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Gene expression and single nucleotide polymorphism of *interleukin-6* in asthenozoospermic men: A case-control study in Iraqi patients

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Abstract

The study was conducted to determine the gene expression and Single Nucleotide Polymorphism (rs 1800795 C/G) of *Interleukin 6* (*IL-6)* gene in semen samples of asthenozoospermic Iraqi men**.** In the present study, following the serum analysis, 125 patients with asthenozoospermia and 40 healthy fertile controls were enrolled .The samples were collected from Biotechnology Research Center / Al-Nahrain university and Kamal Al-Samarie IVF Hospital ,Baghdad, Iraq. through the period from November 2016 to December 2017. The determinations of the SNP (rs1800795) and gene expression were carried out by reverse transcription quantitative polymerase chain reaction(RT-qPCR) of semen samples. The difference in the mean of threshold cycle (ΔCt) of *IL-6* mRNA showed a non significant difference ($p>0.05$) in asthenozoospermic patients groups compared to corresponding means in fertile control group. Assessing the $2^{-\Delta\Delta Ct}$ means in asthenozoospermic patients distributed to subgroups, revealed important finding, that the asthenozoospermic patients with immunological factor(IAS) showed increased expression of folding in *IL6* mRNA comparing to other asthenozoospermic subgroups, but the difference was statistically not significant. Inspecting *IL-6* gene genotypes and allele frequencies in asthenozoospermic and oligoasthenozoospermic patients groups with control group, revealed that there was significant variation in the heterozygous (CG) genotype frequency in (rs1800975).It is concluded that the gene expression of *IL-6* was up-regulated in asthenozoospermic Iraqi patients, Moreover, the SNPs of this gene may have a role in asthenozoospermic patients complaining from idiopathic infertility.

Keywords: *Interleukin-6* gene, Single nucleotide polymorphisms (SNPs), Asthenozoospermic Iraqi patients

1. INTRODUCTION

Development of male infertility is influenced by many background diseases and/or risk factors . infertility affects 13-18% of couples and male factor accounts for up to half of all the cases(1). A substantial increase in the infertility risk is noted mainly in male population, especially when exposed to environmental factors, such as diet and general lifestyle. However, it is important to recognize also genetic variants associated with common complex diseases as male infertility which can be only “one piece of the puzzle” making up an overall risk (2).

Immunological factors are vitally important for the observed sperm motility reduction. Cytokines have been important mediators of the immunity and can be involved in numerous processes in the male genital tract including acting as immunomodulatory elements within the male gonad(3,4)

The relationship between cytokines and human reproduction represents a growing area of investigation because of their involvement in different aspects of reproductive physiology and fertility regulation, including gonadal and sperm functions. A lot of evidence suggests that somatic testicular cells (Sertoli, Leydig and peritubular cells) in physiological conditions produce vast amounts of cytokines, such as *interleukin 1 (IL-1*) and *Interleukin 6 (IL-6),* which take part in spermatogenesis and semen maturation . In consequence, they must also appear as natural components of seminal plasma(5).

Recent reports have described the influence in of seminal cytokines, in particular interleukins(*IL-6)* on the semen parameters especially, sperm motion, which leading to asthenozoospermia condition(6).

IL-6 gene encodes the classic proinflammatory cytokine IL-6 , The human gene for *(IL-6*) was cloned and reported by Hirano (7). It is mapped to 7p15-p21 chromosome and consists of five exons and four introns, the molecular weight of this gene is 23,718 Dalton .

On the other hand ,the biggest difference between SNPs and mutations is that SNPs are inherited. Some of these genetic differences have proven to be very important to human health. If those inherited SNPs are high-risk candidates, they deserve our attention, and their investigation will lead to further mechanistic research to develop new treatment programs(8)

The genetic variation of cytokines can lead to the diversity of immune and inflammatory response. Polymorphisms in the promoter region of IL-6 gene may also results in variation of transcription and expression of the cytokine.(9,10). At our knowledge there are very cease studies concern on the correlation between *IL-6* gene and male infertility. therefore, this study was designed to fount out the role of IL-6 rs 1800975 SNP and gene expression on asthenozoospermic infertile Iraqi men.

2. PATIENTS,MATERIALS AND METHODS

2.1. Patients

The study was designed to be prospective study. The sample size was 165 fertile and infertile men during their visit to Biotechnology Research Center/Al-Nahrain University and Kamal Al- Samaraie IVF Hospital, Baghdad-Iraq, with average ages between(20-46)years old.

The selected 125 men were intentionally divided, according to the type of infertility factor that investigated in the semen analysis, into 48 infertile men with asthenozoospermia(AS) , 32 infertile men with oligoasthenozoospermia(OAS), and 25 men with immunological asthenozoospermia(IAS) .In addition to the patients,40 healthy fertile men(normozoospermia) were also enrolled in this study.

2.2. *IL-6* gene SNPs

The probe for *IL-6* gene was assessed for the SNP in the promoter region, which included rs1800795 (*IL-6* C/G) .
TaqMan fluorescent oligonucleotide probes and primers sequences were designed according to their reference sequence (rs) in the database of NCBI(National Center for Biotechnology Information). the primers and probes and synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). The sequences of each of the probes and primers used in the allelic discrimination experiments are shown in table(1) .

Table (1): Primers and probes used in the study

IL-6(Primer for SNP Genotyping)	
Forward	**ATGACGACCTAAGCTGCACT**
Reverse	**TGAGCCTCAGACATCTCCAG**
VIC- probe	**CCATGCTAAAGGACGT**
FAM- probe	**CGATGCTAAAGGACGTC**
IL-6(Primer for Gene Expression)	
Forward	**CCCTGACCCAACCACAAATG**
Reverse	**CTACATTTGCCGAAGAGCCC**
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase	
Forward	**GAAATCCCATCACCATCTTCCAGG**
Reverse	**GAGCCCCAGCCTTCTCCATG**

The Genomic DNA was extracted from sperm using a DNA extraction Kit(BioTake Corporation, Beijing, China) according to the manufacturer instructions , then the samples were subjected to qRT- PCR .The reaction mix was adjusted to a final volume of 10 µl as suggested by the manufacturer, and included: 5µl GoTaq Prob qPCR Master Mix (Promega/USA), 0.5µl of each primer (10 mM), 0.5µl of each prob, 1 µl DNA, and 2µL nuclease-free water. The mix was transferred to a realtime thermocycler (MIC-4 Real-time PCR System, Australia), which was programmed for the following optimized cycles: initial denaturation for 5 min at 95 _C° (one cycle), 40 cycles of denaturation (20 sec at 95 _C°), annealing (30 sec at 60 _C°) and extension (20 sec at 72 _C°), and finally one cycle of melt curve at 65–90 _C°.

2.3. Gene expression of *IL-6*

The expression of *IL-6* gene was determined by the reverse transcription quantitative polymerase chain reaction (RT-qPCR) method after isolation of total RNA. A ready-to-use reagent (TRIzolTM LS Reagent; Thermo Fisher Scientific; USA) was used to isolate total RNA, and the instructions of manufacture were followed.
The isolated RNA was reversely transcribed to cDNA using WizScript™ RT FDmix Kit. The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl).
The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was carried out using the GoTaq Green Master Mix System Kit (Promega ,USA) and cDNA as a template. forward and reverse oligonucleotide primers of IL-6 gene were designed and showed in table(2-1). The forward and reverse primers of the housekeeping gene GAPDH (reference gene: glyceraldehyde-3-phosphate dehydrogenase) were also given(11) .
The reaction mix was adjusted to a final volume of 10 µl as suggested by the manufacturer, and included: 5µl GoTaq_ qPCR Master Mix (1X), for 2-Step RT-qPCR , 0.5µl of each primer (10 mM), 2 µl cDNA, and 2µL nuclease-free water. The mix was transferred to a realtime thermocycler (MIC-4 Real-time PCR System,Australia), which was programmed for the following optimized cycles: initial denaturation for 5 min at 95 _C° (one cycle), 40 cycles of denaturation (30 ec at 95 _C°), annealing (30 sec at 60 _C°) and extension (30 sec at 72 _C°), and finally one cycle of melt curve at 65–90 _C°.
The expression was given as $2^{-\Delta\Delta Ct}$, which represents the relative fold change. Therefore, the results were expressed as a fold change in the expression level of a target gene that was normalized to endogenous control (housekeeping gene) and relative to a calibrator, which is the target gene in control subjects(12)

Statistical analysis

Data analysis was done by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data were appeared as mean ± standard deviation. Shapiro–Wilk normality test was used to determine whether the studied parameters followed a gaussian distribution.
Variables in which the distribution of data did not conform to normality were first log transformed for analysis and then converted back to standard units for presentation.Categorical variables were analyzed by Chi-square test.Tukey's, Dunnett, and Bonfferoni Post Hoc test for multiple comparison were applied after ANOVA tests.
Hardy-Weinberg equilibrium calculated using a web tool (13). The difference in frequencies of genotypes and alleles between the patient groups and control group were analyzed using the Chi-square test. Odds ratios (ORs) with a 95% confidence interval (CI) were calculated for measuring the strength of the association between the studied genes and male infertility. The association degrees between variables were analyzed by Pearson correlation analysis. A two-tailed p-value less than 0.05 ($p<0.05$) was considered significant (14).

3. RESULTS

3.1. *IL-6* gene SNPs

The SNP of IL-6 gene (C/G rs1800795; located on Chromosome 7:22727026 bp) was presented with three genotypes (CC, CG, GG) and two alleles (C and G).
Analysis of Hardy-Weinberg equilibrium (HWE) in (C) group and (AS) group revealed that the genotypes was consistent with the equilibrium, and significant differences ($p <0.01$) were observed between the observed

and expected genotype frequencies in control group, while the difference was not significant (p>0.05) in patients group (Table 2 A).

Inspecting IL-6 gene genotypes and allele Frequencies in (AS) group and (C) group revealed that there were significant variation between these frequencies, Although a decreased frequencies of C allele (85 vs. 75%) and an increased frequencies of G allele (15 vs. 25 %) were observed in controls compared to patients (Table 2 B).

In CG Polymorphism, the odds ratio for the CG genotype was 0.29(0.095-0.91) with p=0.028 indicating that heterozygous genotype CG was a higher risk of (AS) group than the wild type CC and mutant type GG .

Analysis of Hardy-Weinberg equilibrium (HWE) in (C) group and (OAS) group revealed that the genotypes was consistent with the equilibrium, and there was significant differences (p <0.01) were observed between the observed and expected genotype frequencies in control group, while the difference was not significant(P>0.05) in patients group (Table3 A).

Inspecting IL-6 gene genotypes and allele Frequencies in (OAS) group and (C) group revealed that there were significant Variation in the heterozygous genotype frequency, Although a decreased frequencies of C allele (85 vs. 77.1 %) and an increased frequencies of G allele (15 vs. 22.9 %) were observed in controls compared to patients (Table 3B).

In CG Polymorphism, the odd ratio for the CG genotype was 0.35 (0.12-1.01) with p=0.048 indicating that heterozygous genotype CG was a higher risk of (OAS) group than the wild type CC and mutant type GG.

Table 2A. Number and percentage frequencies of IL-6 gene genotypes and their Hardy-Weinberg equilibrium (HWE) in (C) group and (AS) group.

Genotype	(C) group (n=40)		*P* value	(AS) group (n=32)		*P* value
	Observed n (%)	Expected n (%)		Observed n (%)	Expected n (%)	
CC	31 (77.5)	28.9 (72.25)	*P* < 0.01	18 (56.25)	18 (56.25)	*P* > 0.05
CG	6 (15)	10.2 (25.5)		12 (37.5)	12 (37.5)	
GG	3 (7.5)	0.9 (2.25)		2 (6.25)	2 (6.25)	

1 degree of freedom (d.f.) for Chi-squared distribution.

Table 2B. Genotype and allele frequencies of IL-6 gene in (C) group and (AS) group.

Genotype / Allele	(C) group (n=40)	(AS) group (n=32)	OR	95% CI	*P* value
	no. (%)	no. (%)			
CC	31 (77.5)	18 (56.25)	2.68	(0.97-7.42)	0.05
CG	6 (15)	12 (37.5)	0.29	(0.095-0.91)	0.028
GG	3 (7.5)	2 (6.25)	1.22	(0.19-7.76)	0.84
C	68 (85)	48 (75)	1.88	(0.82-4.35)	0.132
G	12 (15)	16 (25)	0.52	(0.23-1.22)	0.132

OR, odd ratio; CI, confidence interval;

Table 3 A. Number and percentage frequencies of IL-6 gene genotypes and their Hardy-Weinberg equilibrium (HWE) in (C) group and (OAS) group.

Genotype	(C) group (n=40)		*P* value	(OAS) group (n=48)		*P* value
	Observed n (%)	Expected n (%)		Observed n (%)	Expected n (%)	
CC	31 (77.5)	28.9 (72.25)	*P*<0.01	29 (60.4)	28.52 (59.42)	*P* > 0.05
CG	6 (15)	10.2 (25.5)		16 (33.3)	16.96 (35.33)	
GG	3 (7.5)	0.9 (2.25)		3 (6.3)	2.52 (5.25)	

1 degree of freedom (d.f.) for Chi-squared distribution.

Table 3 B. Genotype and allele frequencies of IL-6 gene in (C) group and (OAS) group.

Genotype / Allele	(C) group (n=40)	(OAS) group (n=48)	OR	95% CI	*P* value
	no. (%)	no. (%)			
CC	31 (77.5)	29 (60.4)	2.26	(0.88-5.78)	0.087
CG	6 (15)	16 (33.3)	0.35	(0.12-1.01)	0.048
GG	3 (7.5)	3 (6.3)	1.22	(0.23-6.39)	0.817
C	68 (85)	74 (77.1)	1.69	(0.77-3.66)	0.19
G	12 (15)	22 (22.9)	0.59	(0.27-1.29)	0.19

OR, odd ratio; CI, confidence interval.

Analysis of Hardy-Weinberg equilibrium (HWE) in (C) group and (ASI) group revealed that the genotypes was consistent with the equilibrium, there was significant differences ($p < 0.001$) and ($P<0.05$) respectively, were observed between the observed and expected genotype frequencies in control and patients groups (Table 4A).

Inspecting IL-6 gene genotypes and allele Frequencies in (ASI) group and (C) group revealed that there were no significant variation between these frequencies , Although a decreased frequencies of C allele (85 vs. 78%) and an increased frequencies of G allele (15 vs. 22 %) were observed in controls compared to patients (Table 4B).

3.2. IL-6 gene Expression

In table -8, the ΔCt of IL6 mRNA showed a non significant(P>0,05)difference in asthenozoospermic patients groups when they distributed according to their asthenozoospermic condition as follows, Asthenozoospermia(AS)(5.42±1.87), Oligoasthenozoospermia (OAS)(5.69±1.52) and Immunological Asthenozoospermia (IAS)(5.03±1.45)] compared to corresponding means in fertile control group(5.95±1.37).

Assessing the $2^{-\Delta\Delta Ct}$ means in asthenozoospermic patients distributed to subgroups, revealed important findings, first, all the asthenozoospermic patients groups showed up-regulation in the expression of IL-6 gene, second, the IAS showed increased expression of folding in IL6 mRNA comparing to other asthenozoospermic subgroups, but with no significant differences.(P=0.48) (Table -9).

Table 4A. Number and percentage frequencies of IL-6 gene genotypes and their Hardy-Weinberg equilibrium (HWE) in (C) group and (ASI) group.

Genotype	(C) group (n=40)		*P* value	(ASI) group (n=25)		*P* value
	Observed n (%)	Expected n (%)		Observed n (%)	Expected n (%)	
CC	31 (77.5)	28.9 (72.25)	*P*<0.01	17 (68)	15.21 (60.84)	*P*<0.05
CG	6 (15)	10.2 (25.5)		5 (20)	8.58 (34.32)	
GG	3 (7.5)	0.9 (2.25)		3 (12)	1.21 (4.84)	

1 degree of freedom (d.f.) for Chi-squared distribution.

Table 4B. Genotype and allele frequencies of IL-6 gene in (C) group and (ASI) group.

Genotype / Allele	(C) group (n=40)	(ASI) group (n=25)	OR	95% CI	*P* value
	no. (%)	no. (%)			
CC	31 (77.5)	17 (68)	1.62	(0.53-4.97)	0.40
CG	6 (15)	5 (20)	0.71	(0.19-2.61)	0.60
GG	3 (7.5)	3 (12)	0.59	(0.11-3.21)	0.54
C	68 (85)	39 (78)	1.56	(0.65-3.96)	0.31
G	12 (15)	11 (22)	0.62	(0.25-1.551)	0.31

OR, odd ratio; CI, confidence interval.

Table-8: Expression of IL-6 mRNA in semen of the studied groups

Group ΔCt (mean± SD)		*P* value†	*P* value‡
(C) group 5.95±1.37	(AS) group 5.42±1.87	0.205	0.35
	(OAS) group 5.69±1.52	0.452	
	(ASI) group 5.03±1.54	0.164	

‡ Univariate analysis of variances (ANOVA) significance test; † Post Hoc test for multiple comparison; using Dunnett's test; significant value (*P*<0.05).

Table-9:Expression fold ($2^{-\Delta\Delta Ct}$) of IL-6 mRNA in semen of the infertile men groups.

Groups	$2^{-\Delta\Delta Ct}$ (mean ± SD)	*P* value‡
(AS) group (n=32)	1.39±0.56	0.48
(OAS) group (n=48)	1.16±0.45	
(ASI) group (n=25)	1.82±0.46	

‡ Univariate analysis of variances (ANOVA) significance test; followed by Post Hoc test: Bonferroni correction for multiple comparison; **no significant differences were found**; mean square (error)= 0.256; corrected error= 21.7

4-DISCUSSION

4-1 - *IL-6* gene SNPs

The rs1800795 polymorphism in the promoter of the coding gene *IL-6* can affect the transcription of the gene, and thus alter the cytokine production. This SNP has been discussed in previous association studies on various cancers(15).

In this meta-analysis, the current study targeted this polymorphism to examine its role in the susceptibility to male infertility.

Previous studies have shown that the *IL-6* polymorphism (rs1800795) may predispose to prostate cancer and influence disease severity. The rs1800795 polymorphism located in the promoter of the coding gene IL-6and can affect its gene transcription and thus change serum IL-6 levels (16).

Our results showed that there was a significant correlation between genotypes and cause male infertility of the asthenozoospermic and oligoasthenozoospermic types of sperm abnormalities.

4-2- *IL-6* gene Expression

An up-regulation of *IL6* gene expression was observed in the semen samples of asthenozoospermic patients, and it was more pronounced in patients with immunological factors than other asthenozoospermic men groups. In agreement with such findings, it has been reported that semen level of IL-6 was significantly increased in asthenozoospermic patients. The authors also reported a higher expression of IL-6 gene in the men complaining from male factor infertility than in fertile control (17,18).

The present study highlights the importance of IL-6 in the development of testis and its function that relate generally to male fertility. In the male gonad, cytokines like IL-6 are produced physiologically and are involved in its normal function. In consequence, they must also appear as natural components of seminal plasma(2).

These interleukins control immune cell function within the testis, they also are produced by “non-immune cells” to stimulate and maintain spermatogenesis. Some cytokines (IL-1, IL-6) are also produced by integral testicular somatic cells, such as the Leydig and Sertoli cells. They can affect every aspect of reproductive physiology and fertility regulation(5).

Previous studies showed that the high levels of IL6 in semen may correspond to the quality of semen parameters, such as sperm count, motility and morphology, or sperm-oocyte penetration rates, they found clear relation between increasing IL-6 level with low sperm motility . (19)

In this study it been found that in Iraqi patients, the prominent cytokine associated with immune cell function, like *IL-6* was detected at low concentration in fertile men. This suggests that cellular immune activity is low in the genital tract of normal men. Elevated levels of some of these factors have been detected in semen and sera of infertile men, indicating that cell-mediated immunity may be up-regulated by genital tract infections. Variables that could affect levels of cytokines and other immunologic factors in semen from different ethnic and demographic groups include genetic polymorphisms, differences in diet, hygiene, sexual practices and drug use (20).

It has been reported that, IL-6 inhibits meiotic DNA synthesis during the cycle of the seminiferous epithelium, influences the secretion of transferrin and inhibin B by Sertoli cells and reduces sperm motility and influences the secretion of transferrin and inhibin B by Sertoli cells (21,22)

Results of the present study in accordance with other studies which found increase in the expression of *IL-6* gene in infertile men with immunological factors than other patients groups, they found that IL-6 could perturb Sertoli cell Blood Testes Barrier (BTB)integrity during testicular infection and inflammation, result in presence of antisperm antibodies , a common reason of male infertility (23) .Thus this study conducted that the Iraqi men complaining from asthenozoospermia have up-regulation of *IL-6* gene expression, Moreover, the SNPs of this gene may effect on asthenozoospermic men complaining from idiopathic infertility.

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