Differences in the seminal plasma proteome are associated with oxidative stress levels in men with normal semen parameters

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Objective: To study the seminal plasma proteome in association with semen lipid peroxidation levels in men with normal semen parameters.
Design: Cross-sectional study.
Setting: University andrology and research laboratories.
Patient(s): A total of 156 normozoospermic men.
Intervention(s): Seminal lipid peroxidation levels were assessed in individual samples through thiobarbituric acid reactive substances quantification. Subsequently, lipid peroxidation data were used to divide the samples into the experimental groups: low lipid peroxidation levels (control group, bottom 15%, n = 23) and high lipid peroxidation levels (study group, top 15%, n = 23). Seminal plasma proteins from these groups were pooled (four pools per group, with biological variation between the pools) and used for a shotgun proteomic analysis using a liquid chromatography–tandem mass spectrometry approach. Quantitative data were used for univariate (unpaired Student's *t* test) and multivariate (partial least-squares discriminant analysis, logistic regression, and discriminant analyses) statistical analyses. Significant proteins were also used for functional enrichment analysis.
Main Outcome Measure(s): Seminal plasma protein profile and postgenomic pathways of seminal plasma are associated with seminal lipid peroxidation levels.
Result(s): In total, 629 proteins were quantified in seminal plasma. Of these, 23 proteins were absent or underexpressed and 71 were exclusive or overexpressed in the study group. The main enriched functions in association with seminal lipid peroxidation were unsaturated fatty acids biosynthesis, oxidants and antioxidants activity, cellular response to heat stress, and immune response. Moreover, we suggested mucin-5B as a potential biomarker of semen oxidative stress.
Conclusion(s): The seminal plasma proteome does reflect semen lipid peroxidation status and, thus, oxidative stress. (Fertil Steril® 2015;104:292–301. ©2015 by American Society for Reproductive Medicine.)
Key Words: Lipid peroxidation, oxidative stress, proteomics, seminal plasma, sperm

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Oxidative stress is detected in semen of up to 40% of infertile men (1), and thus it is widely considered one of the main cellular mechanisms of male infertility (2–6). It has also been associated with idiopathic infertility, even in men with normal semen quality (7). Therefore, it is suggested that seminal oxidative stress may affect sperm physiology, especially through impairment of sperm functions (6, 8, 9). Human sperm

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are notably sensitive to oxidative stress, owing to the high membrane concentration of unsaturated fatty acids (8) and to its limited antioxidant and membrane repair capacity (10, 11). Oxidative stress promotes its negative effects on sperm functions particularly by oxidation of seminal plasma and sperm lipids and proteins (9, 12–19). Oxidation of cellular and mitochondrial membrane lipids, known as lipid peroxidation, is a self-propagating process that ultimately leads to the loss of membrane integrity, permeability, and stability and to enzymes inactivation (12). Consequently, lipid peroxidation can lead to reduced sperm mitochondrial activity, acrosome damage, and DNA fragmentation (9, 12, 13).

Additionally, oxidation of seminal plasma and sperm proteins produces carbonyl groups, rendering them highly susceptible to proteolysis (16). Furthermore, sulfhydryl groups of cysteine residues are highly affected by oxidation by reactive oxygen species (ROS), leading to accumulation of oxidized proteins and decreased protein function (14, 15). Thus, loss of free sulfhydryl groups is considered a marker of oxidative stress (20). Several studies have demonstrated a significant reduction in free sulfhydryl levels in seminal plasma of infertile men (18, 19), which was also associated with decreased sperm quality and function (17). It is suggested that the effects of oxidative stress are more pronounced in extracellular proteins, owing to their longer half-life and lower damage repair capacity (15). Therefore, we hypothesized that seminal oxidative stress may alter the seminal plasma proteome, especially because of [1] oxidation of seminal plasma proteins, [2] oxidation of sperm proteins, which are released to seminal plasma, [3] peptides derived from proteolysis of seminal plasma and sperm proteins, and [4] released proteins from dead or altered sperm associated with oxidative stress in the male reproductive tract. To test this hypothesis, a proteomic analysis of seminal plasma in association with seminal lipid peroxidation levels was performed. Our results demonstrated several altered proteins, mostly related to ROS production and metabolism.

MATERIALS AND METHODS

Study Design

This study was approved by the São Paulo Federal University (UNIFESP, Brazil) institutional review board (Research Ethics Committee approval no. 1184/11). Informed written consent was provided by all included patients.

We performed a cross-sectional study with men undergoing semen analysis at the Andrology Laboratory of the Human Reproduction Section, UNIFESP. Semen was retrieved by masturbation after a 2- to 5-day period of ejaculatory abstinence. Only men aged 20–50 years and with sperm concentration $>15 \times 10^6$ sperm/mL were included. Subjects presenting ejaculate volume <1.5 mL, progressive motility <32%, morphology <4%, or leukocyte concentration $\geq 1 \times 10^6$ leukocytes/mL were excluded. Only men with normal semen parameters were included, to avoid bias in our proteomics findings, because some studies have already shown a difference in the seminal plasma proteome in asthenozoospermic and teratozoospermic men (21, 22). Additionally, men with leukocytospermia were also excluded, to avoid the contamination of seminal plasma with leukocytes proteins. Therefore, we tried to isolate our study factor (oxidative stress), to detect the proteome changes indeed associated with it. Between July 2012 and November 2013, 233 samples complied with the inclusion criteria and were prospectively collected. Of these, 77 samples presented alterations in semen analysis and were, thus, excluded. Therefore, this study was carried out including 156 subjects (n = 156).

For all semen samples, an aliquot was used for semen analysis, according to the World Health Organization 2010 manual (23), and the remaining volume was centrifuged at $800 \times g$ for 30 minutes. The obtained seminal plasma was directly frozen and kept at −20°C to be further used for lipid peroxidation evaluation and proteomic analysis. Before these analyses, seminal plasma was thawed and centrifuged at $16{,}100 \times g$ for 1 hour at 4°C to remove cellular debris. All reagents used in this study were obtained from Sigma-Aldrich, unless otherwise described.

Seminal Lipid Peroxidation Evaluation

Seminal peroxidation levels were assessed in individual samples by measuring the thiobarbituric acid reactive substances (TBARS) levels in seminal plasma, as previously described by Oborna et al. (24), with a few modifications. Thiobarbituric acid reactive substances is a red complex with absorbance peak at 540 nm, formed by the reaction of malondialdehyde (MDA), a by-product of lipid peroxidation, with two molecules of thiobarbituric acid (24). Semen lipid peroxidation levels were evaluated instead of sperm ROS levels for the following reasons. [1] ROS in sperm demonstrate only ROS levels generated by sperm, not providing any information regarding the sperm damage caused by ROS (that might be or not generated in sperm), whereas lipid peroxidation levels directly reflect this damage. [2] Reactive oxygen species are capable of oxidizing sperm and seminal plasma lipids (measured by semen lipid peroxidation levels), and proteins, which might lead to alterations on the seminal plasma proteome. Therefore, semen lipid peroxidation levels measured directly in the seminal plasma might be better correlated to seminal plasma proteomics data than ROS levels measured in sperm. [3] Some by-products of lipid peroxidation, such as MDA (measured in our study), are also capable of oxidizing sperm and seminal plasma lipids and proteins. Thus, this measure is more informative about the real effects of oxidative stress on the seminal plasma proteomics than measuring ROS levels.

Briefly, 100 μL of a solution containing 8.1% (wt/vol) sodium dodecyl sulfate (GE Healthcare), 0.8% (wt/vol) thiobarbituric acid, and 20% (vol/vol) acetic acid (Carlo Erba Reagents) were added to 100 μL of seminal plasma. Samples were then incubated in a water bath at 100°C for 1 hour and cooled in ice for 5 minutes to stop the reaction. To separate the TBARS molecules, 250 μL of N-buthanol (LabSynth) were added to each sample, which were then homogenized in vortex for 1 minute and centrifuged at $16{,}100 \times g$ for 15 minutes at 15°C. Finally, 100 μL of the aqueous phase were transferred to a microplate, in duplicate. A standard curve