COMPARATIVE PROTEOMIC ANALYSIS REVEALS DIFFERENTIAL REGULATION OF REDOX HOMEOSTASIS AND PURTURBED OXIDATIVE PHOSPHORYLATION PATHWAY IN UNILATERAL COMPARED TO BILATERAL VARICOCELE CONDITION

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INTRODUCTION
Varicocele is recognized as one of the leading causes of male infertility with a prevalence rate of 15% of the male population and in 40% of infertile men. Unilateral varicocele accounts for approximately 90% of varicocele cases, while 10% occur bilaterally. Infertile men with varicocele are often presented with poor semen quality, increased seminal oxidative stress and sperm DNA fragmentation. These anomalies semen characteristics are most commonly involved in impaired sperm function. However, a cohort of men with varicocele is fertile with normal spermatogenesis and semen parameters. Therefore, the exact molecular mechanisms of varicocele-associated infertility still remain unclear (Panner Selvam and Agarwal, 2019). Varicocele is a pathological dilation of the pampiniform venous plexus within the spermatic cord. Therefore, it could be hypothesized that ischemic-hypoxia and hyperthermia combined with oxidative stress is the mediating machinery for testicular tissue damage in infertile men with varicocele. We have previously shown that spermatozoal mitochondrial proteins, specifically those involved in oxidative phosphorylation and ATP synthesis, are dysregulated in infertile men with varicoceles (Agarwal et al., 2016; Samanta et al., 2018). Seminal plasma harbours a diverse group of proteins responsible for sperm transport, sperm protection and maturation. Disturbances in the seminal plasma proteome affects the fertilizing potential of the male germ cells. The main objective of the present study was to have a proteomic insight into seminal plasma in order to delineate the possible pathways involved in the etiology of sperm dysfunction in unilateral and bilateral varicocele conditions.

MATERIALS AND METHODS
After ethical clearance from the Cleveland Clinic Institution Review Board, semen samples were collected from participants after obtaining written consent. Semen collection and analysis were done according to WHO 2010 guidelines. Pooled seminal plasma samples from n=5 unilateral varicocele patients and fertile healthy men (n=5) were subjected to quantitative proteomic analysis. The detailed workflow of shotgun proteomics carried out is presented in Figure 1. Differentially expressed proteins (DEPs) obtained from three different analysis were subjected to pathway analysis using Ingenuity Pathway Analysis (IPA) software.

Database searching and protein identification
All MS/MS raw files were analyzed using Mascot, Sequest and X! Tandem to search the human reference database (332922 entries). Scaffold (version 4.0.6.1) was used to validate MS/MS-based peptide and protein identifications. Proteins annotation was performed using Gene Ontology (GO) terms from National Center for Biotechnology Information (NCBI).

Quantitative proteomics
The relative quantity of the proteins was determined by comparing the number of spectra, termed spectral counts (SpCs), used to identify each protein. SpCs was used to measure the abundance of proteins in the complex mixture. To overcome the sample-to-sample variation normalization of spectral counts using the NSAF (normalized spectral abundance factor) approach was applied prior to relative protein quantification.

Differentially expressed proteins (DEPs) were obtained by applying different constraints for significance tests and/or fold changes. Proteins were filtered based on the average SpC of the two protein multiple runs, as accurate quantification and determination of real biological change is a function of absolute number of SpCs. The abundance of the proteins was classified as High (Hi), Medium (M), Low (L), or Very Low (VL) based on their average spectral counts among 3 replicate runs.

Functional analysis
Functional annotation and enrichment analysis were performed using publicly available bioinformatic annotation tools and data-bases such as GO Term Finder, GO Term Mapper, Uniprot, Software for researching annotations of proteins (STRAP) and database for annotation, visualization and integrated discovery (DAVID) (http://david.niaid.nih.gov). Proprietary software packages such as IPA (Ingenuity Pathway Analysis) from Ingenuity Systems were also used to obtain consensus-based, comprehensive functional context for the list of proteins involved in the redox homeostasis and oxidative phosphorylation process derived from the dataset.

Bioinformatic analysis of protein-protein interaction
Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to display the functional link between the protein-protein interaction among the proteins involved in reproduction and oxidative stress.

RESULTS
1. Global proteomic analysis identified differential expression of seminal plasma proteins in unilateral, and bilateral varicocele patients compared to control patients (Figure 2).
2. Proteomic analysis revealed the presence of cellular proteins particularly of mitochondrial origin in seminal plasma.
3. Proteins involved in the oxidative phosphorylation pathway of spermatozoa were present (Z score = 3.5) in unilateral varicocele patients (Figure 3).
4. Z score was not available for majority of the proteins associated with oxidative phosphorylation pathway in combined varicocele and bilateral varicocele groups (Table 1).
5. Proteins regulating the cellular antioxidant mechanisms such as SOD2 (Z score = 3.94) and SOD3 (Z score = 9.08) were detected in unilateral varicocele patients.
6. IL-8 signaling pathway was activated in bilateral varicocele group (Z score = 2.236) compared to unilateral varicocele group (Z score = 1.342).

CONCLUSIONS
• Our proteomic results indicate that release of spermatozoa proteins into seminal plasma of unilateral varicocele patients may be due to oxidative damage of sperm membrane or inflammation originating from mitochondrial dysfunction.
• Absence of sperm proteins in seminal plasma of bilateral varicocele patients may be due to apoptosis of spermatozoa that might have been phagocytosed.

REFERENCES