RESEARCH PAPER

The Association Study between Tumor Necrosis Factor- Receptor 1 36 A/G (TNFR1 36 A/G) Gene Polymorphism and Azoospermic Infertile Men in Erbil City

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A B S T R A C T:

Tumor necrosis factor-alpha (TNF-α) is a multifunctional cytokine that controls cellular activities related to spermatogenesis. (TNFR1) affects TNF-α activity and also may leads to gene dysfunction and male infertility. This retrospective study was done to assess the linkage of TNFR1 36 A/G gene polymorphism with idiopathic azoospermia in Erbil City. The present study was carried out on 98 infertile, 109 fertile males as healthy control. TNFR1 36 A/G gene polymorphisms were detected in all study subjects, and their seminal fluids were analyzed. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method is used for identify polymorphism in both cases and control groups. MspA1I restriction enzyme were digested PCR products. Digested and PCR products were analyzed by 3% gel electrophoresis for 45 minutes at 97 V. The results of the present study showed that the incidence of AG genotype in azoospermia male higher than control groups (OR=2.069(1.154-3.708), P=0.014, this study found that G allele frequency in azoospermia men had a significant difference compared to the control group (OR=1.487 (0.992-2.170). Moreover, AG genotype and G allele associated with an increased risk of non-obstructive azoospermia in Erbil City.

KEY WORDS: Male infertility, Tumor necrosis factor-alpha, allele. Azoospermia, Genotype.

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1. INTRODUCTION

Infertility in males is usually due to disturbance in the process of spermatogenesis, which cause no or incompetent spermatozoa (Rajender et al., 2010; Sikka,2001). Some spermatogenic factors cause infertility, such as decreased sperm motility, low sperm count, destruction of sperm DNA, or poor semen quality. Quality of seminal fluid may be compromised due to some factors like high levels of reactive oxygen species (ROS) and oxidative stress (OS) (Harchegani et al., 2019; Aitken, 1999; Armstrong et al., 1999). The world's population is increasing and may reach nine billion in 2050. In addition to that, 15% of couples world-wide remain childless due to infertility. Only a few of these due to genetic causes, also genetic factors are thought to underlie many causes of idiopathic infertility (Bracke et al., 2018). About 30-40% of the male in the reproductive age group have a defect in sperm production. In quality or quantity, 50% of them due to low sperm motility (asthenozoospermia)
and low sperm count (oligospermia) Shamsi et al. (2008).

Azoospermia means a lack of sperm in at least two ejaculate samples (including the centrifuged sediment) (World Health Organization, 2010; Corea et al., 2005). Generally, 40 to 50 % of couple infertile. About 50% of them are male, and 10-20% of these (or 1% of all men in the general population) suffer from azoospermia (Pashaei et al. 2020).

Chromosomal diseases are mostly founded in infertile compared with fertile population (Signore et al. 2020). These chromosomal changes found in 20% of azoospermic and the Klinefelter syndrome (47,XXY) is most frequent genetic causes of azoospermia (or severe oligozoospermia) (Röpke and Tüttelmann, 2017). Therefore, it is better these men doing genetic testing before the use of their sperm for assisted reproductive techniques ART.

Tumor necrosis factor-alpha (TNF-α) is a protein with molecular weight’s around (17KDa) and produced mostly by activated T cells, monocytes, macrophages, and a few non-lymphoid cells such as male germ cells and Sertoli. TNF-α binding to a TNF transmembrans receptor, which recruits cytosolic proteins and transduces the signal. TNF-α is causing Leydig cell steroidogenesis, Sertoli cell-germ cell junction dynamics, and regulates germ cell apoptosis (Xia et al., 2005). TNF-α due to activation of nuclear factor kappa B represses gene expression of the steroidogenic enzyme in Leydig cells (Siu et al., 2003; Hong et al., 2004; Pentikäinen et al., 2001). Both Sertoli and Leydig cells produce a lot of the immunoregulatory cytokine, IL-6, driven by IL-1. Both these cytokines control Sertoli cell and germ cell development (Hedger et al., 2003).

Current studies are yet to show direct connections among cytokines and male infertility. Furthermore, cytokines might be helpful markers for analysis and observing treatment in patients with urogenital infection/inflammation (Sengupta et al., 2020)

Fertility in men relies upon the best possible creation of sperm cells, by means of spermatogenesis, which is extremely intricate and includes the synchronization of numerous components. The nearness of proinflammatory cytokines, TNF-α, interleukin (IL)-1α and IL1β in the male reproductive system, explicitly in testes, epididymis and spermatozoa, have a few critical physiological capacities including immunoregulation in the male reproductive tract.(Azenabor et al., 2015).

The steroidogenic enzymes are causing a decrease in the production of testosterone (Rebourcet et al., 2020). High levels of testosterone hormone cause the depletion of germ cells from the epithelium (Mealy et al. 1990). Increase levels of TNF-α also found in the seminal fluid in cases of leukocyte infiltration of sperm and also found that inflammation-mediated azoospermia is directly related to TNF-α in the seminal fluid (Martínez et al., 2010; Seshadri et al., 2009).

The location of TNFRI gene is on chromosome 12p13.2 (Hong et al. 2004). There are many polymorphisms in TNFRI gene which have a huge effect on the performance of receptor; one of these is 36 A/G, and this receptor has important role in the activity of TNF-α. Because of that it is better to investigate the polymorphisms associated with this gene. The present study found the association of alleles and genotypes of TNFRI 36 A/G polymorphism with azoospermia. The present case-control study was undertaken to identify the Gene polymorphism in male with azoospermia in Erbil City.

2.MATERIALS AND METHODS

2.1 Subjects

The present study performed on 98 non-obstructive azoospermic infertile men (age range 20-50 years) and 109 healthy fertile men as a control groups, from February 2018 to May 2019. This study performed on human subjects that are selected from patients who are attending the infertility center in Erbil City. Those Patients that are married for about one year and having unprotected intercourse were considered for this study. Those patients that have chronic diseases, history of pelvic /spinal injuries, and karyotype abnormalities are excluded in this study. And from all patients, informed verbal consent was obtained, and the ethics committees approved the research of the Medical Research Center/ Hawler Medical University. Assessment of seminal fluid of studied groups were done after 3-5 days of sexual abstinence according to the guideline of the World Health Organization (WHO) to diagnose their infertility status and to know the quality and quantity of sperm. See (Table 3)
2.2 Detection of TNFR1 Gene Polymorphism

Venous blood samples (3ml) were collected via atraumatic antecubital venipuncture into vacutainer tubes containing EDTA. The samples were either used immediately for study of polymorphism or kept at about -20°C until further analysis. The identification of TNFR1 gene polymorphism assessed by using polymerase chain reaction and PCR-RFLP, techniques that are used at the Medical Research Center/Hawler Medical University in Erbil City/ IRAQ. Briefly, DNA was isolated from blood samples (by using 100 prep DNA extraction kit (Fermentas company)), and the polymorphic region is amplified by PCR (Boiron/ Genekam Biotechnology ready to use PCR master mix), concentration and volume of the solutions in PCR reaction were:(15μl Master mix, 5μl DNA sample, 2μl Forward primer, 2μl Reveres primer and 26μl ddH2O).

PCR thermal profile for amplification of single nucleotide 36A/G polymorphisms of TNFR1 gene was: (5 min initiation denaturation at 95°C, 30s denaturation at 95°C, 30s annealing at 69°C, 30s extension at 72°C and 5 min final extension at 72°C) with primers 5'-GAGCCCAAATGGGGAGTGAGAGG-3' and 5'-ACCCAGGCCGCGGAGGAGAG-3'. The PCR products were analyzed by 2% agarose gel electrophoresis (Genekam Biotechnology AG, Germany) to amplify the TNFR1 gene fragment, that 183-bp band was observed. PCR products were subsequently digested with restriction enzyme endonucleasesMspI (BioLabs Inc, NEW ENGLAND), then the samples were analyzed on 3% agarose gel for 45 minutes at 97 V.

2.3 Digestion By MSPA1I Restriction Enzyme

The PCR-RFLP method was used after a PCR reaction, also by using the MSPA1I restriction enzyme for both case and control samples. After optimizing the enzymatic digestion conditions, 8μl PCR products (for each sample) were cut by mixed with 1μl MSPA1I restriction enzyme, 5μl NE Buffer (1X), and 26μl ddH2O. For activation of this enzyme, this 40μl (total volume) was incubated overnight for 37°C. After that, the restriction enzyme inactivated at 65°C for 20min. Through 3% agarose gel, the mixtures were electrophoresed. The polymorphism within amplified products has a length of 183 bp. In this research, it is expected to find three different bands on gel electrophoresis, including homozygous individuals (AA): a fragment in 183-bp region, heterozygous individuals (AG): three fragments in 183-bp, 108-bp, and 75-bp regions, as well as homozygous individuals (GG): two fragments in 108-bp and 75-bp regions.

3. STATISTICAL ANALYSIS

Through the chi-square test using the statistical package, version 20 SPSS, assessment done and found the difference between infertile men and control groups and their relationships with azoospermia. The Odds Ratio (OR), P-value, and confidence interval were calculated for the frequencies and parameters, while quantitative variables were expressed as the mean standard deviation (SD). Differences were considered statistically significant at p≤0.05.

4. RESULTS

The participants are divided into two groups: infertility (n=98) and fertility (n=109) subjects. On 3% gel electrophoresis, three different bands were observed. Including AA genotype (183bp), AG genotype (183bp, 108bp, and 75bp) and GG genotype (108bp, and 75bp). Figure 1 indicating digested products on 3% agarose gel.

The results demonstrated that the frequencies of AA (183bp), AG (183bp), 108bp, and (75bp), and GG (108bp), and (75bp) genotypes were 17.34%, 42.8%, and 39.7% respectively in cases and 34.86%, 26.60%, and 38.53% in control groups. Table 1.

Besides, the frequencies of A and G alleles were 38.3% and 60.6% respectively in the case group, and 48.16% and 51.83% in the control group (Table 2). The present study indicated that the frequency of AG genotype in cases and controls was significantly different (p=0.014). The frequency of AG genotype was higher in cases when compared with controls. The risk of non-obstructive azoospermia was more in men with AG genotype (OR=2.069) (1.154-3.708). The difference in A and G alleles frequency was significant between the two groups (p=0.054), and the risk of azoospermia people with G allele was higher 1.487 (0.992-2.170) when compared with A allele (0.682(0.461-1.008). Table 2.

Chi-square test showed significant differences between genotypes frequencies in cases and controls OR=2.069, Cl; 95%, 1.154-3.708. Chi-square test showed significant differences.
differences between alleles frequencies in cases and controls OR=1.487, CI; 95%, 0.992-2.170.

Regarding semen quality, especially volume of sperm, concentration, total count, motility (motile, and immotile), and morphology of sperm (normal, and abnormal), a highly significant change among fertile men, was defined compared with infertile men (azoospermia) (p=0.000). Table 3.

Table (1) : Frequency distribution of TNFR1 36A/G polymorphism genotypes in infertile men with azoospermia

| Genotypes | Azoospermic men | Controls | P-value | OR (CL;95%) |
|-----------|----------------|----------|---------|-------------|
| AA        | 17 (17.34%)    | 38 (34.86%) | 0.004  | 0.387(0.201-0.744) |
| AG        | 42 (42.8%)     | 29 (26.60%) | 0.014  | 2.069(1.154-3.708) |
| GG        | 39 (39.7%)     | 42 (38.53%) | 0.894  | 1.039(0.594-1.818) |

Table (2): Frequency distribution of TNFR1 36 A/G polymorphism alleles in patients with azoospermia.

| Genotypes | Azoospermic men | Controls | P-value | OR (CL;95%) |
|-----------|----------------|----------|---------|-------------|
| A         | 76(38.3%)      | 105(48.16%) | 0.054  | 0.682(0.461-1.008) |
| G         | 120(60.6%)     | 113(51.83%) | 0.054  | 1.487 (0.992-2.170) |

Table (3): Seminal Fluid Analysis of fertile and infertile men (Azoospermia men).

| Variable       | Fertile men | Infertile men(Azoospermic men) | p. value |
|----------------|-------------|--------------------------------|----------|
|                | No (109)    | No(98)                         |          |
| Volume (ml)    | 4.55±0.95   | 2.014±0.667                    | 0.000³   |
| Concentration (10⁶/ml) | 58.48±18.99   | 0.00±0.000                    | 0.000⁴   |
| Total count (10⁹/ejaculate) | 2.692 ±100.700 | 0.00±0.000                 | 0.000⁵   |
| Motility       | Motile     | 79.17±13.57                    | 0.000³   |
|                | Immotile   | 21.055±10.23                   | 0.000⁵   |
| Morphology     | Normal     | 68.55±10.99                    | 0.000⁵   |
5. DISCUSSION

Azoospermia, which means discharge free of sperm, that influences 1% of all men and up to 15% of men with infertility (Gudeloglu, 2013). Our research was led to survey TNFR1 36 A/G Gene Polymorphism, and it is predominance among patients with idiopathic azoospermia and found that the G allele and AG genotype of TNFR1 36 A/G polymorphism is significantly higher in azoospermic infertile men when compared to control group. However, polymorphism of TNFR1 36 A/G gene, can change in function of the type 1 receptor of TNF-α cytokine, resulting in cytokine dysfunction.

It has been claimed in an Iranian study that the GG genotype associated with non-obstructive azoospermia and they clarified that the increased risk of non-obstructive azoospermia associated with G allele (Ashrafzadeh et al., 2017). NOA happens due to defects in spermatogenesis. However, the etiology of most patients' testicular dysfunction stays obscure (Han et al., 2020).

According to the study in Erbil City the single nucleotide polymorphism (SNP) rs12086634 (T>G) of the HSD11B1 gene is associated with polycystic ovary syndrome (PCOS) significantly, and the G allele of this SNP rs12086634 (T>G) was associated with obesity. (Shareef et al., 2019)

Lazaros et al., 2012 in Greece population investigated the relationship between 36 A/G polymorphism of TNFR1 with male infertility and sperm concentration and motility using PCR-RFLP. Their study showed that the allele A of TNFR1 is associated with increased sperm concentration and motility, and supporting the significance of the TNFR1 gene is semen quality. The study in Kurdistan region demonstrated a significant association between TT genotype of mir-125a G >T polymorphism and In Vitro Fertilization (IVF) failure (Dizay et al., 2019).

The genetic factors and environmental have a role in infertility, and the genetic factors are confirmed in men with idiopathic infertility (Tanoonmad et al., 2019). Table 1, showed that the odds ratio for AG groups was more than one and the p-value was 0.014; it means that this group has increased risk to azoospermia. But the odds ratio for GG, AA groups was less than one, and the p-value was non-significant, and according to these values, the two groups have decreased risk to azoospermia.

Men with NOA have high rates of DNA repair mechanisms defects and irregularities in cell cycle control. (Gunes et al., 2015). The infertility issues in 15% of couples are identified with male factors in 40% of them (Zaimy et al., 2013).

Spermatogenesis is depend on a huge number of cellular signals causing coordination and connection between various cells of the testes. Cytokines, including TNF-α, control this relationship and capacity among the cells and, subsequently it could be related with sperm variations for example, changes in morphology, number, and motility. TNF-α receptors, for example, TNFR1 and TNFR2 exist in sertoli and Leydig cells. The capacity of these cells can be balanced by binding the cytokine to them (Tronchon et al., 2008; Zalata et al., 2013).

Table 1:

| Abnormal | 31.75±4.99 | 0.00±0.000 | 0.000* |

According to the Table 1 and 2: The PCR-RFLP was used by the help of restriction Msap1I enzyme for the 98 blood samples (cases) and 109 blood samples (control groups), and the results demonstrated that the frequency AG genotype and G allele were less in controls than cases.

Primary infertilities’ prevalence is accounted for to be 10% to 12% (World Health Organization 2004). Also, the rate of fertility and number of children is steady decreasing globally (World Health Organization, 2004). Incremental population growth, caused by low fertility rates, is an existential problem for the developing countries in Asia. The northern and western parts of Europe first encountered the risk of law fertility rates, but now Asian countries are haunted by it. Marriage is strongly embodied in the Asian social
systems as a habit or tradition; hence the rates of children born to single mothers are limited. This phenomenon immensely contributed to low fertility rates and the number of children (Matsuda S., 2020).

Kotowicz et al. (2010) determined that increasing the serum levels of Cancer A-125 recommends the need for applying progressively forceful types of treatment. Soluble TNF receptor type I might be of significance in diagnosing patients at the early clinical phases of adenocarcinoma, particularly those with Cancer A-125 focus inside.

Figure 1: 3% agarose gel electrophoresis pattern of some RFLP products of TNFR1 gene polymorphism. Line (1) indicates DNA markers with a 50 bp. Lane (2) is (AG) heterozygous individuals: three bands in 183-bp, 108-bp, and 75-bp areas, it means that the variation could be observed on one strand of DNA but cannot be detected in other once. Lanes (3 and 6, 7) are negative and don’t have the mutation. Lane (4) is homozygous individuals (AA): a fragment in 183-bp region, and no mutation found on this region, As well as lane (5) is homozygous individuals (GG): two bands in 108-bp and 75-bp regions, it means that mutation should be observed on both strands of DNA.

6. CONCLUSION
In conclusion, the study showed that the AG genotype and G allele of 36 AG TNFR1 might be a risk factor for non-obstructive azoospermia in a population of Erbil City

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CONFLICT OF INTERESTS
There are no conflicts of interest regarding this study.

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