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Expression of Heat Shock Protein *HspA2* Gene in Some Iraqi Oligoasthenozoospermia Patients

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Abstract: Among the several heat shock protein (HSP) families implicated in the control of reproductive system development and function, the 70 kDa HSP family has emerged as essential for male fertility. The *HSPA2* is crucial for the development of germ cell differentiation. The present study was designed to compare *HSPA2* gene expression in apparently healthy subjects and oligoasthenozoospermic patient's. This study looked at about 50 Iraqi men with oligoasthenozoospermia from the Kamal Al-Samraee hospital in Baghdad. The period of study was extended from April to September 2021. For this study, 50 apparently healthy men were recruited. The *HSPA2* gene was quantified using Real-Time Polymerase Chain Reaction and the amplification was reported as a Ct value (threshold cycle) . Results revealed that the level of expression of the *HSPA2* gene was reduced in oligoasthenozoospermic patients compared to control depending on the methods Ct, ΔCt, and $2^{-\Delta Ct}$. The fold of the *HSPA2* gene was in oligoasthenozoospermic patients significantly (P<0.05) lower than that of apparently healthy subjects. In conclusion, the findings of this study indicate that the *HspA2* gene expression down-regulated significantly in the semen of Iraqi oligoasthenozoospermic patients.

Keywords: gene expression; *HSPA2*; oligoasthenozoospermia.

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Introduction

Infertility is the inability to have a child after a year or more of regular sexual activity without protection (1).

It is not only a matter of quality of life, but it is also an illness that affects both the female and male reproductive systems. That can be a risk factor for other deadly diseases over the life course, such as cancer, cardiovascular disease, and metabolic (2, 3, 4). About 70% of cases of male infertility remain unexplained despite extensive efforts by researchers to determine their causative factors (5).

The most prevalent reason for male infertility is a low sperm count, often known as oligozoospermia. A term for fertile men whose sperm counts are less than 15 million per milliliter, a sperminogram or sperm analysis can detect oligoasthenozoospermia, which is defined as a reduction in the concentration and percentage of motile spermatozoa in a sperm sample. (1).

Spermatogenesis disruption is a common cause of infertility, and genetic disorders influencing spermatogenesis might be the etiology of many cases of male infertility that go undetected. As a result of technological advancements and the introduction of new methods and approaches, it is believed that many of the causes of male infertility will be recognized and treated in the near future (5).

Heat shock proteins (HSPs) are one of the main molecular chaperone

proteins in eukaryotic cells. Their molecular weights range from 15 to 110 kDa. HSPs in mammals are generally classified into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40, and HSP27 (HSPB) families based on their molecular weight. Mammalian HSPs with molecular weights of 60, 70, 90, and 110 kD have received the most research attention (6). Heat Shock Protein Family A, Member 2(*HspA2*), a testis-specific member of the 70-kDa hsp family, It is mapped to 14q24.1 and is a molecular chaperon that contributes to the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum. However, their expression levels vary depending on cell type (7).

Heat shock proteins-70 (HSP70) were first discovered as proteins that are up regulated in response to and protective against proteotoxic stresses, which increase the fraction of proteins that are in a (partially) unfolded state, and hence increasing their possibility of losing cell function and resulting to cell denaturation and aggregation cascades that can lead to cell death (8, 9). Hsps prevent cellular auto-regulation in response to heat and the homeostatic system by maintaining a balance between protein synthesis and degradation (10).

Studies have shown that the parts of sperm that interact with oocytes are made during spermatogenesis and then change during the maturation and activation of the epididymis in the female reproductive tract and the major regulator of these processes in human spermatozoa has been identified as the molecular chaperone heat shock protein A2 (*HSPA2*) (11).

Phosphorylation of *HSPA2* can influence chromatin remodelling in sperm (12).

Gene expression is a complex multistep process including transcription, translation, and turnover of messenger RNAs and proteins. Low or high gene expression is related to many diseases, including a comparative study about genes expression between fertile and infertile male. The comparison showed that there is a difference in gene expression between patients and healthy subjects. A number of transcripts involved in a variety of cellular activities, including reproduction and development, were found to be expressed differently in fertile and infertile people (13).

There is wide interest in the study of infertility among Iraqi researchers, as many studies have appeared that dealt with the effect of gene expression on different genes and their relationship to infertility in men and women. (14-17).

Disrupted spermatogenesis, decreased sperm motility, and a lack of capacitation and fertilization are all connected to altered expression levels of heat shock-related 70 kDa protein 2 (18).

It has been shown that the relationship of *HSPA2* to male infertility retrieved and the alteration of *HspA2* expression has been involved in spermatogenic impairment (19).

Anomalies in this gene's expression have been attributed to spermatogenic and/or spermiogenic abnormalities, which may play a key role in the pathogenesis of male infertility (20, 7).

In addition to the above roles, it also been established that the *HSPA2* gene can be expressed at a relatively high level in a small proportion of somatic cells. Furthermore, it has the potential to play a variety of roles in different forms of cancer Different studies have found that it is differentially expressed in various malignancies. It has been demonstrated that *HSPA2* is down-regulated in stomach adenocarcinoma and colon cancer (21).

Materials and methods

Subjects and sampling

Semen samples were collected from men who have been diagnosed with oligoasthenozoospermia (n = 50) and men who are fertile (n = 50) as controls. RNA was extracted from sperm pellets using the TRIzol reagent. The men with Oligoasthenozoospermia were already diagnosed by a specialist physician at Kamal AL.Samraee Hospital for Fertility and Infertility. The study protocol was approved by the ethics and research committees of the hospital, and all patients gave informed consent to the study. Both groups' age distribution ranged from 22 to 35 years.

Seminal fluid analysis

A complete semen analysis measures the quantity and quality of the fluid produced during ejaculation. Semen samples contain spermatozoa, which are then examined under a microscope for sperm count, motility, morphology, and the presence or absence of aggregation and white blood cells, pH, volume, viscosity, and liquefaction are also examined according to (WHO, 2010).

Specimen collection

For RNA extraction, 3 ml of freshly ejaculated samples of human semen was obtained by masturbation and collected directly into sterilized specific containers. The pellet was suspended 0.75 ml of TRIzol® LS Reagent for preservation. The ratio (3 TRIzol: 1 seminal pellet). The sample were immediately stored at -20Cº before being processed.

Analysis of gene

Total RNA was extracted from semen samples using the TRIzol ® LS Reagent,

Following the manufacturer's protocol (22). The Nano instrument (Avans/ Taiwan) was used to measure the purity and RNA concentration of the samples by reading its absorbance at two different wavelengths (260 and 280nm). A260/A280 ratios of around 2.0 were indicative of the purity of the RNA sample. The extracted RNA was used as a template to synthesize complementary DNA using (High-Capacity cDNA Reverse Transcription Kit).

Quantitative (qRT–PCR)

For evaluate the *HSPA2* gene's expression levels, has been used a quantitative real-time qRT-PCR SYBR Green technique, a fluorescent dye that identifies any double-stranded DNA, and the amplification was reported as a Ct value (threshold cycle) .

Primer sequences for target gene and control housekeeping gene are shown in Table (1).

Table (1): Sequence of primers used for quantify *HspA2* gene expression

Primers	Sequence (5′ →3′ direction)	Product size (bp)	Reference
HSPA2 gene expression		344	Li and Sarkar, (23)
Forward	TTGTTGGAAGTCTTTGGTATA		
Reverse	CATTTGCATTTATGCATTTGT		
ACTB (reference gene)			
Forward	CGTGACATTAAGGAGAAGCTGTGC	375	Hamel et al., (24)
Reverse	CTCAGGAGGAGCAATGATCTTGAT		

Utilizing the Qiagen Rotorgen Q with qPCR software, QRT-PCR was carried out. The components of the PerfectStartTM Green qPCR SuperMix Kits were used to measure the threshold cycle (Ct), which allowed the levels of gene expression and fold changes to be measured. Components and volumes for

gene expression are shown in Table (2) and Table (3) shows the running program for the thermocycler.

Table (2): Reaction components and volumes for gene expression

Component	Volume / µl
***2× PerfectStart*™ Green qPCR SuperMix**	10
Forward primer	1
Reverse primer	1
Template cDNA	3
Nuclease free water (N.F.W)	5
Total volume	20

Table (3): Stages and temperature of qRT PCR

Cycle step	Temp(°C)	Time	Cycle
Initial Denaturation	94ºC	30 sec	1
Denaturation	94ºC	5 sec	40
Annealing	57ºC	15 sec	
Extension	72ºC	20 sec	
Melting	55-95ºC	1 min	1

Statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. T-test was used to significant compare between means.

Results and discussion

Semen analysis

Semen analysis is a first-line diagnostic method for identifying genetic or environmental variables that contribute to male infertility. Quantitative or qualitative abnormalities in spermatogenesis, such as those influencing sperm concentration, motility, or shape, can cause male infertility (25).

Table (4) displays the semen characteristics of apparently healthy men and patients with oligoasthenozoospermia.

When compared to oligoasthenozoospermic patients, the volume of semen was significantly (p<0.01) higher in men who were fertile (2.29 *versus* 1.84 ml, respectively).

There were no significant changes in semen pH between apparently healthy men and oligoasthenozoospermic patients; both groups' pH levels were within the normal level. The sperm count in controls was statistically (p<0.01) higher than in the oligoasthenozoospermia patients group (40.86 *versus* 4.72 million per ml, respectively)., the percentage of normal sperm was statistically significant higher (p<0.01) in controls than in the patients with oligoasthenozoospermia (55.70% *versus* 24.80%, respectively). Whereas, the percentage of abnormal sperm was significantly lower (p<0.01) in the apparently healthy men than in the patients with oligoasthenozoospermia (44.30 % *versus* 75.20% respectively). The percentages of sperm motility Grade B (progressive movement) in the apparently healthy subjects' group were significantly (p<0.01) higher than in the oligoasthenozoospermic patients (20.80% versus 5.00%, respectively) .

Furthermore, the percentage of sperm motility Grade C (non-progressive movement) in apparently healthy men was significantly (p<0.01) higher than in Oligoasthenozoospermia. The percentages of immotile sperms Grade D in the apparently healthy men were significantly (p<0.01) lower than in the Oligoasthenozoospermic patients (38.60% *versus* 78.30%, respectively).

Table (4): Semen Parameters in oligoasthenozoospermia patients versus apparently healthy controls. (Mean ± SE)

Semen parameters	Controls	Patients	T-test	p- value
Volume (ml)	2.29 ±0.07	1.84 ±0.11	0.269 **	0.0013
pH	7.60 ±0.01	7.63 ±0.02	0.039 NS	0.129
Sperm concentration ($x10^6$)	40.86 ±0.77	4.72 ±0.57	1.886 **	0.0001
Morphologically normal sperm (%)	55.70 ±1.82	24.80 ±1.62	4.844 **	0.0001
Abnormal sperm (%)	44.30 ±1.82	75.20 ±1.62	4.844 **	0.0001
Grad A (rapid linear progressive) (%)				
Grad B (progressive movement) (%)	20.80 ±1.02	5.00 ±0.86	2.649 **	0.0001
Grad C (non- progressive movement) (%)	28.70 ±0.83	16.70 ±1.32	3.098 **	0.0001
Grad D immotile (%)	38.60 ±1.59	78.30 ±1.85	4.849 **	0.0001

NS: No significant; ** means significant at 0.01 level.

Quantitative expression study of *HSPA2* gene by quantitative reverse transcriptase real-time PCR

In terms of gene expression, high Ct values indicate low gene expression and low Ct values indicate high gene expression. In this study the gene expression was normalized to the level of a housekeeping gene (*β-actin*) and quantified by the ΔCt value and folding (2-ΔΔct) method.

Real-time PCR quantification of β-Actin expression

The expression of β-actin gene (housekeeping gene) in apparently healthy subjects and oligoasthenozoospermic patients are presented in Table (5).

Table 5 displays housekeeping gene Ct value in this study. There was no significant difference between the mean Ct values for apparently healthy people and oligoasthenozoospermic patients, which were a mean±SE (22.91 ±1.68) of healthy control and mean±SE (22.94 ±1.77) of oligoasthenozoospermia patients.

Table (5): CT value for housekeeping gene β-Actin

Group	No.	Ct value ± SE
Control	50	22.91 ±1.68
Patients	50	22.94 ±1.77
T-test	--	3.056 NS
P-value	--	0.916

NS: Non-Significant.

As observed in Table (6), the fold of actin gene expression and 2-Ct values for a patient with oligoasthenozoospermia and an apparently healthy person were similar. The values of 2-Ct were (1.24E-07) and (1.26E-07) for oligoasthenozoospermic patients and apparently healthy subjects, respectively. The fold of *β-actin* gene expression was in oligoasthenozoospermic patients and healthy fertile groups (0.98 and 1.00, respectively). However, this result was not statistically significant. The fact that there were only small differences in results between the two groups is indicative of the quality of the PCR technology utilised and *β-actin* works as an appropriate control gene.

Table (6): Comparison of β-Actin fold expression between study groups

Group	Means Ct of β-Actin	2-ct	experimental group/ Control group	The fold of gene expression
Group 1 Patients	22.94	1.24E-07	1.24/1.26	0.98 ±0.07
Group 2 Healthy	22.91	1.26E-07	1.26/1.26	1.00±0.00
T-test	--	--	--	0.337 NS
(P-value)	--	--	--	0.902

NS: Non-Significant.

Real time PCR quantification of *HSPA2* gene expression

The values of Ct, ΔCt, and $2^{-\Delta Ct}$ of *HSPA2* gene for oligoasthenozoospermic patients and apparently healthy subjects are presented in Table (7).

The Ct values of *HSPA2* gene were 28.23, 23.34 in oligoasthenozoospermic patients and healthy fertile groups respectively. As it is explained previously, the higher Ct value indicates a lower quantity of RNA at the start of the process. The ΔCt values of *HSPA2* gene were 5.29, 0.34 in oligoasthenozoospermic patients and healthy fertile groups respectively. As showed in the Table (6) the fold of gene expression was significantly (P<0.05) lower in oligoasthenozoospermic patients compared to control.

Table (7): Fold of *HSPA2* gene expression depending on $2^{-\Delta Ct}$ Method

Group	Means CT of *HSPA2* gene	Means Ct of reference gene	Δct (Means CT of *HSPA2* gene Means Ct of reference gene	$2^{-\Delta Ct}$	Experiment group/control group	Fold of gene expression
Group 1 Patients	28.23	22.94	5.29	0.025	0.025/0.742	0.03 ±0.01
Group 2 Healthy	23.34	22.91	0.43	0.742	0.742/0.742	1.00 ±0.00
T-test	--					0.607 *
(P-value)	--					0.0441

*** (P≤0.05)**

In accordance with the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ values, the expression level of the *HSPA2* gene is provided in Table (8).

Depending on the $2^{-\Delta\Delta Ct}$ method, the values of ΔΔCt was in oligoasthenozoospermic patients and apparently healthy subjects (1.24*versus* -3.62, respectively). Therefore, depending on the $2^{-\Delta\Delta Ct}$ method, the fold of *HSPA2* gene was in oligoasthenozoospermic patients significantly (P<0.05) lowers than that of apparently healthy subjects (0.03 *versus* 1.00, respectively).

It was found that the use of the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ values both show the same result, which is a decrease in the fold of *HSPA2* gene expression in oligoasthenozoospermic patients.

Table (8): Fold of *HSPA2* gene expression depending on $2^{-\Delta\Delta Ct}$ Method

Group	Means Ct of *HSPA2*	Means Ct of β-Actin	ΔCt (Means Ct of *HSPA2* -Means Ct of β-Actin	ΔCt Calibrator	ΔΔCt	$2^{-\Delta\Delta Ct}$	experimental group/ Control group	The fold of gene expression
Group 1 Patients	28.23	22.94	5.29	4.05	1.24	0.423	0.423/12.29	0.03 ±0.01
Group 2 Healthy	23.34	22.91	0.43	4.05	-3.62	12.29	12.29/12.29	1.00 ±0.00
T-test	--	--	--	--	--	--	--	0.607 *
(P-value)	--	--	--	--	--	--	--	0.0441

*** (P≤0.05)**

The findings of this study was consistent with those of previous studies that shown lower gene expression in oligozoospermic patients (26, 27). In addition it has been found *HSPA2* gene expression down-regulated significantly in the semen of oligoteratozoospermic patients(28).

Accumulating evidence indicates that the expression of the *HSPA2* gene is down-regulated in human testes with defective spermatogenesis, and contributes to male infertility (29, 30).

study conducted by (31). Reported that the expression of *HSPA2* differed between fertile and infertile men by the use of flow cytometry, inflorescence microscopy, Western blotting, and reverse transcription polymerase chain reaction on two levels in human sperm mRNA and Protein. The results indicated that *HSPA2* gene expression was significantly correlated with sperm concentration and morphology.

Moreover, (32) Found all cases with a distinct failure in sperm's capacity to interact with zona pellucida (ZP) were have a significant deficiency of *HSPA2*. Demonstrated the importance of *HSPA2* in the assembly and expression of sperm surface receptors that mediate sperm-oocyte interactions.

Also a previous study (33) showed the percentages of *HSPA2* gene expression were lower in infertile individuals compared with healthy individuals, by direct evaluation of *HSPA2* and by using flow cytometry (34) found Deficiency of *HSPA2* in the seminal plasma of azoospermic males as a biomarker of spermatogenesis state.

In a recent study (35) reported an association between down regulation of *HSPA2* protein and reduced semen quality in patients with testicular cancer seminoma. This indicates the altered expression levels of *HSPA2* protein associated with decreased production of normal spermatozoa during spermatogenesis and is consistent with the observed reduction in sperm concentration and total sperm count in seminoma group.

Conclusion

In the *HSPA2* gene, the level of expression of the *HSPA2* gene was reduced in oligoasthenozoospermic patients compared to apparently healthy men.

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