

Glutamine and norepinephrine in follicular fluid synergistically affect the antioxidant capacity of human granulosa cells and the outcome of IVF-ET

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Research Article

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Abstract

Background: An increasing number of studies have demonstrated that changes in metabolic levels in follicular fluid are directly related to oocyte maturation, fertilization, the quality of embryo and pregnancy rates. However, whether the intra-follicular neurotransmitters affect the function of granulosa cells and the outcome of in vitro fertilization and embryo transfer (IVF-ET) is not clear.

Methods: Human follicular fluid and cumulus granulosa cells (GCs) were harvested from large follicles obtained from 27 patients undergoing IVF. Neurotransmitters and steroid hormones in follicular fluid were measured through liquid chromatography–tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography–mass spectrometry (HPLC-MS/MS) respectively. Based on the content of Glutamine (GIn) in follicular fluid, the samples were divided into two groups: high Gln level group and low Gln level group. The expression of proliferation-, steroidogenesis- and antioxidant-related genes in GCs was detected by qRT-PCR. In vitro experiments, KGN cells were cultured with or without Gln and NE followed by western blotting to detect PCNA and IDH1 expression. EdU staining and ELISA were conducted to evaluate GCs proliferation and measure the contents of 17 b-estradiol (E2) and progesterone (Prog) in the conditioned medium. Primary and secondary outcomes were the number of mature and retrieved oocytes, and the ratio of high quality embryos, respectively.

Results: Gln and norepinephrine (NE) were abundant neurotransmitters in follicular fluid. The contents of Gln and NE in follicular fluid exhibited a significantly positive correlation. The contents of Gln and NE in follicular fluid affected the function of granulosa cells, including their proliferative potential, E2 production and antioxidant capacity. Moreover, the concentrations of Gln and NE in follicular fluid showed significantly positive correlation with isocitrate dehydrogenase 1 (IDH1) expression in GCs. Importantly, the increased Gln and NE in follicular fluid partially affected the quality of embryonic development by regulating IDH1 expression in GCs. In vitro studies further demonstrated that Gln and NE played synergistically function in improving GCs proliferation and increasing the E2 production by upregulating IDH1 expression.

Conclusions: These data demonstrate that neurotransmitter metabolic levels in follicular fluid affect the function of cumulus GCs. As important neurotransmitter metabolites, GIn and NE play significant roles in promoting GCs proliferation and increasing E2 production in granulosa cells. In addition, intrafollicular GIn and NE could upregulate the expression of the antioxidative gene IDH1 in cumulus GCs, which could be a good predictor for selecting optimal quality oocytes and evaluating the quality of embryonic development.

Background

Subfertility, also commonly referred to infertility, is a reproductive system disease characterized by failure to establish a clinical pregnancy after normal unprotected sexual intercourse for 12 months or more, or impairment of the reproductive capacity of an individual or with his or her partner [1, 2]. Subfertility has

been recognized as a worldwide public health problem by the World Health Organization (WHO), and approximately half of these cases lack an definitive explanation for pregnancy failure [3]. It is estimated that in western countries, one in six couples has subfertility, which is recognized as the cause of psychological distress in both men and women [4]. Assisted reproductive technology (ART) including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) is a promising option with suitable interventions for the clinical management of subfertility [5]. IVF offers a possibility for achieving pregnancy, but the success rate is still suboptimal [6, 7].

A healthy intrafollicular environment supports the production of developing oocytes and affects oocyte quality by coordinating the cumulus GCs and the follicular fluid [8]. Follicular fluid originates from both plasma and secretion by cumulus GCs. As an important biofluid, follicular fluid provides a special microenvironment for follicular development and impacts oocyte quality, implantation and early embryo development. Follicular fluid is rich in proteins, including sex hormones, nutrient substances, cytokines, growth factors and neurotransmitters, which is critical for oocyte development [9]. Cumulus granulosa cells represent the predominant somatic cell type of follicle development and are involved in steroidogenesis and folliculogenesis. Furthermore, they connect with oocytes via direct gap junctions to support the developing oocyte [10-12]. The process of follicular development involves local biochemical exchanges and substantial modifications in cellular metabolism.

Previous studies have demonstrated that NE in the follicular fluid derived from the sympathetic neurons of the peripheral nervous system in the mammalian ovary takes part in the regulation of ovarian function [13, 14]. Follicular cells such as granulosa and theca express β -adrenergic receptor in respond to the β -adrenergic agonist, leading to a physiological follicular development, ovulation and the synthesis and production of steroid hormones [15]. In addition, NE and its metabolites could induce the generation of reactive oxygen species (ROS) in human GCs [16]. Once the level of NE concentration exceeds the physiological concentration, it may lead to ROS-related pathophysiology events including oxidative stress and cell death [17]. Thus, the content of NE in the follicular microenvironment could be a key physiology or physiological indicator for the signaling in steroidogenic Leydig cells and the process of ovulation.

Amino acids serve as substrates for the synthesis of proteins, not only enhancing embryonic development but also affecting blastocyst formation in mice. A study demonstrated that Glycine (Gly), Glutamine (Gln), alanine (Ala), Glutamate (Glu) and Proline (Pro) were the most abundant amino acids in follicular fluid [18]. The disturbance of follicular amino acid metabolism is related to women with polycystic ovary syndrome (PCOS), which may be the reason for the poor pregnancy outcome in obese patients and increased risk of abortion in PCOS patients [19]. In addition, they are not only components of proteins, but also regulate neurotransmitter homeostasis, such as a metabolite shuttle known as the glutamate/GABA-glutamine cycle describes the release of neurotransmitter glutamate or GABA from neurons and subsequent uptake into astrocytes, play import roles in nervous system [20, 21]. However, whether the neurotransmitters metabolic levels provided by follicular fluid influences the function and hormone synthesis of cumulus granulosa cells need to be further investigated.

Oxidative stress plays an important role in reproductive processes, including follicular development, oocyte maturation, ovulation and fertilization, as well as embryo implantation and embryo development [22]. Excessive generation of ROS, if not efficiently counterbalanced by antioxidative enzymes, may cause damage to oocytes and granulosa cells, leading to poor oocyte quality [11]. In addition, increased levels of endogenous ROS and reduced antioxidant genes expression diminish cellular antioxidant defense capability, resulting in oxidative damage to lipids, proteins and DNA in ovary [23]. In our previous study, psychological stress-induced oxidative stress decreased the expression of the antioxidant gene IDH1 to disturb and compromise the function of granulosa cells, accelerating ovarian senescence [24]. Antioxidant treatment with melatonin can provide reproductive medicine with a way to improve IVF outcomes [25]. Additionally, antioxidant supplementation in IVF culture medium could increase fertilization rates and subsequent healthy embryo development [26]. However, little is known about the impact of neurotransmitters in follicular fluid on oxidative capacity of granulosa cells.

During the process of follicle growth to ovulation, which is mediated by the preovulation production of gonadotropins, metabolic profiles change dynamically in follicular fluid. Thus, a better understanding of the metabolic levels in follicular fluid is important for exploring the metabolic cooperativity between oocytes and cumulus granulosa cells. In recent years, metabolomics, as a new approach, has been used to explore various molecular mechanisms involved in tissues and organs. Among the various metabolomics techniques, LC-MS/MS is widely used as a conventional detection method for analyzing small molecules, which could provide a quantitative evaluation with minimal handling [27].

In this study, we identified the neurotransmitters in follicular fluid from IVF patients by high sensitivity, convenient and fast LC-MS/MS technology and evaluated the impact of neurotransmitter Gln and NE in follicular fluid on the function and antioxidative capacity of cumulus granulosa cells, which could impact the clinical outcome of IVF patients. Additional in vitro cell experiments were performed to explore the effects of Gln and NE on the proliferation and steroidogenesis of granulosa cells.

Methods

Study design

This is an observational study of infertile women before and during IVF treatment at the International Peace Maternity and Child Health Hospital (IPMCH), Shanghai, China. Follicular fluid and cumulus granulosa cells on the day of oocyte retrieval were obtained from 27 patients undergoing IVF at the reproductive center. The subjects were recruited with male factor infertility or tubal factor infertility. Subjects with endometriosis and PCOS were excluded. The age of the patients ranged from 25 to 40 years, and the size of the follicles ranged from 19 to 24 mm.

Collection of GCs and follicular fluid

The size of the follicle was estimated by ultrasound at the time of oocyte retrieval. Follicular aspirates contain oocytes surrounded by cumulus GCs. After the oocytes were removed by the embryologist, the

remaining material was centrifuged at 3000 g for 15 mins to isolate the GCs. The fluid from the first aspirated follicle was used to measure hormone and neurotransmitter metabolic levels.

Quantification of neurotransmitters in follicular fluid by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Human follicular fluid was prepared for neurotransmitters metabolomics detection. Twenty-three metabolites (Ach: Acetylcholine chloride; Gln: Glutamine; Glu: Glutamate; His: L-Histidine; NE: norepinephrine; Tyr: Tyrosine; Trp: Tryptophan; Kyn: Kynurenine; GABA: 4-Aminobutyric acid; HisA: Histamine; PA: Picolinic acid; TyrA: Tyramine; DA: Hydroxytyramine hydrochloride; TrpA: Tryptamine; 5-HT: Serotonin hydrochloride; E: Adrenaline hydrochloride; KynA: Kynurenic acid; 5-HIAA: 5-Hydroxyindole-3-acetic acid; DOPA: Levodopa; XA: Xanthurenic acid; VWA: Vanillymandelic Acid; 5-HTTP: 5-Hydroxytryptophan; MT: Melatonine) were simultaneously reported in LC-MS/MS as described previously[28, 29]. Data analysis was performed with Analyst 1.6 software.

RNA Extraction and qRT-PCR

Granulosa cells were collected in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to previously described methods[24]. A total of 1 mg RNA was converted to cDNA with a Takara kit (Applied Biosystems Foster City, CA, USA; Takara, Shiga, Japan). The genes of interest were amplified with a 7900HT fast real-time PCR system (Applied Biosystems) and SYBR Green Real-time PCR Master Mix (Applied Biosystems/Takara). The PCR primers were designed according to the cDNA sequences in the NCBI database. The primer sequences used are shown in Table 1. The cycling conditions used for the PCR analysis were as follows: 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s (40 cycles). 18S was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was employed to determine the relative mRNA expression level.

Table 1
Primer sequences for qRT-PCR in human granulosa cells

Target genes	Primer sequence(5'-3')	Product Size (bp)
IDH1	F: AGAAGCATAATGTTGGCGTCA	106 bp
	R: CGTATGGTGCCATTTGGTGATT	
GPX1	F: CCGGGACTACACCCAGATGA	101 bp
	R: CGTTCTCCTGATGCCCAAAC	
NRF2	F: TCAGCGACGGAAAGAGTATGA	174 bp
	R: CCACTGGTTTCTGACTGGATGT	
KEAP1	F: CTGGAGGATCATACCAAGCAGG	220 bp
	R: GGATACCCTCAATGGACACCAC	
PCNA	F: CCTGCTGGGATATTAGCTCCA	109 bp
	R: CAGCGGTAGGTGTCGAAGC	
CCND2	F: TTTGCCATGTACCCACCGTC	104 bp
	R: AGGGCATCACAAGTGAGCG	
LHR	F: CACATAACCACCATACCAGGAAA	124/283 bp
	R: AAGTCAGTGTCGTCCCATTGA	
FSHR	F: AAAGCTGCCTACTCTGGAAAAG	170 bp
	R: GACCCCTAGCCTGAGTCATATAA	
STAR	F: GTGGAACCCCAGTGTCAAGA	129 bp
	R: CAGCGCACACTCACAAAGTC	
CYP11A	F: GAGGCCCAGCGATTCATTGAT	94 bp
	R: TCCTGAACAGACGGAACAGGT	
CYP19A	F: TGGAAATGCTGAACCCGATAC	161 bp
	R: AATTCCCATGCAGTAGCCAGG	
HSD-3β	F: CTTGTGCGTTAAGACCCACAT	124 bp
	R: GGGTTGACTGTAGAGAACTTTCC	
HSD-17β	F: GTGCTGGTGTGTAACGCAG	98 bp
	R: GTCCCTACTACATTCACGTCCA	
18sRNA	F: GTAACCCGTTGAACCCCATT	151 bp

Target genesPrimer sequence(5'-3')Product Size (bp)

R: CCATCCAATCGGTAGTAGCG

Hormone metabolic levels in the follicular fluid

The concentrations of twenty kinds of hormones in the follicular fluid were detected by high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) as described previously[30]. In addition, the concentration of testosterone and cortisol in the follicular fluid was detected by the chemiluminescence method[31].

Human Granulosa Tumor Cell Line (KGN) culture and treatment

The human ovarian granulosa cell line was kindly gifted by Dr. Zuwei Yang (IPMCH, School of Medicine, Shanghai Jiao Tong University, Shanghai, China). KGN was initially cultured in Dulbecco minimal essential medium (DMEM)-F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum and antibiotics (100 IU/mL penicillin, 100 μ g/mL streptomycin) in a 5% CO₂ atmosphere at 37°C. After deprivation of glutamine for 24 h, the cells were treated with the corresponding conditioned medium for 48 h.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatant samples were collected and centrifuged for 20 min at 2500 rpm to acquire the supernatant at -4°C. The supernatant was stored at -80°C for further analysis. The concentration of 17β-estradiol and progesterone in cell culture supernatant was measured by ELISA kits (Xinle Biological Technology, Shanghai, China), according to the manufacturer's instruction.

Western blotting

Proteins were extracted from cultured cells with RIPA lysis buffer (Yeasen, Shanghai, China) and protein concentration was measured via a BCA kit (Thermo Scientific, Rockford, IL, USA). The protocol for western blotting is reported previously[24]. The following primary antibodies were used: rabbit antibodies against β -tubulin (1:1000, CST, MA, USA) and anti-PCNA antibody (1:1000, CST, MA, USA). After incubation with primary antibodies at 4°C overnight, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500, CST, MA, USA) for 1 hour at room temperature. The bands were detected by electrochemiluminescence (ECL, Millipore, Billerica, MA, USA). The relative intensity of the target proteins was normalized to β -tubulin using ImageJ software (NIH, Bethesda, MD, USA).

EdU Staining

The proliferation of KGN cell was evaluated by EdU staining (RiboBio, Guangdong, China). Briefly, EdU was added to the cell culture medium at a concentration of 10 µM. Cells were cultured for 24 hours and stained with Alexa 594 and DAPI. Images were captured under a microscope (Leica, WetzlaR, Germany)

and positive fluorescence signals were counted. The number of EdU-positive cells per 100 cells was evaluated.

Statistical analysis

All statistical analyses were performed using Prism software, and a *p* value of <0.05 was considered statistically significant. Means and standard error were used as descriptive statistics. T-tests were used to compare different variables between the low-level and high-level glutamine groups. Linear correlation analysis was used to compare the expression of the IDH1 gene and steroidogenesis in luteinized granulosa cells. In the current study, when a power analysis was performed with 80% power and an α value of 0.05, the number of samples in each group needed to be 13 to confirm statistical significance. **Ethics approval and informed consent**

Informed consents for participating in this study were obtained from all the patients and this study was approved by the Institutional Ethics Committee of the International Peace Maternity and Child Health Hospital. All experiments were performed in accordance with the relevant guidelines and regulations.

Results

Glutamine is positively correlated with norepinephrine content in the follicular fluid of IVF patients

Dynamic metabolic changes within the follicular fluid are important for cumulus expansion, which represents the consequence of oocyte cumulus complex (OCC) metabolic cooperation. In this study, we first measured the content of neurotransmitters in follicular fluid by LC-MS/MS. The results regarding the concentrations of Ach, Gln, Glu, His, NE, Tyr, Trp, Kyn in human follicular fluid are shown in Table 2. The other fifteen neurotransmitters were not detected in most of the follicular fluid samples. Correlation analysis showed that Gln was positively correlated with the NE content in follicular fluid (R=0.5869, P<0.005, Figure 1A). However, there was no relationship between the contents of Gln and Glu in follicular fluid (R=0.2374, P>0.05, Figure 1B).

Concentration of multiple neurotransmitters in human follicular fluid Sample ID Ach Gln Glu His NE Tyr Kyn Trp (ug/ml)(ug/ml) (ug/ml) (ug/ml) (ug/ml) (ug/ml) (ug/ml) (ug/ml)01 7.227 0.238 0.019 45.715 21.989 1.083 0.233 6.431 02 0.025 49.37 10.045 1.472 0.281 6.231 7.373 0.548 03 0.080 60.028 16.639 3.620 0.401 19.822 5.658 0.757 04 0.018 49.313 15.104 1.365 0.251 4.751 6.977 0.307 05 0.019 16.819 7.711 0.350 52.869 1.510 0.240 5.724 06 0.016 54.879 13.450 1.273 0.245 6.297 5.830 0.202 07 0.019 35.712 17.795 1.074 0.253 5.903 6.359 0.361 80 0.027 44.439 11.364 1.389 0.244 6.018 4.618 0.307 09 0.018 12.867 1.329 0.258 6.925 51.359 6.633 0.319 10 7.698 0.243 0.023 60.335 15.089 0.947 0.214 6.458 11 0.020 0.284 44.826 13.077 1.046 0.202 4.362 5.838 12 0.026 42.962 14.758 1.339 0.130 5.837 5.290 0.272 13 0.021 58.259 13.474 1.411 0.235 5.413 5.804 0.225 14 0.017 56.594 16.155 1.208 0.285 7.238 10.141 0.241 9.157 15 0.02 52.631 6.706 1.455 0.142 10.169 0.255 16 10.595 0.455 0.018 44.161 1.417 0.144 5.729 6.591 17 0.017 37.091 14.466 1.146 0.231 4.984 7.835 0.346 18 0.021 33.855 8.973 1.014 0.143 4.525 5.623 0.261 19 0.016 0.180 8.394 0.243 50.998 10.091 1.029 6.335 20 0.014 44.261 11.385 1.012 0.186 4.101 6.881 0.284 21 0.018 1.249 0.205 5.096 0.199 50.524 11.722 6.630 22 0.018 38.522 11.873 1.055 0.096 5.849 4.797 0.265 23 0.017 37.578 10.232 1.099 0.113 4.864 5.351 0.248 24 14.710 1.106 0.158 0.323 0.018 42.237 6.031 5.536 25 0.027 44.286 11.252 1.313 0.109 5.851 6.290 0.262 26 0.022 45.651 11.975 1.157 0.218 7.406 7.377 0.336

Table 2 oncentration of multiple neurotransmitters in human follicular fluic

Sample ID	Ach	Gln	Glu	His	NE	Tyr	Trp	Kyn
	(ug/ml)							
27	0.026	33.927	8.471	1.103	0.127	5.956	5.352	0.348
Ash: Asstulahaling ahlarida: Clay Clutaning: Cluy Clutanate: Llig: L. Lligtiding: NE: norming hring: Tyr								

Ach: Acetylcholine chloride; Gln: Glutamine; Glu: Glutamate; His: L-Histidine; NE: norepinephrine; Tyr: Tyrosine; Trp: Tryptophan; Kyn: Kynurenine

GIn serves as a substrate for the synthesis of proteins in follicular fluid, which directly promotes oocyte nuclear maturation and enhances embryo development. However, the effect of GIn and NE in follicular fluid on the function of cumulus GCs is still unclear. To further investigate the function of GIn and NE in follicular fluid, 27 samples were divided into two groups according to their concentration of GIn, the high GIn level group (>45µg/mL) and low GIn level group (<45µg/mL).

Effect of intra-follicular glutamine/norepinephrine on the function of granulosa cells

The function of GCs involved in a proliferative capacity, receptor expression and steroid hormone synthesis in cumulus GCs was determined by qRT-PCR. Compared with the low Gln level group, those with high Gln level had a significant 2-fold increase in proliferating cell nuclear antigen (*PCNA*) and cyclin D2 (*CCND2*) mRNA levels in the GCs (P<0.001 and P<0.01, Figure 2A-B). However, there were no statistically significant differences in luteinizing hormone receptor (*LHR*) or follicle stimulating hormone receptor (*FSHR*) mRNA levels between the two groups (P>0.05, Figure 2C-D). Notably, the mRNA levels of the steroid hormone synthesis-related genes steroid acute regulatory protein (*STAR*), cytochrome P450 (*CPY11A, CYP19A*) and hydroxysteroid dehydrogenase-3beta (*HSD-3β*) significantly decreased in the low Gln level group (P<0.05, Figure 2E-H). However, there was no significant difference in hydroxysteroid dehydrogenase-17beta (*HSD-17β*) mRNA levels between the two groups (P>0.05, Figure 2I). These data demonstrated that the increased Gln and NE in the follicular fluid could promote the proliferation and steroidogenesis of GCs.

Glutamine/norepinephrine promote estrogen synthesis in the follicular fluid of IVF patients

To obtain an overview of hormone metabolic characteristics in follicular fluid between high and low Gln level groups, targeted metabolic profiling was conducted using HPLC-MS/MS. Nine kinds of hormones were detected in the two groups, including estrone, estriol, 17β -estradiol, progesterone, methyl testosterone, (D)-norgestrel, 19-nortestosterone, epitestosterone and medroxy progesterone. In addition, the concentration of testosterone in follicular fluid was measured by a chemiluminescence immunoassay. The results demonstrated that the concentrations of estrone and 17β -Estradiol were significantly higher in the high Gln level group than in the low Gln level group (P<0.05 and P<0.001, Figure 3A-B). There were no statistically significant differences in the other hormone contents between the two groups (P>0.05, Figure 3C-I). These results revealed that Gln and NE in follicular fluid played important roles in promoting estrogen synthesis and the production.

Glutamine/norepinephrine in the follicular fluid increase the expression of antioxidant genes in granulosa cells

Oxidative metabolism is the major oxygen consumer in the follicle, which could be affected by amino acid metabolism. Antioxidants are indicated as factors that can maintain the balance between ROS reproduction and clearance. Then, we detected the expression levels of the antioxidant genes in GCs via gRT-PCR between the two groups. In the group of high Gln level, antioxidant gene *IDH1* and glutathione peroxidase 1 (GPX1) increased significantly in GCs compared with that in group of low Gln level (P<0.001 and P<0.0001, Figure 4A-B). Moreover, the expression of nuclear factor erythroid 2-related factor 2 (*NRF2*) and its endogenous inhibitor, kelch-like epichlorohydrin-associated protein-1 (KEAP1), significantly increased in the high Gln level group (P<0.01, Figure 4C-D). Notably, the expression level of IDH1 mRNA in GCs showed a positive correlation with the Gln concentration in follicular fluid (R=0.3822, P<0.05, Figure 4E). Furthermore, there was a positive correlation between the NE concentration in follicular fluid and IDH1 expression in GCs (R=0.4009, P<0.05, Figure 4F). To further clarify whether this difference in the expression of antioxidative genes was caused by individual differences, we summarized the clinical data of the IVF patients as Table 3 and found that there were no statistically significant differences in age (P=0.1601), body mass index (BMI, P=0.5927), serum Anti-Mullerian Hormone (AMH, P=0.6033) or thyrotropin (TSH, P=0.8698) between this two groups. These results demonstrated that the contents of Gln and NE in follicular fluid might affect the antioxidant capacity of GCs.

Characteristics	High level of Glutamine	Low level of Glutamine	P-value			
	(n=14)	(n=13)				
Age(years)	31.00[28.75-36.00]	31.00[26.50-33.00]	0.1601			
BMI(Kg/m2)	21.16[19.52-23.00]	19.95[18.31-21.52]	0.5927			
Duration of infertility	2.50[1.00-4.63]	1.00[1.00-3.00]	0.0472			
Previous pregnancy	2.00[1.00-3.00]	0[0-1.00]	0.0164			
Previous delivery	0[0-0.25]	0[0-0]	0.0820			
AMH(ng/ml)	2.35[1.29-4.29]	2.90[1.93-3.81]	0.6033			
TSH(mIU/L)	1.91[1.45-3.03]	2.18[1.83-2.70]	0.8698			
Antral follicle count	9.50[5.75-12.25]	9.00[8.50-12.50]	0.9911			
Indication for IVF			0.3289			
Idiopathic	1(7.14%)	1(7.69%)				
Male factor	1(7.14%)	0(0)				
Endometriosis	4(28.58%)	2(15.38%)				
Tube factor	6(42.86%)	6(46.15%)				
Multiple	2(14.28%)	4(30.77%)				
Data are median (interquartile range (IQR)) or numeric value (%)						
AMH: anti-Mullerian hormone						

Table 3 Characteristics of patients assessed for follicular fluid analysis

IDH1 expression in granulosa cells is related with the quality of embryo development of IVF patients.

To determine whether follicular development and oocyte growth were different between the high Gln level and low Gln level groups, we compared several clinical indices in the two groups, including the number of mature follicles (diameters \geq 14 mm) on the human chorionic gonadotrophin (HCG) injection day, the number of retrieved oocytes, the fertilization rate, and the number and percentage of higher-quality embryos. There were no significant differences between the two groups in the above parameters (P>0.05, Figure 5A). Pearson correlation analysis was used to identify the relationship between the Gln/NE concentration in the follicular fluid and the ratio of higher-quality/cleaved embryos, and IDH1 expression in granulosa cells and the ratio of higher-quality/cleaved embryos. Results showed that there were no significant correlation between the Gln/NE concentration in the follicular fluid and the ratio of higherquality/cleaved embryos (R=0.1158 and R=0.0134, P>0.05, Figure 5B-C). However, a significantly positive correlation was observed between IDH1 gene expression in GCs and the ratio of higher-quality/cleaved embryos (R=0.4766, P<0.05, Figure 5D). These results demonstrated that the IDH1 expression in cumulus GCs is directly related with the quality of oocyte and embryo development in IVF patients.

Glutamine/norepinephrine improve the function of granulosa cells in vitro

Previous studies have reported that Gln is required for oocyte maturation and embryo development [32] and adrenergic activity through NE participates in the control of follicular development and steroidal secretion [33]. To study the roles of GIn and NE in granulosa cells, the proliferation of cultured KGN cells in vitro was evaluated via EdU staining and western blotting. Fluorescent staining showed that the percentage of EdU-positive cells was dramatically reduced in the Gln deprivation group. The addition of NE partially increased the ratio of EdU-positive KGN cells. Importantly, Gln supplementation increased significantly the EdU-positive number (P<0.0001, Figure 6A-B). Moreover, the concentrations of E2 and Prog in the cell culture supernatant were measured by ELISA. The results showed that the concentration of E2 was reduced in the GIn deprivation group, and adding GIn to the medium significantly increased E2 production in KGN cells, which could be further improved by NE supplementation (P<0.01, Figure 6C). Although Gln deprivation reduced the level of Prog in the cell supernatant, Gln and NE supplementation had no obvious effect on Prog production in KGN cells ((P<0.05, Figure 6D). The expression of PCNA protein in KGN cells exhibited similar trends to the morphological results in the different treatment groups. Notably, Gln deprivation obviously reduced IDH1 protein expression in GCs, which could be recovered by NE and Gln supplementation (P<0.0001, P<0.01 and P<0.05, Figure 6E-G). These results revealed that the neurotransmitters GIn and NE could synergistically enhance the cell proliferation of granulosa cells and increase the E2 production.

Discussion

Follicular fluid is an important external environment for the oocyte growth and follicular development. Ideally, follicular fluid, a superfluous, abundant and easily available biological sample in IVF cycles, would be the optimum source of noninvasive prediction of oocyte quality [34]. Currently, investigations of follicular fluid biomarkers for oocyte quality mainly focus on measuring steroid hormones secreted by granulosa cells, such as estradiol and progesterone. Some studies have demonstrated that high levels of Prog and E2 in follicular fluid are positively related to the quality of oocyte and could be indicators for assessing oocyte mature and development. However, other studies have reported no correlation between hormones in the follicular fluid and oocyte quality or that they may even be correlated with a lower quality [35]. Steroid hormone production mainly relies on the function of granulosa cells, which are dynamically regulated by small molecule metabolites in follicular fluid. Therefore, exploring the relationship between metabolites and granulosa cell function may be conducive to search reliable biomarkers for assessing oocyte quality and predicting the outcome of IVF patients.

A metabolic approach is a powerful tool to analyze the complexities of follicular fluid. Previous study reported the application of gas chromatography-mass spectrometry (GC-MS) to detect polybrominated diphenyl ethers (PBDEs) in follicular fluid and found that the presence of PBDEs could be associated with

failed embryo implantation[36]. In the current study, a more simple, fast and efficient LC-MS/MS technology was successfully applied for determination of simultaneous neurotransmitter in human follicular fluid. Consistent with other studies, we found that many different neurotransmitter metabolites exist in human follicular fluid, including Ach, Gln, Glu, His, NE, Tyr, Trp and Kyn, as shown in Table 2. Among them, high levels of Gln and NE have attracted our attention; there was a positive relationship between the contents of Gln and NE in follicular fluid. NE is released from ovarian nerve terminals wherein the ovarian compartment is directly innervated by sympathetic nerves [33]. Whether ovarian sympathetic nerves control the concentration of Gln in follicular fluid needs to be further studied.

Currently, extensive scientific attention has been given to an important metabolite Gln, which is one of the most abundant amino acid in the human body, such as skeletal muscles, plasma [37] and follicular fluid [38]. It has anti-inflammatory and antioxidant functions as well as effects that modulate the heat-shock protein response during stress; moreover, as a precursor of glutamate, Gln participates in multiple neurotransmitter metabolic pathways [37, 39, 40]. In addition, peripheral neurotransmitter NE participates in the control of steroidal secretion from the ovary and follicular development [33]. To study the effect of intra-follicular Gln and NE on cumulus granulosa cell function, we compared the expression of proliferation- and steroid hormone synthesis-related genes between the low Gln level and high Gln level groups. The results showed that Gln and NE could promote granulosa cell proliferation and steroid hormone synthase gene expression. To confirm this relationship, hormone synthetic metabolites were also quantitatively analyzed by LC-MS/MS technology. Among the many hormones, only the concentrations of estriol and 17 β -estradiol were significantly decreased in the low Gln level group. These findings indicated that the content of Gln and NE in follicular fluid plays important roles in maintaining the proliferation of cumulus GCs and E2 production.

An altered follicular microenvironment may directly damage follicle recruitment and growth. Previous studies show that metabolites in the follicular fluid are direct or indirect regulators of oxidative stress and the function of granulosa cells [41]. A research found that Gln was used as an additive substance to enhance antioxidant capacity in the breast muscle of heat-stressed broilers [42]. Moreover, Gln improves the body's resistance to stress [43] and restores the ovarian function of PCOS rats [44]. In addition, Gln, as the main energy supply substance for mitochondria to form ATP, is an important source of energy for oocyte maturation and the development of embryo [38]. Therefore, Gln has become an essential component in embryo culture medium. A study reported that human ovarian tissue was cryopreserved using freezing medium supplemented with cryoprotectants and antioxidants (L-glutamine and taurine) and exhibited good preservation of ovarian vascular integrity and functionality of granulosa cells post thawing [45]. Furthermore, the glutamine supplementation could effectively alleviate inflammatory reaction and oxidative stress in PCOS rats [44] and protect Chinese hamsters from radiation damage [46]. Thus, the relationships among the levels of Gln in follicular fluid, cumulus GC function, and oocyte development are worthy of further study.

Excessive ROS damages DNA, reduces cell proliferation, and induces mitochondrial-mediated apoptosis in GCs, then affecting oocyte development [47, 48]. Studies have demonstrated that there is a correlation

between the apoptosis of GCs and poor oocyte quality and IVF outcomes in patients with PCOS [11, 49]. Many environmental stressors may cause inferior oocyte competence and alter oocyte development and growth by affecting GC function [48, 50, 51]. The bidirectional interplay between cells and microenvironment affects the expression of genes and ultimately alter cellular behaviors by dynamic reciprocity [52]. In the current study, we found that Gln and NE not only upregulated IDH1 gene expression but also increased GPX1 expression in cumulus GCs. Notably, the concentration of Gln and NE in the follicular fluid were positively related to IDH1 gene expression in cumulus GCs, which could be a predictor of high-quality embryos. To date, a clear correspondence between specific metabolites in follicular fluid and measurable embryo-related variables has not been established. Taking the level of Gln and NE in follicular fluid and antioxidant gene expression in cumulus granulosa cells into account will provide more accurate information to help embryologists choose optimal quality oocytes.

There are several limitations in current study. First, the number of clinical samples was insufficient. Although the number of samples in the two groups met the statistical requirements, the limited number of samples influenced the subsequent analysis of reproductive outcomes of IVF patients. The second limitation was that only antioxidant genes expressions were assayed in cumulus GCs, however, other oxidative stress makers including ROS, MDA, GSH did not detected due to lack of enough samples. Finally, only KGN cells were used to confirm the roles of GIn and NE in maintaining granulosa cell function in vitro experiments. Whether appropriate supplementation of GIn and/or NE in animal models with ovarian dysfunction could improve fertility outcomes needs to be explored in future studies.

Conclusions

In summary, our research demonstrates that the concentrations of GIn and NE in follicular fluid influences proliferation and steroidogenesis in human cumulus GCs. Moreover, intrafollicular GIn and NE could upregulate the expression of the antioxidative gene IDH1 in cumulus GCs, which could be a good predictor for evaluating the quality of embryonic development.

Abbreviations

5-HIAA: 5-Hydroxyindole-3-acetic acid; 5-HT: Serotonin hydrochloride; 5-HTTP: 5-Hydroxytryptophan; Ach: Acetylcholine chloride; Ala: alanine; ART: Assisted reproductive technology; AMH: Anti-Mullerian Hormone; BMI: body mass index; CCND2: cyclin D2; DA: Hydroxytyramine hydrochloride; DOPA: Levodopa; E: Adrenaline hydrochloride; E2: 17b-estradiol; FSHR®follicle stimulating hormone receptor; GABA: 4-Aminobutyric acid; GCs: granulosa cells; GC-MS: gas chromatography-mass spectrometry; GPX1: glutathione peroxidase 1; Gln: Glutamine; Glu: Glutamate; Gly: Glycine; His: L-Histidine; HisA: Histamine; HCG: human chorionic gonadotrophin; HPLC: high-performance liquid chromatography; HPLC-MS/MS: high-performance liquid chromatography–mass spectrometry; HSD-3b: hydroxysteroid dehydrogenase-3beta; HSD-17b: hydroxysteroid dehydrogenase-17beta; ICSI: intracytoplasmic sperm injection; IDH1: isocitrate dehydrogenase 1; IPMCH: International Peace Maternity and Child Health Hospital; IVF: in vitro fertilization; IVF-ET: in vitro fertilization and embryo transfer; *KEAP1*: kelch-like epichlorohydrin-associated protein-1; Kyn: Kynurenine; KynA: Kynurenic acid; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LHR: luteinizing hormone receptor; LOD: limit of detection; LOQ: limit of quantification; MRM: multiple reaction monitoring; MT: Melatonine; NE: norepinephrine; NRF2: nuclear factor erythroid 2related factor 2; OCC: oocyte cumulus complex; PA: Picolinic acid; PBDEs: polybrominated diphenyl ethers; PCNA: proliferating cell nuclear antigen; PCOS: polycystic ovary syndrome; Pro: Proline; Prog: progesterone; ROS: reactive oxygen species; STAR: steroid acute regulatory protein; Trp: Tryptophan; TrpA: Tryptamine; TSH: thyrotropin; Tyr: Tyrosine; TyrA: Tyramine; XA: Xanthurenic acid; WHO: World Health Organization; VWA: Vanillymandelic Acid;

Declarations

Consent for publication

All authors have agreed to publish this article.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) with the dataset identifier px-submission #562857 and px-submission #562860.

Competing interests

The authors report no declarations of interests.

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Authors' contributions

Study design: D.L. and Q.Z.; Data collection: Q.Z., L.W., C.Z. and J.S.; Data analysis and interpretations: Q.Z., L.W., C.Z. and J.S.; Drafting the manuscript: D.L., Q.Z. and L.W. All the above authors revised and approved the manuscript and take responsibility for the integrity of the data.

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Figures

Figure 1

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