Relation Between Different Human Sperm Nuclear Maturity Tests and In Vitro Fertilization

MOHAMMAD HOSSEIN NASR-ESFAHANI,^{1,3} SHAHNAZ RAZAVI,² and MOHAMMAD MARDANI²

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Purpose: To consider the relationship between different sperm nuclear maturity tests and in vitro fertilization (IVF) rate, in order to select the most sensitive, specific, and independent factor(s) for prediction of in vitro fertilization.

Methods: Infertile couples (101) were randomly selected from IVF candidates referred to Isfahan Fertility and Infertility center. Semen samples were collected on the day of oocyte recovery. Following routine semen analysis, major portion of the semen was prepared for routine IVF insemination and the remaining was used for following sperm nuclear maturity tests: chromomycin A3 (CMA3), aniline blue, sodium dodecyl sulphate (SDS) test, and acridine orange test with or without heat shock (87°C, 5 min). Sperms (200) were evaluated for each test. The results were recorded and analyzed for their correlation to fertilization rate, using correlation coefficient, logistic regression analysis, student t-test, and receiver operating characteristics (ROC) curve.

Results: Among these tests, aniline blue and CMA3, and semen parameters, sperm morphology, and sperm motility showed a significant correlation with fertilization rate. Using logistic regression analysis, sperm morphology and CMA3 were the only independent factors related to in vitro fertilization. ROC curves showed that among above tests, CMA3 is the most specific and sensitive for sperm nuclear maturity.

Conclusion: Among CMA3, aniline blue, SDS test, and acridine orange, CMA3 was the most sensitive and specific test that can be used along with routine semen analysis for more precise prediction of fertilization rate.

KEY WORDS: Acridine orange; aniline blue; CMA3; IVF; sperm nuclear maturity.

INTRODUCTION

Following the first report of ICSI in human beings, ICSI has become the standard procedure for treatment of severe male infertility (1,2). Thus, the role of sperm functional tests for prediction of in vitro fertilization (IVF), before routine IVF treatment, has become evident (3). Along with routine semen analysis, sperm chromatin nuclear maturity, as a useful tool for evaluation of male infertility and prediction of in vitro fertilization rate, has been well established (4–6). Sperm chromatin maturity takes place during spermiogenesis, epididymal maturation, and finally these structures are stabilized following ejaculation (7,8). In addition, sperm head condensation and finally nuclear shape formation is due to histone–protamine replacement during spermiogenesis (9). The aforementioned structure is stabilized by formation of disulfide bridges between some of thiol groups (–SH) of protamines during the epididymal transition (10). Finally optimal sperm nuclear maturity and stability is obtained by noncovalent bonds between remaining free thiol groups by prostatic Zn²⁺ (8). Heterogeneity of sperm nuclear maturity has been reported in different semen samples, especially between fertile and infertile patients (11). Thus optimal nuclear maturation is suggested to be required for successful in vitro fertilization. Several sperm nuclear maturity tests are available and each of them underlies one of the sperm nuclear maturity defects. Presence of remaining histones can be detected by aniline blue staining, which binds to lysine-rich histones (12). Chromomycin A3 (CMA3) is a fluorochrome, which detects protamine deficiency in loosely packed chromatin and is correlated to extent of nicked DNA (13). Sodium dodecyl sulphate (SDS) analysis reveals sperm chromatin stability,

¹ Isfahan Fertility and Infertility Center, Isfahan, Iran.

² Department of Anatomy, Isfahan University of Medical Sciences, Isfahan, Iran.

³ To whom correspondence should be addressed at Royan Institute, Department of Clinical and Experimental Embryology and Andrology, Tehran, Iran.

which takes place during spermiogenesis, epididymal transition, and finally during ejaculation (14). Acridine orange staining called sperm chromatin structural assay (SCSA) reflects sperm chromatin denaturation (single strand DNA vs. double strand DNA) (15,16). The resistance of DNA to denaturation due to heat or acid stress is known to be dependent on normal sperm maturity (17). Although there are many reports on these tests and their relationship to in vitro fertilization rate, there is still some disagreement and contradictions between the putative potential of each of these tests to predict fertilization (5,15,17,18).

Therefore, the aim of this study was to compare the relationship between the tests just described and semen parameters with in vitro fertilization rate, to choose a chromatin maturity test(s) along with semen parameters for more precise prediction of in vitro fertilization rate.

MATERIALS AND METHODS

Sperm Preparation

Semen samples were obtained from 101 couples referring to Isfahan Fertility and Infertility Center for IVF treatment. Semen samples were collected on the day of oocyte recovery by masturbation after 3–4 days of abstinence. A portion of ejaculate was prepared for IVF insemination, using discontinuous percoll gradient (90:70:50) and the remaining portion was subjected to analysis. After the percoll preparation several smears were prepared from each specimen to record abnormal morphology and chromatin status, using aniline blue, CMA3, acridine orange staining, and SDS treatment. A total of 200 sperms were evaluated per smear in all tests.

Aniline Blue Staining

Sperm smears were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (14 mL NaH_2PO_4 0.2 M + 36 mL Na_2HPO_4 0.2 M, pH 7.2) for 30 min. The smears were stained with 5% aqueous aniline blue (Merck) in 4% acetic acid (pH 3.5) for 5 min (12).

Chromomycin A3 Staining

The semen smears were fixed in carnoy's solution (methanol/glacial acetic acid 3:1) at 4°C for 5 min. For CMA3 (Sigma) staining, each slide was treated for 20 min with 100 μL of CMA3 solution (0.25 mg/mL in McIlvaine buffer: 7 mL citric acid 0.1 M + 32.9 mL

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 M, pH 7.0, containing 10 mM MgCl_2). The slides were then rinsed in buffer and mounted with buffered glycerol (1:1) (19). Microscopic analysis of the slides was performed on a fluorescent Zeiss axioplan microscope (Germany), with the appropriate filters (460–470 nm). Evaluation of CMA3 staining was done by distinguishing between bright yellow stained spermatozoa (CMA3 positive) and dull yellow stained spermatozoa (CMA3 negative).

ACRIDINE ORANGE STAINING

Semen smears were prepared as for CMA3 staining, then fixed overnight in Carnoy's solution. Each smear was stained for 10 min with freshly prepared acridine orange (0.19 mg/mL) in citrate phosphate buffer (80 mL citric acid 0.1 M + 5 mL NaH_2PO_4 0.3 M, pH 2.5) (15). The others smears were stained similarly after being incubated in 20 mL of Tamponade solution (80 mM citric acid + 15 mM Na_2HPO_4 , pH 2.5) at 87°C for 5 min to induce heat stress DNA denaturation. Smears were evaluated on the same day, using fluorescence microscope, with the above filters. The duration of illumination was limited to 40 s per field. The percentage of green, red, orange, or yellow sperms per slide was calculated.

Treatment of Spermatozoa With SDS

Percoll prepared sperms (50 mL) were mixed with 350 μL of 1% SDS in 0.05 M borate buffer (88 mL NaOH 0.1 M + 4.2 mL Na-tetraborate 0.25 M + $\cong$ 400 mL H_2O , pH 9). After incubation for 60 min at 37°C, the reaction was stopped by the addition of equal volume of 2.5% glutaraldehyde in 0.05 M borate buffer. Nuclear swelling was assessed after staining (14). The percentage of stable (uncondensed) spermatozoa was calculated.

Sperm Assessment

Sperm count was calculated by Makler counting chamber. After immobilizing the cells with a fixing solution, the counts were expressed as million per mL. Motility was evaluated by direct microscopic examination and morphology by papanicolau staining technique (20). In addition to assessing the percentage of abnormal morphology, sperms with abnormal heads were subgrouped as follows: macrocephalic, microcephalic, amorphous, elongated, tapering, pear form, duplicated, round, and pinhead.

In Vitro Fertilization

Oocytes were collected by an ultrasound method, isolated in Nunc dish containing 1 mL of IVF medium (Ham's F-10+ 10% human serum albumin) and under 37°C and 5% CO₂ condition. After 3–5 h they were inseminated using 50,000–100,000 motile spermatozoa per oocyte. Fertilization was assessed under an Olympus stereomicroscope (Japan) for presence of pronuclei 17–19 h after insemination. In order to eliminate the female factors effecting fertilization, patients with less than four mature oocytes or with bad quality oocytes were excluded from this study. The percentage fertilization was obtained by multiplying the ratio of fertilized oocytes to total number of oocytes by 100.

Standardization of Results

During preliminary experiment, researchers and sample coefficients of variation (CV) were recorded. Intraassay variations were determined by evaluating 100 cells on 10 different microscopic fields (total 1,000 cells), from the same semen specimen. Interassay variations were accomplished by evaluating staining for nuclear maturity tests by counting 200 cells on five different specimen from the same sperm donor. CV was calculated for both intra- and interassay values, using the following formula: $CV(\%) = (SD/mean) \times 100$. The CV during standardization of results was less than 11% in all nuclear maturity tests, except for acridine orange for which it was 15%.

Statistical Analysis

All statistical calculation including student *t*-test, coefficient of correlation, logistic regression, receiver operating characteristic (ROC) analysis were carried out using statistical package for social studies (SPSS-9) software. For assessing the independent effect of semen parameters and sperm nuclear maturity tests on in vitro fertilization rate, logistic regression analysis was used as suggested by Liu *et al.* (3). The discriminating power of sperm nuclear maturity tests as screening tests for prediction of in vitro fertilization rate and sperm morphology was illustrated using ROC curves.

RESULTS

A substantial variation in the percentage of CMA3, aniline blue, acridine orange, and SDS test was ob-

Table I. Correlation Between Sperm Parameters and Nuclear Maturity Tests With In Vitro Fertilization Rate

Variable	<i>r</i>	<i>P</i> value	Logistic regression
Count (×10/ml)	0.190	NS	NS
Sperm motility (%)	0.196	<.05	NS
Abnormal morphology (%)	−0.665	<.01	<0.001
Amorphous head	−0.445	<.01	NS
Elongated pear-form head	−0.451	<.01	NS
Round head	−0.377	<.01	NS
Chromomycin A3	−0.564	<.01	<0.045
Aniline blue	−0.342	<.01	NS
Acridine orange	0.037	NS	NS
Acridine orange + heat	0.071	NS	NS
SDS-test	0.086	NS	NS

Note. NS: not significant.

served in ejaculated spermatozoa varying between 8.5%–62%, 2.5%–71.5%, 15%–100%, and 0%–92.5%, respectively.

Semen Parameters and Nuclear Maturity

Significant positive correlation was obtained between sperm motility with in vitro fertilization rate. Also a significant negative correlation between sperm abnormal morphology, CMA3, and aniline blue staining with in vitro fertilization was observed (Table I).

To determine which groups of sperm characteristics will independently affect fertilization rates, all of the data were examined by logistic regression analysis. These results showed that after sperm head morphology, only CMA3 staining show independent relation to in vitro fertilization rate (Table I).

Table II shows correlation between sperm parameters and sperm nuclear maturity tests. The highest negative correlation was obtained between sperm morphology with CMA3 and aniline blue staining, respectively.

Table II. Correlation Between Sperm Parameters With Sperm Nuclear Maturity Tests

Sperm parameters	CMA3	AB	AO	AO+H	SDS
Count (×10/ml)	0.246*	0.243*	0.009	0.064	0.190
Motility (%)	0.150	0.009	0.047	0.013	0.053
Morphology (%)	−0.524**	−0.384**	−0.043	0.045	−0.155

Note. CMA3: chromomycin A3. AB: aniline blue, SDS: sodium dodecyl sulfate, AO: acridine orange, AO+H: acridine orange with heat.

* *p* < .05; ** *p* < .01.

Comparative Analysis of Sperm Parameters and Nuclear Maturity Tests

By dividing patients into fertilizing and nonfertilizing groups, the mean of abnormal sperm head, amorphous, round, elongated-pear form head, and CMA3 positivity were significantly different. Whereas, in the other parameters including the mean of sperm concentration, motility, oocyte numbers, male and female age, and so forth were similar in two groups (Table III).

Sperm Morphology

Strong correlation between sperm morphology with in vitro fertilization rate and CMA3 staining was observed. Among sperm head abnormalities, amorphous, elongated-pear form, and round head have higher correlation with fertilization rate and CMA3 staining, respectively (Table I and III).

Discriminative Power of Nuclear Maturity Tests

Table I and III show a negative and strong correlation between in vitro fertilization rate with abnormal sperm morphology and percentage of CMA3 positivity and also a positive correlation between CMA3 staining and sperm abnormal morphology. In order to determine the discriminating power of the above nuclear maturity tests to predict fertilization rate and abnormal morphology, the areas under the ROC curve for these tests were obtained. ROC analysis showed the highest sensitivity and specificity for CMA3 staining among the nuclear maturity tests (Table IV). Dur-

Table IV. Receiver Operating Characteristic (ROC) Analysis of Nuclear Maturity Tests

Nuclear maturity tests	Area under the ROC curve	
	Fertilization rate	Morphology
Chromomycin A3	0.911	0.760
Aniline blue	0.614	0.669
Acridine orange	0.543	—
Acridine orange + heat	0.615	—
SDS test	0.391	—

ing ROC analysis, varying CMA3 and aniline blue cut off values were used to obtain optimum sensitivity and specificity. At the cut off point of 20% for aniline blue, sensitivity and specificity were 62% and 75%, respectively and at the cut of point of 30% for CMA3, sensitivity and specificity were 80% and 72%, respectively.

DISCUSSION

Since the initial reported pregnancy using ICSI (1,2), tremendous progress has occurred in the treatment of male factor infertility, apparently regardless of the severity of the sperm defects. This advancement has emphasized on two major fields in the treatment of infertility: 1) to select patients with low fertilization rate and allow them to take ICSI as their initial route of treatment. 2) During ICSI, sperm membrane–olemma interaction as a selective barrier within a sperm population is no longer relevant. Therefore it emphasizes the quality of the sperm chromatin, with respect to selection of sperm for injection.

Even though the most normal appearing, motile spermatozoa are selected for injection in the ICSI procedure, the quality of semen sample from which sperm has been chosen must also be taken into consideration. In general the ICSI fertilization rate does not exceed 65% in most clinics. Despite the mechanical injection of one sperm into each mature oocyte, a possible explanation of this lower than expected fertilization rate could be that sperm selected from semen of patient with male factor infertility may have damaged DNA (21). Abnormalities such as loosely packaged chromatin and damaged DNA have already been observed in poor quality semen samples (22). Our pervious work and other authors have shown that protamine deficiency measured by CMA3 staining independently effect fertilization (4,5). However abnormality in sperm chromatin can take place at several levels including 1) histone/protamine replacement,

Table III. Comparative Analysis of Mean Sperm Parameters and Nuclear Maturity Tests Between Fertilized and Nonfertilized Groups

	Fertilizing group (Mean $\pm$ SD)	Nonfertilizing group (Mean $\pm$ SD)	P value
Fertilization rate (%)	62.84 $\pm$ 25	00.00 $\pm$ 00	.001
Abnormal head (%)	39.20 $\pm$ 09	51.30 $\pm$ 07	.022
Amorphous head (%)	14.50 $\pm$ 05	20.00 $\pm$ 05	.060
Round head (%)	03.15 $\pm$ 02	04.77 $\pm$ 02	.050
Elongated pear-form head (%)	14.20 $\pm$ 05	19.22 $\pm$ 06	.010
Chromomycin A3 (%)	78.33 $\pm$ 09	59.80 $\pm$ 10	.001
Aniline blue (%)	80.57 $\pm$ 13	75.50 $\pm$ 10	NS
Acridine orange (%)	68.13 $\pm$ 22	67.00 $\pm$ 17	NS
Acridine orange + heat (%)	70.15 $\pm$ 20	63.02 $\pm$ 17	NS
SDS test	80.20 $\pm$ 16	70.60 $\pm$ 24	NS

Note. NS: not significant.

2) absence of protamine, 3) epididymal maturation, and 4) chromatin stability during ejaculation. Therefore during this study we observed that when analyzing sperm chromatin maturity of a sample using different tests along with routine semen analysis or semen parameters, abnormal sperm morphology showed the strongest negative correlation with fertilization. This result is in concordance with many others authors, who have concentrated their attention on the predictive value of morphology, using WHO standards or strict criteria (23–25), and it is felt that normal morphology is indicative of normal sperm function while high level of abnormal spermatozoa in ejaculation is associated with reduce fertilization potential. Even though the role of sperm morphology in prediction of pregnancy and in vitro fertilization outcome is well established in literatures (26,27) and our results, some authors question the sole predictive role of morphology in prediction of male fertility potential (28). Also, when studying the role of sperm morphology in relation to fertilization rate in ICSI, literature shows that there is no clear relation between percentage of spermatozoa with normal form and fertilization, cleavage, and percentage outcome of ICSI (29–31).

Even though the role of sperm morphology in IVF is well established and its limited role in ICSI, the need for emphasis on sperm chromatin has become more profound. In support of these reports our results also show that sperm chromatin assessed by CMA3 can affect fertilization independently of sperm morphology.

The results in relation to different sperm nuclear maturity tests with in vitro fertilization suggest that CMA3, which reflect the degree of protamine deficiency, and aniline blue, which indicates the degree of excess histone in sperm head, show significant correlation with fertilization. These results are in concordance with similar results reported by Lolis (22), Esterhuizen (5), and Iranpour (4). However, these results are in disagreement with reports from Sakkas (31) and Bianchi (32), in relation to IVF. This difference could be due to different patient selection. However, these authors showed a significant correlation between fertilization and CMA3 positivity in SUZI procedure. They suggested that there is higher competition between low and high CMA3 positive sperms due to the number of sperms subzonally injected in SUZI, (18,32). Immature sperm nuclear can be detected by staining with acidic aniline blue, since they contain lysine rich histones. The excess of these proteins may disrupt chromatin condensation, which in turn affects sperm morphology and fertilization. Our results show a significant negative correlation

between sperm morphology and in vitro fertilization rate. However, when using logistic regression analysis of all semen variables including aniline blue, no significant relation to in vitro fertilization was obtained and these results are in agreement with that of Liu (33).

SDS analysis showed no significant relation with in vitro fertilization rate. SDS tests show the degree of chromatin stability and it reflects the epididymal maturation and to a certain extent the normal function of male genital accessory glands. Therefore, there appears to be no relation between chromatin stability and in vitro fertilization rate. Liu (34) also did not observe any relation with in vitro fertilization rate when using SDS alone or with DTT or EDTA. Acridine orange staining also known as SCSA, differentiates single strand and double strand DNA and also reveals the degree of DNA resistance to denaturation, when treated with acid or heat. Our results showed no relation between acridine orange or acridine orange with heat shock. When studying research carried out on acridine orange one can see disagreement between different reports (15,17,26,35,36). This difference is possibly due to different method of acridine orange measurement (simple fluorescent microscopy evaluation vs. flowcytometric procedure) (15,17,36). Recently, Evenson (16) suggested that acridine orange assay under fluorescent microscope provides a general picture of the status of the sperm DNA denaturation susceptibility. This procedure is limited to two or three classifications (green, red, yellow) rather than the 1,024 discrete channel levels of red and green fluorescence/cell by flowcytometry. Most of the studies that show a strong relation between acridine orange and in vitro fertilization rate have used flow cytometry; however, there is a disagreement among those using fluorescence microscopy about the prognostic value of acridine orange for prediction of in vitro fertilization rate (16,37).

Since fertilization is a multifactorial process and in order to determine which of the semen parameters or nuclear maturity tests affects fertilization independently, the data were examined by logistic regression analysis, suggested by Liu (3). The results of analysis suggested that even though many factors were related to fertilization rate, percentage of abnormal morphology and CMA3 positivity were the only two factors that affect fertilization independently. Therefore, one may conclude that it is probable that anomalies of spermiogenesis during protamination renders spermatozoa functionally immature and thus deficient chromatin structure may limit fertilization of oocyte by spermatozoa (38). These results also suggest that

CMA3 positive spermatozoa competes with CMA3 negative spermatozoa in that sample and thereby reduces fertilization rate. Although aniline blue showed a negative significant correlation with fertilization rate, it did not affect fertilization independently. This suggests that CMA3 assay not only detects excess histone, but also reveals absence of protamine where there is no histone present. Sakkas (31) and Selva (39) reported that a high percentage of injected or penetrated oocyte that fails to progress to pronuclear stage contains condensed sperm nuclei in ICSI and SUZI, respectively. One possible explanation of this could be the fact that CMA3 positive spermatozoa after entering the oocyte via zona pellucida or direct injection showed premature chromosomal condensation due to the active maturation promoting factor (MPF) of oocyte (40) or the other possible explanation could be that the absence of protamine on specific region of DNA might prevent decondensation in the other regions. Since sperm morphology was the first factor that shows independent relation to fertilization, the relation between nuclear maturity tests and sperm morphology were obtained, showing that CMA3 and aniline blue were the only nuclear maturity tests that affect sperm morphology. Analysis of discriminative power of these nuclear maturity tests to predict fertilization showed that CMA3 has the strongest sensitivity and specificity to predict sperm morphology and in vitro fertilization rate. In conclusion our data and others show that CMA3 is the most sensitive and specific of nuclear maturity tests for prediction of in vitro fertilization rate and sperm morphology. Since sperm morphology is the first factor and protamine deficiency measured by CMA3 is the second factor independently affecting fertilization rate, it can be suggested that CMA3 can be used as a complementary test along with sperm morphology for prediction of in vitro fertilization rate, especially in the cases with unexplained infertility. Sperm with higher than 30% CMA3 positive should be considered for ICSI and a sperm preparation method such as percoll or pure-sperm that can separate low CMA3 positive sperm from semen is advised (41).

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