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Original Article

Application of co-immunoprecipitation coupled LC-MS/MS for identification of sperm immunogenic membrane antigens

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Abstract: Objective: Antisperm antibodies play a main role in immunological infertility, as they impair sperm function by binding to the sperm membrane. In this study, we conducted screening experiments tried to screen sperm membrane proteins interacting with antisperm antibodies by the method of co-immunoprecipitation and liquid chromatogram mass/mass (LC-MS/MS). Methods: The serum samples from infertile persons were previously screened as positive for ASA in ELISA and reproduced with mixed antiglobulin reaction. The swim-up method was used to separate mature and motile sperm. Hypoosmotic swelling homogenization and sonication were sequentially conducted to extract sperm membranes. The purified human sperm membrane proteins were then mixed with serum from disease group (positive for antisperm antibodies) and control (not containing antisperm antibodies) serum samples. The binding proteins of antisperm antibodies were enriched using co-immunoprecipitation assay. The immunoprecipitates were separated on SDS-polyactylamide gel, then the binding proteins were cut from the gel and analyzed by LC-MS/MS after the enzymolysis. Results: The serum samples from infertile persons (39 females and 17 males) were previously screened as positive for ASA in ELISA and conformed to MAR. The healthy controls (17 females and 14 males) were ASA-negative in ELISA and already possessed healthy offspring. 107 proteins that interacted with antisperm antibodies were obtained from the study via LC-MS/MS. These proteins could be divided into three groups: 13 antigens detected by control serum samples only, 14 antigens recognized by both infertile patients and control sera, 80 antigens specific for patients with antisperm antibodies. 15 novel sperm membrane proteins are CatSper1, CatSper3, CatSper4, SPAG9, Apolipoprotein A-I, Dynein heavy chain 14, axonemal, Cylicin-2 (CYLC2), Izumo sperm-egg fusion protein 4, Thioredoxin domain-containing protein 2, IQ domain-containing protein H (IQCH), IQ domain-containing protein F1 (IQCF1), Spermatogenesis-associated protein 5 (SPATA5), Sperm acrosome membrane-associated protein 1, E3 ubiquitin-protein ligase RNF 114. Conclusions: Fifteen novel sperm membrane proteins discovered with co-immunoprecipitation coupled LC-MS/MS analysis could be referred as male immunoinfertility-related antigens. These proteins may be valuable as an indicator in the clinical diagnosis and monitoring treatment of infertility.

Keywords: Infertility, sperm membrane proteins, co-immunoprecipitation, LC-MS/MS

Introduction

Infertility affects about 15%-20% of reproductive-age couples worldwide, and almost one-half of all cases of infertility depend on male factors [1]. Autoimmune reactions against the sperm cells play a role in fertility impairment [2]. In fact, the plasma membrane structure has a great importance for successful fertilization, given that capacitation, acrosome reaction (AR), and sperm-egg fusion are membrane-associated events [3]. Antisperm antibodies (ASA) have been related to impaired sperm function(s), diminishing the chance of achieving a spontaneous pregnancy [4]. Thus, the identification of antigens of biological relevance involved in fertilization is essential to understand the mechanism by which ASA influence the sperm-fertilizing capacity and to develop reliable additional diagnostic methods for clinically relevant ASA [2, 5].

For a decade, progressing techniques in electrophoresis, chromatography, and mass spec-

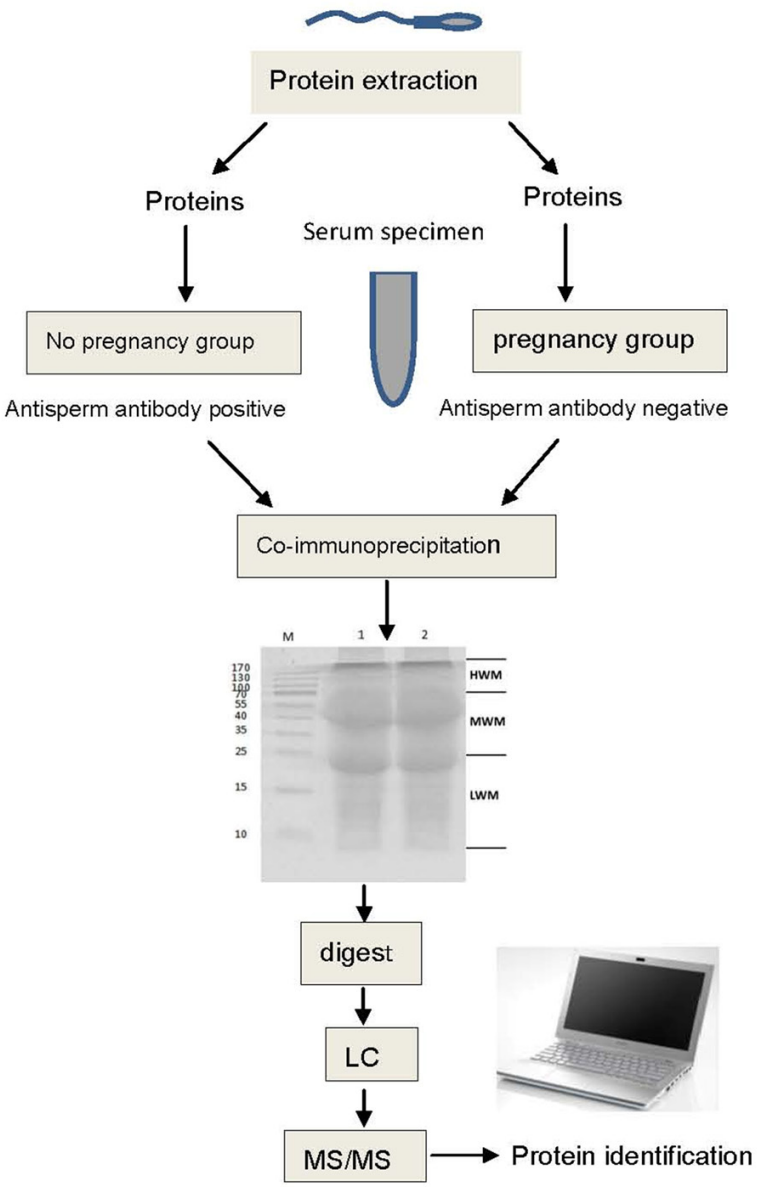

Figure 1. Overall methodological approach used to identify sperm immunogenic membrane antigens. Hypoosmotic swelling homogenization and sonication were sequentially conducted to extract sperm membranes. Sperm membrane proteins were then mixed with serum from disease group and control serum samples. The binding proteins of antisperm antibodies were enriched using co-immunoprecipitation assay. The immunoprecipitates were separated on SDS-polyactylamide gel, and then the binding proteins were cut from the gel and analyzed by LC-MS/MS after the enzymolysis.

trometry (MS) analysis has offered a quick, accurate, and sensitive identification of proteins. Two dimensional (2-D) electrophoresis of sperm extracts exposed to sera and/or seminal plasma from immunoinfertile men allowed the detection of specific sperm proteins [6]. Although the 2D gel electrophoresis is a widely method for analysis of complex mixtures of proteins, it still reflects its limits. First, the multistep nature of 2D gel electrophoresis makes it a labor-intensive and time-consuming technique. Then, the 2D gel electrophoresis assay show limits in analysis for cytosolic proteins, excluding the membrane fraction because of poor resolution of membrane proteins on 2D gels. Finally, proteins with extreme isoelectric points (below 4.0 and above 8.5) were outside the range of resolution of 2D gels [7].

The substantial number of different sperm functions which may potentially be affected by ASA to reduce fertility indicates that several different sperm-surface entities might be involved in immunosubfertility [8]. Although significant efforts have been made to identify immune relative sperm proteins through proteomics studies, it is necessary to develop new strategy or method to look for relative sperm proteins on the immunity infertility.

Currently, most novel surface antigens are identified by performing co-immunoprecipitation with patient's serum followed by mass spectrometry analysis. Application of these technologies identified many autoantigens [9], such as the citrullinated hsp90 isoforms in RA-associated interstitial lung disease [10], M-type phospholipase A2 receptor in idiopathic membranous nephropathy [11, 12], and so on [13, 14].

In this study, we conducted to enrich ASA relative sperm membrane protein with co-immunoprecipitation method, then, the sperm membrane protein were identified by LC-MS/MS (the overall methodological approach refer to **Figure**

1). The identified protein may be helpful to understand the molecular basis of fertilization, better diagnose/treat immunoinfertility and develop immunocontraceptive methods.

Materials and methods

Antisperm antibody testing with indirect mixed antiglobulin reaction (ID-MAR)

For the detection of ASA the sperm MAR-immunoglobulin-G (IgG) test from Anhui Anke Biotechnology Co., LTD (Anhui, China) was used. This test used IgG coated sRBC, which bind to the sperm bound ASA, when an anti-human Fab' fragment is added as a link. The ASA in the patient plasma samples (39 females and 17 males) were tested using the indirect MAR test method. The evaluation of 100 motile spermatozoa and the percentage of spermatozoa with attached sensitive red blood cell (sRBC) represent the test result. Samples containing ASA with MAR test result greater than 10% were considered to be positive.

Donor spermatozoa and membrane preparation

Spermatozoa samples from 48 donors with normal sperm parameters were obtained by masturbation after 3-6-day abstinence for the membrane preparation [15].

All spermatozoa from the donor ejaculates were enriched and purified from other cells by means of a swim up preparation.

The sperm membrane preparation was treated as previously described with some modifications [16]. Briefly, the enriched fraction of motile spermatozoa were diluted at 1:9 with a modified hypoosmotic medium (65 mOsmol fructose, 25 mM sodium citrate (pH 7.3), 10 mM benzamidine, 20 mg/L aprotinin, and 0.5% NP40). The spermatozoa were swollen in this hypoosmotic medium for 2 hr in a water bath (37°C). The spermatozoa membrane was first stripped off by homogenization (Ultra-turrax; 1200 U/min; 2 min; 4°C). The suspension was sonicated in a sonication bath for 2 min (10 kHz) and centrifuged by 4,000 × g (4°C, 15 min) to remove the cell debris and unbroken sperm. The supernatant was centrifuged at 10,000 × g (4°C, 10 min) and the resultant supernatant underwent a further ultracentrifugation at 100,000 g (4°C, 2 hr). The final pellet containing the membrane proteins (plasmalemma, inner and outer membrane) was resuspended, pooled and dissolved in RIPA lysis buffer and stored at -80°C. The quality of protein was monitored by SDS-PAGE.

Co-immunoprecipitation

An immunoprecipitation assay was performed using AsAb positive serum. Briefly, a total of 10 µL of the AsAb positive serum was bound to 30 µL protein A/G Plus sepharose (Santa Cruz Biotech #SC-2003) in 200 µL RIPA buffer, incubated for 1 h at 4°C, and then washed three times with NETN buffer (Hepes, NaCl, Triton-100, NaF). Ab-coated Sepharose beads were mixed with sperm membrane extraction and rotated at 4°C for 1.5 h. After three washes, the beads were resuspended in SDS sample buffer and the co-immunoprecipitated proteins were separated using 12% SDS-PAGE and stained.

LC-MS/MS

The Beijing Biomedical Mass Spectrometry Laboratory at The Beijing Proteome Research Center was responsible for the MS. Briefly, the procedure was as following. The stained SDS-PAGE gel piece was washed and destained. The gel piece was cut into three pieces as high molecular weight, medium molecular weight and low molecular weight gel strips. The gel strips were dehydrated in 200 µl of 75% acetonitrile 5 min, 200 µl ddH_2O 5 min, rehydrated in 50 mM ammonium bicarbonate. Afterwards, the gel strips were grinded and digested in 100 ng/ml sequence-grade modified trypsin in 50 mM ammonium bicarbonate overnight at 37°C. The next morning, the digest was added with 200 µl of 100% acetonitrile and incubated 5 min at room temperature. The supernatant from the elution was collected; the pellet was resolved by addition of 30 µl 0.1% formic acid and 200 µl of 100% acetonitrile. The solution were combined and evaporated in a SpeedVac concentrator for 30 min. The remaining solution volume was adjusted to 10 µl with buffer A (2% ACN, 0.1% formic acid) for LC-MS/MS analysis.

Online chromatography was performed with a Thermo Easy-nLC 1000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled online to the Q Exactive HF instrument with a nano-electrospray ion source (Thermo Fisher

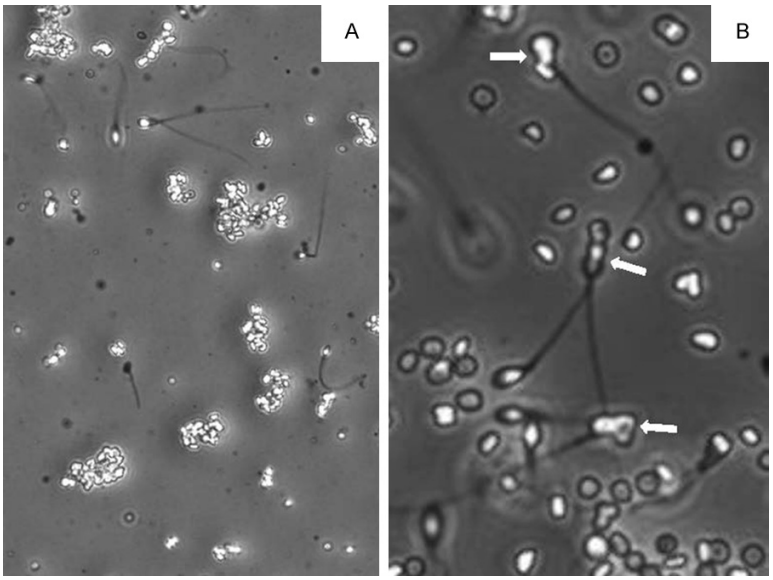

Figure 2. Antisperm antibody testing with indirect mixed antiglobulin reaction. The ID-MAR was considered to be negative (A). The ID-MAR was considered to be positive (spermtozoa with attached sRBC at head or tail (arrow), B).

Scientific). The Thermo Easy column SC200 was 150 μm × 100 mm C_{18} reversed phrase column balanced in buffer A. The peptides were loaded onto the Thermo Easy column SC001 traps (150 μm × 20 mm C_{18} reversed phrase) with buffer A, entered into the column SC200, then separated with buffer B (84% CAN and 0.1% FA) at a flow rate of 600 nL/min. MS data were acquired with a Q Exactive HF instrument. The full MS scans were acquired at a resolution 70000, a maximum ion inject time of 60 ms. Sequencing was done with higher-energy collisional dissociation fragmentation with a target value of 3e6. Scan range was set 300 to 1400 m/z. The data dependent MS/MS (dd-MS_2) were acquired at a resolution 17500, a maximum ion injecting time of 80 ms. Sequencing was done with higher-energy collisional dissociation fragmentation with a target value of 5e4. Normalized collision energy was set to 27.

Proteins identified in different amounts in the two groups were classified according to subcellular localization and biological function(s) using the information available at the UniProt Knowledgebase (UniProtKB/Swiss-Prot) website (http://www.uniprot.org). The list of differential proteins was also analyzed using the bioinformatics tool DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/). The following search parameters were used: two maximum miss cleavage for trypsin; enzyme specificity was set as N-terminal protein acetylation and methionine oxidation as variable modification. Criteria used to accept identification included a minimum of two peptides (and at least one unique peptide) matched per protein, with a false discovery rate of 0.01.

Validation of identified proteins by western immunoblotting

Spermatozoon and its protein extracts from ASA-negative donors were prepared as earlier described. About 100 lg of each sperm protein sample was separated by 12% SDSPAGE and then transferred to polyvinylidene diffluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in 5% skimmed milk (RT) and incubated overnight at 4°C with the following primary antibodies (Abcam, Cambridge, UK): anti-Apolipoprotein A-I (ab52945), anti-CatSper1 (ab203626), and anti-GAPDH (C18-46, Applygen, Beijing, China) as a loading control. The incubation with secondary antibody conjugated with horse-radish peroxidase (Goat Anti-Rabbit IgG H&L, bs-0295G-HRP, Biosynthesis, Beijing, China) was set for 1 h at RT. Proteins of interest were visualized with Amersham ECL kit (Beyotime, P0018) in ChemiDoc XRS+System (Bio-Rad Laboratories).

Results

ASA detection

All the serum samples tested with ID-MAR were ASA-positive in at least one of the following immunoglobulin class IgG with more than 10% of spermatozoa bound to antiglobulin-coated sRBC in ID-MAR (**Figure 2**).

Membrane preparation and one dimensional electrophoresis

The different protein purification steps after separation using one-dimensional SDS poly-

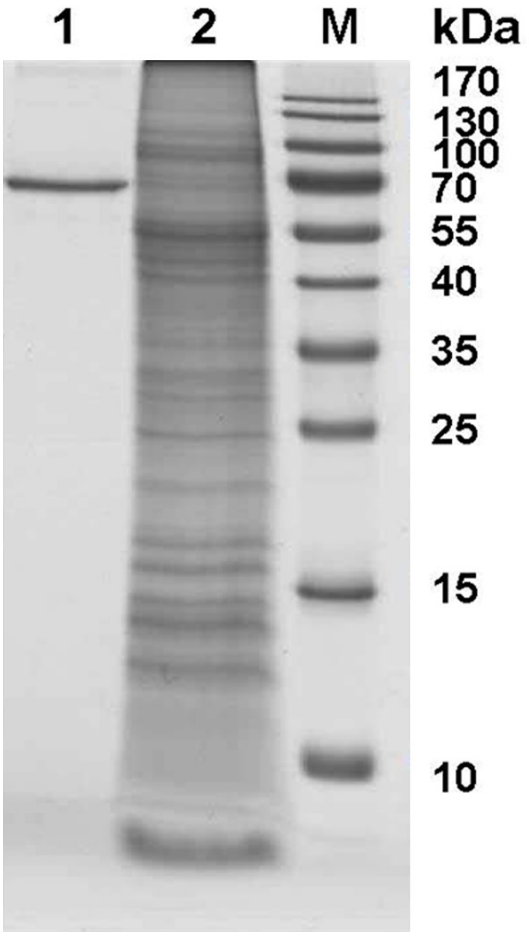

Figure 3. SDS-PAGE of sperm membrane protein on a 12% gradient gel. Lane (1) Bovine serum albumin; (2) Sperm membrane protein. The concentration of the sperm membrane proteins was determined for 1.19 μg/μl by comparing with the concentration of bovine serum albumin (1 μg/μl). (3) Marker.

acrylamide gradient gel and visualization with Coomassie brilliant blue (**Figure 3**). The concentration of the sperm membrane proteins was determined for 1.19 μg/μl by comparing with the concentration of bovine serum albumin (1 μg/μl).

Sperm proteomic identification

The SDS-PAGE gel was cut into three pieces as high molecular weight, medium molecular weight and low molecular weight gel strips (**Figure 4**). Sperm membrane protein extractive was subjected to LC-MS analysis as it seemed to us to be appropriate to extend the insight into sperm antigens immunobiology. We have selected three groups of sperm antigens: 13 antigens detected only by ASA-negative sera, 14

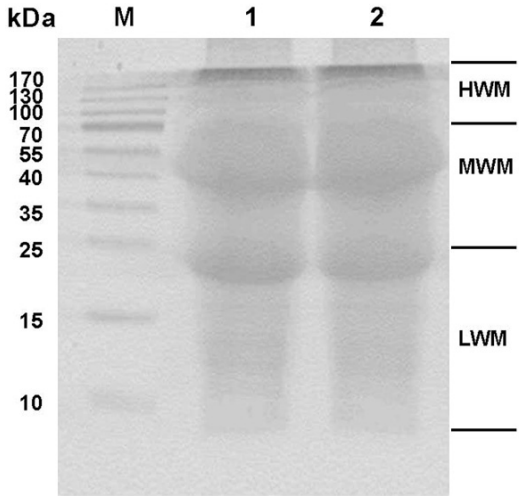

Figure 4. The SDS-PAGE gel was cut into three pieces as high molecular weight (HWM), medium molecular weight (MWM) and low molecular weight (LWM) gel strips.

antigens recognized by both infertile patients and control sera, 80 antigens recognized by ASA-positive sera of patients with fertility problems including 46 cell membrane proteins (**Table 1**) and 34 cytoplasm membrane proteins (**Table 2**). Among proteins detected by ASA-positive sera of infertile patients, there were identified 15 sperm entities known from their involvement in fertilization process (**Table 3**).

Western immunoblotting validation

The results obtained were validated using Western immunoblotting technique. Two of all identified proteins (Apolipoprotein A-I and CatSper1) were proved to be contained in our sperm protein extracts (**Figure 5**). In this study, Apolipoprotein A-I has been a protein identified in human spermatozoon for the first time.

Discussion

Identification of sperm antigens recognized by ASA present in infertile individuals is a step toward understanding the development of autoimmune infertility and may also lead to immune contraception based on the components derived from male gametes when exploring novel detected entities.

In this study, we tried to identify the sperm membrane antigen by means of ASA-positive and ASA-negative serum and co-immunopre-

Table 1. Sperm cell membrane protein detected by ASA-positive individuals

Protein ID	Protein name	Location	M.W. (kDa)	Length (aa)	Role in biology
P02647	Apolipoprotein A-I	Secreted	30.78	267	As part of the SPAP complex, activates spermatozoa motility.
Q1ZYL8-2	Izumo sperm-egg fusion protein 4	Secreted	24.49	214	Play a role in spermatozoa-egg-interaction.
O60271-9	Sperm associated antigen 9	Acrosome	128.61	1177	Play a role in spermatozoa-egg-interaction.
Q0VDD8	Dynein heavy chain 14, axonemal	Cilium axoneme	399.89	3507	Involved in sperm motility; implicated in sperm flagellar assembly.
Q14093	Cylicin-2	Calyx	39.08	348	Possible architectural role during spermatogenesis. May be involved in spermatid differentiation.
Q8N6M8	IQ domain-containing protein F1	Acrosome	23.7	205	Involved in sperm capacitation and acrosome reaction.
Q9HBV2	Sperm acrosome membrane-associated protein 1	Membrane	32.14	294	May be involved in sperm-egg fusion.
Q8NEC5	Cation channel sperm-associated protein 1	Flagellum membrane	90.09	780	Voltage-gated calcium channel that plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
Q86XQ3	Cation channel sperm-associated protein 3	Flagellum membrane	46.42	398	Plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
Q7RTX7	Cation channel sperm-associated protein 4	Flagellum membrane	54.09	472	Plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
O00264	Membrane-associated progesterone receptor component 1	Microsome membrane	21.67	195	Receptor for progesterone.
O14656	Torsin-1A	Nucleus membrane; Peripheral membrane protein	37.81	332	Protein with chaperone functions important for the control of protein folding, processing, stability and localization as well as for the reduction of misfolded protein aggregates.
O14735-3	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	Membrane (Endoplasmic reticulum membrane; Golgi apparatus membrane; Cell membrane)	18.62	168	Catalyzes the biosynthesis of phosphatidylinositol (PtdIns) as well as PtdIns: inositol exchange reaction.
O76024	Wolframin	Endoplasmic reticulum membrane; Multi-pass membrane protein	100.29	890	Participates in the regulation of cellular Ca^{2+} homeostasis.
P11047	Laminin subunit gamma-1	Basement membrane	177.6	1609	Binding to cells via a high affinity receptor, laminin is thought to mediate the attachment, migration and organization of cells into tissues.
P13498	Cytochrome b-245 light chain	Cell membrane	21.01	195	Associates with NOX3 to form a functional NADPH oxidase constitutively generating superoxide.
P14543-2	Nidogen-1	Basement membrane	122.02	1114	It probably has a role in cell-extracellular matrix interactions.
P15428-5	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	Basolateral plasma membrane	21.53	198	Contributes to the regulation of events that are under the control of prostaglandin levels.
P17181-2	Interferon alpha/beta receptor 1	Single-pass type I membrane protein	49.55	434	Can form an active IFNB1 receptor by itself and activate a signaling cascade that does not involve activation of the JAK-STAT pathway.
P19404	NADH dehydrogenase [ubiquinone] flavoprotein 2	Mitochondrial membrane	27.39	249	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis.
P32189-1	Glycerol kinase	Mitochondrion outer membrane; Peripheral membrane protein; Cytoplasmic side	57.49	524	Key enzyme in the regulation of glycerol uptake and metabolism.

P33151	Cadherin-5	Single-pass type I membrane protein	87.53	784	This cadherin may play a important role in endothelial cell biology through control of the cohesion and organization of the intercellular junctions.
P33947	ER lumen protein-retaining receptor 2	Endoplasmic reticulum membrane; Multi-pass membrane protein	24.42	212	Required for the retention of luminal endoplasmic reticulum proteins.
P35613-2	Basigin	Cell membrane	29.22	269	Plays pivotal roles in spermatogenesis, embryo implantation, neural network formation and tumor progression.
P51572	B-cell receptor-associated protein 31	Endoplasmic reticulum membrane	27.99	246	Serves as a cargo receptor for the export of transmembrane proteins. May be involved in CASP8-mediated apoptosis.
P61619	Protein transport protein Sec61 subunit alpha isoform 1	Endoplasmic reticulum membrane; Multi-pass membrane protein	52.26	476	Plays a crucial role in the insertion of secretory and membrane polypeptides into the ER.
Q02246	Contactin-2	Cell membrane; Lipid-anchor > GPI-anchor	113.39	1040	In conjunction with another transmembrane protein, may be involved in cell adhesion.
Q03167-2	Transforming growth factor beta receptor type 3	Single-pass type I membrane protein	93.43	850	Could be involved in capturing and retaining TGF-beta for presentation to the signaling receptors.
Q13546	Receptor-interacting serine/threonine-protein kinase 1	Cell membrane	75.93	671	Activates the MAP3K5-JNK apoptotic cascade.
Q15286	Ras-related protein Rab-35	Cell membrane	20.03	201	The small GTPases Rab are key regulators of intracellular membrane trafficking.
Q15437	Protein transport protein Sec23B	Golgi apparatus membrane; Endoplasmic reticulum membrane	86.48	767	Involved in transport from the endoplasmic reticulum to the Golgi apparatus.
Q16678	Cytochrome P450 1B1	Microsome membrane	60.85	543	This enzyme is involved in an NADPH-dependent electron transport pathway, Contribute to oxidative homeostasis and ultrastructural organization and function of trabecular meshwork tissue through modulation of POSTN expression.
Q7Z6K5-2	Arpin	Lamellipodium	43.88	394	Involved in steering cell migration by controlling its directional persistence.
Q8IY26	Phospholipid phosphatase 6	Membrane	32.19	295	It may be indirectly involved in innate immunity.
Q8J025	Protein APCDD1	Cell membrane	58.8	514	Negative regulator of the Wnt signaling pathway. Inhibits Wnt signaling in a cell-autonomous manner and functions upstream of beta-catenin.
Q8NEN9	PDZ domain-containing protein 8	Membrane	128.56	1154	Plays a role in the regulation of cell morphology and cytoskeletal organization.
Q96J02-2	E3 ubiquitin-protein ligase Itchy homolog	Cell membrane	98.68	862	Involved in the regulation of apoptosis and reactive oxygen species levels through the ubiquitination and proteasomal degradation of TXNIP, and probably plays an important role in the regulation of immune response.
Q9HD26-3	Golgi-associated PDZ and coiled-coil motif-containing protein	Golgi apparatus membrane	35.13	319	May also regulate the intracellular trafficking of the ADR1B receptor. May play a role in autophagy.
Q9NV70-2	Exocyst complex component 1	Plasma membrane	100.28	879	Involved in the docking of exocytic vesicles with fusion sites on the plasma membrane.
Q9UL26	Ras-related protein Rab-22A	Endosome membrane	21.86	194	Plays a role in endocytosis and intracellular protein transport.
Q9Y2I1-4	Nischarin	Cell membrane	56.87	515	Plays a role in protection against apoptosis.
A1A528	Centromere/kinetochore protein zw10 homolog	Endoplasmic reticulum membrane	76.81	672	Involved in regulation of membrane traffic between the Golgi and the endoplasmic reticulum (ER).
H3BVI4	Lipase maturation factor	Endoplasmic reticulum membrane	40.15	350	Involved in the maturation of specific proteins in the endoplasmic reticulum.
O14990	Putative type-1 protein phosphatase inhibitor 4	Protein phosphatase type 1 complex	22.66	202	It inhibits activity of the catalytic subunit of PP1 and weakly inhibits the activity of myosin-associated phosphates.
P47755	F-actin-capping protein subunit alpha-2	Actin cytoskeleton	32.95	286	Bind in a Ca^{2+}-independent manner to the fast growing ends of actin filaments, thereby blocking the exchange of subunits at these ends.
Q5T1C6	Acyl-coenzyme A thioesterase THEM4	Ruffle membrane	27.13	240	Plays a role in the apoptotic process, possibly via its regulation of AKT1 activity.

Table 2. Sperm cytoplasm membrane protein detected by ASA-positive individuals

Protein ID	Protein name	Location	M.W. (kDa)	Length (aa)	Role in biology
Q86VQ3-2	Thioredoxin domain-containing protein 2	Cytoplasm	53.27	486	Probably plays a regulatory role in sperm development.
Q86VS3-2	IQ domain-containing protein H	Unknown	78.87	684	May play a regulatory role in spermatogenesis.
Q8NB90	Spermatogenesis-associated protein 5	Cytoplasm; Mitochondrion	97.9	893	May be involved in morphological and functional mitochondrial transformations during spermatogenesis.
Q9BVQ7-2	Spermatogenesis-associated protein 5-like protein 1	Cytoplasm	66.11	620	Unknown
Q9Y508-2	E3 ubiquitin-protein ligase RNF114	Cytoplasm; Nucleus	21	191	May play a role in spermatogenesis.
O00151	PDZ and LIM domain protein 1	Cytoplasm	36.07	329	May act as an adapter that brings other proteins (like kinases) to the cytoskeleton.
O15131	Importin subunit alpha-6	Cytoplasm	60.35	536	Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1.
O75935-3	Dynactin subunit 3	Cytoplasm	18	158	Together with dynein may be involved in spindle assembly and cytokinesis.
P01042-3	Kininogen-1	Extracellular space	43.82	391	Kininogens are inhibitors of thiol proteases.
P20742	Pregnancy zone protein	Blood microparticle; Extracellular exosome	163.86	1482	Is able to inhibit all four classes of proteinases by a unique 'trapping'mechanism.
P29622	Kallistatin	Extracellular exosome	48.54	427	Inhibits human amidolytic and kininogenase activities of tissue kallikrein.
P43378	Tyrosine-protein phosphatase non-receptor type 9	Cytoplasm	68.02	593	Participate in the transfer of hydrophobic ligands or in functions of the Golgi apparatus.
P63151	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform	Cytosol	51.69	447	Modulate substrate selectivity and catalytic activity, and also might direct the localization of the catalytic enzyme to a particular subcellular compartment.
P78560	Death domain-containing protein CRADD	Cytoplasm	22.75	199	Apoptotic adaptor molecule specific for caspase-2 and FASL/TNF receptor-interacting protein RIP.
P80108	Phosphatidylinositol-glycan-specific phospholipase D	Extracellular region	92.34	840	This protein hydrolyzes the inositol phosphate linkage in proteins anchored by phosphatidylinositol glycans (GPI-anchor) thus releasing these proteins from the membrane.
P80188-2	Neutrophil gelatinase-associated lipocalin	Extracellular region	22.46	198	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development.
Q08629	Testican-1	Extracellular matrix	49.12	439	May play a role in cell-cell and cell-matrix interactions.
Q14141-2	Septin-6	Cytoplasm	48.87	427	Required for normal organization of the actin cytoskeleton. Involved in cytokinesis.
Q15018	BRISC complex subunit Abro1	Cytoplasm	46.9	415	Plays a role in regulating the onset of apoptosis via its role in modulating 'Lys-63'-linked ubiquitination of target proteins.
Q7Z478	ATP-dependent RNA helicase DHX29	Cytoplasm	155.23	1369	ATP-binding RNA helicase involved in translation initiation.
Q8IUI8-2	Cytokine receptor-like factor 3	Cytoplasm	49.35	438	May play a role in the negative regulation of cell cycle progression.
Q8WZ42-3	Titin	Cytoplasm	2292.9	26926	Play a role in chromosome condensation and chromosome segregation during mitosis.
Q96BN8	Ubiquitin thioesterase otulin	Cytoplasm	40.26	352	Acts as a regulator of angiogenesis and innate immune response.
Q96CV9-3	Optineurin	Cytoplasm	59.56	520	Plays an important role in the maintenance of the Golgi complex, in membrane trafficking, in exocytosis, through its interaction with myosin VI and Rab8.
Q9C0B2	Cilia- and flagella-associated protein 74	Cytoplasm	86.96	762	As part of the central apparatus of the cilium axoneme may play a role in cilium movement.
Q9NWB7	Intraflagellar transport protein 57 homolog	Cytoplasm	49.11	429	Plays an indirect role in sonic hedgehog signaling, cilia being required for all activity of the hedgehog pathway.
Q9P2H3	Intraflagellar transport protein 80 homolog	Cytoplasm	88.03	777	Component of the intraflagellar transport (IFT) complex B, which is essential for the development and maintenance of motile and sensory cilia.
Q9UGI8-2	Testin	Cytoplasm	46.91	412	May play a role in cell adhesion, cell spreading and in the reorganization of the actin cytoskeleton.
Q9UHD8-7	Septin-9	Cytoplasm	63.5	567	May play a role in the internalization of 2 intracellular microbial pathogens, Listeria monocytogenes and Shigella flexneri.

Q9Y233	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	Cytoplasm	88.41	779	Plays a role in signal transduction by regulating the intracellular concentration of cyclic nucleotides.
P49448	Glutamate dehydrogenase 2	Mitochondrion matrix	61.43	558	Important for recycling the chief excitatory neurotransmitter, glutamate, during neurotransmission.
O00462	Beta-mannosidase	Lysosome	100.89	879	Exoglycosidase that cleaves the single beta-linked mannose residue from the non-reducing end of all N-linked glycoprotein oligosaccharides.
Q9Y3I1	F-box only protein 7	Mitochondrion	58.5	522	Plays a role downstream of PINK1 in the clearance of damaged mitochondria via selective autophagy (mitophagy) by targeting PARK2 to dysfunctional depolarized mitochondria. Promotes MFN1 ubiquitination.
Q16134-3	Electron transfer flavoprotein-ubiquinone oxidoreductase	Mitochondrion	62.82	570	Accepts electrons from ETF and reduces ubiquinone.

Table 3. Identified 15 sperm entities known from their involvement in fertilization process

Protein ID	Name	Location	Molecual weight (kDa)	Length (aa)	The role of biology
P02647	Apolipoprotein A-I	Secreted	30.78	267	As part of the SPAP complex, activates spermatozoa motility.
Q1ZYL8-2	Izumo sperm-egg fusion protein 4	Secreted	24.49	214	Play a role in spermatozoa-egg-interaction.
O60271-9	Sperm associated antigen 9	Acrosome	128.61	1177	Play a role in spermatozoa-egg-interaction.
Q0VDD8	Dynein heavy chain 14, axonemal	Cilium axoneme	399.89	3507	Involved in sperm motility; implicated in sperm flagellar assembly.
Q14093	Cylicin-2	Calyx	39.08	348	Possible architectural role during spermatogenesis. May be involved in spermatid differentiation.
Q8N6M8	IQ domain-containing protein F1	Acrosome	23.7	205	Involved in sperm capacitation and acrosome reaction.
Q9HBV2	Sperm acrosome membrane-associated protein 1	Membrane	32.14	294	May be involved in sperm-egg fusion.
Q8NEC5	Cation channel sperm-associated protein 1	Flagellum membrane	90.09	780	Voltage-gated calcium channel that plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
Q86XQ3	Cation channel sperm-associated protein 3	Flagellum membrane	46.42	398	Plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
Q7RTX7	Cation channel sperm-associated protein 4	Flagellum membrane	54.09	472	Plays a central role in calcium-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction and chemotaxis towards the oocyte.
Q86VQ3-2	Thioredoxin domain-containing protein 2	Cytoplasm	53.27	486	Probably plays a regulatory role in sperm development.
Q86VS3-2	IQ domain-containing protein H	Unknown	78.87	684	May play a regulatory role in spermatogenesis.
Q8NB90	Spermatogenesis-associated protein 5	Cytoplasm; Mitochondrion	97.9	893	May be involved in morphological and functional mitochondrial transformations during spermatogenesis.
Q9BVQ7-2	Spermatogenesis-associated protein 5-like protein 1	Cytoplasm	66.11	620	Unknown
Q9Y508-2	E3 ubiquitin-protein ligase RNF114	Cytoplasm; Nucleus	21	191	May play a role in spermatogenesis.

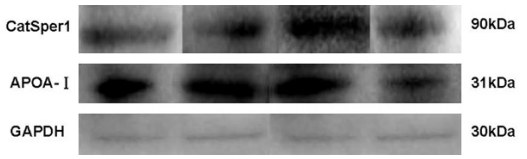

Figure 5. Two of all identified sperm membrane proteins were selected for subsequent validation: ApoA-I and CatSper1. GADPH protein was used as a loading control. These proteins were reproducible detected in the protein sample of each sperm donor (n = 4), separately.

cipitation approach. In fact, to our knowledge, this is the first study evaluating sperm membrane antigens, together with the co-immunoprecipitation assay and the LC-MS/MS analysis.

We had chosen three groups of sperm antigens: the antigens integrated by ASA-negative sera, the antigens integrated by ASA-positive sera and ASA-negative sera and the antigens combined specifically only by ASA-positive sera. This distribution clearly refers to the sperm antibodies/sperm antigens classification previously proposed by Nowicka-Barer et al. [17].

Membrane antigens detected by sera from the first group seemed to be nonspecific proteins of low immunoreactivity nature. However, there were four of proteins, testis-specific serine/threonine-protein kinase 2, mitochondrial fission 1 protein, and serine/threonine-protein phosphatase, lysosome-associated membrane glycoprotein 1 have been reported to be involved in fertilization process.

According to K. Nowicka-Bauer and Paradisi [17, 18], the second group of proteins commonly detected by ASA-positive and ASA-negative sera consists of antigens not necessarily related with fertility status. Observed immunoreactivity of heat-shock proteins could support this interpretation, especially due to the fact that these proteins seem to be localized on the sperm surface; therefore, their high reactivity can be connected with 'molecular mimicry' between heat-shock proteins and some micro-organisms.

The third group of antigens was exclusively detected by ASA-positive sera of patients with reproductive problems. Following K. Nowicka-Bauer et al. and Paradisi et al. classification, these proteins can be referred as male immunoinfertility-related antigens and candidates for therapeutic means and/or immunocontraception. What is interesting, in this group we could select 15 proteins known to be involved in fertilization process, mainly in capacitation and spermatozoon-oocyte interaction, such as the Cation channel sperm-associated protein 1 [19, 20], Cation channel sperm-associated protein 3, Cation channel sperm-associated protein 4 and the Izumo sperm-egg fusion protein 4, et al. Cation channel of sperm (CatSper), a sperm-specific ion channel, is unique in orchestrating the events for fertilization, and seems to be exclusively evolved for sperm function and male fertility [21, 22]. Izumo sperm-egg fusion protein 4 is subjective to the izumo sperm-egg fusion protein 1 (izumo1) family [23], also called Sperm 22 kDa protein c113. After the acrosome reactome the sperm has passed through the cumulus cells and the zona pellucida. The membrane of the sperm head and the membrane of the oocyte are drawn together through the interaction of the sperm-bound protein Izumo and the ooctye CD9 membrane protein. It is not difficult to predict CatSper and Izumo play a special role in fertility.

Interestingly, we also found some previous not reported sperm membrane proteins perhaps involving in fertility. Cylicin-2 is possible architectural role during spermatogenesis, which may be involved in spermatid differentiation [24]. Thioredoxin domain-containing protein (Txndc2, Txndc3) probably plays a regulatory role in sperm development, which may participate in regulation of association with the spermatozoa after spermiation and potentially play an important role in regulating the redox status of the mature gamete [25]. IQ domain-containing protein F1 is probably involved in sperm capacitation and acrosome reaction [26]. Spermatogenesis-associated protein 5 may be involved in morphological and functional mitochondrial transformations during spermatogenesis [27]. E3 ubiquitin-protein ligase RNF-114 may play a role in spermatogenesis [28]. The apolipoprotein A-I molecule was reported bound in sperm activating protein (SPAP) that supporting sperm motility [29]. These proteins are involved in various mechanisms previous to fertilization including recognition of the oocyte by sperm, the acrosin reaction and the union of sperm and oocyte.

In conclusion, in the present study we have identified some sperm membrane proteins involved in fertilization with co-immunoprecipi-

tation coupled LC-MS/MS strategy. The strategy was one efficient approach to identify the sperm membrane proteins involving immunological infertility. These proteins may justify further investigation into the mechanisms involved in male immunoinfertility and an effort toward the development of an antispermcontraceptive vaccine. Furthermore, these proteins may be valuable as an indicator in the clinical diagnosis and monitoring treatment of infertility.

Disclosure of conflict of interest

None.

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