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Proteomic Analysis of Testicular Interstitial Fluid in Men with Azoospermia

I-Shen Huang [a,b,c], Li-Hua Li [d,e], Wei-Jen Chen [a,c], Eric Yi-Hsiu Huang [a,c], Chi-Chang Juan [b,*,†], William J. Huang [a,b,c,†,*]

[a] Department of Urology, Taipei Veterans General Hospital, Taipei, Taiwan; [b] Department of Physiology, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan; [c] Department of Urology, College of Medicine and Shu-Tien Urological Science Research Center, National Yang Ming Chiao Tung University, Taipei, Taiwan; [d] Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, Taipei, Taiwan; [e] School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan

Article info

Abstract

Background: The primary microenvironment of the testis comprises testicular interstitial fluid (TIF) surrounding the seminiferous tubules and testicular interstitial tissue. The pathological alterations of germ and Sertoli cells could affect the TIF composition and might contain putative biomarkers for monitoring active spermatogenesis.

Objective: We identified differentially expressed proteins in the TIF of patients with obstructive (OA) or nonobstructive (NOA) azoospermia to elucidate the underlying etiology of defective spermatogenesis.

Design, setting, and participants: We prospectively enrolled nine patients, including three men with OA and six with NOA with ($n = 3$) and without ($n = 3$) successful sperm retrieval. Their TIF was collected during the testicular sperm extraction procedure.

Outcome measurements and statistical analysis: TIF was analyzed using liquid chromatography-tandem mass spectrometry to identify differentially expressed proteins specific to OA and NOA with or without successful sperm retrieval. The dysregulated protein was further validated using Western blotting.

Results and limitations: Among the 555 TIF proteins identified in NOA patients, 14 were downregulated relative to OA patients. These proteins participate in biological processes such as proteolysis, complement activation, and immune responses; complement and coagulation cascade pathways were also enriched. Furthermore, 68 proteins with significantly higher levels were identified in the TIF of NOA patients with successful sperm retrieval than in those with failed sperm retrieval; these are mainly implicated in oxidation-reduction processes. The expression of

† Professors Chi-Chang Juan and Dr. William J. Huang contributed equally to this article.
* Corresponding authors. Department of Urology, Taipei Veterans General Hospital, No 201, Section 2, Shipai Rd, Taipei 112, Taiwan, ROC. Tel. +886-2-2875-7519; Fax: +886-2-2875-7540 (W. J. Huang); Department of Physiology, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan. Tel. +886-2-2826-7802; Fax: +886-2-2826-4049 (C.-C. Juan).
E-mail addresses: ccjuan@ms.ym.edu.tw (C.-C. Juan), williamjshuang@gmail.com (W.J. Huang).

calreticulin, which can distinguish successful and failed testicular sperm retrieval in the NOA group, was validated by Western blotting.

Conclusions: We provide the first scientific evaluation of TIF protein composition in men with azoospermia. These findings will help identify the physiological and pathological roles of each protein in regulating sperm production. Thus, our study underscores the potential of TIF in sperm retrieval biomarker discovery and would serve as a foundation for further studies to improve treatment strategies against azoospermia.

Patient summary: Using a proteomic approach, we identified and analyzed the total protein content of testicular interstitial fluid in humans with defective spermatogenesis for the first time and discovered altered protein expression patterns in patients with nonobstructive azoospermia (NOA). Proteins related to oxidation-reduction processes were upregulated in NOA patients with successful sperm retrieval compared with those with failed sperm retrieval. This can aid the development of novel diagnostic tools for successful testicular sperm retrieval.

1. Introduction

Testicular interstitial fluid (TIF) surrounding the seminiferous tubules and testicular interstitial tissue constitutes the primary microenvironment of the testis, in which its hormone milieu and immune homeostasis play a critical role in promoting germ cell differentiation [1,2]. The TIF dynamics are largely regulated by testicular vasomotion (rhythmic contraction and relaxation of testicular blood vessels), an androgen-mediated mechanism governing microvascular permeability, which controls the passage of fluid and macromolecules between the vascular system and the interstitial space [3–5]. Functional Sertoli cells provide a niche suitable for appropriate development and function of the intricate testicular vascular network essential for TIF homeostasis [6]. Such an organization is vital as it supplies the nutritional base for testicular somatic and germ cells, as well as transports gonadotropins, which are instrumental to the integrity of spermatogenesis. It also provides a specialized niche microenvironment essential for germline stem cells [7].

Western blotting was applied to identify two germ cell–derived proteins, phosphatidylethanolamine-binding and androgen-regulated proteins, in TIF, by one early pilot study [8]. Liquid chromatography tandem mass spectrometry (LC-MS/MS)-based methods helped determine qualitatively and quantitatively the total protein content of TIF in rats; approximately 276 proteins, including those secreted by Sertoli, Leydig, peritubular myoid, and germ cells, were identified [9]. Therefore, the TIF proteome can theoretically reflect spermatogenic activity within the testis and may be used as a surrogate for predicting the presence of spermatozoa.

With the rapid advancements in accurate MS and data analysis algorithms, a range of proteomic-based approaches that examine the entire proteome expressed by the genome have been adopted to examine the biochemical pathways responsible for altered spermatogenesis in men with infertility. The altered proteomic profiles of seminal plasma in infertile men to identify biomarkers that correlate with conventional sperm parameters and male fertility potential have been investigated [10]. These studies will aid in acknowledging the mechanisms by which various proteins in the reproductive tract regulate sperm motility, capacitation, acrosome reaction, and sperm-oocyte interaction [10]. Although these data provide epigenetic insights explaining male infertility in those with normal and abnormal standard semen parameters, it provides limited details about testicular microenvironment deregulation, as the testes contribute only <10% to the overall proteome of seminal plasma [11]. Therefore, TIF would provide a representative snapshot of the spermatogenesis microenvironment as it baths the seminiferous tubules and testicular interstitium required to further understand the etiology and prognosis of nonobstructive azoospermia (NOA). As approximately 70% of men diagnosed with NOA are classified as idiopathic [12], a proteomic analysis may help provide insights into the epigenetic alteration involved in the etiology and pathophysiology of defective spermatogenesis. Additionally, this may help develop novel tools for effective treatment strategies.

We hypothesized that the proteomic profile of TIF in men with NOA differs from that in men with obstructive azoospermia (OA), and that men with NOA and viable sperm within seminiferous tubules display distinct protein TIF profiles that reflect specific aspects or cellular functions involved in spermatogenesis. Therefore, we adopted a proteomic approach to identify potential predictive biomarkers likely to be associated with successful testicular sperm retrieval.

2. Patients and methods

2.1. Study population

Following institutional review board approval (IRB: 2022-01-036AC), we prospectively collected TIF from azoospermic men undergoing testicular sperm extraction. Azoospermia was diagnosed according to the

World Health Organization criteria, and the etiology of OA or NOA was determined by the stage of spermatogenic differentiation. In the present study, patients diagnosed with OA had undergone vasectomy, were proved fertile, and underwent testicular sperm extraction for in vitro fertilization. Patients with NOA were dichotomized as having successful or failed sperm retrieval. All the enrolled patients had a normal karyotype (46, XY), and the Y chromosome microdeletion analyzed by multiplex polymerase chain reaction was negative for known mutations.

2.2. Microdissection testicular sperm extraction and TIF collection

A short scrotal incision (3–4 cm) was made longitudinally on the median raphe. After opening the layers of the scrotum and performing an incision of the tunica vaginalis, the testis was exposed. To minimize the potential contamination of TIF with blood during the fluid aspiration procedure, we took several precautions. First, we firmly compressed the testis prior to incising the tunica albuginea to minimize bleeding. Second, we made a small incision (1 cm) and immediately aspirate TIF with a 20-gauge *Jelco* peripheral IV catheter–attached syringe. If necessary, we also cauterized testicular blood vessels using microbipolar cautery with fine-tipped forceps. Third, we carefully monitored the aspirate during the procedure and avoided aspirating fluid containing blood (Fig. 1). After collecting the TIF, we immediately placed the samples on ice, centrifuged them at 300g for 10 min, and stored the supernatant at –80°C for further examination.

Regarding the microdissection testicular sperm extraction procedure, the incision made on the tunica albuginea was extended to allow exposure and direct examination of the testicular parenchyma using an operating microscope at 20–25× magnification. Seminiferous tubules with larger diameters and enhanced opacity were excised using a microsurgical technique and evaluated intraoperatively [13]. The procedure was considered successful if at least one spermatozoon was collected. All surgical procedures were carried out by the same surgeon (W.J.H.).

2.3. LC-MS/MS proteomic analysis of TIF

The procedure begins with sample preparation steps by reduction and alkylation for improved protein extraction and peptide coverage. Subsequently, an LC-MS/MS analysis was performed on an Orbitrap Fusion Lumos Tribrid quadrupole-ion trap-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nanospray ion source. The peptides were separated on an Ultimate 3000 nanoLC system (Thermo Fisher Scientific, Bremen, Germany). Next, peptide mixtures were loaded onto a 75-µm ID, 25-cm long C18 Acclaim PepMap NanoLC column (Thermo Fisher Scientific) packed with 2-µm particles with a pore size of 100 Å. After the completion of the operation, MS/MS data were subjected to a label-free quantification analysis using the MaxQuant platform (v1.6.7.10) [14]. This analysis enabled the identification and quantification of 831 proteins in nine samples on an Orbitrap Fusion Lumos starting from only a few to 50 µg of sample. Details regarding sample preparation, instrument settings, instrument operation, data analysis, and Western blotting are provided in the Supplementary material. Absolute protein amounts were determined using the intensity-based absolute quantification (iBAQ) method. This method calculates the sum of intensities of all peptide peaks and divides it by the number of theoretically observable peptides to obtain an iBAQ value that reflects absolute protein quantification [15]. Detailed protocols are provided in the Supplementary Material.
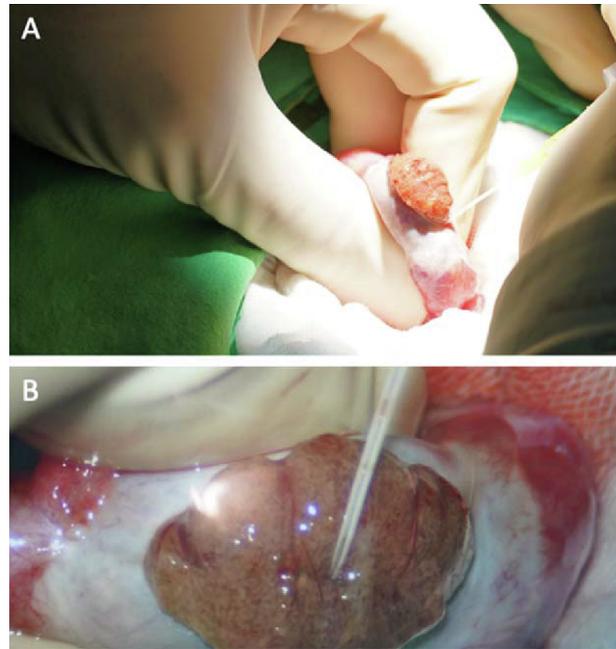
Fig. 1 – Obtaining testicular interstitial fluid (TIF) while performing microdissection testicular sperm extraction. (A) Maintain firm compression on testis using the thumb and index finger from the start of the procedure, prior to incising the tunica albuginea, throughout the entire TIF collection process. (B) Magnified image obtained during the TIF collection procedure, using a 20× magnification to provide a more detailed view of the steps involved.

2.4. Statistical analysis

All data are expressed as the mean ± standard error of each group. The data were compared using the one-way analysis of variance and Student t test to determine statistical significance. Statistical significance was set at $p < 0.05$.

3. Results

3.1. General characteristics of the study participants

The general characteristics of the nine patients in each group (group 1, OA; group 2, NOA with unsuccessful sperm retrieval; and group 3, NOA with successful sperm retrieval) are summarized in Table 1. Each group consisted of three patients. Testicular histology revealed normal spermatogenesis, Sertoli cell only (implying the depletion of all germ cells), and hypospermatogenesis (implying a reduction of the degree of normal spermatogenic cells) in groups 1, 2, and 3, respectively (Table 1). Notably, we did not include any patients in this study whose testicular histology pattern showed maturation arrest (germ cells with arrest at the primary spermatocyte or up to the level of spermatid). Furthermore, we successfully retrieved spermatozoa from all OA patients. The mean collected TIF volume was 97.7 µl (range 23–210 µl) from a total of nine patients, which was adequate for an analysis.

Table 1 – Demographic data of men with OA and NOA

	Group 1	Group 2	Group 3
Case no.	3	3	3
Age (yr)	39.0 ± 5.0	35.7 ± 6.0	33.7 ± 3.1
Testis size (ml)	17.7 ± 3.8	6.7 ± 2.1	12.3 ± 4.6
Endocrine levels			
FSH (mIU/ml)	5.4 ± 2.6	23.1 ± 5.5	18.3 ± 4.5
LH (mIU/ml)	2.7 ± 1.0	10.1 ± 2.5	6.4 ± 2.2
Testosterone (ng/ml)	5.2 ± 2.7	2.4 ± 0.7	2.3 ± 0.7
Prolactin (ng/ml)	8.7 ± 1.4	11.8 ± 1.8	9.8 ± 7.7
Estradiol (pg/ml)	21.7 ± 9.8	10.1 ± 3.6	13.7 ± 5.0

FSH = follicle stimulating hormone; LH = luteinizing hormone; NOA = nonobstructive azoospermia; OA = obstructive azoospermia. Values are presented as mean ± standard deviation.
Group 1: patients diagnosed with obstructive azoospermia.
Group 2: patients diagnosed with nonobstructive azoospermia but unsuccessful sperm retrieval.
Group 3: patients diagnosed with nonobstructive azoospermia and successful sperm retrieval.

3.2. Analyses of TIF from patients with OA and NOA with unsuccessful surgical sperm retrieval

We performed a label-free MS-based proteomic analysis to compare the relative abundance of proteins in TIF between OA and NOA patients (Fig. 2) to obtain insights into the biological processes involved in azoospermia. Among the 862 proteins identified in groups 1 and 2, only 373 were shared between the groups. Approximately 156 proteins were significantly dysregulated in group 2 than in group 1, among which 14 were downregulated and 142 were upregulated. The 14 downregulated proteins in group 2 were associated with biological processes of proteolysis (42.9%), complement activation (21.4%), classical pathway (21.4%), and immune response (21.4%). In contrast, the group 2–specific upregulated proteins were associated with cell-cell adhesion, glycolytic process, canonical glycolysis, and gluconeogenesis (Fig. 3).

3.3. Analyses of TIF in NOA patients with or without successful surgical sperm retrieval

We identified 555 proteins in six patients with NOA, among which 364 were differentially expressed between groups 2 and 3, as illustrated by Venn diagrams (Fig. 4). Among these, 68 were significantly upregulated and 16 were significantly downregulated in TIF in group 3 compared with those in group 2. A gene ontology (GO) enrichment analysis performed on the 68 upregulated proteins revealed that proteins related to oxidation-reduction processes (23.8%) were most enriched in the biological process category, and oxidoreductase (13.5%) was most enriched in the molecular function category. The top ten differentially expressed proteins are listed in Table 2. Furthermore, the proteins with increased levels involved in the oxidation-reduction process in the TIF of NOA patients with successful (group 3) versus unsuccessful (group 2) surgical sperm retrieval are presented in Table 3.

3.4. Kyoto Encyclopedia of Genes and Genomes pathway enriched in TIF

Among the 14 proteins downregulated exclusively in group 2, a Kyoto Encyclopedia of Genes and Genomes (KEGG)
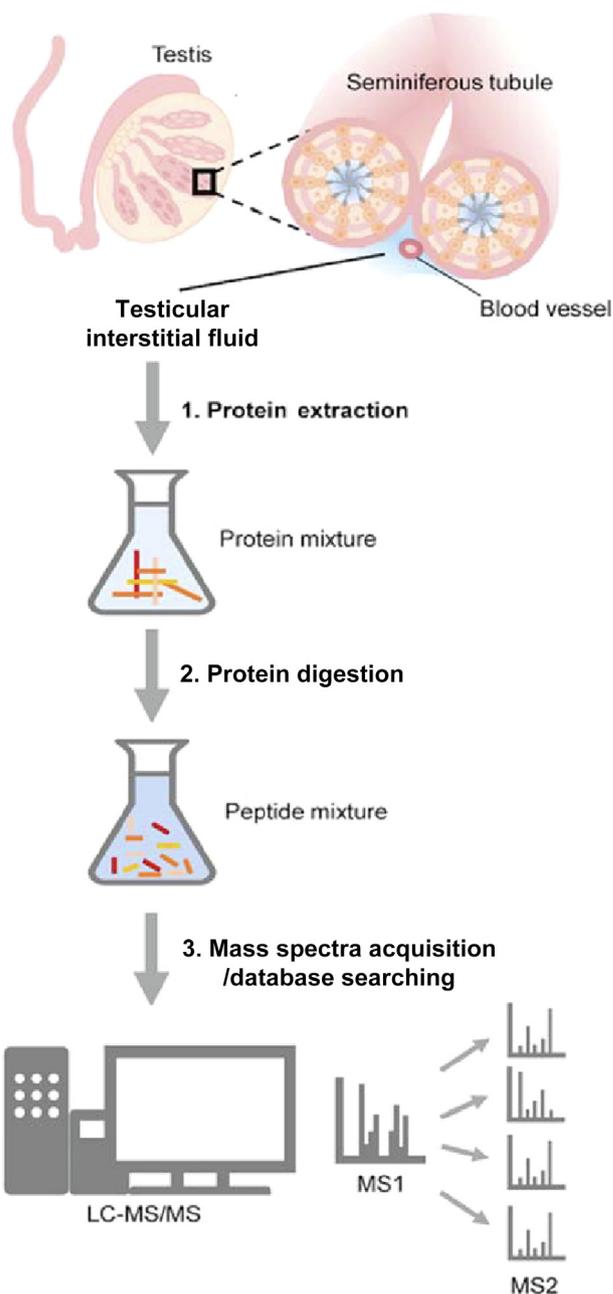
Fig. 2 – Schematic workflow for evaluating the TIF composition in men with azoospermia. LC-MS/MS = liquid chromatography tandem mass spectrometry; TIF = testicular interstitial fluid.

pathway analysis demonstrated an over-representation for the "complement and coagulation cascades," corresponding to the complement C1q subcomponent subunit B, immunoglobulin kappa variable 1–5, and immunoglobulin lambda variable 6–57 protein. The 68 proteins upregulated in group 3 compared with those in group 2 were primarily involved in the biosynthesis of antibiotics, glycolysis/gluconeogenesis, and the carbon metabolism pathway.

3.5. Complement levels in TIF

The association of TIF levels of C3 and C4 with spermatogenesis was examined in patients with normal spermatogen-

Fig. 3 – TIF proteome result of the label-free experiment. (A) Venn diagram comparing TIF proteins found to be enriched ± two-fold in group 2 versus group 1. (B) Enriched GO biological process, cellular component, and molecular function in 14 downregulated and 142 upregulated proteins. GO = gene ontology; NOA = nonobstructive azoospermia; OA = obstructive azoospermia; TIF = testicular interstitial fluid.

Fig. 4 – Comparison of TIF proteins identified in groups 2 and 3. (A) Venn diagram comparing TIF proteins found to be enriched ± two-fold in group 3 versus group 2. (B) Enriched GO biological process, cellular component, and molecular function in 16 downregulated and 68 upregulated proteins. GO = gene ontology; NOA = nonobstructive azoospermia; TIF = testicular interstitial fluid.

esis (group 1), defective spermatogenesis, and unsuccessful surgical sperm retrieval (group 2). C4 levels were elevated in TIF in group 2 patients relative to those in group 1 (15.2 ± 2.3 vs 10.4 ± 0.2, $p = 0.023$), whereas those of C3 did not differ significantly (68.2 ± 4.9 vs. 76.8 ± 10.3, $p = 0.258$; Fig. 5).

Table 2 – Top ten differentially expressed TIF proteins between NOA men with successful surgical sperm retrieval (group 3) and those with unsuccessful surgical sperm retrieval (group 2)

Accession no.	Gene name	Protein description	Fold change Group 3/group 2	iBAQ in group 2 Patient no. 4	iBAQ in group 2 Patient no. 5	iBAQ in group 2 Patient no. 6	iBAQ in group 3 Patient no. 7	iBAQ in group 3 Patient no.8	iBAQ in group 3 Patient no. 9
Q15392	DHCR24	Delta (24)-sterol reductase	7.03	1 061 500	0	0	138 360 000	0	0
Q8IVL1	NAV2	Neuron navigator 2	6.88	0	0	232 690	0	0	27 467 000
P27797	CALR	Calreticulin	5.63	336 440	0	0	16 697 000	0	0
P63104	YWHAZ	14-3-3 Protein zeta/delta	5.61	0	746 610	424 740	55 530 000	0	1 756 500
P68104	EEF1A1	Elongation factor 1-alpha 1	4.80	6 118 800	0	0	129 140 000	0	40 982 000
O95340	PAPSS2	Bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase 2	4.52	906 680	0	0	18 395 000	0	2 338 700
P21399	ACO1	Cytoplasmic aconitate hydratase	4.39	289 940	0	0	6 085 300	0	0
P04040	CAT	Catalase	−5.50	10 942 000	0	21 740 000	721 680	0	0
P02746	C1QB	Complement C1q subcomponent subunit B	−5.45	328 470	34 668 000	38 674 000	682 920	0	1 004 200
P69891	HBG1	Hemoglobin subunit gamma-1	−5.23	362 350 000	8 253 600	85 485 000	9 616 800	2 522 000	0
P04632	CAPNS1	Calpain small subunit 1	−4.94	0	754 110	54 746 000	916 680	0	890 870

iBAQ = intensity-based absolute quantification; NOA = nonobstructive azoospermia; TIF = testicular interstitial fluid.

Table 3 – List of proteins with increased levels involved in oxidation-reduction processes in the testicular interstitial fluid of NOA men with successful surgical sperm retrieval (group 3) versus those with unsuccessful surgical sperm retrieval (group 2)

Accession no.	Gene name	Protein description	Fold change Group 3/group 2	iBAQ in group 2 Patient no. 4	iBAQ in group 2 Patient no. 5	iBAQ in group 2 Patient no. 6	iBAQ in group 3 Patient no. 7	iBAQ in group 3 Patient no. 8	iBAQ in group 3 Patient no. 9
Q15392	DHCR24	Delta(24)-sterol reductase	7.03	1 061 500	0	0	138 360 000	0	0
P00387	CYB5R3	NADH-cytochrome b5 reductase 3	4.14	2 654 600	0	2 074 000	73 919 000	0	9 731 600
Q06278	AOX1	Aldehyde oxidase	4.13	128 410	0	0	2 027 200	0	223 280
P08294	SOD3	Extracellular superoxide dismutase [Cu-Zn]	3.68	538 150	0	0	0	5 679 300	1 243 600
O14756	HSD17B6	17-Beta-hydroxysteroid dehydrogenase type 6	3.32	5 208 300	0	0	52 233 000	0	0
P07195	LDHB	L-lactate dehydrogenase B chain	3.28	7 171 400	14 382 000	2 470 600	117 350 000	10 841 000	106 040 000
P00167	CYB5A	Cytochrome b5	3.03	19 434 000	0	0	159 560 000	0	0
P30044	PRDX5	Peroxiredoxin-5, mitochondrial	2.97	5 595 900	0	3 105 000	68 210 000	0	0
P49327	FASN	Fatty acid synthase	2.58	455 860	0	0	2 727 500	0	0
Q99536	VAT1	Synaptic vesicle membrane protein VAT-1 homolog	2.56	4 157 700	2 839 900	0	14 612 000	883 570	26 015 000
P49189	ALDH9A1	4-Trimethylaminobutyraldehyde dehydrogenase	2.55	4 880 000	1 688 600	0	30 475 000	861 180	7 377 600
P15121	AKR1B1	Aldo-keto reductase family 1 member B1	2.48	4 130 500	2 044 600	1 311 100	19 094 000	0	22 729 000
P05093	CYP17A1	Steroid 17-alpha-hydroxylase/17,20 lyase	2.45	26 270 000	3 519 500	2 722 600	178 570 000	0	0
Q9UBM7	DHCR7	7-dehydrocholesterol reductase	2.36	5 766 000	0	0	29 672 000	0	0
P00352	ALDH1A1	Aldehyde dehydrogenase 1A1	2.14	9 868 200	7 231 300	1 152 900	51 610 000	940 730	27 961 000
P00390	GSR	Glutathione reductase, mitochondrial	2.05	264 990	0	616 500	1 247 800	0	2 403 400

iBAQ = intensity-based absolute quantification; NOA = nonobstructive azoospermia.

3.6. Western blot analysis of calreticulin in TIF

Calreticulin in TIF was downregulated in NOA compared with that in OA ($p < 0.05$). Additionally, Western blot results confirmed a five-fold downregulation in the calreticulin protein levels ($p < 0.05$) in the TIF in NOA with unsuccessful sperm retrieval (group 2) than in samples from NOA with successful sperm retrieval (Fig. 6).

4. Discussion

The analysis of TIF in men with azoospermia helped identify altered expression (upregulated and downregulated) of several proteins involved in primary gonadal failure–related biological processes. A comparative proteomic analysis revealed that a network of proteins associated with comple-
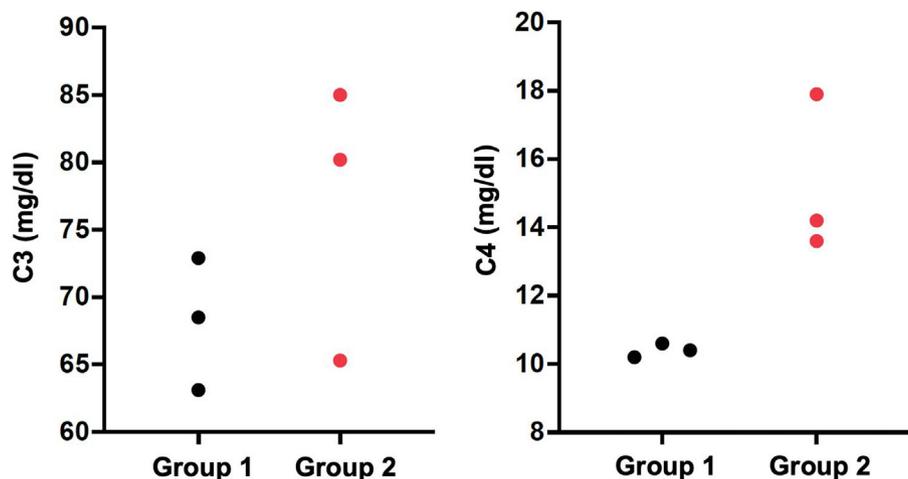
Fig. 5 – C3 and C4 levels in TIF in OA and NOA patients. Group 1: obstructive azoospermia. Group 2: nonobstructive azoospermia with unsuccessful sperm retrieval. NOA = nonobstructive azoospermia; OA = obstructive azoospermia; TIF = testicular interstitial fluid.
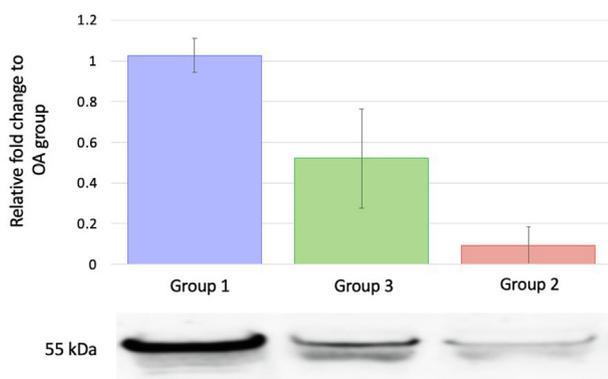
Fig. 6 – Western blot analysis of calreticulin in the TIF of OA and NOA patients. Group 1: obstructive azoospermia; group 2: nonobstructive azoospermia with successful sperm retrieval; and group 3: nonobstructive azoospermia without successful sperm retrieval. NOA = nonobstructive azoospermia; OA = obstructive azoospermia; TIF = testicular interstitial fluid.

ment activation was downregulated in the TIF of patients with NOA. This may at least partly explain its association with defective spermatogenesis. Furthermore, the chance of successful sperm retrieval in patients with NOA may be influenced by TIF proteins primarily involved in the oxidation-reduction process. Collectively, our findings may have potential clinical applications in detecting active spermatogenesis in men with NOA.

In addition to the central nervous system and anterior eye chamber, the testis is recognized as one of the few immune-privileged sites in the body. It has adopted a unique immunological environment that protects developing allogenic germ cells from immune attack under physiological conditions [16]. Such immune regulation is achieved through anatomical sequestration of the highly antigenic germ cells in the adluminal compartment by the blood-testis barrier, a physical barrier created by the basal tight junction between adjacent Sertoli cells and several molecules involved in immunosuppression and immunomodulation [16]. Experimental data from cellular and animal

models suggest that testosterone and M2 macrophages are crucial in maintaining various homeostatic, immunological, and inflammatory processes in the testis, primarily by inducing the expansion of immunosuppressive regulatory T cells and increased production of the anti-inflammatory cytokine Interleukin-10 [17,18].

Additionally, the role of complements and their regulators in the immunoregulatory function of the testicular microenvironment has been discussed less often. However, their importance in regulating humoral immunity has been acknowledged. Sperm are endowed with several complement regulatory proteins, including the CD46 (membrane cofactor protein), CD55 (decay accelerating factor), and CD59 (protectin), on the inner acrosomal membrane, selectively expressed during maturation and development [19]. These membrane-bound complement regulatory CD molecules play a complex role in reproduction. Additionally, these may protect against excessive complement activation to facilitate sperm survival within the female reproductive tract [19]. Similarly, Sertoli cells inhibit the activation of the complement cascade via classical, lectin, and alternative pathways by expressing the complement regulatory proteins mentioned earlier and secreting several immunosuppressive factors [20]. Therefore, we speculated that the overexpression of complement regulatory CD molecules on Sertoli cells in patients with NOA hinders the complement activation process in TIF; however, the exact mechanism of this observation warrants further investigation.

Our GO and KEGG enrichment analyses revealed the enrichment of GO terms related to proteolysis, complement activation, immune response, and over-representation of the KEGG pathway for complement and coagulation cascade. The relatively high C4 level in TIF in NOA patients, which is involved in the classical and lectin pathways, provided further evidence to support the attenuated complement activation process compared with OA patients.

Our study provided insights into the relationship between oxidative stress and the chance of successful sperm retrieval. Oxidative stress is characterized by an imbalanced generation of reactive oxygen species (ROS)

that causes oxidative damage to vital cellular macromolecules, including lipids, proteins, and DNA, resulting in compromised cell function [21]. The testis relies on the major ROS-scavenging enzymes and small-molecular-weight antioxidants to resist oxidative insults, thus avoiding germ cell attrition and preserving spermatogenesis [22]. Although normal physiological ROS levels are essential for fertilization, extensive evidence from observational studies based on various direct or indirect oxidative stress markers suggests that excessive ROS generation correlated significantly with altered semen parameters [13,23]. Concordantly, peripheral blood leukocyte ROS were used as a surrogate model for systemic oxidative stress measurement in patients with NOA. Although the leukocyte ROS levels were comparable between NOA patients with or without successful sperm retrieval, this study reported pathologically elevated ROS levels in peripheral leukocytes in men with NOA compared with that in fertile controls [24]. In our study, we identified that TIF proteins mainly related to oxidation-reduction processes were upregulated in men with successful sperm retrieval (group 3) compared with those in group 2. These upregulated proteins include glutathione reductase, 4-trimethylaminobutyraldehyde dehydrogenase, aldehyde oxidase, peroxiredoxin-5, and synaptic vesicle membrane protein VAT-1 homolog. Collectively, our data highlight oxidative stress within the testes as a pivotal factor in sperm retrieval success. Therefore, counteracting oxidative stress within the testes before surgical sperm retrieval could theoretically facilitate optimal sperm recovery.

We evaluated calreticulin as a diagnostic indicator of spermatogenesis owing to its correlation with male infertility [25]. Calreticulin reportedly regulates various essential biological processes involved in protein folding and antigenic peptide cross-presentation, in addition to its well-recognized role in maintaining adequate calcium levels in organisms [26]. In our study, calreticulin was underexpressed in TIF in men with NOA, especially those with unsuccessful sperm retrieval. This may be attributed to the transport of this resident protein from the endoplasmic reticulum into the acrosome of spermatids during spermatogenesis [27]; it may also be released into the extracellular environment (testicular interstitial space). Nevertheless, the exact mechanism underlying this signature remains unexplored.

The small number of patients in our study is a potential limitation that could impact the generalizability of our findings. To address this limitation, a larger sample size is needed to confirm our results and enhance their applicability to the wider population. Additionally, not all the differentially expressed TIF proteins were universally expressed between NOA men with or without successful sperm retrieval, which further limits the generalizability of our findings. Moreover, the majority of uniquely expressed proteins in patients with NOA with or without successful sperm retrieval were not analyzed, and therefore, further investigations are necessary to determine whether these TIF proteins contribute to successful or failed sperm retrieval. Such evaluations may provide new insights into the complexities associated with the etiology and treatment of azoospermia.

Despite these limitations, our study sheds light on the diverse protein expressions in the testicular microenvironment of NOA men with or without successful sperm retrieval, which warrants further investigation.

5. Conclusions

We identified and analyzed the total protein content of TIF in humans with defective spermatogenesis using a proteomic approach for the first time and discovered altered protein expression patterns in NOA patients. Knowledge of the TIF protein composition in patients with impaired spermatogenesis will help understand the physiological and pathological roles of each protein in regulating sperm production and develop targeted therapy for patients with azoospermia. We have highlighted the potential of TIF for application in sperm retrieval biomarker discovery and provided pilot data for further studies. Further studies are required to comprehensively evaluate the testicular microenvironment. Therefore, we plan to use a microwestern array targeting a particular molecular pathway to identify proteins associated with successful sperm retrieval. This will enhance our understanding of the molecular mechanisms underlying defective spermatogenesis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euros.2023.06.004.

References

[1] Smith LB, Walker WH. The regulation of spermatogenesis by androgens. Semin Cell Dev Biol 2014;30:2–13.

[2] Chen Q, Deng T, Han D. Testicular immunoregulation and spermatogenesis. Semin Cell Dev Biol 2016;59:157–65.

[3] Damber JE, Maddocks S, Widmark A, Bergh A. Testicular blood flow and vasomotion can be maintained by testosterone in Leydig cell-depleted rats. Int J Androl 1992;15:385–93.

[4] Collin O, Bergh A, Damber JE, Widmark A. Control of testicular vasomotion by testosterone and tubular factors in rats. J Reprod Fertil 1993;97:115–21.

[5] Welsh M, Sharpe RM, Moffat L, et al. Androgen action via testicular arteriole smooth muscle cells is important for Leydig cell function, vasomotion and testicular fluid dynamics. PLoS One 2010;5: e13632.

[6] Rebourcet D, Wu J, Cruickshanks L, et al. Sertoli cells modulate testicular vascular network development, structure, and function to influence circulating testosterone concentrations in adult male mice. Endocrinology 2016;157:2479–88.

[7] Yoshida S, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 2007;317:1722–6.

[8] Turner KJ, McKinnell C, McLaren TT, et al. Detection of germ cell-derived proteins in testicular interstitial fluid: potential for monitoring spermatogenesis in vivo. J Androl 1996;17:127–36.

[9] Stanton PG, Foo CF, Rainczuk A, et al. Mapping the testicular interstitial fluid proteome from normal rats. Proteomics 2016;16:2391–402.

[10] Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reprod Biomed Online 2014;29:32–58.

[11] Batruch I, Lecker I, Kagedan D, et al. Proteomic analysis of seminal plasma from normal volunteers and post-vasectomy patients identifies over 2000 proteins and candidate biomarkers of the urogenital system. J Proteome Res 2011;10:941–53.

[12] Peña VN, Kohn TP, Herati AS. Genetic mutations contributing to non-obstructive azoospermia. Best Pract Res Clin Endocrinol Metab 2020;34:101479.

[13] Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. Hum Reprod 1999;14:131–5.

[14] Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res 2011;10:1794–805.

[15] Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. Nature 2011;473:337–42.

[16] Li N, Wang T, Han D. Structural, cellular and molecular aspects of immune privilege in the testis. Front Immunol 2012;3:152.

[17] Fijak M, Schneider E, Klug J, et al. Testosterone replacement effectively inhibits the development of experimental autoimmune orchitis in rats: evidence for a direct role of testosterone on regulatory T cell expansion. J Immunol 2011;186:5162–72.

[18] Walecki M, Eisel F, Klug J, et al. Androgen receptor modulates Foxp3 expression in CD4+CD25+Foxp3+ regulatory T-cells. Mol Biol Cell 2015;26:2845–57.

[19] Harris CL, Mizuno M, Morgan BP. Complement and complement regulators in the male reproductive system. Mol Immunol 2006;43:57–67.

[20] Kaur G, Thompson LA, Dufour JM. Sertoli cells—immunological sentinels of spermatogenesis. Semin Cell Dev Biol 2014;30:36–44.

[21] Pizzino G, Irrera N, Cucinotta M, et al. Oxidative stress: harms and benefits for human health. Oxid Med Cell Longev 2017;2017:8416763.

[22] Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 2008;1:15–24.

[23] Kao SH, Chao HT, Chen HW, Hwang TIS, Liao TL, Wei YH. Increase of oxidative stress in human sperm with lower motility. Fertil Steril 2008;89:1183–90.

[24] Cito G, Becatti M, Natali A, et al. Redox status assessment in infertile patients with non-obstructive azoospermia undergoing testicular sperm extraction: a prospective study. Andrology 2020;8:364–71.

[25] Tokuhiro K, Ikawa M, Benham AM, Okabe M. Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male fertility [corrected]. Proc Natl Acad Sci U S A 2012;109:3850–5.

[26] Raghavan M, Wijeyesakere SJ, Peters LR, Del Cid N. Calreticulin in the immune system: ins and outs. Trends Immunol 2013;34:13–21.

[27] Nakamura M, Moriya M, Baba T, et al. An endoplasmic reticulum protein, calreticulin, is transported into the acrosome of rat sperm. Exp Cell Res 1993;205:101–10.