 and BMP15 with live birth and the correlation between AMH with live birth. Two-tailed P value < 0.05 was considered as statistically significant.

Results

Basic characteristics of the study population

No patients dropped out. Among the young patients with low prognosis, 11 had abnormal menstrual cycles. Compared with the control group, their AFC and AMH were significantly lower, whereas abnormal menstrual cycle rate, basal FSH, basal E_2, and FSH/LH ratio were significantly higher in the low prognosis group ($P < 0.05$). The average age, duration of infertility, BMI, basal LH, P, TT, PRL, fasting insulin (FINS), fasting plasma glucose (FPG), and HOMA-IR did not significantly differ between the two groups ($P > 0.05$) (Table 2).

Controlled ovarian stimulation and outcomes of IVF-ET treatment

Oocyte retrieval was canceled due to failure of follicle growth in one patient from the low prognosis group. No oocytes could be retrieved during follicle aspiration in one patient from the low prognosis group. There were no transferable embryos in 3 patients from the low prognosis group (no normal fertilization or cleavage) (Fig. 1).

Fresh embryo transfer was cancelled for one patient from the control group due to high levels of progesterone. Fresh embryo transfer was cancelled for two patients from the low prognosis group due to abnormal endometrium. All these patients underwent frozen-thawed embryo transfer at least one month later, which resulted in a clinical pregnancy for one patient from the low prognosis group.

Compared with the control group, the E_2 level on the trigger day, the number of oocytes, the implantation rate, the ongoing pregnancy rate, and LBR per treatment cycle started in the low prognosis group were significantly lower, whereas their dosage of rFSH and canceled cycle rate were significantly higher ($P < 0.05$). The duration of COS, number of embryos per ET, rate of maturation, fertilization, cleaved embryo, higher-quality embryo, ongoing pregnancy and live birth per ET cycle, miscarriage, and multiple pregnancy did not significantly differ between the two groups ($P > 0.05$). No

Table 2 Basal characteristics of the study population

	Control group ($n = 51$)	Low prognosis group ($n = 52$)	P value
Age (yrs)	29.49 ± 2.66	30.37 ± 3.49	0.156
Abnormal menstrual cycle	0/51	21.15% (11/52)	0.001
Duration of infertility (yrs)	3.90 ± 2.67	4.15 ± 3.08	0.658
BMI (kg/m^2)	21.55 ± 2.66	22.38 ± 3.02	0.142
AFC	14.90 ± 4.60	5.46 ± 1.26	< 0.001
FSH (mIU/mL)	6.15 ± 1.59	8.26 ± 2.52	< 0.001
LH (mIU/mL)	4.85 ± 2.51	4.20 ± 1.48	0.112
FSH/LH ratio	1.59 ± 0.96	2.14 ± 0.86	0.003
E_2 (pg/mL)	40.45 ± 16.24	52.02 ± 23.49	0.005
P (ng/mL)	0.30 ± 0.29	0.32 ± 0.20	0.723
TT (ng/mL)	0.26 ± 0.16	0.22 ± 0.13	0.193
PRL (μIU/mL)	201.30 ± 48.86	206.72 ± 56.82	0.605
AMH (ng/mL)	3.29 ± 1.48	0.82 ± 0.46	< 0.001
FPG (mmol/L)	4.65 ± 0.33	4.70 ± 0.33	0.429
FINS (micro IU/L)	9.85 ± 3.26	10.07 ± 3.28	0.743
HOMA-IR	2.03 ± 0.64	2.10 ± 0.69	0.581

Continuous variables are presented as mean ± SD. Categorical characteristics are presented as numbers and percentages. Abbreviations: *BMI*, body mass index; *AFC*, basal antral follicle count; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; E_2, estradiol; *P*, progesterone; *TT*, total testosterone; *PRL*, prolactin; *AMH*, anti-Müllerian hormone; *FPG*, fasting plasma glucose; *FINS*, fasting insulin; *HOMA-IR*, homeostatic model assessment of insulin resistance. Continuous variables were compared with independent-samples *t* test. The rate of abnormal menstrual cycle was compared with Chi-square test. $P < 0.05$ was considered as statistically significant

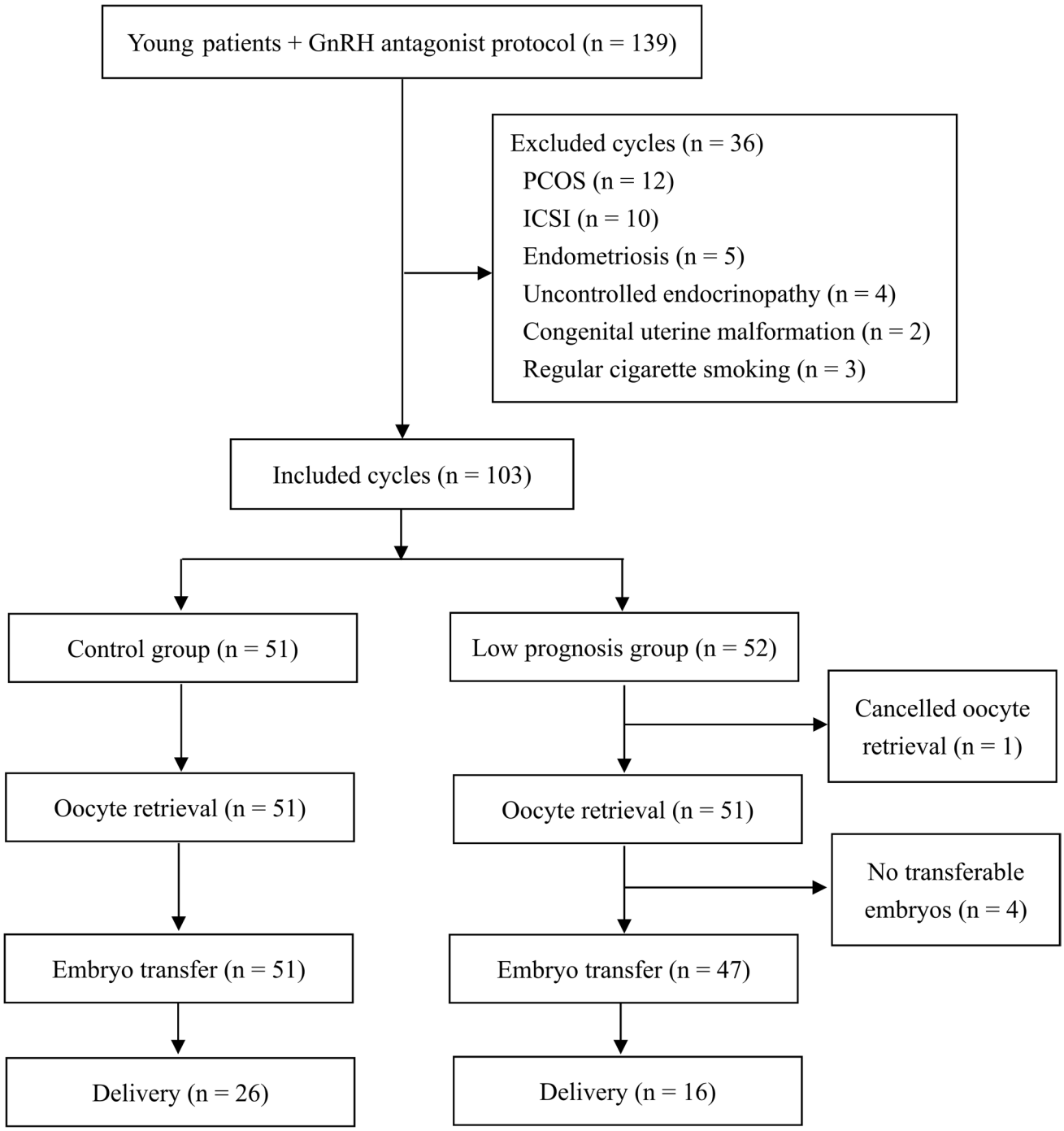

Fig. 1 Flowchart for patient selection and treatment

moderate and severe OHSS and no ectopic pregnancy were reported in the study population (Table 3).

No pregnancy complication was found in these patients. The mean gestational age at delivery was 38 weeks (range, 35 ~ 40 weeks) and the mean birth weight was 3089 g (1800 ~ 3950 g). One woman from the control group had delivered at 35 weeks. No birth defect was noted among the 49 live-born infants.

Decreased concentration of GDF9 and BMP15 in FF from young patients with low prognosis

The concentrations of GDF9 (1026.72 ± 159.12 pg/mL *vs.* 1298.06 ± 185.41 pg/mL, $P < 0.001$) and BMP15 (685.23 ± 143.91 pg/mL *vs.* 794.37 ± 81.79 pg/mL, $P < 0.001$) were significantly lower in the FF derived from the low prognosis group as compared with the control group (Table 3).

Table 3 Controlled ovarian stimulation, IVF outcome, and the concentration of GDF9 and BMP15 in the FF

	Control group ($n = 51$)	POR group ($n = 52$)	P value
Treatment cycles	51	52	
Oocyte retrieval cycles	51	51	
ET cycles	51	47	
Cycle cancellation rate (%)	0/51	9.62% (5/52)	0.023
Dosage of rFSH (IU)	1720.59 ± 577.06	2319.23 ± 656.17	< 0.001
Duration of COS (days)	9.67 ± 1.76	9.25 ± 1.77	0.234
E_2 on the trigger day (pg/mL)	1990.01 ± 606.05	887.59 ± 395.51	< 0.001
Endometrial thickness (mm)	9.96 ± 1.77	9.65 ± 2.11	0.417
Numbers of oocytes retrieved	12.04 ± 4.24	4.25 ± 1.91	< 0.001
Maturation rate (%)	86.64% (532/614)	87.62% (177/202)	0.721
Fertilization rate (%)	73.78% (453/614)	70.30% (142/202)	0.334
Cleaved embryo rate (%)	82.12% (372/453)	79.58% (113/142)	0.496
Higher-quality embryo rate (%)	39.29% (178/453)	32.39% (46/142)	0.139
Numbers of embryos per ET	1.84 ± 0.37	1.87 ± 0.34	0.684
Implantation rate (%)	37.23% (35/94)	22.73% (20/88)	0.033
Ongoing pregnancy rate per treatment cycle started (%)	50.98% (26/51)	30.77% (16/52)	0.037
Ongoing pregnancy rate per ET cycle (%)	50.98% (26/51)	34.04% (16/47)	0.091
Miscarriage rate (%)	13.33% (4/30)	11.11% (2/18)	0.822
Multiple pregnant rate (%)	16.67% (5/30)	11.11% (2/18)	0.598
LBR per treatment cycle started (%)	50.98% (26/51)	30.77% (16/52)	0.037
LBR per ET cycle (%)	50.98% (26/51)	34.04% (16/47)	0.091
In FF			
GDF9 (pg/mL)	1298.06 ± 185.41	1026.72 ± 159.12	< 0.001
BMP15 (pg/mL)	794.37 ± 81.79	685.23 ± 143.91	< 0.001

Continuous variables are presented as mean ± SD. Categorical characteristics are presented as numbers and percentages. Abbreviations: *ET*, embryos transferred; *COS*, controlled ovarian stimulation, E_2, estradiol; *LBR*, live birth rate; *FF*, follicle fluid; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15

Continuous variables were compared with independent-samples *t* test. Chi-squared test was used to compare the rates of maturation, fertilization, cleaved embryo, higher quality embryo, cycle cancellation, implantation, miscarriage, multiple pregnancy, and ongoing pregnancy rate between the two groups. $P < 0.05$ was considered as statistically significant

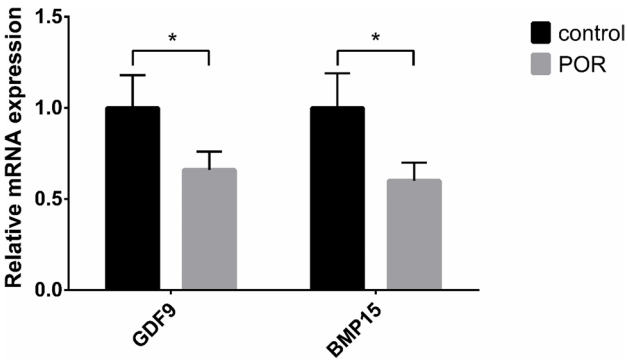

Fig. 2 Decreased mRNA levels of *GDF9* and *BMP15* in the GCs derived from young low prognosis patients undergoing IVF-ET treatment. The mRNA level of *GDF9* and *BMP15* was significantly decreased in the low prognosis group compared with the control group ($P < 0.05$). *GAPDH* was used as the internal control. Only patients with oocyte retrieval were included. The relative mRNA level was compared by using the independent-samples *t* test

Decreased expression of GDF9 and BMP15 in the GCs from young patients with low prognosis

As shown in Fig. 2, the mRNA level of the *GDF9* and *BMP15* was significantly lower in the GCs derived from the low prognosis group compared with the controls. Only patients with oocyte retrieval were included. The relative mRNA level was compared by using the independent-samples *t* test. *GAPDH* was used as the internal control.

The expression of GDF9 and BMP15 in ovary positively correlated with live birth in young patients with low prognosis

As shown in Table 4, the expression of protein and mRNA of GDF9 and BMP15 in the ovary was positively correlated with

Table 4 Correlation between GDF9 and BMP 15 with AMH and live birth in patients with low prognosis

	FF GDF9 (pg/mL)		FF BMP15 (pg/mL)		*GDF9* in GCs		*BMP15* in GCs		AMH	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Live birth	0.349	0.016	0.341	0.019	0.363	0.014	0.313	0.036	-0.175	0.238
AMH (ng/mL)	-0.125	0.403	0.073	0.624	0.042	0.785	-0.171	0.262	-	-

Abbreviations: *FF*, follicle fluid; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; *GCs*, granulosa cells; *AMH*, anti-Müllerian hormone; *r*, correlation coefficient

Spearman rank correlation was applied to analyze the correlation between GDF9, BMP 15, and AMH with live birth. Pearson's correlation was applied to analyze the correlation between GDF9 and BMP 15 with AMH. $P < 0.05$ was considered as statistically significant

live birth in young patients with low prognosis by Spearman rank correlation ($P < 0.05$), whereas it had no correlation with AMH by Pearson's correlation analysis ($P > 0.05$). AMH had no correlation with live birth by Spearman rank correlation ($P > 0.05$).

Discussion

This study found significantly decreased expression of GDF9 and BMP15 in FF and GCs derived from young patients with low prognosis, accompanied by a higher cycle cancellation rate, retrieval of fewer oocytes, lower ongoing pregnancy rate, and LBR per treatment cycle started compared with young patients with normal ovarian reserve. Even in young patients with low prognosis, GDF9 and BMP15 expressions were positively correlated with live birth regardless of the AMH levels.

GDF9 and BMP15 can promote folliculogenesis by acting on the GCs from the following aspects: (1) to promote cell proliferation and suppress apoptosis [26], (2) to augment the effects of FSH and insulin-like growth factor I (IGF-I) and promote the synthesis and secretion of E_2 [27], (3) to promote the differentiation and expansion of cumulus cells in response to the LH surge [28], and (4) to promote glycolysis for oocyte development [29]. Previous studies have found that the follicular development was stagnant in the primary follicular stage, while the meiotic competence in the peri-ovulatory stage was jeopardized in GDF-9-deficient mice [30, 31].

In the present study, we found that both *GDF9* and *BMP15* were decreased in the FF and GCs derived from young patients with low prognosis, which was in keeping with results reported by others. Wang et al. [15] have identified a *GDF9* p.R146C mutation along with reduced production of mature GDF9 protein among young women (< 37 years old) with poor ovarian reserve. The ability of GDF9 to stimulate GC proliferation was also reduced. Variants of the *GDF9* and *BMP15* genes have also been identified among patients with the POI phenotype [7, 15]. Such variants may result in abnormal expression and protein structure, along with abnormal follicular development [32, 33]. Decreased *GDP9* and *BMP15* expressions may also account for the retrieval of fewer oocytes among the young patients with low prognosis in our study. Nevertheless, Zhong et al. [34] have noted that BMP-15 expression in the FF was slightly increased among such patients. A plausible explanation to this inconsistency include (1) the average age of the subjects in their study (34 years old) was older than our study (30 years old), while the change of *GDF9* and *BMP15* expressions with age is not completely clear; (2) the smaller sample size in Zhong' study (14 poor responders) [34]; and (3) the difference in the controlled ovarian stimulation (COH) protocols (mid-luteal long agonist protocol *vs.* antagonist protocol). Riepsamen et al. [14] have found serum BMP15, but not GDF9 expression, to be lower in women over 55 years ($n = 25$) compared with those of reproductive age ($n = 6$). Their sample size was relatively small, and only part of the samples were tested. They have also tested BMP15 and GDF9 expressions in FF but did not report the results as the samples were diluted to various degrees by media/saline during oocyte retrieval. In addition, the expression of BMP15 and GDF9 in the GCs and outcome of IVF-ET were not reported, though such data are critical for assessing the impact of BMP15 and GDF9 expression on poor responders. Our study also revealed the expression of *GDF9* and *BMP15* to be decreased in GCs from young patients with low prognosis, which coincided with the alteration in FF [18]. Notably, in both the low prognosis and control groups, *BMP15* expression was lower than *GDF9* expression in both FF and GCs, which was in keeping with a previous report that the expression of *BMP15* in gonads was lower compared with *GDF9* [35].

In this study, the quality of oocytes and embryos was not significantly decreased among the young patients with low prognosis. Galey-Fontaine et al. [2] also discovered that age can be used as predictive factors for the outcome of IVF-ET treatment in poor responders. They also found that young poor responders (< 36 years old) had more oocytes retrieved and MII oocytes compared with older poor responders (≥ 36 years old). Our previous study found that the expression of *GDF9* and *BMP15* declined along with increased age, especially in those over 40, which was also associated with poorer oocyte quality [18]. It may therefore be possible that the degree of reduction in the expression of *GDF9* and *BMP15* may not be enough to influence the quality of oocytes in young patients with low prognosis. Another plausible explanation for this may be the age-related decline of mitochondrial function, which can affect the quality of oocytes [36].

A high cycle cancellation rate can prevent some patients with low prognosis to reach the embryo transfer stage, which may in part explain the significantly decreased ongoing pregnancy rate and LBR per treatment cycle started. Saldeen et al. [3] also noted that poor responders had significantly lower rates for pregnancy and live birth, whereas higher cancellation rate compared with normal to high responders in both younger (≤ 37 years) or elder (> 37 years) patients. In our study, the ongoing pregnancy rate and LBR per ET cycle of the young patients with low prognosis were not significantly decreased. However, Bai et al. [37] in a larger study found that the clinical pregnancy rate per ET cycle was decreased in 166 young poor responders (< 35 years old) compared with 409 normal responders.

Some have reported that age, ovarian reserve (basal FSH level), and gonadotrophin dosage could predict the outcome of IVF-ET in poor responders [2, 3]. In this study, we found that in young patients with low prognosis, the expression of GDF9 and BMP15 in the ovary was positively correlated with live birth regardless of AMH levels, suggesting that the two markers may be used as predictors for the outcome of IVF-ET treatment. Other researchers have also proposed that these may be used as biomarkers for predicting the potential of oocyte development [10, 12]. Furthermore, we also found that AMH was not correlated with live birth in young patients with low prognosis, similar with other study [38]. GDF9 and BMP15 are mainly secreted by oocytes and AMH is secreted by GCs, which may be the reason that GDF9 and BMP15 are valuable for predicting the outcomes of IVF-ET.

In summary, our study has shown decreased expression of *GDF9* and *BMP15* in the ovaries of young patients with low prognosis, which was positively correlated with live birth. We propose that *GDF9* and *BMP15* are involved in the pathogenesis of early-onset poor ovarian reserve, though the underlying mechanism is not yet fully understood. Further research, including *in vitro* experiments, is warranted. Augmentation of the two proteins may be beneficial for young patients with low prognosis, but this will require thorough research as well as ethical approval.

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Author contribution Tian-hong Huang wrote the manuscript. Jesse Li-Ling and Kun Zhang critically read and revised the manuscript. Ya-nan Zhang and Jia-jing Wei helped with sample collection. Jiu-zhi Zeng helped with patient enrollment. Fu-rui Chen, Fang-yi Long, and Qiao-ying Zhu carried out the laboratory work. Shi-qi Chen conducted the data analysis. Yan Gong designed the study. All authors have read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This study was approved by the Medical Ethics Committee of Sichuan Provincial Women's and Children's Hospital. The procedures used in this study have adhered to the tenets of the Declaration of Helsinki.

Consent to participate Written informed consent has been obtained from all participants.

Conflicts of interest The authors declare that there is no conflict of interest.

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