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Modified sperm stress test: a simple assay that predicts sperm-related abnormal in-vitro fertilization*

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Loss of sperm motility is associated with the process of sperm senescence and occurs at different rates within a given normal or abnormal sperm population. Reactive oxygen species attack cell membrane phospholipids, generating fatty acid peroxides and other degradation products, that also have deleterious effects on sperm motility and fertilizing ability. The objective of this investigation was to study a modification of the original sperm stress test (MOST), changing the culture medium to one offering transitional metals and shortening the total test time, to ascertain whether it can predict fertilization under these laboratory conditions. A total of 41 semen samples was obtained from patients undergoing in-vitro fertilization (IVF) at our institution. Semen samples were grouped into those producing total fertilization rates (FR) within normal limits (>50%) and those showing low total FR (<50%). The normal FR group had a significantly greater MOST mean value than the low FR group (0.71 versus 0.44). Furthermore, there was a statistically significant correlation between the MOST score and ungrouped fertilization rates ($r = 0.53$, $P = 0.0004$). Diagnostic statistics for MOST ratio values predicting <50% FR showed an optimal threshold of 0.39. Collectively, sensitivity, specificity, positive predictive value and negative predictive value have their largest values at this threshold. Taking into account the above mentioned threshold figures, there is a significant association between MOST and FR categories ($P = 0.0009$). In conclusion, MOST is a simple assay that has significant predictive value for sperm related IVF abnormalities.

Key words: culture media/IVF/motility/spermatozoa

Introduction

Among basic semen parameters, sperm rapid linear progressive motility (Comhaire *et al*., 1987) and morphology judged by strict criteria (Kruger *et al*., 1987) are two of the best predictors of human in-vitro fertilization (IVF). Other more elaborate tests, either because they lack accuracy and consistency or because the techniques are cumbersome, have not become part of routine semen investigation. A simple test that can predict IVF results with an acceptable level of accuracy is still being looked for.

Normal sperm motility requires an adequate sperm structure and metabolism. Loss of sperm motility is associated with sperm senescence and/or structural or metabolic alterations; all these processes may occur at different rates within a given normal or abnormal sperm population. It has been demonstrated that reactive oxygen species (ROS) have beneficial or detrimental effects on sperm functions, depending on the nature and concentration of the ROS involved (de Lamirande and Gagnon, 1995). ROS action on cell membrane phospholipids generates fatty acid peroxides and other degradation products that, in turn, have deleterious effects on sperm motility and fertilizing ability (Aitken *et al.*, 1989).

Endogenous lipid peroxidation is one of the most important pathophysiological mechanisms involved in sperm motility loss. Polyunsaturated fatty acids, a component of sperm membrane phospholipids, are easily oxidized under aerobic conditions. As a consequence of ROS contact, toxic lipid peroxides are produced, inducing immobilization, senescence and death of the spermatozoa (Alvarez and Storey, 1984).

Two major enzyme systems have been found in human spermatozoa, protecting them from lipid peroxidation, namely superoxide dismutase (Menella and Jones, 1980) and glutathione peroxidase (Alvarez and Storey, 1989). Despite the antioxidant systems or because of their malfunction, excessive generation of ROS has been detected in abnormal spermatozoa (Aitken and Clarkson, 1987) and may be responsible for the derangement of function and motility in this type of spermatozoa.

It has been demonstrated that the rate of motility loss *in vitro*, even in normal human spermatozoa, is positively correlated with the endogenous lipid peroxidation potential at temperatures between 24 and 40°C (Alvarez *et al.*, 1987). Sukcharoen *et al*. (1996) found a significant correlation between some sperm movement characteristics (beat cross frequency and straightness), sperm morphology and ROS and the fertilizing capacity of human spermatozoa *in vitro*.

In fact, based on the previous considerations, Alvarez *et al*. (1996) recently developed a test, called the 'sperm stress test', which appears to predict pregnancy rates in assisted reproduction.

A modification of Alvarez *et al*.'s technique developed in our laboratory (modified sperm stress test; MOST) shortens

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the total test time and appears to predict fertilization rather than pregnancy rate when assisted reproduction methods are used.

The present investigation was undertaken to determine the value of MOST in the prediction of human fertilization *in vitro* and to establish the threshold value that should be used clinically to separate samples with good fertilization potential from those with low capacity.

Materials and methods

Reagents and supplies

Ham's F10 nutrient mixture, a balanced salt medium containing L-glutamine, lactate, pyruvate, streptomycin, penicillin, and sodium bicarbonate was obtained from Gibco BRL (Grand Island, NY, USA) and prepared in our laboratory. This culture medium has no antioxidants in its composition. Bovine serum albumin (BSA) (Fraction V) and Percoll were obtained from Sigma (St Louis, MO, USA). The Makler counting chamber was purchased from Safi-Medical Instruments Ltd (Haifa, Israel). Polystyrene test tubes (13×100 mm) were obtained from Falcon Labware (Oxnard, LA, USA).

Semen collection and analysis

A total of 41 semen samples obtained from men (age 20–40 years) undergoing IVF was included in the study. Semen samples were collected by masturbation after 3 days of sexual abstinence and were processed within 1 h of collection.

Semen specimens were allowed to liquefy at room temperature and following liquefaction, basal sperm concentration and motility were measured at 37°C using a computer-aided sperm analyser (CASA) (Cellsoft Automatic Semen Analyzer series 3000; Cryo Resources Ltd, Montgomery NY, USA) according to a previously established protocol (Calamera *et al.*, 1989).

Sperm morphology was assessed following the criteria described in the WHO Manual (1992).

The samples studied presented no significant sperm agglutination, pyospermia or hyperviscosity.

Motile sperm fraction separation

After 30 min of liquefaction at room temperature, motile spermatozoa were isolated using discontinuous (47.5–95%) Percoll gradients (Mortimer, 1994). Following centrifugation for 20 min at 300 *g*, the isotonic 95% Percoll fraction was recovered and the spermatozoa were then washed twice using Ham's F10 medium with 0.3% BSA.

The final pellet was resuspended in the same medium and used for both IVF insemination (Veeck *et al*., 1983) and MOST.

Motility parameters

A 5 µl aliquot of the sperm suspension was placed into a 10 µm depth Makler chamber prewarmed at 37°C and computer-assisted sperm motion analysis was performed. The settings used during analysis were: number of frames analysed per second = 20; frequency = 30 Hz; threshold velocity = 10 µm/s; minimum sampling for motility = 1 frame; minimum sampling for velocity = 4 frames; pixel scale = 0.688 µm.

The following sperm parameters were obtained: (i) sperm concentration, (ii) number of motile and non-motile spermatozoa, (iii) percentage of motile spermatozoa, (iv) concentration of motile spermatozoa, (v) curvilinear velocity (VCL), (vi) linear velocity (VSL) and (vii) linearity (LIN).

IVF procedure

Ovarian stimulation was performed using a combination of leuprolide acetate and human menopausal gonadotrophins as described previously (Abdalla *et al*., 1990). Follicular growth was monitored by transvaginal ultrasonic examinations and serum oestradiol measurements. When the diameter of the two leading follicles was >16 mm, 10 000 IU of human chorionic gonadotrophin (HCG) was given. Oocyte retrieval was performed under vaginal ultrasonic guidance 36 h thereafter.

Following oocyte retrieval, the oocytes were scored for quality and maturity and incubated at 37°C in 5% CO_2 in air in Nunc culture dishes (Copenhagen, Denmark) containing Ham's F10 with 0.3% BSA. Four to 6 h later, methaphase II oocytes were inseminated with 125 000–150 000 spermatozoa per oocyte in cases of normal samples and 500 000 spermatozoa per oocyte in cases of abnormal ones. Pronuclear assessment was performed at 16–18 h post-insemination.

A threshold figure of 50% total fertilization rate was used to categorize the normal and the low fertilization rate groups (Acosta, 1992). This figure was derived by using mean total fertilization rate minus 2 SD. Similar figures have been reported by the Tygerberg group and by our own laboratory.

The culture medium was changed after pronuclei assessment 12–18 h later. No more than four embryos (2–6-cell stage) were transferred to the uterine cavity 48 h after insemination.

On the day after oocyte retrieval, intravaginal micronized progesterone was administered in doses of 800 mg per day until a pregnancy test was performed.

Sixty-two oocytes were inseminated and used to obtain the fertilization rate in the low fertilization rate group and 151 oocytes were included in the group of normal fertilization.

Modified sperm stress test

A 100 µl aliquot of the isotonic 95% Percoll sperm fraction was used to perform the test.

Prior to beginning with MOST incubation, a 5 µl aliquot of sperm suspension was placed in a Makler chamber and scored with CASA to determine percent motility (initial motility). The polystyrene tubes with the sperm suspensions were incubated at 40°C in a water bath for exactly 4 h. Following incubation another 5 µl aliquot was transferred to a Makler chamber and motility scored again with CASA (final motility). In the basal and post-incubation aliquots >300 spermatozoa were studied. MOST ratios were calculated by dividing final motility over initial motility.

Statistical analysis

Logistic regression was used to establish correlations and contributions of all the sperm parameters to fertilization rates. Optimal cut-off for MOST ratios predicting poor fertilization was estimated by applying receiver operating characteristics (ROC) curve analysis and calculating diagnostic statistics [sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)] for significant variables from the logistic regression. Semen parameters were compared using two sample tests and the non-parametric Mann–Whitney *U*-test.

Results

Using 50% as the cut-off figure for the minimum normal total fertilization rate (FR) for the inseminated oocytes (Acosta, 1992), semen samples were grouped into those showing normal total FR (>50%) and those showing low total FR (<50%). Thirty-two of the 41 samples (78%) showed FR >50% and nine (22%) had FR <50%. A total of 151 oocytes was inseminated in the first group and 127 fertilized (84%); 62 were inseminated in the second group and seven fertilized (11%).

Table I presents mean standard errors and medians of the

Table I. Original post-Percoll, and post-incubation sperm parameters and IVF results for semen samples showing normal (>50%) and low (<50%) total fertilization rates. Sperm parameters were studied on spermatozoa from the 95% Percoll fraction, immediately after separation (basal Percoll) and after 4 h of incubation in supplemented Ham's F10 medium at 40°C (post-incubation). MOST ratios were calculated dividing post-incubation motility by basal Percoll motility. Motion parameters were determined in a CASA system. NS, not significant. M–W, Mann–Whitney test

Fertilization groups	*n*		Original semen			Basal Percoll			
			Concentration $\times 10^6$/ml	Motility %	Morphology WHO %	Concentration $\times 10^6$/ml	Motility %	Velocity μm/s	Linearity
>50%	32	Mean	129.0	58.0	34.70	53.1	66.8	64.3	5.98
		±SEM	14.4	3.0	8.70	6.5	4.1	3.4	0.23
		Median	115.0	60.0	36.00	45.0	68.0	62.0	6.00
<50%	9	Mean	77.3	45.0	30.20	30.7	45.6	50.9	4.89
		±SEM	27.2	7.1	7.35	9.1	8.0	5.4	0.40
		Median	50.0	50.0	26.00	19.0	47.0	49.0	5.00
		P (*t*-test)	NS	NS	NS	NS	0.0198	NS	0.0468
		P (M–W)	0.0418	NS	NS	NS	0.0224	NS	0.0445

Fertilization groups	*n*		4 h Post-incubation				MOST Ratio	IVF		
			Concentration $\times 10^6$/ml	Motility %	Velocity μm/s	Linearity		Inseminated oocytes	Fertilized oocytes	Fertilization rate
>50%	32	Mean	50.6	49.1	52.1	3.90	0.71	5.20	4.50	0.87
		±SEM	6.6	4.2	3.7	0.30	0.03	0.42	0.40	0.03
		Median	39	47.0	48.0	4.00	0.78	5.00	4.00	0.95
<50%	9	Mean	28.1	23.1	46.9	4.63	0.44	5.10	0.44	0.08
		±SEM	7.3	8.7	4.0	0.22	0.09	0.70	0.24	0.05
		Median	20	10	50	4.50	0.35	5.00	0.00	0.00
		P (*t*-test)	NS	0.0071	NS	NS	0.0011	NS	< 0.0001	< 0.0001
		P (M–W)	NS	0.0053	NS	NS	0.0055	NS	< 0.0001	< 0.0001

sperm parameters for each FR group. The normal FR group had a significantly greater basal (post-Percoll) motility than the low FR group (66.8 versus 45.6%), and a significantly greater post-incubation motility (49.1 versus 23.1%). The normal FR group had a higher original mean concentration than the low FR group, although the figures did not differ significantly. However, non-parametric test indicated a significant difference in medians (115 versus 50). In addition, the normal FR group had significantly greater basal linearity than the low FR group. None of the other sperm parameters differed significantly. The normal FR group had a significantly greater MOST mean than the low FR group (0.71 versus 0.44). Furthermore, there was a statistically significant correlation ($r = 0.53$, $P = 0.0004$) between the MOST score and ungrouped fertilization rates.

The MOST ratio was the best predictor of low FR ($R^2 = 0.21$, $P < 0.0014$) followed by post-incubation motility ($R^2 = 0.17$, $P < 0.0041$). No other parameters were either significant or had R^2 values >0.15. MOST ratio was also the best predictor of very poor FR (<10%) ($R_2 = 0.33$, $P < 0.0001$) followed closely by post-incubation motility ($R_2 = 0.32$, $P < 0.0001$). Original concentration was also significant, but not as good a predictor ($R_2 = 0.21$, $P < 0.0012$). No other parameters were statistically significant or had R_2 values >0.15.

Figures 1 and 2 show the plotted ROC curves for MOST ratios predicting 50 and 10% FR respectively.

Table II presents diagnostic statistics for MOST ratio values predicting <50% FR. An optimal threshold figure would appear to be 0.39. Collectively, sensitivity, specificity, PPV and NPV have their largest values at this threshold figure. In this case, PPV is predicting a negative outcome (FR <50%). An optional threshold predicting <10% FR would also be 0.39 with sensitivity of 0.83, specificity of 0.97, PPV of 0.83, and NPV of 0.97. Taking into consideration the above mentioned thresholds, there is a significant ($P = 0.0009$) association between MOST and FR categories (Table III).

None of the sperm parameters studied was a significant predictor of pregnancy ($P > 0.14$ for all).

Discussion

The most common method of semen evaluation involves the performance of routine semen analysis using standardized protocols. Traditionally, the emphasis is placed on the number, motility and morphology of the spermatozoa in the ejaculate (WHO, 1992). However, the results of basic semen analysis do not provide accurate predictive information on fertilization *in vitro*.

The recognition that male infertility can be treated by techniques such as IVF prompted the rapid development of a number of tests that attempted to predict fertilizing ability (Liu and Baker, 1992). Over the last few years, many new tests of human sperm function have been developed and introduced into the andrology laboratory practice (Liu and Baker, 1994). The clinical value of some of them, for diagnosis and prognosis of male infertility, remains to be confirmed. It was demonstrated that sperm rapid forward progressive motility and strict morphology are the two best predictors for IVF (Enginsu *et al.*, 1993). However, neither of them evaluates the changes that spermatozoa may undergo after prolonged incubation before actual fertilization of the oocytes occurs.

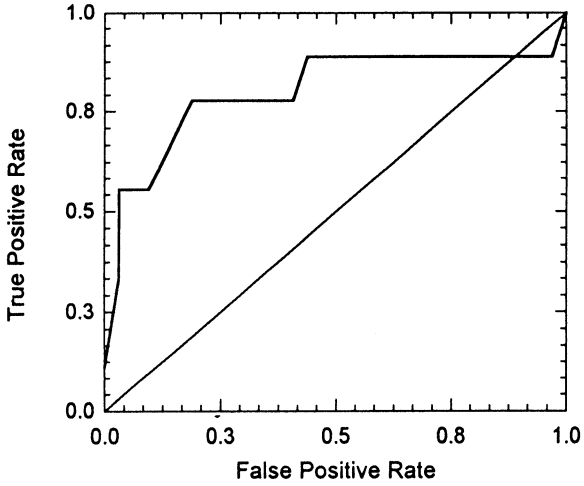

Figure 1. ROC curve for MOST ratio predicting <50% total fertilization.

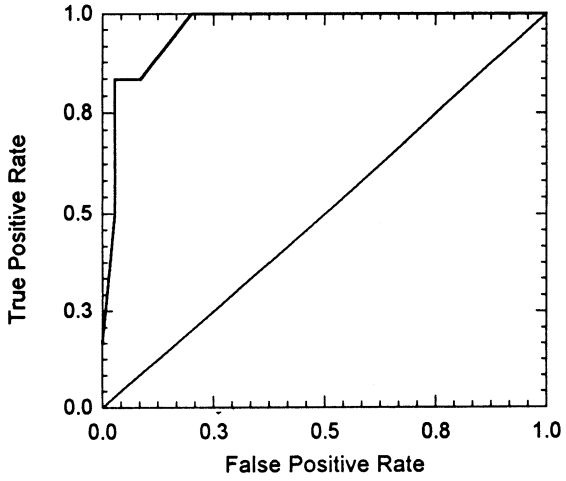

Figure 2. ROC curve for MOST ratio predicting <10% total fertilization.

Table II. Diagnostic statistics for stress test ratio predicting fertilization <50%. TP, true positives; FP, false positives; FN, false negatives; TN, true negatives; Sen, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value

MOST ratio	TP	FP	FN	TN	Sen	Spe	PPV	NPV
0.30	3	1	6	31	0.3333	0.9688	0.75	0.8378
0.34	3	1	6	31	0.3333	0.9688	0.75	0.8378
0.39	5	1	4	31	0.5556	0.9688	0.8333	0.8857
0.43	5	3	4	29	0.5556	0.9063	0.6250	0.8788

Despite the complex aetiology of male infertility, one stereotypical feature appears in clinic. This is the production of abnormal levels of oxygen derived free radicals or ROS (Aitken *et al*., 1989). When this phenomenon is uncontrolled, it causes lipid peroxidation in spermatozoa and alters membrane function, motility and metabolism. Based on this type of dysfunction, we developed the MOST in an attempt to predict oocyte fertilization *in vitro* in the context of assisted reproductive technologies.

The major significant result obtained in this study was the high PPV of MOST for poor fertilization *in vitro*. Low values of MOST probably indicate an increase in lipid peroxidation that may interfere with sperm fertilizing capacity.

MOST false positive results suggest that the test does not take into account other factors necessary to achieve fertilization, e.g. characteristics of the oocytes and other properties of the spermatozoa. MOST low values, in turn, suggest that insemination of oocytes with >61% spermatozoa that lose motility after incubation (4 h at 40°C) is incompatible with normal fertilization rates, probably due to the high lipid peroxidation potential of such a sperm sample.

Alvarez *et al*. (1996) published an assay named the 'sperm stress test'; its results correlated well with pregnancy rates in assisted reproduction. Although similar in nature, MOST has some notable methodological differences, not only in the reduction of the incubation time, but also in the intrinsic mechanism of the reaction. In Alvarez's methodology, the authors used human tubal fluid medium, a balanced salt medium lacking transitional metal supplementation. It is known that sperm function may be further compromised by the presence of transitional metals such as iron and copper that can act as donors and acceptors of free electrons. In our work, we used Ham's F10, a commonly used IVF culture medium containing 52 separate constituents, including transitional metal supplements in the form of copper sulphate and ferrous sulphate.

Table III. Association between MOST score and total fertilization rate (FR) categories

MOST score	FR <50%	FR >50%	Total
<0.39	5 (12.2)	1 (2.4)	6 (14.6)
>0.39	4 (9.8)	31 (75.6)	35 (85.4)
	9 (22.0)	32 (78.0)	41 (100)

Values in parentheses are percentages.
Two-sided $P = 0.0009$ (Fisher's exact test).
Odds ratio: 38.75.

The role of transitional metals in the genesis of peroxidative damage in different culture media was recently investigated. It was demonstrated that Ham's F10 medium induced a marked loss of sperm motility associated with a significant increase in peroxidative damage. Moreover, it was suggested that some other components of the Ham's F10 medium, in addition to the iron, can stimulate lipid peroxidation (Gomez and Aitken, 1996).

In our opinion, the mechanism involved in MOST is probably more related to a forced lipid peroxidation than to a spontaneous one. Therefore, what we see after an incubation of 4 h at 40°C is accelerated sperm peroxidative damage and its consequence, decreased motility, phenomena that would take longer under

standard IVF conditions but would eventually lead to reduced fertilization rates.

We cannot be certain at this time if the reduced fertilization is due to decreased sperm motility, altered zona and/or oolemma binding, or perhaps to oocyte damage, and we are currently investigating these possibilities.

If this test interpretation is correct, patients with semen samples showing MOST ratios <0.39 should benefit from ICSI procedures; however, the impact of a potentially pre-existing peroxidative damage on the spermatozoon chosen for ICSI remains to be established.

In conclusion, MOST is a simple assay that seems to have significant predictive value for sperm related in-vitro fertilization abnormalities.

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