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Evaluation of DAZ1 Gene Expression in Spermatogenic Failure among Infertile Non-Obstructive Azoospermic Iraqi Males

I. Abd Ali Al-Humairi (PhD)[*1], H. A. Karbal (MD)[1], A. A. Abid Albdairi (MD)[2]

1.Hammurabi College of Medicine, University of Babylon, Babylon, Iraq.

2.Teba IVF & Genetic Center, Babylon, Iraq.

•**Corresponding Author: I. Abd Ali Al-Humairi (PhD)**

Address: Hammurabi College of Medicine, University of Babylon, Babylon, Iraq.

Tel: +964 (780) 3678193. **E-mail:** saleh.cho@yahoo.com

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ABSTRACT

Background and Objective: Non-obstructive azoospermia (NOA) is one of the causes of male infertility. Male infertility can be induced by changes in the expression pattern of a significant number of genes in spermatogenic cells. So, analysis of gene expression profiles of testicular tissues is essential in these patients. Since DAZ1 is an essential gene in spermatogenesis, this study aimed to evaluate the role of DAZ1 gene expression in NOA Iraqi males.

Methods: This cross-sectional study enrolled a total of 50 infertile men with NOA attending Teba IVF and genetic Centre from December 2018 to December 2019. All patients had bilateral testicular sperm aspirate (TESA) under local anesthesia and biopsy. The quantitative detection and gene expression analysis of DAZ1 were carried out using the qPCR technique. The correlations between DAZ1 expression levels, testicular spermatogenic patterns, and clinical hormonal indicators were evaluated. The patients' histological phenotype and hormonal profile were also evaluated in gene expression data.

Findings: The mean age of the patients was 35.1±6.6 years. DAZ1 gene expression was different in faulty spermatogenesis testes. Hypospermatogenesis patients had the greatest expression level of DAZ1. DAZ1 transcript expression was significantly lower in the six spermatogenic samples without germ cells or Sertoli cells ($p<0.05$). Men with the worst histology (maturation arrest and no germ cell and Sertoli cell) had higher mean blood FSH levels (36.6±3.9) ($p<0.05$). DAZ1 expression in testicular samples demonstrated significant positive correlation with the spermatogenic score ($p<0.0001$).

Conclusion: The differential expression of spermatogenesis-related gene (DAZ1) across the various spermatogenic patterns suggests that this gene may be a molecular marker for NOA spermatogenesis assessment.

Keywords: *DAZ1 Protein, Histopathology, Male Infertility, Spermatogenesis, Infertility, Nonobstructive Azoospermia.*

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Introduction

Infertility is a significant health issue affecting around 10%–15% of couples worldwide. About one half of this percentage is attributed to male infertility (1). Non-obstructive azoospermia (NOA) is one of the causes of male infertility (10%) as a result of testicular failure which means absence of mature sperm in semen (2). Infertile azoospermic patients might be able to become fertile and have their own children using artificial reproductive technology (ART), including testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) (3). Testicular tissue is made up of a variety of cell types that serve as an appropriate environment for the development of germ cells in male through successive meiotic and mitotic divisions involved in spermatogenesis, eventually leading to the production of mature sperms. Male factor infertility can be induced by changes in the expression pattern of a significant number of genes in spermatogenic cells which ultimately influence the effective progression of spermatogenic process and the discharge of fully mature sperm (4). Thus, analysis of gene expression profiles of testicular tissues is very essential among patients suffering from infertility. Infertile males with azoospermia are found to have deletion of several genes that play a key role in spermatogenesis. These genes are located in a region along Y chromosome called male specific region of the Y chromosome (MSY). Of those, the Deleted in Azoospermia (DAZ) gene deletion occurs with high frequency, specifically among azoospermic infertile men. Perhaps, the location of DAZ in the azoospermia factor c region (AZFc) have made it one of the critical players in the spermatogenic process (5). DAZ gene family comprises four genes (DAZ1, DAZ2, DAZ3 and DAZ4) with high degree of sequence homology (6). They code for an RNA-binding protein specific to germ cells that may bind to many unidentified transcripts and regulate their translation process throughout gametogenesis (7). It has been reported that men who have complete deletions of AZFc have poor outcomes following assisted reproduction (8). However, deletions of DAZ family does not affect the ICSI outcomes (9, 10). Hence, this study aims to evaluate the state of spermatogenesis in males with NOA by examining DAZ1 gene expression pattern; the critical player in spermatogenesis. The significance of gene expression data to the histological phenotype and hormonal profile of the patients were also considered.

Methods

This cross-sectional study enrolled a total of 50 infertile patients with NOA attending Teba IVF and genetic center between December 2018 and December 2019. The center's specialist performed a physical examination on each patient to rule out obstructive azoospermia and assessed their hormone levels for testosterone, LH, and FSH. The research was done based on ethical code of University of Babylon (2019:3:23/129).

Testicular sperm aspiration (TESA): An effective way to evaluate spermatogenesis status in NOA patients is TESA biopsy (11). All patients had a bilateral testicular sperm aspirate (TESA) under local anesthesia, then the testicular specimen was kept in Bouin's fixative for histological evaluation. Two expert pathologists assessed the samples, and spermatogenesis scoring was conducted according to histological classification. Patients were divided into five groups based on the histological findings including Hypospermatogenesis, Round spermatid maturation arrest, Spermatocyte maturation arrest, Sertoli cell only and No germ cell & Sertoli cell.

Quantitative Polymerase Chain Reaction (qPCR): The quantitative detection and gene expression analysis of DAZ1 were carried out using the qPCR technique and standardized with the use of housekeeping gene (actin) in formalin-fixed paraffin-embedded (FFPE) tissue samples. This was performed according to the method described by Lavery et al. (12) and includes the following steps:

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Total RNA extraction: The formalin-fixed paraffin-embedded (FFPE) tissue samples were sliced into thin pieces and the wax layers were removed by xylene and washed by absolute ethanol. Using the (easy-BLUETM Total RNA Extraction Kit) and following manufacturer guidelines, total RNA was extracted.

Estimation of the total RNA extracted: Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer was used to measure the amount of RNA (ng/L) in the extracted total RNA. The quality of the RNA was examined at (260/280 nm) absorbance.

Treatment with DNase I: Using a *DNase I* enzyme kit and following the instructions provided by the Promega business (USA), the extracted RNA was processed to eliminate the small amounts of genomic DNA from the eluted total RNA.

The combination was then incubated at 37 °C for an additional 30 minutes. After that, 1µl of the stop reaction was added, and the DNase enzyme action was inactivated by incubating at 65 °C for 10 minutes.

Synthesis of cDNA: The AccuPower® RocketScriptTM RT PreMix kit was used in accordance with the manufacturer's instructions as follows to create cDNA from the DNase treated total extracted RNA samples utilizing mRNA transcripts.

The components of the RT mix were then added to the AccuPower® RocketScriptTM RT PreMix kit strip tubes, which also contain all the additional components required for the synthesis of cDNA (5x Reaction Buffer, Reverse Transcriptase, DTT, dNTP, and RNase Inhibitor). The ExiSpin vortex centrifuge was used to spin all of the strip tubes for 3 minutes at 3000 rpm, after which they were incubated in a Thermocycler (BioRad-USA) under the parameters listed in Table 1.

Table 1. Thermocycler conditions for cDNA synthesis protocol

Stage	Temperature	Duration
Synthesis of cDNA	42 °C	60 minutes
Inactivation of Heat	95 °C	5 minutes

Primers for qPCR: The NCBI-Genbank database and primer3 plus online are used to design the qPCR primers that were utilized in this work. These primers were supplied by the Korean company Macrogen as shown in Table 2.

Table 2. qPCR primers designed to measure the expression of the DAZ1 and Actin genes

Primer	Sequence (5'-3')	Product Size	Genbank
DAZ1 gene			
F	TGAGCAGTTCAAAGGCCAAG	132bp	NM_004081.6
R	TGAAAGAAGGGCCAGAAAGC		
Actin gene			
F	TCGTGCGTGACATTAAGGAG	133bp	NM_001101.5
R	TTGCCAATGGTGATGACCTG		

Creating the master mix for qPCR: The RealMODTM Green SF 2X qPCR mix Kit, which is based on SYBER green dye amplification in Real-Time PCR systems, was used to create the qPCR master mix, and it was created as follows:

Components of qPCR master mixture: qPCR master mix for housekeeping and target genes was made. Following that, the aforementioned qPCR master mix components were put in qPCR white plate strip tubes, mixed with an ExiSpin vortex, centrifuged for five minutes, and then put in a MiniOpticon Real-Time PCR system.

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Thermocycler conditions for qPCR: Thermocycler conditions for qPCR were set up in accordance with the instructions provided with the kit, and the primer annealing calculation was performed online using Optimase ProtocolWriterTM as shown in Table 3.

Table 3. Thermocycler conditions for qPCR

Step of qPCR	Temperature	Duration	No. of cycles
First-order denaturation	95 °C	5 min	1
Denaturation	95 °C	20 sec	40
Annealing\Extension	58 °C	30 sec	40
Melting	65-95°C		1

qPCR data analysis: Expression analysis for target and housekeeping genes (relative gene expression) was performed using the equations listed in (The Livak approach) by Livak et al. (13):
CT (target gene, test) – CT (HKG gene, test)= ΔCT (Test)
CT (target gene, control) – CT (HKG gene, control)= ΔCT (Control)
ΔCT (Test) – ΔCT (Control)= ΔΔCT
Relative gene expression OR Relative quantification (RQ) = 2-CT ΔΔCT.

Since we did not include normal sample and we do not need absolute values for DAZ1 gene expression, we chose the sample with the highest Ct value as a calibrator (control). The expression value of DAZ1 in the control sample is 1. Relative gene expression (RQ) of rest of the samples was calculated accordingly. Data were analyzed using SPSS V.23 and ANOVA and correlation coefficient analysis was used to compare data between variables. A p-value below 0.05 was considered as significant.

Results

Patient clinical characteristics: A total of 50 infertile men with non-obstructive azoospermia were considered in this cross-sectional study. The patient's age range was (35.1±6.6) years. Based on the histopathological examination, our 50 NOA patients were distributed as 17 spermatocytes maturation arrest (MA, 34%), 15 sertoli cell only (SCO, 30%), 8 round spermatid maturation arrest (RSMA, 16%), 6 no germ cells and no sertoli cells (NGCNSC, 12%) and 4 hypospermatogenesis (HS, 8%) according to their TESA score as shown in Figure 1.

Quantitative expression of DAZ1 gene in testicular samples: Analysis of DAZ1 gene expression profile demonstrated significant expression difference among testicular tissue samples clustered according to histological score. The highest expression pattern was observed in patients within Hypospermatogenesis group (Figure 2).

Evaluation of plasma hormones: Reproductive hormones including FSH, LH and Testosterone were measured and analyzed according to TESA spermatogenesis scoring groups. The mean LH, FSH and testosterone levels were 6.454 IU/L, 20.23 IU/L and 383.98 ng/dl, respectively. Significant variation existed between the means of spermatogenic score and hormones. We observed a marked increase in LH and FSH hormones in patients with no germ cells and Sertoli cells. Another noticeable increment of FSH is spotted in patients with maturation arrest as shown in Table 4.

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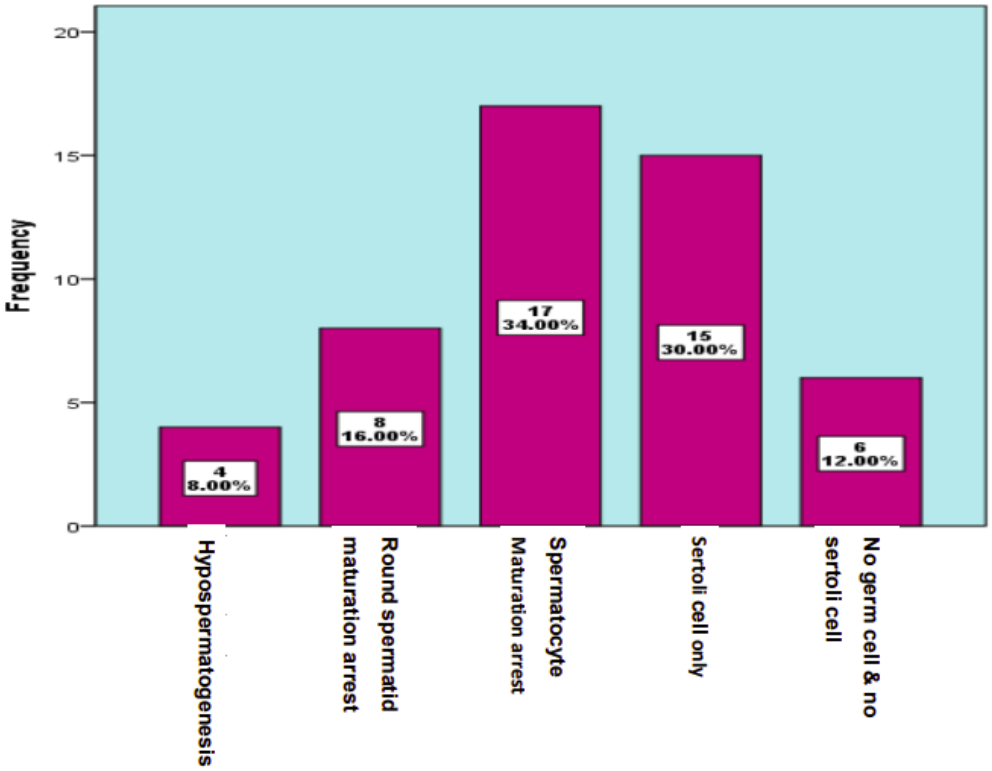

Figure 1. Distribution of patients according to histological score

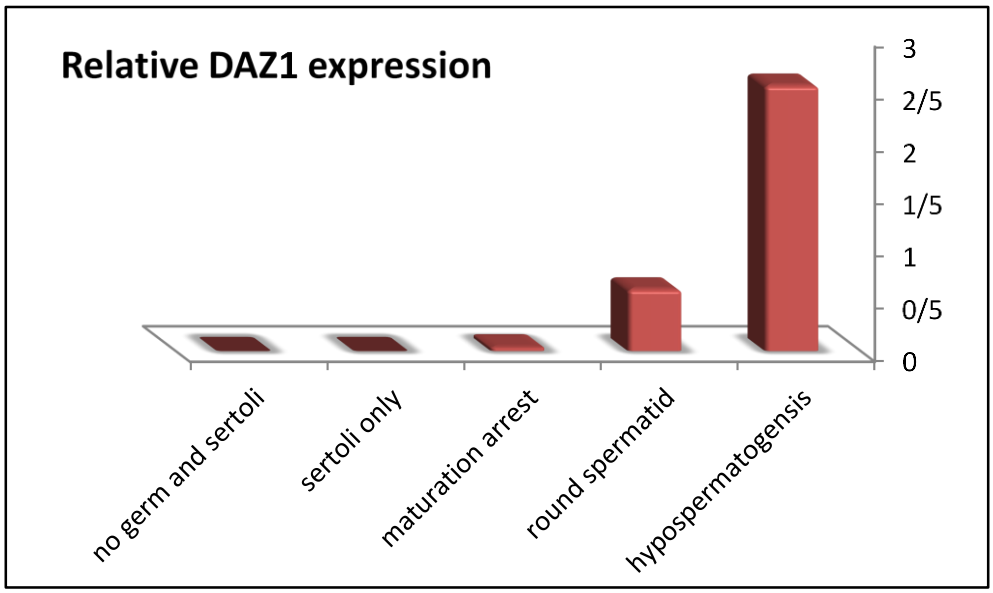

Figure 2. The relative expression of DAZ1 gene in five different histopathological types of infertile NOA patients (p=0.0001)

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Table 4. Difference between the mean spermatogenesis score according to hormones

Variables	Number	Mean±SD	p-value
Testosterone			
Hypospermatogenesis	4	471.750±12.5	0.46
Round spermatid maturation arrest	8	457.625±12.2	
Spermatocyte maturation arrest	17	351.941±11.5	
Sertoli cell only	15	386.533±13.6	
No germ cell & Sertoli cell	6	311.667±10.9	
Total	50	383.980±12.5	
LH			
Hyperspermatogenesis	4	5.100±3.5	0.04
Round spermatid maturation arrest	8	4.337±1.5	
Spermatocyte maturation arrest	17	6.853±2.1	
Sertoli cell only	15	5.787±0.8	
No germ and Sertoli cell	6	10.717±2.3	
Total	50	6.454±1.2	
FSH			
Hyperspermatogenesis	4	12.500±.85	0.007
Round spermatid maturation arrest	8	13.550±1.2	
Spermatocyte maturation arrest	17	21.229±1.5	
Sertoli cell only	15	18.160±2.8	
No germ and Sertoli cell	6	36.633±3.9	
Total	50	20.230±5.6	

Quantitative expression of DAZ1 in relation to testicular spermatogenic scoring patterns: There is a significant association between the mean spermatogenesis score according to expression; patients classified as hypospermatogenesis group were found to have the highest DAZ1 expression levels in comparison with the other spermatogenic groups as summarized in Table 5. FSH, LH and testosterone serum levels were compared, and total number of testicular fragments were analyzed; comparisons between cases gave no significant differences except for testosterone which was high (Table 6).

Table 5. Difference between the mean of score according to expression

Score	Number	Mean±SD	p-value
Hypospermatogenesis	4	2.53±0.5	0.0001
Round spermatid maturation arrest	8	0.59±0.2	0.0001
Spermatocyte maturation arrest	17	0.04±0.02	0.0001
Sertoli cell only	15	0.001±0.004	0.0001
No germ cell & Sertoli cell	6	0.0007±0.003	0.0001

Table 6. Variables comparison in Testosterone, LH and FSH

Variable	Hypersperm atogenesis	Round spermatid maturation arrest	Spermatocyte maturation arrest	Sertoli cell only	No germ and Sertoli cell	Total	p-value
Testosterone	471.750	457.625	351.941	386.533	311.667	383.980	0.46
LH	5.100	4.337	6.853	5.787	10.717	6.454	0.04
FSH	12.500	13.550	21.229	18.160	36.633	20.230	0.007

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Associations of DAZ1 expression with clinical parameters of infertile NOA patients: DAZ1 expression in testicular samples demonstrated significant positive correlation with the spermatogenic score (p=0.0001) but was negatively correlated with other factors including age, testosterone, LH and FSH ([p=0.279], [p=0.134], [p=0.828] and [p=0.603]), as summarized in Table 7.

Table 7. Correlation between expression and other variables

	Age	Testicular biopsy score	Testosterone	LH	FSH
RQ_DAZ1 expression					
R	0.156	0.669	0.215	0.031	-0.075
p-value	0.279	0.0001	0.134	0.828	0.603
Total	50	50	50	50	50

Discussion

The current research findings showed low levels of DAZ1 gene expression in NOA patients and a significant positive correlation with the spermatogenic score.

Y chromosome microdeletions were the most common hereditary cause of spermatogenesis dysfunction (14, 15). The testicular transcriptome, which includes both somatic and germ cell expression patterns of different critical genes, has been extensively researched in recent years (16, 4).

Among those, DAZ gene family was identified as a potential candidate influencing spermatogenesis (17). The majority of the pertinent research that have been published have looked at the potential impact of several Y chromosome genes on sperm maturation and reproductive status, but knowledge of DAZ1 gene expression in NOA is still limited.

The overarching aim of the present research was to study the potential impact of DAZ1 gene expression as a spermatogenic molecular marker and to assess the possibility of employing this marker for evaluating the spermatogenic levels in testicular samples of NOA infertile Iraqi patients.

To the best of our knowledge, only a few studies have used real-time PCR or other quantitative methods to measure the copy number variation of the DAZ1 genes. Nonetheless, no report has explicitly investigated this gene in infertile men in the Iraqi population.

Our real-time PCR analysis demonstrated low levels of DAZ1 expression in 92% (n=46/50) of the infertile NOA patients. Shafae et al. (18) showed that 10% of Egyptian people with idiopathic NOA have a deletion of the DAZ1 gene. Another study showed that 40% of infertile people with azoospermia had lower levels of DAZ gene expression including DAZ1 (12). This difference might be attributed to racial, geographic, patient sample selection, methodological, and ethnic characteristics.

Downregulation of DAZ1 gene was linked to the abnormal testicular histology varying among five different histopathological types of NOA infertile patients. The highest expression was detected among hypospermatogenic tissues compared to tissues with various degrees of spermatogenic maturation arrest and SCO. Interestingly, examination of the testicular histology from the six patients with no germ cells and no sertoli cell demonstrated significantly reduced expression of DAZ1 transcript.

This can be explained by the fact that DAZ1 gene is predominantly expressed in germ cells and at the same time signify the importance of this gene in spermatogenesis. Hence, a universal loss of germ cells could be the cause of a decreased DAZ1 transcript count in patient with spermatogenic failure (19). These findings are highly in agreement with the reports that has been previously documented; significantly low DAZ gene expression profile in infertile patients with various forms of spermatogenic failure (14, 17, 18,

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20, 21). Our results also showed that DAZ1 gene transcripts were also found in all Sertoli cell only specimens. There could be a few foci of germ cells in these specimens. That is why numerous accounts of males with SCO syndrome have successfully underwent TESE (22).

In addition, marked elevation of gonadotropins serum levels (mainly FSH) was noticed among infertile patients with histopathologically unfavorable testicular biopsies; namely no germ cells and Sertoli cells only groups showing statistically significant correlation with p values of 0.004. In agreement with these results, Kavoussi et al. reported significant association between high serum levels of FSH and the more severe testicular patterns, i.e. maturation arrest and Sertoli cell only patterns (23). FSH is essential for starting spermatogenesis as well as spermatozoa maturation (24). Adamczewska et al. showed increased serum levels of FSH in substantial number of their SCOS biopsies denoting that higher FSH levels are thought to be a reliable sign of damaged germinal epithelial tissue of azoospermic infertile subjects (25). The molecular findings and hormone levels in NOA men, however, do not appear to be significantly correlated in our data.

Using RT-PCR, we determined the patterns of DAZ1 gene expression in the tissues of the testicles from infertile patients suffering from NOA. Reduced transcripts may be a side effect of germ cell death in specimens with spermatogenic failure. Additionally, a decrease in sperm count and sterility may have resulted from the increase in FSH serum levels interfering with the spermatogenic process.

This study may have certain limitations. First is the sample size of infertile men with NOA. Secondly, normal men were not included in our research because of the difficulty of obtaining testicular biopsy from them. In Iraqi community, men are generally not willing to take part in fertility analysis and studies unless they have reproductive issue. One last limitation is the lack of patient follow up in terms of fertilization outcomes.

Conflict of interest: The authors declare there is no conflict of interest.

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