Functional Sperm Testing and the Role of Proteomics in the Evaluation of Male Infertility

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The limitations of conventional semen analysis testing in the diagnosis and management of male factor infertility have been well documented. A number of more sophisticated assays including measurements of sperm deoxyribonucleic acid (DNA) fragmentation rates, seminal oxidative stress, and antioxidant capacity have been increasingly used in the evaluation of male infertility. Moreover, in the past few years, tremendous advancements in the burgeoning field of sperm proteomics promise to revolutionize the andrologist’s diagnostic armamentarium, as will be discussed in this review. UROLOGY 84: 255–261, 2014. © 2014 Elsevier Inc.

A pproximately 10% of couples are infertile, with male factors contributing to as many as 50% of these cases.1 Over the past 50 years, semen analysis has represented the cornerstone of the laboratory evaluation for male infertility with little substantive change over time, serving as a surrogate measure of male reproductive potential. Although this test has a profound influence on the work up and treatment of male infertility, its use is plagued by significant limitations. Men with abnormal semen parameters frequently achieve spontaneous pregnancies, and only half of infertile men have a recognizable cause detected by conventional semen analysis.2 The limited ability of this important tool to predict fecundity risks misclassifying a subject’s true fertility status and compromises its value as a basic screening test. Fortunately, the advent of more sophisticated assays, including measurements of seminal oxidative stress, antioxidant capacity, and sperm deoxyribonucleic acid (DNA) fragmentation rates, as well as recent advances in the field of sperm proteomics, have provided promising alternative tests to better assess male reproductive potential.

LIMITATIONS OF CONVENTIONAL SEMEN ANALYSIS

The clinical utility of conventional semen analysis results is severely limited by several important factors, including (1) inherent variability from one ejaculate to the next, (2) racial and geographic variations in observed semen characteristics, and (3) imprecise correlations between semen analysis results and observed male reproductive potential.

Variability of Semen Parameters With Repeat Testing

Semen analysis results vary for a given patient because of preanalytic influences (eg, the duration of abstinence), variation in the method of analysis or the evaluator, and inherent biological variability.5 In the largest analysis of variability and reproducibility of semen analysis results, Leushuis et al3 analyzed the data of more than 5000 men with 2 routinely performed semen analyses. Comparison of within-subject results revealed a coefficient of variation ranging from 28% to 34% for all semen parameters, confirming the limited reproducibility of semen analyses as reported elsewhere.4,5

Ethnic and Geographic Variations in Semen Parameters

Multiple studies have also demonstrated ethnic differences with respect to semen parameters. For example, a national study of Chinese men who conceived within the past year demonstrated that 7%, 7%, 79%, 52%, 46%, and 10% of men had values below the World Health Organization (WHO) criteria for sperm concentration, total sperm count, rapid progressive motility, sperm progressive motility, sperm viability, and normal morphology, respectively.6 Similarly, fertile Japanese men have been shown to have lower levels of semen quality than European men,7 whereas African-American men have been shown to have significantly lower semen parameters than White and Latino men.8

Imprecise Correlation Between Semen Parameters and Male Reproductive Potential

Interpretation of semen analysis results is further complicated by uncertainty regarding the appropriate cutoff conventional reference values. The WHO has published multiple editions of its laboratory manual in the past 3 decades (WHO 1987, WHO 1992, WHO 1999, and WHO 2010), defining and redefining the reference
values for all semen parameters. Although the WHO works to establish reference norms, it is known that fertile men display tremendous variation in all of the semen parameters and that extensive overlap exists between fertile and infertile men with respect to all semen parameters. For example, a review of 166 men by Agarwal et al found that sperm concentration only yields a sensitivity of 0.48 in distinguishing men with male factor infertility. Moreover, although the percentage of men with normal strict morphology was low in most of the infertile. Moreover, although the percentage of men with normal strict morphology was low in most of the male factor infertility patients (sensitivity, 0.83), almost half of the fertile men examined also presented with abnormal morphology (specificity, 0.51). Fertile and infertile men also overlapped with respect to motility, giving this parameter a sensitivity of only 0.74 and a specificity of 0.90. Therefore, in the absence of azoospermia, none of the semen parameters is diagnostic of infertility.

Although semen analysis testing continues to be the mainstay in the evaluation of infertile men, physiologic variability with repeat testing, racial and geographical variations, and the wide range of semen parameters observed in normal fertile men significantly limit the utility of this test. Fortunately, additional diagnostic tests now serve as useful adjuncts to the basic semen analysis. Each of these will be discussed in detail in the following.

OXIDATIVE STRESS

A growing body of evidence suggests that excessive production of reactive oxygen species (ROS) can overwhelm the total antioxidant reserves in semen, leading to abnormal semen parameters and DNA damage in the sperm nuclear and mitochondrial genomes. When used in conjunction with conventional semen analysis, measurements of oxidative stress can help (1) distinguish between fertile and infertile men, (2) identify subgroup men who are most likely to conceive over time, and (3) identify the subgroup of infertile men who may benefit the most from antioxidant supplementation.

Physiological levels of ROS are of paramount importance for certain sperm functions (eg, capacitation); however, excessive ROS pose a threat to sperm plasma membrane integrity. For this reason, spermatozoa and seminal plasma contain a variety of enzymatic and nonenzymatic antioxidant systems capable of countering the negative effects of ROS. However, a number of lifestyle (eg, smoking), environmental (eg, pesticides, air pollution, electromagnetic radiation), and health (eg, chemotherapy exposure, urogenital tract infections, neutrophil or macrophage infiltration) factors significantly raise production of ROS, overwhelming the total antioxidant capacity (TAC) of sperm. This imbalance, termed oxidative stress, disrupts sperm plasma membrane fluidity, impairs sperm motility, and interferes with membrane fusion events.

Multiple assays for measuring seminal ROS levels have been developed; however, the chemiluminescence method is the most common technique used to measure the ROS generated by spermatozoa. The ROS level for men with normal semen parameters is $1.5 \times 10^4$ cpm/20 million sperm/mL, and infertile men may be classified as being oxidative stress positive if they exhibit an ROS level higher than this level. The seminal TAC, representing the total antioxidant protection in seminal plasma, may also be measured using chemiluminescence assays that quantify the ability of seminal antioxidants to block oxidation of specific reagents. Moreover, Sharma et al have validated a novel composite ROS-TAC score, taking into account both sperm ROS and seminal TAC measurements to more accurately reflect the level of oxidative stress as a balance between these measured values.

There is growing body of evidence that infertile men possess lower levels of individual antioxidants, a reduced TAC, and increased ROS levels relative to fertile men. Elevated ROS levels are detected in the semen of 25%-40% of infertile men, and infertile men have been shown to have TAC levels that are 30%-43% lower than those measured in fertile controls. Furthermore, when compared with normal controls, infertile men have significantly lower ROS-TAC scores. Thus, infertile men as a whole are prone to an imbalance between the harmful effects of excess ROS and the protective functions of seminal antioxidants.

When the generation of ROS overwhelms the ROS scavenging system, the resulting oxidative stress compromises sperm function and viability and leads to sperm DNA fragmentation (discussed in the following). Men who exhibit excessive ROS demonstrate higher rates of sperm abnormal morphology and head defects. Conversely, TAC level has been positively associated with each of the semenogram parameters, including sperm concentration, motility, and morphology. Oxidative stress also produces DNA strand breaks; in fact, every 25% increase in seminal ROS levels is associated with a 10% increase in sperm DNA fragmentation rate.

Cumulatively, these structural, functional, and DNA defects impair sperm fertilization potential. Not surprisingly, men with elevated ROS have been shown to be less likely to achieve spontaneous pregnancy. Moreover, infertile men with favorable ROS-TAC scores are more likely to ultimately initiate a successful pregnancy than infertile men with unfavorable scores.

The aforementioned relationship between ROS levels, TAC, and sperm fertilization potential has formed the basis for antioxidant therapy of infertile men—the goal being to restore the balance between ROS-generating and antioxidant defense systems in patients. A number of studies have evaluated the efficacy of various oral antioxidants (eg, vitamins C and E, zinc, selenium, folate, and carnitine) on sperm quality, pregnancy rate, and live birth rate in infertile men. In fact, it has been reported that men taking oral antioxidants have more than 4 times the pregnancy rate and an almost 5-fold higher live birth rate when undergoing assisted reproductive technique.
and breakage. Although the overall effectiveness of oral antioxidant therapy and the optimal regimen has not yet been established, the available literature suggests that there is at least a mild benefit to antioxidant supplementation in infertile men.

DNA FRAGMENTATION RATE
Sperm chromatin integrity is vital for both transmission of paternal genetic information and proper embryo development. Since it was initially described in 1993, measurement of the sperm DNA fragmentation rate has been suggested as an independent marker of male fertility and has been shown to better predict the outcome of both spontaneous pregnancy and ARTs than traditional semen parameters. Spermatozoal chromatin is highly compacted by nuclear proteins called protamines, and this high degree of chromatin condensation protects the DNA from stress by nuclear proteins called protamines, and this high degree of chromatin condensation protects the DNA from stress and breakage. Despite this protection, sperm DNA damage can still occur in the form of single or double-strand DNA breaks, inter- or intra-strand cross linkage, or base deletions or modifications. A number of different factors have been shown to produce these DNA effects, including tobacco use, chemotherapy, malignancy, leukospermia, and high measured levels of oxidative stress.

Many tests of sperm DNA damage are available, including both direct measures of DNA fragmentation and indirect tests of sperm chromatin compaction. The 2 most commonly used assays that measure sperm DNA damage are the terminal deoxynucleotidyl transferase deoxyuridine Triphosphate (dUTP) nick end labeling assay and the sperm chromatin structure assay (SCSA). Although the terminal deoxynucleotidyl transferase dUTP nick end labeling assay directly measures single- and double-strand DNA fragmentations, the SCSA analyzes DNA damage indirectly using a flow cytometer to estimate the percentage of spermatozoa with DNA denaturation.

Clinically, high rates of DNA fragmentation are negatively correlated with bulk semen parameters and have been associated with several infertility phenotypes: (1) longer time for natural conception, (2) idiopathic infertility, (3) recurrent intrauterine insemination (IUI) and in vitro fertilization (IVF) failure, and (4) spontaneous miscarriage. Thresholds for DNA damage tests have not yet been determined in men with unexplained infertility. A DNA fragmentation index (DFI) cutoff of 19.25% can distinguish infertile men with DNA damage from healthy controls with a sensitivity of 65%. However, raising the DFI cutoff to 30% lowers the sensitivity to 15% but achieves a specificity of 96%. Although the optimal reference range has not yet been defined, it is clear that men with a high percentage of spermatozoa with DNA damage have a lower potential for natural fertility and a prolonged time to natural pregnancy. Moreover, high DNA fragmentation rates have been associated with lower biochemical pregnancy, clinical pregnancy, and delivery rates after IUI. In fact, in the largest reported study on the predictive value of SCSA in relation to the outcome of IUI, Bungum et al demonstrated that clinical pregnancy rates were ten fold higher for men with DFI ≤30% compared with men with DFI >30%. Moreover, DNA fragmentation rates of >30% have been associated with approximately half the likelihood of success with conventional IVF methods.

In addition to lower pregnancy rates, high levels of DNA fragmentation are associated with greater rates of pregnancy loss. For example, a meta-analysis of couples conceiving spontaneously or via assisted conception in the form of IUI, IVF, or intracytoplasmic sperm injection (ICSI) has demonstrated that men with high DNA damage show a greater than 2-fold increase in miscarriage rate compared with patients with normal levels of DNA damage. Thus, although men with a high DFI experience a modest reduction in conception rates with conventional IVF and little effect with ICSI, they are 2-3 times more likely to experience a pregnancy loss than men with a normal DFI.

DNA fragmentation testing may be used in combination with the conventional semen analysis to help explain idiopathic male factor infertility, to confirm adverse effects of oxidative stress (see the following), and to establish the DNA integrity of sperm before undertaking more expensive ART procedures. Measurements of DFI may also influence which ART technique is used because it has been shown that the odds of biochemical pregnancy are 3 times higher with ICSI than with IVF when the DFI exceeds 30%. Thus, when high levels of DNA fragmentation are detected, ICSI is preferred over conventional IVF. Moreover, because significant DNA damage can occur during epididymal transit, some studies have suggested that men with high DFI may benefit from using their intratesticular spermatozoa rather than using ejaculated sperm or sperm obtained by epididymal aspiration.

Although a number of studies have highlighted the relationship between DNA damage and male factor infertility, it is noteworthy that The Practice Committee of the American Society for Reproductive Medicine at present does not recommend assessment of DNA fragmentation routinely for clinical use. In its most recent appraisal of the evidence pertaining to the clinical utility of sperm DNA integrity testing, this guideline committee concluded that the data fail to demonstrate a relationship between abnormal DNA integrity and reproductive outcomes. For example, there has been no study to date demonstrating that sperm DNA integrity testing performed well in advance of IUI is predictive of pregnancy. Moreover, a systematic review and meta-analysis of ICSI studies indicate that sperm DNA damage is not associated with ICSI pregnancy rates. The clinical utility of DNA fragmentation testing is further limited by the fact that neither a standard DNA fragmentation test nor a universally agreed upon cutoff level have been established, both of which confound the implementation of this valuable tool in daily practice. Thus, despite the aforementioned value of DNA damage testing, the American Society for
Reproductive Medicine has concluded that DNA integrity testing alone does not predict pregnancy rates achieved through natural conception or via IUI, IVF, or ICSI25—highlighting the need for new molecular markers that are better capable of predicting reproductive outcomes.

**PROTEOMICS**

Proteomics refers to the comprehensive analysis of all the proteins expressed by a cell, tissue, or organism; the field of sperm proteomics specifically characterizes the entire human sperm protein repertoire.23 In just the past decade, the field of sperm proteomics has grown dramatically as researchers have realized that understanding the differential expression of sperm proteins holds the key to understanding which cellular pathways are implicated in male infertility.27,28 Although no more than 100 different sperm proteins had been identified as recently as the 1970s, novel proteomic tools developed over the past few decades have led to the discovery of more than 6000 different sperm proteins. These developments have elucidated the structure and physiology of the male gamete and laid the groundwork for the even newer field of comparative sperm proteomics. Although this field is still in its infancy, comparing the proteome of different quality sperm samples makes it possible to identify unique sperm proteins that contribute to male infertility.28

The human spermatoozon lends itself particularly well to proteomic evaluation because it is easily acquired and purified. Sperm proteomic assessment begins with isolation and purification of sperm. Sperm proteins are then separated by one of several methods, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), standard 2-dimensional gel electrophoresis, or 2-D fluorescence difference gel electrophoresis and analyzed by liquid chromatography-mass spectroscopy.29 Proteins are identified using a protein database (eg, Mascot or SEQUEST) and their roles in various cellular pathways may be assessed by cross-referencing Reactome, a free online knowledge base that breaks down biological pathways into a series of molecular events.28

Although initial proteomic studies were aimed at identifying small numbers of specific sperm surface proteins,28 the first detailed proteomic analysis of human sperm was published as recently as 2005.30 Since then, high throughput techniques have enabled researchers to identify and catalog 6198 proteins (an estimated 78% of the entire sperm proteome) at present.28

Spermatozoa may also be divided into subcellular fractions such as heads or tails by differential centrifugation, and each of these fractions may be analyzed independently. This approach, dubbed “subcellular proteomics,” gives a more thorough assessment of spermatozoal content and helps pinpoint which cellular compartments the various sperm proteins localize to.29

For example, assessment of isolated tail fractions has led to the identification of numerous metabolic enzymes essential for motility, and analysis of sperm membrane fractions has uncovered various membrane integral proteins that play a role in spermatozoa-oocyte interaction.28

With the sperm proteome nearing completion in the coming years, researchers have begun to make proteomics comparisons of different quality sperm samples with the hopes of identifying novel biomarkers of male infertility. Among the first of these was a pilot study by Thacker et al4 in which the sperm protein profile was compared between 1 fertile and 3 infertile men using the aforementioned proteomic techniques. Four unique proteins were predominantly present in the semen of fertile men; however, 2 of these were not identified in the semen of infertile men, suggesting unique differences in the spermatozoa protein profiles of fertile and infertile men. This study served as proof of principle that proteomics may be useful in the assessment of nonfunctional sperm and has paved the way for larger-scale proteomics comparisons.

A number of studies have subsequently characterized proteomic anomalies in infertile men with asthenozoospermia. Zhao et al31 compared sperm protein expression profiles in asthenozoospermic patients with those of normozoospermic donors and identified 10 differentially expressed proteins. Martínez-Heredia et al32 similarly reported 7 proteins downregulated and 10 proteins upregulated in asthenospermic men compared with controls. In total, comparative proteomic studies have thus far cataloged a total of 109 proteins whose levels differ in men with asthenospermia.28

Multiple studies have also highlighted the differential expression of sperm proteins found in men with various other semen abnormalities. Hosseinifar et al13 compared semen samples from normospermic men without varicoceles with oligospermic men with varicoceles and noted 15 consistent differences in protein expression between the groups. More recently, Zylbersztejn et al34 found that apoptosis regulation proteins were overexpressed in semen from adolescents with varicocele and abnormal semen quality whereas spermatogenesis proteins were overexpressed in adolescents with varicocele and normal semen quality. Interestingly, Xu et al35 recently compared sperm from 10 infertile men with normal semen parameters against sperm from 10 fertile donors and identified 24 differentially expressed proteins even in the absence of apparent semen abnormalities.

Proteomic analyses have also revealed that the nuclear protamine content differs between fertile and infertile men, suggesting that sperm from the latter may have reduced DNA integrity and be more susceptible to oxidative damage.36 De Mateo et al37 were the first to report a correlation between abnormal protamine content and DNA integrity. These authors identified 7 proteins that correlate with protamine content and 8 proteins that correlate with DNA integrity in infertile men compared with controls. Intasqui et al38 expanded on these findings, identifying 71 proteins that are exclusively or overexpressed in men with low rates of DNA fragmentation and 23 proteins that are exclusively or overexpressed in men with high rates of DNA fragmentation. Moreover, a
recent proteomic analysis demonstrated that the expression profile of proteins present in human spermatozoa differs in men with high and low levels of ROS. Ten proteins are reportedly overexpressed and 5 are underexpressed in men with high levels of ROS, suggesting that these proteins play an important role in the management of oxidative stress.39

Various other studies have highlighted major groups of sperm proteins implicated in cases of ART failure. For example, Pixton et al40 compared the sperm protein expression profile from a patient who experienced failed fertilization during IVF with 3 fertile controls and demonstrated 20 consistent differences in protein expression. A similar study by Frapsauce et al41 that compared the sperm proteome of men who experienced IVF fertilization failure with that of men who experienced normal fertilization/cleavage during IVF identified 14 proteins that were differentially expressed. Zhu et al similarly profiled proteins in 6 men whose sperm resulted in a clinical pregnancy after ART and compared these with sperm proteins from 6 men whose semen did not result in a clinical pregnancy after ART. Their analysis identified 21 proteins that were differentially expressed (>1.2-fold) in men whose sperm resulted in a clinical pregnancy.42

Seminal plasma contains numerous seminal-specific proteins critical for sperm function,44 and a number of studies have assessed the proteomic profile of this acellular fluid conglomerate rather than spermatozoal proteins. As early as 1987, Ayayagari et al44 evaluated the postliquefaction proteolytic breakdown of seminal plasma proteins and found that a group of proteins were present in the seminal plasma of oligospermic but not in that of normospermic or azoospermic men. Wang et al45 compared seminal plasma protein profiles from asthenospermic patients and normal controls and their analysis revealed 45 proteins that were 3-fold upregulated and 56 proteins that were 3-fold downregulated in men with asthenospermia. In a more recent analysis of seminal plasma, Sharma et al46 identified 7 additional seminal proteins with levels that varied in men with abnormal sperm counts and/or morphology.

At present, differentiating cases of obstructive azoospermia (OA) from nonobstructive azoospermia (NOA) is oftentimes indeterminate based on clinical criteria (eg, testis size and FSH level), requiring a surgical procedure to make a definitive diagnosis. A number of studies have therefore specifically assessed seminal plasma proteins with the hopes of identifying novel protein biomarkers to differentiate men with OA and NOA. Yamakawa et al47 compared seminal plasma samples of fertile and azoospermic men and identified a number of differences in protein expression, including 4 protein markers for NOA and 1 candidate marker for OA. More recently, Batruch et al referenced 2048 proteins in seminal plasma from men with NOA (the “NOA proteome”) against previously cataloged proteomes of fertile male controls and vasectomized men. This comparison yielded 34 proteins elevated and 18 proteins decreased in controls relative to men with NOA, as well as 59 proteins elevated and 16 proteins decreased in men with NOA compared with vasectomized men. These distinct proteomic differences suggest that 1 or more of these seminal proteins may one day be used as noninvasive biomarkers capable of distinguishing men with OA and NOA.48

Several seminal plasma proteins have already been evaluated as potential biomarkers for genital duct patency. For example, Légaré et al49 evaluated levels of specific cysteine-rich secretory protein in seminal plasma of men with OA and NOA and identified a threshold cutoff for seminal cysteine-rich secretory protein level, which distinguished between OA and NOA with a specificity of 85% and a sensitivity of 92%.49 Heshmat et al50 similarly focused their attention on a specific seminal plasma protein, lipocalin-type prostaglandin D synthase (L-PGDS). When L-PGDS levels were compared in men with normal semen parameters, OA, NOA, and in vasectomized men, the authors found that seminal L-PGDS level was significantly lower in men with obstruction than in any of the other groups. In fact, all men with OA had a measured L-PGDS level <100 μg/L, whereas 30% of men with NOA had an L-PGDS level >100 μg/L—thus allowing the diagnosis of NOA to be made in nearly a third of men without a testis biopsy. Although no biomarker is yet commercially available, these and other novel seminal protein biomarkers may one day be used to differentiate men with OA and NOA, allowing clinicians to predict the likelihood of successful sperm retrieval without relying on surgical intervention for a conclusive diagnosis.

In <10 years, the sperm and seminal fluid proteomes have been largely defined including a number of proteins with altered expression in men with semen abnormalities or poorly functioning sperm. Although sperm and seminal fluid proteomics research is still in its early stages, the aforementioned studies point to the exciting potential of this relatively new field. First, identification and characterization of sperm proteins promises to augment our understanding of sperm function at the subcellular level as well as to shed light on the various protein interactions necessary for the events of fertilization (eg, capacitation, sperm-oocyte fusion, etc). Second, each new protein found to be over- or under-expressed in the seminal plasma of men with poor sperm quality represents a potential novel proteomic biomarker for male infertility. Earlier proteomic research has laid the groundwork by identifying, cataloging, and comparing protein profiles in men with various semen qualities; subsequent analysis of each individual candidate biomarker will identify those proteins with the greatest diagnostic/prognostic value. Already, researchers have successfully identified novel protein markers that can discriminate between men with OA and NOA without the need for surgery. Hopefully, new markers will emerge with the potential to identify infertile men most likely to ultimately conceive naturally, men most likely to benefit from medical or surgical intervention.
interventions (eg, varicocele repair, empiric medically therapy, and antioxidant therapy), and men who are most likely to benefit from ART rather than more conservative approaches.

**CONCLUSION**

Although conventional semen analysis remains a valuable tool in the evaluation of male factor infertility, this basic screening test may not accurately reflect reproductive potential. Fortunately, measurements of sperm DNA damage, oxidative stress, and TAC enable clinicians to predict pregnancy and reproductive outcomes more accurately. Meanwhile, the burgeoning field of sperm proteomics promises to enhance our knowledge of the numerous cellular pathways necessary for sperm function and to help identify those with the greatest biological significance in men suffering from infertility. Moreover, by studying the differential expression of sperm and seminal plasma proteins in fertile vs infertile men and in men with OA vs NOA, this field holds the key to the development of novel diagnostic and prognostic protein biomarkers for the evaluation of infertile men. Together, the combined application of these new tools will profoundly expand the andrologist’s diagnostic armamentarium for male factor infertility in the coming years.

**References**


