Antisperm protein targets in azoospermia men

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ABSTRACT

BACKGROUND: The number of couples that meet the definition of infertility at reproductive ages is increasing worldwide. One of the most known conditions of infertility in males is azoospermia, defined as complete absence of spermatozoa in the semen. Azoospermia manifests in two forms, namely obstructive and non-obstructive azoospermia. Although the presence of antisperm antibody (ASA) has been reported in 88% of the patients with obstructive azoospermia (OA), interestingly, there is no data regarding ASA targets in OA individuals. **AIM:** The present study aimed to identify sperm antibody targets in a group of OA men. **SETTINGS AND DESIGN:** The present study was carried out on 27 OA infertile men and 27 healthy fertile age-matched males as cases and controls, respectively. **SUBJECTS AND METHODS:** The sperm proteome was separated using two-dimensional gel electrophoresis technique, transferred onto the polyvinylidene fluoride membrane, and blotted with the sera of a group of OA men. Then, it was compared with the membranes blotted with the sera of a group of healthy fertile men. Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass spectrometry was used to identify the different blotted spots and finally the results of the mass analysis were confirmed using reverse transcriptase polymerase chain reaction method. **RESULTS:** The results indicated that OA patients might produce antibody against two sperm proteins, Tektin-2 and triose phosphate isomerase. Moreover, the expressions of the two targeted proteins were confirmed at RNA level. **CONCLUSIONS:** The findings of the present study revealed two functionally important sperm proteins as antibody targets in azoospermic men.

KEY WORDS: Azoospermia, Tektin-2, triose phosphate isomerase, two-dimensional polyacrylamide gel electrophoresis, Western blot

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INTRODUCTION

The number of couples that meet the definition of infertility at reproductive ages is increasing worldwide. One of the most prevalent conditions of infertility in males is azoospermia, defined as complete absence of spermatozoa in the semen.[1] Azoospermia manifests in two forms, namely obstructive and non-obstructive azoospermia. Obstruction of posttesticular genital tracts may lead to high-level reduction of sperm count in seminal plasma due to the problems with sperm delivery, a condition which is called OA.[2] OA accounts for around 40% of azoospermia cases.[2] Naturally, human sperms are not exposed to the immune system. However, when sperm antigens come into contact with immune cells, antisperm antibody (ASA) formation occurs. In OA, due to the problems with the ductal system or issues with ejaculation, blood–testis barrier might breakdown and the immune system meets the sperm cells resulting in the production of ASA. In spite of the fact that ASAs may be produced in fertile couples, the chance of ASA production in infertile couples is at least 5 times more than the fertile ones.[3] Therefore, it seems that not all ASAs cause infertility. Interestingly, the presence of ASA is reported in 88% of the patients with OA.[4] Up to now, reports are available regarding the production of antibody against several sperm antigens, including sperm head protein 1, sperm

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flagella protein (SFP) 2, SFP3, SFP4, heat shock 70 kDa protein 1-HOM, protein disulfide isomerase ER-60 precursor, caspase-3, and SPRASA.[5-7] Nonetheless, there is no data regarding ASA targets in OA males. Moreover, despite the fact that several ASA targets are reported until now, there is controversy about the importance or the role of these antibodies in several forms of human infertility. Indeed, it seems that only those antibodies that affect sperm functions or fertilization capacities might affect the fertility rate. Regarding OA, there is no report directly addressing the sperm immune targets for ASAs. Up to now, several techniques, including gelatin agglutination test, tray agglutination test, immunobead test, immunofluorescence assay (IFA), ELISA, and mix antiglobulin reaction have been used to find sperm targets for ASAs.[8] Yet using two-dimensional gel electrophoresis (2-DE) combined with Western blot may give more comprehensive information about ASA targets.[9] Shetty *et al*. in 2001 used this approach to find the sperm target antigens for ASAs.[10] They identified eight sperm proteins as immune contraceptive candidates. Using liquid chromatography-mass technique, Domagala *et al*. identified 35 sperm proteins as immunogenic antigens in 2007.[11] Interestingly, only a few studies have addressed the sperm immune targets in OA males. Lee *et al*. investigated the value of ASAs in the diagnosis of OA and found that the presence of serum ASA was highly accurate in predicting OA, although they did not identify the targets of these ASAs.[4] In the present study, we used 2-DE technique to separate sperm immune proteome and Western blot method to identify ASA targets in a group of OA patients.

SUBJECTS AND METHODS

Serum samples

Serum samples were obtained from 27 OA infertile without any history of infection and 27 healthy fertile age-matched males as cases and controls, respectively. The diagnosis of OA was based on the 2010 WHO guideline's semen analysis criteria.[12] The clinical condition of the patients and the type of azoospermia were obtained from their medical records, checked, and confirmed by an urologist. All OA cases were selected among men with primary idiopathic epididymis obstruction with normal hormonal profile, testis size, and spermatogenesis. Excluding criteria for OA cases were azoospermia due vas deferens or ejaculatory duct. Moreover, patients with genital infections, vasectomy, or other iatrogenic injuries to the male reproductive tract were excluded from the study. All fertile subjects had a normal semen analysis report and also had at least two children with the same partner. On the other hand, the infertile subjects had not experienced fertility with their partners. None of the participant had an ASA positive test checked by using an IFA test (Euroimmune, Germany).

Sperm samples

Semen samples were collected by masturbation after 1 week of sexual abstinence from four fertile men with high sperm counts and pooled to form a whole sperm sample. All the sperm donators had a normal semen analysis according to the WHO's semen analysis criteria.[13] Written informed consent for using the sera or sperm was obtained from all the participants. This study was approved by the Local Ethics Committee of Shiraz University of Medical Sciences.

Sperm isolation

Pure sperm (Nidacon International AB, Mölndal, Sweden) density gradient centrifugation method was used to isolate the mature sperms.[14] At first, the semen samples were liquefied by incubation at 37°C for 30 min. Then, 2 ml of liquefied semen was carefully loaded over a two-layer pure sperm density gradient consisting of 2 ml of the 80% at the bottom layer and 2 ml of the 40% on the top layer. After centrifugation at 500 g at room temperature for 21 min, the sperm pellet was collected and washed for 2 times in pure sperm medium by centrifugation at 300 g at room temperature for 7 min. Afterward, the sperm cells were counted and evaluated by light microscopy. The washed sperms showed more than 95% and 99% motility and purity, respectively. After separation, the sperm pellets were stored in nitrogen tank until protein extraction.

Protein extraction

For protein extraction, 32 × 107 sperm cells were loaded in 4 ml sperm lysis buffer containing 2 M thiourea, 7 M urea, 75 mM dithiothreitol (DTT), 40 mM tris, 1% ampholine, and 4% CHAPS. After that, they were incubated in a dark room at room temperature for 2 h (every 15 min, the lysate was vortexes for 5 s). Then, the lysate was centrifuged at 10,000 × g at 4°C for 30 min and the supernatant was collected. The protein concentration was measured by bradford assay (Quick Start™ Bradford Protein Assay Kit, Bio-Rad, Hercules, CA, USA). The protein supernatant was aliquoted and stored at − 70°C until use.

Two-dimensional polyacrylamide gel electrophoresis

Linear precast 18 cm IPG strips (pH: 3–10 and 4–7, Bio-Rad, Hercules, CA, USA) were used for active isoelectric focusing (IEF). Briefly, 150 µg of the sperm protein extracts in a total volume of 340 µl mixture containing 8 M urea, 2% CHAPS, 2% DTT, 2% IPG buffer, and 0.001% bromophenol blue were applied on the IPG strips and rehydrated at 60V for 16 h. After rehydration, the strips were focused at the total voltage of 65,000 Vh (IPGphore 2 IEF System, Amersham, UK). The focused strips were equilibrated in two steps. In the first step, 65 mM DTT at 37°C for 15 min and in the second step, 135 mM iodoacetamide at room temperature for 15 min were used to equilibrate the focused strips in a equilibration buffer containing 6 M urea, 50 mM Tris–HCl pH 8.8, 30% v/v glycerol, 2% SDS, and 0.001% bromophenol blue. The equilibrated strips were placed on the top of 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels (SCIE Plus Twin Electrophoresis System, UK) and run at a constant voltage of 200 volt until the tracking dye ran off the gel.

Dot blot technique

Dot blot technique was used to screen the sera. Briefly, sperm protein extracts were applied on the 54 separate pieces of polyvinylidene fluoride (PVDF) paper as a dot and air dried. PVDF papers were then blocked overnight with 3% bovine serum albumin (BSA) in tris-buffered saline with Tween 20 (TBST) buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH = 7.6). Afterward, the blocked papers were incubated with the sera (1/50 diluted in blocking solution) for 2 h and after 5 times washing with TBST buffer, incubated with goat polyclonal anti-human horseradish peroxidase (HRP) conjugated antibody (1/1000 diluted in the blocking solution, ab98567, Abcam, Al Ain, UAE) for 1 h. Besides, DAB (ACROS, Organics, USA) was used as chromogen and H_2O_2 as substrate to develop the blot reactions.

Western blot technique

Western blot was done using the selected sera based on the dot blot results as previously described.[15] Ten sera from the cases and ten from the controls were used for immunoblotting. At first, sperm proteins were separated using 2-DE as described in the previous section. Then, the separated protein spots were transferred onto the PVDF membranes using a semi-dry blotter system (Pharmacia, Uppsala, Sweden). Two two-dimensional-PAGE (2D-PAGE) gels were prepared and transferred simultaneously to minimize the variation due to the technical issue. After that, the membranes were blocked by incubating with 3% BSA in TBST buffer (overnight at 4°C). After blocking, the membranes were incubated with pooled (each from 3 to 4 individuals) sera from the cases and controls (1/50 diluted in blocking solution) and incubated at room temperature for 2 h. Finally, three pooled sera from the cases and controls were tested and all the tests were done in duplicate. The membranes were then incubated with the secondary anti-human-HRP antibody and developed as described in dot blot section.

Gel staining and spot detection

Colloidal coomassie brilliant blue (CBB) G-250 technique described by Neuhoff *et al*. was used for gel staining.[16] In addition, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry technique was used for identification of the targeted spots.[17] The targeted spots were selected after comparing the PVDF membranes of the cases to those of the controls. The selected spots were picked from the CBB-stained gel manually and were identified using mass technique after digestion with trypsin. Mass spectrometry analysis was performed at proteomics technology facility, Department of Biology, York University, UK, using Ultraflex III version 1.0 MALDI-TOF/TOF Proteomics Analyzer Instrument (Bruker Daltonik GmbH, Bruker Daltons, Germany). For data-based search, the MASCOT program search algorithm (http://www.matrixscience.com), based on the National Center for Biotechnology Information Database (NCBI: http://www.ncbi.nlm.nih.gov), was used. In data search, one missed cleavage with trypsin and two modifications (carbamidomethylation of cysteine and oxidation of methionine) were allowed in search setting.

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to confirm the presence of the identified sperm proteins (Tektin-2 [TEK2] and triose phosphate isomerase [TPI]) at RNA level. Total sperm RNA from the healthy subjects was extracted using total RNA extraction kit (Jena Bioscience GmbH, Jena, Germany). Moreover, 10×10^6 pure isolated sperm cells were used for extraction according to the manufacturer's instructions. The concentration of the extracted RNA was determined using a Nano drop instrument, and cDNA was synthesized from total RNA using Easy™ cDNA Synthesis Kit (Pars Tous, Iran). The presence of TEK2, TPI, and β-actin transcripts was assessed using the following specific designed primers (using primer blast tool, http://www.ncbi.nlm.nih.gov/tools/primer-blast): 5'-GGAAAGGAGGTGGTGTCTGTG-3' (forward) 5'-GGCATTGGTGGATAGCAGGT-3' (reverse) and 5'-TATGGAGGCTCTGTGACTGG-3' (forward) 5'-GTTTGGCATTGATGATGTCC-'3 (reverse) for TEK2 and TPI, respectively, while 5'-GGCGGCACCACCATGTACCC-3' (forward) and 5'-GGAGGGGCCGGACTCGTCAT-3'(reverse) primers were used to amplify β-actin as the control transcript.

Statistical analysis

Descriptive statistics were used to compare the cases and controls regarding the blotted membranes.

RESULTS

Dot blot

Dot blot results indicated that 23 out of the 27 tested sera from azoospermic cases had strong or moderate reactivity with sperm proteins, whereas the remaining 4 showed low or negative reactivity. Interestingly, 22 out of the 27 sera from the healthy fertile men also indicated reactivity with the sperm proteins, most of which showing moderate reactivity. In addition, 5 sera from the healthy participants had low or negative reactivity with the sperm proteins.

Two-dimensional polyacrylamide gel electrophoresis and Western blot

At first, the IPG strip in the pH range of 3–10 was used to separate the sperm protein spots. More than 1000 sperm protein spots were detected in this range when stained with silver nitrate technique, most of which laying at pH = 4–7 [Figure 1]. To increase the resolution of 2D-PAGE gels, the IPG strips within the pH range of 4–7 were used for Western blot. Western blot results indicated that several sperm proteins were targeted with the sera from both the OA patients and healthy controls. Among these proteins, four were targeted only with the patients' sera and showed reproducibility in three separate rounds of blotting with different pooled sera. To identify the targeted protein spots, a twin 2D-PAGE gels in the pH range of 4–7 was run. One gel was stained with CBB and the other was blotted with the pooled patients' sera. The stained gel was compared with the blotted paper and three out of the four targeted spots were successfully picked and identified using MALDI-TOF/TOF mass spectrometry technique. Mass results indicated that two sperm proteins, TEK2 and TPI, were specifically targeted with the patients' sera [Table 1 and Figure 2].

Reverse transcriptase polymerase chain reaction

RT-PCR results confirmed the expression of two targeted proteins at RNA level. Based on the expected product size (145 bp and 121 bp for TEK2 and TPI, respectively), the bonds are presented in Figure 3.

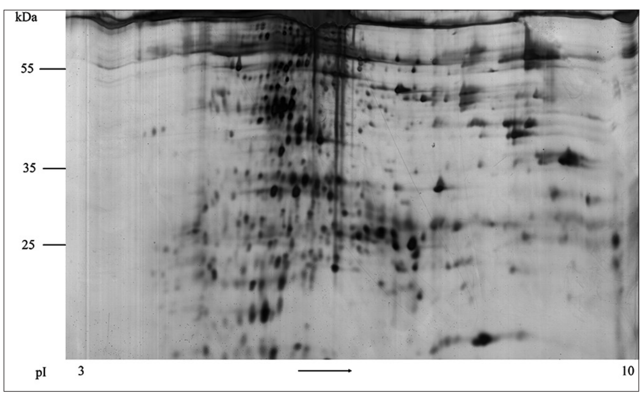

Figure 1: Representative picture of a silver nitrate-stained two-dimensional polyacrylamide gel electrophoresis (12%, pI 3–10 linear)

DISCUSSION

Sperm antigens are good targets for antibody responses and may affect the infertility rate. Interestingly, ASAs are also found in many fertile men and women.[18] The presence of ASAs in fertile couples indicated that not all ASAs cause infertility. Moreover, this fact indicates the inadequacy of our knowledge regarding the importance and role of ASAs in human infertility. Therefore, investigation of specific sperm immune targets may shed light on the roles and mechanisms of action of these antibodies in infertility. Previous published studies indicated that the chance for producing ASAs in OA was significantly higher in comparison to other forms of infertility.[4] However, the targets of these antibodies have not been addressed yet. Nevertheless, even in OA males, not all ASAs affect infertility and consequently, identification of specific sperm targets is essential. In the present study, using Western blot technique combined with mass analysis, for the 1st time, we identified two specific sperm antigens that were targeted by the immune system in the OA patients. TPI is the first targeted sperm protein reported in the current study. TPI is a 36 KDa protein that is expressed in almost all the tissues and is an important enzyme in carbohydrate glycolation that interconverts dihydroxyacetone phosphate and glyceraldehyde 3 phosphate.[19] TPI is present all over the acrosomal membranes of nonreacted spermatozoa and plays a role in the acrosome reaction and sperm binding to the zona pellucida.[20] Moreover, TPI is essential for glucose metabolism, and glucose is necessary for sperm capacitation and acrosome reaction.[21] Although as an immunodominant sperm auto-antigen, the production of antibody against TPI has been reported before.[20,22] The presence of anti-TPI antibody in the OA males was reported in the present study for the 1st time. Interestingly, our Western blot results [Figure 2] regarding the location of TPI blotted spots is in line and comparable with the

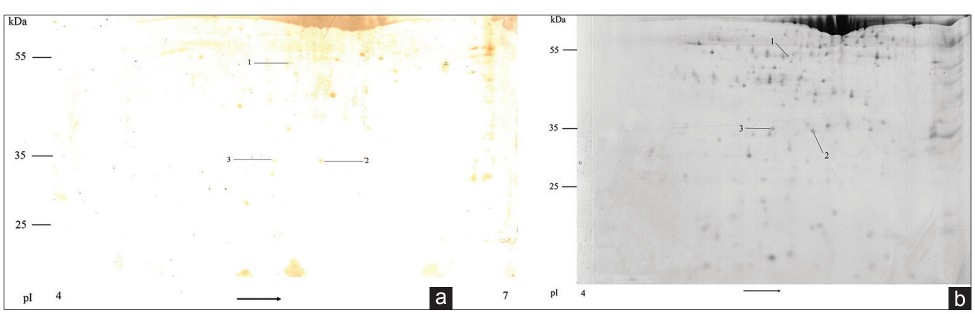

Figure 2: (a) Representative picture of polyvinylidene fluoride membranes (12%, pI 4–7 linear) blotted with pooled sera from obstructive azoospermia patients in which the location of the differentially blotted spots (1: Tektin-2, 2 and 3: Triose phosphate isomerase) is indicated. (b) Representative picture of a two-dimensional polyacrylamide gel electrophoresis stained with coomassie brilliant blue in the same pH range (12%, pI 4–7 linear) in which three corresponding proteins related to the spots in Figure 2a (Tektin-2 and triose phosphate isomerase) are labeled

Table 1: Differentially blotted protein spots with obstructive azoospermia and control sera, which were picked from coomassie blue-stained gel and identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass technique

Spot number[a]	Protein name	Mr[b]	pI[b]	Score[c]	Match peptides	Positions	Sequence coverage %	Association number
1	TEKT2	50	5.4	444	NLPLDVAIECLTLR	107-120	14	GI: 16507950
					VPDGSTTLQQWDDFSR	208-223		
					VATEFAFR	259-266		
					NTLEEIAELQEDIR	285-298		
					FVPEVDTFTR	404-413		
2	TPI	36	5.6	356	FFVGGNWK	7-14	22	GI: 4507645
					VPADTEVVCAPPTAYIDFAR	34-53		
					DCGATWVVLGHSER	86-99		
					VVLAYEPVWAIGTGK	161-175		
3	TPI	36	5.2	356	FFVGGNWK	7-14	22	GI: 4507645
					VPADTEVVCAPPTAYIDFAR	34-53		
					DCGATWVVLGHSER	86-99		
					VVLAYEPVWAIGTGK	161-175		

[a]Spot numbers are the same as the spot labels in Figure 2, [b]Experimental/mass (kDa) or PI. [c]Protein scores of >66 were considered as statistically significant (*P*<0.05). TPI=Triose phosphate isomerase, TEK2=Tektin-2, PI=pH Isoelectric

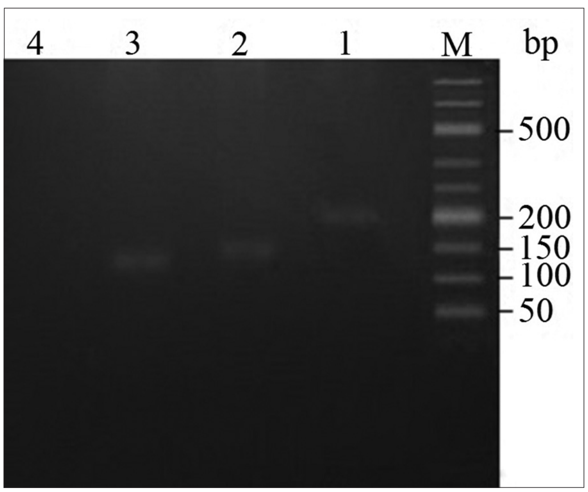

Figure 3: Representative picture of an agarose gel (3%) that indicates the electrophoresis results of reverse transcriptase polymerase chain reaction products for Tektin-2, triose phosphate isomerase, and β-actin. M, size marker, line 1, β-actin as positive control (204 bp), line 2, triose phosphate isomerase (121 bp), line 3, Tektin-2 (145 bp), and line 4 as negative control

report by Auer *et al*.[20,22] The production of auto-antibody against TPI might prevent acrosome reaction and/or sperm binding to the zona pellucida and as a result, affect fertility.[22] The second sperm immune target which was reported in this study is TEK2. TEKs1–5 are a group of cytoskeleton proteins that are present in cilia, flagella, basal bodies, and centrioles.[23] TEKs are associated with sperm motility.[24] TEK2 is a sperm-specific protein that is expressed in the sperm flagella microtubules.[25,26] It has been reported that the level of TEK2 is positively correlated with spermatozoa motility and is also associated with fertilization rate, embryo quality, and pregnancy rate.[27] In line with the role of TEK2 in sperm motility, reduced sperm motility has been reported in case of lower expression of TEK2.[27] The level of TEK2 is also downregulated in the spermatozoa of oligoasthenozoospermic men compared to normozoospermic controls.[27] Furthermore, TEK2 has been shown to play a role in the capacitation and hyperactivation of spermatozoa in hamsters.[24] There is no report regarding the production of antibody against TEK2 in infertile men. Yet the findings of the present study revealed anti-TEK2 antibody as a marker for OA. Considering the significant role of TEK2 in sperm motility and fertility rate, the production of antibody against TEK2 in OA patients might affect fertility. Thus, it may be accounted as a new marker for the diagnosis of OA. In conclusion, our study results indicated two functionally important sperm proteins as markers and antibody targets in azoospermic men. Of course, further studies are required to confirm the results of the present study and to assess the role of these antibodies in OA. Investigation of probable mechanisms of these antibodies in the induction of OA or the use of Abs and targeted antigens in the treatment of OA men is in need for future works.

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Conflicts of interest

There are no conflicts of interest.

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