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Impact of seminal plasma heat shock 70 biomarker and its effect on seminal fluid parameters in infertile men

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Abstract
Background: Infertility is a global problem can affect both man and women couples worldwide characterized as a failure to achieve a clinical pregnancy following ≥ 12 months of having regular unprotected sex. Heat Shock Protein 70 (HSP70) is a protein have been established as a protein participated in male germ cell development and fertility and was elevated in male infertility. The study aims to investigate HSP70 in the seminal plasma of infertile Iraqi men.
Method: This study included 83 semen samples from 53 infertile men and 30 healthy controls, collected at Kamal Al-Samraei Hospital, Baghdad, from March 2023 to June 2024. Patients (aged 19-50 years) underwent clinical evaluation, and semen analysis followed WHO 2010 guidelines. Samples were centrifuged, and supernatants were stored at -80°C for HSP70 assessment using ELISA (Elabscience, Cat. E-ELH1863).
Results: In the study, 53 male patients were divided into three groups: infertile oligozoospermic OZ (n=17), asthenozoospermia AZ (n=18), and oligoasthenozoospermic OAS (n=18), and fertile control (n=30). ELISA was used to measure HSP70 in seminal plasm. The semen infertile man had a considerably higher mean ±SD of HSP70 (11.83±1.42) compared to the control group (6.37±1.25) (p=0.000). It was substantially higher in AZ and OAS than OZ. HSP70 ROC showed a flawless male patient diagnosis from healthy at cutoff value (>8.48 ng/ml). It offers AUC (1, p<0.001) with 100% sensitivity and specificity.
Conclusion: the higher expression of HSP70 in infertile patient might be considered as a therapeutic agent for infertility and acquires the test a perfect diagnostic identity for male infertile.

Keywords: Asthenozoospermia, HSP70, oligozoospermic, male infertile

Introduction

Infertility is a significant global health issue affecting approximately 15-20% of couples worldwide, with male infertility contributing to nearly half of these cases due to poor semen quality [1, 2]. It is defined as the inability to achieve a clinical pregnancy after at least 12 months of regular unprotected intercourse [3], with a global incidence of 15% and male-related factors responsible in 40% of cases [3, 4]. Male infertility is categorized into primary (79%) and secondary (21%), where primary infertility refers to the inability to conceive a first child, whereas secondary infertility occurs when a man is unable to father another child after a previous successful conception [3, 5]. Multiple factors contribute to male infertility, including genetic causes, environmental exposures to chemicals and endocrine disruptors, as well as lifestyle factors such as smoking and alcohol consumption [4, 6, 7]. The primary causes are abnormalities in semen and sperm parameters, including oligozoospermia (low sperm count), azoospermia (absence of sperm), asthenozoospermia (reduced sperm motility), necrozoospermia (low sperm viability), teratozoospermia (abnormal sperm morphology), and aspermia (absence of semen) [8, 9]. Semen analysis remains the primary method for diagnosing male infertility, as semen is composed of testicular secretions containing spermatozoa (5% of semen volume) and seminal plasma (95%) [10]. Male infertility is linked to poor seminal parameters in approximately 50% of cases, with regional variations in prevalence [11, 12]. While semen analysis is traditionally used for diagnosis, its limitations have led to the search for novel biomarkers to better assess male reproductive health [2]. Seminal plasma contains proteins, lipids, hormones, and bioactive molecules crucial for sperm function, making it a potential source for diagnostic biomarkers [2, 13]. Infertility has been associated with the presence or absence of specific sperm and seminal plasma proteins, highlighting the need for advanced molecular analysis in semen evaluation [6].

Heat shock proteins (HSPs) are molecular chaperones essential for protein folding and cellular protection against stress. Initially identified in 1974, HSPs are highly conserved and categorized by molecular weight (HSP40, HSP60, HSP70, HSP90, and HSP100) [14-16]. These proteins maintain intracellular stability and play a key role in cellular responses to oxidative stress, hypoxia, and apoptosis, particularly in reproductive tissues such as the testes and placenta [15, 17, 18]. HSP70, the most abundant HSP, exists in two forms: HSC70 (73 kDa), constitutively expressed under normal conditions, and HSP70 (72 kDa), which is stress-induced and acts as an intracellular and extracellular chaperone [16]. These proteins are found in spermatogenic cells and are involved in sperm production and differentiation [15]. Heat or osmotic stress disrupts HSP70 expression, impairing normal sperm function and autophagy, leading to reduced sperm count and viability [17]. Moreover, HSP70 undergoes dynamic redistribution during sperm capacitation and acrosome reactions [15]. Studies have shown that elevated HSP70 levels in seminal plasma correlate with male infertility, making it a promising biomarker for diagnosing reproductive dysfunction [6, 19, 20]. The current study aims to highlight on the HSP70 as markers of male infertility via identifying it as a biomarker for the diagnosing and monitoring of male reproductive ability.

Method

This study included 83 semen samples were obtained from 53 infertile men and 30 healthy controls. The samples were collected from Iraqi male patients who attended to Kamal Al -Samraei Hospital for infertility diagnosis and assisted reproductive technologies in Baghdad, Iraq from the period between March 2023 and June 2024. The patients age range between (19- 50) year, they agree to participate in study, infertile group had history of infertility more than one year, control group had at least one child, abstinence period 72hours, exclude patients who had operation, refused participate in the study, or have medical disease, or history of infectious disease last 6 months, or taking drugs or supplement vitamins. Examination was done by andrologist due to social factor, examination by our colleague andrologist for (BP, vital signs, abdominal& pelvic examination). All patients sent for complete blood count, fasting blood sugar were instructed to collect their semen sample in a dry, sterile and wide- mouthed plastic container. The demographic information including (name, age, type of fertility, abstinence period, time of sample collection) wa5s obtained from patients and labelled with precise details. Before conducting the study, ethics approval was obtained by Kamal Al -Samraei Hospital for infertility diagnosis and assisted reproduction. Materials: human heat shock protein (HSP Elabscience Cat. E-ELH1863). Methods: After liquefaction of semen sample, the examination was carried out according to the WHO 2010. Every collected sample was divided into three parts after examination. The samples were centrifuged at a constant 3000 rpm speed and for 10 min. Supernatant was separated and aliquoted into Eppendorf tube and stored at-80 °C until the completion the collection of all samples to assess the HSP70 using enzyme-linked immunosorbent (ELISA) assay according to the manufacturer's instructions. The data was analyzed statistically by SPSS version 24 software and introduced as mean ±SD and frequency n (%). Significant differences between the two groups were analyzed using a T-test, either assuming equal variances or unequal variances based on the variances assessed by Levene's test for equality of variances. For more the two mean comparison the ONEWAY-ANOVA test was suitable to use. The significance difference between groups was considered at $P<0.05$. The receiver operating curve (ROC) analysis used to discriminate between two varied situations like infertile and fertile men.

Results

Total of 53 infertile men were included in this case-control study with (mean ±SD) of age (29±6.5) years and 30 age-matched healthy men fertile their age was (30.5±8). The mean ±SD of patient's infertile duration was (9.1±0.644) year. Infertile patients' group were diagnosed as 32.07%, n=17 of oligozoospermic (OZ), 33.9%, n=18 of asthenozoospermic (AZ) and 33.9%, n=18 of infertile oligoasthenozoospermic men (OAS). Generally, as seen in Table (3.1), the results showed a significant difference ($p<0.05$) in HSP level and other laboratory tests for seminal fluid including motility and number of sperm.

Table 1: Sociodemographic and HSP70 maker differences between patients and the control groups

Tested Parameter	Studied groups (mean ±SD)		P - value
	Control fertile men (n= 30)	Patients' infertile men (n= 53)	
Age (year)	30.5±8	29±6.5	0.435
Infertility duration (year)	9.1±0.644	---	----
HSP70 (ng/ml)	6.37±1.25	11.83±1.42	0.00

* Significant at the 0.05 level (2-tailed), * * Significant at the 0.01 level (2-tailed).

As we previously reported, the patient infertile group was characterized as OZ, AZ and OAS depending on the number of the sperms and their activities. As illustrated in Table (3.2), the level of HSP70, no. of sperm and motility showed a significant difference within the three those subgroups and also when compared to normal control group.

Table 2: HSP70 and laboratory tests levels in infertile men groups

Analyzed Parameters (mean ±SD)	Normal fertile men	Infertile Men groups (n= 53)			Sig.
		OZ (n= 17)	AZ (n= 18)	OAS (n= 18)	
HSP70 (ng/ml)	6.3±1.25	10.6±0.67	12±1.53	12.8±0.94	0.000**
No. of sperm / million	54.17±14.12	12.71±6.04	58.33±7.75	6.0±3.144	0.000**
Motility %	72.87±11.4	46.65±10.2	9.00±6.088	5.78±3.173	0.000**

* Significant at the 0.05 level (2-tailed). * * Significant at the 0.01 level (2-tailed).

The pairwise findings, as shown in Figure 1, showed that the mean ±SD of HSP for OZ, AZ, and OAS patients were elevated significantly in comparison to healthy normal fertile men (P=0.000). Furthermore, its level was increased in AZ and also in OAS infertile patients' groups when compared to OZ group (P=0.009, 0.000 respectively). While, there was a nonsignificant difference (P=0.242) for the level of protein appeared between the AZ and OAS. As in Figure (1).

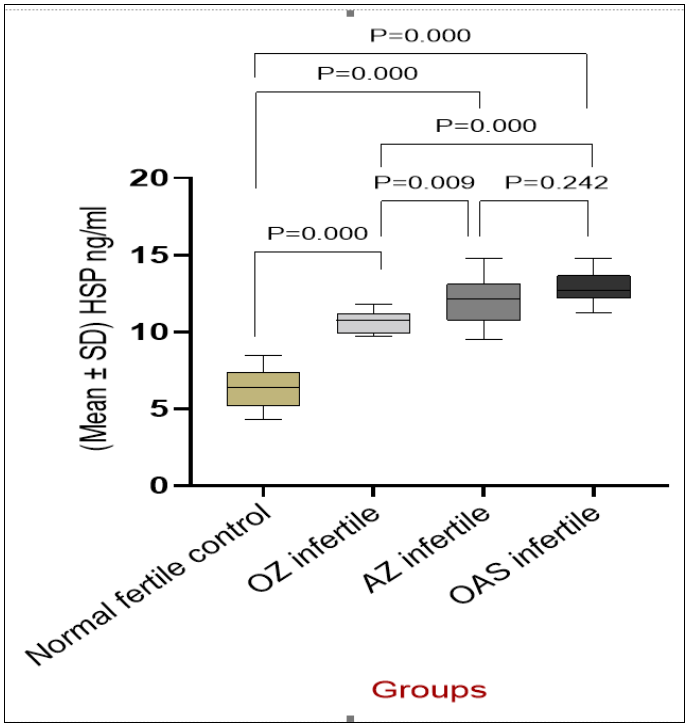

Fig 1: Box plot scheme of HSP in OZ, AZ, OAS infertile men and normal fertile men groups.

Firstly, the OZ group showed mean ±SD for the no. of sperm was 12.71±6.04, and significantly reduced than control group. And, the percentage motility was 46.65±10.2 and also significantly decreased than control group but it still at the normal limit for the reproductive process. Further, AZ infertile men showed the mean ±SD for no. of sperm was 58.33±7.75, and it non-significantly differed from control group (p=0.556). As well as, the mean ±SD for percentage of motility was 9.00±6.088 and it significantly reduced than healthy group (p=0.000). Lastly, the OAS infertile men group revealed the mean ±SD for no. of sperm was 6.0±3.144 and for motility ratio was 5.78±3.173 and the both examinations were reduced significantly than healthy fertile men group (p=0.000). The distinguishing between infertile patients' men and healthy fertile groups was analyzed by receiver operating curve (ROC) test. It is providing the cutoff, standard error (ES), sensitivity, specificity, positive and negative predictive values (+PV & -PV) and accuracy. This diagnostic assay can be carried out by various software to ascertain the presence or absence of a disease state, which is crucial in clinical practice. It is essential to determine the patient's health status clarifying if the patient is infected or not. The area under curve AUC represents the quality of the diagnostic test of the selected biomarker. It is categorized to different levels including: 1.0 level is a perfect, excellent (0.9-0.99), good (0.8-0.89), fair (0.7-0.79), poor (0.51-0.69), and 0.5 is of no value. Our results, as seen in Figure (2), found the HSP biomarker had a perfect discrimination between the fertile and infertile men with AUC (1, p<0.001).

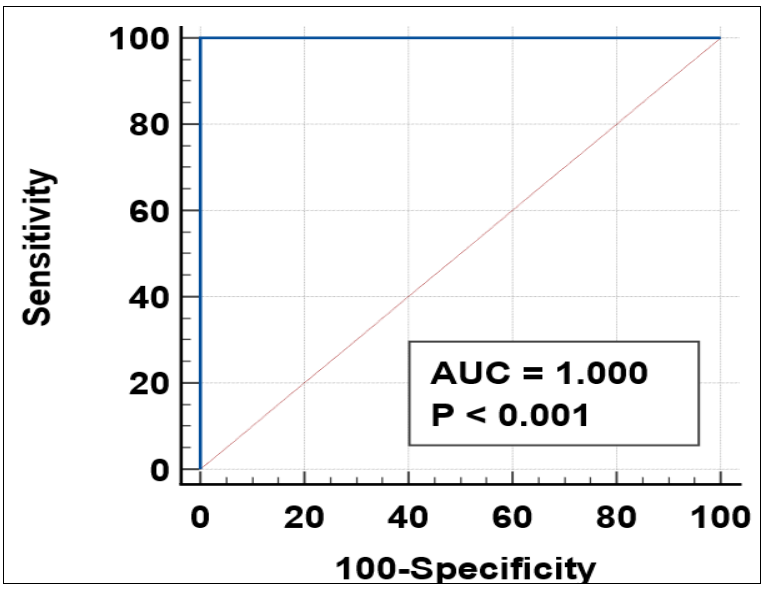

Fig 2: ROC curve of HSP for discrimination the infertile men from normal fertile men.

It gave a cutoff value was (>8.48 ng/ml) and the test provide 100% sensitivity and specificity as shown in Table (3).

Table (3): ROC analysis of HSP70 to distinguish the infertile from fertile men

Parameter	AUC	SE	Sig.	Cutoff	95% CI	Sens. %	Spec. %	Accuracy (Youden index J)	+PV	-PV
HSP70 ng/ml	1.00	0.00	<0.001	>8.48	0.957-1.0	100	100	1.0	100	100

Characteristic of Mela Age Effect on the Levels of HSP70 Biomarker

The results of this study, as seen in Table (4), showed there was a no significant difference in the level of HSP70 at different stage of age in both fertile and infertile men groups.

Table 4: ROC analysis of HSP70 to distinguish the infertile from fertile men

Age stages	(Mean ±SD) HSP ng/ml level			
	Fertile men	N	Infertile men	n
< 20 year	5.56±0.45	3	11.97±0.66	4
20-24 year	7.47±1.16	4	10.87±1.54	10
25-29 year	6.91±1.21	8	10.19±1.31	19
30-34 year	6.54±1.68	6	11.21±1.87	10
35-39 year	8.59±2.68	7	11.66±2.33	4
40-45 year	9.78±0.2	2	10.61±1.5	6
p-value	0.052 ns		0.211 ns	

Discussion

The seminal parameters like volume, motility, and morphology of sperms are all known to decrease with aging [22]. Mitochondria is the major site might be targeted for apoptosis, which in turn give rise to somatic cells aging and reduced fertility in a germ cell [23]. Subsequently, high temperature, chemical or physical stress, viral infections, medical drugs, and modifying agents can cause HSP production in cells. The HSP synthesis in cells could appear via hyperthermia, oxidative and metabolic stress, and aging [24]. Furthermore, the physiological functions like proliferation, differentiation, development, and aging, may stimulate HSP production [25]. Many earlier studies demonstrated the increasing of HSP70 semen levels in male infertility when compared to normal physiological fertile men as we forementioned previously. Abdel Jail *et al.* found its level was higher in patients than control (P<0.001) and the level of the protein was higher in oligozoospermic (OZ) class of infertility than asthenospermic (AZ) infertile (P = 0.008) [19]. Further, the study of Erata *et al.* showed significant increase of HSP70 protein of asthenozoospermic (AZ) (P= 0.042) and oligoasthenospermic (OAS) (P= 0.017) men fertile compared to control fertile men [20]. In addition, the study of Saeed *et al.* also showed increase level of HSP70 in men infertile than healthy men (P<0.001) and found at higher level in OAS group than OZ [12]. Also, the researchers found that the HSP70 capable for an excellent prediction of the fertile from infertile men via ROC analysis. The AUCROC curve was 0.89 and the test has 81.4% sensitivity and 90% specificity. In addition, Aworu *et al.* study showed increase level of HSP70 protein in the infertile patients than healthy men. The higher level was seen in the oligospermic OZ compared to azoospermic and normo-spermic subjects [6]. Our study was agreed to above mentioned studies regard the general level increasing of protein in infertile men in comparison to fertile men. However, we found the HSP70 concentration was in a higher level in AZ than OZ group, but the study of Abdel Jail and colleagues demonstrated vice to versa. We demonstrated also the predictive ability of HSP70 biomarker was perfect level of discrimination between fertile person and infertile men with AUC level was 1 and 100% sensitivity and specificity as seen in Figure (1). Polymorphisms of the Hsp70 gene were observed in individuals with shorter lifespans, and its gene expression was observed in isolated heat-treated blood cells. These results support the hypothesis that increased longevity is associated with decreased expression of the HSP gene [23]. Nevertheless, our study found that the age progress of men had no effect on the HSP70 level production in both fertile and infertile men as seen in Table (4).Our findings that revealed the over expression of HSP 70 are consistent with its function of in protection of protein structure damage and unfolding, and that substantiates the presence of testicular damage by (infection, hypoxia, oxidative stress, immunological reaction insult) But it does not differentiate among these causes. Other markers could differentiate among these causes eig CRP, or presence of pyospermia, or increase oxygen radicals.

Conclusion

The current study concludes that the HSP70 protein was highly expressed in male infertile spermatozoa. Its level was observed in a higher level in asthenozoospermic and oligoasthenozoospermic than oligozoospermic patients. It has a perfect capability for the diagnosis of infertility disease in male, and it might be considered as a therapeutic target.

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