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A Test of the Human Sperm Acrosome Reaction Following Ionophore Challenge

Relationship to Fertility and Other Seminal Parameters

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ABSTRACT: Acrosome reaction capacity was tested on semen samples from 53 fertile and 26 subfertile men. Preparations were divided into two aliquots after 3 or 24 hours of culture. One aliquot received 10 μmol/L calcium ionophore A23187 in dimethyl sulfoxide (DMSO) and the other received DMSO alone. Acrosome reactions were scored on ethanol-permeabilized smears using fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* lectin. The following factors were analyzed: the spontaneous reation rates (control); induced reaction rates (ionophore-challenged); and the difference between the two, being the proportion of spermatozoa in the population capable of reacting in response to calcium influx (acrosome reaction to ionophore challenge [ARIC]). While spontaneous reactions bore no relation to fertility, induced reactions and ARICs were significantly reduced or absent in subfertile men, indicating acrosomal dysfunction as a likely cause of fertilization failure. The test was shown to have a predictive value for fertility comparable to that of the hamster ovum sperm penetration assay and to be a simple and cost-effective addition to existing semenology.

Key words: Fertilization, infertility, human spermatozoa, calcium ionophore.

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Before fertilization can occur, mammalian spermatozoa need to capacitate and undergo the acrosome reaction (Yanagimachi, 1981). Capacitation normally occurs in the female reproductive tract, but now it can be achieved *in vitro*. Many infertility laboratories use zona-free hamster oocytes to test the capacitation and potential fertilizing ability of human semen samples (Yanagimachi et al, 1976) and to karyotype spermatozoa (Rudak et al, 1978). However, the zona-free sperm penetration assay (SPA) is time-consuming and expensive to run, and can only test the fertilizing capacity of a small subset of the sperm in any population. The incidence of false-positive and false-negative results is also of concern (Gould et al, 1983; Albertsen et al, 1983; Bronson and Rogers, 1988). Moreover, even fertile men vary considerably in the rate at which their sperm will capacitate and penetrate in this system (Yanagimachi, 1984). Finally, the test is not readily available in Australia because importation of hamsters is prohibited and costs preclude the use of cryopreserved oocytes for most laboratories. In this article, we explore an alternative method of assessing the ability of spermatozoa to undergo the acrosome reaction *in vitro*, a necessary prerequisite to sperm–egg fusion.

It is known that the rates of spontaneous acrosome reactions in human spermatozoa cultured under capacitating conditions are low and variable (Byrd and Wolf, 1986), although they can be increased by contact with oocyte-cumulus complexes (Stock et al, 1989) or with acrosome reaction-inducing factors such as follicular fluid (Mortimer and Camenzind, 1989). Capacitated spermatozoa of some men with unexplained infertility show a reduced ability to elicit an acrosome reaction in response to follicular fluid (Calvo et al, 1989). This phenomenon may indicate a specific lesion in this aspect of sperm function that may be important in infertility. Tesarik (1989) has suggested that tests for acrosomal function should distinguish between spontaneous acrosome reactions and those produced by a suitable physiologic stimulus. We examined the ability of capacitated spermatozoa to respond to the challenge of calcium ionophore and showed that this capacity is closely related to fertility. This study concentrated on the basic technique; further work is being prepared that relates the findings to different etiologic factors in male infertility.

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Materials and Methods

Subject Classification

The men were all subjected to a detailed andrological investigation. They were classified as fertile (53 cases) or subfertile (26

cases). The fertile group was composed of the following: known fertile donors (35), men who achieved fertilization and pregnancy in *in vitro* fertilization (IVF)-related cycles (17), and one man who achieved fertilization and pregnancy following intrauterine artificial insemination of his partner with washed spermatozoa (1). All men in the infertile group had a history of infertility and repeated IVF failure with no evidence that such failure was due to oocyte deficiencies. One half (13/26) of these men achieved fertilization in one assisted conception cycle, but they had a poor fertilization rate of less than 50% (fertilization being defined by the observation of two pronuclei in zygotes 18 to 20 hours after insemination). These isolated *in vitro* successes were only seen following treatment of their spermatozoa with the phosphodiesterase inhibitor and motility enhancer pentoxifylline (1-{5-oxohexyl}-3-7-dimethylxanthine, marketed as Trental®: supplied as a pure compound by Hoechst Australia Ltd., Melbourne, Australia; Yovich et al, 1990). As no significant differences could be detected in any of their seminal parameters, including the acrosome reaction tests described below, all 23 subfertile men were grouped together for analysis.

Sperm Preparation

Sperm samples were obtained by masturbation following at least 3 days of abstinence from the known fertile men and subfertile men who participated in assisted conception procedures. These procedures included *in vitro* fertilization and embryo transfer (IVF-ET), tubal embryo stage transfer (TEST; Yovich et al, 1989), and pronuclear stage tubal transfer (PROST; Yovich et al, 1987). Some specimens were obtained as surplus sperm suspensions following an *in vitro* fertilization procedure.

Routine semen analysis was performed according to World Health Organization guidelines (World Health Organization, 1987), except that a subjective motility score of 1 through 3 was also allocated. Sperm were washed twice by centrifugation (200 × g, 7 minutes) and layered under culture medium (see below). Supernatant was collected after 30 minutes to 1 hour, a procedure that normally provides a high proportion of progressively motile spermatozoa (Leung et al, 1984). For severely oligozoospermic samples, washed suspensions were sedimented under 1 ml of culture medium in one well of a four-well tissue culture dish (Nunc, Roskilder, Denmark), and motile sperm were harvested from the supernatant after 1 hour (Matson et al, 1987). The aim of sperm preparation was to arrive at a final concentration of approximately 1 to 5.0 × 10^6 motile spermatozoa/ml for ionophore challenge. The procedures are essentially the same as those used in our IVF laboratory before IVF-related procedures. Specimens from some men did not allow for sufficient sperm to be recovered to carry out the ionophore challenge (see Results).

Media

Sperm suspensions were cultured in a modified Hepes-buffered Tyrode's solution (HT9; composition in g/l: NaCl, 5.689; KCl, 0.356; $CaCl_2.2H_2O$, 0.294; $NaHCO_3$, 2.100; $MgCl_2.6H_2O$, 0.096; $Na_2.HPO_4$, 0.048; Na pyruvate, 0.055; glucose, 1.000; penicillin G, 0.060; Hepes, 5.206; phenol red, 0.005; and Na lactate, 2.0 ml in 60% solution). Osmolarity was 280 to 290 mOsm; pH was 7.3 in air. For sperm culture, medium was supplemented with 10% heat-inactivated human serum. All chemicals were Analar grade from Sigma Chemical Co. (St. Louis, MO) or Mallincrodt, Inc. (Paris, KY). Some suspensions used in IVF attempts were cultured in 5% carbon dioxide: 5% oxygen: 90% nitrogen in human tubal fluid medium (HTFM; Quinn et al, 1985).

Preparation of Stock Solutions

Calcium ionophore A23187 (Sigma) was prepared as 5 mmol/L stock in dimethyl sulfoxide (DMSO). Aliquots of 300 μl were stored frozen at −20°C until use. Before use, stock was diluted 1 to 10 with protein-free HT9; 10 μl of this was used to challenge each 500 μl of sperm suspension, giving a final ionophore concentration of 10 μmol/L (Cross et al, 1986; Cross and Meizel, 1989). Control suspensions were treated with the same volume of DMSO diluted 1 to 10 with protein-free HT9.

Fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* lectin (PSA: catalog number L 0770, Sigma) was made in ultrapure water (resistivity, 18 MΩ/cm from a Millipore "Milli-Q" system; Millipore Australia, Lane Cove, Australia) at 1 mg/ml and stored frozen (−20°C) until use. For staining slides, this solution was diluted 1 to 10 with ultrapure water and kept in the dark at 4°C.

Ionophore Challenge

For each ionophore challenge, 1 ml of prepared sperm suspension with at least 2.0 × 10^5 motile sperm/ml was used. This was divided into two aliquots. One was challenged with 10 μl of A23187 stock in DMSO (10 μmol/L final concentration), and the other was treated with diluted DMSO alone to serve as a control. After 1 hour at 37°C, each sample was layered over 300 μl of 70% Percoll density gradient medium (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in a 1.5-ml microcentrifuge tube and centrifuged at 600g for 12 minutes. Supernatant and density gradient medium were removed, and the sperm pellet was resuspended in 50 μl of 96% ethanol at 4°C. The suspension was fixed for at least 30 minutes at 4°C before staining. All specimens were ionophore challenged after 3 hours of culture. Where there were sufficient spermatozoa, subsamples were further cultured for a total of 24 hours. This was only possible for 26 fertile and 7 subfertile cases. Motility remained better than 70% for the duration of the challenge.

Lectin Staining

Ethanol-fixed sperm suspensions were thoroughly suspended by repeated pipetting. Aliquots of 20 μl were dropped onto clean glass slides, and the ethanol was allowed to evaporate. Each was covered with a 10-μl drop of FITC-PSA (100 μg/ml) and placed in a dark humidified chamber at 4°C for 10 to 15 minutes. Excess stain was removed by gently immersing the slide in a beaker of ultrapure water at an angle of 45° approximately 15 times and allowing it to dry. The smear was then mounted under phosphate-buffered glycerol (1:9; pH, 7.4) before examining it under epifluorescence microscopy using an oil immersion objective. Slides were stored in the dark at 4°C to prevent bleaching.

Categorization of Staining Patterns

Four main staining patterns were recorded: 1) complete staining of the acrosome; 2) partial or patchy staining of the apical segment of the acrosome with complete staining of the equatorial segment; 3) staining of the equatorial segment only; and 4) faint

staining of the whole sperm, other irregular pattern, or abnormally formed acrosome. Only patterns 1 through 3 were considered to form part of the physiologically "normal" acrosome reaction or loss. This normally culminates in the exposure of oocyte plasma membrane fusion sites of the inner acrosomal membrane and leaves an intact equatorial segment that is thought to be essential for penetration of the zona pellucida (Yanagimachi, 1981; Bedford, 1982). The proportion of sperm demonstrating a complete acrosome reaction was expressed as the number of pattern 3 expressed as a percentage of 1 + 2 + 3 and calculated for both control and ionophore-challenged suspensions. The difference between the two (ionophore less spontaneous) was considered to be the percentage of sperm in the population capable of responding to ionophore, which will hereafter be called the acrosome reaction following ionophore challenge (ARIC). Thus, a typical result would give a spontaneous acrosome reaction rate of 10% with an induced rate of 35%; the derived ARIC would be 25%. For some cases with a low response, counting variability meant that the observed incidence in ionophore-stimulated suspensions was less than in the controls. In these cases, the ARIC was negative and should therefore be considered to be essentially zero.

Statistical Analysis

Data were examined using Macintosh software: FASTAT (SYSTAT, Inc., Evanston, IL) and MASS (Western Statistical Computing Associates Pty. Ltd., Nedlands, Australia). Distributions were tested for normality, kurtosis, and skewness. Where appropriate, nonpaired t tests were used to compare sample means between fertile and subfertile men. Non-normally distributed data were evaluated using the Mann-Whitney nonparametric U test. Quartile distributions were analyzed using stem-and-leaf analysis with FASTAT.

Predictive Value of the ARIC

The value of a clinical test must be evaluated in terms of its ability to detect problems prospectively. Thus, a test will give both true- and false-negative predictions and true- and false-positive predictions. In evaluating the effectiveness of a test, one valuable technique is to use receiver-operator-characteristic (ROC) curves in which the sensitivity of the test is plotted against the rate of false-positive results (Vinatier and Monnier, 1988). A test that has no predictive value will show a one-to-one relationship between the two factors, and a 45° line graph will result. Conversely, a predictive test will show high levels of sensitivity and low levels of false-positive results. The value of the ARIC test in predicting poor fertilization outcome was analyzed retrospectively on the data presented here. For this test, various cut-off values were considered. Subfertile patients, defined as those with fertilization problems, with ARICs below the cut-off point counted as "true positives" and fertile patients below the cut-off counted as "false positives."

Results

ARIC and Fertility Status

The ARIC value was determined after 3 and 24 hours in culture. Full results in relation to fertile status are presented in Table 1. Whereas spontaneous reactions were lower for the fertile group at 3 hours and higher at 24 hours, the differences were not statistically significant. However, a much higher response to ionophore was seen for fertile men at both 3 and 24 hours, with mean sperm reaction rates of 35% and 46%, respectively, compared with rates of 19% and 23% in the subfertile group, respectively. When the ARIC was calculated (from the difference between spontaneous and induced rates for the same samples), the differences between fertile and subfertile men were even more striking and statistically significant at both 3 and 24 hours ($P < 0.001$ by t test). Thus, the 25% and 75% quartile hinges of the data distribution for fertile men were ARICs of 14.5% and 43.2%, respectively; those for subfertile men were only 3.3% and 15.7%, respectively.

Predictive Value of the ARIC

The results of the ARIC are demonstrated in Figure 1 and Table 2. Clearly, the ARIC test is highly accurate in predicting poor fertilization: an ARIC cut-off of 10 is 54% sensitive in predicting subfertility while giving only 15% false-positive results. Lower ARIC values, while less sensitive, give even better exclusion of false-positive results. No fertile men were found with an ARIC value of less than

Table 1. *Spontaneous and induced acrosome reactions and ARIC values at 3 and 24 hours for fertile and subfertile men**

Acrosomal factor measured	Fertile men	Subfertile men	Significance of any difference
Spontaneous Reactions at 3 h (Mean, SD, Range)	5.1 ± 4.00 (0–20.4: n = 53)	7.5 ± 6.12 (0–21.2: n = 26)	Not significant by Mann-Whitney
Induced reactions at 3 h	34.8 ± 19.7 (3.3–72.8)	19.0 ± 11.80 (1.4–50.0)	$P < 0.001$ by t test
Difference: ARIC at 3 h	29.7 ± 18.37 (3.3–68.4)	11.5 ± 11.53 −2.0†–42.1	$P < 0.001$ by t test
Spontaneous reactions at 24 h	7.4 ± 7.88 (0–35.1: n = 24)	3.4 ± 3.4 (0–10.2: n = 7)	Not significant
Induced reactions at 24 h	46.4 ± 20.32 (10.3–79.8)	22.8 ± 11.14 (7.5–38.9)	$P < 0.001$ by t test
Difference: ARIC at 24 h	39.0 ± 20.28 (−8.3†–79.8)	19.4 ± 9.03 (6.4–33.0)	$P < 0.001$ by t test

* All results are presented as the mean, standard deviation (SD), and range.

† ARIC values for some individuals were negative due to counting errors at low levels, ie, more reactions were observed in the control suspensions than in the ionophore-challenged suspensions.

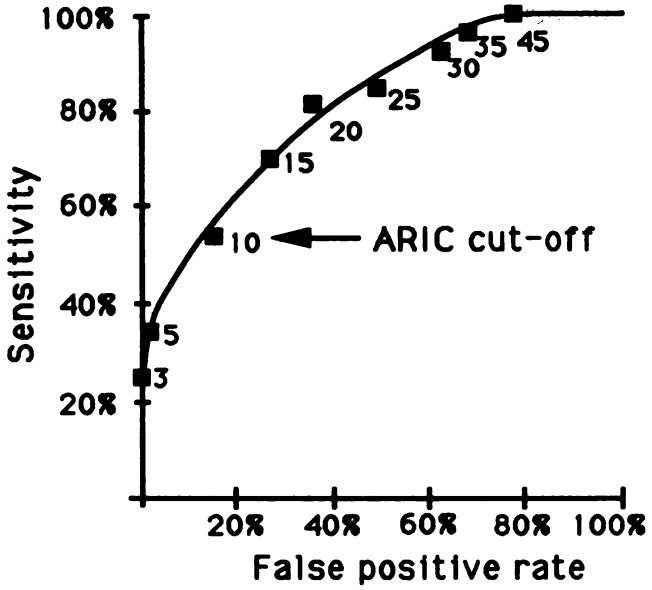

FIG. 1. Receiver–operator-characteristic (ROC) curve for 3-hour ARIC results as a predictor of poor fertilization *in vitro*. The graph is based retrospectively on the fertile and subfertile groups described in Materials and Methods. It plots sensitivity (true positives as a percentage of true positives plus false negatives), against the incidence of false positives. True positives are those men whose ARIC value lies below the cut-off point and who are subfertile, whereas false positives are fertile men with ARIC values below the threshold. A poor ROC curve would show a 1:1 relationship (ie, 45° straight line). See also Table 3.

5%, but 9 of the 26 subfertile men (35%) fell in this range. Only three of these men succeeded in fertilizing ova following sperm stimulation with pentoxifylline.

Other Seminal Parameters

Other seminal parameters are summarized in Table 3. Subfertile men had significantly lower total sperm counts, motile and progressively motile sperm counts, and grade of motility (measured on a subjective scale of 1 to 3) than fertile men. However, these measures were statistically less successful for discriminating between fertile and subfertile men than the ARIC test.

Limitations of the Technique

Successful ARIC estimations could be achieved on samples that provided at least 2.0×10^5 motile spermatozoa/ml after preparation. On one occasion, a count of only 1.0×10^5 gave measurable results. However, during this study period, 23 semen samples (from 22 men) were encountered that could not be studied because sufficient spermatozoa could not be recovered for counting. One of these instances was due to technical error. In the remaining 22 samples, the mean progressively motile sperm count was 1.86×10^6/ml before preparation (range, 0.1 to 9.0 10^6/ml), with a mean motility rating of 1.7 (range, 1 to 2). Thus, all these specimens were classified as oligozoospermic/asthenospermic based on WHO criteria (World Health Organization, 1987). Elevated seminal viscosity was noted in 9 cases and teratospermia (fewer than 50% morphologically normal forms) in 13 cases. Seven of the samples showed multiple defects. The fertility status of 10 of the men (as defined by *in vitro* fertilization) was not fully resolved; however, eight were classified as infertile (no fertilization) and four as subfertile (less than 50% *in vitro* fertilization). Two of the "subfertile" men achieved successful IVF pregnancies following their unsuccessful ARIC assessment: one had a history of fluctuating sperm counts, and a successful outcome was obtained for the other after using pentoxifylline (Yovich, et al, 1990).

Table 2. *Various threshold ARIC levels applied retrospectively to the fertile and subfertile groups in terms of capacity to predict poor fertilization outcome* in vitro

ARIC threshold (%)	3	5	10	15
Sensitivity (%)	23	35	54	69
Predictive value (%)	100	90	64	56
Specificity (%)	100	98	85	74
95% Confidence intervals of specificity (%)	—	94.6–100	76.9–93.5	63.2–84.0
False-positive rate (%)	0	2	15	26

Discussion

The ability of sperm to undergo a complete acrosome reaction in response to an ionophore challenge (ARIC as defined here) is significantly related to fertility status and is depressed or even absent in subfertile men. It seems likely that acrosomal dysfunction may be a major cause of fertilization failure and, therefore, of human infertility. Our findings support Tesarik's (1989) hypothesis that fertile spermatozoa will show enhanced levels of acrosome reactions following a suitable stimulus.

In this series, 86% (48 of 56) of the fertile men had ARIC values greater than 10%, and the 25% and 75% percentiles of the fertile population had ARIC values of 14.5% and 43.2%, respectively. In practice, we now counsel men with ARIC values of 5% or less that they are likely to experience problems with fertilization *in vitro*, and we take care to adopt suitable strategies in preparing their spermatozoa. Such strategies include routinely using pentoxifylline in preparing spermatozoa (Yovich et al, 1990), increasing the sperm concentration for insemination from 1.0×10^5 to 1.5×10^5 sperm/ml, and requiring two ejaculates to be produced 1 hour apart to maximize the numbers of spermatozoa available (Yovich et al, 1986; Yovich et al, 1989)

For simplicity and ease of evaluating sperm smears, the technique, as described here, did not include a vital stain for distinguishing live from dead sperm (Cross et al, 1986; Cross and Meizel, 1989). Obviously abnormal acrosomal morphologies, however, were not counted in determining

Table 3.

Seminal parameter	Fertile men	Subfertile men	Significance of any difference
Seminal volume ml (mean, SD, range)	3.8 ± 1.76 (1.5–10.1)	3.5 ± 1.61 (1.0–6.5)	Not significant
Total conc. 10^6/ml	88.1 ± 61.08 (11.0–277.0)	55.1 ± 42.29 (12.3–212.0)	$P < 0.01$ by Mann-Whitney
Motile conc. 10^6/ml	56.1 ± 40.01 (6.2–173.0)	29.3 ± 29.6 (1.1–148.0)	$P < 0.001$ by Mann-Whitney
Progressive motile conc. 10^6/ml	39.5 ± 29.99 (2.6–128.3)	18.9 ± 23.17 (0.7–111.0)	$P < 0.001$ by Mann-Whitney
Total progressive motile sperm in sample × 10^6	127.4 ± 82.54 (9.1–396.7)	79.1 ± 136.31 (1.75–677.1)	$P < 0.001$ by Mann-Whitney
Percentage normal forms	63.5 ± 13.20 (24–85)	59.6 ± 10.62 (23–76)	Not significant
Grade of motility from 1 to 3*	2.19 ± 0.40 (1.5–3.0)	1.87 ± 0.35 (1.2–2.5)	$P < 0.01$ by Mann-Whitney

*

the ARIC, so this should probably minimize the inclusion of "false" acrosome reactions. Moreover, the sperm suspensions used always contained high proportions (70% to 80%) of motile cells. While this is probably not a serious technical deficiency, we are currently evaluating the feasibility of improving the ARIC determination by including a vital stain.

The other major limitation of the technique is the need for at least 2.0×10^5 motile sperm/ml following sperm preparation. While this is probably close to the limit for successful IVF fertilization using current techniques, techniques such as sperm microinjection (Bongso et al, 1989) or culture in very small volumes (van der Ven et al, 1989) will probably prove feasible for severely oligozoospermic men in the future, and it would be useful to be able to screen in advance for acrosomal function for these cases. We are currently evaluating a recently reported micro-method based on capturing small numbers of sperm on a filter (Morales and Cross, 1989). This would allow us to measure acrosome reaction capacity for men with even lower sperm counts.

The test we describe takes about 3½ hours: 3 hours to prepare and incubate spermatozoa and another half-hour to evaluate the findings. It is thus considerably simpler and cheaper than the hamster ovum sperm penetration (SPA) assay (Gould et al, 1983; Rogers, 1985) and may indeed give very similar information about the sperm population: ie, that sperm are capable of undergoing the acrosome reaction. Although the ARIC test does not give information on the ability of sperm to fuse with an exposed oocyte surface, this may not be clinically relevant since abundant evidence suggests that a capacity to undergo the acrosome reaction is a basic prerequisite for this function (Yanagimachi, 1981, 1988). Direct comparison with the SPA assay is not possible as hamsters are prohibited animals in Australia. However, Albertsen et al (1983) found that a threshold of 14% hamster egg penetration would give a specificity of 80% in identifying infertile men. As presented in Figure 1 and Table 3, the ARIC test would give this level of specificity at a threshold of around 15%. The tests would thus appear to be broadly comparable, but further work is needed to evaluate them more critically.

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