Original Article

USP7 and *SET9* Expression in The Oligospermic Human Semen: A Case-Control Study

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Abstract

Background: Oligospermia is defined as a less than 15 million per milliliter sperm in each ejaculation of semen. Proper and complete spermatogenesis requires the expression of a large number of genes. As a result, stopping the expression of any of them may lead to disrupt the process of spermatogenesis. In order to understand the disorders of spermatogenesis, it is necessary to study expression of effective genes in the spermatogenesis process. Therefore, in the present study, *USP7* and *SET9* (*SETD7*) gene expression was examined in the healthy and oligospermic men.

Materials and Methods: In this case-control study, semen samples of individuals with normal sperm and oligospermia were collected from men who referred to the Roya Clinic (Qom, Iran) according to World Health Organization (WHO) parameters after obtaining consent. Then the expression of *USP7* and *SET9* genes in two groups was analyzed using quantitative polymerase chain reaction (qPCR).

Results: There was no difference forage between the healthy and oligospermic individuals (P=0.889). The data showed that, *USP7* gene expression in the patients was 3.99 times higher than the control group (P<0.001). The expression of *SET9* gene in the patient was 1.28 times less than the control group, which was not significant (P=0.231). The results indicated that *USP7* gene expression was increased in the 84% of oligospermic individuals.

Conclusion: The *USP7* gene can be considered as one of the molecular markers in the development of oligospermia.

Keywords: Apoptosis, Male Infertility, Oligospermia, Ubiquitination

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Introduction

Infertility refers to a couple's inability to conceive after at least one year of intercourse without the use of contraceptives. According to the World Health Organization (WHO), today, almost 80 million couples suffer from infertility worldwide (1). The prevalence of infertility among Iranian couples ranges from 10.3 to 24.9% of which 50% is related to male causes (2).

Male-induced infertility is a complex disorder that affects a large portion of the male population, however many of its causes are unknown. Studies have shown that different genes affect the process of spermatogenesis and incomplete spermatogenesis is one of the important factors in the male infertility.

Oligospermia is not an uncommon disorder and is seen in about 5% of infertile couples. Also, 10-20% of infertile men are diagnosed with abnormal semen analysis. In recent years, our understanding of the genetic etiology of oligospermia has been advanced. Genetic factors cause more than 20% of oligospermia (3). If a gene is expressed at a certain stage of spermatogenesis, it is possible to predict how spermatogenesis will progress through molecular methods and its adaptation to histopathological findings. So far, the role of different genes in this category has been investigated and it has also been observed that many autosomal genes may be involved in male infertility. The two genes, *USP7* and *SET9*, can play a role in the spermatogenesis process (4, 5). The *USP7* gene is located at chromosomal position 16p13.2 and has 35 exons and seven RNA transcripts are made from it and the *SET9* gene is situated on chromosome 4q31.1 and has 10 exons and five RNA transcripts are made from it.

The *SET9* and *USP7* genes interfere in the FOXO (forkhead box O) signaling pathway. FOXO is one of the pathways involved in the process of spermatogenesis through involvement in ubiquitination. Also, FOXO as a critical factor influences the PI3K/AKT pathway in the spermatogonial cells (6).

The *SET9* has numerous activities such as chromatin

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binding, cellular response to DNA degradation, histone lysine N-methyltransferase activity, binding to P53, protein binding and lysine protein N-methyltransferase. Also, this gene is effective in the epigenetic process. One of the causes of oligospermia is epigenetic disorders which their importance has recently been realized (7).

The USP7 or ubiquitin specific protease 7 is a highly common deubiquitinating enzyme (DUB) involved in the cellular process such as apoptosis and DNA damage (8). USP7 has methyltransferase property and inhibits FOXO by methylation. Previous studies have documented the specific role of two factors, FoxO1 and FoxO3a in the fertility of mice (9). Since Foxo is a vital factor in the PI3K/AKT pathway in the spermatogonial stem cells, regulatory factors of this pathway like USP7 can be considered as a candidate for oligospermia in the diagnosis and treatment process. Therefore, in this research, the expression of *SET9* and *USP7* genes in the oligospermic individuals was studied and compared with the fertile men.

Materials and Methods

Sampling

This case-control study was approved by the Research Ethics Committee of Islamic Azad Tehran Medical Science University, Tehran, Iran, (IR.IAU.PS.REC.1398.325). Also, written consent was just obtained from all volunteer participants.

Fifty infertile men with oligospermia who referred to the Roya Fertility Clinic (Qom, Iran), between October and December 2020, were invited to this case-control study.

Also, 50 fertile men with at least one child and no family history of infertility were considered as a control group.

Exclusion criteria in the case group were varicocele, high agglutination in the semen sample and unhealthy karyotype and/or Y chromosome microdeletion. Also, all participants were asked for habitats concerning alcohol consumption, smoking, taking any herbal and chemical medication or special treatment such as radiotherapy and chemotherapy.

Semen samples were collected from the participants after 3-4 days of sexual abstinence. The samples were incubated for 20 minutes at 37°C for liquefaction and evaluated following the WHO criteria (1).

RNA extraction and cDNA synthesis

Semen samples were washed with phosphate-buffered saline (P5-119, Sigma, Germany) and then the total RNA content was extracted using a GeneAll Biotechnology kit (404-304, GeneAll, South Korea). Quantity of the extracted RNA was determined using the NanoDrop spectrophotometer (NanoDrop Thermo Scientific, USA). RNA purity and concentration was indicated by measuring the absorbance ratio (260/280 and 260/230), after adjusting pH of the solution. Quality of RNA was monitored by electrophoresis on 1% agarose (116801, Merk, Germany) gel followed by ethidium bromide (111615, Merk, Germany) staining. The gel was visualized under the gel doc system at 260 nm UV wavelength (Fig.1). cDNA was synthesized by HyperScript TM RT premix with the Random Hexamer kit (501-025, GeneAll, South Korea) according to the manufacturer's protocol. A total amount of 1 µg of RNA was used for cDNA synthesis. In order to ensure cDNA synthesis, the reverse transcription polymerase chain reaction (RT-PCR) product was used as a template for amplification of *GAPDH*, *SET9* and *USP7* gene. Then the PCR product was run on 2% agarose gel (Fig.2).

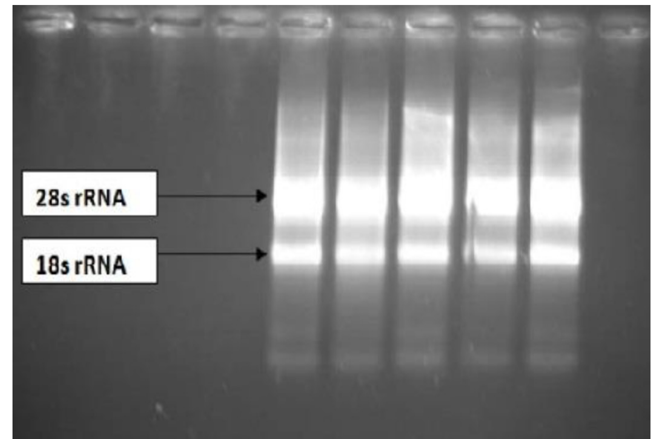

Fig.1: RNA gel electrophoresis.

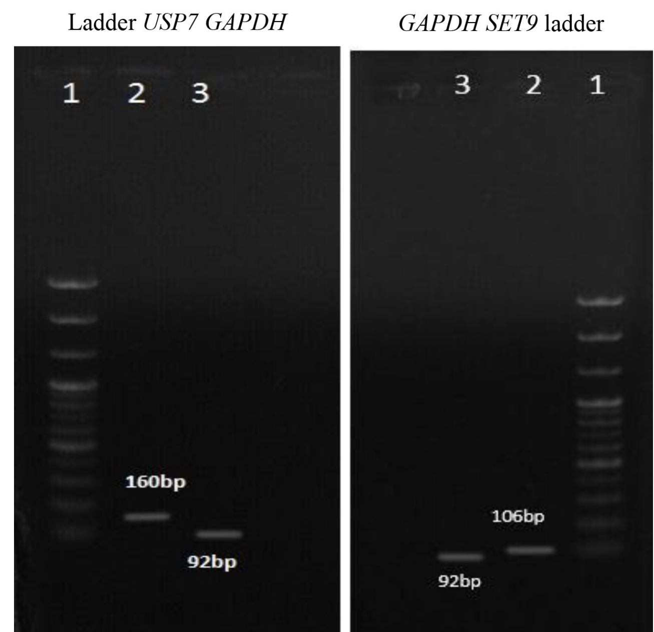

Fig.2: cDNA gel electrophoresis.

Real-time polymerase chain reaction

FASTA sequences of *USP7* and *SET9* genes and housekeeping gene, *GAPDH*, were obtained from NCBI website, and then primers were designed and blasted using Oligo 5 software (Table 1). The efficiency of all primer pairs was calculated using the standard curve method.

Table 1: Sequences of the primers

Genes	Primer sequence (5'-3')	Accession	Product length (bp)	Annealing temperature (◦C)
Usp7	F: GGGAGGAGGAGGAGGAGG R: GCTTTCTGCTGCTGCTGC	NM_003470.3	160	60
SET9	F: TACGGGCGGTCCAAGTGTC R: GGCCCGTCAGCGTTTCTCT	NM_030648.3	106	61
GAPDH	F: TGGCTACAGCAACAGGGTG R: CTCTTGTGCTCTTGCTGGG	NM_001289746.2	92	58

Each PCR contained 10 µl of real Q-plus 2x Master mix SYBR Green High ROX (A325402, , Ampilicon, Denmark), 0.6 µl of forward primer and 0.6 µl of reverse primer (5 µm concentration), 2 µl of cDNA and deionized water in a final volume of 20 µl. Amplification was carried out on a STEP ONETM Real Time PCR (4376375, Applied Biosystems, USA) with the following program: 95°C for 10 minutes and 1 cycle for primary denaturation, 40 cycles of secondary denaturation 93°C for 20 seconds, Annealing 58°C for *USP7* and 61°C for *SET 9* for 35 seconds, and extension 72°C for 20 seconds. All runs were followed by a melting curve analysis at 55-59°C for 10 seconds. Non-template controls (deionized water) and Non-RT controls (without RT enzyme) were used to assess genomic DNA contamination.

After the end of the reaction, melting curve analysis was performed in order to confirm the specificity of the reaction product and the absence of non-specific products such as primer dimer. Finally, the expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method. The *GAPDH* was used as reference gene in each sample to standardize the results. All products were analyzed at least in three technical replicates.

Statistical analysis

The independent t test was used for the mean comparison in the control and oligospermic groups using SPSS software version 22 (BMI SPSS statistics version 22, USA).

The raw data were extracted as Ct from the Real time PCR device and analyzed using REST 2009 software by independent t test. P<0.05 was considered as significant.

Results

The mean age of control and oligospermic individuals was 31.12 ± 2.14 years. and 31 ± 1.69 years, respectively. The age difference was not significant between our groups (P=0.889).

The results of the absorbance ratio confirmed purity of RNA. A ratio ≥1.8 and ≤2.2 was interpreted as a pure RNA sample. All qPCR experiments were conducted with three replications.

Table 2 shows the frequencies and values of USP7 and SET9 genes expression in all three modes (increase, decrease and non-significant) in the oligospermic group (n=50).

Table 2: Frequency of genes expression

Genes	Total cases	Frequency of up expression cases (value)	Frequency of down expression cases (value)	Frequency of non-significant cases (value)
USP7	50	84% (5.93)	-	16% (1.41)
SET9	50	20% (3.28)	38% (3.125)	42% (1.02)

Figure 3 demonstrates the mean expression levels of *USP7* and *SET9* genes in the oligospermic and control groups. The *USP7* gene expression in the oligospermic group increased 3.99 times in comparison with the control group, which was significant (P<0.001). While, the expression of *SET9* gene in the oligospermic group was 0.783 ± 0.06 which decreased 1.28 times in comparison with the control group and was not significant (P=0.231).

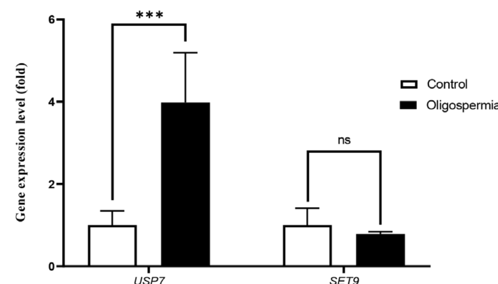

Fig.3: Expression levels of *USP7* and *SET9* genes. ***; P<0.001 and ns; No significant. All experiments were performed in triplicate.

Discussion

Our results found that the *SET9* gene expression was reduced in the oligospermic group in comparison with the control group, but this decrease was not significant. This gene is involved in the histone methylation process which is a type of epigenetic process. Epigenetic factors can play an important role in the reproductive process by affecting the developmental stages of spermatozoa. Protamine-DNA interaction leads to spiraling of sperm DNA into cyclic subunits that each subunit consists of 50,000 bp (10). Each histone protein contains a central nucleus surrounded by DNA (nucleosome) and N-terminal tails protruding outwards and subjected to post-translational changes such as methylation, acetylation, phosphorylation and ubiquitination. The combined effect of these post-translational modifications are a key to DNA regulation, such as replication, repair, and activation or inhibition of DNA (11). Given that the *SET9* gene (*SETD7*) can influence the sperm lysine methylation process. We observed lysine methylation reduction in the oligospermic group that also was associated with sperm count decrease.

In this study, it was found that *USP7* gene expression was significantly increased in our oligospermic participants in comparison with the healthy fertile participants. This gene is involved in the deubiquitination process. USP7, which is primarily located in the nucleus, regulates the stability of several proteins involved in the various cellular processes, including DNA damage response, transcription regulation, epigenetic control of gene expression, and apoptosis (12). USP7 has been documented that regulates the cell level of p53 tumor inhibitor protein in most cancers due to its role in the ubiquitination process (13). Ubiquitin (Ub) is a labeling cellular proteolytic peptide that plays a controlling role in the complex functions of human and animal cells. Protein labeling for degradation is its main function through the proteasomal system. It also controls the stability, function, and location of intracellular proteins (14, 15).

P53 and E3Ub ligase MDM2 are USP7 targets. There are reports of switch-like manner function of USP7 in the these factors regulation. Under normal condition, USP7 associates with MDM2 to protect the E3Ub ligase from auto-ubiquitination. Therefore, MDM2 ubiquinates P53 and allows it for proteasomal degradation. However, under stress signals or patient circumstances, such as oligospermia that leads to DNA damage the expression of USP7 increases and it preferentially binds p53, stabilizes it through deubiquitination process and causes induction of the apoptosis pathway (16). This can be a good reason for reduction of sperm cells in the oligospermic men.

After the spermatozoa leave the testicle, they are stored in the epididymis, where they reach their final maturity (17). These stages of maturation and stabilization, protects sperm from oxidative damage during storage and after releasing into the female reproductive tract. The Ub enzymes exist in human semen plasma as well as in damaged spermatozoa that are secreted by epididymal epithelial cells during the epididymal passage (18). Damage to the DNA structure or other structures in the testicle may trigger apoptosis pathways, and ubiquitinated these sperms. Also, improper twisting of sperm surface antigens or the loss of their structure may cause ubiquitination of damaged sperm (19).

Conclusion

Overall, our data showed that the RNA expression of *SET9* did not change significantly in the oligospermia. On the other hand, a significant increase in the *USP7* gene expression, RNA level, was observed and this increase was not related to the age. The *USP7* by involving in deubiquitination may cause a defect in the spermatogenesis process due to trigger apoptosis pathway. Therefore, the number of sperm is reduced as we observed in our oligospermic patients. Our results illustrated important effect of this gene that directed to further study on the oligospermia issue. The *USP7* can be used as a reference gene in the prognosis, detection and screening of oligospermia.

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Authors' Contributions

M.R.; Participated as supervisor, study design, and data interpretation. Z.P.; Participated as advisor, conducted molecular experiments and PCR analysis. M.F., Z.Y.; Participated in doing experiments and statistical analysis. M.R., Z.P.; Drafted and revised the manuscript. All authors read and approved the final manuscript.

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