What Makes Some ROS-Positive Men Fertile? A Comparative Proteomic Study

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ABSTRACT

Elevated levels of reactive oxygen species (ROS) in a major cause of male infertility. ROS are considered as an independent initiator of sperm function. Spermatic tissues are sensitive to elevated ROS levels due to their rich endoplasmatic reticulum, and can lead to oxidative stress. This study used high throughput proteomics analysis to investigate why some men with elevated ROS levels boast fertility.

INTRODUCTION

The ability of spermatozoa to generate reactive oxygen species (ROS) is a universal property of mammalian sperm. It is a major contributor to the oxidative stress responsible for defective sperm function. The mechanisms by which oxidative stress limits the functional competence of mammalian spermatozoa involves the peroxidation of lipids, the induction of oxidative DNA damage, and the formation of protein adducts. ROS production in these cells involves extraction leakage from the sperm mitochondria. This is triggered by a number of factors that enhance stress in ROS+ men.

RESULTS

1. Enhanced metabolism as evidenced by better oxidative stress and enhanced antioxidant defense.

Experimental profile of differentially expressed proteins

1. Of the 340 differentially expressed proteins (DEP), 12 were overexpressed, 95 were underexpressed, and 16 were uniquely expressed in ROS+ group. On the other hand ROS- samples have 137 unique proteins expressed in the spermata.

2. DAVID enriched functional analyses results are shown in Figures 1 A-D.

3. In terms of protein abundance, 86 high abundance proteins, 247 medium abundance proteins, 303 low abundance proteins and 76 very low abundance proteins were identified.

Functional annotations and pathway analysis

1. Transcript variants of NADH dehydrogenase, NDUFS3 and 5 were overexpressed in the high ROS group.

2. Mitochondrial respiratory dependent proteins and transport related transmembrane protein 4 were uniquely expressed in ROS+ group.

Bioinformatics analysis

Functional annotations of proteins were obtained using bioinformatics tools and pathway databases.

MATERIALS AND METHODS cont.

Sample preparation and Global proteomic analysis

Sample preparations were pooled from 5 subjects in each group for proteomic analysis. Only those samples that had equal amount of protein contributed by similar number of ejaculated sperm in each patient and the fertile group were equal. Equal amount of proteins from each group was subjected to a 1D SDS-PAGE. After in gel digestion extracted peptides were resequenced in a final volume of ~30 μL for LC-MS analysis. All MS/MS samples were analyzed using Mascot and SEQUEST Altsynth.

Criteria for Protein Identification

To validate ROS/RS-based peptide and protein identifications was done by Scaffold (established at >99.0% confidence and 95% for proteins by the Peptide Prophet algorithm with Scaffold delta mass correction to achieve a false discovery rate (FDR) of <1.0%). Proteins were annotated with gene ontologies using the Online Mendelian Inheritance in Man (OMIM) and the National Center for Biotechnology Information (NCBI).

Quantitative Proteomics

For proteomic analysis, the relative quantity of the protein was determined by comparing the number of spectra (termed as spectral counts SpCs), used to identify each protein. Different constraints for significance tests (t-value) and fold change cutoffs or normalized spectral abundance factor (NSAF) ratio were accepted as shown below:

- v. Low abundance: spectral count range 1-7.7; p ≤ 0.001 and NSAF ratio ≥ 2.5 for underexpressed proteins
- ii. Low abundance: spectral count range 8-19; p ≤ 0.01 and NSAF ratio ≥ 2.5 for overexpressed, ≤ 0.4 for underexpressed proteins
- iii. High abundance: spectral counts >80; p ≤ 0.05 and NSAF ratio ≥ 1.5 for overexpressed, ≤ 0.4 for underexpressed proteins

Bioinformatics analysis

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MATERIALS AND METHODS

After institutional Review Board approval, semen samples were collected from 31 fertile donors. Semen analysis was performed. Leukocyte spermatozoa was related when the mean cell concentration was > 1 x 10^6/mm^3. In the sample, it was confirmed by the eosin or the Endo test. Specimens that were positive for the Endo test (>1 x 10^6/white blood cells/mm^3) were not included in the study.

Reactive oxygen species (ROS) measurement

ROS formation was measured by chemiluminescence assay in the semen using 5 mM luminol (5,12-dimethyl benzene-1,4-diol) and 0.05% 3,3'-dihydroxy-1,1'-binaphthyl as the probe continuously at 15 min using a Berthold luminometer (Biotop/Fluor 953, Oelde, TH). Samples were divided into two groups based on the ROS levels:

- ROS negative group: ROS levels ≤ 0–9.93 RLU/10^6 sperm
- ROS positive group: ROS levels > 93.02 RLU/10^6 sperm

Figure 1: DAVID enrichment of proteins

A. Overexpressed
B. Underexpressed
C. Unique to ROS + group
D. Unique to ROS - group

Figure 2: String analysis drawing

A. Protein involved in ROS generation
B. Proteins involved in Antioxidant defense

1. 1Enhanced metabolism as evidenced by better androgen response and augmented energy metabolic enzymes may be the cause for elevated oxidative stress in ROS+ men.

2. Based on the comparative proteome profiles, we conclude that fertility in men with high seminal ROS may be enhanced antioxidant defense.

3. String protein-protein interaction analysis of all DEPs reveal a complex protein-protein interaction such as expression of heat shock proteins, proteosomes, energy metabolism etc. along with a rich mitochondrial antioxidant defense. This may result in men with high ROS level in the spermatozoa to be fertile.