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RESEARCH ARTICLE

Association of Heat Shock Proteins (HSP70), Reactive Oxygen Species and Inflammatory Mediators in Male Infertility

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Abstract

Heat shock protein 70 (HSP70) is considered as a gene which affects semen quality traits. The present study attempted to investigate the relationship between the HSP70 expression level and physiological status in Normozoospermia and an Asthenozoospermi. The current study was planned to investigate the relationship between the HSP70 gene polymorphism and infertility in infertile males with normal sperm parameters. A complete of sixty patients and thirty control subjects were collected between February to May 2019. The genotyping of the HSPA1L gene was performed using polymerase chain reaction (PCR) in addition to restriction fragment length polymorphism (RFLP). We tend to determine vital variations within the genotype frequencies of HSPA1L asthenozoospermia patients and controls. Our results showed highly significant differences between asthenozoospermia patients and control groups in levels TNF, IL-6, and ROS, ($p<0.05$ was, 0.001, 0.04, 0.004 respectively).as well showed that the value of the area under the curve (AUC) was the highest for IL-6 (0 .766) and was the p-value ($p< 0.05$) .And the genetic study showed significant differences in frequency of (TT) genotype ($p<0.05$) between asthenozoospermia patients and controls (71.6%versus 60%, respectively). While the frequency of (CT) Genotype showed differences between asthenozoospermia patients and controls but no significant differences ($p>0.05$, 23.3%versus 20%respectively).While allele frequency(C) showed significant differences ($p<0.05$). Conclusion: It has been well established that heat shock proteins (HSP) is involved in wide varieties of physiological regulation process in Asthenozoospermia.

Keywords: *Male infertility; HSP70; RFLP; TNF- **α**; IL-6; Oxidative stress.*

Introduction

The infertility is a main problem almost 7% of men from the general populace are infertile and in at least 15% of cases this condition is associated with genetic disorders, including both chromosomal and single-gene changes Genetic causes can be detected in all essential etiologic categories of male infertility (pre-testicular, testicular and post-testicular forms) and genetic tests became a part of the recurring diagnostic procedure in selected agencies of patients [1].

Asthenozoospermia may be a common reason of male infertility, that is characterized by method of minimized spermatozoa forward motility (progressive motility<32%) [2]. Asthenozoospermia has been established, consisting of disturbances of the mitochondrial sheath and axonemal complicated formation in the course of spermiogenesis, impaired characteristic of accessory sex glands providing compounds required for moves or epididymis accountable for the maturation of spermatozoa, genetic defects, and hormonal disturbances. All of these ought to induce the incidence of asthenozoospermia [3, 4]. HSPs are elementary proteins current in all living matters from small organism to man, the place they defend against varied stresses. HSPs are recognised to be anxious in many steps of the generative manner and should additionally induce a reaction response that in all probability impairs semen quality and sperm fertility [5, 6].

HSPs are differentially expressed in human gamete and are involved in sperm vitality, motility, apoptosis, and capacitation reaction. HSPs are related to stress, and also the expression of those proteins is upregulated once cells are uncovered to accumulated

temperatures or different stresses like pathogens, cytokines (interleukin-1, interleukin-2 and etc.) [7], and physical and chemical factors Cytokines mediate inflammatory responses, are necessary in intercellular communication, and play a multifarious role in the procreative physiology of men and female [8, 9]. Exogenous factors which will contribute to the increased concentrations of ROS are each environmental factors (high temperatures, nonparticulate radiation, pesticides and pollution) and style factors (advanced age, alcohol consumption, smoking, stress, avoirdupois and poor diet [10].

The heat shock proteins (HSPs), a family of endogenous, protecting proteins, are positioned in the cytoplasm and nucleus (e.g. HSP70 and HSP90 respectively) to preserve regular cell function. ROS, cytotoxic lysosomal enzymes and cytoskeletal variations are able to set off HSP expression [11]. HSP70 expression degree led to the decline of antioxidant enzymes things to do in the cells, quite greater ROS injured sperm; it induced sperm motility decrease [12].

Materials and Methods

Sixty (60) blood samples were collected from infertility patients with asthenozoospermia patients who visited Kamal Al-Samraee hospital for fertility, infertility and in vitro fertilization-Baghdad-Iraq. And thirty (30) samples Normozoospermia as control.

Interleukin-6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α) assay Procedure

Human **IL**-6 and TNF-α was measured by using enzyme linked immunosorbent assay (sandwash elisa) technology according manufacturing company (Elascience - China).

Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) determined using method [13].

DNA Extraction

Genomic DNA from whole blood cells was extracted and purified using Extraction and purification Kit from Favergen Company (Taiwan).

Genotypic Identification using RFLP-PCR Amplification

The targeted sites of DNA were amplified using specific designed primers: primer obtained from Bioneer, IDTDNA (USA). Primer: Forward sequence was 5-CATATGGGGCTGCGGTACAA -3 and the reverse sequence was 5-TGATGTTTGAAGATGAGGGGAATG -3 PCR was done in 20ul reaction volumes containing 1ul from reverse and forward primer, 12.5 ul of Green Master Mix, 3 ul of Genomic DNA and the volume of reaction was finished up to 20 ul by including 2.5 ul of Nuclease free water. Intensification was completed in a thermo-cycler customized for one minutes at 94°C; for 35 cycles 1 minute each at 94°C, one moment at 62 °C and one minutes at 72°C; and a last expansion of 7 minutes.

PCR items were electrophoresed utilizing gel electrophoresis in 1% agarose at 75 V for 1 hour and pictured by ethidium bromide. Photographs were taken utilizing gel documentation framework. The PCR item was cut utilizing NcoI restriction, the PCR-RFLP method was achieve steady with Promega Company Protocol. After digestion with NcoI reaction were electrophoresed utilizing gel electrophoresis (Cleaver Scientific, UK) in 3% agarose gels at 75 V for 1 hour and 8 %Polyacrylmide gel electrophoresis control connected:75 V, 20 Am for 160 min. after that gels pictured by ethidium bromide. Photographs were taken utilizing gel documentation system (EBOXCX, UK).

Statistical Analysis

Statistical analysis was carried out using SPSS version 23, where data were expressed as the Means, Standard Error, One-sample T Test, One-way ANOVA test and Chi-square test, Odds ratio to identify the danger factors of male infertility with their 95% confidence interval (CI), were used to find the association between the categorical variables, P value ($P \leq 0.05$) was considered statistically significant.

Result and Discussion

Results are summarized in Tables (1) Values were presented as mean ± standard error (S.E.) and $P < 0.05$ was considered statistically significant .The levels of two proinflammatory cytokines, namely tumor necrosis factor a (TNF-a) and interleukin 6 (IL-6), were investigated in serum of normozoospermia men (n=25) and infertile

(n=25) men to evaluate the relationship between these biomarkers and HSP70A1 gene in this study.

Table 1: Mean differences of serum levels of TNFα, IL-6 and ROS between Asthenozoospermia men and control group

Groups / Biomarker	Control(n=25)	Patients Asthenozoospermia(n=25)	t-test	P-value
IL- 6 (pg/ml	10.66± 0.37	21.93 ±3.98	1.98*	0.04
TNF-a (pg/ml)	96.25±5.21	160.02 ±17.56	4.039*	0.001
ROS	2.61±0.37	4.17±0.31	3.25*	0.004

*P ≤ 0.05; SE: Standard error

The mean value (mean ± SE) of serum TNF- and IL6 levels estimated in Asthenozoospermia patients was (160.02±17.56), (21.93±3.98) and controls (96.25±5.21). (10.66±0.37). A statistically significant difference was found in the serum TNF- and IL6 levels between patients and controls (p< 0.001, p<0.04 and 0.004) Cytokines mediate inflammatory responses, are necessary in intercellular communication, and play multifaceted position in the reproductive physiology of men and female [1, 2].

The role of interleukin-6 in leukocyte recruitment, its consequences on the coagulation cascade, and on the endothelium and on different elements of the vasculature might also additionally be essential and Various cytokines are concerned in acute-phase protein synthesis, which includes tumor necrosis factor, (TNF-)and interleukin -6 (IL-6). The present results were approved with prior studies [3].

And Estrada et al [14].Who found the relative increase in TNFa polymorphism frequency in infertile patients was associated with asthenospermia in the absence of genital infection. In this context, it should be noted that TNF a has been reported to modify sperm motility. For example, TNFa in vitro causes a significant reduction in both progressive and total sperm motility [4]. The found several pathologic effects of ROS on the male reproductive system have been demonstrated by several studies [5, 6]. On the other hand, some research confirmed no significant relationship between sperm motility and the tiers of ROS production [7]. Although the mechanisms of action of the cytokine on sperm quality (particularly on motility) stay to be elucidated, cytokines, and especially TNFa, can result in reactive oxygen species formation, which may also be a substantial purpose for sperm quality alteration main to male infertility [8].

One of the important sources of endogenous ROS in semen is ordinary spermatozoa [9], and an excessive mitochondrial manufacturing of ROS from these cells is recognised to correlate properly with faulty sperm function, particularly sperm motility [10].Data from Table (2) show that the value of area under the curve (AUC) was the highest for IL-6 followed by that of ROS that of and finally that of serum TNF; 0 .766, 0 .619 and 0.538 respectively.

This indicates that IL-6 is the best in discrimination between normozoospermia and Asthenozoospermia subjects followed by IL-6, ROS and TNF. The best serum IL-6, TNF and ROS discriminative concentrations were≥11.54 pg/ml (sensitivity 85%, specificity64%), ≥ 91.85 pg/ml (sensitivity 54%, specificity 55%), 0.84 (sensitivity 100%, specificity 18%) respectively.

Table 2: Analysis of Receiver operating characteristics curves for serum TNF, IL-6 and ROS for Asthenozoospermia patients

Test Result Variable(s)	AUC	Std. Error	Sensitivity	Specifity	Criterion	p-value	Asymptotic 95% Confidence Interval
ROS	0.618	0.123	100%	18%	≥ 0.84	0.168	0.318-0.806
IL-6	0.765	0.102	85%	64%	≥11.54	0.004	
TNF	0.538	0.128	54%	55%	≥91.85	0.382	0.481-0.904
							0.242-0.742

*P ≤ 0.05; SE: Standard error

In the present study, receiver operating characteristic curve analysis was performed to find a cut off value that could be used for diagnostic purposes in case of male factor infertility. We suggest a cut off of ≥11.54 as a diagnostic or screening tool in general to diagnose male factor infertility. Interleukin-6 is a 26 kDa cytokine, produced by many different cells in the body, including lymphocytes, monocytes, fibroblasts and endothelial cells. Interleukin-6 was initially known by a variety of names reflecting its multitude of actions [11]. The HSPA1L gene polymorphism was studied in Asthenozoospermia cases and controls. HSPA1L T>C, The distribution observed in HSPA1L gene polymorphism in control group and cases groups are showed in Table (3): the highest genotype in control group was TT mutant homozygote genotype (60%) followed by heterozygote genotype TC (20%) homozygote genotype CC (20%) and. In Asthenozoospermia disease, the highest genotype was TT homozygote two bands (71.6%) the genotyping distribution pointed out that heterozygote TC was more than wild type CC, which reached to (23.3%), (3%) respectively.

Table 3: The Genotype distribution and odd ratio of HSP70A1 gene polymorphism between the (patient Asthenozoospermia with control)

Genotype	control No. (%)	patients Asthenozoospermia No. (%)	*P*-value	OR (95%)
CC [a]	6 (20%)	3 (5%)		
TT	18 (60%)	43(71.6%)	0.03*	0.2 (0.04-0.93)
CT	6 (20%)	14(23.3%)	0.07	0.25 (0.04-1.36)
Total	30(100%)	60(100%)		
Allele frequency				
	control	patients		
T Allele	18	20		
C Allele	42	100	0.05*	2.14(1.03-4.45)

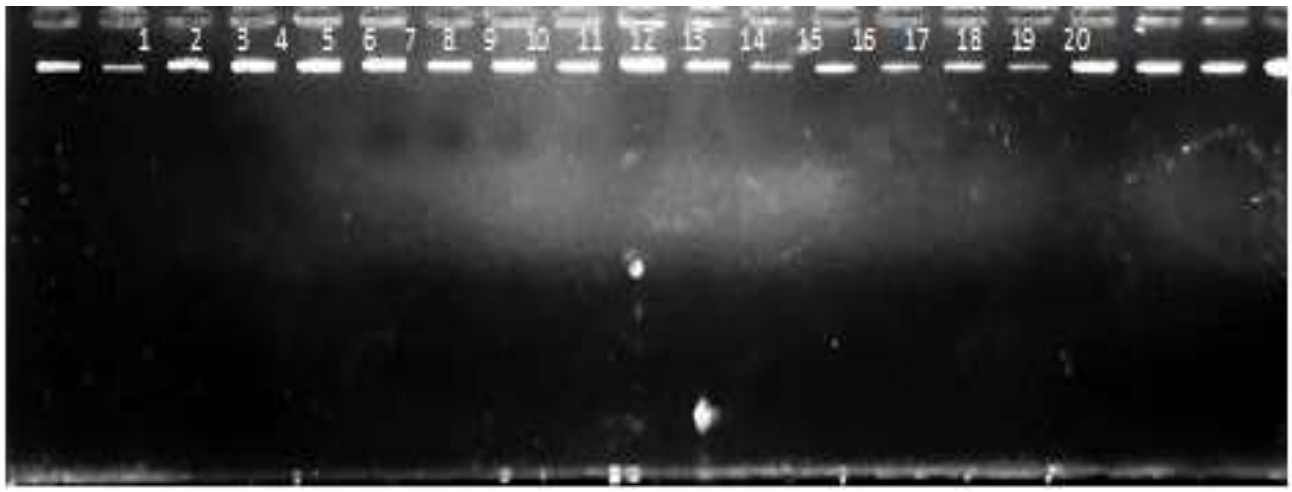

Figure 1: The electrophoresis pattern of DNA extracted from blood Lane 1 - lane 20 refers to extracted DNA from blood samples; Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h (10 µl in each well)

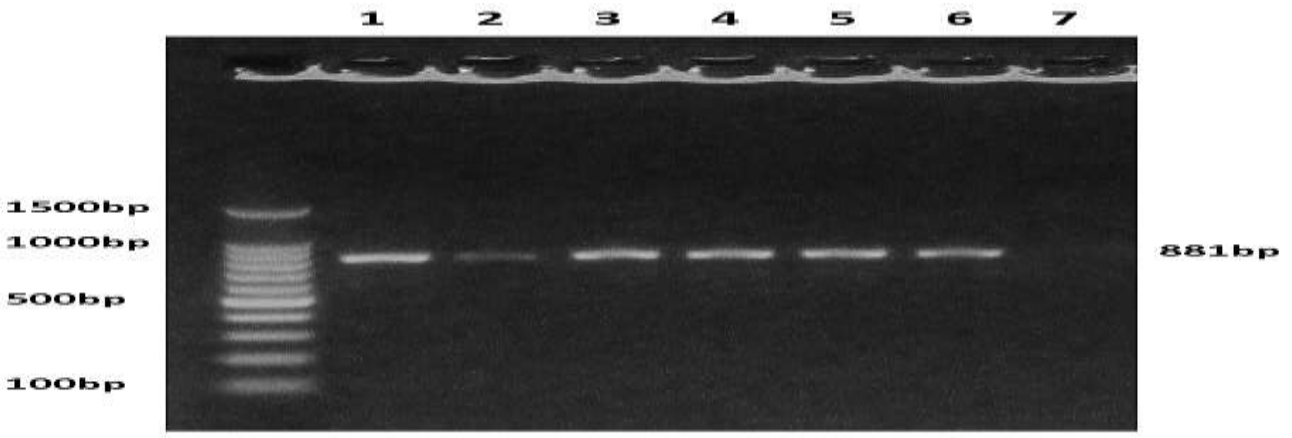

Figure 2: Agarose gel electrophoresis of HSP70A1L amplification products. The amplified products were one band 881 bp in size. Electrophoresis conditions: agarose concentration 1%, power applied: 75 V, 20 mA for 120 min. Staining method; precast ethidium bromide

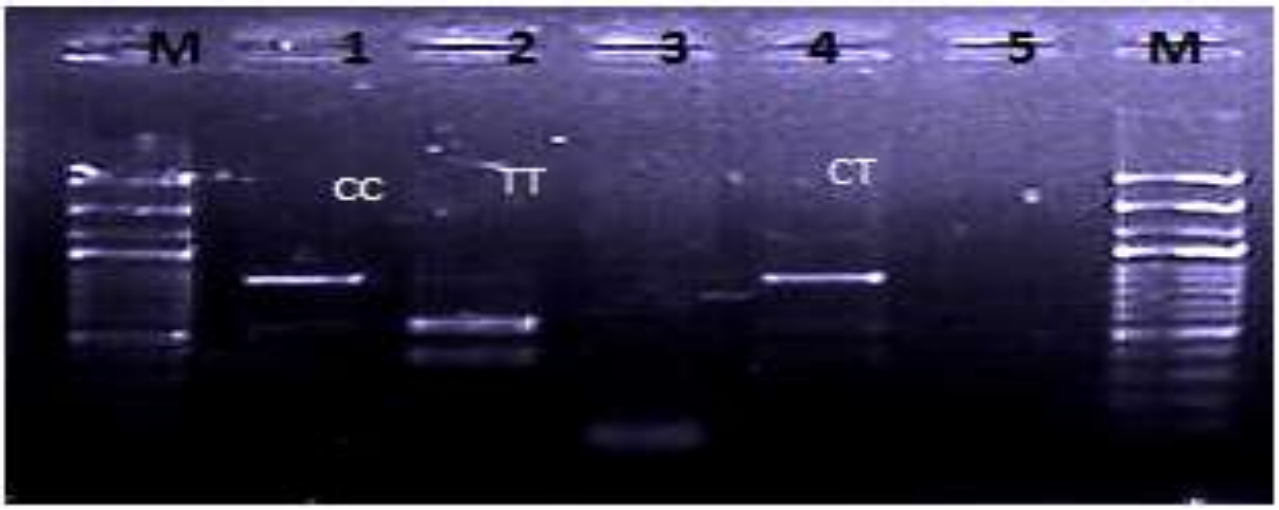

Figure 3: The electrophoresis pattern of RFLP-PCR for PCR product (881 bp) with restriction enzyme NCOI, 3% agarose, 75 V, 20 Am for 120 min. (10 µl in each well).Lane M: DNA ladder 100 bp Lane **1, showing wild homozygote (CC 881bp) genotype. Lane 2, showing mutant homozygote (TT530, 351bp) genotype. Lane 4, showing mutant heterozygote (CT 881,530,351) genotype**

The results of this study are disagreeing with the results of Ciftci *et al* [15]. Who found, in Turkey, that infertility in males with normal sperm parameters was not significantly associated with HSPA1L: gene polymorphisms [15]. Also, in Iran, Kohan and Tabiee [16] showed that the HSPA1L polymorphism is associated with the idiopathic male infertility risk and that the individuals with TC and CC genotype had an increased risk of male infertility [16]. The Hsp70 loci within the major histocompatibility complex (MHC) class III region on chromosome 6 contain three intronless genes.

The HSPA1A and HSPA1B genes are 12 kb apart and encode an identical heat-inducible protein, but have divergent 50 and 30 untranslated region (UTR) sequences. The third gene, HSPA1L is located 4 kb telomeric to HSPA1A. HSPA1L encodes a constitutively expressed protein that shares 90% identity with HSPA1A, the sequences differing most in the C-terminal 100 amino acids [17].

The polymorphism of *hsp70 gene*, in several genes are associated with male infertility [18]. The *h*sp70 gene encodes a highly inducible stress protein, which is fundamentally important in protein transport and folding [18]. HSPs can modify many of the physiological functions of numerous different proteins and therefore critical for cell survival [19]. Despite the fact that the HSPs are often related to the cellular stress response, they also play an important function in supporting normal cell processes which include development and differentiation [20].

HSPs are involved in the different developmental stages of spermatogenesis such as dramatic transformations and cellular differentiation [21]. The importance of heat shock proteins for sperm development also extends to synergistic roles associated with the functional transformation of these cells that occurs during their successive phases of post-testicular maturation within the epididymal maturation of male and capacitation of female reproductive tracts [21].

Male infertility is a multifactorial syndrome that accounts for about 50% of all infertilities [1]. It is a heterogeneous disorder, with several genetic, environmental and behavioural factors contributing to impaired spermatogenesis [22]. Despite progress, mainly in the field of genetics, the etiology is still unknown in about 50% cases, and the condition is termed 'idiopathic infertility'. It is currently accepted that genetics contributes to spermatogenetic failure in about 30%cases of idiopathic infertility in males [22].

Over the past decades, many genetic studies have investigated the association between male infertility and genetic polymorphisms related to metabolic enzymes [23]. Increasingly, the evidence suggests that polymorphisms, including the HSP70 polymorphism, in several genes are associated with male infertility.17TheHSP70 gene encodes a highly inducible stress protein, which is fundamentally important in protein transport and folding [18].

In conclusion, the present population-based case-control study indicated that both

HSPA1L gene polymorphisms affect the risk of male infertility in Iraqi population [24, 33].

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