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Cholesterol metabolism decreased in patients with diminished ovarian reserve

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#These authors' contributions are the same

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Abstract

Research question: The aim of this study was to investigate the role of decreased

cholesterol metabolism in the pathogenesis of diminished ovarian reserve (DOR).

Design: The current research included 72 women with normal ovarian reserve (NOR), 86 women with DOR. Then, the data of cholesterol metabolism in granulosa cells (GCs) of these women were analyzed.

Results: On the day of human chorionic gonadotropin injection, estradiol (E_2) and progesterone (P) in DOR group were significantly lower than those in the control group ($P < 0.05$). There were no significant differences in total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) between NOR and DOR groups. The cholesterol-regulated genes SREBF and SCAP, SCAP in GCs from women with DOR were downregulated ($P < 0.05$). Cholesterol synthesis and transport genes (e.g. IDI1, FDFT1, CYP51A1, SRB1 and STARD1) were also significantly decreased ($P < 0.05$). In females with DOR, cholesterol-related substances such as coprostanone, 11A-acetoxyprogesterone, hydroxyprogesterone were significantly reduced ($P < 0.05$). CYP19A1, a key steroidogenesis gene, were significantly reduced. 17α-hydroxyprogesterone (17OH-P) and E_2 decreased ($P < 0.05$).

Conclusion: Decreased cholesterol metabolism affect steroid hormone synthesis in GCs, which might be a possible mechanism for DOR.

Keywords

Diminished ovarian reserve, Cholesterol metabolism, Steroid hormones

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1. Introduction

Diminished ovarian reserve (DOR) is defined as a decline in the quantity and quality of the retrieved oocytes, with clinical manifestations of oligomenorrhea, menorrhagia, amenorrhea, and infertility. The prevalence of DOR ranges from 6% to 64% at different ages(Mutlu and Erdem 2012). DOR is associated with a reduction in the live birth rate and clinical pregnancy rate and an increase in abortion rate(Broekmans et al. 2006). Although, genetic, idiopathic, autoimmune, iatrogenic and familial factors can account for most cases(Richardson et al. 2014), the exact etiology of DOR remains further investigation. Thus, young women patients seeking for assisted-reproduction but with DOR are one of the greatest challenges for in vitro fertilization (IVF).

Human folliculogenesis relies on the precise control of the oocyte maturation and the proliferation of surrounding granulosa cells (GCs). GCs provide necessary nutrients and promote maturation signals for the oocyte(Dumesic et al. 2015). In contrast, oocyte-secreted factors accelerate GCs differentiation and mucification(Gilchrist et al. 2004). Several studies have supported that energy generation for oocyte maturation is through GCs, particularly in bovine(Steeves and Gardner 1999), feline(Spindler et al. 2000) and porcine oocytes(Herrick et al. 2006). Sanchez and colleagues(Sanchez et al. 2014) reported the apoptosis rate of GCs linked to oocytes quality. For these reasons, analyzing and comparing GCs changes could be a key to clarify the etiologies of DOR. Current evidence has demonstrated that plasma lipoproteins are the main source of cholesterol for steroid production in the ovaries(Azhar et al. 2003). Existing reports indicated that dyslipidemia in patients with DOR, but specific parameter variations are controversial(Tehrani et al. 2014; Verit et al. 2016; Verit et al. 2014). In GCs, it is essential to determine whether cholesterol metabolism can affect oocyte development in DOR.

As a cholesterol precursor, steroid hormone maintains normal reproductive development and function, as well as bodily homeostasis(van Montfoort et al. 2014). It is known that alterations in the metabolic status may have a negative impact on

female fertility(Li et al. 2018). The arrest of antral follicle growth in women with polycystic ovarian syndrome is associated with altered steroidogenesis(Franks et al. 2008). Cholesterol homeostasis is crucial for cell viability and the coordinator node could regulates uptake, synthesis, efflux and storage of cholesterol(Cruz et al. 2013). Sterol regulatory element-binding factor (SREBF) and its ligand (SCAP) could control cellular cholesterol homeostasis. The mevalonate pathway (or HMG-CoA reductase pathway), a rate-limiting step in cholesterol synthesis, a component of endogenous cholesterol biosynthesis(Lu et al. 2001). The processes of activation and regulation of converting mevalonate to cholesterol involve with many genes, such as IDI1, FDFT1, SQLE, CYP51A1, MSMO1, SC5D, etc(Sharpe and Brown 2013). Lipoprotein receptors act as conduits for cholesterol delivery to cells. SR-B1 and LDLR carry HDL and non-HDL plasma lipids into cells, respectively(Danilo et al. 2013; Munir et al. 2014). Additionally, cholesterol binding to membrane proteins transport to other organelles by two mechanisms: vesicular and non-vesicular(Luo et al. 2017). Cholesterol transport to endosomal/lysosomal membranes is achieved by the synergistic action of NPC1 and NPC2. Cholesterol incorporates into mitochondria by steroidogenic acute regulatory protein (StAR/STARD1)(Elustondo et al. 2017). What's more, steroid hormones are derivatives of cholesterol which maintain oocyte development. Hormone synthesis is primarily accomplished by CYP11A1, CYP17A1, CYP19A1, members of the cytochrome P-450 (CYP) family of enzymes(Chatuphonprasert et al. 2018). So, cholesterol synthesis, transport, and steroid hormone transformation are all essential for oocyte development.

The aim of this study is to investigate the effects of cholecterol metabolism on DOR. Ultra-high performance liquid chromatography-multiple reaction monitoring-mass spectrometry (UHPLC-MRM-MS) was used to quantify steroid substances in our GCs. RT-qPCR was used to estimate the genes expression related with cholesterol metabolism in DOR. These results would help us understand whether infertility in DOR patients are related with cholesterol metabolism and could offer a new potential strategy for DOR diagnoses and treatment.

2. Materials and methods

2.1 Patients

A total of 158 patients under 35 years old who undergoing IVF/intracytoplasmic sperm injection (ICSI) cycles were recruited from the Centre of Reproductive Medicine. The study protocol was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL 2015-0008). All women were fully counseled and written informed consents were obtained from all participants. The inclusion criteria for women with DOR included: (a) age ≤ 35 years, (b) AMH ≤ 1.1ng/mL, (c) total antral follicle count ≤ 7, and (d) 12IU/L ≤ FSH ≤25IU/L. Women were excluded if they had (a) a history of ovarian cystectomy or oophorectomy, (b) received cytotoxic chemotherapy, and (c) received pelvic irradiation.

2.2 Ovarian stimulation and GCs collection

Patients received the gonadotropin-releasing hormone (GnRH) antagonist protocol. Recombinant FSH or human menopausal gonadotropin (HMG) were administered on day 2 of menstrual cycle. Follicle growth was monitored by transvaginal ultrasound. In the stage of 14 mm diameter follicle size, GnRH-antagonist were injected daily until human chorionic gonadotropin (hCG) triggering. When at least three follicles > 16 mm in diameter, GCs and follicular fluid were obtained after ovulation induction.

In total, GCs (the samples of our research) were collected from the dominant follicle of each patient and these patients were then divided into two groups: 72 NOR patients and 86 DOR patients. The sample of each person were detected in each different experiment. GCs were separated from follicular fluid by density gradient centrifugation through 50% Percoll (Solarbio) for 15 min at 1000 g. The GCs were harvested in the intermediary layer and washed twice with phosphate buffered saline (PBS), then centrifuged for 5 minutes at 1000g. The supernatants were discharged and GCs were stored at -80 °C.

2.3 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) Analysis

Untargeted analysis is the most frequently utilized method in LC-MS/MS

analysis. Accurate quantification of metabolite concentration is challenging due to narrow linear ranges, complex data processing, and limited scanning time(Chen et al. 2013).

We randomly examined the GCs from 3 patients with DOR and 3 patients with NOR (n = 6) through LC-MS/MS analysis. The samples were analyzed by Waters 2D UPLC (Waters) connected with a Q-Exactive mass spectrometer (Thermo Fisher Scientific) which equipped with a heated electrospray ionization (HESI) source. The Waters ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters) was utilized for experiments. Chromatographic separation was at a flow rate of 0.35 mL/min. The injection volume was 5 μL and the column temperature was maintained at 45 °C. 0.1% formic acid (A) and acetonitrile (B) were used in the positive mode and 10 mM ammonium formate (A) and acetonitrile (B) were used in the negative mode. The multistep gradient program was as follows: 0-1 min, 2% B; 1-9 min, 2%-98% B; 9-12 min, 98% B; 12-12.1 min, 98% B to 2% B; and 12.1-15min, 2% B. The positive/negative ionization modes parameters were set as follows: spray voltage, 3.8/−3.2 kV; aux gas flow rate, 10 arbitrary units (arb); sheath gas flow rate, 40 arb; capillary temperature, 320 °C; aux gas heater temperature, 350 °C.

2.4 Ultra-high performance liquid chromatography-multiple reaction monitoring-mass spectrometry (UHPLC-MRM-MS) Analysis

The common method for UHPLC-MRM-MS analysis is targeted analysis, characterized by a wide linear range, sufficient scanning time, and good sensitivity and accuracy, which is the gold standard for metabolite quantification(Chen et al. 2013).

We randomly selected GCs from 3 DOR patients and 3 NOR patients (n = 6), and detected them by UHPLC-MRM-MS analysis. The UHPLC separation was performed using an ACQUITY UPLC-I/CLASS (Waters) equipped with a XBridge ® BEH C8 (2.1 × 100mm, 2.5 μm, Waters). The mobile phases consisted of 0.5 mmol/L NH4F (phase A) and methal (phase B). The UHPLC system injection volume was 10 μL and the column temperature was set at 50°C. The autosampler temperature was

conditioned at 10°C. A Xevo TQ-S triple quadrupole mass spectrometer (Waters) was used for assay development. The MS conditions parameters were: capillary voltage 2kV, desolvation temperature 550 °C, cone gas flow 150 (L/Hr), esolvation gas flow 1100 (L/Hr), nebuliser gas flow=7.0 (Bar). The MassLynx Work Station Software (Version 4.1) was employed for MRM data acquisition and processing.

2.5 RNA isolation from GCs and RT-PCR

The samples of GCs from 33 NOR patients and 40 DOR patients were used for RT-PCR experiments. In order to ensure the specific expression of genes occurred not only in this experiment, but also in other groups, we recollected GCs from 33 NOR patients and 40 DOR patients and repeated the same experiments. The sequences of the primers used were given in Table 1S.

Total RNA was extracted from Clinical GCs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 2 μL of RNA was extracted for cDNA synthesis using high-capacity cDNA reverse transcription kit (Code No. RR036A, Takara). The concentration of cDNA obtained by reverse transcription was 50 ng/μL. The reverse transcriptase polymerase chain reaction mixture contained 2 μL of cDNA, 10 μL SYBR Premix Ex Taq II (Code No. RR820A, Takara), 0.5 μL of each the forward and reverse primer and nuclease-free water to 20 μL. The cycling parameters were carried by 40 cycles (95°C for 15 s, 58°C for 15 s, 72°C for 20 s). The relative gene expression was normalized to GAPDH transcript content and was calculated as $2^{-\Delta\Delta Ct}$.

2.6 Statistical analysis

Data were analyzed using SPSS 26.0 statistical software. The data were presented as the mean ± SD. Student's t-test were used for statistical differences between groups, with $p < 0.05$ was considered as significant.

3. Results

3.1 Parameters evaluated in the study group

The baseline characteristics for the 158 women were demonstrated in Table1: 72 young women with normal ovarian reserve (NOR), 86 young women with DOR.

There were no marked differences in the body mass index (BMI), duration of infertility, basal serum levels of estradiol (E_2), progesterone (P), follicle luteinizing hormone (LH) and thyroid stimulating hormone (TSH) between these 2 groups. We found statistically significant differences in these parameters, such as age (29.53 ± 2.98 vs 30.98 ± 2.81 years, $P < 0.01$), basal serum levels of follicle stimulating hormone (FSH) (6.14 ± 1.51 vs 15.34 ± 2.79 IU/L, $P < 0.001$), anti-Mullerian hormone (AMH) (4.46 ± 2.88 vs 0.44 ± 0.28 μg/L, $P < 0.001$), antral follicle count (AFC) (15.94 ± 6.75 vs 4.08 ± 2.15 n, $P < 0.001$), E_2 on hCG (4428.60 ± 1566.18 vs 1152.74 ± 850.85 ng/L, $P < 0.001$), P on hCG (1.48 ± 0.70 vs 0.64 ± 0.70 ng/L, $P < 0.001$), number of follicles ≥ 14 mm on triggering day (15.63 ± 3.96 vs 2.97 ± 1.74 n, $P < 0.001$) and rate of high-quality embryos (54.23 vs 36.36 %, $P < 0.001$).

3.2 Decreased cholesterol in GCs of DOR patients

The results of serum lipid concentrations in the two groups of NOR (n = 72) and DOR (n = 86) are displayed in Table 2 and described as follows: there were no significantly differences in total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG) levels in both groups.

To investigate if the cholesterol decrease was accounted by decreased mRNA expression of the corresponding genes, we performed RT-PCR analysis of thirteen genes involved in cholesterol metabolism in the NOR and DOR groups. Several genes relevant with cholesterol biosynthesis (IDI1, FDFT1, SQLE, CYP51A1, MSMO1, SC5D) were examined. Of these, IDI1, FDFT1 and CYP51A1 were significantly downregulated in the DOR group ($p < 0.05$) (Figure 1A). Cholesterol uptake by GCs occurs via the LDLR and SRB1 receptors. SRB1 identified from cell surface is the main receptor which recognizes and uptakes the HDL-CE(Acton et al. 1996). SRB1 expression levels in GCs were significantly decreased in the DOR group ($p < 0.05$), while LDLR was not significant. The expression of STARD1, which is the rate-limiting step in the progesterone synthesis from cholesterol(Stocco and Clark 1996), was reduced by 2.62 fold in DOR group. Lysosomal cholesterol egress requires two proteins, NPC1 and NPC2(Sleat et al. 2004). These proteins encoded by genes

had no significant difference between NOR and DOR groups (Figure 1B). Sterol regulatory element binding transcription factors (SREBFs) is controlled automatically for processing and stability by SCAP (SREBF chaperone). They performed an increasingly important role in maintaining intracellular cholesterol homeostasis(Weber et al. 2004). Compared with the control group, SCAP gene were remarkably decreased in DOR group ($p < 0.05$) (Figure 1A).

In this study, we assessed 3 patients with DOR and 3 normal controls by LC-MS/MS analysis to screen and identify potential metabolites. To further explore the differences in the metabolic profile between NOR and DOR groups, multivariate (PCA and PLS-DA) statistics and univariate (Fold-change and Student's t test) were combined to obtain the differential metabolites, following the criteria (Fold-change $\geq$ 1.2 or $\leq$ 0.83 and $p < 0.05$). Comparing metabolites between the NOR group and DOR group, 8 differential metabolites related to cholesterol metabolism were screened and identified as 11A-acetoxyprogesterone, hydroxyprogesterone, progesterone, coprostanone, 4-cholesten-3-one, 7-oxocholesterol, chenodeoxycholic acid and deoxycholic acid (Table 3). In DOR group, 11A-acetoxyprogesterone (0.032-fold), hydroxyprogesterone (0.052-fold), progesterone (0.009-fold) and coprostanone (0.145-fold) decreased significantly. Chenodeoxycholic acid, deoxycholic acid, 4-cholesten-3-one and 7-oxocholesterol were no statistical difference. Coprostanone, chenodeoxycholic acid and deoxycholic acid were the main metabolite of cholesterol. In addition, 4-cholesten-3-one and 7-oxocholesterol were cholesterol oxidation products. Therefore, we speculated that cholesterol decreased in patients with DOR.

3.3 Effect of steroid hormones secretion in GCs

Three key genes (CYP11A1, CYP17A1 and CYP19A1) are involved in converting cholesterol into the steroid hormone biosynthesis pathway. So, we selected these genes to explore the causes of steroid hormones synthesis disorder in GCs of DOR patients compared with NOR patients. As shown in Figure 2, we found that the expression of CYP19A1 was statistically significantly different in DOR group ($p <$

0.05).

The level of endogenous steroid hormones in human are usually between nmol/l and pmol/l ranges, so it is a challenge to quantify accurately. UHPLC-MRM-MS analysis have enabled accurate identification and quantification of metabolites such as 17α-hydroxyprogesterone, androstenedione, 11-deoxycorticosterone, estrone, estradiol, progesterone and pregnenolone. Steroid hormones concentrations in NOR (n = 3) and DOR groups (n = 3) were summarized in Table 4. GCs steroid hormone concentrations were reduced in DOR patients compared with normal controls, 17α-hydroxyprogesterone (17OH-P) (6-fold), 11-deoxycorticosterone (Doc) (11-fold), estrone (E_1) (5-fold), estradiol (E_2) (4-fold), progesterone (P) (5-fold), pregnenolone (Preg) (2-fold). Among them, 17OH-P and E_2 were statistically significantly different ($p < 0.05$), and the rest were not. Other relevant information regarding the formula, molecular weight, precursor and product ions, LLOD, LLOQ and linearities are shown in Table 2S. Collectively, lower concentrations of steroid hormones were observed in DOR group compared to the control group.

4. Discussion

DOR is a multifactorial disease, and it is widely known that there are no special therapy for DOR(Fusco et al. 2011). Precise interplay between GCs and the oocyte is indispensable for follicle development and oocyte maturation(Chang et al. 2016). Cholesterol biosynthesis are involved in several biological processes, e.g. steroidogenesis, membrane constitution and repair. Thus, we speculated that the abnormal cholesterol metabolism of GCs may be responsible for oocyte quality in women with DOR. The purpose of this study was to explore the cholesterol changes in GCs of DOR.

There is a convincing evidence that a link between lipid profile such as TC, TG, HDL, LDL and ovarian reserve. Verit et al. showed that lower HDL levels and higher TG, LDL levels among patients with DOR(Verit et al. 2016; Verit et al. 2014). But it is claimed that LDL decreased less, while TC, HDL increased more in DOR women compared with NOR women in Tehrani's study(Tehrani et al. 2014). More studies are

needed to investigate the lipidemia levels in patients with DOR. Through this study, it is found that there was no significant difference for the patients with DOR on lipidemia. However, according to our LC-MS/MS analysis, cholesterol was significantly reduced in GCs of DOR patients compared with GCs of NOR (Table 3). It may be due to decreased expressions of cholesterol transport genes. In porcine oocytes, Wang et al. identified the most suitable genes for normalization, GAPDH/18S and YWHAG/BACT genes, for GCs and oocytes, respectively(Wang et al. 2017). Thus, GAPDH served as a housekeeping gene to study GCs changes in our study. We found that the key cholesterol transport genes SRB1, STARD1 were significantly downregulated, and other genes (e.g. LDLR, NPC1 and NPC2) were not significant (Figure 1B). LDLR and SRB1 played a vital role in importing cholesterol. LDLR deficient mice does not affect fertility(Ishibashi et al. 1993), whereas knockout mice (Scarb1$^{-/-}$) displays suboptimal luteal steroid genesis and ovarian pathology(Jiménez et al. 2010). Significantly decreased SRB1 expression may contribute to oocyte developmental disorders in women with DOR. STARD1 is a key gene, which has a role in transporting cholesterol from the outer to the inner mitochondrial membrane(Miller 2007). Cholesterol transport in GCs may be essential elements in the development of DOR.

Some studies suggest that intracellular cholesterol content may reflect oocyte developmental potential, especially in porcine(Watanabe et al. 2013) and mice oocytes(Comiskey and Warner 2007). Our previous study had indicated that cholesterol synthesis was reduced in GCs of DOR patients(He et al. 2020). We found, in agreement with previous study, that cholesterol synthesis genes including IDI1, FDFT1, CYP51A1 expressions presented striking difference in DOR group ($P < 0.05$) (Figure 1A). As mentioned above, cholesterol transport was decreased in GCs of DOR patients (Figure 1B). In mammalian cells, sterol regulatory element-binding protein 2 (SREBP2) and its sterol-sensitive partner, SCAP, maintain a delicate cholesterol balance with a complex feedback mechanism(Brown et al. 2018). SREBP, the protein encoded by SREBF1, is the transcription factors that regulate key genes expression of

cholesterol biosynthesis and import. Compared with the control group, our results showed that SCAP expression dropped much more ($P < 0.05$). These results implied that aberrant regulation of SREBF/SCAP may decrease cholesterol synthesis and transport in GCs.

Cholesterol functions as a precursor molecule in the synthesis of steroid hormones(Greaves et al. 2014). In the present study, we found steroidogenic mediators CYP11A1, CYP17A1 and CYP19A1 were altered in the DOR group (Figure 2). CYP19A1 expression decreased significantly ($P < 0.05$). Cholesterol is converted to progesterone which is catalyzed by CYP11A1; furthermore, CYP17A1 and CYP19A1 metabolize progesterone to estradiol(Ke et al. 2004; Sahmi et al. 2004). The steroid hormone concentrations in GCs of the DOR showed that the 17OH-P, Doc, E_1, E_2, P and Preg were decreased compared with the control group (Table 4). Our results significantly lower P and E_2 on the day of hCG triggered injection in DOR patients may be one reason for steroid hormone reduction in GCs (Table 1). Steroid hormone changes are associated with oocyte development. P and E_2 jointly delayed progression of oocytes through prophase I(Burks et al. 2019). Low P synthesis is associated with reduced conceptus elongation in cattle(Forde et al. 2011), sheep(Nephew et al. 1991) and pig(Trout et al. 1992). In our study, there was a significant difference in the rate of high-quality embryos between the two groups ($P < 0.05$). Thus, lower steroid hormones in GCs of DOR patients have been postulated to be a predictor of oocyte quality.

In conclusion, this study verified that the downregulation of cholesterol biosynthesis and transport may account for the pathogenesis of DOR by decreasing steroid hormones in GCs. Decreased cholesterol metabolism plays an important role in the development of DOR. This result provides a potential resolvent for clinical early diagnosis and timely treatment.

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Author contributions

Xiulan Yang and Xiaolei Liang designed the research and prepared the manuscript. Zhongying Zhao, Qigang Fan and Hongli Li performed all the experiments and analyzed the data. Lihui Zhao, Chang Liu and Xiulan Yang revised the manuscript. Xiaolei Liang provided financial support for the research. All authors have approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2015-0008).

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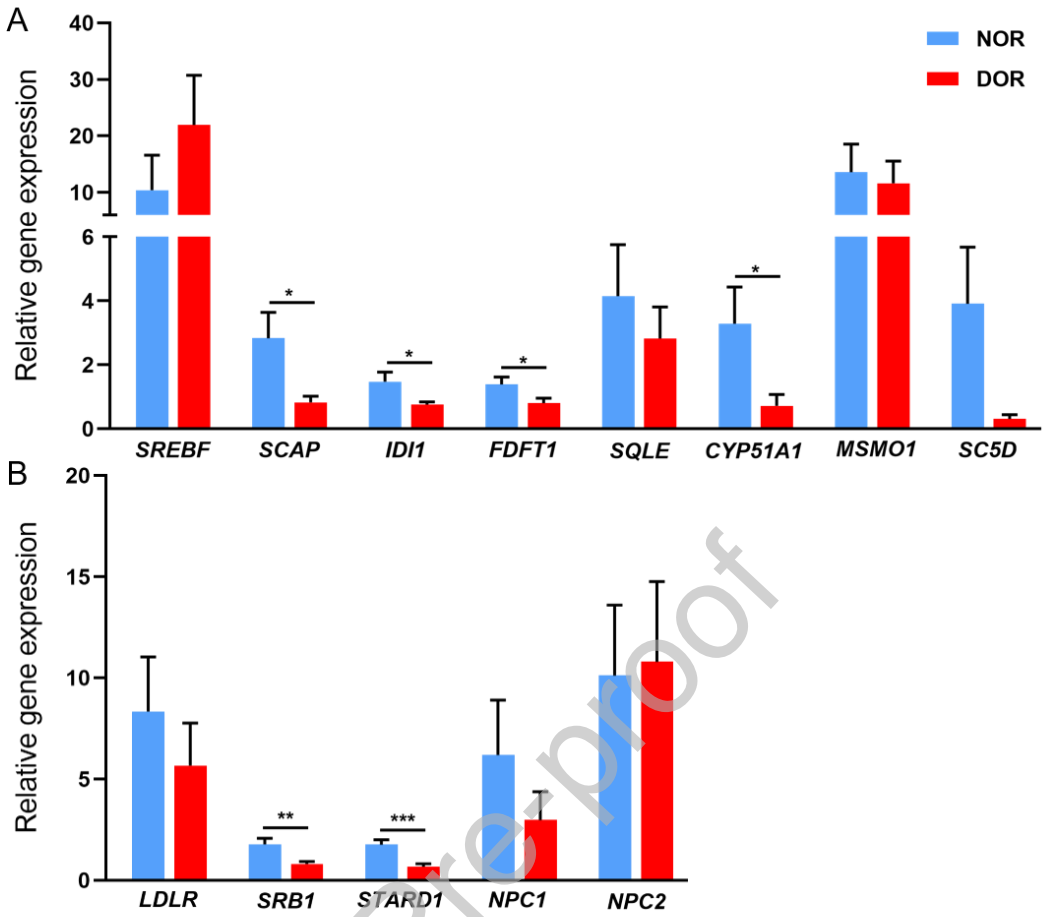

Fig.1 Normalized expression levels of (A) Cholesterol regulation and synthesis genes, (B) cholesterol transporter genes in NOR (n=33) and DOR (n=40) groups. Values were expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001.

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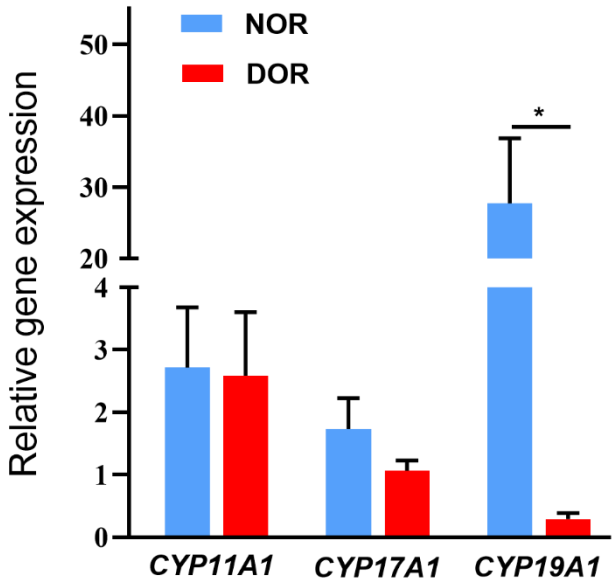

Fig.2 Normalized expression levels of CYP11A1, CYP17A1 and CYP19A1 genes in NOR (n=33) and DOR (n=40) groups. Values were expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001.

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Table 1. Baseline characteristics of NOR (n=72) and DOR (n=86) groups

	NOR (n=72)	DOR (n=86)	p-value
Age (years)	29.53 ± 2.98	30.98 ± 2.81	0.002
BMI (kg/m^2)	22.69 ± 3.39	21.93 ± 2.27	0.108
Duration of infertility (years)	3.81 ± 2.50	3.37 ± 1.98	0.220
Basal LH (IU/L)	5.74 ± 3.35	4.95 ± 2.72	0.101
Basal FSH (IU/L)	6.14 ± 1.51	15.34 ± 2.79	0.000
Basal E_2 (ng/L)	49.07 ± 31.02	47.57 ± 41.68	0.802
Basal P (ng/mL)	18.38 ± 8.30	18.23 ± 8.56	0.917
TSH (mU/L)	3.37 ± 1.57	3.19 ± 1.98	0.541
AMH (μg/L)	4.46 ± 2.88	0.44 ± 0.28	0.000
AFC (n)	15.94 ± 6.75	4.08 ± 2.15	0.000
E_2 on hCG (ng/L)	4428.60 ± 1566.18	1152.74 ± 850.85	0.000
P on hCG (ng/L)	1.48 ± 0.70	0.64 ± 0.70	0.000
Number of follicles ≥14mm (n)	15.63 ± 3.96	2.97 ± 1.74	0.000
Rate of high-quality embryos (%)	54.23(391/721)	36.36(48/132)	0.000

Notes.

Data are expressed as mean ± SD; BMI, body mass index; LH, luteinizing hormone; FSH, follicle stimulating hormone; E_2, estrogen; P, progesterone; TSH, thyroid stimulating hormone; AMH, anti-Mullerian hormone; AFC, antral follicle count; hCG, human chorionic gonadotropin

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Table 2. Biochemical parameters of NOR (n=72) and DOR (n=86) groups

Lipid profiles	NOR(n=72)	DOR(n=86)	P-value
TC(mg/dl)	155.30 ± 31.76	155.89 ± 33.69	0.937
TG(mg/dl)	112.33 ± 74.58	101.15 ± 61.81	0.502
HDL(mg/dl)	53.59 ± 11.01	51.87 ± 10.78	0.498
LDL(mg/dl)	78.98 ± 23.96	79.04 ± 22.84	0.992

Notes.

Data are expressed as mean ± SD; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein

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Table 3. Situation of cholesterol metabolites in NOR (n=3) and DOR (n=3) groups

Metabolite	Formula	Mean-NOR(n=3)	Mean-DOR(n=3)	VIP[a]	FC[b]	P-value[c]	Class	Label
11A-acetoxyprogesterone	$C_{23}H_{32}O_4$	31549343.86	999085.19	1.602	0.032	0.0269*	Gluco/mineralocorticoids, progestogins and derivatives	down
Hydroxyprogesterone	$C_{21}H_{30}O_3$	64135225.71	3341650.24	1.575	0.052	0.0337*	Gluco/mineralocorticoids, progestogins and derivatives	down
Progesterone	$C_{21}H_{30}O_2$	92445245.68	790713.94	1.652	0.009	0.0154*	Gluco/mineralocorticoids, progestogins and derivatives	down
Coprostanone	$C_{27}H_{46}O$	43882473.34	6382705.53	1.803	0.145	0.0008**	Cholesterols and derivatives	down
4-cholesten-3-one	$C_{27}H_{44}O$	36657203.22	5504884.02	1.312	0.150	0.0707	Cholesterols and derivatives	down
7-oxocholesterol	$C_{27}H_{44}O_2$	10749383 2.71	5241668.51	1.201	0.049	0.0592	Cholesterols and derivatives	down
Chenodeoxycholic acid	$C_{24}H_{40}O_4$	26326968.09	458004.02	1.493	0.0174	0.0539	Dihydroxy bile acids, alcohols and derivatives	down

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Deoxycholic acid	$C_{24}H_{40}O_4$	39	19600535.17	17514334.267	0	0.8936	0.8152	Dihydroxy bile acids, alcohols and derivatives	do wn

Notes.

[a]VIP values obtained from contribution of m/z to the PLS-DA model

[b]FC, fold change

[c]p-value obtained from T test method

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Table 4. The difference of 7 steroid hormones in NOR (n=3) and DOR (n=3) groups

Steroid metabolite	NOR (n=3)	DOR (n=3)	P-value
17OH-P	2616.91 ± 14.62	391.83 ± 245.06	0.004**
A_4	76.51 ± 38.88	143.82 ± 2.37	0.103
Doc	304.33 ± 232.33	27.59 ± 3.05	0.208
E_1	472.62 ± 307.88	91.13 ± 14.99	0.195
E_2	995.00 ± 226.21	251.34 ± 240.24	0.039*
P	113139.80 ± 98210.70	20538.53 ± 8494.12	0.179
Preg	17546.14 ± 12966.43	7529.12 ± 7429.52	0.310

Notes.

Data are expressed as mean ± SD; 17OH-P, 17α-hydroxyprogesterone; A_4, androstenedione; Doc, 11-deoxycorticosterone; E_1, estrone; E_2, estradiol; P, progesterone; Preg, pregnenolone

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KEY MESSAGE

In granulosa cells of DOR women, downregulation of cholesterol biosynthesis and transport decreasing steroid hormones levels, leading to oocyte dysplasia and infertility.

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