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Metabolic characterization of asthenozoospermia using nontargeted seminal plasma metabolomics

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ABSTRACT

Background: Asthenozoospermia (AS) is a common cause of male infertility. Due to the limitation of routine semen analysis, metabolic alterations associated with the disease are unclear. We applied a metabolic profiling strategy as a surrogate method to accurately assess and provide new insights into the etiologies of asthenozoospermia.
Methods: Seminal plasma samples from patients diagnosis with asthenozoospermia (n = 33) and healthy subjects (n = 30) were investigated using a nontargeted metabolomics approach based on proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The spectral data were then subjected to multivariate and univariate analyses to identify metabolites that were correlated with asthenozoospermia. The disturbed metabolic pathways which the biomarkers were involved in were analyzed.
Results: Nineteen metabolites including up-regulation or down-regulation of several amino acids, changes in lipids metabolism, phospholipids (choline) metabolism, cholesterol metabolism, nucleoside metabolism, the Krebs cycle and energy metabolism were identified and associated with asthenozoospermia. In particular, the elevation of oxysterols such as 5α-cholesterol and 7-ketocholesterol in seminal plasma of patients with asthenozoospermia was an important finding, indicating the important role of oxidative stress in the mechanism of asthenozoospermia.
Conclusions: The excellent performance of this metabolomics approach offer a highly novel means of etiological diagnosis of asthenozoospermia.

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1. Introduction

Infertility affects ~10–15% couples worldwide and male infertility contributes to approximately 50% of these infertile cases [1–4]. Semen analysis in idiopathic patients reveals a decrease in sperm motility, a phenotype known as asthenozoospermia (AS) [5]. Routine semen analysis may indicate defective sperm production or diminished sperm motility. However, it has become evident that basic semen analysis alone is insufficient to determine the male fertility status [6]. The etiologies of male infertility are very limited, and a systematic understanding of asthenozoospermia remains a challenge.

Recently, the possibility of using metabolomics as a diagnostic tool for various diseases has been investigated in a number of studies [7–13]. Increasing evidence has shown that abnormal metabolic phenotypes in body fluids reflect the pathogenesis and pathophysiology of disease [14–16]. These body fluids include seminal plasma. Like most body fluids, the seminal plasma represents a good sample for metabolomic analysis in the evaluation of male fertility/infertility, alterations at the molecular level in spermatozoa and seminal plasma can affect male fertility [17–20], and when combined with metabolomics studies, the seminal plasma analysis should be more useful for studies of male infertility. Regrettably, it is a pity that the seminal plasma has not yet received much attention from the metabolomics community, and comparative metabolomic analysis of male infertility-associated seminal plasma has not been well documented to date.

In the current study, the seminal plasma from patients with asthenozoospermia and healthy donors was subjected to proton nuclear magnetic resonance (^{1}H NMR) spectroscopy based metabolomic analysis. To our knowledge, this is the first report about human seminal plasma metabolomics study. The aim of this study was to determine if metabolomics analysis of human seminal plasma can provide new insights into the etiologies of asthenozoospermia, and if the molecular markers identified in the study can serve as potential biomarkers for asthenozoospermia. We propose that such markers may help clinicians to better diagnose causes of asthenozoospermia.

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2. Materials and methods

2.1. Clinical samples collection

The present work was approved by the Regional Committee for Medical Research Ethics and the Human Ethics Committee of Shenzhen Second People's Hospital, and carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/en/30publications/10policies/b3/index.html. Each subject studied gave their informed consent.

Semen was obtained from healthy donors or infertile patients according to World Health Organization Criteria [21]. Subjects with a family history of endocrine or anatomical disorders were excluded from the study. Semen collection was done by masturbation after 3–5 day-sexual abstinence. After complete liquefaction at 37 °C for 30 min, parameters including semen volume, sperm concentration, sperm motility and viability were analyzed by a computer assisted semen analysis (CASA) system under 200× magnification. Sperm morphology was assessed by SpermFunc® Diff-Quik Staining kit according to manufacturer's manual (BRED Life Sciences). Healthy controls were selected according to the following criteria: spontaneous pregnancy achieved within less than one year of pregnancy expectation and with the last pregnancy less than one year prior to study; a sperm concentration of $\geq 20 \times 10^6$ spermatozoa/ml, sperm motility with forward progression ≥32%, and normal morphology ≥4%. Asthenozoospermia was selected according to the following criteria: sperm motility with forward progression <32% in addition, including both rapid, slow progressive and sluggish motility in the same class within 60 min of ejaculation during the past 3 months. The concentration of sperm, and percentage of morphologically normal sperm, were equal to or above the lower reference limits.

Finally, a healthy control and an asthenozoospermia group comprising 33 and 30 age-matched controls and cases were chosen for the next metabolomic analysis respectively. The average age of the men was 31 years in the healthy control group (range 21 – 40 years) and 32 years old in the asthenozoospermia group (range 21 – 40 years). Both the seminal plasma samples from patients with asthenozoospermia and healthy controls were stored at −80 °C until analysis.

2.2. ^{1}H NMR spectroscopy analysis

All the seminal plasma samples were thawed at room temperature and homogenized using a vortex mixer. Then, 200 μl of 0.2 mol/l sodium phosphate buffer (0.2 M Na_2HPO_4/0.2 mol/l NaH_2PO_4, pH 7.4) containing 1 mmol/l sodium-3-trimethylsilyl-[2,2,3,3-$2H_4$]-propionate (TSP) in D_2O (99.9%) was added to 400 μl of each seminal plasma. After centrifugation at 12,000 rpm for 5 min at 4 °C to remove any precipitate, aliquots of 550 μl of the resulting supernatants were transferred into 5-mm NMR tubes and stored at 4 °C until analysis.

The ^{1}H NMR spectra for all specimens were acquired in a random order at 298 K on a Bruker AVANCE 600 spectrometer (Bruker Biospin) equipped with a 5 mm TCI cryogenic probe. One-dimensional ^{1}H NMR spectra of all seminal plasma samples were acquired using the standard NOESYpr1d pulse sequence with water pre-saturation. Water resonances were suppressed by using a 90°-3 μs-90°-100 ms-90° pulse sequence with irradiation at the water frequency during both the relaxation delay of 2 s and the mixing time of 100 ms. For each sample, a total of 64 transients were collected into 32,000 data points with a spectral width of 9590.7 Hz and an acquisition time of 1.60 s.

2.3. Data processing

All ^{1}H NMR free induction decays (FIDs) were imported into MestReNova NMR Suite Version 6.0.2 software package (Mestrelab Research, S.L.) for processing and binning. The FID was zero filled to 64 K and an exponential weighting factor corresponding to a line broadening of 1.0 Hz was applied prior to Fourier transformation. Both phase and baseline correction were automatically performed carefully, and the spectra were referenced to the chemical shift of TSP at δ 0.0 ppm. The NMR spectra covering the region of δ 8.5–0.0 ppm were data-reduced to 202 consecutive non-overlapped regions (bins) with equal width of 0.04 ppm. Each segment consisted of the integral of the associated NMR region except the region of δ 5.2–4.7 ppm (containing the residual peak from the suppressed water resonance), which was set to zero integral in the analysis. Subsequently, the integrated spectra segments for each NMR spectrum were normalized according to the total area of the spectrum. Then, the resulting normalized datasets were submitted to multivariate analysis.

2.4. Multivariate statistical analysis

After mean-centering and unit-variance (UV) scaling the datasets, principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection on latent structure discriminant analysis (OPLS-DA) were performed to identify metabolic differences between patients with asthenozoospermia and healthy donors using SIMCA-P ver. 12.0 software package (SIMCA-P + 12.0, Umetrics). First, PCA analysis was performed to observe intrinsic clusters and find obvious outliers. Then, PLS-DA and OPLS-DA were employed to visually discriminate between patients with asthenozoospermia and healthy donors. PLS-DA, a supervised extension of PCA, was performed to maximize group (class) separations, simplify interpretations, and find potential biomarkers. OPLS-DA is a supervised pattern-recognition procedure that combines the existing theory of PLS-DA and orthogonal signal correction (OSC). The OPLS-DA model removes variability not relevant to class separation, and normally, only one predictive component is used for the discrimination between 2 classes [22–24].

To guard against the OPLS-DA-derived model over-fitting, internal cross-validation (ICV) was performed. For ICV, permutation tests with 100 iterations using the 7-fold cross-validation method were performed. This rigorous test compares the goodness of fit of the original model with that of randomly permuted models [25]. The ICV test provides model validity in the form of the explained variance parameter (R2) and the cross validation parameter (Q2), which indicates goodness of fit and accuracy of prediction, respectively, in the OPLS-DA model. The cumulative values of total Y explained variance (R2) and the Y predictable variation (Q2) approaches 1 indicated proper modeling. In parallel, CV-ANOVA (analysis of variance testing of cross-validated predictive residuals) tests were performed to determine significant differences between asthenozoospermia and the healthy control group in the OPLS-DA models. The Pearson product-moment correlation coefficient and level of significance were determined as described [26].

Based on a variable importance in projection (VIP, a measure of their relative influence on the model) threshold >1 from the OPLS-DA model together with the V-plot (plot constructed with the VIP value versus p (corr) value of each metabolite), a number of metabolites responsible for discriminating between the metabolic profiles of asthenozoospermia and healthy controls could be obtained. Then, different discriminant metabolites obtained were validated at a univariate level using student's t-test from SPSS, version 17.0. A $p < 0.05$ was considered to be statistically significant. The normalized amount of each metabolite was plotted in a histogram using the Origin software package (ver 8.0).

2.5. Prediction models and receiver operating characteristic (ROC) curves analysis

To evaluate the predictive and diagnostic ability of the significantly different metabolites, external cross-validation was carried out. Two-thirds of the total samples (22 healthy controls and 20 asthenozoospermia) were randomly selected from each group as the test set, and the remaining one-third of the samples (11 healthy controls and 10 asthenozoospermia) constituted the training set for validation. Then, the

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