Serum Metabolomic Signature Predicts Ovarian Response to Controlled Stimulation

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Research

Keywords: Serum metabolomics, response, COS

Posted Date: November 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1082463/v1>

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Abstract

Introduction In in vitro fertilization (IVF) cycles, some patients fail to adequately respond to ovarian stimulation. Finding novel biomarkers predicting ovarian response in advance would be meaningful.

Objective To identify serum metabolomics predicting the growth of follicles after controlled ovarian stimulation (COS).

Methods Blood samples were collected at the start of pituitary downregulation and on the fifth day after controlled ovarian stimulation. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods were used to quantify metabolites. Demographic data were calculated with SPSS version 22.0 software. Multivariate statistics were used to analyze metabolomics dataset. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic model.

Results The number of retrieved oocytes was higher in the group of Follicle-to-oocyte index (FOI) ≥ 1 group. Analyses revealed 50 different metabolomics between the pre- and post- COS groups. Compared with baseline, amino acids increased significantly following COS. At baseline, acetylglycine was more abundant in the FOI <1 group, while glycine and lipids were more abundant in the FOI ≥ 1 group. After COS, glycine, N-acetyl-L-alanine, D-alanine, and 2-aminomuconic acid were increased in those with FOI ≥ 1 , but L-glutamine was increased in those with FOI <1 . ROC curves indicated that the combination of glycine, acetylglycine and lipids predicts different responses to controlled ovarian stimulation (AUC =0.866).

Conclusion Serum metabolism might reflect the response to ovarian stimulation. Higher glycine and PC may be a good predictor for response to COS.

Introduction

Controlled ovarian stimulation (COS) is a critical step for in vitro fertilization (IVF) and several different protocols have been developed for controlled ovarian stimulation, including agonists, antagonists and mini-stimulation. In most cases, women achieve sufficient follicle growth after proper ovarian stimulation. However, some patients who fail to adequately respond to stimulation and cannot obtain ideal results, with an incidence that varies from approximately 6.7-25% [1-4]. The factors that predict ovarian response to COS include the woman's age, basal antral follicle counts (AFCs) and anti-Mullerian hormone (AMH) levels. Women are classified according to the number of retrieved oocytes as poor responders, normal responders or hyper-responders[4]. Traditionally, reproductive doctors repeatedly monitor ovarian follicle growth via ultrasonography during COS and correspondingly adjust the dose of gonadotropin to obtain an adequate ovarian response. However, ultrasonographic features are always delayed with respect to the effects of gonadotropin and cannot predict the final retrieved oocytes. In 2011, Genro et al. introduced a new model named the follicle output rate (FORT) to determine the hyporesponsiveness during ovarian stimulation [5]. Based on the concept of FORT, the Follicle-to-oocyte index (FOI) was proposed to predict ovarian resistance to gonadotropin stimulation [6]. Nevertheless, these methods cannot help in designing

gonadotrophin stimulation protocols at the beginning or in adjusting medication dosages during COS and therefore, have low clinical application value. Therefore, it is urgent to find novel biomarkers that can predict late ovarian response and guide the protocol of gonadotrophin stimulation, which will be meaningful for individual treatment and increasing the success rate in IVF.

Metabolomics is a powerful tool for systematically studying all chemical processes concerning metabolites and can measure dynamic multiparametric metabolic responses to pathophysiological stimuli or genetic modifications. Indeed, this tool is gaining rapid attention in the clinical arena and displays great potential in disease diagnosis and prediction. In the field of assisted reproduction arena, metabolomics has been applied for assessing the underlying pathology of endometriosis [7], polycystic ovary syndrome (PCOS)[8], some infertility conditions [9] and the development of follicles and embryos. With respect to the prediction of follicle and embryo reproductive potential, most studies have focused on follicle fluid and the embryonic metabolome, but such sampling is invasive and difficult to implement in clinical applications. Only one study found that significant changes in serum metabolomics changes might be the main reason for ovulatory dysfunction in PCOS patients [10]. As the serum metabolome in controlled ovarian stimulation has not been well characterized. The current study aimed to investigate the serum metabolomics profile to identify variation in metabolomics composition during the growth of follicles after controlled ovarian stimulation. We hypothesized that ovarian response to controlled stimulation might be characterized using serum metabolomics.

Materials And Methods

Study group

This retrospective study was conducted at the reproductive medicine center of Northwest Women's and Children's Hospital. The protocol was reviewed and approved by the Ethics Committee for the Clinical Application of Human Assisted Reproductive Technology of Northwest Women's and Children's Hospital. Written informed consent was obtained from each patient. Fifty-seven patients who underwent their first IVF cycle using controlled ovarian stimulation with a gonadotropin-releasing hormone agonist (GnRH-a) long protocol were enrolled. The patients were divided into two groups based on the Follicle-to-oocyte index (FOI). The first group comprised patients with $FOI < 1$; the other group was patients with $FOI \geq 1$. Patients with polycystic ovary syndrome, endometriosis and other inflammatory diseases were excluded.

Controlled ovarian stimulation

Daily injection of 0.1 mg triptorelin was used for pituitary downregulation from the mid-luteal phase of menstrual cycle. Downregulation was confirmed 14 days later (follicle diameter < 5 mm, serum E_2 (estrogen) < 50 pg/mL, serum LH (lutein hormone) < 5 IU/L).

During ovarian stimulation, gonadotropin (Gn, 75-300 IU/day) was administered for almost 10-15 days. The dosage was adjusted according to the woman's age, basal serum FSH (follicle stimulation hormone) level, and response of follicle growth. Follicle growth was monitored by transvaginal ultrasound and

serum estrogen (E_2) levels every 1-5 days. When at least two follicles reached a mean diameter of 18 mm or three follicles reached a mean diameter of 17 mm, human Chorionic Gonadotropin (HCG) 5000-10000 U was applied to trigger the final maturation of follicles. Oocyte retrieval was performed at 36 hours after HCG administration. Standard IVF or ICSI was used for fertilization and embryo transfer was performed on 3-5 days after ovum pick up.

Serum sample preparation

Following overnight fasting, venous blood samples were collected from all patients at two time points: at the start of pituitary downregulation; and on the fifth day after controlled ovarian stimulation. The blood samples were allowed to clot at room temperature and then centrifuged to collect the serum, which was stored at -80 °C until further analysis. Before performing liquid chromatography–tandem mass spectrometry (LS-MS/MS) experiments, 50 µL of sample was thawed, transferred to an EP tube, vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at -40 °C to precipitate proteins. The sample was centrifuged at 12000 rpm for 15 min at 4 °C. The resulting supernatant was transferred to a fresh glass vial for analysis. A quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all samples.

Quantification of metabolites

Biochemical metabolites were determined by Biotree, Inc., using liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods with positive and negative ion–mode electrospray ionization (ESI). Plasma peaks were detected and the plasma remaining after relative standard deviation de-noising were assessed. Missing values were filled up by half of the minimum value. An internal standard normalization method was applied for this data analysis. The final dataset was imported into SIMCA16.0.2 software package (Sartorius Stedim Data Analytics AB, Umea, Sweden) for multivariate analysis.

Statistical analysis

For demographic data, SPSS version 22.0 software was used for analysis. Student's *t* test was employed for continuous variables, and the χ^2 test for categorical variables; $P < 0.05$ was considered statistically significant. For metabolome data analyses, supervised orthogonal projections to latent structures–discriminate analysis (OPLS-DA) was applied to visualize group separation and identify significantly changed metabolites. The value of variable importance in the projection (VIP) of the first principal component in OPLS-DA analysis was obtained to summarize the contribution of each variable to the model, and the OPLS-DA regression model was validated by cross-validation to determine the prediction ability. Metabolites with VIP >1 were considered significantly changed. Boxplot charts were used to display the data distribution for each group, and a heatmap was generated to visualize metabolite abundances in individual samples. In addition, commercial databases including KEGG (<http://www.genome.jp/kegg/>) and MetaboAnalyst (<http://www.metaboanalyst.ca/>) were used for pathway enrichment analysis. A receiver operating characteristic (ROC) curve was also generated to evaluate the efficacy and accuracy of the diagnostic model.

Results

Demographics

The demographic characteristics of the two groups in the present study are shown in Table 1. No statistically significant differences were found between the two groups in terms of maternal age, duration of infertility, basal FSH, basal LH, basal E2, Gn duration and Gn dose. The number of retrieved oocytes was significantly higher in the $\text{FOI} \geq 1$ group ($P < 0.05$).

Metabolomic differences between pre-COS and post-COS

There were 615 differentially enriched serum metabolites between pre- and post- COS with variable importance for the projection ($\text{VIP} > 1$ and $P < 0.05$). Of these, fifty metabolomics have been annotated (Fig.1a). The OPLS-DA score was calculated to evaluate the overall metabolite profile difference, revealing a clear separation of the metabolome between the two groups (Fig.1b). In the OPLS-DA permutation regression model, R^2Y was 0.708 and Q^2Y 0.418 (Fig.1c), indicating that the model was credible for interpreting differences between the two groups.

Various types of metabolites altered between the two groups, with the majority of differentially-expressed metabolomics being in the amino acid pathway. Compared with baseline, amino acids significantly increased following COS. (Figure.1d).

Metabolomic differences between $\text{FOI} < 1$ and $\text{FOI} \geq 1$ groups at baseline

The baseline metabolomic profile showed nine different metabolites between the $\text{FOI} < 1$ and $\text{FOI} \geq 1$ groups (Fig.2a). The majority of significantly different metabolites between these two groups derive from amino acids (including glycine and acetylglycine) and some lipids. Acetylglycine was more abundant in the $\text{FOI} < 1$ group, whereas glycine and lipids were more abundant in the $\text{FOI} \geq 1$ group (Fig.2b).

Metabolomic differences between $\text{FOI} < 1$ and $\text{FOI} \geq 1$ groups after COS

After COS, fifteen different metabolites were found between the $\text{FOI} < 1$ and $\text{FOI} \geq 1$ groups (Fig3a). The variable metabolites between the two groups mainly included glycine, N-acetyl-L-alanine, D-alanine, L-glutamine and 2-aminomuconic acid, which are all in the amino acid pathway. Glycine, N-acetyl-L-alanine, D-alanine, and 2-aminomuconic acid were increased in those with $\text{FOI} \geq 1$ but L-glutamine was increased in those with $\text{FOI} < 1$ (Fig. 3b).

Prediction model of metabolomics for the response to controlled ovarian stimulation

To identify metabolites that can distinguish response to controlled ovarian stimulation, a ROC curve was used to build a classification model based on nine metabolites that varied significantly at baseline between the two groups with different FOIs. The ROC curves indicated that the combination of glycine,

acetylglycine and lipids can predict different responses to controlled ovarian stimulation (AUC =0.866, Fig. 5).

Discussion

The results of this study demonstrate that controlled ovarian stimulation influences the metabolic signature of human serum, especially in the amino acid pathway, lipid pathway and fatty acid pathway. For the first time, we established a model of serum metabolites to predict response to controlled ovarian stimulation, as judged by FOI. We found that glycine and acetylglycine, combined with lipids, can predict different responses to controlled ovarian stimulation.

A proper response to ovarian stimulation is crucial for IVF success. The Follicle-to-oocyte index (FOI), as a qualitative marker, can reflect the nature of follicle growth and its response to gonadotropin [11]. This index is usually applied to describe women who have a hypo-response to ovarian stimulation and to predict the success of IVF cycles for these women [12]. In general, a normal FOI is defined as > 0.5 and low as ≤ 0.5 [6]. Nevertheless, accurate biomarkers that can predict the response to ovarian stimulation are still needed for women with a normal or hyper response such that proper oocytes and better clinical results can be obtained. The patients in our research were all young and had good ovarian reserve; for all, there were at least thirteen antral follicle counts and eleven follicles retrieved. Therefore, we used $\text{FOI} \geq 1$ and $\text{FOI} < 1$ to separate groups.

Glycine is a conditional essential amino acid for humans [13], and deficiency in this amino acid causes immune defects, low growth rates and altered nutrient metabolism [14]. Moreover, low circulating glycine has been associated with type 2 diabetes [15], insulin resistance [16] and metabolic syndrome [17]. Glucose metabolism is critical for follicle growth. Cumulus cells convert glucose to pyruvate; and lactate, which are then metabolized via the tricarboxylic pathway (TCA) followed by oxidative phosphorylation to provide the energy needed for oocyte development [18,19]. Any alteration in glucose metabolism may affect follicular growth. Insulin is produced by pancreatic beta cells, increasing plasma glucose levels [20]. Insulin resistance correlates with a decrease in insulin sensitivity and disables the ability of cells to take up and utilize glucose [21]. A higher concentration of insulin, which correlates with T2DM and metabolic syndrome, does not elicit an appropriate response to stimulate glycogen synthesis [22] and causes abnormal follicle development. Our study found lower circulating glycine in patients with $\text{FOI} < 1$, which would result in a poor response to ovarian stimulation via glucose metabolism pathway. This finding was in accordance with the study of Chahal, et al [23].

Phosphatidylcholines (PCs), a kind of lipid, play vital roles in membrane construction and energy storage. One study [24] compared the follicular fluid between poor and normal responders and found increased PCs in the latter, suggesting that alterations in lipid balance might reflect ovarian response to hormones. Follicle fluid lipid profiling also demonstrated that PCs are increased in PCOS patients and those with hyper response to controlled ovarian stimulation compared to subject with a normal COS response [25]. Interestingly, our results also showed abundant PCs in women with $\text{FOI} \geq 1$, and PC is an accurate

biomarker able to predict response to controlled ovarian stimulation, in accordance with Montani's research [26]. A possible explanation for this is the increase LH that accompanies the development of follicles, which may stimulate PC generation. Increased PC in human cumulus cells may be a consequence of a proper response to LH administration during IVF [27]. Moreover, two previous studies have demonstrated that LH supplementation influences follicular fluid steroid composition and contributes to improving of ovarian response in poor- responding women [28,29].

There are some limitations in our study. First, this was a retrospective study, which might cause some selection bias. However, the baseline characteristics of the patients were comparable between the two groups, which would minimize selection bias. Second, although our data indicated some alterations in the amino pathway, further investigations using large sample size are still needed for confirmation.

In conclusion, our study shows that serum metabolism can reflect the response to ovarian stimulation. Higher glycine and PC may be good predictors of response to gonadotropin in ovarian stimulation via glycose and lipid pathways. Further randomized controlled trials may provide strong proof for this, and new therapies will be explored to improve the outcome of controlled ovarian stimulation.

Declarations

Ethics approval and consent to participate

Ethical approval

The study received approval by the Ethics Committee for the Clinical Application of Human Assisted Reproductive Technology of Northwest Women's and Children's Hospital. Informed consent was obtained from all individual participant include in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This study was supported by the Basic Research Project of Natural Science Fund of Shaanxi Province (2016JQ8026).

Authors' contributions

Xin Mu: design the work, make contribution to the work and write manuscript.

Mei-li Pei: Data analysis and write part of manuscript.

Feng Zhu: Revise manuscript and interpretation of data.

Ya-juan Fan: Acquisition and analysis of data.

Juan-zi Shi and Pei-jun Liu: Project guide, drafted the work and substantively revised manuscript.

Acknowledgements: We thank all the clinicians, scientists and embryologists in the Northwest Women and Children's Hospital for their assistance with data collection, and all the patients for their contribution to this study.

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Tables

Table 1 Demographic and laboratorial data between FOI <1 and FOI ≥1.

Group	FOI<1	FOI≥1	t/χ^2	<i>P</i>
Maternal age (years)	28.37±3.83	29.28±3.76	-0.892	0.376
Maternal BMI (kg/m ²)	20.95±2.77	21.01±2.39	-0.086	0.932
Paternal BMI (kg/m ²)	23.92±2.92	23.82±3.16	0.120	0.905
Duration of Infertility (years)	3.04±2.30	3.48±3.24	-0.575	0.567
Basal AFC (n)	15.33±3.89	13.62±4.80	1.460	0.150
Basal FSH (IU/L)	6.76±1.27	6.14±1.16	1.916	0.061
Basal LH (IU/L)	6.09±4.28	5.97±2.76	0.129	0.898
Basal E2 (pg/ml)	40.41±20.19	48.51±38.46	-0.976	0.334
Gn dose (IU)	2025.93±696.54	2143.97±575.65	-0.693	0.491
Gn time (day)	10.04±1.31	10.41±1.12	-1.157	0.252
Number of Retrieved oocytes (n)	11.15±4.52	17.66±5.32	-4.914	0.000
Number of transferred embryos (n)	1.87±0.34	1.74±0.45	1.079	0.287
Implantation rate (%)	22/43(51.16%)	16/33(48.48%)	0.054	0.817
Clinical pregnancy rate (%)	17/23(73.91%)	14/19(73.68%)	0.000	0.987
Live birth rate (%)	14/23 (60.87%)	11/19 (57.89%)	0.038	0.845

p Values in bold indicate statistical significance ($p \leq 0.05$)

Figures

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Figure 2

(a) Heatmap visualizations of metabolomics between groups with FOI <1 and FOI ≥1 at baseline. Higher abundances are represented by red shades whereas lower intensities are represented by blue shades. (b) Box plot of highlighted metabolites between two groups. HF before = FOI ≥1 at baseline. LF-before = FOI <1 at baseline.

Figure 3

(a) Heatmap visualizations of metabolomics between groups with FOI <1 and FOI ≥1 after COS. Higher abundances are represented by red shades whereas lower intensities are represented by blue shades. (b) Box plot of highlighted metabolites between two groups. HF after = FOI ≥1 after COS. LF after = FOI <1 after COS.

Figure 4

ROC analysis shows area under the curve for glycine, PC, beryllium lysoPC and acetylglucine. The area under the curve for the combination of these nine metabolites was 0.866.