Sexual Function/Infertility

Proteomic Signatures of Sperm Mitochondria in Varicocele: Clinical Use as Biomarkers of Varicocele Associated Infertility

Luna Samanta, Ashok Agarwal,* Nirlipta Swain, Rakesh Sharma, Banu Gopalan, Sandro C. Esteves, Damayanthi Durairajanayagam and Edmund Sabanegh

From the American Center for Reproductive Medicine, Department of Urology (LS, AA, NS, RS) and Department of Urology (ES), Cleveland Clinic Foundation, Cleveland, Ohio, Redox Biology Laboratory, Department of Zoology, School of Life Sciences, Ravenshaw University (LS, NS), Odisha, India, Yorg Corp. (BG), Plano, Texas, ANDROFERT, Andrology and Human Reproduction Clinic, Referral Center for Male Reproduction (SCE), Campinas, Brazil, and Discipline of Physiology, Faculty of Medicine, Universitá Teknologi MARA (DD), Sungai Buloh, Malaysia

Purpose: Varicocele may disrupt testicular microcirculation and induce hypoxia-ischemia related degenerative changes in testicular cells and spermatozoa. Superoxide production at low oxygen concentration exacerbates oxidative stress in men with varicocele. Therefore, the current study was designed to study the role of mitochondrial redox regulation and its possible involvement in sperm dysfunction in varicocele associated infertility.

Materials and Methods: We identified differentially expressed mitochondrial proteins in 50 infertile men with varicocele and in 10 fertile controls by secondary liquid chromatography-tandem mass spectroscopy data driven in silico analysis. Identified proteins were validated by Western blot and immunofluorescence. Impaired mitochondrial function was associated with decreased expression of the proteins (ATPase1A4, HSPA2, SPA17 and APOA1) responsible for proper sperm function, concomitant with elevated seminal oxidation-reduction potential in the semen of infertile patients with varicocele. Liquid chromatography-tandem mass spectroscopy data were corroborated by Western blot and immunofluorescence. Impaired mitochondrial function was associated with decreased expression of the proteins (ATPase1A4, HSPA2, SPA17 and APOA1) responsible for proper sperm function, concomitant with elevated seminal oxidation-reduction potential in the semen of infertile patients with varicocele.

Conclusions: Impaired mitochondrial structure and function in varicocele may lead to oxidative stress, reduced ATP synthesis and sperm dysfunction. Mitochondrially differentially expressed proteins should be explored for the...
development of biomarkers as a predictor of infertility in patients with varicocele. Antioxidant therapy targeting sperm mitochondria may help improve the fertility status of these patients.

**Key Words:** testis, spermatozoa, mitochondria, varicocele, proteomics

**VARICOCELE** is found in approximately 16% of healthy male adolescents and adults, and in 20% to 40% of all infertile men.\(^1\) Stagnation of testicular microcirculation induced hypoxic-ischemic degenerative changes in testicular cells may impair semen quality and result in DNA fragmentation and OS induced infertility. However, the underlying pathophysiological mechanisms are not fully understood.

Impaired sperm motility is associated with defects in the sperm mitochondrial ultrastructure.\(^2\) Mitochondrial dysfunction occurs not only in the genome but also at the transcriptome and proteome levels.\(^3\) In fact, considerable differences exist in the sperm proteomic profiles of men with varicocele compared to those without varicocele,\(^4\) fertile donors,\(^5-7\) or patients who underwent varicocelectomy.\(^8\) In our previous studies we identified 99 DEPs in the spermatozoa of men with varicocele relative to fertile controls.\(^5-7\)

Mitochondria, which are the principal sites of ROS production, produce superoxide radical during hypoxia.\(^9\) Mitochondrial proteins, especially the ETC subunits, have a significant role in sperm quality.\(^10\) Furthermore, the activity of sperm mitochondrial enzymes influences semen characteristics such as sperm count, vitality and motility.\(^11,12\) Therefore, it is plausible that hypoxic-ischemic shock in varicocele cases may alter the sperm mitochondria proteome, resulting in abnormal spermatozoa in the ejaculate and ultimately infertility.

The goal of this study was to elucidate the role of mitochondria in sperm dysfunction in infertile men with varicocele using a combination of wet laboratory and in silico techniques.

**MATERIALS AND METHODS**

**Patients and Specimens**

Following institutional review board approval semen specimens were obtained from 50 infertile men with clinical varicocele, which was unilateral in 33 and bilateral in 17, and from 10 fertile normozoospermic men without clinical varicocele who had fathered at least 1 healthy child naturally during 2015 to 2016. Specimens were collected by masturbation after 2 to 3 days of ejaculatory abstinence. Varicocele was diagnosed by physical examination. Semen samples with a sperm concentration less than $10^9$/ml containing $1 \times 10^9$/ml or greater leukocytes, a history of fever within 3 months before enrollment and female factor infertility were excluded from analysis (supplementary materials 1, [http://jurology.com/](http://jurology.com/)). All participants provided written consent before being enrolled in this prospective study.

**Semen Analysis**

Semen analysis was performed according to WHO criteria, 5th edition, to determine sperm concentration, motility, presence of round cells and morphology. Specimens with $1 \times 10^9$/ml or greater round cells were tested for leukocytospermia by the Endtz test.

**Oxidation-Reduction Potential Measurement**

ORP was assayed by galvanoostat based technology using the MiOXSYS\(^{\text{TM}}\) System as previously reported.\(^13\) ORP is expressed in mV/10\(^6\) sperm per ml.

**Mitochondrial Differentially Expressed Protein In Silico Analysis and Protein Selection for Validation**

The selection of proteins for the validation study was based on published work and the results of the mentioned bioinformatics analysis. We used certain criteria, including 1) mitochondrial proteins from published data sets that were differentially expressed in infertile patients with varicocele compared to fertile controls, 2) proteins associated with mitochondrial redox regulation and sperm function according to previous publications and 3) proteins involved in human infertility, particularly varicocele, according to published literature.

Functional annotation and enrichment analysis were performed with publicly available bioinformatics annotation tools and databases, including the GO Term Finder, the GO Term Mapper, UniProt, the STRAP (Software for Researching Annotations of Proteins) and the DAVID (Database for Annotation, Visualization and Integrated Discovery). Protein-protein interaction was analyzed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) Network. Pathway analyses were done with MetaCore\(^{\text{TM}}\) Pathway Maps and Disease Marker Sets.

**Select Protein Expression Validation Studies**

**Western Blot.** The DEPs of interest were verified in each semen specimen using WB as previously described (supplementary materials 1, [http://jurology.com/]).\(^7\) Seven specimens were obtained from fertile men and 6 were obtained from infertile patients, including 3 with unilateral and 3 with bilateral varicocele. In addition to individual WB analyses, pooled specimens from patients with varicocele, combined into 3 pools of 3 individuals each, were run against pooled samples from the fertile group to maintain biological variability. Samples were normalized to the protein concentration in each group.

**Immunocytochemistry-Immunofluorescence.** Spermatozoa from 3 fertile men, 3 infertile men with unilateral
varicocele and 3 infertile men with bilateral varicoceles were processed using a standard protocol (supplementary materials 1, http://jurology.com/). Fluorescence intensity measurements were performed in 300 spermatozoa per group.

Statistical Analysis
The Shapiro-Wilk test was used to assess data normality and the Levene test was used for homogeneity of variance. Analyses were performed with SPSS®, version 25. The 2-tailed Student t-test was applied to compare WB intensity readings. The Wilcoxon rank sum test was used for semen parameters and IF between the study groups with significance considered at p < 0.05. Multivariate statistical analysis with NSAF for each DEP was done for LC-MS/MS data.

PCA was performed with mean centered scaling and full cross-validation using The Unscrambler®, version 10.1. Results are presented as 3-D PCA score plots with each point representing an individual LC-MS/MS data set, including 3 each for the fertile control, the infertile unilateral group and the bilateral varicocele group. Unsupervised hierarchical clustering using the Pearson correlation and a heat map were drawn using the open source clustering software Cluster 3 (http://bonsai.hgc.jp/~mdehoon/software/cluster/). To compare protein expression the intensity values of WB and sperm images of the infertile varicocele groups were taken together and compared with those of the fertile control group.

RESULTS

Semen Analysis and Sperm Oxidative-Reduction Potential
Supplementary materials 2 (http://jurology.com/) show the distribution of varicocele grade and laterality, and reduced sperm concentration and motility. Seminal ORP was increased in infertile patients with varicocele compared to fertile controls (fig. 1, A).

Mitochondrial Protein Expression
A total of 141 mitochondrial proteins were identified in the study population, of which 22 were differentially expressed in the varicocele group compared to the fertile group (fig. 1, B). The GO annotations and the DAVID analysis revealed their involvement in mitochondrial organization, electron transport and metabolism. Hierarchical clustering performed on the z-score of 22 mitochondrial proteins and the 2 subunits of cAMP dependent protein kinase showed a separate cluster between the fertile and varicocele groups (fig. 1, C). Similarly the 3-D PCA score plot generated from the unsupervised PCA of NSAF from label-free high throughput mass spectrometry data of the proteins revealed a separate grouping for fertile men and men with varicocele (fig. 1, D).

Although the expression profile of the DEPs assayed by WB and IC/IF demonstrated a declining trend in the varicocele group compared to the control group, the difference between the unilateral and bilateral varicocele groups was not significant. Therefore, for subsequent reporting of results patients with varicocele were combined for comparison with the control group.

Protein Interaction Studies
The protein-protein interaction studied by the STRING and MetaCore analyses revealed strong interactions between detected DEPs in the data set concerning protein kinase (enrichment p = 0, supplementary tables 1 to 4 and supplementary fig. 1, http://jurology.com/). The critical biological processes resulting from protein interaction were 1) aerobic respiration, 2) energy derivation by oxidation of organic compounds, 3) the tricarboxylic acid cycle, 4) cellular respiration and 5) the oxidation-reduction process. The top molecular functions resulting from protein interaction were 1) coenzyme binding, 2) oxidoreductase activity acting on the aldehyde or oxo- group of donors and disulfide as the acceptor, 3) oxidoreductase activity, 4) NAD binding and 5) catalytic activity (supplementary fig. 1, http://jurology.com/).

The predicted upstream transcription regulations of DEPs using MetaCore indicated that the top transcription factors were involved in the regulation of cellular metabolism, energy production, reproductive processes, meiosis, steroid hormone action, ROS and antioxidant response (supplementary fig. 2, http://jurology.com/). These transcription factors showed cross-talk among the regulatory pathways via mitochondrial ETC (fig. 2).

Select Protein Western Blot and Immunocytochemistry
The expression profile of the 5 mitochondrial ETC complexes revealed a declining trend in the varicocele group compared to the control group. The arbitrary intensity unit values obtained from densitometric analysis of WB revealed decreased expression of the proteins NDFSU1, UQRC2 and COX5B, corroborating the NSAF values of the LC-MS/MS data (fig. 3, A to D). Similar results were observed for PKR1A when analyzed by WB and IC/IF (fig. 3, E to G). The expression profile of all proteins involved in sperm function processes, including ATPase1A4, HSPA2, SPA17 and APOA1, were down-regulated in the sperm of patients with varicocele compared to controls (fig. 3, H to S).

DISCUSSION
To our knowledge this is the first report of the high throughput identification of mitochondrial proteins
Figure 1. ORP level in spermatoza of fertile controls and infertile patients with varicocele. Asterisk indicates p<0.05 (A). Differentially expressed proteins and log2 NSAF in fertile control and infertile varicocele groups (B). Heat map shows z-score of 22 mitochondrial proteins and 2 subunits of cAMP-dependent protein kinase type I (α regulatory subunit and catalytic subunit α isoform 2) measured independently in triplicate and with respect to fertile controls (CN), infertile men with unilateral varicocele (UVN) and infertile men with bilateral varicoceles (BVN) (C). Three-dimensional PCA score plot generated from unsupervised PCA of NSAF shows separate groups for fertile controls, infertile men with unilateral varicocele and infertile men with bilateral varicoceles (D). NSAF of proteins measured independently in triplicate were clustered together, revealing consistent data reproducibility.
Figure 2. Cross-talk between transcription factors in nucleus and DEPs involved in mitochondrial function regulation. Layout is based on cellular location of proteins.
Figure 3. Expression profile of mitochondrial complex proteins and PKR1A in fertile controls (c) and infertile patients with varicocele (v). Western blot of complex I subunit NDFSU1 and OXPHOS cocktail with positive control human heart tissue lysate-mitochondrial extract and tubulin (A). Densitometry analysis of Western blot signal intensity of complex I-NDFSU1 (p < 0.01) (B), complex III-UQCR3 (p < 0.04) (C) and complex IV-COXII (p < 0.03) (D). Western blot with corresponding densitometry analysis of α-tubulin of PKR1A (E), ATPase1A4 (H), HSPA2 (K), SPA17 (N) and APOA1 (Q) (p < 0.05). Confocal images of single and sperm cluster of PKR1A (F), ATPase1A4 (I), HSPA2 (L), SPA17 (O) and APOA1 (R). Quantification of fluorescence intensity/integrated (Int.) density of PKR1A (p < 0.0001) (G), ATPase1A4 (J), HSPA2 (M), SPA17 (P) and APOA1 (S) (p < 0.01). a.u., arbitrary units. DAPI, 4',6-diamidino-2-phenylindole. DIC, differential interference contrast.
along with validation of expression levels in the spermatozoa of infertile men with varicocele. The expression profile of ETC complexes reflects testicular germ cell mitochondria and, thus, may provide important clues regarding anomalies during spermatogenesis. Germ cell mitochondria undergo transformation throughout spermatogenesis. Spermatogonia and early spermatocytes have canonical mitochondria while late spermatocytes, spermatids and spermatozoa have more condensed and metabolically more efficient mitochondria. Our data mining results showed under expression of important ETC subunits, particularly NDFSU1, UQRCRC2 and COX5B.

This was further corroborated by the in silico data obtained, which showed that principal proteins involved in mitochondrial organization were under expressed in the varicocele group. LETM1 protein, which is crucial for maintenance of the mitochondrial tubular networks, the assembly of super complexes of the respiratory chain and the maintenance of the tubular shape and cristae organization, was also down-regulated. Similarly the MICOS complex subunit MIC60, a mitochondrial inner membrane protein with crucial roles in the maintenance of cristae junctions, inner membrane architecture and the formation of contact sites to the outer membrane, was under expressed in the varicocele group. Under expression of the mitochondrial import receptor subunit TOM22 suggested impaired protein transport into the mitochondria, which may result in improper transport of nuclear encoded mitochondrial proteins.

Under expressed enzymes of the metabolic pathways in the varicocele group, such as 1) PDHA1 and PDHA2 somatic and testis specific forms, 2) Krebs cycle (ACO2 [aconitate hydratase], IDH3B [iso-citrate dehydrogenase NAD subunit β] and OGDH [2-oxoglutarate dehydrogenase]), 3) cholesterol and fatty acid metabolism (ACAT2 [acetyl-CoA acetyl-transferase], HADHA [trifunctional enzyme subunit α], ACADS [short-chain specific acyl-CoA dehydrogenase], ECHS1 [enoyl-CoA hydratase] and ECH1 [Δ(3,5)-Δ(2,4)-dienoyl-CoA isomerase]), and 3) amino acid valine metabolism ([H1BADH] 3-hydroxyisobutyrate dehydrogenase) further point toward metabolic dysregulation of sperm mitochondria in varicocele. In fact, reduced mitochondrial activity in varicocele was proposed to be associated with sperm DNA fragmentation and OS induction. Based on dianinobenzidine assay the rate of sperm mitochondrial oxygen consumption and ETC complex activity showed a decrease in patients with varicocele accompanied by an increase in OS. In this study under expression of critical subunits of ETC revealed that the decline in mitochondrial activity was essentially due to a decline in protein expression. This was further corroborated by increased ORP, which implies an impaired electron flow resulting in increased ROS.

The expression profile of ETC complexes was assayed in this study to understand mitochondrial redox regulation. Although paradoxical, complex I and III of the ETC produce ROS during hypoxia when substrate O₂ availability is low. The flavin linked sites and iron-sulfur centers are the principal sites of superoxide generation in complex I, which highly depends on mitochondrial membrane potential. Sperm mitochondrial membrane potential in varicocele is hyperpolarized, resulting in augmented superoxide production via reverse electron flux from the ubiquinone pool into the complex.

Genetic studies implicating complex III mediated superoxide release were reported. However, the production of superoxide by complex I is always toward the matrix side, while that of complex III may be toward the matrix and into the intermembrane space. After superoxide release into the intermembrane space, it is dismuted by SOD1. The resulting H₂O₂ is proposed to activate transcription factor HIF-1α (hypoxia inducible factor-1α) for metabolic adaptation to hypoxia. Thus, complex III is proposed to be an oxygen sensor. HIF-1α in turn induces PDK1 (PDH kinase), which inhibits PDH activity, suggesting that respiration is decreased by substrate limitation. In fact, we observed a significant decrease in the expression of UQRCRC2, the core protein required for assembly of the complex. This is suggestive of poor hypoxia sensing and failure of adaptation, which was further supported by mass spectra data showing decreased expression of the PDHA1 and PDHA2 subunits.

The decrease in the major mitochondrial enzymes of carbohydrate, lipid and amino acid valine metabolism suggests less availability of reducing potentials along with the disruption of mitochondrial electron transport, which implies an ATP deprivation state. This scenario will lead to decreased production of cAMP which, together with decreased expression of regulatory PKR1A and catalytic subunits, may lead to a decline in the activity of cAMP-dependent protein kinase A, disturbing cAMP mediated signaling. Heterozygotes for PKR1A were reported to be infertile and had a lower sperm count with morphologically aberrant spermatozoa.
We analyzed a series of proteins crucial for sperm function which were selected from the mass spectra data. ATPase1A4 is a unique isoform of the testis specific α-subunit of Na,K-ATPase and sperm motility is reported to depend on it.24 HSFA2 is a testis specific chaperone and an established marker of human sperm cellular maturity, function and fertility.25 Its expression profile correlates with the assisted reproductive technology outcome26 and was reported to be degraded by OS.27 SPA17 is a 20 kDa membrane protein which is cleaved proteolytically after acrosome reaction, particularly at the C-terminal calmodulin domain, to a 17 kDa peptide. This cleaved peptide binds to the extracellular matrix of oocyte during fertilization.28,29 APOA1, part of the SPAP (sperm activating protein) complex, activates spermatozoa motility and was reported to be a sperm immunogenic membrane antigen.30 A decrease in the expression profiles of these proteins in the spermatozoa of the varicocele group clearly indicated sperm dysfunction associated infertility.

CONCLUSIONS
Based on our findings we conclude that under expression of regulatory and catalytic subunits of cAMP-dependent type I protein kinase results in impaired protein kinase mediated signaling. In turn this affects the overall protein turnover, including under expression of mitochondrial structural and functional proteins, thus leading to increased ROS and decreased ATP production. Under expression of UQCRC2 leads to the failure of hypoxia sensing, which may then impair the adaptive response.

Augmented ROS levels in turn induce secondary damage to mitochondria and spermatozoa, impairing their function. A decrease in ATP may result in substrate limited decreased production of cAMP, leading to further suppression of protein kinase activity, thereby inducing a vicious cycle.

Our hypothesis will be strengthened by further evaluation of isolated sperm mitochondria using LS-MS/MS and protein validation in a larger cohort. Overall our data suggest that mitochondrial ETC proteins (NDFSU1, UQCRC2 and COX5B) together with testis specific PDH may serve as noninvasive biomarkers of sperm function in patients with varicocele and may assist in the development of mitochondria targeted antioxidant based approaches.

ACKNOWLEDGMENTS
Researchers who critically read the manuscript and provided useful suggestions: Drs. Ahmad Majzoub, Haitham Elbardisi and Mohamed Arafa, Hamad General Hospital, Doha, Qatar; Dr. Ralf Henkel, University of the Western Cape, Bellville, South Africa; Dr. Juan Alvarez, La Coruna, Spain; Dr. Eva Tvrda, Slovak University of Agriculture, Nitra, Slovak Republic; and Dr. Manesh Kumar Panner Selvam, Tania R. Dias and Ana D. Martins, American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, Ohio. Dr. Belinda Willard, Director, Proteomic Core Laboratory, Lerner Research Institute, assisted with proteomic analysis. GeneGo provided MetaCore Pathway Maps and Disease Marker Sets.

REFERENCES
15. Tamai S, lida H, Yokota S et al: Characterization of the mitochondrial protein LTM1, which
maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. J Cell Sci 2008; 121: 2588.


EDITORIAL COMMENT

OS has been a topic of great interest in the study of varicocele associated infertility in the last 2 decades. Studies have confirmed the association of sperm DNA damage with OS and mitochondrial inactivity (reference 17 in article).1 In this quest sperm mitochondrial proteomic studies provide insight into the structural and functional irregularities which in turn may explain the apparent sperm dysfunction.

The authors of this study examined the expression profiles of key enzymatic proteins as well as mitochondrial structural proteins in infertile men with varicocele as well as in normozoospermic fertile men. They found that several mitochondrial structural proteins and metabolic enzymes of interest were under expressed in the varicocele group. The implication of these findings is a state of ATP deprivation and oxidative stress. Furthermore, specific proteins important for sperm function were also under expressed in the varicocele group.

The results of this study provide a new understanding of mitochondrial dysregulation from a proteomic and metabolic standpoint. It would be worthwhile to compare the differential expression of proteins in normozoospermic men with varicocele to elucidate whether certain proteins are more predictive of varicocele associated infertility. As the authors suggest, this would further clarify which proteins may be candidates for noninvasive biomarkers.

Akash A. Kapadia and Jason C. Hedges
Department of Urology
Oregon Health and Science University
Portland, Oregon

REFERENCE