Research Paper

Investigating the Relationship between Tumor Necrosis Factor Alpha-308 and Interleukin-1beta C+3953T Polymorphisms with Male Fertility in Erbil Province

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Male infertility is influenced by psychological, biological and environmental factors. Nowadays, the morphological change of genes and biological molecules are the focus of attention. Therefore, this study was conducted with the aim of investigating the relationship between tumor necrosis factor alpha-308 and interleukin-1beta C+3953T polymorphisms with male fertility in Erbil province. This study was conducted between 2020 and 2021 on 270 men who referred to the infertility center in Erbil, Iraq, with infertility problems. The sampling was purposeful and the samples were divided into three groups of 90 people with the characteristics of the first group without fertility problems, the second group with primary infertility and the third group with secondary infertility. Blood samples were taken from each sample and the samples were sent to the laboratory for deoxyribonucleic acid extraction and allele analysis. A polymerase chain reaction machine was used for this purpose. Statistical package for the social sciences version 23 software and descriptive statistical tests, chi-square, odds ratio and conditional regression were used to analyze the obtained data. The results of tumor necrosis factor alpha-308 genotype showed the frequency of alleles in three groups of men, it can be concluded that there is a significant difference between the three groups of men in terms of mutation frequency and fertility ($p \leq 0.001$). The results of interleukin-1beta C+3953T genotype have shown that there is a significant difference in mutation frequency and finally fertility in three groups of men ($p \leq 0.001$). The tumor necrosis factor alpha-308 genotype GA and GG alleles have a significant relationship with male fertility. In genotype interleukin-1beta C+3953T, CT and CC alleles have a significant relationship with male fertility. The results of this study showed that tumor necrosis factor alpha-308 genotype and interleukin-1beta C+3953T genotype were identified as important risk factors in male infertility.

Key words: Male infertility, tumor necrosis factor alpha, polymorphism, interleukin-1beta C+3953T

According to the World Health Organization (WHO), infertility is the inability to get pregnant after at least a year of frequent, unprotected sex[1]. According to estimates, 8 %-12 % of couples in the reproductive age group struggle with infertility, which is a serious health issue on a global scale. Males are discovered to be primarily to blame for infertility and this constitutes 20 %-30 % of infertility cases, while they contribute to 50 % of cases overall[2]. Infertility affects >186 million persons worldwide, the majority of who live in developing nations[3]. Iraq's current fertility rate in 2022 is 3.493 births per woman, reduced to 1.33 % from 2021, according to a United Nations-World population prospects estimate[4].

Male infertility has been attributed to several factors, including problems in spermatozoa caused by insufficient numbers (azoospermia/oligospermia), poor motility and incorrect structure/morphology. Systematic drops in sperm counts have been noted in some articles[5,6]. Based on Okonofua *et al.* study, four broad causes and risk factors of male infertility identified were biological/physiological/genetic causes;

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behavioral/lifestyle risk factors; environmental factors and socio-demographic risk factors[5]. The genetic and biological factors in male infertility that have been identified so far include chromosomal disorders, monogenic diseases, meiosis and endocrine disorders[7].

Other biological factors influencing in male fertility include the presence of cytokines, chemokines and growth factors in human sperm such as Interleukins (ILs), Tumor Necrosis Factor alpha (TNF-α); TNF-related apoptosis-inducing ligand, soluble receptors and antagonists; granulocyte and macrophage colony-stimulating factors and interferon's; alteration of chemotactic monocyte growth factors and activating factors[8]. The IL-1 family plays an essential role in balancing and regulating spermatogenesis, as well as in cell survival and proliferation. This group has several members, including IL-1α, IL-1beta (β) and Interleukin 1 Receptor Antagonist (IL-1RA). Alteration of IL-1β with TNF-α ends in decreased sperm motility and asthenozoospermia phenotype[9].

Study of Zamani-Badi *et al.* and Aziziaram *et al.*, showed that the C3953T polymorphism of the IL-1β gene as a synonymous exon polymorphism can change the structure and process of Ribonucleic Acid (RNA) binding and affect fertility[9,10]. But in terms of the effect of other cytokines, as well as their simultaneous effect on infertility, no much information has been mentioned. The aim of this study was to investigate the relationship between TNF-α-308 and IL-1β C+3953T polymorphisms with male fertility in Erbil province.

MATERIALS AND METHODS

Study design and settings:

This study was conducted between 2020 and 2021 in the infertility center of Erbil, Iraq.

Participants:

A total of 270 samples were collected at Runahi *In Vitro* Fertilization (IVF) center, Erbil, Iraq. According to the type of fertility problem, the samples were divided into three equal groups, 90 people in the primary infertility group, 90 people in the secondary infertility group and 90 people in the fertile group, infertile participants were free of other parameters of infertility such as cystic fibrosis, Klinefelter syndrome, varicocele, Y chromosome microdeletion and chemotherapy. Also, men with smoking and drinking were excluded from the study. In order to examine the blood samples, 10 CC of each patient were sampled and the samples were stored at -20° until further analysis. Informed consent was taken from all participants and the study was approved by local ethics committee.

Procedure:

Genomic extraction: Genomic Deoxyribonucleic Acid (DNA) from blood specimens was prepared using a DNA extraction kit (Thermofisher, USA) and following the manufacturer's instructions with minor modification. Briefly, qualification and quantification of DNA concentration was performed by using Nano Drop (ND-1000, USA). Samples of genomic DNA with (A260-A320)/(A280-A320) ratio more than 1.7 and outputs more than 30 ng/µl were obtained.

Primer design: A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. Selection of primers was dependent on the target region in the sequence. A pair of primer of sequences for TNF-α and IL-6 genes were designed. Online primer design program was employed. The sequence of the primers, annealing temperature and the length of Polymerase Chain Reaction (PCR) products are exhibited in Table 1.

PCR amplification: PCR is the amplification of target sequences of DNA and DNA *in vitro*. PCR technique was used in this research to amplify the TNF-α and IL-1β genes. Two pairs of primers were designed by SDSC workbench online primer design program. All materials in PCR reaction were sterilized and PCR mixture was prepared in the sterile cabin. PCR reaction was performed by using MJ Research, AB Applied Biosystem thermal cyclers (Table 2).

The thermocycling program was set to run 35 cycles according to the following parameters as shown in Table 3.

Data analysis:

Determining the significant difference between the studied groups, it was investigated with statistical analysis including mean, standard deviation and correlation coefficient. The difference in the frequency of alleles and genotypes was performed using chi-square test in Statistical Package for the Social Sciences (SPSS) software version 23. The Odds Ratio (OR) was estimated with a 95 % Confidence Interval (CI). Chi-square and conditional regression were used to analyze the data and a significance level of less than 0.05 was considered.

TABLE 1: PRIMER SEQUENCES, PCR PRODUCT SIZE OF THREE TARGETS REGION OF TNF-α AND IL-1β GENES AND OPTIMAL ANNEALING TEMPERATURE

Primer name	Sequence from 5' to 3'	Optimal annealing temperature	PCR product size
TNF-α gene			
Forward primer	5′-GGTGCTTGTTCCTCAGCCTC-3′	54.1°	273 bp
Reverse primer	5′-AGATGATCTGACCTGCCTGGG3′		
IL-1ß gene			
Forward primer	5′-ATTCTGCGCAGCTTTAAGGA-3′	55.4°	394 bp
Reverse primer	5′-AACAACAATCTGAGGTCGCC-3′		

TABLE 2: THE COMPONENTS OF PCR REACTION AND THEIR QUANTITIES IN 25 µl TOTAL VOLUME

Chemical substances	Quantity (µl)
Distilled water (dH_2O)	15.875 µl
10x PCR buffer Ammonium sulfate $(NH_4)_2SO_4$	2.5 µl
25 mM Magnesium chloride ($MgCl_2$)	2 µl
2 mM deoxynucleotide triphosphates (dNTPs)	2 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
5 U/ml Taq DNA polymerase	0.125 µl
DNA template	2 µl
Mixture	25 µl

TABLE 3: CONDITIONS OF PCR REACTION

Steps	Temperature	Time
Pre denaturation	94°	5 min
Denaturation	94°	30 s
Primer annealing	72°	30 s
Extension	72°, 25 cycles	30 s
Final extension	72°	5 min
Hold	4°	0

RESULTS AND DISCUSSION

After the PCR reaction on the extracted DNA, the samples of 270 men, which included 90 primary infertility men, 90 secondary infertility men and 90 fertile men, determined the genotypic and allelic frequencies for them. The percentage frequency and allelic genotype of TNF-α-308 and IL-1β C+3953T gene mutation in all three groups of primary infertility, secondary infertility and fertile men were determined. The difference of alleles and the roles of alleles that play a role in male infertility were determined.

The mean age of men is 32.714±55.4 (31.66-33.8). The results of TNF-α-308 genotype showed that out of 90 men with primary infertility, 15 people have AA allele, 21 people have GA allele and 54 people have GG allele. Also, in secondary infertility, 9 people have AA allele, 13 people have GA allele and 68 people have GG allele, and in fertile, 0 people have AA allele, 9 people have GA allele, and 81 people have GG allele. According to the frequency of alleles in three groups of men, it can be concluded that there is a significant difference between the three groups of men in terms of mutation frequency and fertility ($p \leq 0.001$). The results of IL-1β C+3953T genotype showed that in primary infertility 12 people have TT allele, 32 people have CT allele and 46 people have CC allele. Also, in secondary infertility, 6 people have TT allele, 19 people have CT allele and 56 people have CC allele, and in fertile, 1 person has TT allele, 9 people have CT allele, and 81 people have CC allele. The results showed that there is a significant difference in mutation frequency and finally fertility in three groups of men ($p \leq 0.001$) (Table 4).

Based on the results of this study, it was shown that TNF-α-308 genotype GA allele has a significant relationship with male fertility (OR=1.254, CI 95 %=1.15-1.34; p=0.002) and this relationship is also significant in the GG allele. It was seen with fertility in men (OR=1.524, CI 95 %=1.36-1.69; p=0.003). In

genotype IL-1β C+3953T, the relationship between CT and CC alleles with male fertility was also investigated. A significant relationship was seen in the CT allele with fertility (OR=2.5, CI 95 %=1.25-5.36; p=0.001) and a significant relationship was seen in the CC allele with male fertility in this study (OR=1.45, CI 95 %=1.36-3.23; p=0.001) (Table 5).

Considering the high prevalence of infertility in men, there is still little information about the causes of this infertility. In recent years, with the progress in the identification of gene sequences, a large number of point mutations have been identified. Despite the identification of genetic factors such as monogenic and mutagenic defects, about 40 % of the causes of infertility in men have not been identified yet[11,12]. Therefore, in this study, TNF-α-308 genotype and C IL-1β C+3953T genotype was investigated and the types of alleles affecting male infertility were determined. The results showed that GA and GG alleles in TNF-α-308 genotype and CT and CC alleles in IL-1β C+3953T genotype play a role in male fertility.

The effect of TNF-α-308 genotype on male infertility has been investigated in different populations, which shows the effect of this genotype on infertility[13]. TNF-α is a cytokine family that plays a role in the biological function of spermatogenesis as a pro-inflammatory cytokine. There are also TNF-α receptors in the testis, Sertoli and Leydig cells can regulate the secretion of these cells. It has been shown that high levels of TNF-α in infertile men are associated with reduced sperm motility. Several nucleotide polymorphisms in the TNF-α promoter region have been investigated. Studies have shown that the 308 mm polymorphism has a role in infertility[14]. The results showed that GA and GG alleles have a significant effect on male infertility. The systematic review study of Mostafa *et al.*[15], was conducted with the aim of the effect of TNF-α-308 polymorphisms in male infertility. In this study, it was shown that GA and CT alleles are directly related to the risk of infertility in men and are considered as an important risk factor in male infertility, which is in line with the results of the present study.

TABLE 4: DISTRIBUTION OF THE GENOTYPES ACCORDING TO MUTATIONS IN PRIMARY INFERTILITY, SECONDARY INFERTILITY AND FERTILE MEN

Type	Genotype/Allele (308G>A TNF-α)			p-value*
	AA	GA	GG	
Primary infertility	15	21	54	
Secondary infertility	9	13	68	0.001
Fertile	0	9	81	
Type	Genotype/Allele (IL-1β C+3953T)			p-value*
	TT	CT	CC	
Primary infertility	12	32	46	
Secondary infertility	6	19	65	0.001
Fertile	1	10	79	

Note: *p-values were obtained from chi-square test

TABLE 5: ODDS RATIOS FOR MALE INFERTILITY ACROSS DIFFERENT GENOTYPES

Type	OR (95 % CI)	p-value*
Genotype/Allele	308G>A TNF-α	
AA	1 (ref)	
GA	1.254 (1.15-1.34)	0.002
GG	1.524 (1.39-1.69)	0.003
Genotype/Allele	IL-1β C+3953T	
TT	1 (ref)	
CT	2.5 (1.25-5.36)	0.001
CC	1.45 (1.36-3.23	0.001

Note: *p-values were obtained from chi-square test

In the study conducted by Ashrafzadeh *et al.*[16], it was conducted with the aim of the effect of TNFR1 36 A/G polymorphism on idiopathic azoospermia in the Iranian population. In this study, 108 infertile men and 119 fertile men were examined. The results showed that the frequency of GG and AG genotypes in the group of infertile men was higher than that of the fertile group, and finally it was found that the genotype GG and the G allele were related to infertility in Ramadan, and the results of the present study also showed that the G allele can be one of the important factors in male infertility.

In this study, the effect of IL-1β C+3953T genotype on male infertility showed that CT and CC alleles play an important role in infertility. In the study of Zamani-Badi *et al.*[10] who aimed to investigate the relationship between C3953T single nucleotide polymorphism in the 5th exon of IL-1β gene and idiopathic male infertility, 230 fertile men and 207 infertile men were examined. In this study, the T allele was identified as an important allele in male infertility and this allele was also identified as the allele involved in infertility in the present study. These results show that the C3953T polymorphism can be a potential biomarker for the genetic diagnosis of infertility where men should be considered.

Cytokines play a role in the regulation of spermatogenesis, which play a role in the differentiation of germ cells during cell division. Members of the IL-1 family are pleiotropic cytokines involved in inflammation, immune regulation and there are other homeostatic functions of ILs such as IL-1β that are normal in the testis under homeostasis and are increased by inflammatory infection. In the study of Jaiswal *et al.*[17] the effect of IL-1β gene on male infertility was investigated. The results showed that the genotype CT and allele C were significantly more in unbelieving men and played a role in male infertility, which is consistent with the results of the present study. In other studies, the CT and CC genotypes, which are in line with the results of the present study, were identified as one of the important factors of infertility in men[18-20].

The results of this study showed that TNF-α-308 genotype and IL-1β C+3953T genotype were identified as an important risk factor in male infertility. Also, it seems necessary to conduct more studies. In this regard, it is necessary to pay attention to the sample size and ethnicities, as well as considering environmental factors.

Author's contributions:

All authors passed the criteria for the authorship contribution based on recommendations of the International Committee of Medical Journal Editors.

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Conflict of interests:

The authors have no conflicts of interest to declare.

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