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Research Article

THE CORRELATION BETWEEN HSPA2, CHOROMATIN DAMAGE, FERTILIZATION, AND EMBRYO QUALITY IN ASTHENOTERATOZOOSPERMIA INDIVIDUALS UNDER INTRACYTOPLASMIC SPERM INJECTION

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ABSTRACT. A testis-specific member of the HSP70 family is heat-shock protein A2 (HSPA2), which is recognized as a factor for succession ART (assisted reproductive technology) in male factor infertility and correlate with sperm maturation. This study aimed to investigate the correlation between HSPA2, chromatin damage, fertilization, and embryo quality in individuals with Asthenoteratozoospermia undergoing intracytoplasmic sperm injection (ICSI). Sperm were provided from 50 Asthenoteratozoospermia (ATZ), and 45 Normospermia (NS) ejaculations. Sperm parameters (concentration, motility, and morphology), DNA fragmentation index (DFI), protamine deficiency, and HSPA2 level on sperm, as well as the correlation between HSPA2 content and sperm parameters and embryo quality, were evaluated in the groups. ICSI was applied to achieve a successful outcome. The results indicated a significant decrease in HSPA2 in the ATZ group compared to the NS group, resulting in a decrease in sperm quality and DNA integrity ($p<0.001$). Fertilization rate ($p<0.05$), cleavage ($p<0.05$), and embryo quality ($p<0.001$) were significantly reduced in ATZ individuals compared to NS individuals. There is a significant correlation between HSPA2 expression with sperm motility and normal morphology ($p<0.05$). There was also a significant correlation between HSPA2 expression and DFI, protamine deficiency, and embryo quality ($p<0.001$). Aberrant HSPA2 expression has a key role in chromatin damage and fertilization processes.

Keywords: *Asthenoteratozoospermia, heat-shock protein A2, intracytoplasmic sperm injection, quality of embryo.*

INTRODUCTION

An increasing number of infertile couples are turning to assisted reproductive technology. About 40-50% of all infertility cases are associated with male factors [1]. Intracytoplasmic sperm injection (ICSI) is the best therapeutic option [2]. Sperm quality is a key determinant of ART success, which is usually assessed by semen analysis such as sperm motility, and sperm DNA fragmentation [3]. Large numbers of mRNAs in human sperm influence spermatogenesis events and sperm quality [4]. The correlation between sperm quality and gene expression has been studied in animals [5]. As a study, testicular gene analysis is an important marker for determining the function of genes in spermatogenesis and male infertility [6].

Heat shock protein family A member 2 (HSPA2), a molecular chaperone, is expressed within spermatogenesis [7]. An important role demonstrated for the HSPA2 gene in

human testis involves repairing DNA strand breaks, replacement of protamine during nuclear compression, and removal of cytoplasm during the later stages of sperm maturation [8]. This protein also functions as a calcium-binding protein in the capacity formation process and as a proper collector of sperm membrane proteins, i.e., channels [9]. Therefore, protein (HSPA2) expression is essential for sperm functions, including motility, acrosome reaction, and oocyte fertilization [10].

In both IVF and ICSI treatments, targeted gene disruption of HSPA2 resulted in increased chromosomal aneuploidy, meiosis failure, apoptosis, DNA fragmentation, and ultimately fertilization failure [11, 12]. The problem in determining functional sperm from individuals with low competence to fertilize oocytes reduces the success of the processes [13]. The role of HSPA2 in human fertility is not well understood despite its importance in spermatogenesis as well as fertility [14]. A critical requirement for ART is the introduction of effective genes as biomarkers for human fertility. Thus, this research aimed to investigate HSPA2 expression in sperm and to determine the correlation between HSPA2, chromatin damage, fertilization, and embryo quality in individuals with asthenoteratozoospermia undergoing intracytoplasmic sperm injection (ICSI).

MATERIALS AND METHODS

Ethical approval

The study was confirmed by the Institutional Review Board, and those who donated semen samples for this study completed an informed consent form. The Azad Medical University Human Research Ethics Committee (IR.IAU.Qom.REC.1396.55) confirmed this investigation.

Study design and sperm preparation

Sperm samples were collected from 50 Asthenoteratozoospermic (ATZ). 45 fertile subjects without clinical signs of infertility such as normozoospermia (NS) who attended the Infertility Research Center at the Academic Center for Education, Culture, and Research (ACECR), Qom, Iran, for ICSI. Only couples with Astheno-terato-zoospermic infertility participated in the study. Couples with other causes were excluded. All samples (2-5 ml) were analyzed for morphology (Papanicolaou stain), motility using the CASA system (LABOMED, SDC313B, Germany), and concentration (counting chamber) according to (WHO) guidelines [15]. After the semen was liquefied at room temperature for 30 minutes, a portion of the semen sample was rinsed with Ham's F-10 solvent. The sperm pellet was tested for DNA fragmentation, protamine deficiency by the TUNEL technique, as well as Chromomycin A3 staining and HSPA2 measurement by Western blot. Subsequently, the remaining samples were used for processing and motile, morphologically normal sperm were isolated by density gradient centrifugation (DGC) at 300 g for 20 minutes using gradient 80% and 40% (Origio, Denmark).

Assessing HSPA2 localization in sperm

In the NS and ATZ groups, the attendance of HSPA2 on the sperm surface was compared. 1×10^6 sperm were rinsed twice in FCM buffer (ice-cold PBS pH=7.2, consisting of 1% goat serum together with 2% FCS) at 300 g for 10 minutes. 100 µl of an affinity-purified rabbit anti-biomarker antibody was added. Spermatozoa were incubated with 100 µl of FITC-conjugated goat anti-rabbit antibody (Sigma-Aldrich- AP101P-

USA) for 30 min at 4°C. Sperm fractions were marked by propidium iodide (PI) to assess sperm vitality (Sigma-Aldrich- C-2587-USA). Samples were used without primary and secondary antibodies (evaluation of autofluorescence) or with primary antibodies only (negative control). A flow rate of FCM was used to analyze 10,000 sperm per sample (Partec PAS, Germany). FlowJo 7.5.4 (Tree Star, Ashland, USA) was selected to perform the analysis [16].

Assessment of HSPA2 level in sperm by RT-PCR

Real time polymerase chain reaction (RT-PCR) was carried out according to Lima et al [17]. Briefly, RNA was extracted by the RNXplus reagent (Cinnagen, Tehran, Iran) and treated with DNase I to remove DNA contamination. cDNA was synthesized by treating 2 µg of RNA with Re-vertAid H Minus M-MuLV reverse transcriptase (Fermentase Corporation, Vilnius, Lithuania). In order to detect the relative expression of target genes, RT-PCR was carried out by SYBR-Green/ROX qPCR master mix assay (Thermo scientific, USA) by gene-specific primers. *β-actin*: forward, 5′-CGT GAC ATT AAG GAG AAGCTG TGC-3′; reverse, 5′-CTCAGG AGG AGC AAT GAT CTTGAT-3′, and *HspA2*: forward, 5′-TTG TTG GAA GTC TTT GGT ATA-3′and reverse, 5′-CAT TTG CATTTA TGC ATT TGT-3′. Relative gene expression was determined as the abundance ratio of each target gene relative to β-actin. PCR was performed through administering heat for a period of 10 minutes at 94°C to achieve DNA denaturation, along with 36 complete cycles (1 min. at 94 °C, 1 min. at 57 °C, 1 min. at 72 °C), and finally an extension for 10 minutes at 72 °C. The Applied Biosystems Real-Time PCR System software was used to perform a melting curve analysis to verify the specificity of RT-PCR products. Moreover, the ΔΔCt method was used to measure the relative quantification of mRNA expression.

Assessing DNA fragmentation in sperm

The percentage of DNA fragmentation was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL-Promega Kit-G3250, USA). 200 sperm per slide at 100X magnification was counted by a fluorescence microscope (BX51, Olympus-Japan). Red-headed sperm had intact DNA. Sperm with a green head consisted of fragmented DNA [18].

Assessing protamine deficiency in sperm

Chromomycin A3 (CMA3) (Sigma-Aldrich- C2659-USA) staining was performed according to Nasr-Esfahani, et al. [19]. CMA3 estimated the predominance of chromatin in sperm samples. Initially, samples were rinsed in PBS and placed in Carnoy solvent. Each slide was treated with 100 µl of the CMA3 solution for 20 minutes. The spermatozoa with bright yellow heads (CMA3+) were defined as positive, while spermatozoa without brightness (CMA3-) were classified as negative. The percentage of CMA3+ sperm in each sample was described.

Assessment of the fertilization and embryo quality

After oocyte collection, oocytes were incubated in culture media and treated with hyaluronidase (Hyase; Vitrolife, Gothenburg, Sweden) in G- MOPS medium (Vitrolife, Gothenburg, Sweden). They were rinsed in fresh G- MOPS and transferred to G-IVF

(Vitrolife, Gothenburg, Sweden) under oil until the time of insemination. For ICSI, the DGC-processed sperm were placed in a central drop of polyvinylpyrrolidone solvent. They were chosen according to their morphology and motility. They were injected into the oocytes using an Eppendorf micromanipulator installed on a Nikon inverted microscope. To evaluate fertilization and embryo quality, oocytes were measured 48-72 hours after the ICSI technique using morphological parameters such as blastomeric fragmentation and regularity [20]. The fertilized oocytes showed two PNs. The quality of the embryos was divided into A-C. Grade A is the highest quality with equally symmetrical blastomeres as well as minor fragmentation (less than 10%). Grade B means moderate quality and equally symmetrical blastomeres with moderate fragmentation (25-30%). Grade C represents low quality, unevenly sized blastomeres, and high cytoplasmic fragmentation (up to 50%) [21].

Statistical analysis

Statistical analysis was performed with SPSS version 21 (SPSS Inc, Chicago, IL. USA). For the analysis, the two paired and independent paired t-tests were performed between the two groups. $P<0.05$ was considered significantly different. They were expressed as mean±SD. Spearman's technique was used to calculate the correlation coefficients.

RESULTS AND DISCUSSION

Assessment of sperm parameters

The data showed a significant decrease in sperm concentration ($P<0.001$), percentage of total motility (TM) ($P<0.001$), percentage of progressive motility (PM) ($P<0.001$), and normal morphology ($P<0.001$) in the sperm of the ATZ group compared to the sperm of the NS group. But immotile ($P<0.001$), percentage of DNA fragmentation ($P<0.001$) (Table 1, Fig. 1), and protamine deficiency ($P<0.001$) in the sperm of ATZ group were significantly increased compared to the sperm of NS group (Table 1).

Table 1. *The findings from sperm parameters between NS and ATZ groups.*

Sperm Parameters	ATZ group (n=50)	NS group (n=45)	P-value
Sperm concentration (10^6/mL)	36.52±1.80	51.06±2.51*	0.001
TM (%)	31.42±9.60	70.18±11.21*	0.001
PM (%)	20.48±8.57	44.54±10.08*	0.001
Immotile (%)	65.34±9.57	22.11±7.11 *	0.001
Normal morphology (%)	95.02±3. 16	98.02±1. 16*	0.001
DFI (%)	19.34±2.47	5.14±2.46*	0.001
Protamine deficiency (%)	47.33±6.09	20.77±4.28*	0.001

Values are mean±SD. NS: Normozoospermia; ATZ: Asthenoteratozoospermia; TM: Total Motility; PM; Progressive Motility; DFI: DNA fragmentation index.

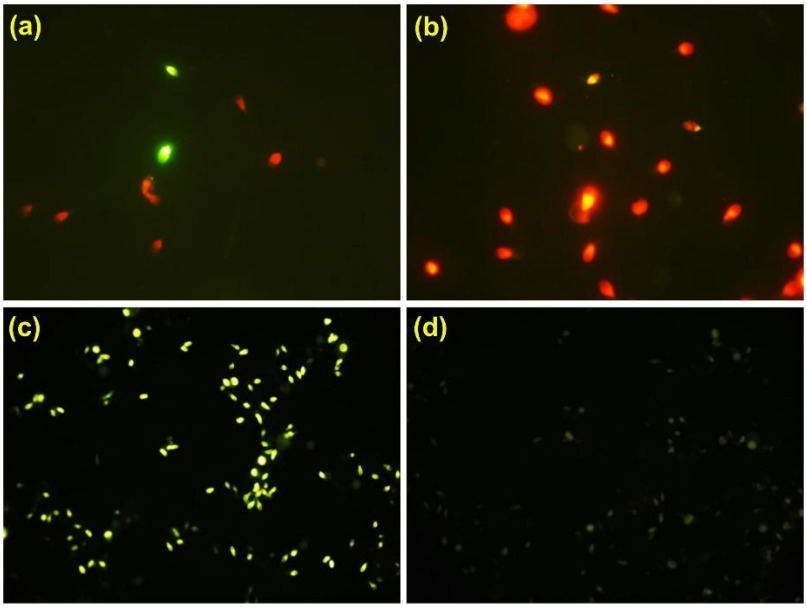

Fig. 1. *Comparing sperm DNA fragmentation to protamine deficiency in ATZ, NS groups. DNA fragmentation, as well as protamine deficiency levels in sperm from ATZ ejaculates (a, c), had significant enhancement in comparison to those in sperm from NS ejaculates (b, d). DNA fragmentation was evaluated via TUNEL assay. Red-staining sperm exhibits intact DNA, although green-staining sperm exhibit DNA fragmentation. Spermatozoa having bright yellow staining (c) were characterized by protamine deficiency (CMA3 positive). Those having dull yellow staining (d) exhibit normal amounts of protamine (CMA3 negative). NS: Normozoospermia; ATZ: Asthenoteratozoospermia.*

The level of HSPA2 localization

Immunostaining was selected to evaluate the localization of HSPA2 in the NS and ATZ groups. The percentage of HSPA2 in the spermatozoa was 3.7% (total head), 6.4% (posterior head), 11.16% (equatorial head), 9.14% (anterior head), 17.87% (midpiece), 6.95% (tail), 4.89% (cytoplasm), and 16.74% (membrane) (Fig. 2). Therefore, the localization of this protein should be compared between the NS and ATZ groups, whose percentages of HSPA2 in the anterior (13.88±3.56 for NS, 4.4±1.88 for ATZ; p=0.037) as well as equatorial regions (18.85±4.06 for NS, 3.47±1.09 for ATZ; p=0.008) showed a significant difference. Assessment of HSPA2 in samples revealed a significantly higher percentage of HSPA2 in fertile (NS) and infertile (ATZ) individuals (13.1±1.35 NS 5.6±0.83; P=0.017) and infertile (Fig. 3).

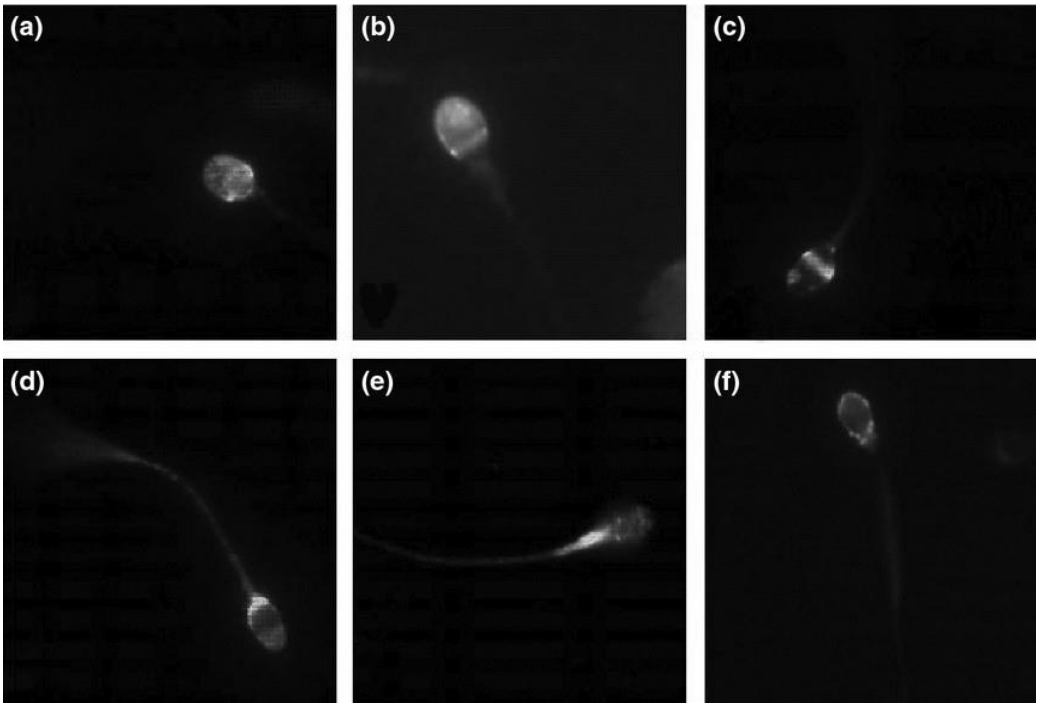

Fig. 2. *Measurement of HSPA2-positive sperm via immunocytochemistry. HSPA2 Localization on sperm by rabbit anti-HSPA2 as the primary antibody, goat anti-rabbit-FITC as the secondary antibody (a–f). (a) Total head, (b) anterior, (c) equatorial, (d) posterior along with tail, (e) midpiece along with the tail, (f) membrane.*

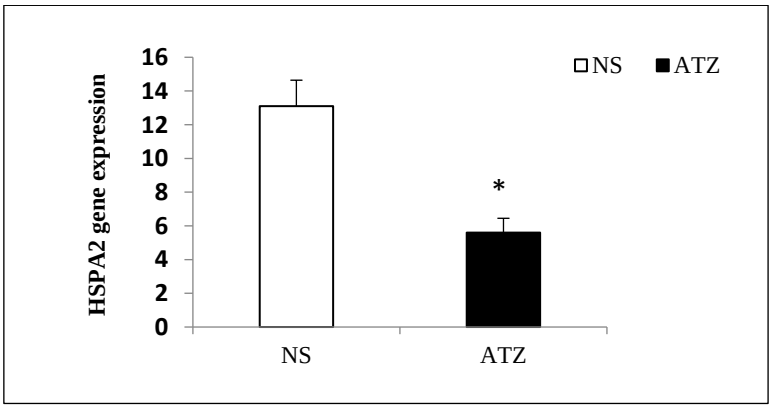

Fig. 3. *Comparison of relative expression of HSPA2 in fertile (NS) and infertile (ATZ) individuals by RT-PCR. NS: Normozoospermia; ATZ: Asthenoteratozoospermia. * P<0.01 is significant.*

Fertilization rate and embryo quality

ICSI results in both groups are presented (Table 2). We indicated the mean percentage of fertilization rate, cleavage stage ($P<0.05$), and grade A, B, and C class embryos ($P<0.05$) showed significant differences between the groups.

Table 2. *Comparing ICSI outcome between NS and ATZ groups.*

Parameters	ATZ group (n=50)	NS group (n=45)	P-value
Fertilization rate (%)	69.65±13.63	79.23±22.04	**0. 03**
Cleavage rate (%)	63.30±18.69	75.05±21.02	**0.02**
Embryo quality (grade A) (%)	27.09±8.01	50.27±5.87	**0.001**
Embryo quality (grade B) (%)	10.30±6.45	14.52±11.78	**0.01**
Embryo quality (grade C) (%)	5.3±1.6	1.9±0.6	**0.01**

Values reported are mean ± SD. Embryo with no fragmentation: grade A; Embryo fragmentation <20%: grade B; embryo fragmentation >20%: grade C. ICSI: Intracytoplasmic sperm injection; NS: Normozoospermia; ATZ: Asthenoteratozoospermia.

Correlation between HSPA2 level with sperm parameters, fertilization, and embryo quality

HSPA2 level significantly correlated with total motility (P<0.01), normal morphology (P<0.05), DNA fragmentation (P<0.001), and protamine deficiency (P<0.001). In addition, sperm HSPA2 level significantly correlated with fertilization rate (P<0.01), cleavage rate (P<0.01), and embryo grade A (P<0.001) (Table 3).

Table 3. *Correlations between HSPA2 level with sperm parameters, fertilization, and embryo quality.*

Correlation	HSPA2	
	r	P-value
TM	0.34*	**0.007**
Normal morphology	0.31*	**0.01**
DFI	-0.66**	**0.001**
Protamine deficiency	-0.53**	**0.001**
Fertilization	0.31*	**0.01**
Cleavage	0.27*	**0.01**
Embryo grade A	0.43**	**0.001**
Embryo grade B	0.08	0.35
Embryo grade C	0.07	0. 33

r: shows the Pearson correlation coefficient. The statistically significant correlation was p<0.05. Total motility (TM), DNA fragmentation index (DFI), grade A (embryo with no fragmentation), grade B (embryo fragmentation <20%). * Correlation at the 0.05 level (2-tailed) is significant. ** Correlation at the 0.01 level (2-tailed) is significant.

Due to the prominent role and function of HSPs in sperm, they can be introduced as one of the important markers of infertility. The main results from the study include: (a)

HSPA2 levels were decreased in the Asthenoteratozoospermia group compared to the Normozoospermia group, (b) the significant correlation between HSPA2 and sperm quality (sperm motility and normal morphology), sperm chromatin integrity, fertilization rate, and embryo quality. It has been indicated that HSPs play a key role in fertility when absent or abnormally expressed [8]. HSPs have two functions: First, they physiologically function as molecular chaperones (intracellular housekeeping proteins) by mediating the transport of other intracellular proteins [22, 23]. Second, they are activated as a result of cellular stress, such as the presence of oxygen free radicals [24]. Our results showed that the percentage of sperm expressing this protein in the anterior or equatorial regions increased significantly in the normozoospermia group compared with the asthenoteratozoospermia group. This difference has implications for the fertility of these individuals. It is consistent with other studies that have established the localization of HSPA2 as a key molecule for sperm-egg interaction on the surface of human spermatozoa and an association with sperm motility and fertility potential [10, 14, 25, 26].

These results suggest that sperm in the asthenoteratozoospermia group had decreased HSPA2 levels, increased DNA fragmentation, and abnormal chromatin packaging. This is consistent with studies [27] that examined HSP70 expression and found a significant association with sperm DNA fragmentation. Sperm parameters (motility, morphology) correlated significantly with HSPA2 levels in sperm. In the field of research on the relationship between HSPA2 and sperm motility. HSPA2 was found to be crucial for the appropriate function of the alkalization-activated selective Ca^{2+} channel, which affects sperm capacitation, especially sperm hyper-activation [28]. According to the results, fertilization, cleavage rate, and embryo quality were decreased in the asthenoteratozoospermia group. Also, the correlation between HSPA2 and ICSI outcomes. Several studies have shown that decreased HSPA2 levels in sperm cause impaired sperm-egg recognition as well as fertilization [29, 30].

There are two reasons that HSPA2 levels on spermatozoa plays a role in determining fertilization success rate following ICSI: (i) HSPA2 is required as a spermiogenesis-activating factor between sperm and oocytes for ICSI-based fertilization and embryonic cleavage; (ii) the hyaluronic acid ligand for HSPA2 of the Arg-Gly-Asp sequence (RGD) tripeptide receptor facilitates the common use of this biomarker in ARTs [30, 31]. On the other hand, studies have indicated a negative correlation of sperm DNA damage with pregnancy rate or embryo quality. Our results are consistent with previous studies that showed a decrease in ICSI outcomes in ejaculations with asthenoteratozoospermia due to HSPA2 and ADAM2 levels [31]. Current semen analysis can suggest male fertility, but the parameters are not able to determine exact defects, including oocyte fertilization and embryo development [32]. Therefore, it is necessary to develop modern techniques to determine effective genes in ART clinics.

CONCLUSION

Overall, HSPA2 level in sperm correlates with DNA integrity and ICSI outcomes. The data indicate that sperm HSPA2 content is predictive in determining sperm quality in infertile men. Indeed, sperm selection is based on HSPA2 level with the least chromatin damage for ART. However, more investigations are required to completely describe the efficiency of this biomarker in improving ART implications.

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Conflict of Interest. The authors declared that there is no conflict of interest.

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