High fertilization prediction by flow cytometric analysis of the CD46 antigen on the inner acrosomal membrane of spermatozoa

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The study was set up to determine the relationship between the human sperm acrosome reaction and fertilization in couples undergoing routine in-vitro fertilization (IVF) treatment. Prospective data analysis was carried out on all IVF patients during a 6 month period. Exceptions were those patients having insufficient sperm concentration to allow both acrosome reaction determination and insemination.

The main outcome measures were the prediction of fertilization in IVF patients using flow cytometric analysis of the spontaneous and ionophore-induced acrosome reaction [giving the acrosomal response to ionophore challenge (ARIC) score] in the male partner's spermatozoa versus standard analytical methods of sperm motion parameters and morphology. Stepwise logistic regression indicated only two independent factors predictive of fertilization: ARIC score ($\chi^2 = 109.6, P < 0.0001$) and post-Percoll % motility ($\chi^2 = 8.8, P < 0.003$). Of patients with an ARIC score of >10, 92% had >30% of oocytes fertilized; 100% of patients with an ARIC score of <10 had <30% fertilization of oocytes. The sensitivity and specificity of the assay system were 1.00 and 0.82 respectively. The results would indicate that the ARIC test as measured by flow cytometric analysis of CD46 binding is a sensitive and specific assay for use in the prediction of fertilization in IVF patients, thus enabling direct channelling of those patients with ARIC scores of <10 into the more invasive micro-assisted fertilization schemes.

Key words: acrosome reaction/CD46/fertilization prediction/flow cytometry

Introduction

Recently, we reported a novel assay technique for the objective quantitation of the spontaneous and ionophore-induced acrosome reaction in human spermatozoa (Carver-Ward et al., 1994). The principle of the technique depends on the binding of the CD46 antibody to the corresponding antigen present only on the inner acrosomal membrane of human spermatozoa; thus, expression and detection of the antigenic determinant can be measured only after the spermatozoon has undergone the acrosome reaction (D’Cruz and Haas, 1992). Work conducted by several authors (Andersen et al., 1993; Liszewski et al., 1991) has shown that CD46 (membrane cofactor protein/L TLX antigen/HuLy-m5) is a C3b/C4b-binding cell surface glycoprotein acting as an inhibitor of complement activation on cells. CD46 is one of six proteins of the RCA (regulators of comple ment activation) gene cluster which inhibit activation of complement through interaction with fragments of C3 and/or C4. CD46 is present on a wide range of membranes, i.e. all peripheral blood cells (with the exception of erythrocytes), epithelial, endothelial and fibroblast cells, trophoblastic tissue and spermatozoa. Fenchel et al. (1989) discovered that a monoclonal antibody (GB24) to CD46 bound to spermatozoa in the region of the equatorial segment and the inner acrosomal membrane, and only if the spermatozoon had undergone the acrosome reaction. A possible role for the presence of CD46 on the inner acrosomal membrane of human spermatozoa was presented by Anderson et al. (1993). Briefly, the function of CD46 on human sperm inner acrosomal membranes is to protect the spermatozoon from complement-mediated lysis. Further, antibodies binding to CD46 inhibit sperm penetration of hamster ova in the zona-free hamster oocyte penetration assay. Thus, it would appear that there is a role for CD46 in gamete interaction, based on the hypothesis that C3b binds to both a C3 receptor on the oolemma and to a C3b-binding protein (i.e. CD46) on the equatorial segment of an acrosome-reacted spermatozoon (Cervoni et al., 1992).

By evaluating this binding potential using flow cytometry, rapid assessment of large numbers of spermatozoa with regards to viability and acrosomal status is possible, thereby eliminating possibilities of measuring the ‘false’ acrosome reaction, a parameter often overlooked in other methodologies (Cummins et al., 1991; Takahashi et al., 1992; Pampiglione et al., 1993; Calvo et al., 1994a,b; Payne et al., 1994). Takahashi et al. (1992) described the kinetics of the acrosome reaction and correlated the results with fertilization in in-vitro fertilization (IVF) cycles. However, the study examined only the spontaneous acrosome reaction over a period of time, rather than looking at the inducible acrosome reaction, which is possibly more related to sperm function. Conversely, Pampiglione et al. (1993) found a good correlation between the ionophore-induced acrosome reaction and fertilization in IVF. This is in agreement with Aitken et al. (1993), who studied the effects of ionophore in enhancement of the hamster oocyte penetration assay and concluded that a group of ‘poor responders’ were
also ‘poor hamster egg penetrators’ Thus, the ability of spermatozoa to undergo the acrosome reaction would appear to be vital to sperm–zona binding in the human and mouse (Wassarman, 1987), and absence of an acrosome reaction would point to a poor prognosis for IVF (Calvo et al., 1994b).

Further work by Brandelli et al. (1995) demonstrated prediction of fertilization utilizing N-acetylglucosamine-neoglycoprotein (GlcNAc); when they compared its action with that of calcium ionophore, they found no significant differences between the two acrosome reaction inducers. Also, use of the assay for predicting fertilization in IVF cycles was encouraging, but demonstrated high false positive and false negative rates (9 and 22% respectively). However, the number of patients included in the study was quite small. Further, the study was performed utilizing a fluorescent slide assay technique and also did not take into account the live/dead populations of spermatozoa. The use of flow cytometric analysis has been well investigated by Tao et al. (1993), who examined the binding of a monoclonal antibody, MH61, to the sperm inner acrosomal membrane. Tao et al. (1993) also demonstrated the necessity of utilizing a supravital stain concomitant with the flow cytometric analysis. However, it is still uncertain whether this antibody is specific for the CD46 antigenic determinant expressed on the inner acrosomal membrane of acrosome-reacted spermatozoa. In contrast, Ohashi et al. (1995) demonstrated a simple assay for the acrosome reaction utilizing Accro beads (latex beads coated with MH61 antibody); however, again the assay, although giving good correlation with the sperm penetration assay as well as IVF rates, was based on subjective analysis and lacked the use of a supravital stain.

In the study presented here, we have assessed the spontaneous and ionophore-induced acrosome reactions immediately prior to oocyte insemination in normal IVF cycles and have correlated these data with the outcome of fertilization. This approach was taken in order to eliminate any variability between semen specimens, i.e. by assessment of the sperm population used for insemination. Also, by utilizing flow cytometry we were able to analyse a minimum of 5000 spermatozoa per patient (instead of the usual 100–200 assessed when using the fluorescent slide assays), thereby providing a sensitive and wholly objective analysis of sperm status (Carver-Ward et al., 1994), along with isolation (and removal from the analysis) of the dead and dying spermatozoa. Concomitant analysis of all other sperm parameters was also carried out (motility parameters) by computer-aided semen analysis (CASA), and sperm morphology by strict criteria (Kruger et al., 1986) in order to cross-correlate the acrosome reaction with other parameters in relation to fertilization outcome.

Materials and methods

Reagents and stock solutions

Calcium ionophore (A23187) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A stock solution of 5 mmol/l in dimethylsulphoxide (DMSO, Sigma) was stored at –40°C in 0.2 ml aliquots The same batch was used throughout the protocol. A working stock was obtained by diluting the stock 1:10 in human tubal fluid (HTF) medium as previously described (Carver-Ward et al., 1994). Bovine serum albumin (BSA), fraction V, low endotoxin, was obtained from Irvine Scientific (Santa Ana, CA, USA).

Anti-human CD46 monoclonal antibody (AMAC Inc., Westbrook, ME, USA), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig; Becton Dickinson, San Jose, CA, USA) and ethidium bromide (Sigma) were also made up as previously described (Carver-Ward et al., 1994).

Sperm preparation

Semen specimens were obtained from patients undergoing IVF treatment at the time of oocyte retrieval. All specimens were collected by masturbation into sterile containers after 72 h of sexual abstinence. All specimens were processed within 1 h of collection and aliquots processed for flow cytometry immediately prior to oocyte insemination.

Semen analysis was carried out using the Makler chamber (Sefi Medical Instruments, Haifa, Israel) in conjunction with a CASA system (Cellsoft 2000; Cryo Resources Ltd., New York, NY, USA).

All specimens were prepared by a modification of the standard Percoll separation technique (Dravland and Mortimer, 1985; Carver-Ward et al., 1994). Briefly, 0.5 ml each of 95% and 47.5% Percoll were gently layered into 15 ml conical centrifuge tubes. Between 0.3 and 1.0 ml of liquefied semen was gently layered onto each gradient and the whole tube centrifuged at 350 g for 20 min. Resulting sperm pellets were made up to 5 ml with HTF-BSA (Quinn et al., 1985) and centrifuged for 10 min. Resulting pellets were made up to 0.2 ml with HTF-BSA and incubated in 5% CO₂ at 37°C until the time of insemination. All semen parameters and morphological analysis (Kruger et al., 1986) were measured on fresh, post-preparation and pre-insemination (for ease of analysis, post-Percoll will henceforth refer to the pre-insemination sample) specimens. An aliquot of ~200 000 spermatozoa was taken for acrosome reaction testing.

Assessment of ARIC (acrosomal response to ionophore challenge) score

The pre-insemination aliquot of spermatozoa was treated as previously described (Carver-Ward et al., 1994). The time interval between post-Percoll preparation and oocyte insemination was ~4 h (Jaroudi et al., 1994). Briefly, the aliquot was divided equally between two tubes and each portion made up to 0.5 ml with HTF-BSA. To portion A was added 10 ml of HTF-BSA, and to portion B was added 10 ml of calcium ionophore (final dilution 10 μM). Each tube was capped lightly and incubated at 37°C/5% CO₂ for 45 min. At the end of this period, each was made up to 6 ml with HTF-BSA and centrifuged at 300 g for 10 min. Supernatants were aspirated and the wash repeated. Final pellets were made up to 0.5 ml and taken for flow cytometric analysis.

Note: It should be noted that no diluent controls were utilized in this study (DMSO as diluent for the calcium ionophore). Preliminary studies (unpublished observations) indicated that the final dilution of DMSO present in the sperm incubation sample was ~0.5% and that there was no demonstrable effect of this concentration on sperm viability, motility, morphology, or the acrosome reaction, as compared to untreated (culture media only) controls, after testing on 150 routine semen specimens.

Flow cytometry

Sperm suspensions were made up to 2.0 ml with phosphate-buffered saline (PBS, Sigma) and centrifuged at 500 g for 5 min. Pellets were resuspended in 20 ml of reconstituted anti-human CD46 monoclonal antibody (final concentration = 10 mg/ml). Tubes were incubated at room temperature for 30 min followed by centrifugation, as above, with 2 ml of PBS Supernatants were discarded and 4 ml of FITC-
conjugated goat anti-mouse Ig was added to each 0.1 ml pellet. Suspensions were incubated for 30 min at room temperature in the dark, followed by two washes in PBS (as above). Final pellets were resuspended in 1 ml of PBS and analysed on the FACSScan flow cytometer (Becton Dickinson Immunocytometry Systems). Immediately before analysis, 20 ml of ethidium bromide (50 mg/ml) was added to give a final concentration of 1 mg/ml, in order to stain any dead or dying spermatozoa and thereby allow 'gating' to remove these 'false acrosome reactions' from the data pool.

Data were collected and analysed as previously described (Carver-Ward et al., 1994); a minimum of 5000 spermatozoa were analysed for each sample. The ARIC (acrosome response to ionophore challenge) score was calculated by subtracting the spontaneous acrosome reaction from the ionophore-induced acrosome reaction

**Ovarian stimulation**

Ovarian stimulation was performed as previously described (Jaroudi et al., 1993) Transvaginal oocyte retrievals were carried out 34 h after human chorionic gonadotrophin (HCG) administration. Oocytes were cultured for ~4 h prior to insemination with 80,000–100,000 spermatozoa (Jaroudi et al., 1994). Only mature oocytes, according to the criteria of Veeck (1986), were included in the study.

**Statistical analysis**

The results are expressed as mean values ± SD. The fertilization rates were compared using the $\chi^2$ test. Comparison between groups was performed using the Mann–Whitney test. Because there was no linear relationship between semen parameters, ARIC scores and fertilization rate per cycle, Spearman correlation coefficients were utilized to test the correlation between sperm parameters and fertilization rate; stepwise logistic regression analysis was used to determine which of these parameters would independently predict the probability of fertilization. A level of $P < 0.05$ was considered significant.

Continuous variables (ARIC score and post-Percoll motility) were dichotomized since their relationship with outcome (probability of fertilization) was better approximated by a binary rather than a linear function. The selection of threshold values for dichotomization was carried out using a modified receiver operating characteristic (ROC) curve analysis (Silverberg et al., 1991). In this method, the sum of the true positive rate and true negative rate is plotted as a function of different threshold values of a continuous variable. The cut-off points for dichotomization correspond to the peaks of such plots. The ARIC score was classified as negative if $\leq 10$ and positive if $>10$. Motility was classified as negative if $<50\%$, and positive if $>49\%$.

**Results**

A total of 33 ARIC-negative cycles achieved an average fertilization rate per cycle of 3.9 ± 8.0%. This was significantly lower than 96 ARIC-positive cycles with a fertilization rate per cycle of 73.9 ± 26.1% ($P < 0.00001$).

Except for ARIC scores, all sperm parameters of pre- and post-preparation and post-Percoll (pre-insemination) preparations were analysed. None of the pre-Percoll or post-preparation parameters had any correlation with fertilization (data not shown); only post-Percoll (pre-insemination) data are presented. Table I demonstrates the correlation between the tested sperm parameters and fertilization rate.

The sperm parameters which correlated with fertilization (ARIC score, motility, normal morphology, concentration and maximum amplitude of lateral head displacement) were entered as independent variables in a stepwise logistic regression analysis to formulate a model that would predict fertilization. The step selections were based on the maximum likelihood method.

Logistic regression analysis indicated only two independent factors were associated with fertilization: ARIC score ($\chi^2 = 109.6, P < 0.0001$) and post-Percoll motility ($\chi^2 = 8.8, P < 0.003$); their presence provided significant improvement over the model with no parameters other than the constant.

The 129 treatment cycles (consisting of 129 individual, non-repeated patients) were categorized according to the cause of infertility as follows: 63 were male factor, 52 tubal factor, and 14 unexplained infertility. Of the 63 male factor cycles, 28 were ARIC-negative and 35 were ARIC-positive. The ARIC-positive and -negative male factor cycles were comparable with respect to sperm parameters and number of oocytes inseminated (Table II). Male factor was defined as described previously (Jaroudi et al., 1994) — briefly, this consisted of a minimum of three previous semen analyses exhibiting one or more of the following criteria: an average sperm density of $<20 \times 10^6/ml$, average motility $<40\%$ (World Health Organization, 1992), and average abnormal morphology $>86\%$ according to Kruger et al. (1986). Although sperm parameters were comparable, including motility, the 35 ARIC-positive male factor cycles gained a significantly higher average fertilization rate per cycle than the 28 ARIC-negative male factor cycles ($61.3 \pm 29.4$ and $4.6 \pm 8.5\%$ respectively; $z = 6.0,$

| Table I. Correlation between fertilization rate and sperm parameters |
|-----------------------------|-----------------|-----------------|
| Parameter                  | $r$             | $P$             |
| Acrosome reaction (%)      | 0.68            | $<0.0001$       |
| Motility (%)               | 0.46            | $<0.0001$       |
| Concentration ($\times 10^6$) | 0.36           | $<0.001$       |
| Mean amplitude of lateral head displacement (mm/s) | 0.05 | NS |
| Maximum amplitude of lateral head displacement (mm/s) | 0.12 | $<0.04$ |
| Beat cross frequency       | -0.04           | NS              |
| Linearity (VCL)            | -0.17           | NS              |
| Normal morphology (%)      | 0.37            | $<0.001$       |
| Velocity (mm/s)            | 0.09            | NS              |

*Spearman correlation coefficient between sperm parameters and oocyte fertilization rate*

NS = not statistically significant

| Table II. Analysis of male factor cycles with negative and positive scores in acrosomal response to ionophore challenge (ARIC) test. There was no significant difference between the two groups |
|-----------------------------|-----------------|-----------------|
| Parameter                  | Negative (n = 28) | Positive (n = 35) |
| Oocytes (%)                 | 9.4 ± 4.4        | 9.2 ± 6.0       |
| Motility (%)                | 43.7 ± 17.5      | 50.6 ± 26.1     |
| Concentration ($\times 10^6$) | 31.1 ± 35.1      | 77.0 ± 107.8   |
| Mean amplitude of lateral head displacement (mm/s) | 2.5 ± 0.7 | 2.3 ± 0.6 |
| Maximum amplitude of lateral head displacement (mm/s) | 3.2 ± 0.8 | 3.0 ± 0.7 |
| Beat cross frequency        | 16.4 ± 3.0       | 15.6 ± 2.5      |
| Average linearity (VCL)     | 51.1 ± 14        | 45.5 ± 12       |
| Normal morphology (%)       | 58.0 ± 14        | 52.0 ± 16.5     |
| Average velocity (mm/s)     | 53.4 ± 16.5      | 50.5 ± 12.6     |

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Table III. Number of oocytes retrieved and fertilized in relation to acrosome reaction in patients with tubal, male factor or unexplained infertility

<table>
<thead>
<tr>
<th>Type of infertility</th>
<th>Tubal</th>
<th>Tubal</th>
<th>Male factor</th>
<th>Male factor</th>
<th>Unexplained</th>
<th>Unexplained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrosome reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>50</td>
<td>2</td>
<td>35</td>
<td>28</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>negative</td>
<td>462</td>
<td>11</td>
<td>323</td>
<td>265</td>
<td>89</td>
<td>46</td>
</tr>
<tr>
<td>No (%) oocytes fertilized</td>
<td>360 (77.9)</td>
<td>0</td>
<td>166 (51.4)</td>
<td>11 (4.1)</td>
<td>67 (75.3)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Significantly different from male cycles with negative acrosome reaction, P < 0.0001
*Significantly different from unexplained cycles with negative acrosome reaction, P < 0.0001

Discussion

In this study we have shown that the ionophore-induced acrosome reaction of human spermatozoa may be used to predict the outcome of fertilization in IVF cycles. As stated previously, several authors (Liszewski et al., 1991; Andersen et al., 1993) have demonstrated that CD46 antibody specifically binds to the CD46 antigenic determinant present on the inner acrosomal membrane of spermatozoa, and this antigenic determinant is expressed only after the spermatozoon has undergone exposure of the inner acrosomal membrane (i.e. the acrosome reaction). Further, it is thought that the presence of this antigenic determinant protects the spermatozoon from complement-mediated lysis during its passage through the zona pellucida and the perivitelline space of a mature oocyte (Liszewski et al., 1991; Cervoni et al., 1992; Andersen et al., 1993). We previously defined an assay system based on flow cytometric analysis of CD46 antibody binding to the inner acrosomal membrane of acrosome-reacted spermatozoa (Carver-Ward et al., 1994), whose results were in agreement with those of D'Cruz and Haas (1992), and have extended the study to develop a screening assay for use in prediction of fertilization.

The results presented here demonstrate that the cut-off point for the ARIC score is 10, giving highly significant positive and negative predictive values as well as high specificity and sensitivity. The cut-off point was decided after examining the dichotomized data and the ROC curve — the curve expressing % AR can be seen to be highly linear around the value of 10% (Figure 1) — thereby indicating that this point should be the deciding factor when defining positive and negative values. Conversely, the curve expressing % motility can be seen to have no expressly defined cut-off point, the cut-off may be arbitrarily assigned to the 50% motility value. Further, previous work has indicated that the normal range for normal fertile patients with respect to ARIC score is usually >20%, with values between 10 and 20% being inconclusive, depending on the assay system (Cummins et al., 1991; Carver-Ward et al., 1994, 1996; Brandelli et al., 1995). Obviously, long-term
multicentre studies need to be conducted in order to define the precise range of the ARIC score, with the appropriate 95% confidence limits, in individual normal fertile males, in order to be able to identify consistently the patients for whom routine IVF would be unsuitable, if it were to be solely based on the use of acrosome reactivity. Thus, conducting this assay would appear to be valuable for the predictor of fertilization, especially where failure of, or low fertilization cannot be anticipated, i.e. in the presence of normal semen parameters. For example, 28 of the cycles whose sperm characteristics could not be distinguished from those of the other male factor cycles exhibited a negative ARIC score (Table II); therefore, these patients could immediately be excluded from IVF treatment and passed directly to the advanced micromanipulation techniques available, such as intracytoplasmic sperm injection (ICSI), thus reducing the need to offer IVF screening cycles to patients. Further, the same analysis could be applied to those patients undergoing intrauterine insemination (IUI) treatment, especially in cases of unexplained infertility where repeated failure of conception could thus be attributed to an abnormal or subnormal acrosome response. In this study, the unexplained infertility patients with a negative ARIC score had 0% fertilization.

When used in conjunction with the assisted reproductive technologies, the method we describe gives a positive predictive value of 0.92, i.e. the possibility of >30% fertilization occurring in a given IVF cycle is predictable in 92% of cases, when based on ARIC score at the time of insemination. The 30% cut-off for fertilization is based on our own criteria for patient treatment, i.e. those patients achieving <30% oocyte fertilization in a previous cycle will immediately be offered ICSI rather than a further cycle of IVF, especially in those cases whose semen parameters are within the normal range. Conversely, the negative predictive value, i.e. the possibility of <30% of oocytes achieving fertilization, is 1.00, or 100%.

Unlike other studies (Cummins et al., 1991; Albert et al., 1992; Calvo et al., 1994b; Liu and Baker, 1994), we preferred to examine the population of spermatozoa used for insemination purposes for two reasons: (i) to reduce the possibility of variability in a given patient’s ARIC score and (ii) to allow precise correlation between ARIC score and fertilization. Further, concomitant examination of sperm viability, as performed in our assay, and the acrosome reaction by flow cytometry allows precise and objective quantification of a minimum of 5000 spermatozoa, thereby ruling out the ‘false’ acrosome reaction displayed by dead or dying spermatozoa. Thus, in contrast with other studies (Takahashi et al., 1992; Pampiglione et al., 1993; Liu and Baker, 1994; Payne et al., 1994), we have discarded the lectin assays in favour of flow cytometric analysis using CD46 antibody binding to the inner acrosomal membrane. As reported by Taylor et al. (1994), CD46 plays an essential role in the process of spermatozoan–oocyte fusion at the region of the oolemma, as explained above, possibly preventing premature sperm destruction (Andersen et al., 1993). Thus, by detecting patients who are unable to respond to an acrosome reaction inducer, either pharmacological or physiological, we may immediately propose such patients for the more invasive treatments such as ICSI. Thus, we would remove futile cycles from the treatment protocols, thereby improving and enhancing patient care, as well as increasing chances of obtaining successful fertilization and embryo development.

Studies are in progress to examine variability in ARIC scores longitudinally, i.e. in subsequent cycles of IVF, especially where previous fertilization failure has occurred, to examine whether the defective acrosome reaction is a temporary and/or reversible anomaly.

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