The significance of the ionophore-challenged acrosome reaction in the prediction of successful outcome of controlled ovarian stimulation and intrauterine insemination

Guneet Makkar, Ernest Hung Yu Ng, William Shu Biu Yeung and Pak Chung Ho

Department of Obstetrics & Gynecology, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong Special Administrative Region, People’s Republic of China

BACKGROUND: This prospective study compared the acrosome reaction following ionophore challenge (ARIC) versus conventional sperm parameters and sperm velocities in predicting successful outcome following ovarian stimulation and intrauterine insemination. METHODS: All patients were offered a maximum of three treatment cycles. Conventional semen analysis was performed and sperm velocities were measured using computer-aided sperm analysis. Acrosome-reacted sperm were stained using chlortetracycline after ionophore challenge. Multiple logistic regression analysis and the receiver–operator characteristic curve analysis were applied to determine the best predictive variables and their cut-off values. RESULTS: ARIC score was the most significant variable in predicting pregnancy, followed by the percentage of induced acrosome-reacted sperm, serum estradiol levels on the day of hCG and sperm morphology by strict criteria. Higher spontaneous acrosome reaction had a negative relationship with pregnancy. ARIC score of 10% had a sensitivity of 85.3% and a specificity of 85.5%. The positive and negative predictive values were 64.2 and 96.6% respectively and the false positive and negative rates were 14.7 and 14.5% respectively. CONCLUSION: ARIC score was a better predictor of pregnancy than conventional sperm parameters and sperm velocities.

Key words: acrosome reaction/ARIC/calcium ionophore/intrauterine insemination

Introduction

Intrauterine insemination (IUI) in conjunction with controlled ovarian stimulation (COS) is usually offered to infertile couples having patent Fallopian tubes, prior to other assisted reproductive methods. The role of basic semen parameters in predicting successful outcome of COS/IUI is still controversial (Bolton et al., 1989; Horvath et al., 1989; Hinney et al., 1993; Nuojua-Huttunen et al., 1999). The acrosome plays an important role in the process of human fertilization, both in vivo and in vitro. The acrosome reaction (AR) is essential for sperm penetration through the zona pellucida and for preparing fusion of sperm with the oolemma because sperm that are incapable of undergoing the AR will not penetrate either zona-intact or zona-free oocytes (Yanagimachi, 1994). AR must be precisely timed to ensure successful fertilization (Tesarik, 1989). The ability of human sperm (even from fertile men) to undergo spontaneous AR when cultured under capacitating conditions is low and variable (Byrd and Wolf, 1986; Fenichel et al., 1991). The study of spontaneous AR gives little indication as to the fertilizing ability of sperm and therefore discrimination between fertile and infertile individuals is most unlikely (Pilikian et al., 1992).

AR can be induced by chemicals such as calcium ionophore A23187 by generating an intracellular calcium signal which sets in motion a sequence of membrane-associated changes culminating in AR (Irvine and Aitken, 1986). The AR ionophore challenge (ARIC) test, as well as the concept of acrosomal inducibility (Henkel et al., 1993) has been used as a predictor of sperm fertilizing ability (Tasdemir et al., 1993; Yovich et al., 1994; Carver-Ward et al., 1996; Avrech et al., 1997).

The aim of this study was to compare the outcome of the ARIC score versus conventional sperm parameters and sperm velocities in predicting successful outcome following COS/IUI.

Materials and methods

Infertile patients attending the Assisted Reproduction Unit at the Department of Obstetrics and Gynaecology, University of Hong Kong for COS/IUI were invited to participate in the study. During infertility
work-up, they all underwent a standard protocol of history-taking, physical examination and investigations including conventional semen analysis on at least two occasions, serum mid-luteal progesterone concentration and diagnostic laparoscopy with chromotubation. Patients had to satisfy the following inclusion criteria: (i) age of women <40 years; (ii) duration of infertility >2 years; (iii) regular ovulatory cycles as shown by mid-luteal progesterone concentrations ≥30 nmol/l; (iv) bilateral tubal patency and absence of peri-tubal adhesion confirmed by diagnostic laparoscopy with chromotubation and (v) total number of motile sperm in the ejaculate during work-up ≥10 x 10^6. Exclusion criteria were: (i) previous artificial insemination cycles and (ii) total number of motile sperm in the ejaculate <10 x 10^6. Every patient was extensively counselled and gave an informed consent prior to participating in the study, which was approved by the Ethics Committee, Faculty of Medicine, The University of Hong Kong. A maximum of three treatment cycles was offered.

All patients underwent COS by hMG (Pergonal; Serono, Aubonne, Switzerland). On the second day of the treatment cycle, serum estradiol level (E2) was checked and a baseline transvaginal ultrasound scan was performed. When E2 was <220 pmol/l and there was no ovarian cyst on the scan, 150 IU of hMG was given i.m. daily from day 3 onwards. The ovarian response was monitored by both transvaginal scanning and serum E2 level on a regular basis. 10 000 IU of hCG (Profasi; Serono) was given when the leading follicle was >18 mm in diameter and there were not more than three follicles of >16 mm in diameter. Cycles with excessive responses were cancelled because of a higher risk of multiple pregnancies.

After an abstinence of 2–3 days, the husband was asked to submit a semen sample in a sterile plastic container ~2 h before the IUI procedure. The sample was allowed to liquefy completely at room temperature, usually within 30 min. After liquefaction, sperm preparation was done by a discontinuous density gradient centrifugation method using ISolate sperm separation media (Irvine Scientific, Santa Ana, CA, USA) (Makkar et al., 2001). The pellet obtained after centrifugation was washed twice with Earle’s balanced salt solution (EBSS; Sigma, St Louis, MO, USA) supplemented with 0.35% Plasmanate (PPF; Bayer Corporation, Elkhart, USA) or 8% patient’s serum. The resulting sperm pellet after washing was overlaid with the same medium to give a final volume of 0.3–0.5 ml.

IUI was performed 38 h after hCG using a Tomcat catheter (Monoject, St Louis, MO, USA) as described by Ho et al. (1992). The patient was asked to rest in a supine position for 15 min after IUI and thereafter to resume her routine activities. The luteal phase was supported by two further doses of 1500 IU hCG on day 5 and day 10 after the ovulatory hCG injection. Serum E2 and progesterone levels were checked on day 10 and a pregnancy test performed on day 20 after the ovulatory hCG injection. A pelvic ultrasound was arranged to confirm the presence of intrauterine pregnancy and to determine the number of gestational sacs after a positive pregnancy test. The end point was determined as when the patient became pregnant or had completed three treatment cycles without success. IVF treatment was offered if the patient was still not pregnant after three treatment cycles.

Semen analysis
All the tests were performed on the aliquots of the same ejaculates that were processed for insemination and 50 μl from the prepared sample was taken for the following tests. Sperm concentration, progressive motility and percentage of sperm with normal morphology were evaluated according to World Health Organization, (World Health Organization, 1992) guidelines, both before and after semen preparation. Morphology was also assessed using strict criteria (Kruger et al., 1987).

Computer-aided sperm analysis (CASA) was done using the Hobson Sperm Tracker System (HST; Hobson Tracking Systems Limited, Sheffield, UK). The system details, the set-up parameters of HST and the technical details were similar to those stated previously (Makkar et al., 1999). The following parameters were determined: mean curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), mean straight line velocity (VSL, μm/s), mean linearity [LIN (VSL/VCL), %], mean straightness [STR (VSL/VAP), %], mean amplitude of lateral head displacement (ALH, μm), head beat cross frequency (BCF, Hz) and percentage of sperm exhibiting hyper-activated motility [HA, % (based on the following criteria: LIN <65, VSL >100 μm/s, ALH >7 μm].

Chlorotetracycline assay
The chlorotetracycline (CTC) staining fluorescence pattern (DasGupta et al., 1993) was used to stain the acrosome of sperm immediately after processing of the semen sample, with and without prior challenge with calcium ionophore A23187 (Cummings et al., 1991). Calcium ionophore A23187 (Free Acid; Sigma Chemical Co., St Louis, MO, USA) stock solution was prepared as 5 mmol/l stock in dimethylsulphoxide. Aliquots of 50 μl were frozen at −20°C. Before use this was thawed, diluted 1:10 with EBBS and added to the sperm giving a final ionophore concentration of 10 μmol/l. For each ionophore challenge, 20 μl sperm solution was taken in a 1.5 ml microcentrifuge tube, and diluted with EBSS to give a volume of 500 μl. A total of 10 μl of A23187 diluted stock was added to it (10 μl/final concentration), and the tube was incubated at 37°C. After 30 min it was centrifuged at 600 g for 12 min. Supernatant with EBSS was removed and 20 μl of sperm pellet was obtained.

The CTC working solution contained CTC–HCl (Sigma Chemicals Co.) at 750 μmol/l in a buffer containing 20 mmol/l Tris, 130 mmol/l NaCl, 5 mmol/l cystine that had been filtered using a 0.2 μm filter and was adjusted to pH 7.8 ± 0.05 using 1 mol/l HCl. The CTC working solution was prepared fresh each time and stored in a light-shielded container at 4°C. A 20 μl aliquot of sperm suspension was mixed thoroughly in a light-protected Eppendord tube with an equal volume of warmed CTC working solution. This CTC-stained sperm sample was then immediately fixed using pre-warmed 2 μl of 12.5% glutaraldehyde in 1 mol/l Tris buffer (pH 7.8), to give a final concentration of 0.06% glutaraldehyde. The tubes were centrifuged at 600 g for 5 min. About 20–30 μl of supernatant was discarded and slides were prepared by placing 5 μl of the sperm suspension onto a clean glass slide. A cover slip was added and minimum pressure was applied to orientate sperm heads flat for observation.

The slides were examined immediately at ×600 magnification with a Zeiss Axioskop epifluorescence microscope (Zeiss, Oberkochen, Germany) with a BP 450–490 excitation filter and LP 520 barrier filter, and CTC fluorescence was observed through a FT 510 chromatin beam-splitting mirror. The sperm were considered as ‘acrosome-reacted’ when there was lack of fluorescence on the head and those sperm showing fluorescence on any part of, or over, the entire head were treated as ‘unreacted’ (Perry et al., 1995). For each sample, 100 sperm were observed and percentage of acrosome-reacted sperm was determined. The ARIC score was the difference between induced (+A23187) and spontaneous (−A23187) acrosome-reacted sperm.

Statistical analysis
Only clinical pregnancies were considered in this study. A clinical pregnancy was defined as the presence of intrauterine gestational sac(s) on scanning or products of conception on histological examination in the case of miscarriages. The total number of motile sperm were obtained by multiplying semen volume, concentration and percentage of progressive motile sperm whereas the total number of
normal sperm was obtained by multiplying semen volume, concentration and percentage of normal morphology (World Health Organization, 1992) criteria.

Statistical analysis was performed using Statistical Program for Social Sciences (SPSS Inc., Chicago, IL, USA) as described by Nie et al. (1975). Two-tailed $P < 0.05$ was considered statistically significant. Because semen parameters did not follow Gaussian distribution, results are presented in median (range). Comparison between pregnant and non-pregnant cycles of various characteristics was done by Mann–Whitney $U$-test. $\chi^2$-Analysis was used to compare the different pregnancy rates. Multiple logistic regression analysis and the receiver–operator characteristic (ROC) curve analysis were performed to determine the best predictive variables and their cut-off values (Metz, 1978; Altman and Bland, 1994). Logarithmic transformation of semen parameters was used in the multiple logistic regression analysis.

## Results

Ninety-seven couples underwent 204 cycles of COS/IUI between July 1997 and July 1999. Seventy-seven couples (79.4%) had primary infertility and 20 (20.6%) had secondary infertility. Thirty-four pregnancies were achieved giving the pregnancy rate (PR) of 16.7% per treatment cycle and 35.1% per couple. The multiple PR (twins and triplets) was 20.1% (7/34). PR was similar in different causes of infertility (male, unexplained and endometriosis) and in different treatment cycles (data not shown). There were no differences in age of women, age of husband, duration of infertility, doses of hMG required and size of the largest follicle between pregnant and non-pregnant cycles. Significantly higher E2 level on day of hCG and more follicles with diameter of >16 mm were present in pregnant than non-pregnant cycles (Table I).

Comparing the conventional sperm parameters, percentage of normal forms assessed by both WHO and strict criteria in the neat and inseminated semen samples and total number of normal sperm in the inseminated semen were significantly higher in pregnant than that in non-pregnant cycles. Seminal volume, concentration of sperm, percentage motility and number of total motile sperm were similar in both pregnant and non-pregnant cycles (Table II). None of the velocities measured by CASA or the BCF of sperm showed any difference between pregnant and non-pregnant cycles (data not shown).

### Table I. Comparison of demographic parameters and ovarian responses between pregnant and non-pregnant cycles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pregnant ($n = 34$)</th>
<th>Non-pregnant ($n = 170$)</th>
<th>$P$-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of women (years)</td>
<td>33.0 (29–37)</td>
<td>34.0 (28–46)</td>
<td>NS</td>
</tr>
<tr>
<td>Age of husband (years)</td>
<td>37.0 (32–46)</td>
<td>36.0 (28–50)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>4.0 (2–10)</td>
<td>4.0 (2–11)</td>
<td>NS</td>
</tr>
<tr>
<td>Doses of hMG (ampoules)</td>
<td>15.5 (8–46)</td>
<td>16.0 (5–87)</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol level on hCG day (pmol/l)</td>
<td>3154 (701–10 504)</td>
<td>1819 (463–10 274)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No. of follicles &gt;16 mm diameter</td>
<td>2.0 (1–3)</td>
<td>1.5 (1–4)</td>
<td>0.041</td>
</tr>
<tr>
<td>Size of the largest follicle (mm)</td>
<td>19.0 (16–24)</td>
<td>19.0 (16–25)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are median (range).  
*Mann–Whitney $U$-test.  
NS = non-significant.

### Table II. Comparison of conventional sperm parameters of neat and inseminated semen between pregnant and non-pregnant groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Neat semen</th>
<th>Inseminated semen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pregnant</td>
<td>Non-pregnant</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>2.8 (0.3–6.5)</td>
<td>3.0 (0.3–8.5)</td>
</tr>
<tr>
<td>Concentration ($\times 10^6$/ml)</td>
<td>80.5 (23.0–410)</td>
<td>69.0 (6–500)</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>51.0 (6–86)</td>
<td>51.0 (0–84)</td>
</tr>
<tr>
<td>Total motile sperm ($\times 10^6$)</td>
<td>112.0 (14–836)</td>
<td>100.7 (0.1–1083)</td>
</tr>
<tr>
<td>Normal morphology (WHO criteria)* (%)</td>
<td>8.5* (2–16)</td>
<td>6.0* (0–21)</td>
</tr>
<tr>
<td>Normal morphology (strict criteria) (%)</td>
<td>42.0* (6–42)</td>
<td>18.0* (0–55)</td>
</tr>
<tr>
<td>Total normal sperm ($\times 10^6$)</td>
<td>41.8 (4.6–508)</td>
<td>30.2 (0.0–669)</td>
</tr>
</tbody>
</table>

Values are median (range).  
*P < 0.01 between pregnant and non-pregnant cycles.  
*P < 0.05 between pregnant and non-pregnant cycles.

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Table III shows the AR status of sperm in the inseminated semen. Semen samples associated with pregnancy demonstrated a significantly higher percentage of induced acrosome-reacted sperm (+A23187) compared with non-pregnant. Moreover, the ARIC score was also significantly higher in pregnant cycles. The percentage of spontaneous acrosome-reacted sperm (±A23187) demonstrated similar values between pregnant and non-pregnant cycles.

Those parameters exhibiting significant difference between pregnant and non-pregnant cycles were entered as independent variables in a stepwise regression analysis model predicting pregnancies. The most significant variable in the prediction of successful outcome was ARIC score with $\beta = 0.7067$ (standard discriminant function coefficient) ($P < 0.0001$). This was followed by the percentage of induced acrosome-reacted sperm ($\beta = 0.2494; P < 0.05$), serum E2 levels on the day of HCG ($P < 0.001$), and sperm morphology by strict criteria ($P < 0.01$). The percentage of spontaneous acrosome-reacted sperm demonstrated a negative relationship with pregnancy with ($\beta = -0.2006, P < 0.05$).

ROC curve analyses
ARIC score was a better predictor compared with percentage of induced acrosome-reacted sperm (partial area under the curves 0.885 and 0.761 respectively) (Figure 1). A threshold value of 10% for ARIC had the highest sensitivity and highest negative predictive value with a specificity of 85.5%. The positive and negative predictive values were 64.2 and 96.6% respectively and the false positive and negative rates were 14.7 and 14.5% respectively. The threshold value for the percentage of induced acrosome-reacted sperm was 15% with a sensitivity of 76.4% and a specificity of 61.2%. The positive and negative predictive values for the test were 37.8 and 92.6% respectively.

Discussion
The significance of ARIC score in predicting successful fertilization in IVF treatment is well documented in the literature. A low percentage of acrosome-reacted sperm after induction has been shown to be associated with a low fertilization rate in IVF (Schill, 1991; Calvo et al., 1994a; Sukcharoen et al., 1995) and IUI (Bielsa et al., 1994). Moreover, AR was significantly related to fertilization rates in vitro in teratozoospermic patients in male infertility (Liu and Baker, 1998). On the other hand, no correlation between acrosome status and fertilizing ability of sperm was also observed in some studies (Plachot et al., 1984; De Jonge et al., 1988; Wolf, 1989; Coetzee et al., 1994). These controversial results could be due to the selection of spontaneous acrosome-reacted sperm or small sample size in some of the studies. Combined parameters of acrosome-reacted sperm, total number of motile and morphologically normal sperm had predictive value for pregnancy for infertile couples in IUI (Bielsa et al., 1994) and IVF (Parinaud et al., 1995).
We performed our tests on the same population of sperm used for insemination. The advantage was to reduce the possibility of variation among the sperm variables and to have a precise correlation between them and pregnancy. AR was conducted only on inseminated semen after sperm preparation since sperm in raw semen are incapable of undergoing induced AR (Lee et al., 1987). Our results clearly indicate that the ARIC score and the percentage of induced acrosome-reacted sperm were significantly higher in pregnant than non-pregnant cycles. This is the first study to demonstrate that the ARIC score in the inseminated semen sample had the best power to predict successful pregnancy following COS/IUI.

These findings support the hypothesis of Tesarik (1989) that fertile sperm will show enhanced levels of AR following a suitable stimulus. AR, a prerequisite to successful fertilization (Calvo et al., 1994a), is reported to be impaired in men suspected of being infertile (Calvo et al., 1994b; Oehninger et al., 1994). Significantly more frequent acrosome alterations are present in infertile men (Bielsa et al. 1994). The percentage of acrosome-reacted sperm differs significantly between fertile and infertile men, and AR provides additional information on the quality of semen specimen, not derived from the conventional semen analysis alone (Calvo et al., 1994b).

A negative relationship with pregnancy was established with a higher percentage of spontaneous acrosome-reacted sperm in our study. More sperm with spontaneous AR have been previously shown to be negatively correlated with fertility in artificial insemination with frozen sperm (Marshburn et al. 1992). Good sperm samples have low spontaneous AR and premature AR are the result of artificial disturbances in which sperm are less fertile and result in failed fertilization (Tesarik, 1989). Therefore, a higher rate of spontaneous AR found in the capacitated sperm populations reflects a decreased rather than increased fertilizing performance of the sample. More of these sperm lose their acrosomal membrane during their passage through the cumulus oophorus and so are unable to bind to zona pellucida and fertilize the oocyte (Takahashi et al., 1992).

Tests for acrosomal function should distinguish between spontaneous AR and those produced by a suitable stimulus. While human sperm in capacitation media have the ability to undergo spontaneous AR (4–10%) (Smullen et al., 1992), there is no direct relationship with fertilization rates in vitro (De Jonge et al., 1988; Wang et al., 1993; Krausz et al., 1996) and in vivo (Cummins et al., 1991). Calcium ionophore A23187 was used to induce AR of sperm in the present study. Ionophore is a known inducer substance for AR and it significantly enhanced AR rates in culture dishes in a concentration-dependent manner without any effect on sperm motility (Aitken et al., 1984; Tesarik, 1985). Moreover, A23187 was the most effective AR inducer as compared with progesterone and follicular fluid (Jaiswal et al., 1999).

The threshold values for the ARIC score and the percentage of induced acrosome-reacted sperm in this study were found to be 10 and 15% respectively. Similarly, fertilization failure in IVF was more likely when ARIC score was ≤5–10% (Cummins et al., 1991; Henkel et al., 1993; Yovich et al., 1994; Carver-Ward et al., 1996; Krausz et al., 1996; Tesarik, 1996) or the percentage of induced acrosome-reacted sperm was <3–31% (Fenichel et al., 1991; Henkel et al., 1993; Pampiglione et al., 1993; Parinaud et al., 1995; Esterhuizen et al., 2001). Discrepancies of the threshold values among these studies may be related to the methods used to induce acrosomal exocytosis (zona pellucida, low temperature, progesterone and calcium ionophore by rest) and the methods used to evaluate AR (using fluorescent-labelled lectins and antibodies or triple stain). Definitions for AR and fertilization rates also varied in these studies.

Both induced AR and sperm–zona binding assays were demonstrated to be predictors of fertilization (Oehninger et al., 2000) but the sperm–zona binding assay has many limitations in the clinical settings such as the supply of human oocytes. AR assays appear to be equally predictive compared with sperm– zona binding assays (partial areas under the ROC curve; 82 versus 85% respectively; meta-analysis; Oehninger et al., 2000), and have the advantage of being simpler in method. Many researchers have therefore chosen this practical approach and use ARIC score to predict successful fertilization in IVF.

In summary, the ARIC score was a better predictor of pregnancy following COS/IUI when compared with conventional semen parameters and various sperm velocities measured by CASA. The significant variables in the prediction of successful outcome were ARIC score, the percentage of induced acrosome-reacted sperm, serum E2 levels on the day of HCG, and strict criteria of assessing morphology. The threshold values for ARIC and percentage of induced acrosome-reacted sperm were 10 and 15% respectively. The ARIC score may be used to select patients for COS and IUI.

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