Raman Spectrum of Follicular Fluid: a Potential Biomarker for Oocyte Developmental Competence in Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in reproductive women where abnormal folliculogenesis is considered as a common characteristic. Our aim is to evaluate the potential of follicular fluid (FF) Raman spectra to predict oocyte development and pregnancy outcome, so as to prioritize the best promising oocyte for implantation, reducing both physiological and economical burdens of PCOS patients. In addition, the altered metabolic profiles will be identified to explore the aetiology and pathobiology of PCOS.

Methods: In this study, follicular fluid samples obtained from 150 PCOS and 150 non-PCOS women were measured with Raman spectroscopy. Individual Raman spectrum was analyzed to find biologic components contributing to the occurrence of PCOS. More importantly, the Raman spectra of follicular fluid from the 150 PCOS patients were analyzed via machine-learning algorithms to evaluate their predictive value for oocyte development potential and clinical pregnancy.

Results: Mean-centered Raman spectra and principal component analysis showed global differences in the footprints of follicular fluid between PCOS and non-PCOS women. Two Raman zones (993-1,165 cm^{-1} and 1,439-1,678 cm^{-1}) were identified for describing the largest variances between the two groups, with the former higher and the latter lower in PCOS FF. The tentative assignments of corresponding Raman bands included phenylalanine and β -carotene. Moreover, it was found that FF, in which oocytes would develop into high-quality blastocysts and obtain high clinical pregnancy rate, were detected with lower quantification of the integration at 993-1,165 cm^{-1} and higher quantification of the integration at 1,439-1,678 cm^{-1} in PCOS. In addition, based on Raman spectra of PCOS FF, the machine-learning algorithms via the fully connected artificial neural network (ANN) achieved the overall accuracies of 90% and 74% in correctly assigning oocyte developmental potential and clinical pregnancy, respectively.

Conclusions: The study suggests that the PCOS displays unique metabolic profiles in follicular fluid which could be detected by Raman spectroscopy. Specific bands in Raman spectra have the biomarker potential to predict the oocyte development and pregnancy outcome for PCOS patients. Importantly, these data may provide some valuable biochemical information and metabolic signatures that will help us to understand the abnormal follicular development in PCOS.

Background

Polycystic ovary syndrome (PCOS) is the most common and complex endocrinopathy, which affects more than 10% of women of reproductive ages [1]. It is a multi-factorial and heterogeneous syndrome with variable phenotypes, including hyperandrogenism, menstrual irregularity and polycystic ovarian morphology [2]. The clinical and biochemical characteristics of PCOS have typical heterogeneity, however, abnormal follicular development to induce anovulation is still the basic important characteristic of PCOS [3, 4]. Due to menstrual disorder or anovulation, PCOS patients often have to obtain pregnancy through assisted reproductive technology. However, in clinical practice, sufficient oocytes are usually retrieved

from PCOS patients who were under controlled ovarian stimulation (COS) during in vitro fertilization (IVF), but high-quality mature oocytes or embryos are limited in number [5, 6]. Therefore, understanding the causes of follicle abnormalities and selecting the most promising embryos for transplantation are the prominently urgent tasks to improve the pregnancy rate of PCOS patients.

It is known that metabolomics is an attractive approach to identify and quantify small molecules and could describe both the physiological and pathological states of the organism [7]. As the important microenvironment for follicular development and oocyte maturation, follicular fluid (FF) is the medium for bi-directional communication between oocyte and the surrounding cells [8]. Accordingly, the metabolomics of follicular fluid will truly reflect the folliculogenesis. In fact, the levels of cytokines, growth factors, proteins, metabolites and non-coding RNAs in FF have been reported to be associated with oocyte quality and pregnancy outcome [9–11]. The paucity of high-quality mature oocytes of PCOS patients may due to abnormal endocrine and intra-ovarian paracrine interactions in the follicular fluid microenvironment. Therefore, metabolic profiles of follicular fluid from PCOS patients will not only monitor the smallest biochemical changes in the oocyte development, but also help to provide a specific test for the PCOS diagnosis or embryo selection [12, 13].

As a noninvasive and label-free method for metabolomics, Raman spectroscopy has started to be increasingly applied in biomedical science. It describes the inelastic scattering of light that provides the unique molecular fingerprints of relevant biological molecules [14]. As it should be, Raman spectroscopy shines a new light on reproductive medicine by investigating complex biochemical interactions or evaluating of living cells and tissue [15]. The first Raman investigations of reproductive organs focused on oncology. In 1992, human cervical, uterine, endometrial and ovarian tissues were examined by Raman technology, and four specific regions of the Raman profile between normal/benign and cancerous states were identified [16]. There is, nevertheless, a scarcity of studies using Raman spectroscopy to investigate the relationship between metabolic changes and embryo development or its pregnancy outcome during in vitro fertilization (IVF) process. In 2007, Seli et al. firstly investigated the Raman profiles of spent IVF culture media from Day 3 human embryos that implanted or not. Their results confirmed its strong association with the metabolomic profile and clinical outcome [17]. Later, a retrospective study reported that the changes of metabolic footprints in embryo growth medium that could be detected by Raman spectroscopy was related with chromosomal abnormalities in embryos [18]. Recently, Raman spectroscopy was used in detecting the metabolic changes associated with reproductive related diseases (i.e. endometriosis [19], PCOS [20]). It was found that the vibrational Raman spectroscopy characterization of granulosa cells (GCs) from patients affected by unilateral ovarian endometriosis was abnormal. The altered GCs metabolism and biochemical composition impaired the overall ovarian functions [19]. For PCOS, the Raman profile of FF was found to be different from that of normal women [20], but its predictive value for oocyte development and pregnancy outcome has not been established yet.

In this study, we performed Raman spectroscopy combined with multivariate statistical methods to detect the metabolic changes in follicular fluid from women with PCOS. Based on the specific spectral bands in

FF, we also investigated the possibility and accuracy of the Raman biomarkers, and implemented an artificial intelligence (AI) approach to predict the oocyte development and clinical outcome for PCOS patient undergoing IVF. Besides the biomarker potential of Raman profiles, the results also suggest the changes of metabolism in FF of PCOS patients. To some extent, it will also reveal the pathogenesis of abnormal folliculogenesis and offer a new potential strategy for PCOS therapy.

Materials And Methods

This study was approved by the Scientific and Ethical Committee of the Shanghai First Maternity and Infant Hospital affiliated to Tongji University. Written informed consents were obtained from all the participants.

Participants and sample collection

A total of 300 participants (150 non-PCOS and 150 PCOS) who underwent in vitro fertilization (IVF) in Shanghai First Maternity and Infant Hospital between October 2017 and December 2019 were recruited to obtain informed consent. Eligibility inclusion criteria were: (1) 25–35 years of age; (2) diagnosis of PCOS, according to the revised Rotterdam European Society of Human Reproduction and Embryology / American Society for Reproductive Medicine Criteria [2], where at least two of the followings were fulfilled: chronic oligo-ovulation or anovulation, androgen excess, and polycystic ovaries. The control group had regular menstrual cycles, normal ovary sonographs and normal ovulating; (3) no history of drugs affecting glucose and lipid metabolism, and without any known medical conditions or diseases, including Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumors and endometriosis; (4) undergoing the first IVF treatment with GnRH antagonist protocol; (5) husband with normal sperm to avoid the influence of spermatozoa on embryo development; (6) one blastocyst transferred in fresh cycle or freezing-thawing cycle afterwards, in order to track the pregnancy outcome. The clinical characteristics of the PCOS and non-PCOS controls are summarized in Table 1.

Table 1
The clinical characteristics of women with PCOS and non-PCOS control.

	PCOS group (n = 150)	Non-PCOS group (n = 150)	P value
Age (y)	29.68 ± 3.19	30.64 ± 3.25	0.711
BMI (kg/m ²)	24.04 ± 3.36	21.54 ± 2.66	0.034
Duration of infertility (y)	1.7 ± 1.3	1.6 ± 1.5	0.84
Primary infertility, n (%)	38(59.3%)	41(56.9%)	0.78
FSH (mIU /mL)	6.64 ± 1.95	6.75 ± 1.21	0.23
LH (mIU /mL)	10.31 ± 8.47	6.62 ± 1.30	< 0.001
Basal LH /FSH	2.25 ± 0.71	1.21 ± 0.22	< 0.001
E ₂ (pg /mL)	56.01 ± 13.11	45.59 ± 12.62	0.41
T (ng /mL)	0.72 ± 0.22	0.38 ± 0.13	< 0.001
P (ng /mL)	0.69 ± 0.21	0.54 ± 0.23	0.29
PRL (ng /mL)	14.85 ± 7.77	13.84 ± 6.26	0.118
AFC	18.24 ± 3.25	9.90 ± 2.31	< 0.001
Note: PCOS, polycystic ovary syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteotrophic hormone; E ₂ , oestradiol; T, testosterone; P, progesterone; PRL, prolactin; AFC, antral follicle count. Data are presented as the mean ± SD.			

All patients who participated in the current study underwent the same long GnRH antagonist stimulation protocol. The details of the stimulation cycle procedure were previously described [21]. When two or more follicles were at least 18 mm in diameter and the serum E₂ levels were at least 300 pg/mL per dominant follicle, the patients received 250 µg hCG (Profasi; Serono). Follicular fluid (2–3 mL) was collected from one of the dominant follicles (> 18 mm) by vaginal puncture under ultrasound echo-guidance 36 h after hCG administration. Then, the corresponding oocyte isolated from the collected FF was rinsed and inseminated individually to culture and evaluate the development potential. The collected FF samples were centrifuged at 1,000 g for 10 min at 4 °C to remove the cells and debris. The supernatant was stored at -80 °C for Raman spectra test.

Embryo cultivation and assessment of blastocyst quality / IVF outcome

The embryos were cultured in sequential media of SAGE (CooperSurgical, Leisegang Medical, Berlin) in a single droplet culture (25 µL) covered by mineral oil. Embryos were cultured to the blastocyst stage on Day 5–6. And, the transferrable blastocysts (≥ 3BC) were measured with the Gardner scoring system [22]

and divided into two subgroups: high-quality ($\geq 4\text{BB}$) blastocysts and low-quality ($\geq 3\text{BC}$ and $\leq 4\text{BC}$). The transferrable blastocysts were transferred in fresh cycles or freezing-thawing cycles. Vaginal progesterone was given for two weeks after the oocyte retrieval. The presence of an intrauterine gestational sac is defined as clinical pregnancy.

Raman spectroscopy

Raman spectra were collected with the use of a Raman spectroscopy system (Basecare Raman 200) equipped with a 532 nm laser. The size of the laser spot was 100 μm . Briefly, the FF samples from $-80\text{ }^{\circ}\text{C}$ storage were thawed at room temperature ($25\text{ }^{\circ}\text{C}$) for 30 minutes before analysis. A total of 7 μL of the FF sample was dropped onto a clean place on the surface of the sample test substrate without touching the substrate. Raman signals were obtained in standard mode with the use of a charge-coupled device camera with an integration time of 20 seconds. The spectral range was from 50 to 2000 cm^{-1} with a spectral resolution at 1 cm^{-1} . The background control spectrum was subtracted from each sample spectrum. Five replicates were taken for each aliquot.

Data processing and analysis of Raman spectra

In this study, spectral analysis relied on machine-learning data sets and bioinformatics methods. The full-spectrum pattern recognition was drawn and the characteristic peaks of Raman spectrum which contains abundant material structure information were identified. The raw Raman spectra were preprocessed by subtracting the dark signal background from each spectrum. Spectral normalization was finished by vector normalization of the fingerprint region from 600 to $1,800\text{ cm}^{-1}$ by using Labspec 6 software (Horiba). Mean-centered Raman spectra were obtained by subtracting the means of the spectra from each sample spectrum. Data analysis, statistics and visualization were done in the R environment with the use of in-house scripts. The Raman data was analyzed by the unsupervised PCA method, which could extract key features, reduce the high dimensionality of the data and determine principal components (PCs). The specific wavenumber of Raman shift derived by PCA analysis were corresponding to the molecular bonds or metabolites. Quantification of metabolite concentration was done by integrating individual Raman bands and presented as box plots.

Machine-learning classification

Besides feature extraction to find biological information for PCOS, screening oocyte with high developmental potential and more promising clinical pregnancy rates are often desirable for IVF purposes. In this study, referring to the methods described in the previous report [23], the fully connected artificial neural network (ANN) was established to classify oocyte development potential and IVF outcome based on original Raman spectra of follicular fluid.

The schematic architecture of the fully connected ANN is illustrated in Fig. 1. It was composed of one input layer, 2 hidden layers that contained 64 neurons and 64 neurons, as well as one output layer. An activation function relu was associated with each neuron that summed up the outputs from that neuron

and transferred them to the next layer in the network. The relationship between input (i.e., Raman spectral pattern of follicular fluid) and output (i.e., the high-quality versus low-quality blastocyst, or pregnancy success versus pregnancy failure) could be learned from the recorded data (training data set) and used to predict the unknown sample (prediction data set). The error threshold was set as 1%, indicating that the difference between the predicted value and the true value should be less than 1% after the model was well-trained. The prediction ability of fully connected ANN analysis was evaluated by “leave-one-out” cross-validation. Briefly, all the data points except one were employed to train the model and a prediction was made for that one at each time. The information of sample ID, replicate number, morphological score and the pregnancy outcome were added as features to train the fully connected ANN models.

For each classification model, the area under the receiver operating characteristic (ROC) curve (AUC), the precision, sensitivity, F1 score as well as an overall accuracy rate were calculated. All models were constructed in a Python 3.3 environment. Specifically, precision is the ability of a classifier to not label a negative sample as positive. Sensitivity is the ability of a classifier to find all of the positive samples in one class. F1 score is a weighted mean of the precision and the sensitivity, where 1 is the best value and 0 the worst. The definitions are as follow: TP, true positives; FP, false positives; TN, true negatives; and FN, false negatives.

$$\text{Precision} = \text{TP} / (\text{TP} + \text{FP})$$

$$\text{Sensitivity} = \text{TP} / (\text{TP} + \text{FN})$$

$$\text{F1 score} = 2 \times (\text{Sensitivity} \times \text{Precision}) / (\text{Sensitivity} + \text{Precision})$$

$$\text{Accuracy} = (\text{TP} + \text{TN}) / (\text{TP} + \text{TN} + \text{FP} + \text{FN})$$

Results

Raman spectral analysis unveils metabolic differences in follicular fluid of PCOS

In this study, Raman spectra of 150 PCOS and 150 non-PCOS patients were obtained by analyzing follicular fluid samples. The fingerprint region from 600 cm^{-1} to 1800 cm^{-1} was calculated for analysis, which typically contained the most essential biological information [14]. In accordance with the previous study [20], the raw Raman spectra data of the FF samples from PCOS patients showed no significantly different standard deviation/ variation by compared to those of non-PCOS patients (Fig. 2A). Then, Raman spectra from all samples were averaged and subtracted from each sample at each wavenumber, and the mean values were assembled for each group to obtain mean-centered spectra. The results show that the mean-centered spectra are significantly different between the two groups (Fig. 2B). The corresponding Raman bands and their tentative assignments are summarized in Table 2. Some signature bands can be assigned to phenylalanine ($1,003 \text{ cm}^{-1}$), C-C, C-N stretching (protein) ($1,156 \text{ cm}^{-1}$), β -carotene accumulation (C-C stretch mode) ($1,516 \text{ cm}^{-1}$), and C = O ($1,668 \text{ cm}^{-1}$) [24].

Table 2 Peak assignments in the Raman spectra from follicular fluid of PCOS patients.

Peak (cm ⁻¹)	Assignment
758	Tryptophan Ethanolamine group Phosphatidylethanolamine
852	Proline, hydroxyproline, tyrosine Tyrosine ring breathing Glycogen
936	C-C stretching mode of proline & valine & protein backbone (α-helix conformation)/glycogen (protein assignment) P(CH3) terminal, proline, valine + v(CC) α -helix keratin (protein assignment)
1003	Phenylalanine, C-C skeletal
1156	C-C, C-N stretching (protein)
1516	β-carotene accumulation (C-C stretch mode)
1518	v (C = C), porphyrin Carotenoid peaks due to C-C & conjugated C = C band stretch
1590	Carbon particles
1627	C _α =C _α stretch Amide C = O stretching absorption for the β-form polypeptide films
1668	Carbonyl stretch (C = O) Cholesterol ester

Unsupervised PCA was then applied to all of the spectra to reduce the high-dimensional Raman dataset and transform data into appropriate variables that conferred biologic information. A spectral range of 600-1,800 cm⁻¹ was used to minimize the effects of uneven baseline. As shown in Fig. 2C, PCOS and non-PCOS have two clusters with some overlap. The overlap can be explained by the close similarity of follicular fluid between PCOS and non-PCOS patients. Herein, we sought to find biomarkers which can best separate the two groups and provide diagnostic advice for PCOS. Because the difference between groups was mostly significant along dimension 1, a loading plot was made to assign each Raman wavenumber to indicate significant Raman bands along dimension 1. Two Raman zones were identified for describing the largest variances between the two datasets: 993-1,165 cm⁻¹ and 1,439-1,678 cm⁻¹

(Fig. 2D). The quantification of metabolite concentration between PCOS and non-PCOS groups were presented at box plots by integrating individual Raman bands at the two Raman zones ($993\text{-}1,165\text{ cm}^{-1}$ and $1,439\text{-}1,678\text{ cm}^{-1}$). The results showed that the quantification of integration at $993\text{-}1,165\text{ cm}^{-1}$ was found to be higher in PCOS FF samples (0.0286 ± 0.001) compared with non-PCOS FF samples (0.0274 ± 0.001 ; $P < 0.001$). And, the quantification of the integration at $1,439\text{-}1,678\text{ cm}^{-1}$, on the other hand, showed a higher content in non-PCOS FF samples (0.0337 ± 0.001) than in PCOS FF samples (0.0319 ± 0.001 ; $P < 0.001$) (Fig. 3). All the results suggest that there are real different metabolic patterns in follicular fluid of PCOS patients.

Special Raman spectral as biomarkers for predicting oocyte development and IVF outcome in PCOS patients

As the follicular fluid is the direct environment of oocyte, the changes of metabolism in FF will affect oocyte development. In order to investigate whether the Raman spectra were predictive to the oocyte development, the special Raman spectra were compared between two subgroups of the transferrable PCOS blastocysts ($n = 150$), according to their morphological scores: (1) high-quality blastocysts group (HQ, $\geq 4\text{BB}$) ($n = 75$), and (2) low-quality blastocysts group (LQ, $\geq 3\text{BC}$ and $\leq 4\text{BC}$) ($n = 75$). The results showed that there were significant differences in two Raman positions ($993\text{-}1,165\text{ cm}^{-1}$ and $1,439\text{-}1,678\text{ cm}^{-1}$) between HQ and LQ groups ($P < 0.001$) (Fig. 4). In details, the quantification of the integration at $993\text{-}1,165\text{ cm}^{-1}$ was lower in HQ group (0.0316 ± 0.001) compared with LQ group (0.0328 ± 0.001 ; $P = 1.07 \times 10^{-5}$). On the other hand, the quantification of the integration at $1,439\text{-}1,678\text{ cm}^{-1}$ showed a higher content in HQ group (0.0292 ± 0.001) than LQ group (0.0279 ± 0.001 ; $P = 1.92 \times 10^{-6}$).

Embryo quality is a key factor affecting pregnancy outcome. In this study, we further analyzed the relationship of the special Raman spectra and IVF outcome in PCOS. By tracking the pregnancy outcome of each transferred blastocyst, the 150 Raman spectra of PCOS samples were divided into pregnancy success group ($n = 85$) and pregnancy failure group ($n = 65$). As shown in Fig. 5, the quantification of Raman spectra ($993\text{-}1,165\text{ cm}^{-1}$ and $1,439\text{-}1,678\text{ cm}^{-1}$) are significantly different between pregnancy success and pregnancy failure groups ($P < 0.001$). In line with the trend of the same two Raman zones correlating with blastocyst development, the quantification of the integration at $993\text{-}1,165\text{ cm}^{-1}$ was lower in pregnancy success group (0.0316 ± 0.001) compared with pregnancy failure group (0.0335 ± 0.002 ; $P = 1.36 \times 10^{-5}$), and the quantification of the integration at $1,439\text{-}1,678\text{ cm}^{-1}$ was higher in pregnancy success group (0.0289 ± 0.001) than that in pregnancy failure group (0.0278 ± 0.001 ; $P = 1.98 \times 10^{-6}$).

Machine-learning models based on Raman spectra classify blastocysts development and pregnancy outcome with high performance in PCOS

In this study, based on the 150 Raman spectra of PCOS follicular fluid samples, the two fully connected ANN classification models were computed to predict the blastocyst development and clinical pregnancy, respectively. The spectra of different subgroups (HQ or LQ blastocyst; pregnancy success or pregnancy

failure) were split into training set and the testing set in a ratio of 4:1. The training set was used to train a classification model and the testing set was used to evaluate the model performance. Specifically, for the classification model to predict blastocyst development, Raman spectra of 150 PCOS samples were divided into high-quality blastocysts (HQ) (n = 75) and low-quality blastocysts (LQ) (n = 75) groups. Of them, Raman spectra of 100 samples (50 high-quality blastocysts and 50 low-quality blastocysts) were used to train a classification model and the remaining 50 spectra (25 high-quality blastocysts and 25 low-quality blastocysts) were input as the testing set to evaluate the model performance. At the same time, to construct the classification model to predict the IVF outcome, the same Raman spectra of 150 PCOS samples were divided into pregnancy success (n = 85) and pregnancy failure (n = 65) groups. Of them, 100 spectra (50 pregnancy success and 50 pregnancy failure) were used to train a classification model and the remaining 50 spectra (35 pregnancy success and 15 pregnancy failure) were input as the testing set to evaluate the model performance.

The results of ANN models were presented in Table 3. As shown in Table 3A, 23 out of 25 high-quality blastocysts (F1 score 0.9020) and 22 out of 25 low-quality blastocysts (F1 score 0.8980) were assigned correctly. For classifying clinical pregnancy outcome (Table 3B), the ANN model was able to correctly assign 25 out of 35 pregnancy success spectra and 12 out 15 pregnancy failure spectra, with F1 scores of 0.7937 and 0.6486, respectively. Noticeably, the accuracy of the ANN model for predicting the blastocysts development is 90.00%, while 74.00% for predicting the IVF outcome. As shown in Fig. 6, ROC analysis resulted in AUCs of 0.88 for the blastocysts developmental ANN model (Fig. 6A) and 0.75 for the ANN model predicting the IVF outcome (Fig. 6B).

Table 3 Confusion matrix and performance evaluation of ANN classification models for an independent testing set of 50 Raman spectra.

A

Sample count	Confusion matrix		Performance evaluation			
	Predicted HQ- blastocysts	Predicted LQ- blastocysts	Precision	Sensitivity	F1 score	Accuracy
Actual HQ- blastocysts	23	2	88.46%	92.00%	0.9020	90.00%
Actual LQ- blastocysts	3	22	91.67%	88.00%	0.8980	

B

Confusion matrix			Performance evaluation			
Sample count	Predicted pregnancy success	Predicted pregnancy failure	Precision	Sensitivity	F1 score	Accuracy
Actual pregnancy success	25	10	89.29%	71.43%	0.7937	74.00%
Actual pregnancy failure	3	12	54.55%	80.00%	0.6486	

Note: Models were trained from a training set of 100 Raman spectra.

ANN is artificial neural network.

Discussion

To our best knowledge, this is the first report to explore the metabolomics profiles with the use of Raman spectroscopy to perform the machine-learning models for embryo viability selection in PCOS patients. Currently in clinical practice, visual morphology assessment is routinely used for evaluating of embryo quality and selecting the “considerably good” blastocyst for transfer. However, such subjective assessment has nonnegligible inconsistency among embryologists, and as a result, the success rate remains unsatisfactory. Moreover, in the situation where multiple blastocysts have the same morphological scores, the “blind selection” will increase the chances of pregnancy failure and repeated transplants, heavying the financial and psychological burden on patients. Therefore, a more objective and accurate prediction method or model is needed for embryo selection.

Machine-learning approaches are increasingly applied to improve prediction models for clinical decision making [25], and are also thought to have a significant advantage to predict embryo quality reliably [26]. In this study, we implemented an AI approach based on the fully connected artificial neural network (ANN), which is a state-of-the-art machine-learning architecture with higher computational efficiency, stronger fault tolerance, and better resistance to over-fitting [27]. The effectiveness and success of the machine-learning is attributed to several factors, including the sensitivity of the Raman system, the quality of the spectra, the choice of the machine-learning algorithm and the sufficient training datasets. Hereon, the accuracy of the ANN model for predicting the blastocysts development potential is as high as 90%, and the AUC is also up to 0.88. Although our research is still somewhat limited by moderate sample size (FF is more often pooled from multiple follicles of the same patient in clinical practices, and thus N = 300 individual FF is so far the largest sample size in literatures), the results indicated that the machine-learning of ANN model is highly effective and promising. It is conceivable that in the future this approach will be more sensitive and robust with higher quality of Raman spectroscopy, ever-improved and advanced algorithms, and increased training datasets. As expected, the accuracy for predicting IVF

outcome is less well-performed, as more factors beyond follicles, including genomic stability of the embryo and the endometrial receptivity, are also involved to affect the pregnancy outcome.

The outstanding feature of metabolomics is that, unlike genomics and proteomics, it indicates not only a genetically determined phenotype, but also the differences induced by other factors (i.e., age, diet, or physical activity) [28–31]. The metabolic profiles of follicle fluid from PCOS patients reflect both the pathological and physiological states of the abnormal follicle development. Consistent with previous reports [32, 33], our findings proved that high-quality blastocysts development depends on nutrients in follicular fluid and a complex metabolic activity is involved. The proper metabolic activity will further affect pregnancy outcomes. It is worthy to mention that two prominent spectra ($1,003\text{ cm}^{-1}$ and 1516 cm^{-1}) located in the two clinically relevant Raman zones ($993\text{--}1,165\text{ cm}^{-1}$ and $1,439\text{--}1,678\text{ cm}^{-1}$) are assigned to phenylalanine and β -carotene, respectively [24]. Agreeing with the previous opinion [34], we suggest that the two prominent spectra ($1,003\text{ cm}^{-1}$ and 1516 cm^{-1}) are related with the metabolic activity of embryos and considered as biomarkers for embryonic development.

Most meaningfully, the metabolomic analysis of follicle fluid not only helps us to find potential biomarkers to select the high-quality embryos and predict the IVF outcome, but also, to some extent, reveals the abnormal mechanism of follicle development of PCOS. For instance, phenylalanine is one of the amino acids that inhibit hamster 1-cell embryo development in vitro and is harmful for blastocyst formation in pigs [35, 36]. Moreover, the concentrations of phenylalanine in embryo culture media are used as biomarkers for clinical pregnancy in humans [37]. Our results also indicate that the greater vibration at 1004 cm^{-1} of phenylalanine in FF is a warning sign for oocyte development in PCOS, and are in line with the previous views that blastocyst development is modulated by amino acids, and the ideal environment for embryonic development needs the supplement of suitable amino acids [38]. It's worth mentioning that abnormal amino acids are associated with the occurrence of chromosome aneuploidy during human preimplantation embryo development in vitro [39]. It requires further study of whether the elevated phenylalanine in PCOS FF causes chromosomal abnormalities in oocytes, which affects blastocyst development.

Another substance, β -carotene, is noticeably greater in follicular fluid for the oocytes with high developmental potential in PCOS patients. β -carotene, a precursor to vitamin A, has been postulated to contribute to follicular growth. A positive correlation has been observed between the plasma vitamin and β -carotene concentrations and the number of transferable embryos in vitro fertilization process [40]. And, it has been proposed that β -carotene acts as an antioxidant in lipid phases by quenching singlet oxygen and scavenging the peroxy radical [41]. As known, chronic low-grade inflammation along with increased oxidative stress has been suggested as a key contributor of the pathogenesis and development of PCOS [42]. Inappropriate microenvironment conditions can decrease the oxidative metabolism and further underpins the complexity in linking embryo metabolism and viability [43]. Based on the fact that high-quality blastocyst needs more β -carotene, we propose that enough extracellular β -carotene in the follicular fluid could protect oocytes from reactive oxygen species-mediated cytotoxicity, thereby enhancing the developmental competence of oocytes. Besides antioxidation, β -carotene also has other unique roles

in reproduction. The corpus luteum is a steroidogenic tissue that is highly rich in β -carotene, and β -carotene plays a very important role in regulating the luteal function [44]. The levels of β -carotene in follicular fluid has been reported to positively correlated with plasma progesterone level as well [45]. Similar positive relationship also exists in the corpus luteum [46]. Therefore, we also propose that lower β -carotene levels of follicular fluid might be the main reason for luteal insufficiency and difficult pregnancy in PCOS patients.

Conclusion

To clarify the effect of abnormal follicular microenvironment on oocyte development in PCOS patients, we developed a noninvasive and label-free rapid strategy to use Raman spectroscopy to detect metabolic footprint of follicular fluid. Analysis based on Raman spectra showed significant differences in metabolic profiles of follicular fluid between PCOS and non-PCOS patients. Two Raman zones ($993\text{--}1,165\text{ cm}^{-1}$ and $1,439\text{--}1,678\text{ cm}^{-1}$) were identified for describing the largest variances between the two groups. The tentative assignments of corresponding Raman bands $1,003\text{ cm}^{-1}$ (phenylalanine) and $1,516\text{ cm}^{-1}$ (β -carotene accumulation) were proposed to be highly promising biomarkers to evaluate blastocysts viability and predict the IVF outcome. The fully connected ANN models successfully classified blastocysts development potential with high accuracy. In summary, our data suggest that noninvasive method of Raman spectroscopy might be a useful tool to identify and predict the embryo developmental potential, and more importantly, to prioritize the best promising oocyte for IVF, reducing both physiological and economical burdens of PCOS patients by avoiding unnecessary implanting cycles.

Abbreviations

PCOS: Polycystic ovary syndrome; FF: follicular fluid; ANN: the fully connected artificial neural network; COS: controlled ovarian stimulation; IVF: in vitro fertilization; GCs: granulosa cells; AI: artificial intelligence; PCA: principal components analysis; ROC: receiver operating characteristic; AUC: the area under the receiver operating characteristic curve; TP: true positives; FP: false positives; TN: true negatives; FN: false negatives; HQ: high-quality blastocysts group; LQ: low-quality blastocysts group

Declarations

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Authors' contributions

Xin Huang, Ling Hong and Yuanyuan Wu drafted the manuscript, completed the experiments and performed statistical analysis. Xin Huang, Zhiyun Wei and Xiaoming Teng designed the experiment and revised the article. Miaoxin Chen, Ling Hong and Pengcheng Kong helped with collection of follicular fluid

samples and acquisition of data. Pengcheng Kong and Jingling Ruan confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the Institutional Ethical Review Board of Tongji University School of Medicine (Reference: TJUSM-2017-0318). Written informed consent was obtained from all patients and the study was approved by the Ethics Review Board of Shanghai First Maternity and Infant Hospital (Reference: 2017-RM-0920).

Consent for publication

All authors have consented to the publication of this article.

Competing interests

The authors declare that they have no competing interests.

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Figures

Fig. 1

Figure 1

The schematic architecture of the fully connected artificial neural network (ANN) for classification models using Raman spectra. Follicular fluid of PCOS patient were collected and transferred onto a gold-coated microarray slide, and tested using Raman spectroscopy. The structure of the ANN includes one input layer, 2 hidden layers that contain 64 neurons and 64 neurons, as well as one output layer. An activation function relu is associated with each neuron that sums up the outputs from that neuron and transfers them to the next layer in the network. Details can be found in the Materials and Methods section.

Fig. 2

Figure 2

Raman profiling of follicular fluid of PCOS and non-PCOS. (A) Averaged Raman spectra of follicular fluid of PCOS (n=150) and non-PCOS (n=150) patients. Shaded areas represent the standard deviations. (B) Mean-centered Raman spectra by subtracting means from all samples, showing differences between PCOS and non-PCOS groups. (C) Principal component analysis (PCA) plot of all Raman spectra, showing clustering of PCOS and non-PCOS. (D) Raman wavenumber loading plots of contributions along

dimension 1 of the PCA, where grey boxes indicate the Raman bands responsible for the largest variances between the two groups.

Fig. 3

Figure 3

Boxplots of Raman band integration of PCOS and non-PCOS samples. The significant differences at 993-1,165 cm⁻¹ (left) and 1,439-1,678 cm⁻¹ (right) were shown between PCOS and non-PCOS groups. The rectangle in the box plots represents the second and the third quartiles, with the line inside representing the median. The lower and upper quartiles are drawn as lines outside the box. Sample means were compared by Welch 2-sample t test for unequal variances. *** indicates $P < 0.001$.

Fig. 4

Figure 4

Boxplots of Raman band integration of high-quality blastocysts group and low-quality blastocysts group in PCOS. The significant differences at 993-1,165 cm⁻¹ (left) and 1,439-1,678 cm⁻¹ (right) were shown between high-quality blastocysts and low-quality blastocysts groups in PCOS. The rectangle in the box plots represents the second and the third quartiles, with the line inside representing the median. The lower and upper quartiles are drawn as lines outside the box. Sample means were compared by Welch 2-sample t test for unequal variances. *** indicates P<0.001.

Fig. 5

Figure 5

Boxplots of Raman band integration of successful pregnancy group and failed pregnancy group in PCOS. The significant differences at 993-1,165 cm⁻¹ (left) and 1,439-1,678cm⁻¹ (right) were shown between the two groups in PCOS. The rectangle in the box plots represents the second and the third quartiles, with the line inside representing the median. The lower and upper quartiles are drawn as lines outside the box. Sample means were compared by Welch 2-sample t test for unequal variances. *** indicates $P < 0.001$.

Fig. 6

Figure 6

Receiver operating characteristic (ROC) curves of the ANN classification models over all testing sets. (A) ROC analysis of the ANN model for classifying the blastocysts developmental potential. (B) ROC analysis of the ANN model for classifying the IVF outcome. AUC, the area under the ROC curve.