Research Paper

Assessment of Fibronectin protein as a new biomarker for human sperm selection in ART

ABSTRACT

Fibronectin (FN), a multifunctional diametric glycoprotein on the surface of sperm, plays an important role in sperm-oocyte interaction and fertilization process. The aim of this study was to assess the FN levels as a sperm surface biomarker for sperm selection in assisted reproductive technology (ART). Polyclonal antibody against human FN was produced in rabbit. Its quality, purity and immune reactivity were assessed by SDS-PAGE and western blotting (WB). Also, the presence of FN on sperm surface was assessed using immunocytochemistry (ICC) and flow cytometry (FCM). The amount of FN and the sperm quality were assessed in normozoospermia (N) (42 men) and asthenoteratozoospermia (AT) (72 men) groups through Sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA) and chromatin maturation index (CMI). The results showed the FN distribution on the equatorial region of human sperm. Statistically significant differences were found in the FN levels of sperm surface between the two groups with 24.64±9.08% in N and 16.90±7.27% in AT (p≤0.0001). Also, FN level correlated negatively with SCD (p≤0.0001), SCSA (p≤0.0001), and CMI (p≤0.001). A threshold of FN level and DFI percentage respectively were 16 and 30, and were identified as a cut-off value to determine N with specificity of 83.3 and 81.0%, and sensitivity of 16.8 and 19.0%. A specificity and sensitivity of FN-DFI were 91.2 and 8.8%. It seems that FN can be used for selection of sperm with suitable quality, although future studies are recommended.

Key words: Sperm selection, fibronectin, normozoospermia, asthenoteratozoospermia, sperm chromati.

Mahnaz Heidari[1], Mahsa Darbandi[1], Sara Darbandi[1], Niknam Lakpour[1], Forog Torabi[1], Ghazaleh Cheshmi[1], Amirhasan Zarnani[1], Hodjat Zeraati[2], Mohammad Mehdi Akhoni[1] and Mohammad Reza Sadeghi[1,3]*

[1]Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.
[2]Departments of Epidemiology and Biostatistics, Medical Sciences, University of Tehran, Tehran, Iran.
[3]Monoclonal Antibody Research Center, Avicenna Research Institute (ARI), ACECR, ShahidBeheshti University, Evin, Tehran, Iran.

*Corresponding author. E-mail: mahsasaraa@hotmail.com. Tel: +98 2122432020; Fax: +982122432021.

INTRODUCTION

It has been estimated that around 15% of couples in reproductive-age are infertile; approximately half of them have male factor infertility. Assisted reproductive techniques (ART) such as intracytoplasmic sperm insemination (ICSI) and *In Vitro Fertilization* (IVF) are suitable and essential for most of male infertility treatment

(Organization, 2010). Recently, sperm selection is usually based on morphometric criteria such as motility and morphology, while sperm DNA integrity and chromatin maturity would not be assessed in ART (Zini et al., 2008). It has been demonstrated that sperm with normal motility and morphology may have DNA damage (Said and Land, 2011, Torki-Boldaji et al., 2017). Additionally, some sperm abnormalities, especially at the molecular levels, cannot be detected using routine methods of sperm selection in ARTs (Paasch et al., 2007). So, it is necessary to introduce new methods that enable selection of sperm without DNA fragmentation and chromatin integrity (Torki-Boldaji et al., 2017). To overcome these constraints, many sperm selection methods based on sperm surface biomarkers including electrophoresis, Zeta charge, hyaluronic acid (HA), Annexine-V sperm sorting by magnetic activated cell sorting (MACs) and flow cytometer (FCM) are available and used at clinical level for the diagnosis and treatment of severe male factor infertility especially in cases of repeated implantation failure (RIF) (Simon et al., 2013; Parmegiani et al., 2010; Saylan and Duman, 2016; Grunewald and Paasch, 2013; Gil et al., 2013; Barrier Battut et al., 2016; Gaucher et al., 2010). Some of the important potential biomarkers involved in zonapellucida penetration, sperm binding and fertilization of oocyte, are heat shock protein(HSPA2), serum amyloid P compound(SAP), ubiquitin (Ub), fibronectin (FN), cysteine-rich secretory proteins (CRISP), fertilinbeta (ADAM2), PH-20,DJ-1(PARK7) and epididymis P34H protein (Widlak and Vydra, 2017; Torki-Boldaji et al., 2017). Detection of these molecules on the sperm surface and separation of sperm according to them are dependent on the availability of a specific ligand for designing a commercial test. Today, hyaluronic acid is the only specific ligand available for sperm selection (Witt et al., 2016). Also, specific antibodies, as powerful ligands for detection, separation and measurement of other suggested biomarkers, were used in different studies (Capkova et al., 2016; Kaya and Memili, 2016; Rahman et al., 2017). Among them, FN is a diametric multifunctional glycoprotein presents on sperm surface and base membrane of the seminiferous tubules (Capkova et al., 2016). Binding of FN to the sperm surface is mediated by integrin presents in extracellular matrix (ECM) through recognizing the tripeptide amino acid sequence Arg–Gly–Asp (RGD) in FN. FN is incorporated into the sperm membrane developing during late stages of spermatogenesis in the testis and sperm maturation in epididymis which appears over the surface of human sperm after capacitation (Miranda and Tezon, 1992; Ekhlasi-Hundrieser et al., 2007; Fusi et al., 1992). The presence of FN receptor integrin on human oocyte plays an important role in sperm-oocyte interaction and fertilization process (Bronson and Fusi, 1996). The aim of this study was to assess the FN level of the sperm surface after capacitation, its relation with sperm quality and its potential for the detection of sperm defects in ART.

MATERIALS AND METHODS

Sperm collection and preparation

Semen specimens were obtained from 42 normozoospermic (N) men and 72 asthenoteratoozoospermic (AT) men of infertile couples, who were referred to the Avicenna Infertility Clinic affiliated to Avicenna Research Institute (ARI), Tehran, Iran. This study was approved by the bioethics committees of Avicenna Research center. Informed consent was obtained from each healthy donor. Semen samples were collected after 48–72 h of sexual abstinence. Briefly, individual semen samples were allowed to liquefy at room temperature and the sperm were separated from seminal plasma, immature germ cells and non-sperm cells through density gradient centrifugation (DGC) (300g for 20 min) using PureSperm® solutions (Nidacon, Gothenberg, Sweden). The sperm pellet containing normal sperm was washed twice using phosphate buffered saline (PBS), then capacitated sperm were prepared by incubating the previously washed sperm in Ham's F10 medium (Sigma, Germany) supplemented with 3.5% human serum albumin (HSA) for 3 h at 37°C and the aliquots were used freshly in subsequent techniques. Semen analysis was performed according to the World Health Organization guideline (WHO) (Organization, 2010) manual to determine semen volume, pH, morphology and sperm concentration. To analyze sperm motility, a computer-assisted semen analyser (CASA) system was used.

Spermatozoa morphology

From the sperm samples, smears were made, fixed in methyl alcohol, and stained using the Papanicolaou staining method for manual morphologic analysis and evaluated under the light microscope. A total of 200 spermatozoa were scored per slide with an oil immersion objective (Aksoy et al., 2012).

Production and characterization of polyclonal antibody against FN

Polyclonal antibodies of the FN against human sperm surface protein were generated in Avicenna Research Institute (ARI) Tehran, Iran. Briefly, following immunization of rabbits, anti-FN antibody was purified by protein G-affinity chromatography column (Amersham Pharmacia Biotech). To assess the reactivity of anti-FN antibody against FN, Immunochemical assays [ELISA, SDS-PAGE,WB, ICC] and FCM were carried out and compared with commercial FNantibody. As a positive control of FN, the FITC- conjugated goat anti-rabbit (Abcam, Germany) were used as the secondary antibody in all immunoassays

and human liver cells (HepG2 cell line).

Detection of the FN level on sperm

The presence of FN on sperm surface was compared in N and AT groups. Firstly, 1×10^6 sperm were washed twice at 300 g for 10 min and 4°C with FCM buffer (ice-cold PBS pH 7.2, containing 1% goat serum and 2% FCS). Then 100 µl of affinity purified rabbit anti-FN antibody (10 µg/ml) was added to each fraction and incubated for 60 min. Sperm were washed as described above and incubated with 100µl FITC-conjugated goat anti-rabbit (GIBCO, Inc. USA), for 30 min at 4°C. To assess sperm viability, all sperm fractions were labelled with 100 µl propidium iodide (PI) (Sigma-Aldrich, Germany) at a final concentration of 15 µg/ml for 5 min at room temperature. As an isotype control, we used sampled rabbit's IgG (Abcam, Germany) without any primary and secondary antibodies (evaluation of auto-fluorescence) or without just primary antibodies (negative control). Ten thousand sperm were analyzed per sample with a flow rate of FCM (Partec PAS, Germany). FCM analysis was performed (Partec PAS, Germany) by excitation lasers 488 nm (Coherent Saphire*488- 20* DPSS, filter *525/50, DM 505LP*) and *561* nm (MellesGriot 85- YCA- 25, filter 585/15, DM 565LP) to measure the fluorescent intensity in the Alexa Fluor 488 and Alexa Fluor *555* channels. Ten thousand sperms were analyzed per sample with a flow rate. Analysis was performed using FlowJo*7.5.4*. Software (TreeStar Inc., Ashland, OR, USA)(Evenson et al., 1982).

Sperm chromatin dispersion(SCD)

This test was performed using SDFA kit (Dain bioassay, Iran), according to the manufacture`s instruction. Briefly, 50 µl semen was diluted in Ham·s F10 medium and semen aliquot was mixed with 50 µl agarose (6.5%). Then 20µl of the mixture was loaded onto a pretreated glass slide and placed on a cold surface (4°C) for 5 min. Thereafter, the slides were treated with denaturing solution for 7 min and the lysing solution for 15 min. Following this step, the slides were washed with distilled water for 5 min and dehydration was perform using increasing concentrations of ethanol (70, 90 and 100%) and finally air dried slide was stained. At least 200 sperm were assessed in 100X magnification of microscope. Sperm with large or medium halo were classified as intact chromatin and those with no halo or small halo were classified as sperm with fragmented DNA. The results were presented as sperm DNA fragmentation index (DFI) (Fernandez et al., 2005).

Sperm chromatin maturation index (CMI)

The FCM based CMI staining assay was adapted from the

slide based method for assessment of sperm chromatin maturation index. Semen samples were washed with PBS and diluted to reach 10^6 sperm/ml concentration. Each sperm sample was evaluated with the fluorescent microscopy and FCM based CMI staining assay. The fluorescent microscopy was used to prepare the smear for slide-based method. Briefly, 10^6 sperm/ml sperm were washed using PBS (300 g, 5 min) and fixed with Carnoy solution (methanol and glacial acetic acid) for 5 min at 4°C.The fixed sperm were used for the preparation of thin smears. Each slide was treated for 20 min with 100 µl of 0.25 mg/ml Chromomycin A3 (CMA3) (Abcam, USA) solution in McIlvaine buffer (0.1M Citric acid, 0.2M $Na_2HPO_4.7H_2O$ and 10mM $MgCL_2$) at room temperature. The stained slides were then washed and mounted for microscopic assessment with the appropriated filter of 460-470 nm. (Olympus, Japan). In the next step, 200 sperm were assessed on each slide. CMI reacted sperm (CMI^+), as immature *sperm* with protamine deficiency were shown with *bright yellow* stain; however, *mature protaminated sperm* (CMI^-) were observed with *yellowish green* stain. For FCM, the concentration of sperm was adjusted to 10^6 sperm/ ml, and subsequently centrifuged and fixed with Carnoy's solution. The pellet was resuspended in 200 µl of CMA3 solution (0.25 mg/ml) for 1 h. Then, samples were washed twice with PBS and used for FCM. Fluorescence from CMA3 stained sperm was collected in fluorescence detector FL-3 with a 585/42 nm band pass filter. At least 10000 sperm were analyzed for each sample. Positive control was prepared by pre-incubating sperm with 200 mM dithiothreitol (DTT)(Sigma-Aldrich, Germany) at 37°C for 10 min (Shamsi et al., 2011).

Sperm viability assay

Sperm viability was analyzed by FCM in Rhodamine (Rh 123) stained sperm. Semen aliquot was washed and 10^6/ml sperm were incubated in PBS containing 0.01 mg/ml Rodamine123 (R123) (Invitrogen, USA) at 25°C for 10 min in the dark. The stained sperm were washed, centrifuged (300 g for 10 min) and incubated in PBS and immediately analysed by FCM. The FCM analysis was performed using an argon laser at 488 nm for excitation (Graham et al., 1990).

Evaluating of mitochondrial function

Semen aliquot was washed and 10^6 sperms were incubated with Rodamine123 (R123, Sigma, Germany) (0.01 mg/ml in water) at 25°C for 10 min, without light exposure. The stained sperm was washed and centrifuged (300g for 10 min), subsequently PI was added as previously described. Flow cytometric analysis was performed using an argon laser at 48 nm for excitation. Filter set up included a 515 nm long pass filter with a 457-505 nm laser blocker; a 550 nm dichroic beam splitter and a combination of a 525 nm
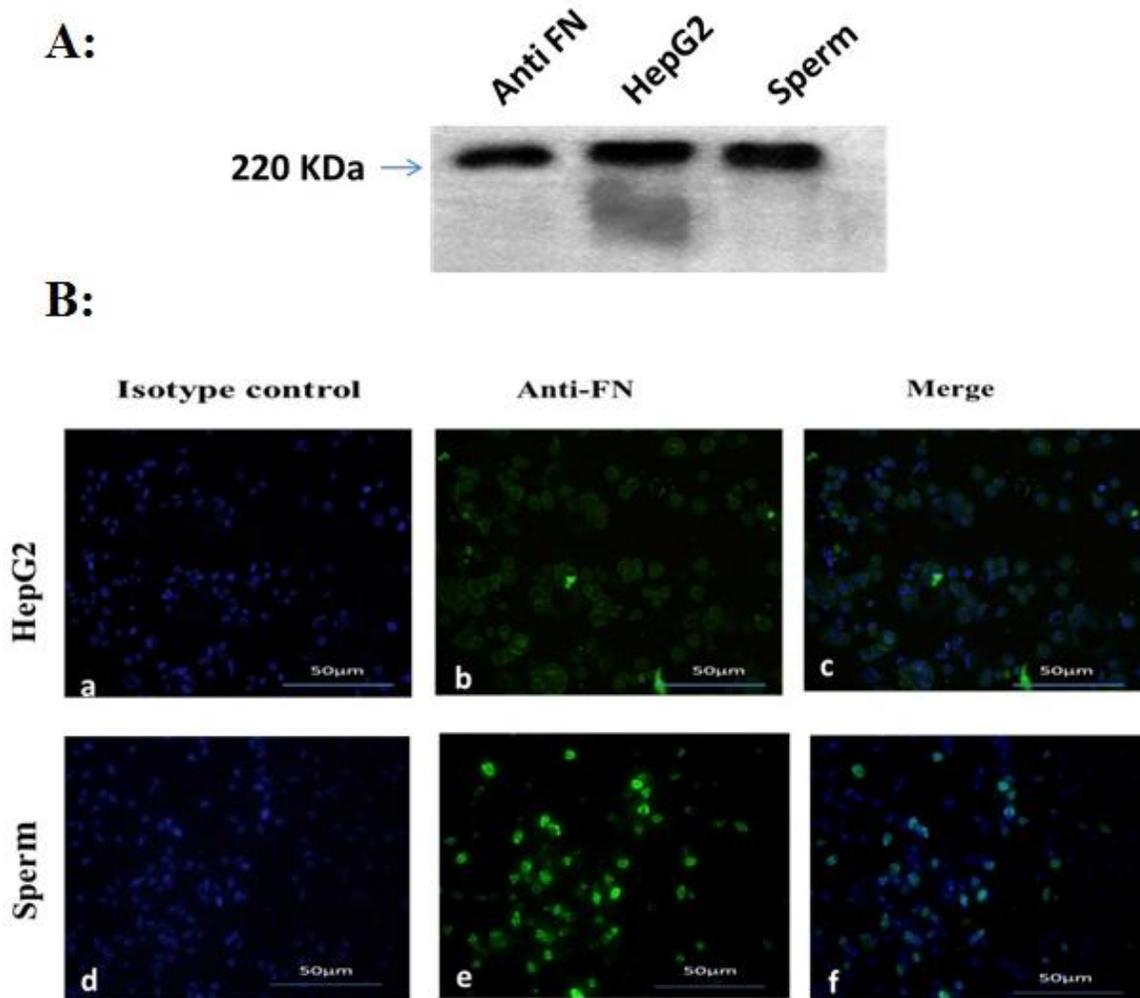
Figure 1: Affinity purification of FNin the sperm samples and positive control (HepG2 and FN protein). A:Position of the FNprotein recognized by Western blot**.**B:Immunocytochemistry (ICC) assay using anti FN antibody. Anti FN antibody was used to detect the presence of relevant proteins on the surface of HepG2 (a, b, c) and sperm (d, e, f). FITC (green color) and DAPI (blue color), a and d (the negative controls).

band pass with a 560 nm short pass filter for R123. Sperm samples were treated with DDW to validate the assay. It changes with mitochondrial activity which is associated with changes in R123 fluorescence intensity.

Sperm chromatin structure assay (SCSA)

Semen sample were prepared for SCSA and all succeeding steps were performed at 4°C. Samples were diluted with TNE buffer (0.15 mol/l NaCl, 0.01mol/l Tris, 0.001mol/l EDTA, pH7.4) to obtain concentration of about 2×10^6 sperm/ml. A 200µl aliquot was removed and admixed with 400 µl of a low-pH detergent solution (0.15 mol/l NaCl, 0.08N HCl, 0.01% Triton X-100, and pH 1.4). After 30 s, 1.2 ml staining solution (6 µg/ml AO, $0.2MNa_2HPO_4$, 1 mM disodium EDTA, 0.15M NaCl, 0.1M Citric acid monohydrate, pH 6.0) was added, and the stained sperm was placed into

the FCM sample chamber. Abnormal chromatin structure, defined here as an increased susceptibility to acid or heat-induced denaturation *in situ*, was quantitated by FCM measurement of the metachromatic shift from green to red fluorescence (Evenson, 2016). The percentage of abnormal DNAs was reported as DFI:

$$DFI\% = = \frac{\text{Red flurescene}}{\text{Total (red + green)} fluorescence}$$

Statistical analysis

All statistical analyses were carried out using the Statistics Package for Social Sciences (SPSS) version 19 (SPSS Inc., USA). Summarized statistics were presented as mean± SD and median. The two-paired t-test and independent paired

t-test were used for analyzing data, computed correlation coefficients (r) between the individual parameters and methods were tested. The P ≤ 0.05 was considered to be significant.

RESULTS

Characterization of the anti-FN antibody

Production of antibody against FN protein in rabbit and its reactivity against pure FN protein were examined by ELISA before and during immunization. The reactivity of affinity purified antibody was observed as a single band at 220 KDa by western blot (Figure 1A). Indirect Immunofluorescence assays were performed using affinity purified anti-FN antibodies, the green fluorescence on the surface of sperm and HepG2 cell line was the indicator for the presence of FN (Figure 1B).

Semen analysis and FN detecting

A total of 114 samples (42inN and 72in AT groups) were assessed and summarized in Table 1. The data showed that sperm concentration (P≤0.0001), progressive motility (P≤0.001) and normal morphologically (P≤0.001) in AT group had significant differences as compared with N group. The percentage of FN level in N group (24.64±7.43%) was significantly higher than the AT group (16.90±7.27%) as is shown in Table 1 and Figure 2 (P≤0.0001). As shown in Table 2, the cut off value of FN expression was estimated to be 16 with 83.3% specificity and 16.7% sensitivity in N and 47.2% specificity and 52.8% sensitivity in AT group.

Sperm chromatin integrity

In N and AT groups, the sperm chromatin integrity was reported by CMI and DFI using SCD and SCSA with the percentage of CMI (23.79±6.34 vs 32.33±11.15), SCD (21.10±7.75 vs 30.42±15.13) and SCSA (21.98±10.46 vs 31.32±14.16). The data showed that sperm CMI (P≤0.0001), SCD (P≤0.0001), and SCSA (P≤0.001) percentages in AT group had significant differences as compared with the N group (Table 1).

Vitality

Sperm vitality was assessed by eosin-nigrosin and Rhodamin123. The percentage of sperm vitality in the group N and AT, respectively were (81.43±8.019 vs 77.45±10.99) and (79.44±10.72vs 66.76± 9.08). As was shown in Table 1, the sperm vitality percentage was not

significant between the two groups (P>0.05). To determine the possible effect of FN level on sperm quality, the correlations between FN expression level and chromatin integrity index were analyzed (Table 3). Significant negative correlations were found between FN expression level and the SCD(r=-0.722, P<0.0001), CMI (r=-0.742, P<0.0001) and SCSA(r=-744, P<0.0001).

DISCUSSION

In the present study, we detected the sperm chromatin maturity and sperm DNA integrity by SCSA, SCD and CMI assays and compared the relation of FN level on the sperm surface with sperm vitality, chromatin compaction and maturation. This findings showed that AT men as compared with N, had lower percentage of sperm FN level, higher level of sperm DNA damage and abnormal chromatin packaging. It seems that the expression level of this protein depended on sperm DNA integrity and chromatin maturity. Based on the fact that the sperm FN level has very wide range in men with normal and abnormal sperm parameters, so we demonstrated a cut-off level for categorizing individuals and sperm based on FN levels, with value estimated to be 16 with specificity 83.3 and 47.2%, and sensitivity 16.7 and 52.8% respectively in N and AT men. It seems that FN can be used for selection of sperm with suitable quality and chromatin compaction. To the best of our knowledge, these results had not been reported by other researchers. Today, several studies presented new biomarkers to separate mature sperm with the best DNA compaction and maturation, including positive and negative biomarkers. If negative biomarkers were used, only defected sperm were removed from their population, while positive could select the best forms for ICSI. Although these methods could select the more healthy sperm from defect and abnormal, most of them damaged sperm motility and functions and therefore need to be improved and optimized before being applied routinely in daily practice of ART (Sutovsky et al., 2015). FN is expressed during late stages of spermatogenesis in the testis, and localized on testicular and epididymal sperm (Fusi and Bronson, 1992; Fusi et al., 1992; Schaller et al., 1993). Previous studies had shown the presence of FN on sperm, and semen played a significant role in fertilization process (Martinez-Leon et al., 2015). FN is a multifunctional adhesive glycoprotein, widely distributed in human tissues (Martinez-Leon et al., 2015). It is known to assist various cellular regulation processes, including cell growth and migration, differentiation, hemostasis and male reproduction. It can exist in both plasma and cell surface, with similarity in the physiological functions of cell adhesion to various biological molecules (Attia et al., 2011). Also FN on the sperm head can bound to integrins and a specific RGD sequences on the oocyte surface (Bronson and Fusi, 1996). To evaluate the efficacy of FN as a positive or negative

Table 1: The results of parameters evaluated between N and AT groups.

Parameter		N (n=42)	AT(n=74)	P value
Semen volume(ml)		4±1.04	4.54±1.76	n.s
Age(y)		32.54±4.21	31.98±4.66	n.s
Sperm concentration(x106/ml)		45.57±11.42	32.40±9.41	0.001
Normal morphology%		5.60±1.73	2.54±1.09	0.001
Progressive motility%		50.57±10.00	28.57±11.30	0.001
FN (%)		23.88±6.93	16.76±7.35	0.000
DFI (%)	SCD (%)	21.10±7.75	30.42±15.13	0.000
	SCSA (%)	21.98±10.46	31.32±14.16	0.000
CMI- (%)		23.79±6.34	32.33±11.1567	0.001
Rh123 (%)		77.45±10.99	66.76±9.08	0.000
viability (%)		81.43± 8.019	77.45±10.99	NS

Values are mean±SD, NS: no significant, normozoospermia (N), asthenoteratozoospermia (AT), sperm chromatin structure assay (SCSA), chromatin maturation index (CMI), Rhodamine (Rh 123), DNA fragmentation index (DFI), Fibronectin (FN).
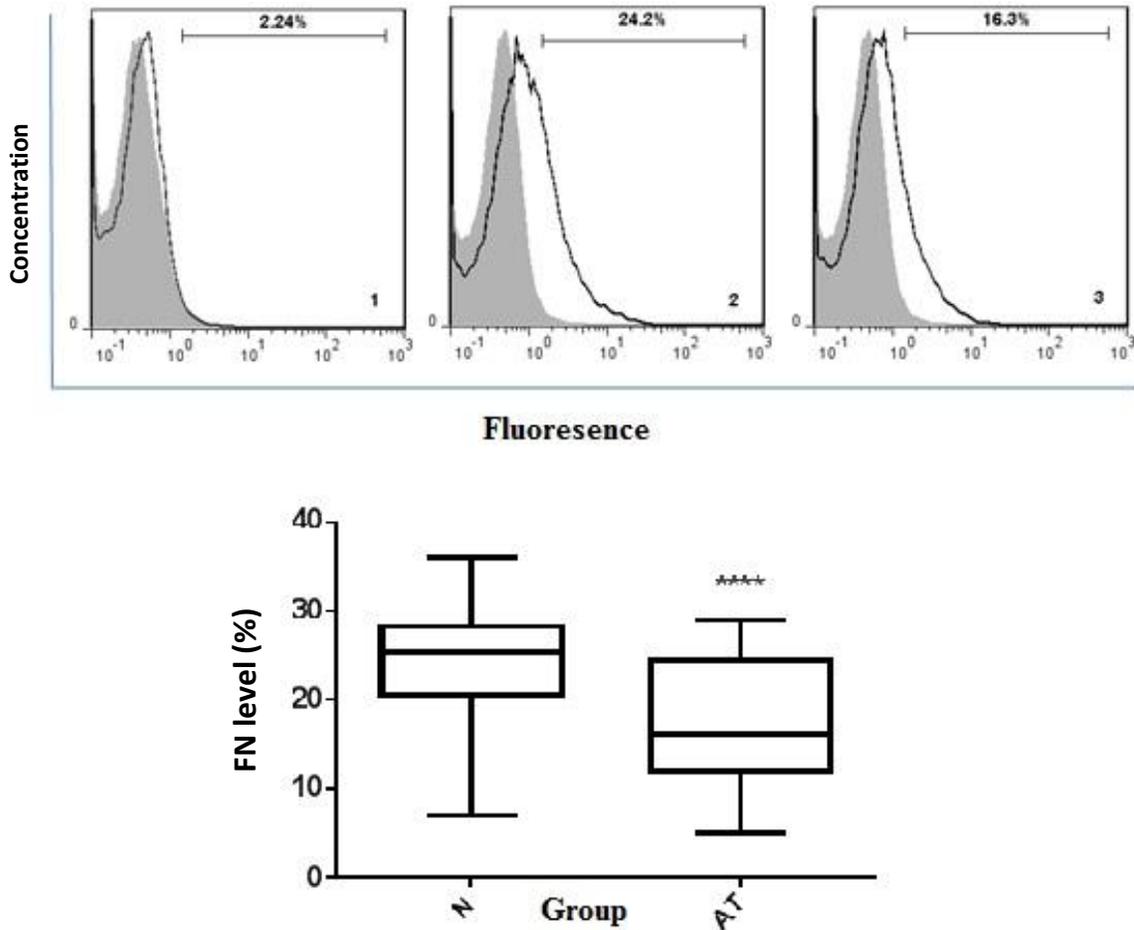

Figure 2: The level of FN on sperm surface 1. Isotype control sperm; 2. Sperm sample of N group; 3. Sperm sample of AT group.

Table 2: The cut-off values in A and AT groups.

Group		FN	DFI		FN-DFI
			SCD	SCSA	
N (n=42)	Cut-off value	>16	<30	<30	FN>16 & DFI<30
	Specificity (%)	83.3%	81.0%	81.0%	91.2%
	Sensitivity (%)	16.7%	19.0%	19.0%	8.8%
AT (n=72)	Cut-off value	<16	>30	>30	>30
	Specificity (%)	47.2%	51.4%	51.4%	81.1%
	Sensitivity (%)	52.8%	48.6%	48.6%	18.9%

Normozoospermia (N), asthenoteratozoospermia (AT),sperm chromatin structure assay (SCSA), DNA fragmentation index (DFI), Fibronectin (FN).

Table 3: The correlation between the sperm FN level sperm and sperm parameters.

Parameter		r	P value
Sperm concentration(x106/ml)		0.053	0.656
progressive		0.315**	0.007
morphology		0.192	0.106
CMI		-0.626**	0.000
DFI	SCSA	-0.660**	0.000
	SCD	-0.738**	0.000
Rhodamin123		0.280	0.017
mortality		-0.398**	0.001

r; indicates the Pearson correlation coefficient. The statistically correlation P ≤ 0.05 was considered to be significant.

biomarker, we need a gold standard of sperm function and male fertility. However, as mentioned previously, there are no such efficient standard methods until now and just parameters such as morphology, concentration and motility are used as standard. These parameters do not have suitable reliability to accurately evaluate male fertility. Therefore, we evaluated FN levels as a usable biomarker to select sperm according to their fertilizablity. As the high levels of FN were found in healthy sperm, therefore FN can be regarded as a positive biomarker to select more functional sperm in ARTs especially in ICSI. Significant negative correlation between FN level on sperm and its DNA fragmentation, chromatin maturation and chromatin integrity confirmed that FN could be a positive biomarker for selection of an intact sperm in ART. We assessed the level of FN not only related to routine parameters of human sperm but also related to functional parameters of sperm such as mitochondrial status, chromatin maturation and DNA integrity. This result is in accordance with studies which showed that localized FN on human sperm surface is an essential factor for sperm-egg adhesion that can be correlated with its normal morphology and functional integrity of sperm (Pinke et al., 1997; Glander et al., 1987;

Hoshi et al., 1994). The positive correlation between FN level, chromatin maturation and DNA integrity in normozoospermia may be due to the role of FN in sperm protamination and suitable folding of chromatin during sperm maturation in the testis and epididymis (Huszar et al., 1997; Varghese et al., 2009). While significant lower level of FN in defect sperm in AT may be indicator for spermatogenesis failure, leading to disorder in sperm function and male infertility. One of the essential steps in spermatogenesis is DNA packaging and condensation which is synchronic with FN expression on sperm during spermatogenesis (Miranda and Tezon, 1992; Gaucher et al., 2010). The defect in chromatin packaging using protamine during spermatogenesis and increasing DNA fragmentation in AT sperm, may be simultaneous with lower FN level which can directly or indirectly affect the process of fertilization (Ni et al., 2016). Moreover, our results were in accordance with studies which estimated that infertile men with low sperm motility and morphology had increased DNA fragmentation, DNA damage and protamine deficiency (Lopes et al., 1998). Another concern was that unlike the PH-20 receptor, which binds to hyaluronic acid ligand in PICSI dish, anti FN antibody was used in our assays despite

the presence of integrin and other FN receptors in the connective tissue. Since this antibody had an animal origin (mouse or rabbit), it was not possible to use in routine preselection or processing of human sperm in ARTs. Accordingly, the alternative method for the use of antibodies with animal origin was the use of RGD sequences receptors for FN *in vitro*. Future studies could be designed using three peptides sequence Arg–Gly–Asp (RGD) on a suitable support of stationary phase to select normal and healthy sperm with FN at its surface for sperm selection in ARTs.

CONCLUSION

It can be concluded that sperm chromatin maturation and DNA integrity correlated with FN level in sperm. The present data strongly suggest that FN levels in sperm had a predictive value in determining the quality of sperm in infertile men. This study showed that the FN levels in sperm could be potentially used as a biomarker in sperm selection and assessing the quality of sperm in ART. These results might help clinicians and scientists to further understand the clinical values of FN levels. However, further studies are needed to fully explain the feasibility and efficacy of this biomarker in improving ART outcomes. In addition, further studies on fertile men, unexplained infertility, pure asthenozoospermia and teratozoospemia failures can further determine the position of this biomarker in male infertility and sperm selection in future.

REFERENCES

Aksoy E, Aktan TM, Duman S, Cuce G (2012). Assessment of spermatozoa morphology under light microscopy with different histologic stains and comparison of morphometric measurements. Int. J. Morphol. 30(4):1544-1550.

Attia A, Hassan A, Zalata A, Hagag M, Yousef K ,Mostafa T (2011). Seminal fibronectin in fertile and infertile males. Andrologia. 43(6):387-391.

Barrier Battut I, Kempfer A, Becker J, Lebailly L, Camugli S ,Chevrier L (2016). Development of a new fertility prediction model for stallion semen, including flow cytometry. Theriogenology. 86(4):1111-1131.

Bronson RA ,Fusi FM (1996). Integrins and human reproduction. Mol. Hum. Reprod. 2(3):153-168.

Capkova J, Kubatova A, Ded L, Tepla O, Peknicova J (2016). Evaluation of the expression of sperm proteins in normozoospermic and asthenozoospermic men using monoclonal antibodies. Asian J. Androl. 18(1):108.

Ekhlasi-Hundrieser M, Schäfer B, Philipp U, Kuiper H, Leeb T, Mehta M, Kirchhoff C, Töpfer-Petersen E (2007). Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related. Gene. 392 (1):253-265.

Evenson DP (2016). The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim. Reprod. Sci. 169: 56-75.

Evenson DP, Darzynkiewicz Z, Melamed MR (1982). Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J. Histochem. Cytochem. 30 (3):279-80.

Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, Lafromboise M, De Jonge C (2005). Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil. Steril. 84(4):833-42.

Fusi F, Bronson R (1992). Sperm surface fibronectin expression following capacitation. J. Androl. 13(1): 28-35.

Fusi F, Lorenzetti I, Vignali M ,Bronson R (1992). Sperm surface proteins after capacitation. J. Androl. 13(6):488-497.

Gaucher J, Reynoird N, Montellier E, Boussouar F, Rousseaux S ,Khochbin S (2010). From meiosis to postmeiotic events: the secrets of histone disappearance. FEBS J. 277(3): 599-604.

Gil M, Sar-Shalom V, Melendez Sivira Y, Carreras R ,Checa MA (2013). Sperm selection using magnetic activated cell sorting (MACS) in assisted reproduction: a systematic review and meta-analysis. J. Assist. Reprod. Genet. 30(4): 479-485.

Glander HJ, Herrmann K, Uf H (1987). The Equatorial Fibronectin Band (EFB) on Human Spermatozoa-a Diagnostic Help for Male Fertility? Andrologia. 19(4): 456-459.

Graham JK, Kunze E ,Hammerstedt RH (1990). Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod. 43(1): 55-64.

Grunewald S, Paasch U (2013). Sperm selection for ICSI using annexin V. Spermatogenesis: Methods and Protocols. pp. 257-262.

Hoshi K, Sasaki H, Yanagida K, Sato A, Tsuiki A (1994). Localization of fibronectin on the surface of human spermatozoa and relation to the sperm-egg interaction. Fertil. Steril. 61(3):542-547.

Huszar G, Sbracia M, Vigue L, Miller DJ ,Shur BD (1997). Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1, 4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol. Reprod. 56(4):1020-1024.

Kaya A, Memili E (2016). Sperm macromolecules associated with bull fertility. Anim Reprod Sci. 169: 88-94.

Lopes S, Jurisicova A, Casper RF (1998). Gamete-specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. Human reproduction (Oxford, England). 13(3): 703-708.

Martinez-Leon E, Osycka-Salut C, Signorelli J, Pozo P, Perez B, Kong M, Morales P, Perez-Martinez S, Diaz ES (2015). Fibronectin stimulates human sperm capacitation through the cyclic AMP/protein kinase A pathway. Hum. Reprod. 30(9): 2138-2151.

Miranda PV, Tezon JG (1992). Characterization of fibronectin as a marker for human epididymal sperm maturation. Mol. Reprod. Dev. 33(4): 443-450.

Ni K, Spiess AN, Schuppe HC, Steger K (2016). The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis. Andrology. 4(5):789-99.

Organization WH (2010). Examination and processing human semen. World Health Edition, F. 286

Paasch U, Grunewald S, Glander H (2007). Sperm selection in assisted reproductive techniques. Soc. Reprod. Fertil. Suppl. 65: 515-525.

Parmegiani L, Cognigni GE, Ciampaglia W, Pocognoli P, Marchi F ,Filicori M (2010). Efficiency of hyaluronic acid (HA) sperm selection. J. Assist. Reprod. Genet. 27(1):13-16.

Pinke LA, Swanlund DJ, Hensleigh HC, Mccarthy JB, Roberts KP ,Pryor JL (1997). Analysis of fibronectin on human sperm. J. Urol. 158(3):936-941.

Rahman MS, Kwon WS, Pang MG (2017). Prediction of male fertility using capacitation-associated proteins in spermatozoa. Mol Reprod Dev. 84(9): 749-759.

Said TM, Land JA (2011). Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Hum. Reprod. Update. 17(6):719-733.

Saylan A, Duman S (2016). Efficacy of Hyaluronic Acid in The Selection of Human Spermatozoa with Intact DNA by The Swim-up Method. Cell J. 18 (1):83-8.

Schaller J, Glander H, Dethloff J (1993). Andrology: Evidence of β1 integrins and fibronectin on spermatogenic cells in human testis. Hum Reprod. 8(11):1873-1878.

Shamsi MB, Imam SN, Dada R (2011). Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. J. Assist. Reprod. Genet. 28(11):1073-1085.

Simon L, Ge SQ, Carrell DT (2013). Sperm selection based on electrostatic charge. Methods Mol Biol. 927: 269-278.

Sutovsky P, Aarabi M, Miranda-Vizuete A, Oko R (2015). Negative biomarker-based male fertility evaluation: sperm phenotypes associated with molecular-level anomalies. Asian J. Androl. 1 (4):554.

Torki-Boldaji B, Tavalaee M, Bahadorani M ,Nasr-Esfahani MH (2017). Selection of physiological spermatozoa during intracytoplasmic sperm injection. Andrologia. 49 (1):

Varghese A, Bragais F, Mukhopadhyay D, Kundu S, Pal M, Bhattacharyya A, Agarwal A (2009). Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. Andrologia. 41 (4): 207-215.

Widlak W, Vydra N (2017). The Role of Heat Shock Factors in Mammalian Spermatogenesis. Adv. Anat. Embryol. Cell Biol. 222: 45-65.

Witt KD, Beresford L, Bhattacharya S, Brian K, Coomarasamy A, Cutting R, Hooper R, Kirkman-Brown J, Khalaf Y, Lewis SE, Pacey A, Pavitt S, West R, Miller D (2016). Hyaluronic Acid Binding Sperm Selection for assisted reproduction treatment (HABSelect): study protocol for a multicentre randomised controlled trial. 6 (10):e012609.

Zini A, Boman JM, Belzile E, Ciampi A (2008). Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum. Reprod. 23(12): 2663-2668.