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Comparative Analysis of Interindividual Variations in the Seminal Plasma Proteome of Fertile Men With Identification of Potential Markers for Azoospermia in Infertile Patients

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Abstract

Seminal plasma is a potential source of biomarkers for many disorders of the male reproductive system. The identification and characterization of proteins in the seminal plasma by using two-dimensional (2-D) difference gel electrophoresis (DIGE) serve as a basis for estimating male infertility. However, individual variation remains an impediment to identifying disease-associated proteins. Therefore, it is necessary to examine the interindividual variations in the seminal plasma proteome of fertile men. The present study analyzed seminal plasma samples from 10 fertile men. The results from silver-stained 2-D DIGE were averaged to reduce gel to gel variations. Up to 501 spots (polypeptides) were detected on the averaged gels for the fertile men. The coefficient of variation (CV) of standardized abundance was calculated for 63 spots that were common to all 10 samples; the CV values ranged from 24.5% to 129.9% with a median value of 63.1%. These results demonstrate that the variability in protein expression among different fertile men is relatively high in seminal plasma compared with that in other human tissue samples. The 63 matched spots were compared with those from 10 patients with azoospermia. There was no common spot that was lost in all of the 10 patients, and 16 of these spots (25%) were present in each of the patients.

We identified 4 and 1 candidate markers for nonobstructive and obstructive azoospermia, respectively. These results validate our method of identifying differences in the proteomic profiles of seminal plasma samples and provide an important basis for protein expression profiling and comparative proteomics of infertility.

Key words: Male infertility, two-dimensional (2-D) difference gel electrophoresis (DIGE), nonobstructive, obstructive

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Seminal plasma, the liquid component of semen, provides a favorable environment for sperm. It contains many proteins originating from the testis, epididymis, and accessory glands. From a medical perspective, many attempts have been made to establish a link between the seminal plasma proteins and cancer of the reproductive tissues—particularly prostate cancer (Banez et al, 2005)—and male infertility (Nishimune and Tanaka, 2006). Of the various instances of male infertility, the most severe cases with regard to natural pregnancy are azoospermia. This is a disorder in which the semen is completely devoid of sperm; it may occur due to spermatogenesis abnormalities, seminal tract obstruction, or inadequate hormonal stimulation (hypogonadotropic hypogonadism). The main clinical findings used to determine the etiology of azoospermia include testicular size, the presence of the vasa by palpation, and the levels of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Azoospermia is mainly classified as either obstructive azoospermia (OA) or nonobstructive azoospermia (NOA). Epididymal duct obstruction of unknown origin and congenital bilateral absence of the vas deferens are common causes of OA. A majority of patients with OA exhibit normal spermatogenesis. On the other hand, NOA is mainly caused by idiopathic spermatogenic disturbances. It is histologically diagnosed as Sertoli cell only (SCO), maturation arrest, or hypospermatogenesis (Sigman and Jarow, 2002).

The evaluation of azoospermia patients should be geared toward determining whether azoospermia is caused by lack of spermatogenesis or by seminal tract obstruction. The first step in the diagnosis of azoospermia is frequently semen analysis. The final step is to centrifuge the semen specimen and check for the absence of sperm. The presence of any sperm in the pellet rules out complete bilateral seminal tract obstruction. NOA patients generally have a small testicular volume and FSH serum levels 2 to 3 times higher than normal (Sigman and Jarow, 2002). For further examination, a conventional testicular biopsy should be performed for diagnosis. Prior to the advent of assisted reproductive technology (ART), patients diagnosed with NOA could not produce offspring. However, ART makes it possible for NOA patients to become fathers. Sperm can be retrieved in some NOA patients by multiple testicular sperm extractions (TESE). Therefore, a specific biochemical marker that predicts the presence of sperm is important and necessary when performing multiple TESE. For this purpose, we have been searching for a suitable marker that would serve as a useful predictor in TESE for NOA patients. Serum and seminal inhibin-B levels, FSH levels, and testicular volume have been discussed previously as potential markers (Ballesca et al, 2000). However, these are not sufficiently reliable to be regarded as specific markers. This leads us to identify the cause of spermatogenic disturbance as well as a sperm retrieval predictor in the seminal plasma protein.

The range of protein concentrations in seminal plasma averages between 35 and 55 mg/mL, making it a rich and easily accessible source for protein identification. In recent years two-dimensional (2-D) gel studies have been combined with mass spectrometric (MS) identification of protein spots that change abundantly in different clinical stages related to infertility (Starita-Geribaldi et al, 2001, 2003). A study using 2-D and 1-D gel electrophoresis and both matrix-assisted laser desorption ionization–time of flight MS and liquid chromatography–tandem mass spectrometry (LC-MS/MS) reported the identification of 61 different proteins (Fung et al, 2004). In a recent study employing

1-D gel electrophoresis and high-confidence identification, 923 proteins were identified in the seminal plasma derived from a single individual (Pilch and Mann, 2006). However, there is no study focusing on intraindividual and interindividual variations that is comparable to other proteomic studies, including those on cerebrospinal fluid (Hu et al, 2005) and liver (Zhang et al, 2006). To identify disease-related markers, it is important to examine the diversity of individual samples. For this purpose, we applied a proteomic approach based on 2-D difference gel electrophoresis (DIGE) to select a normalized standard map of normal seminal plasma proteins from fertile men. We then used this standard map to identify the differences in the expression of polypeptides between fertile men and azoospermia patients.

Materials and Methods

- Top
- Abstract
- Materials and Methods
- Results
- Discussion
- References

Samples

Samples of seminal plasma and sera from 10 fertile men (N1–N10) and 10 infertile patients (ie, 7 NOA [P1–P7] patients and 3 OA patients [P8–P10]) (Table 1) were collected. The testis size was assessed by using a wooden orchidometer (Pharmacia & Upjohn, Hillerød, Denmark). The concentrations of FSH, LH, and testosterone in the sera of fertile men were determined by time-resolved immunofluorometric assay (DELFIA; Wallac, Turku, Finland). Serum levels of FSH and LH of infertile patients were determined with kits from SRL (Tokyo, Japan); a kit from Diagnostic Products Corp (Los Angeles, Calif) was used for testosterone. This study was approved by the Ethical Committee/Institutional Review Board of St Marianna University School of Medicine. Informed consent was obtained from all donors before the use of their semen and blood for research. Semen samples were collected by masturbation in sterile containers after at least 48 hours of abstinence. Each semen sample was liquefied for 15 to 30 minutes at 37 °C prior to the assessment of sperm parameters according to the World Health Organization guidelines (1992). Subsequently, seminal plasma was prepared by centrifuging (12 000 x *g* for 15 minutes at 4 °C) the liquefied semen to eliminate solid materials. The supernatants were used as seminal plasma, and aliquots were frozen at −80 °C until analysis. Each seminal plasma sample was analyzed by 2-D gel electrophoresis in triplicate. The 2-D gel maps of the seminal plasma obtained from the 10 fertile men were compared, and 63 common spots were selected. These spots were used for constructing a standard map. The averaged map of each patient was compared with this standard map.

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Table 1. *Clinical parameters of semen samples*[*]

Apparatus

Separation in the first dimension of 2-D gel electrophoresis was carried out on an Ettan IPGphor isoelectric focusing unit (Amersham Pharmacia Biotech, Uppsala, Sweden). The wet gels were scanned and subsequently processed by the software ImageMaster (Amersham Pharmacia Biotech). The mass spectra of the tryptic digests were acquired by nanoscale capillary LC-MS/MS analysis on a capillary LC system (MAGIC 2002; Michrom BioResources, Inc, Auburn, Calif) connected to an in-line nanoelectrospray mass spectrometer (LCQ Advantage; Thermo Fisher Scientific, Waltham, Mass) equipped with a silica-coated glass capillary (PicoTip; New Objective, Inc, Woburn, Mass).

Reagents

Urea and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad (Hercules, Calif). Silicon oil fluid and immobilized pH gradient (IPG) 3–10 strips were obtained from Amersham Pharmacia Biotech, Inc. 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), and iodoacetamide were obtained from Sigma-Aldrich (St Louis, Mo). Sequencing-grade modified porcine trypsin was obtained from Promega (Madison, Wis), and bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, Ill).

Sample Preparation

A fraction of each sample was used for microscale protein determination using the BCA assay (Smith et al, 1985). The samples were diluted in rehydration buffer containing 8 M urea, 2% CHAPS, 18 mM DTT, 0.5% IPG buffer, and 0.002% bromophenol blue. Analytic separation was performed with 50 µg of seminal fluid proteins on pH 3–10 IPG strips (70 mm in length). For semipreparative separations, 0.5 mg of each protein was separated on pH 3–10 IPG strips (130 mm in length).

Isoelectric Focusing in Immobilized pH Gradients

In routine analysis, the entire IPG gel was used for sample application, during which the protein entered the gel during rehydration. The rehydration volume was 125 µL of rehydration buffer for a gel with the following characteristics: width, 3 mm; thickness, 0.5 mm; length, 70 mm; pH 3–10; nonlinear. Isoelectric focusing was carried out using IPGphor at 20 °C. For analytic purposes, electrophoresis was performed with a voltage gradient of 8000 V to attain a total of 40 kVh for a pH 3–10 IPG strip.

SDS Gel Electrophoresis

Prior to the resolution of the IPG gel by SDS gel electrophoresis using a 10% to 20% polyacrylamide gradient gel, it was equilibrated in a solution for IPG containing 30% (wt/vol) glycerol, 6 M urea, 2% (wt/vol) SDS, and 0.002% (wt/vol) bromophenol blue in 0.05 M Tris-HCl at pH 8.8. The first step of the equilibration was carried out using 65 mM DTT. This was followed by a second equilibration step that was carried out using 260 mM iodoacetamide. Both equilibration steps were performed for 15 minutes at room temperature.

Detection of the 2-D Pattern

Silver staining of the 2-D gels was performed using a silver staining kit (Wako Chemicals, Osaka, Japan). Coomassie brilliant blue R detection was considered a good quantitative indication for spot excision. The gels were scanned, and volumetric determination of the detected spots was performed by computer analysis. Seminal plasma possesses a feature common to many other body fluids—a high dynamic range of protein abundance; this renders analysis of low-abundance components difficult. Therefore, we applied 50 µg of the protein sample per strip (70 mm in length), an amount excessive for silver staining. The abundant proteins, namely transferrin, albumin, prostatic acid phosphatase (PAP), and prostate-specific antigen (PSA), were excluded from this analysis. We then performed 2-D gel electrophoresis 3 times for each sample. Matching was performed using the gel N1-1 as a reference gel. For each sample, the spots that were present in at least 2 gels were considered as the average gel spots.

In-Gel Protein Digestion

The protein spots were excised from the gel. The gel pieces were then washed in water, followed by destaining with 50 mM ammonium bicarbonate containing 50% methanol. The procedure was repeated until the gel was completely destained. Finally, the pieces were homogenized, dried in a vacuum

centrifuge, and stored at −20 °C until trypsin digestion. The dried gel pieces were reswollen with a minimum amount of trypsin solution, depending on the amount of protein (typically 20 µL of a 1 pmol trypsin/10 µL solution in 50 mM Tris-HCl at pH 8.8). When necessary, further buffer was added until the gel piece was completely rehydrated. The digestion was performed for 10 hours at 37 °C. The peptides were extracted with 50% to 80% acetonitrile, and 0.1% trifluoroacetic acid (TFA), and the organic solvent was evaporated in a vacuum centrifuge.

LC-MS/MS and Data Analysis

The concentrated proteolytic peptide mixture was added to 35 µL of 2% acetonitrile and 0.1% TFA and subjected to nanoscale capillary LC-MS/MS analysis. The spectra were collected as MS and MS/MS scans. The MS scan defined the ion composition at an *m/z* range of 450 to 2000, and the MS/MS scan acquired the mass spectrum of the parental ion upon collision-induced dissociation. The acquired collision-induced dissociation spectra were analyzed by direct inspection using Bioworks Browser software (Thermo Fisher Scientific). A list of peptide masses was obtained for each protein digest. This peptide mass fingerprint was then submitted to the MASCOT program (Matrix Science, Inc, Boston, Mass) for protein identification.

Results

2-D Gel Electrophoresis Analysis of Seminal Plasma From Fertile Men

The typical 2-D pattern of the seminal plasma obtained from 10 fertile men is presented in Figure 1A. The mean number of spots detected using the method described in "Materials and Methods" was 386 ± 43, and the number of matched spots was 263 ± 66. We selected 63 spots that were common in the samples from the 10 fertile men (Figure 1B). The coefficient of variation (CV) of these spots ranged from 24.5% to 129.9% with a median value of 63.1%.

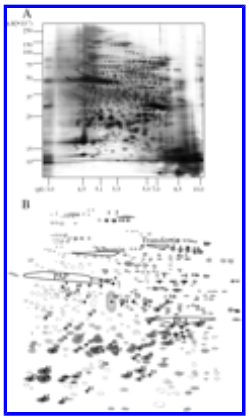

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Figure 1. (**A**) Silver-stained 2-D electrophoretic separation of human seminal plasma. Typical gel from a fertile man (N1), which was used as a reference map for matching. The detected spots are outlined. (**B**) The 63 spots that were matched across all 10 gels from fertile men. They were described on the gel of matched spots of a fertile man (N1) without background. Transferrin, albumin, prostatic acid phosphatase, prostate-specific antigen, and the specific spots on the gel are excluded. The 63 representative matched spots of a fertile man (N1) are indicated with a black asterisk and number. The 16 spots that were present in all patients are indicated with a white asterisk.

2-D Gel Electrophoresis Analysis of Seminal Plasma From Azoospermia Patients

The 63 spots from the fertile men were compared with those of each gel from an azoospermia patient. The mean number of spots detected from the gels of these patients was 393 ± 67. There was no spot

that disappeared from all the gels of the patients, whereas there were 16 spots that were present in the gels of all the patients. The 63 spots were grouped with the CV values of the fertile men, and the heat map indicated that the number of deletions increased with the CV value (Figure 2). With a decrease in the CV values, there was an increase in the number of spots that had larger volumes than the normal samples. The number of spots absent was the smallest in P1; this was the only case in which sperm was detected by TESE (Table 1). Regarding the OA samples, there were 2 spots—numbers 45 and 51—that were absent in all OA samples but were present in all NOA samples. Both spots were determined to be the epididymal secretory protein E1 (Swiss-Prot accession number P61916) based on LC-MS/MS and a MASCOT search (Table 2). Seven spots, numbers 3, 9, 18, 39, 48, 58, and 63, were absent in more than 3 patients with NOA. Among these spots, numbers 3, 18, and 63 were believed to be artifacts of the 2-D gel electrophoresis. Spot number 3 was located in a high pH and molecular-weight region of the gel where separation was poor. Spot number 18 was located near number 16 and was determined to be clusterin (Swiss-Prot accession number P10909) based on LC-MS/MS and a MASCOT search. Spot number 16 was in the form of a cluster. Since this cluster spread as a smear (of spots), it affected the separation of spot number 18. Spot number 63 was near the protein separation front, and unknown factors around pI 4.8 disturbed the pattern of electrophoresis in this region, particularly in the patient samples (data not shown). We eventually selected the remainder, 4 spots, numbers 9, 39, 48, and 58, as candidate markers for NOA. These proteins were identified based on LC-MS/MS and a MASCOT search (Table 2 and Figure 3).

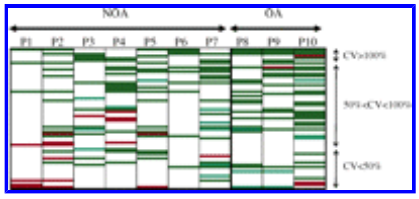

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Figure 2. The pattern of changes in the 63 selected spots. Green or pale green bars indicate absent or significantly reduced spots. Red bars indicate significantly increased spots when compared with the normal samples. The spots were aligned according to their coefficient of variation values.

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Table 2. *Identification of the spots selected for confirming the presence of NOA or OA markers*[*]

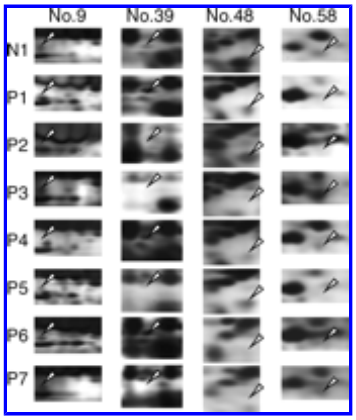

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Figure 3. Typical images of the spots of the candidate markers for nonobstructive azoospermia (NOA). An arrowhead indicates each spot that was absent in more than 3 patients with NOA.

Discussion

- Top
- Abstract
- Materials and Methods
- Results
- Discussion
- References

The identification of plasma/serum protein alterations by 2-D gel electrophoresis has enabled disease diagnosis on the basis of protein map modifications (Tissot et al, 1991). 2-D gel electrophoresis maps have been reported for different human fluids such as blood plasma (Anderson and Anderson, 1991), cerebrospinal fluid (Yun et al, 1992), and amniotic fluid (Liberatori et al, 1997). Understanding the degree of biologic variation present in different individuals is an important basis for proteome expression profiling. It is also essential for comparative proteomic studies designed for detecting authentic differences. In this study, we assessed interindividual variations in the seminal plasma proteome by analyzing 10 normal seminal plasma samples using 2-D gel electrophoresis. Although several studies have used proteomic technologies to analyze the seminal plasma proteome in healthy and diseased states, to the best of our knowledge, this is the first work to assess biologic variations in the seminal plasma proteome among healthy fertile individuals. Our results demonstrated that the abundance variation of 63 protein spots shared by all 10 individuals ranged from 24.5% to 129.9%, with a median value of 63.1%. In all, 46 protein spots, approximately 73% of the total 63 protein spots, exhibited CV values greater than 50%. These results illustrated several properties of the seminal plasma proteome. According to a previous review (Anderson and Anderson, 1991), the average intraindividual and interindividual CV values for a series of plasma proteins are 23% and 45%, respectively. A further study demonstrated that for bacterial and established mammalian cell lines, the biologic variation ranged from 24% to 29%; for cells grown as primary cultures, the biologic variation measured in terms of average CV was between 39% and 47% (Molloy et al, 2003). With regard to the normal liver proteome, the CV values ranged from 6.4% to 108.5%, and the median CV value was 19%. This demonstrated that protein expression in the normal liver among different individuals was relatively stable (Zhang et al, 2006). When compared with these studies, our results demonstrated that the protein expression in liquefied seminal plasma among different individuals was relatively variable. This was due to the fact that almost all of these proteins were extracellular proteins and the proteolysis effects were highly variable. Moreover, the seminal plasma we examined, from fertile men as normal healthy samples, contained 2 cases (N2 and N4; Table 1) that were not normozoospermic. The spots that were selected as candidates for NOA had CV values ranging from 60.4% to 129.9% in

normal individuals. This emphasizes the fact that these proteins behave in a broader dynamic range in the normal physiologic state and provide us with a basic reference to distinguish authentic disease-associated changes from individual variations.

Recently the identification of proteins separated by 1-D gel electrophoresis has been well documented for human seminal plasma (Pilch and Mann, 2006). Pilch and Mann (2006) examined the seminal plasma preparation of a single individual, and proteolytic liquefaction was suppressed by the use of a protease inhibitor. This preparation is suitable for the documentation of proteins; however, the seminal plasma samples were usually obtained in a liquefied and proteolyzed form after the assessment of sperm parameters in infertility clinics. Therefore, the present study used liquefied seminal plasma to discover potential clinically available markers. Another study produced a 2-D gel electrophoresis map of liquefied seminal plasma and compared it with vasectomized and SCO syndrome samples (Starita-Geribaldi et al, 2001). These authors also identified PAP and PSA in the seminal plasma. However, they neither showed the interindividual variations nor identified the potential markers. Some proteins that are abundantly present in the serum, such as albumin, transferrin, lactoferrin, and α-1-antitrypsin, have also been characterized in seminal plasma (Edwards et al, 1981); however, their functions with respect to male infertility remain unclear. Many attempts have been made to identify the constituents of seminal plasma; however, there have been very few studies designed to identify disease-related markers in seminal plasma, particularly those related to male infertility. Here, we identified 4 candidate markers for NOA, namely stabilin 2 (STAB2), 135-kd centrosomal protein (CP135), guanine nucleotide–releasing protein (GNRP), and prolactin-inducible protein (PIP). Of these, STAB, CP135, and GNRP have high molecular weights and exist as membrane or intracellular proteins. There have been no reports on these proteins with respect to male infertility. The origin of these proteins is expected to be the testis or epididymis; however, further investigations are necessary to confirm these origins. PIP is probably a secreted protein that is expressed in several exocrine tissues, particularly those of the mammary gland (Murphy et al, 1987) and seminal vesicle (Autiero et al, 1997). Our result showed that the PIP spot was absent in all OA patient samples, supporting the hypothesis that PIP is derived from the testis or epididymis. The PIP spot was also absent in some NOA patients. This suggests that the origin of PIP is not only the seminal vesicle but also the testis or epididymis. It was reasonable to consider that the 2 spots for OA markers were epididymal secretory protein E1 (Niemann-Pick disease C2 protein [NPC2]). NPC2 is a major secretory protein of the epididymis (Kirchhoff et al, 1996), and its expression level is decreased after vasectomies (Legare et al, 2006). This expression level should be examined in a greater number of seminal plasma samples from NOA and OA patients to determine its application as a clinical marker in distinguishing NOA from OA.

In conclusion, the present study provides the first evidence for interindividual variations in the human seminal plasma proteome and demonstrates that 2-D gel electrophoresis is useful for the identification of clinically available markers for azoospermia. The present data have indicated several possible candidate markers for OA and NOA. Further investigations will be necessary to identify these markers, to employ them in clinical diagnosis, and to clarify the causes of male infertility.

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Footnotes

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