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Research Article

Alterations in Heat Shock Protein 70 (Hsp70) Expression in Sperm of Individuals with Oligozoospermia and Severe Oligozoospermia

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Abstract

Aim:*Infertility, defined as the inability to achieve pregnancy after one year of regular, unprotected sexual intercourse, is categorized as either female or male infertility. Male infertility is often associated with impairments in sperm quality and function. Heat shock proteins (HSPs), particularly HSP70, play a critical role as molecular chaperones, protecting cells from stress-induced damage by stabilizing proteins and ensuring proper folding. However, the relationship between HSP70 expression and male infertility has not been fully elucidated. This study aimed to investigate HSP70 expression in sperm samples from individuals with normal, oligozoospermic, and severe oligozoospermic profiles using immunocytochemical techniques.*

Material and Methods: *Sperm samples were obtained from the In Vitro Fertilization Unit of Erciyes University and divided into three groups: Control (>20 million/ml sperm), Oligozoospermic (<15 million/ml), and Severe Oligozoospermic (<5 million/ml). Samples were stained for HSP70, and hormone levels (testosterone, FSH, and LH) were evaluated.*

Results: *HSP70 expression was significantly elevated in the Oligozoospermic group compared to the Control group but decreased in the Severe Oligozoospermic group, reaching levels comparable to the Control group. Hormonal analyses revealed altered levels of testosterone, FSH, and LH in both oligozoospermic groups.*

Conclusion: *These findings suggest increased HSP70 expression in oligozoospermic individuals reflects intracellular disruptions, potentially linked to hormonal dysregulation and organ dysfunction. Such alterations may affect sperm parameters, including morphology, motility, and count. While this study demonstrates a relationship between HSP70 expression and sperm abnormalities under stress conditions, further research is needed to confirm these mechanisms and explore their broader implications.*

Key Words: *HSP70, Male infertility, Oligozoospermia*

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Introduction

According to the World Health Organization (WHO), infertility is defined as the inability to achieve pregnancy after 12 months or more of regular, unprotected sexual intercourse. This condition may involve male or female factors, or in some cases, both partners. Male infertility can result from testicular and hypothalamic-pituitary disorders (such as cryptorchidism, orchitis, genital tract infections, varicocele, obstruction of the male genital tract, and hypogonadism), genetic conditions (including Kallmann or Klinefelter syndromes, globozoospermia, and Y chromosome microdeletions), cancer, systemic diseases, medical treatments, or exposure to endocrine disruptors (1). Additionally, lifestyle-related factors such as smoking, alcohol and drug use, high-energy diets, obesity, and psychological stress negatively impact male reproductive potential (2). However, in 10-15% of cases, the etiology remains unclear and is categorized as idiopathic infertility (3). Semen analysis is the primary step in assessing male fertility potential and is widely used in the initial evaluation of couples presenting with infertility (4). This analysis evaluates parameters such as semen volume, color, viscosity, pH, sperm concentration, motility, and morphology (5). Oligozoospermia is defined as a sperm concentration of less than 15 million/ml and is classified as mild, moderate, or severe based on sperm count (5). Heat shock proteins (HSPs), defined as intracellular molecular chaperones, are a family of proteins responsible for protein folding and translocation during cell growth and development (6). HSPs assist in protein refolding and stabilization under stress conditions (7). They are classified based on their structure, molecular weight, and functions (8). While some HSPs are continuously synthesized in cells, the synthesis of others increases under stress conditions. HSP70 belongs to the group of proteins whose synthesis is upregulated during stress (9). HSP70 proteins, located in the cytosol, mitochondria, or endoplasmic reticulum, perform various functions depending on their localization (10). HSP70 plays a significant role in testicular function, with its importance increasing with age, and it is continuously involved in spermatogenesis, a critical process (11).

Understanding the mechanisms underlying infertility is essential for developing and optimizing treatment options. Therefore, the present study aimed to investigate the relationship between HSP70, a molecule known to increase in expression under stress conditions, and oligozoospermia and severe oligozoospermia.

Method

Ethical approval for this study was obtained from the Erciyes Clinical Research Ethics Committee (approval number: 2022/191, dated 23.02.2022). Male patients attending the IVF Unit Clinic at Erciyes University Faculty of Medicine for infertility evaluation were categorized based on spermiogram results: individuals with a sperm count above 20 million/ml were included in the Control group, those with a sperm count below 15 million/ml were classified as the Oligozoospermia group, and those with a sperm count below 5 million/ml were placed in the Severe Oligozoospermia group (5). Sperm samples used in this study were obtained from 100 patients aged 20–50 years undergoing In Vitro Fertilization (IVF) treatment at the Erciyes University Faculty of Medicine IVF Unit. Samples remaining after routine IVF procedures were used. Among these individuals, 30 patients within the sperm count range determined in the study were selected and grouped, with 10 patients in each group.

Blood test results routinely monitored in the Urology Clinic were recorded. These included measurements of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels.

Patients were instructed to adhere to a three-day period of sexual abstinence before providing samples. After the abstinence period, semen samples were collected through masturbation at the clinic, ensuring no use of water, soap, creams, saliva, or similar substances. Samples were collected in wide-mouth, sterile plastic containers. Each container was labeled with the patient's name, surname, and date, and patients were verbally informed about the procedures to follow during sample collection.

Semen samples collected through ejaculation were incubated at 37°C for 1 hour to allow liquefaction. Following liquefaction, the samples were evaluated by an experienced embryologist in accordance with WHO criteria for parameters such as volume, pH, count, motility, and morphology. Sperm count and motility were assessed using a Makler counting chamber. For morphology evaluation, a drop of semen (approximately 10 µl) was placed on a slide, smeared, and air-dried.

Prepared samples were smeared on poly-L-lysine-coated slides by applying 500 µl of the sample to each slide. After air-drying at room temperature, samples were stored at room temperature until staining. Before staining, the prepared samples were fixed in 70% ethanol at -20°C and washed twice with distilled water. Antigen retrieval was performed with citrate buffer at high temperature, followed by cooling.

Phosphate buffer solution was used as the washing solution. Subsequent steps were carried out using the Ultravision detection kit (Ultra Vision Detection System Large Volume TP-125-HLX, Thermo Scientific HRP, CA) following the manufacturer's protocol. HSP70 (sc-33575, Santa Cruz Biotechnology, CA) was stained to visualize the target protein in sperm. Samples were passed through a graded series of alcohol and xylene, then mounted with Entellan (12).

Ten randomly selected fields from each slide were photographed under a microscope (Olympus BX51; Olympus, Tokyo, Japan). The HSP70 staining intensity in the sperms included in the images was measured one by one using ImageJ Software (version 1.45s). Thus, HSP70 expression was measured in a total of 350 sperm in each group.

Statistical analyses for the data in this study were performed using GraphPad Prism 7 software. The normality of data distribution was assessed using the D'Agostino & Pearson normality test. Data with a normal distribution were analyzed using the one-way ANOVA test, followed by Tukey's post-hoc test for multiple comparisons between groups. For non-parametric data, median (minimum-maximum) values were reported, and the Kruskal-Wallis test was used for analysis. Dunn's multiple comparison test was applied to determine statistical differences between groups. A p-value of <0.05 was considered statistically significant.

Results

Semen samples from participants were collected following sexual abstinence and evaluated macroscopically and microscopically. The macroscopic analysis of the Control group samples revealed a more viscous consistency and more intense color and odor compared to the other groups. In contrast, semen samples from the Oligozoospermia and Severe Oligozoospermia groups exhibited a lighter color, milder odor, and lower viscosity compared to the Control group.

In terms of sperm count, a statistically significant reduction was observed in the Oligozoospermia and Severe Oligozoospermia groups compared to the Control group ($p<0.05$). While the sperm count in the severe Oligozoospermia group was lower than in the Oligozoospermia group, this reduction was not statistically significant ($p>0.05$) (Table 1).

The analysis of testosterone, FSH, and LH levels revealed no statistically significant differences between the groups ($p>0.05$). Testosterone levels were higher in the Oligozoospermia group compared to the Control group. In the Severe

Oligozoospermia group, testosterone levels were slightly lower than in the Control group but remained similar overall.

Table 1. Results of semen analysis in the experimental groups

	Control	Oligozoospermia	Severe Oligozoospermia	*p*
Spermiogram	121±48.3[a]	9±2.4[b]	2.11±1.1[b]	0.0001

*Values are expressed as *10⁶/ml. Data with a normal distribution are expressed as mean ± standard deviation. No statistically significant difference was observed between groups denoted by the same letter(a,b). A p-value of <0.05 was considered statistically significant.*

FSH levels were higher in both the Oligozoospermia and Severe Oligozoospermia groups compared to the Control group, although these differences were not statistically significant. When LH levels were evaluated, both the Oligozoospermia and Severe Oligozoospermia groups showed higher levels compared to the Control group, but these differences also lacked statistical significance.Testosterone, FSH, and LH levels for all experimental groups are summarized in Table 2.

Table 2. Testosterone, FSH, and LH measurement results for the experimental groups.

	Control	Oligozoospermia	Severe Oligozoospermia	*p*
Testosterone	403.8±126.2[a]	513.1±223.4[a]	407±139[a]	0.2856
FSH	4.17±2.7[a]	6.98±3.5[a]	9.49±9.2[a]	0.1758
LH	4.92(0.3-54)[a]	5.98(3.62-10.1)[a]	6.52(8.24-22.8)[a]	0.8286

Data with a normal distribution are expressed as mean ± standard deviation, while non-normally distributed data are expressed as median (minimum-maximum). No statistically significant difference was observed between groups denoted by the same letter(a). A p-value of <0.05 was considered statistically significant.

HSP70 expression measurements were performed on preparations from all experimental groups, and statistical analysis was conducted on the obtained measurements. HSP70 expression was observed to be localized in the head region of the sperm samples, and measurements were made only from this area.

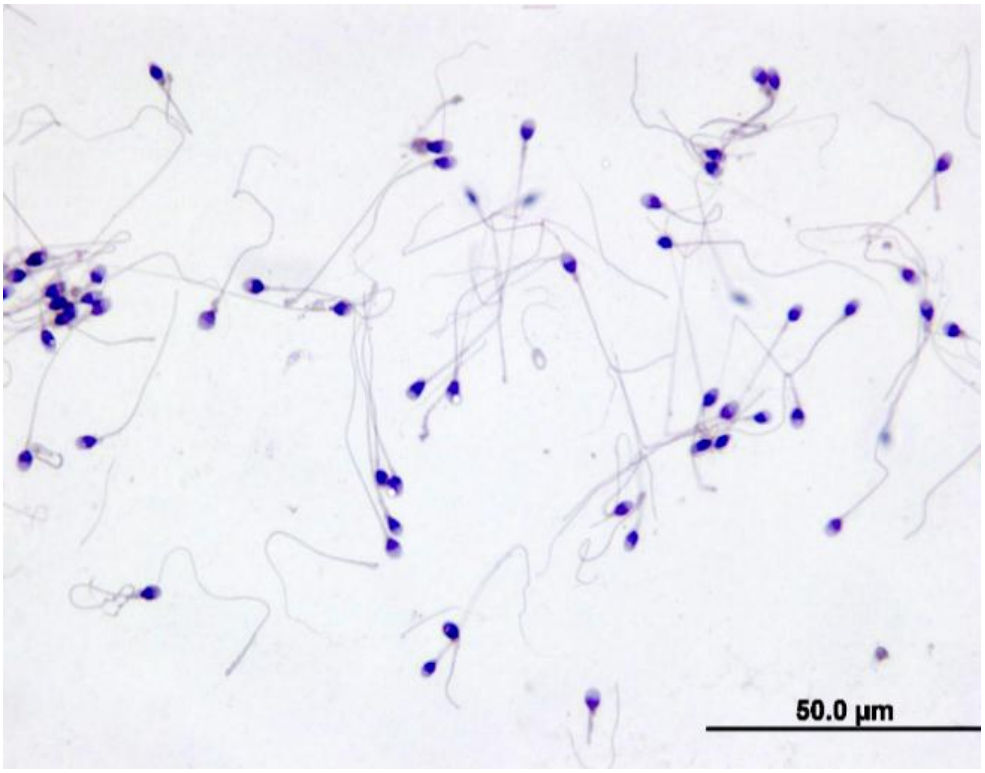

Figure 1. Sperm cells from the Control group displayed after staining. 100X objective. Scale bar: 50.0 µm.

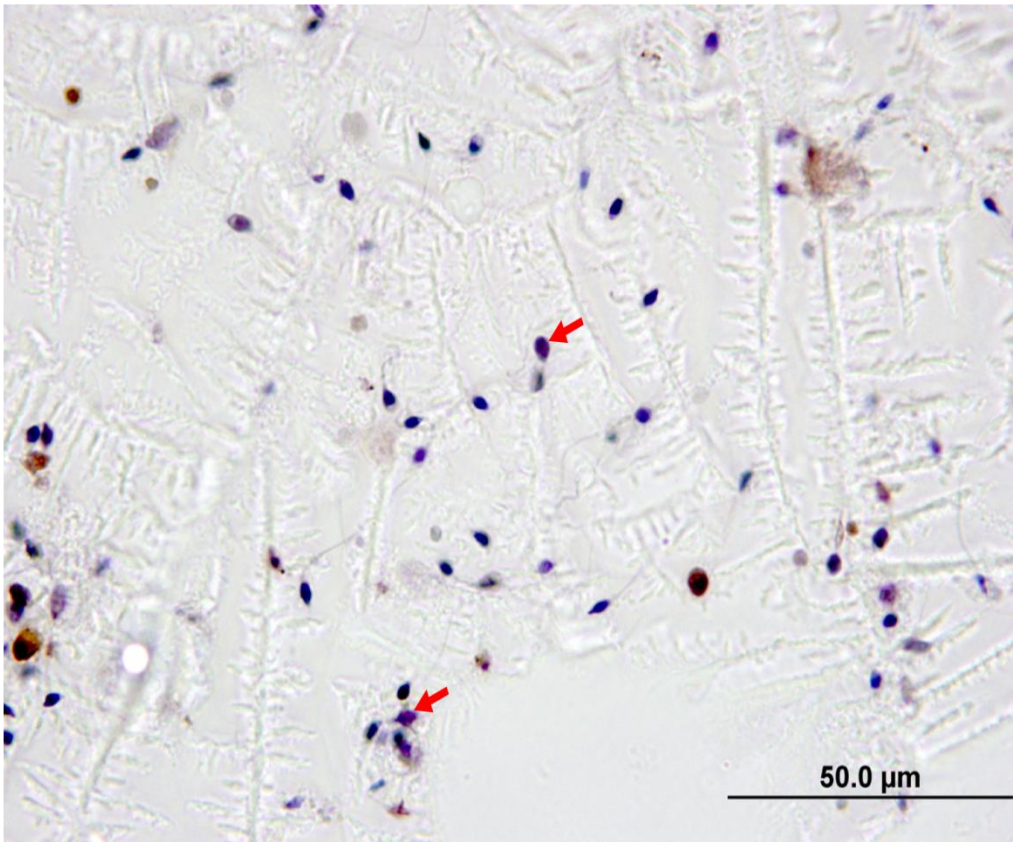

Figure 2. Sperm cells from the Oligozoospermia group displayed after staining, showing HSP70 expression (red arrow). 100X objective. Scale bar: 50.0 µm.

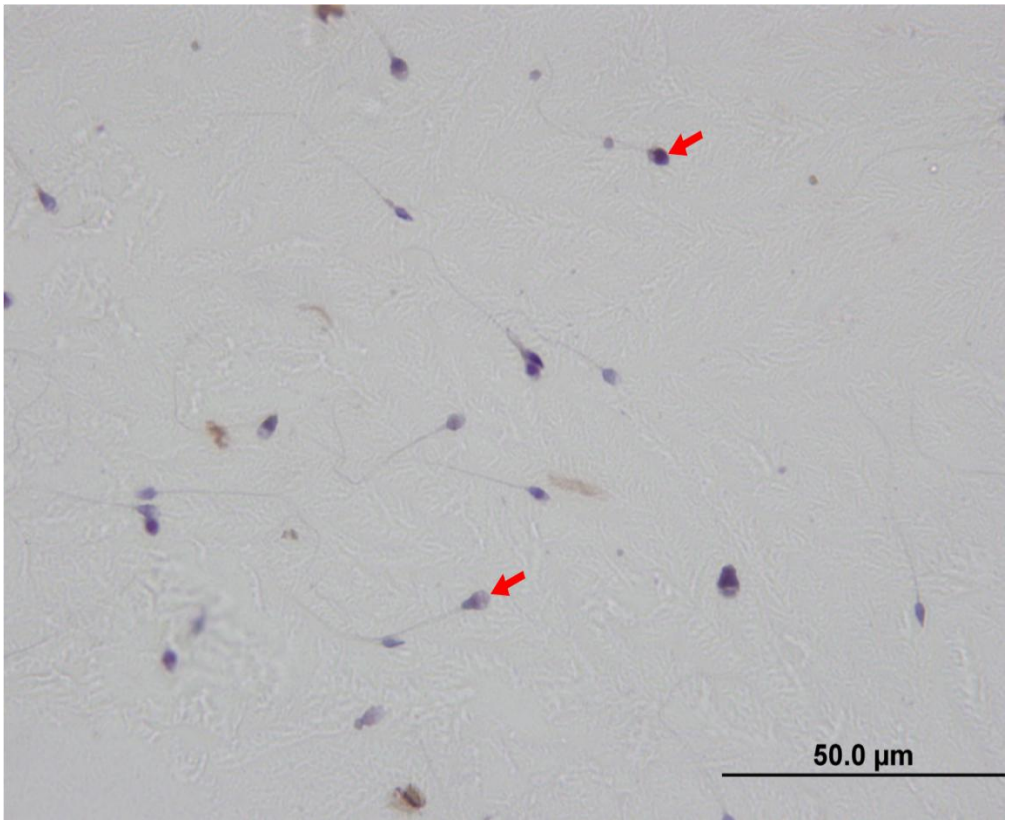

Figure 3. Sperm cells from the Severe Oligozoospermia group displayed after staining, showing HSP70 expression (red arrow). 100X objective. Scale bar: 50.0 µm.

The analysis revealed that HSP70 expression was higher in the Oligozoospermia group (Figure 2) compared to the Control group (Figure 1), and this difference was statistically significant ($p<0.05$). In contrast, HSP70 expression in the Severe Oligozoospermia group was lower compared to the Oligozoospermia group, and this difference was also statistically significant ($p<0.05$) (Figure 3). The HSP70 expression in the Severe Oligozoospermia group was similar to that in the Control group, with no statistically significant difference observed ($p>0.05$). The statistical data for HSP70 expression in all experimental groups are presented in Table 3.

Table 3. HSP70 expression measurement results for the experimental groups.

	Control	**Oligozoospermia**	**Severe Oligozoospermia**	***p***
HSP70	116.8(92.0-166.3)[a]	127.2(101-217.7)[b]	116.9(94.3-220.1)[a]	0.0001

Non-normally distributed data are expressed as median (minimum-maximum). No statistically significant difference was found between groups denoted by the same letter(a). A p-value of <0.05 was considered statistically significant.

Discussion

Clinical infertility refers to a couple's inability to achieve pregnancy despite attempting to conceive for 12 months. It is estimated that around 10–20% of couples worldwide experience infertility (12). Moreover, the concept of reproductive success is defined at the couple level, meaning that infertility etiology may be attributed to one or both members of the couple (13). Many studies estimate that male factors contribute to 30–50% of infertility cases (13, 14). Male infertility can arise from various factors, including genetics, testicular dysfunction, endocrinopathies, lifestyle factors (such as smoking and obesity), congenital anatomical factors, gonadotoxic exposures, and aging (15). Furthermore, studies have shown that an imbalance between high levels of reactive oxygen species (ROS) and insufficient antioxidant defense in semen can impair sperm function. ROS are naturally produced during cellular metabolism, and while low levels play a role in sperm capacitation, excessive ROS can damage sperm DNA, lipids, and proteins, leading to decreased motility, viability, and fertility potential (16, 17).

Male fertility is typically defined by semen quality. Clinical guidelines indicate that semen analysis should be performed during the initial evaluation of a couple experiencing infertility (4). Semen analysis is divided into two categories: macroscopic and microscopic. Macroscopic analysis evaluates characteristics such as volume, viscosity, color, odor, liquefaction time, and pH, while microscopic analysis involves parameters such as sperm count, concentration, morphology, motility, and the examination of leukocytes (5, 18).

The endocrinological evaluation of serum FSH and testosterone is not routinely recommended as a laboratory test for all infertile men, but it is necessary when oligozoospermia or azoospermy is present (19). In men, androgen production is primarily carried out by Leydig cells in the testes, and the production of testosterone, a steroid hormone, is crucial for spermatogenesis, male reproduction, and sexual function (20, 21). Testosterone concentrations in the testes are significantly higher than in serum, with one study reporting levels 80 times higher in the testes (22, 23). The lack of a significant difference in serum testosterone levels across groups in this study may be attributed to this. Testosterone is necessary for sperm production, sexual activity, and the development of secondary sexual characteristics. Its production is mediated by the hypothalamic-pituitary-gonadal axis, regulated by gonadotropin-releasing hormone (GnRH), LH, and FSH. This axis

ensures that testosterone produced in the testes provides negative feedback to the hypothalamus and pituitary, thereby reducing LH and FSH secretion (24). Considering that the normal range for total testosterone levels in our clinic is 300-1200 ng/dl, the testosterone levels in our experimental groups were within the normal limits. FSH and LH levels were found to be higher in both the Oligozoospermi and Severe Oligozoospermi groups compared to the Control group. Recent studies have shown that high FSH levels in idiopathic oligozoospermia and severe oligozoospermia may be associated with Leydig cell dysfunction and Sertoli cell dysfunction. Additionally, despite stable testosterone levels, the tendency for LH to increase suggests that Leydig cell function may not be entirely impaired, and instead, a compensatory mechanism may be at play to maintain androgen production (25, 26). Disruption or alteration of the hormonal balance is crucial for the regulation of organ functions, as complex physiological processes are required for organs to perform their duties. One such process, oxidative stress, is vital for maintaining structure and integrity, especially when accompanied by an impaired hormonal balance.

One of the intracellular mechanisms, molecular chaperones known as heat shock proteins (HSPs), has become an important research topic in various areas, particularly in infertility, cancer, and autoimmune diseases (27). Studies on the overproduction of HSP70 in several cell types support the idea that HSP70 increases in cells heading toward tumor development (28). Heat and stress resulting from varicocele activate HSPs, and increased expression of HSP90 in sperm from oligozoospermic men has been observed regardless of varicocele presence (29). In a study by Dangi et al., investigating the HSP gene profile in goats across different seasons, they found that HSP60 mRNA expression was not statistically significant across age groups in the winter but increased with age in the summer (30). Therefore, it was concluded that HSP60 expression may increase with age (31). A testis-specific member of the HSP70 family, HSPA2, has been reported to have decreased expression in infertile men, whereas supplementation with N-acetylcysteine increased its expression (32). Additionally, HSP70 presence has been shown in sperm with capacitation and acrosome reactions in wild boars and mature bulls (33). In a study attempting to elucidate the relationship between HSP70 and male infertility, it was reported that HSP70 expression may have increased in the sperm of infertile men as a protective mechanism against apoptosis (34).

In the present study, HSP70 expression in

sperm samples from individuals diagnosed with Oligozoospermia and Severe Oligozoospermia was higher in the Oligozoospermi group compared to the Control group, while in the Severe Oligozoospermi group, it was lower and similar to that of the Control group. This result is consistent with the testosterone levels observed in our study, suggesting a relationship between HSP70 expression and testosterone levels. Some studies have shown that azoospermia may develop in patients with severe oligozoospermia, while it does not occur in those with mild oligozoospermia. It is also known that initial FSH levels in severe oligozoospermic individuals differ from those of oligozoospermic patients (35). Moreover, the deletion of Nlrp14, a key regulator of primordial germ cell-like cell (PGCLC) differentiation, has been shown to cause reproductive failure, severe oligozoospermia, sperm abnormalities, and decreased HSP70 expression in both male and female mice (36). In oligozoospermic patients with varicocele, the HSP70 gene was found to be decreased compared to patients with varicocele but normal sperm count (37). Considering these results along with the findings of the present study, it is suggested that individuals with decreased HSP70 gene expression may not be able to perform the protective function of HSP70 proteins in response to environmental stress, which may lead to a reduction in sperm count. The results of this study further emphasize that HSP70 expression increases in the sperm of oligozoospermic men, but it does not increase in severe oligozoospermic patients. When the necessary conditions for maintaining the functionality of various intracellular mechanisms are not met, cellular damage from disturbed intracellular structures may lead to irreversible processes. The decrease in HSP70 expression in sperm from severe oligozoospermic men may indicate the presence of dysfunctional intracellular mechanisms. While sperm samples from patients diagnosed with severe oligozoospermia are analyzed visually, detailed mechanistic techniques for assessing functionality have not been employed.

The findings of this study, demonstrating decreased HSP70 expression in sperm samples, suggest that intracellular molecular chaperone mechanisms may be disrupted. To clarify this issue further, it is concluded that both mRNA expression and the HSP70 protein levels should be evaluated in conjunction with sperm count.

Conclusion

The production of healthy sperm relies on the collaboration of many complex physiological processes that create a harmonious microenvironment. Organ

functionality continues under the influence of hormones; however, disrupted intracellular mechanisms during production pave the way for diagnostic criteria such as sperm morphology, motility, and count, which are obtained in the laboratory. The results of this study revealed that there were no significant differences in the levels of FSH, LH, and testosterone hormones among different patients diagnosed with oligozoospermia. Additionally, the differing HSP70 expression in sperm samples from oligozoospermic and severe oligozoospermic patients indicates the presence of intracellular dysfunctional mechanisms. As a molecular chaperone, HSP70 plays a role in correcting disrupted functionality by increasing its expression within the cell. Although this study sought to identify the potential dysfunction of molecular chaperone mechanisms in the Severe Oligozoospermia group through immunohistochemical analysis of HSP70, further detailed analyses are needed to support these findings.

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During the preparation of this work the authors used ChatGPT 3.5 in order to improve language and readability, with caution. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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