Molecular analysis of testis biopsy and semen pellet as complementary methods with histopathological analysis of testis in non-obstructive azoospermia

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Abstract

Purpose Non-obstructive azoospermia (NOA) is one the many causes of male infertility (10 %) resulting from testicular failure. Multiple testicular biopsies fail to find mature sperm in at least 50 % of cases. Therefore, hunting for sensitive and specific biomarkers of spermatogenesis that could better determine the fertility status in NOA can lead to improved management of male infertility. Therefore, we evaluated sperm production through analyses of germ cell-specific transcripts (DAZ, TSPY1, SPTRX3 and SPTRX1) in semen and testicular biopsies of men with azoospermia.

Methods We collected semen ($N=83$) and testis biopsies ($N=31$) from men with non-obstructive azoospermia. We later extracted RNA and synthesized cDNA using washed semen precipitate and testicular tissues. We also performed semi-

nested PCR with designed specific primers. Using H&E method, an expert pathologist performed the histopathological evaluation. Having categorized the patients into three groups based on histopathological results, we calculated the agreement between molecular results of semen and tissues with histopathological findings for each patient using Kappa statistical test.

Results Molecular findings of precipitated semen and testicular tissues were in disagreement with histopathological results in most cases. Molecular analysis of testis biopsies showed significant difference (Kappa coefficient=0.009, P value=0.894) with histopathological results; TSPY1, DAZ, SPTRX3 and SPTRX1 were respectively detected in 94 %, 94 %, 17.6 % and 52.9 % of men diagnosed with germ cell aplasia.

Capsule The molecular analysis of testicular tissues and semen precipitates for the presence of germ cell-specific transcripts is a diagnostic test that could be of help in the detection of active spermatogenesis in men with non-obstructive azoospermia.

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Conclusions Molecular analysis of semen does not provide sufficient sensitivity and specificity to be used as a screening test at the present time, but it is a useful adjunct to histopathological methods in men with NOA. Spermatid/sperm specific transcripts indicated the possibility to find mature sperm following repeated multiple testicular sperm extraction (TESE) or microdissection TESE (mTESE).

Keywords Male infertility · Non-obstructive Azoospermia · Spermatogenesis · Germ cells · Testis · Transcript expression analysis

Introduction

Non-obstructive azoospermia (NOA) due to the failure of spermatogenesis is seen in about 10 % of infertile men and it is defined as the lack of mature sperm in semen [1, 2]. The introduction of testicular sperm extraction (TESE) in combination with intracytoplasmic sperm injection (ICSI) raised hope in men with azoospermia to conceive their own biological child [3]. In the management of men with NOA, it is crucially important to develop procedures capable of predicting the presence of mature sperm, assessing its quality and successfully recovering testicular sperm from patients with NOA. Testis biopsy and its related histopathological evaluations are already being used for the prediction of TESE outcome and provision of somewhat valid information about the presence and quality of sperm to be used for ICSI cycles [4].

As here are only a few foci of spermatogenesis in the testis of most men with NOA; therefore, finding these locations and extracting adequate sperm may require multiple testicular biopsies. However, sperm cannot be extracted in at least 50 % of the cases following multiple biopsies [5, 6]. On the other hand, invasiveness and inaccuracy of multiple blind random biopsies are accompanied with several complications including persistent pain, swelling, infection, hydrocele, hematoma, transient or even stable reduction of serum testosterone and rare cases of testicular atrophy [7].

Spermatogenesis leads to the production of haploid round spermatids through sequential mitotic and meiotic divisions across seminiferous tubules, subsequently leading to the release of mature sperm into the lumen of seminiferous tubules.

Immature germ cells (IGCs) are distributed across seminiferous tubules and spermatogenic cells and spermatocytes are located close to the basement membrane and spermatid and mature sperm adjacent to the lumen of tubules. Due to unknown reasons, all immature spermatogenic cells including spermatogonia, spermatocytes and spermatid cells shed into the lumen of seminiferous tubules and subsequently semen of fertile and infertile men except in infertile men with germ cell aplasia [8].

More than 90 % of non-sperm cells in the ejaculate of fertile or infertile men are immature germ cells. Detection of IGCs in semen could predict the status of spermatogenesis in testes [9, 10]. Therefore; differential detection of IGCs using immunochemical, cellular or molecular methods provide a valuable diagnostic test for the management of NOA.

The successful progress of spermatogenesis and release of mature sperm is related to the expression of a large number of genes in spermatogenic cells. Many of these genes are testis/germ cell-specific, which can be used for the differential evaluation of gene expression in testis. In addition, assessment of these transcripts in IGCs shed in the lumen of seminiferous tubules could indicate the status of spermatogenesis in corresponding testis and may have the potential for a non-invasive diagnostic biomarker.

We used previously identified testis/germ cells and stage-specific genes in this study. As an example, testis-specific Y-encoded protein 1 (TSPY1), which is located on Yp, is expressed absolutely in germ cells. TSPYs are expressed in spermatogonia and spermatocytes [11, 12]. Due to its expression in spermatogonia, TSPY1 can be used as a marker for presence of spermatogonial cells. Spermatocyte/spermatid-specific thioredoxin-3 (SPTRX3) is related to thioredoxin protein family and is expressed in pachytene spermatocytes and round spermatids [13]. Spermatid-specific thioredoxin-1 (SPTRX1) is another member of thioredoxin protein family and is expressed exclusively in round and elongating spermatid [14]. The Deleted in Azoospermia (DAZ) is a testis-specific gene located in AZFc region on Y chromosome. DAZ is seen to be deleted in about 10 % of men with azoospermia and severely oligozoospermia [15]. DAZ gene is expressed in all stages of spermatogenesis, from spermatogonia to mature sperm [16]. Stage-specific expression of the above genes and their expression patterns are shown in Fig. 1.

Therefore, the aim of this study was to evaluate the status of spermatogenesis in men with NOA through the analysis of above-said testis/germ cell-specific transcripts (DAZ, TSPY1, SPTRX3, and SPTRX1) in seminal fluid and testis biopsies using semi-nested RT-PCR.

Materials and methods

Semen and testicular tissues

Semen samples ($N=83$, age= 33.02 ± 6.42 years) were collected from men with non-obstructive azoospermia attending Avicenna Infertility Clinic, Tehran, Iran, during October 2010 to June 2011. We could collect testicular biopsies in 31 men ($N=31$, age= 32.06 ± 6.01 years) as 52 men were left out of study due to previous testicular biopsies and histopathological evaluations.

Fig. 1 The stage-specific expression of the four germ cell-specific genes in three categories. TSPY1 was expressed in spermatogonial cells, and SPTRX3 in both spermatocytes and spermatid cells. SPTRX1 was expressed in round and elongated spermatid cells and DAZ throughout spermatogenesis

Semen analysis was performed according to the World Health Organization criteria [17]. The diagnosis of patients was based on the absence of mature sperm in seminal fluid and also in seminal sediment (5,000 rpm, 15 min) in at least two separate semen analyses. The patients with obstructive azoospermia, leukocytospermia, and those undergoing chemotherapy or radiotherapy were excluded from the study. We obtained normal testicular tissue from a fertile patient who suffered from prostate cancer and was treated by orchiectomy. The testis sample was used as a positive control for all the experiments. Seminal fluid of a clinically-approved vasectomized man was applied as a negative control. The study was approved by Avicenna Research Institute's Ethics and Human Rights Committee. All patients signed an informed consent form permitting use of remnant of their semen and testis biopsies in the study.

Semen samples are allowed to liquefy for 30 min at 37 °C before analysis. To prepare semen with no sperm in direct wet preparation, the total semen was centrifuged at 5,000 rpm for 15 min. The pellets were washed 2–3 times using sterile phosphate buffered saline (PBS, 0.5 M) at 2,000 rpm for 5 min. The final pellets were searched for mature sperm and stored at –70 °C or immediately were mixed with Tripure reagent (Roche, Germany) following a harsh homogenization and later stored at –70 °C. Surplus testicular biopsies were wrapped in aluminum foil and immersed in liquid nitrogen (LN2) while transferring them from operation room to Avicenna Research Institute. The tissues were stored in LN2 (–198 °C) or immediately mixed with Tripure reagent for

RNA extraction. The estimated size of testicular samples was 2×2×2 mm.

RNA extraction and RT-PCR

The total RNA of semen pellets and testicular tissues were extracted using Tripure reagent (Roche, Germany) according to the manufacturer's instruction. Total RNA was quantified by means of UV absorbance at 260 nm using Picodrop spectrophotometer (Picodrop Limited, UK). Aliquots were kept frozen at –80 °C for cDNA synthesis and further analysis. RNA integrity was assessed using agarose gel (1 %) electrophoresis to determine the presence of 28S and 18S ribosomal RNA bands. Complementary DNA (cDNA) was synthesized as previously reported [18]. Briefly, DEPC-treated water was added to 1 µg of total RNA to a final volume of 11.5 µl. Then, it was incubated at 65 °C for 5 min and chilled on ice, and other reagents including 4 µl of 5× buffer, 20U of RNase inhibitor (Roche, Mannheim, Germany), 2 µl of 10 mM dNTP (Roche, Mannheim, Germany), 20 pM of random hexamer accompanied with specific primers and 200U of M-MuLV RT (Roche, Mannheim, Germany) were added to the mixture. The process of reverse transcription was carried out at 42 °C for 1 h, at 70 °C for 10 min, and finally cooled to 4 °C. The cDNA was stored at –20 °C for further assessment of testis stage-specific genes. The efficiency of RNA extraction was evaluated by analyzing the presence of housekeeping gene (HPRT) cDNA using the following designed primers:

F : CCTGGCGTCGTGATTAGTGAT, R : AGACGTTTCAGTCCTGTCCATAA

Table 1 The sequence of PCR primers used for semi-nested RT-PCR of germ cell-specific genes

Gene	Primer	Sequences	Product length (bp)
DAZ	F1 (External)	TTCTACCTCCGAGGGTTCGC	196
	F2 (Internal)	TTCCTTACACCTTAGCCTTTGG	164
	Reverse (External)	GATTTGCAGCAGACATGGTG	
TSPY1	Forward (External)	ATGACCCCAGAGTCTGCACC	
	R1 (External)	ATGAACAGGATGCTTCTCTCTC	285
	R2(Internal)	GTCAGTGATCAGGGCTGACATCTG	219
SPTRX3	F1 (External)	CCTGTTTTCATGCTATGTCTG	214
	F2(Internal)	GGAGCTGGCTGAACTTGTC	143
	Reverse (External)	CTGCAAGACACAGGTTGCTC	
SPTRX1	F1 (External)	GGAGCCACCTTGAAGCTCTC	394
	F2 (Internal)	GTGAAGGTGATGCTAATGAAAGC	132
	Reverse (External)	TGTGGAACGTGTGGCTGAC	

Primer design

The designed primers for semi-nested PCR included two external primers for primary PCR and one internal primer for secondary PCR. *SPTRX1* primers amplified a sequence of alternative transcript 2 (GenBank accession No. NM_001098529.1), but the alternative transcript 1 (GenBank accession No. NM_032243.5) could not be amplified. *DAZ* primers were designed from part of the common sequences found in transcripts of the four *DAZ* genes (GenBank accession No. NM_004081.5). Primers for amplification of *SPTRX3* and *TSPY1* were designed from sequences with accession numbers NM_001003936.2 and NM_003308.3, respectively. The sequence for PCR primers is shown in Table 1.

The specificity of primers was checked on Basic Local Alignment Search Tool (BLAST). All primers designed from two different exons spanning an intron to avoid genomic DNA amplification through RT-PCR.

Semi-nested RT-PCR

Semi-nested RT-PCR amplifications were performed in 25 μ l of reaction mixture composed of forward and reverse external and/or internal primers (10 pM) (Cinagene, Iran), 10 mM dNTPs (Roche, Germany), 1 IU Taq polymerase (Roche, Germany), 25 mM $MgCl_2$ (Roche, Germany), and 2.5 μ l 10 $\times$ PCR buffer (Roche, Germany) using 2 μ l of the prepared cDNA as a template in primary PCR. The template for secondary PCR was 1 μ l of the PCR product of the primary PCR.

The temperature at the initial cycle was 95 $^{\circ}C$ for 3 min followed by a final extension at 72 $^{\circ}C$ for 5 min. The PCR conditions for primary and secondary PCRs have been shown in Table 2. Amplification of *DAZ* gene was set up as a touch-down PCR. The positive (cDNA was synthesized from normal testicular tissues) and negative controls (water) were used in all primary and secondary PCR reactions. PCR products were run on a 2 % agarose gel for electrophoresis separation and,

subsequently, the DNA bands were visualized under ultraviolet light by ethidium bromide staining of the gel. The sequences of PCR products were confirmed using *Msp*I (Biolabs, England), *Hph*I and *Alu*I (Fermentas) restriction enzymes for 16 h at 37 $^{\circ}C$ and subsequently the evaluation of digestion products was completed using agarose gel electrophoresis.

Histopathological evaluation

Testicular biopsies of men with azoospermia were evaluated using a routine histopathological method. Paraffin-embedded thin sections of testicular biopsies were prepared and stained using hematoxylin/eosin (H&E) method [19, 20]. Depending on the size of each tissue biopsy, 20 to 200 seminiferous tubules were examined in each patient. In atrophic testes, the size of the samples and consequently the number of tubules were diminished.

We fixed testicular tissues in Bouin's fixative and embedded them in paraffin blocks. While preparing 4 μ m sections, we used one out of every 4–5 sections for H&E staining. An expert pathologist evaluated about 10–20 sections in each sample. We later divided the histopathological results into four major categories: (1) sertoli cell only-syndrome (Germ cell aplasia) containing only Sertoli cells without spermatogenic cells, (2) maturation arrest (MA), in which maturation of spermatogenic cells halts at different stages of spermatogenesis, (3) hypospermatogenesis, accompanied by decline in spermatogenesis, and (4) normal spermatogenesis (Obstructive azoospermia).

Statistical analysis

Statistical analysis was performed using SPSS v. 13.0 Software (Chicano, Illinois, USA). The results of single specific-stage gene were compared with histopathological findings using McNemar's test. Furthermore, the agreement between molecular results and histopathological outcomes

Table 2 The cycles and temperature profiles for PCR1 and PCR2 of germ cell-specific genes

Gene	Cycle profile	
	PCR1	PCR2
TSPY1	95 °C/30 s; 60.9 °C/45 s; 72 °C/30 s (27 cycles)	95 °C/30 s; 60.9 °C/20 s; 72 °C/30 s (30 cycles)
SPTRX3	95 °C/30 s; 59 °C/30 s; 72 °C/30 s (34 cycles)	95 °C/30 s; 55.4 °C/30 s; 72 °C/30 s (35 cycles)
SPTRX1	95 °C/30 s; 60.9 °C/30 s; 72 °C/1 min (34 cycles)	95 °C/30 s; 60.9 °C/45 s; 72 °C/45 s (35 cycles)
DAZ (Touchdown)	95 °C/30 s; 63.3 °C/30 s, decreased 0.5 °C per cycle; 72 °C 20 s (20 cycles) 95 °C/30 s; 56.3 °C/30 s; 72 °C/20 s (20 cycles)	95 °C/30 s; 61.7 °C/30 s, decreased 0.5 °C per cycle; 72 °C 30 s (9 cycles) 95 °C/30 s; 54.7 °C/30 s; 72 °C 20 s (30 cycles)

was analyzed by Kappa test. An alpha level of 0.05 was used to indicate statistical significance. Sensitivity and specificity of molecular results for all studied genes were determined according to relevant histopathological gold standards. We performed the statistical analyses only for samples with positive HPRT1 expression.

Results

The status of spermatogenesis in men with NOA was evaluated through detection of transcripts for germ cells and testis specific genes (DAZ, TSPY1, SPTRX3 and SPTRX1) in the seminal plasma pellets and testicular tissues. Semen samples from 83 men with NOA were used for RNA extraction, following conversion of mRNA to cDNA. Only 60 synthesized cDNA libraries contained HPRT1 transcript as a house-keeping gene. Integrity of synthesized cDNA and specific bands of genes are shown in Fig. 2.

The PCR product was confirmed using its digestion with specific restriction endonucleases and determining the fragment length for each amplified gene. The synthesized cDNA library from normal testis was used as a positive control for all reactions. The histopathological results of testis biopsies for 60 men with NOA were categorized into three groups including germ cell aplasia (70 %), maturation arrest (21 %) and hypo-spermatogenesis (9 %) (Fig. 3).

Detection of transcripts in semen and testis

Presence of DAZ, TSPY1, SPTRX3, SPTRX1, and HPRT1 gene transcripts was studied in 60 HPRT1 positive in a total number of 83 semen samples. Among 60 HPRT1 positive samples, 42 were diagnosis with germ cell aplasia, 13 samples with maturation arrest and 5 samples with hypo-spermatogenesis. TSPY1 transcript was found in 84 % and 60 % of maturation arrest and hypo-spermatogenesis groups, respectively, while DAZ transcript was found in 61.5 % and 40 % of maturation arrest and hypo-spermatogenesis groups, respectively. In men diagnosed with germ cell aplasia, DAZ, TSPY1, SPTRX1 and SPTRX3 transcripts were respectively found in 16.5 %, 12 %, 9.5 % and 2.4 % of the cases (Table 3). TSPY1 and DAZ transcripts were detected in higher numbers of samples than other gene transcripts.

Statistical analysis based on McNemar's test (P value=1) did not show significant differences between detection of DAZ, TSPY1 and SPTRX1 transcripts in semen and the histopathological diagnosis of cases with germ cell aplasia. Moreover, there was no significant agreement between cases with maturation arrest and hypo-spermatogenesis. SPTRX3 transcript was not detected in most samples with the diagnosis of maturation arrest (85 %, 11/13) and hypo-spermatogenesis (80 %, 4/5). The sensitivity of TSPY1 and SPTRX1 were 77.8 % and 57.1 % for the diagnosis of sperm maturation arrest in men with azoospermia.

Fig. 2 Semi-nested RT-PCR results of DAZ, TSPY1, SPTRX3, and SPTRX1 genes. **a** in testicular samples of patients with NOA (Products of PCR2). The reference gene, HPRT1. Lanes 1, 2 and 3: Three patients with NOA. Lane 4: Obstructive azoospermia. **b** in control testis samples (Lanes 1 to 6)

Fig. 3 Histopathological pattern from testicular samples stained by H&E method **a** Sertoli cell-only syndrome; **b** Maturation arrest; **c** Hypo-spermatogenesis

Expression of the afore-mentioned genes was evaluated in the testicular biopsies of 31 men with azoospermia, among which 55 % (17) had germ cell aplasia, 35 % (11) had maturation arrest and, finally, 10 % (3) had hypo-spermatogenesis according to histopathological findings. *DAZ*, *TSPY1*, *SPTRX1* and *SPTRX3* were expressed in all testis biopsies with the diagnosis of hypo-spermatogenesis. The four *DAZ*, *TSPY1*, *SPTRX3* and *SPTRX1* transcripts were respectively detected in 100 %, 100 %, 36.4 % and 81.8 % of testis biopsies with the diagnosis of maturation arrest. The same transcripts were detected in 94 %, 94 %, 17.6 % and 52.9 % of testicular tissues with the diagnosis of germ cell aplasia, respectively (Table 3). Using McNemar's test, the difference between results of *DAZ*, *TSPY1* and *SPTRX1* transcripts and histopathological findings for the evaluation of spermatogenesis status was significantly different (P value < 0.05).

Comparison of histopathological and molecular findings

Based on RT-PCR findings, men with azoospermia were categorized into three groups: 1) those with *DAZ* and *TSPY1* transcripts as maturation arrest up to spermatogonia stage and samples with *DAZ*, *TSPY1* and *SPTRX3* transcripts as maturation arrest up to spermatocyte stage, and all these samples were classified as group with maturation arrest; 2) Samples with detection of all transcripts categorized as hypo-spermatogenesis group; and 3) Samples with *HPRT1* expression but without the detection of other transcripts were classified as group with germ cell aplasia.

The association between molecular detection of the transcripts and histopathological diagnosis was analyzed using Kappa coefficient test. Statistical findings did not show significant agreement between molecular findings of semen samples and histopathological diagnosis of the disease in testis biopsies. Although, there was agreement in RT-PCR results with histopathological findings in men with the diagnosis of germ cell aplasia (P value < 0.001, Kappa coefficient = 0.327). Furthermore, comparison of molecular findings and histopathological findings showed no significant agreement (P value = 0.894, Kappa coefficient = 0.009). Comparison of RT-PCR findings and histopathological findings are shown in Tables 4 and 5. In men with the diagnosis of germ cell aplasia using histopathological assessment, RT-PCR analysis of semen samples showed germ cell aplasia in 32 (53.3 %), maturation arrest in 6 (10 %) and hypo-spermatogenesis in 4 (6.7 %) cases. In men with histopathological assessment of maturation arrest (21.7 %), molecular results of semen showed germ cell aplasia in 2 (3.3 %), maturation arrest in 5 (8.3 %) and hypo-spermatogenesis in 6 (10 %) of cases. In addition, molecular analysis of semen samples of 5 (8.3 %) men with azoospermia with histopathological diagnosis of hypo-spermatogenesis showed germ cell aplasia in 2 (3.3 %), maturation arrest in 1 (1.7 %) and hypo-spermatogenesis in 2 (3.3 %) cases. However, RT-PCR results of testicular biopsies in men with azoospermia and histopathological diagnosis of germ cell aplasia were identified as germ cell aplasia in 1 (3.2 %), maturation arrest in 7 (22.6 %) and hypo-spermatogenesis in 9 (29 %) cases. In 11 (35.5 %) cases with the diagnosis of maturation arrest using

Table 3 Results for gene expression of *DAZ*, *TSPY1*, *SPTRX3*, *SPTRX1*, and *HPRT1* in semen and testicular samples of men with NOA

Histopathological groups	Samples	N	Gene expression				
			<i>DAZ</i>	<i>TSPY1</i>	<i>SPTRX3</i>	<i>SPTRX1</i>	<i>HPRT1</i>
Germ cell aplasia	Semen	42	7(16.5 %)	5 (12 %)	1 (2.4 %)	4 (9.5 %)	42
	Testis	17	16 (94 %)	16 (94 %)	3(17.6 %)	9(52.9 %)	17
Maturation arrest	Semen	13	8(61.5 %)	11(84 %)	2(15.4 %)	6(45 %)	13
	Testis	11	11(100 %)	11(100 %)	4(36.4 %)	9(81.8 %)	11
Hypo-spermatogenesis	Semen	5	2(40 %)	3(60 %)	1(20 %)	2(40 %)	5
	Testis	3	3(100 %)	3(100 %)	3(100 %)	3(100 %)	3

Table 4 Comparison of molecular and histopathological results of semen samples in each group of men with azoospermia

Evaluation results			Molecular			Total
			Germ cell aplasia	Maturation arrest	Hypospermatogenesis	
Histopathology	Germ cell aplasia	Count	32	6	4	42
		% of total	53.3 %	10.0 %	6.7 %	70.0 %
	Maturation arrest	Count	2	5	6	13
		% of total	3.3 %	8.3 %	10.0 %	21.7 %
	Hypospermatogenesis	Count	2	1	2	5
		% of total	3.3 %	1.7 %	3.3 %	8.3 %
Total	Count		36	12	12	60
	% of total		60.0 %	20.0 %	20.0 %	100.0 %

The statistical test: Kappa test

histopathological assessment, molecular findings showed germ cell aplasia in none (0 %), maturation arrest in 2 (6.5 %) and hypo-spermatogenesis in 9 (29 %) cases. The molecular findings and histopathological findings of the third group diagnosed with hypo-spermatogenesis were compatible in all of them (100 %); however, this similarity was not seen in the two groups with germ cell aplasia and maturation arrest.

Considering histopathological evaluation of testicular biopsies as the Gold standard for predicting the presence of mature spermatozoa, molecular evaluation yielded 5.9 % sensitivity and 100 % specificity in doing so in men with germ cell aplasia. However, in men with hypo-spermatogenesis, molecular evaluation showed 100 % sensitivity and 35.7 % specificity and in men with maturation arrest, it showed 18.2 % sensitivity and 65 % specificity.

Discussion

More than three decades of infertility treatment corroborates that IVF/ICSI is effective, safe, and successful in treating male or female factor infertility [21]. Hence, azoospermia is the

cause of infertility in more than 10 % of men with infertility [1, 2]. At the present time, testis biopsy is a more reliable and effective procedure in assessing the presence of mature sperm in NOA testis and its quality for use in fresh or frozen TESE/ICSI cycles. Evaluation of testis biopsies are performed by histopathological examination, which is a simple, reliable and world-renowned method [22]. Though, testicular biopsies and histopathological assessments have some limitations due to the presence of focal spermatogenesis in NOA testes and the low probability of finding these foci despite multiple random biopsies of both testes. In addition, histopathological assessment is performed on a limited number of seminiferous tubules [23].

The molecular analysis of semen proposed as a non-invasive method for the determination of spermatogenesis efficiency is often compared with histopathological findings of testicular biopsies. Detection of stage-specific gene transcripts in the semen of men with NOA indicates the presence of spermatogenic cells in the semen, and thus the status of spermatogenesis in the corresponding testis. Previous studies have used flow cytometry as a non-invasive method for the detection of spermatogenic cells in the seminal fluid of men with azoospermia

Table 5 Comparison of molecular and histopathological results of testicular biopsies in each group of men with azoospermia

Evaluation results			Molecular			Total
			Germ cell aplasia	Maturation arrest	Hypospermatogenesis	
Histopathology	Germ cell aplasia	Count	1	7	9	17
		% of total	3.2 %	22.6 %	29.0 %	54.8 %
	Maturation arrest	Count	0	2	9	11
		% of total	.0 %	6.5 %	29.0 %	35.5 %
	Hypospermatogenesis	Count	0	0	3	3
		% of total	.0 %	.0 %	9.7 %	9.7 %
Total	Count		1	9	21	31
	% of total		3.2 %	29.0 %	67.7 %	100.0 %

The statistical test: Kappa test

before testis biopsy [24, 25]. Yeung et al. showed that quantification of seminal IGCs in azoospermia using flow cytometry cannot predict TESE outcome. According to their conclusion, this inability could be due to the limitations of flow cytometry in differentiating between germ cells and other artifacts in semen precipitates [26]. In our previous study, we demonstrated that concomitant detection of DAZ and PRM2 transcripts by RT-PCR in seminal fluid is able to predict the presence of spermatid/sperm in testis of NOA men [18]. More recently, Li and his colleagues performed RT-PCR on cell-free seminal mRNA (cfs-mRNA) of men with azoospermia for the detection of DDX4 (a germ cell-specific gene), SEMG1 (a seminal vesicle-specific gene) and TGM4 (a prostate-specific gene) mRNAs. They can differentiate between obstructive and non-obstructive azoospermia by detecting the presence of germ cells in seminal fluid using cfs-mRNAs as non-invasive biomarkers. However, they could not predict the exact stage of spermatogenesis arrest due to application of only one germ cell-specific gene (DDX4) [27]. In addition, Wu and his colleagues showed that seminal plasma microRNAs can be regarded as potential biomarkers for the evaluation of spermatogenesis. The miR-19b and let-7a miRNAs have shown aberrant over-expression in the seminal plasma of men with idiopathic NOA compared with fertile controls that may be an indicator of spermatogenic failure [28].

More recently, the study on seminal plasma of men with NOA identified potential biomarkers including specific proteins of post-meiotic germ cells, whose presence in seminal plasmas could be monitored by clinicians for better evaluation of spermatogenesis in men with infertility [29].

In this study, the seminal and histopathological findings were not in agreement. Moreover, the expected sensitivity and specificity of the method were not high enough to be used instead of histopathological methods, although there was agreement between RT-PCR results and histopathological findings in men with germ cell aplasia.

We hypothesized that patients with germ cell aplasia would only have HPRT1 transcripts in their semen pellets without any other germ-cell specific transcripts. However, in a few cases with the diagnosis of germ cell aplasia, we observed DAZ, TSPY1, SPTRX3 and SPTRX1 transcripts to be respectively 16.5 %, 12 %, 9.5 % and 2.4 %. Furthermore, we expected that all or most of the germ cell-specific transcripts would be detected in cases with maturation arrest and hypospermatogenesis but these transcripts were not found in some of the cases (Table 3). This discrepancy may be due to the limitations of histopathological and molecular methods in predicting the presence of active spermatogenesis in NOA testis; the limited number of seminal fluids and the very rare number of spermatogenic cells in semen could be other reasons. In addition, RT-PCR results were not compatible with histopathological findings. On the other hand, TSPY1, DAZ, SPTRX3 and SPTRX1 were, respectively, detected in 94 %,

94 %, 17.6 % and 52.9 % of men with germ cell aplasia (Table 3). This discrepancy is due to the presence of focal spermatogenesis in NOA testis detected with sensitive and specific semi-nested RT-PCR method; however, histopathological evaluation which relies on a limited number of sections of a small number of seminiferous tubules could not find these foci of spermatogenesis or mature spermatozoa there. The heterogeneity of testis and arguably inadequate processing of tissues before histopathological evaluation and small unrepresentative biopsy may be other possible causes of these discrepancies. It is important to confirm the absence of mature sperm in cases with germ cell aplasia through selection of the best diagnostic procedures. Hence, we could not confirm the absence of mature sperm by random multiple TESE and subsequent histopathological methods. Moreover, although molecular detection of spermatid/sperm specific transcripts could predict the presence of spermatid/sperm in testis but the treatment plan could not be solely based on molecular findings of testis biopsies or semen precipitates. But instead, treatment plan of couples should be based on methods with more sensitivity and more specificity.

In addition to the presence of mature sperm, the quality of the sperm is of high importance too. Following TESE/ICSI cycles, fertilization rate, embryo quality, implantation and pregnancy outcomes are all dependent on the quality of retrieved spermatozoa [30]. Therefore, cytological study of testicular biopsy is necessary for the selection of the best treatment plan and better understanding of the prognosis of TESE/ICSI cycle in testis with mature sperm. For that reasons, the proposed testis biopsies and histopathological evaluation, accompanied by RT-PCR for the analysis of testis biopsies as a complementary method, can provide reliable data on the selection of the best treatment plan for infertile men with NOA.

There seems to be the need for more studies before using molecular analysis of germ cell-specific gene in different stages of spermatogenesis on semen precipitate or testicular tissue of NOA as a discriminatory test. But molecular finding related to testicular tissue are sensitive enough to be used for decision making in cases with NOA.

In conclusion, the molecular analysis of testicular tissues and semen precipitates for the presence of germ cell-specific transcripts is a diagnostic test that could be of help in the detection of active spermatogenesis in men with NOA. Molecular analysis of immature germ cell in semen does not have enough sensitivity and specificity to be used as a screening test at present but it needs further studies to be used as a discriminatory test in the future.

Molecular findings from testicular tissue evaluation seem to be in disagreement with histopathological findings in most cases. This disagreement is more important to exhibit in cases diagnosed with germ cell aplasia or maturation arrest through histopathological analyses. Spermatid/sperm-specific transcripts

in testicular tissue indicate the possibility of finding mature sperm following repeated multiple TESE or microdissection TESE (mTESE). Therefore, molecular analysis of testis biopsies can be used as a complementary method to histopathological analysis in men with NOA.

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