

## Evaluating the Potential of Three Sperm Surface Antigens as Egg-adhesion Biomarkers for Human Sperm Selection

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### Abstract

**Background:** The selection of sperm with good genomic integrity and surface antigens is suggested for improving assisted reproductive technology (ART) outcome. The aim of this study was evaluating the heat shock protein (HSPA2), Dj-1 and serum amyloid P compound (SAP) three sperm surface proteomes as biomarkers for this purpose.

**Methods:** In this study, semen samples were obtained from 114 men who presented at Avicenna Fertility Clinic for their treatment. The semen characteristics, DNA fragmentation Index (DFI), chromatin maturation index (CMI), biomarker levels, and their embryo quality were considered. The paired-samples t-test and independent-samples t-test were used for analyzing the data and p-values<0.05 were considered significant.

**Results:** Outcomes exhibited the major reduction in HSPA2, DJ-1 and SAP following reduction in sperm quality and DNA integrity (p<0.001) with cut-off value of 14% (HSPA2), 12% (DJ-1) and 10% (SAP). The specificity of these three biomarkers was 95.2, 73.8 and 88.1%, respectively. Also, DFI (p<0.001), CMI (p<0.05), cleavage (p<0.05), and embryos quality (p<0.001) decreased significantly in abnormal spermogram (ANS) group in compared with normal spermogram (NS) group. It was shown that DFI was 97.1% in HSPA2, 76.5% in DJ-1 and 94.1% in SAP, and CMI was 95.0%, 75.50% and 87.5%, respectively. The significant correlation was found between of the three biomarkers and CMI (p<0.001), DFI (p<0.001) and embryos quality (p<0.001).

**Conclusion:** By comparing the efficiency of these three biomarkers for selecting sperm with the lowest level of chromatin damages, it seems that selection based on HSPA2 has significance over others.

**Keywords:** DJ-1, Heat shock protein A2, Serum amyloid P component, Sperm DNA.

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### Introduction

Condensing the sperm DNA over 10-fold has a vital role in keeping paternal DNA integrity compared with somatic cell nuclei (1). Studies proved that sperm DNA integrity is linked with sperm quality and male fertility (2). Furthermore, a strong reverse relation has been found between sperm DNA fragmentation and the rate of

clinical pregnancy in ARTs (3). Accordingly, more advanced techniques are required to evaluate the optimal quality of sperm and recognize the functional sperm from malfunctioning ones. While sperm preparation in ARTs with density gradient centrifugation (DGC) has been thought to result in enrichment of sperm with intact chromatin, based

on our knowledge, till now there is no reliable method for the clinical selection of sperm based on its DNA integrity and chromatin maturity and more improvements are necessary to progress it (3-6). Sperm-membrane proteins have a direct role in sperm-egg adhesion and fusion through fertilization (7). Many sperm selection methods exist based on sperm surface characteristics including electrophoresis, zeta potential, hyaluronic acid (HA), Annexine-V sperm sorting by magnetic activated cell sorting (MACs) and flow cytometer (FCM) these methods are applied at clinical level for diagnosis and treatment of severe male infertility particularly in cases of repeated implantation failure (RIF) (8-11). The selection of sperm with good genomic integrity and surface antigens is suggested for improving assisted reproductive technology (ART) outcome (12-16). Some of the main potential biomarkers involved in zona pellucida penetration, sperm binding and fertilization of oocyte, are heat shock protein (HSPA2), serum amyloid P compound (SAP), cysteine-rich secretory proteins (CRISP), fertilin  $\beta$  (F $\beta$ ), PH-20, DJ-1 and epididymis P34H protein (17-21). Presence of these molecules on the sperm surface and separation of sperm based on them depend on the accessibility of a specific ligand for planning a commercial test. Specific antigens like HSPA2, DJ-1 and SAP, as potent ligands for finding, splitting-up and measuring other recommended biomarkers were used in different investigations (22-24). These three biomarkers are assimilated with the sperm membrane developing through late steps of spermatogenesis in the testis and sperm maturation in epididymis and they improve capacitation which is involved in fertilization (25, 26). Presence of their receptors on human oocyte shows an essential character in sperm-oocyte communication and fertilization process (27). Fixing the DNA strand breaks, replacement of protamine through nuclear compaction and eliminating the cytoplasm in the last stages of sperm maturation are the particular functions for these three proteins in human testis (28). These three biomarkers also express in the equatorial piece of the matured sperm head and/or the tail through spermatogenesis in the testis (29, 30). Moreover, DJ-1 has a main role in androgen receptor-dependent transcriptional activity and oxidative stress (31-35). SAP in human shows both physiological and pathological participation in inflammation, immunity and apoptosis (36). In this study, the cut-off values of HSPA2, DJ-1 and SAP and their potential for sperm selec-

tion in ARTs and also, the ability of each biomarker to separate sperm according to its DNA safety considerations and their embryo cleavage and quality were evaluated as well.

### Methods

**Semen collection and preparation:** Semen specimens were obtained from 114 men at Avicenna Fertility Clinic affiliated to Avicenna Research Institute (Iran, Tehran). This study was approved by the bioethics committees of Avicenna Research Institute (Tehran, Iran) and written informed consent was gained from each donor. All subjects had no history of radiotherapy, chemotherapy, taking chronic medicine or varicocele and semen samples were collected from men after 48–72 *hr* of sexual abstinence. Semen was permitted to liquefy at 37 °C for 30 *min*. An aliquot of the sample was used for semen analysis and the mature sperm were separated by DGC method (300 *g* for 20 *min*) with PureSperm® (Nidacon International AB, Sweden). The sperm pellet was washed twice and aliquoted for using in following procedures. Semen parameters were measured by computer-aided sperm analysis (CASA) according to WHO guidelines (37). Sperm parameters were considered normal when sperm concentration was  $\geq 15$  million/ml, viability  $\geq 58\%$ , normal sperm morphology  $\geq 4\%$  and total sperm motility  $\geq 40\%$ . The specimens were classified according to normal and abnormal spermogram into two groups of NS group with normal spermogram and ANS group with abnormal spermogram.

**Biomarker levels:** The presence of HSPA2, DJ-1 and SAP on sperm surface was compared in NS and ANS groups. Firstly,  $1 \times 10^6$  sperm were washed twice at 300 *g* for 10 *min* with FCM buffer (Ice-cold PBS pH=7.2, containing 1% goat serum and 2% FCS). Then, 100  $\mu$ l of affinity purified rabbit antibody against biomarkers was added. Anti-HSPA2, anti-DJ-1 and anti-SAP (Avicenna Research Institute, Tehran, Iran) and incubated according to its protocol. Sperm were washed as described above and incubated with 100  $\mu$ l FITC-conjugated goat anti-rabbit (Abcam, Germany), for 30 *min* at 4 °C. In order to assess sperm viability, all sperm fractions were labelled with propidium iodide (PI) (Sigma, Germany). As a control, samples without any primary and secondary antibodies (Evaluation of auto-fluorescence) or without just primary antibodies (Negative control) were used. Ten thousand sperm were analyzed per sample with a flow rate of FCM (Partec PAS,

Germany). The differences among individual samples in the percentage of sperm above the set threshold level of fluorescence intensity were assessed and statistically compared. The analysis was done by FlowJo 7.5.4 software (Tree Star, Ashland, USA) (22).

**Sperm DNA fragmentation index (DFI) and chromatin maturity index (CMI):** The percentage of abnormal DNA and mature chromatin was reported as DFI and CMI. Semen samples were washed with PBS and diluted to reach  $1 \times 10^6$  sperm/ml concentration. The assessment of DFI and CMI were respectively approved by sperm chromatin structure assay (SCSA) (38) and Chromomycin A3 staining assay (39) according to the protocols. The procedure of SCSA involved Acridine Orange (AO) (Sigma, Germany) staining of sperm and subsequent flow cytometric measurement (40). To perform the assay, semen samples were placed on liquid ice; all succeeding steps were performed at 4°C. Samples were diluted with TNE buffer (0.15 mol/l NaCl, 0.01 mol/l Tris, 0.001 mol/l EDTA, pH=7.4) to obtain the sperm concentration of  $<2 \times 10^6$  sperm/ml. A 200- $\mu$ l aliquot was removed and admixed with 400  $\mu$ l of a low-pH detergent solution (0.15 mol/l NaCl, 0.08N HCl, 0.01% Triton X-100, pH=1.4). After 30 s, 1.2 ml of staining solution (6  $\mu$ g/ml AO, chromatographically purified in 0.2 mol/l  $\text{Na}_2\text{HPO}_4$ , 1 mmol/l disodium EDTA, 0.15 mol/l NaCl, 0.1 mol/l citric acid monohydrate, pH=6.0) was added, and the stained sample was placed into the flow cytometer sample chamber. Abnormal chromatin structure, defined as an increased susceptibility to acid or heat-induced denaturation in situ, was quantitated by FCM measurement of the metachromatic shift from green (Native DNA) to red (Denatured, single-stranded DNA) fluorescence (Olympus, Tokyo, Japan). Final result was presented as DFI (%).

The pellets were processed with a fixed Carnoy's solution for 5 min at 4°C and then were stained with 100  $\mu$ l of 0.25 mg/ml CMA3 (Sigma, Germany) solution at room temperature. After flow cytometric based CMA3 staining assay, samples which were exposed to CMA3 were washed twice with PBS and assessed by Partec PAS flow cytometer, using an argon laser with an excitation wave length of 488 nm. Flow cytometer from Chromomycin A3 stained sperm was collected in fluorescence detector-2 (FL-3) with a 585/42 nm

band pass filter. A minimum of 10000 sperm were examined for each assay and analyzed using flowjo software. A positive control was obtained by pre-incubating the spermatozoa with 200 mmol di-thiothreitol, a disulphide reducing agent, at 37°C for 10 min. The percentage of immature sperm chromatin was reported as CMI (%).

**Sperm viability and mitochondrial function assay:** Sperm viability was assessed by eosin-nigrosin assay according to WHO protocols by mixing approximately equal volumes of semen and stain (37). Sperm mitochondrial function was assessed by Rodamine123 (Rh123) (Sigma, Germany) staining based on its protocol (41).

Mitochondrial function was analyzed by FCM in Rodamine (Rh 123) stained sperm. Semen aliquot was washed and  $10^6$ /ml sperm were incubated in PBS containing 0.01 mg/ml Rodamine123 (R123) at 25°C for 10 min in dark place. The stained sperm were washed, centrifuged (300 g for 10 min) and incubated in PBS and immediately analyzed by FCM. The FCM analysis was performed using an argon laser at 488 nm for excitation (27).

**Fertilization and embryo quality:** Ovarian hyperstimulation was done according to the long luteal suppression protocol which uses GnRH $\alpha$  and with a combination of human menopausal gonadotropin (hMG). Ovulation was triggered by the administration of human chorionic gonadotropin (hCG). Oocytes were collected 36 hrs post hCG, using a simple lumen aspiration needle. Oocytes were retrieved by transvaginal ultrasound guided follicle aspiration. The oocytes containing cumulus cells were collected from clear follicular fluid. Granulosa cells were detached from collected oocytes using enzymatic and mechanical digestion. For all samples, sperm were injected (ICSI) to metaphase II (MII) oocytes to evaluate fertilization rate and embryo quality. The fertilized oocytes were evaluated by the presence of two pronuclei (2PN) in fertilized oocytes 18 hr after the injection. Embryo quality was estimated by morphological principles documented according to the fragmentation degree and the regularity of blastomeres 48–72 hr post-ICSI technique. Briefly, the embryos were categorized as grade A (Lacking fragmentation), grade B (Fragmentation<20%), and grade C (Fragmentation>20%) based on their quality (42). The embryos were transferred either at 48 or 72 hr stage.

**Statistical analysis:** All statistical analysis was done by the statistical package for social sciences (SPSS) version 19 (SPSS Inc, USA). The K.S test was used for controlling normal distribution and normal data presented as mean±SD. The paired-samples t-test and independent-samples t-test were used for analyzing and data. The p-values of <0.05 were considered significant. Pearson’s correlations were used for parametric variables. For the calculation of a cut-off point, differences in the expression rates were designed and cross-tab was used.

**Results**

The outcomes of all 114 semen samples are exhibited in table 1. With comparison of NS and ANS groups, it has been shown that the average male age (32.55±0.74 and 32.40±1.11, respectively in both groups), female age (31.38±0.73 and 32.40±0.56) and semen volume (4.37±1.04 and 4.94±1.76) had no statistically significant changes between two groups. But, the documents demonstrated that sperm concentration (p<0.001), progressive motility (p<0.001), normal morphology (p<0.001), DFI (p<0.001), CMI (p<0.001), viability (p<0.001), mitochondrial function (0.01), HSPA2 (p<0.001), DJ-1 (p<0.001) and SAP (p<0.001) were meaningfully lower in ANS compared with NS. The fertilization and embryo features were considered through visualizing two-PN and cleavage stage in one, two and three days after ICSI. The outcome indicated that there were statistically significant changes in embryo cleavage phase (p<0.05) and grade A embryo quality (p<0.001) among these two groups (Table 1).

In addition, the outcome exhibited the significant decrease of HSPA2, DJ-1 and SAP with regard to cut off value of 14%, 12% and 10%, respectively following the reduction in sperm quality. The specificity of these three biomarkers was 95.2, 73.8 and 88.1 % in NS group. In addition, among the sperm with biomarker levels higher than cut off 97.1% with HSPA2, 76.5% with DJ-1 and 94.1% with SAP normal DNA integrity was ob-

**Table 1.** The results of parameters evaluated between normal spermogram (NS) and abnormal spermogram (ANS) groups

Parameter	NS (n=42)	ANS (n=74)	p-value
Concentration (×10 <sup>6</sup> /ml)	45.57±1.76	32.40±1.11	p<0.001
Viability (%)	81.43±1.24	77.46±1.02	p<0.001
Mitochondrial function (%)	77.45±1.69	66.76±1.07	0.016
CMI (%)	20.62±0.86	23.51±0.82	0.029
DFI (%)	21.10±1.196	30.42±1.78	p<0.001
DJ-1 (%)	16.77±0.94	12.32±0.81	p<0.001
SAP (%)	15.81±0.83	10.43±0.68	p<0.001
HSPA2	27.88±6.58	15.01±7.27	p<0.001
Cleavage (%)	75.05±21.02	62.30±26.69	0.020
Grade A (%)	49.27±25.87	29.09±32.01	0.001
Grade B (%)	14.52±14.78	12.30±16.45	0.470
Grade C (%)	1.65±5.26	8.70±15.69	0.010

Note: Values are reported by mean±SD, DNA fragmentation index (DFI), chromatin maturation index (CMI), grade A (Embryo without fragmentation), grade B (Embryo fragmentation<20%), and grade C (Embryo fragmentation >20%)

served, but normal chromatin maturation was observed with levels higher than cut offs 95.0% with HSPA2, 75.50% with DJ-1 and 87.5% with SAP. The sperm analyzed with these three approaches in ANS group showed that, 78.4% with HSPA2, 67.6% with DJ-1 and 70.3% with SAP had normal DNA integrity, but 59.6% with HSPA2, 39.7% with DJ-1 and 44.11% with SAP had normal chromatin maturation (Table 2). The probable influence of these three biomarker levels on sperm and embryo quality were analyzed and are manifested in table 3. The major correlation was detected in DJ-1 and SAP level in sperm population with CMI (p<0.001), DFI (p<0.001), and grade A embryo (p<0.001). Moreover, the results presented while these three biomarkers had altered in their levels in NS and ANS groups, HSPA2 meaningfully has greater level (p<0.001) compared with others. After evaluating the correlation, the important correlations were detected between HSPA2 level in sperm population and progressive motility (r=0.32, p<0.01), CMI (r=-0.63, p<0.001), DFI (r=-0.66, p<0.001), cleavage (r=0.33, p<0.01), grade A embryo (r=0.52, p<0.001) and grade B embryo (r=0.28, p<0.05) which is shown in table

**Table 2.** The HSPA2, Dj-1 and SAP and the cut-off values in normal spermogram (NS) and abnormal spermogram (ANS) groups

Groups		HSPA2			DJ-1			SAP		
		Cut off 14%	DFI	CMI	Cut off 12%	DFI	CMI	Cut off 10%	DFI	CMI
NS (n=42)	Specificity (%)	95.2	97.1	95.0	73.8	76.5	75.50	88.1	94.1	87.5
	Sensitivity (%)	4.8	2.9	5.0	26.2	23.5	25.0	11.9	5.9	12.5
ANS (n=72)	Specificity (%)	48.6	78.4	59.6	43.1	67.6	39.7	48.6	70.3	44.11
	Sensitivity (%)	51.4	21.6	40.4	56.9	32.4	60.3	51.4	29.7	55.2

Note: DNA fragmentation index (DFI), chromatin maturation index (CMI)

**Table 3.** The correlation of HSPA2, Dj-1 and SAP with sperm parameters, fertilization and embryo quality

Parameters	HSPA2		DJ-1		SAP	
	r	p-value	r	p-value	r	p-value
Concentration ( $\times 106/ml$ )	0.05	0.656	0.17	0.164	0.19	0.180
Progressive	0.32	0.007**	0.14	0.232	0.13	0.283
Morphology	0.19	0.106	0.23	0.061	0.20	0.095
Vitality	0.08	0.509	0.008	0.94	0.29	0.011
Mitochondria function	0.28	0.173	0.11	0.360	0.09	0.00
DFI	-0.66	0.000***	-0.64	0.000***	-0.65	0.000***
CMI	-0.63	0.000***	-0.61	0.000***	-0.67	0.000***
Cleavage	0.31	0.008**	0.20	0.095	0.18	0.132
Grade A	0.52	0.000***	0.40	0.000***	0.40	0.000***
Grade B	0.28	0.020*	0.19	0.102	0.14	0.240

Note: r; indicates the Pearson correlation coefficient. The value of  $p < 0.05$  was considered significant. Sperm concentration (Concentration), DNA fragmentation index (DFI), chromatin maturation index (CMI), grade A (Embryo without fragmentation), and grade B (Embryo fragmentation  $< 20\%$ ). \*, \*\* and \*\*\* means  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , respectively

3. Also, DJ-1 and SAP levels had correlation with CMI ( $r = -0.64$ ,  $r = -0.65$ , respectively,  $p < 0.001$ ), DFI ( $r = -0.65$ ,  $r = -0.67$ ,  $p < 0.001$ ), and grade A embryo ( $r = 0.40$ ,  $r = 0.40$ ,  $p < 0.001$ ).

### Discussion

In this paper, the levels of HSPA2, Dj-1 and SAP were determined in sperm population in two groups of men with normal and abnormal spermogram. In addition, the correlations of sperm chromatin maturity-integrity and fertilization rate with these biomarkers were considered. Furthermore, the cut-off value of three biomarkers was compared in relation with DFI, CMI and embryo quality to find potential biomarkers for sperm selection with the lowest level of chromatin damage. These findings exhibited that, regarding the alteration of these biomarker levels in sperm of men with normal and abnormal semen parameters in ANS group, the sperm had lower levels of HSPA2, Dj-1 and SAP and higher level of DNA damage and abnormal chromatin packaging in comparison with NS group. This outcome was in line with other studies illustrating the localization of HSPA2, Dj-1 and SAP as an important sperm-egg interaction factor in human sperm surface and the correlation of them with sperm normal morphology, concentration, DNA fragmentation and fertility potential (43-45). In this paper, DNA fragmentation levels were correlated with abnormal sperm parameters which was reported previously by others (46, 47), although some investigators showed this relation with normal semen parameters too (48). As these three biomarker levels were very different in sperm of men with normal and abnormal semen parameters, so a representative

cut-off level for classifying characters and sperm based on these antigens appears to be an important step. As there was no transportation over the female reproductive tract in ICSI, a better understanding of the relationship between sperm DNA integrity and embryonic developmental potential is necessary. While modern semen analysis could predict male fertility, these parameters could not distinguish exact failings such as sperm immature chromatin and DNA damage. Accordingly, searches for identifying the conventional parameters to progress the predictability are required to find functional sperm from others (45). Sperm preparation for ARTs with differential DGC has been found to result in enhancement of sperm with intact chromatin, which in turn was likely to progress the risks of a successful clinical outcome (49). Whereas success rates were known to vary usually through clinics, more advances are necessary to develop it. Currently, new selecting sperm methods have been constructed on sperm molecular characterization and function. Selecting sperm binding to antibody for ICSI was thought to be one such advance. Numerous studies have presented that the sperm DNA damage was negatively correlated to pregnancy rate and embryo quality (50). The reduced level of these biomarkers from the human sperm led to a reduced power for sperm-egg recognition and fertilization following ARTs (12-16). This model was drawn considering the fact that these were taken in mature sperm and were preferably situated in the head area to be shared in oocyte (12, 13, 51). As indicated previously, there was no such standard in its place of routine sperm parameter test (Like morphology, count, motility, etc.), even though these parame-

ters do not have the necessary and reliable efficacy to accurately evaluate male fertility. Based on the outcomes of the current study, reduction in these biomarkers might account as two characteristics of sperm in ANS men. Our results were in agreement to earlier studies that had separately investigated these parameters in infertile men (44, 52). Regarding the outcomes of the current study, the reduction of these three biomarkers clearly exhibited chromatin abnormalities in ANS samples as was shown previously by others (53, 54). By comparing the efficiency and cut off of these three biomarkers with the lowest level of chromatin damages, it seems that selection resulting in HSPA2 has significance over others. Major negative correlation between the HSPA2, Dj-1 and SAP level and the sperm DNA fragmentation, and chromatin maturation might be apparent due to its role in sperm protamination and suitable folding of chromatin during sperm maturation in testis manifests that these biomarkers might be a good choice for selecting the intact sperm for ARTs (55). Hence, it seems that fixing the DNA strand breaks, replacement of protamine during nuclear compaction and eliminating the cytoplasm during the last stages of sperm maturation were the specific functions that were planned for these three biomarkers in human testis. However, HSPA2 has also a crucial role in these functions (44, 56).

### Conclusion

In conclusion, it is suggested that while HSPA2, Dj-1 and SAP had the potential to be a method for selecting sperm with the lowest level of chromatin damages in ARTs, it seems that sperm selection based on HSPA2 level can be improved for this purpose. As HSPA2 is induced in response to today's environmental agents like stress, air pollution, and oxidative stress (57, 58), this method may be helpful for selecting sperm with the lowest damage of reactive oxygen and nitrogen species.

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### Conflict of Interest

The authors declare that they have no competing interests.

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