Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF

A.D.Esterhuizen¹, D.R.Franken², J.G.H.Lourens¹ and L.H.van Rooyen¹

¹Andrology Laboratory, Drs du Buisson and partners, Pretoria and ²Department of Obstetrics and Gynaecology, University of Stellenbosch, Tygerberg, South Africa

The study aimed to establish zona pellucida induced acrosome reaction response (ZIAR) among 35 couples with normal and G-pattern sperm morphology and repeated poor fertilization results during assisted reproduction treatment. ZIAR tests were performed using 0.25 zona pellucida/µl co-incubated with spermatozoa for 60 min. Acrosome reactions were measured with FITC–PSA staining, and expressed as the difference between stimulated and unstimulated (spontaneous) sperm populations. Results were compared with IVF rates of metaphase II oocytes. Interactive dot diagrams divided the patients into two groups, i.e. ZIAR <15% and ZIAR > 15%, with mean fertilization rates of 49 and 79% respectively. The sensitivity and specificity for ZIAR results versus fertilization were 93 and 100% respectively. The area under the curve was 99% and the 95% confidence interval did not include 0.5 which implies that the ZIAR test is able to predict fertilization failure among IVF patients. In conclusion, the ZIAR test has diagnostic potential since it can assist the clinician to identify couples that will benefit from intracytoplasmic sperm injection therapy.

Key words: acrosome reaction/failed fertilization/zona pellucida

Introduction

The astounding success rates achieved by intracytoplasmic sperm injection (ICSI) (Van Steirteghem et al., 1993), emphasized the need to refine sperm function evaluation. This is particularly true in cases of profound male factor infertility and therefore contemporary andrology laboratories should be able to select the most appropriate form of treatment for each couple (Oehninger et al., 1991, 1997; Kruger and Coetzee, 1999). Moreover, the past 10–15 years have brought not only an explosion in the number of laboratory tests for human sperm functions, but also the belief among many clinicians that sperm function testing is now irrelevant due to the advances in IVF technology. Ideally, an accurate and inexpensive test would be used to determine which men require ICSI and which do not. The current success with ICSI has stifled rather than stimulated the search for such a test. Also the history of abuse of sperm function tests has not helped; far too many have been heralded by their advocates as the best diagnostic approach and clinicians have been too quick to pronounce men fertile or infertile on the basis of a single favourite test.

At a consensus workshop in advanced andrology (Consensus Workshop, 1996) it was suggested that because of their validation and unquestioned clinical value, the homologous sperm–zona pellucida binding tests should be incorporated in the advanced stages of the work-up. Sperm–zona binding reflects multiple sperm function events depicting spermatozoa–zona pellucida interaction, i.e. completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand-induced acrosome reaction. A diagnostic test underlining the ability of the spermatozoa to undergo the acrosome reaction in response to homologous zona pellucida would be a valuable additional tool in the male fertility work-up schedule (Franken et al., 1990; Oehninger et al., 1992; Liu and Baker, 1992).

During the consensus workshop it was agreed that better standardization of the currently-used acrosome reaction techniques should be implemented prior to their introduction as a routine clinical tool. The present report is an effort towards development and standardization of the acrosomal response as a clinical tool in the assisted reproductive programme. The most widely utilized method is the acrosome reaction (AR) ionophore challenge test (ARIC), during which the acrosome reaction is induced by calcium ionophore and then identified and defined by lectins in combination with indirect immunofluorescence (Consensus Workshop, 1996). The rationale for the development of this test was manifested in the precise timing of acrosomal response (Tesarik, 1989, 1996; Cummins et al., 1991). Guidelines for the interpretation of the acrosome response during the ARIC test defined AR prematurity in cases where >20% of spermatozoa show spontaneous AR after 3 h incubation under capacitating conditions. AR was reported to be normal if >15% difference existed among spontaneous and
induced AR; <10% difference is abnormal and indicates a possible impairment of fertilization (WHO, 1999).

The ARIC test as well as the concept of acrosomal inducibility (Henkel et al., 1993, 1998) was used as a predictor of sperm fertilizing ability as compared with tests that simply measure the frequency of spontaneous AR.

The zona pellucida (ZP), both intact and solubilized, has been demonstrated to be a powerful and physiological inducer of the AR (Cross et al., 1988; Florman et al., 1989; Bielfeld et al., 1994; Liu and Baker, 1994; Franken et al., 1996, 1997). During fertilization, AR failure can be caused by multiple factors, such as: (i) inadequate sperm capacitation; (ii) an inability of the sperm membrane to undergo specific structural–functional changes after binding to the ZP; or (iii) an impaired capacity of the ZP of a specific oocyte to induce the acrosomal cascade.

This study aimed to evaluate and establish, among non-male factor couples with repeated ‘poor’ or no fertilization, their AR to solubilized human ZP during IVF treatment. The results will be used to define: insufficient AR, i.e. where the difference between spontaneous and zona induced AR (ZIAR) is <15%; and normal AR response where the difference between spontaneous AR and induced AR is ≥15%.

Materials and methods

Patients

All patients in the study signed an informed consent form after Institutional Review Board approval was obtained. The inclusive criteria for patients accepted into the study were: (i) females diagnosed as tubal factors; (ii) having a normal FSH/LH ratio on day 3 of the menstrual cycle (<10 U/l); (iii) producing three or more pre-ovulatory metaphase II oocytes at retrieval; (iv) having husbands with normal sperm parameters as well as either normal or G-pattern (4-14% normal forms) morphology; and (v) a history of repeated poor (<30% metaphase II oocytes fertilized) fertilization or complete fertilization failure, during two previous cycles. The mean (±SD) age and number of oocytes retrieved per patient was 30 ± 3 years and 8.3 ± 2 oocytes respectively. During the 12 month study period, a total of 35 from 382 (9%) couples adhered to the above inclusive criteria.

Ovulation induction

Ovulation induction protocols were adapted to individual needs. Thirty-three patients in the study were treated with a ‘short’ protocol, while two who did not respond well on the short protocol were treated with the ‘long’ protocol. The long protocol started with 0.5 mg/day Buserelin® [gonadotropin releasing hormone (GnRH) analogue; Suprefact, Hoechst IHD, Johannesburg, Republic of South Africa] or Lucrin® (GnRH analogue; Abbott IHD, Johannesburg, Republic of South Africa) on day 21 of the menstrual cycle (<10 U/l); (iii) producing three or more pre-ovulatory metaphase II oocytes at retrieval; (iv) having husbands with normal sperm parameters as well as either normal or G-pattern (4-14% normal forms) morphology; and (v) a history of repeated poor (<30% metaphase II oocytes fertilized) fertilization or complete fertilization failure, during two previous cycles. The mean (±SD) age and number of oocytes retrieved per patient was 30 ± 3 years and 8.3 ± 2 oocytes respectively. During the 12 month study period, a total of 35 from 382 (9%) couples adhered to the above inclusive criteria.

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Oocyte evaluation

Metaphase I and II oocytes were identified according to their nuclear maturity status. In metaphase II oocytes a first polar body was present while in metaphase I oocytes no polar body was observed. Following oocyte retrieval, great care was taken to ensure that the evaluation and classification of the oocytes were meticulously carried out by the embryologist. Under microscopic vision, metaphase II oocytes were characterized by their round, even shape and presence of light colour and homogeneous granularity. Metaphase II oocytes were always associated with expanded, luteinized cumuli and ‘sun-burst’ corona radiata. The membranes of the granulosa cells harvested along with the metaphase II oocytes were also luteinized, loosely aggregated and had mature features (Veeck, 1988).

In-vitro fertilization

Metaphase II oocytes were cultured as suggested in the IVF Science Product Manual (Granberg, 1999). Briefly, culture dishes (Falcon 1006®, Becton Dickinson SA Scientific, Randburg, Republic of South Africa) were prepared using 24 h pre-equilibrated culture media (IVF-50; Scandinavian IVF Science Products, Gothenburg, Sweden and universal IVF medium; Medi-Cult, Mollehaven, Jyllinge, Sweden) and Sigma mineral oil (Sigma-M-8410, embryo tested; Sigma-Aldrich, Johannesburg, Republic of South Africa). A total of 3 ml IVF-50 medium was incubated in tissue culture grade plastic tubes (Falcon 2058®, SA Scientific, Randburg, Republic of South Africa) for the final swim-up procedure. Spermatozoa were washed in ASP-100 (Scandinavian IVF Science Products) and layered with 1.5 ml IVF-50 medium. Oocyte insemination was performed with standard sperm concentration of 100 000 motile spermatozoa/ml/oocyte in 3 ml IVF-50 culture medium. On day 1, oocytes were denuded and evaluated by the embryologist for fertilization status.

Rescued intracytoplasmic sperm injection

The oocytes of patients where no fertilization was reported 1 day after aspiration were subjected to rescued ICSI. Prior to injection the unfertilized oocytes were placed in 10 μl HEPES buffered droplets under oil in 1006 Falcon Petri dishes. Rescued ICSI was performed as a therapeutic procedure and before the zona pellucida induced acrosome reaction response (ZIAR) results were available. Briefly, a small volume (2–3 μl) of prepared spermatozoa was deposited in a droplet of polyvinylpyrrolidone (PVP, Mediculit®; Harrilabs, Randburg, Republic of South Africa). Narishige micromanipulators mounted on a Nikon inverted microscope were used to perform the sperm injection. The oocyte was stabilized by applying negative suction to a holding pipette (Cook, Brisbane, Queensland, Australia). An individual morphologically normal spermatozoon was immobilized and sucked into a Cook injection pipette. The spermatozooa was injected into the ooplasm and the injected oocyte was then incubated according to the IVF protocol.

Preparation of zonae pellucidae

Solubilization of ZP was performed using 20 oocytes at a time depending on the number of ZIAR tests to be performed. This method was developed during previous reports and results revealed it to be optimal in our laboratory, since (i) the small volumes of solubilized zona can be stored (7–10 days) at 4°C (Franken et al., 1996, 2000), thus avoiding loss of precious zona material and (ii) since the ZIAR tests were performed blindly on the day of the oocyte retrieval, the use of small volumes of zonae was more practical. Unpublished data from our laboratory showed that the acrosome inducing potential of the different ZP batches did not differ between batches. Unfertilized oocytes (no pronuclei or second polar body) donated by diagnosed male factor couples from the in-vitro fertilization
Preparation of spermatozoa

The semen from 10 fertile sperm donors (14 samples) were used, during a pilot study, as controls to establish the baseline AR response among a fertile population, not attending an assisted reproductive programme. Spermatozoa from 35 patients taking part in our IVF programme with normal and G-pattern morphology were used. Liquidified semen was washed in ASP-100 (Scandinavian IVF Science Products) for 10 min at 400 g. ASP-100 medium is a modified human tubal fluid culture medium containing standard concentrations of 25 mmol/l sodium bicarbonate supplemented with 10 mg/ml human serum albumin. The sperm pellet was layered with 1.5 ml IVF-50 medium (Scandinavian IVF Science Products) and incubated for 30 min at 37°C and 5% CO₂ to achieve a swim-up separation of motile cells. After the incubation period, 1.0 ml of the top sperm suspension was aspirated, a 0.2 ml portion of highly motile spermatozoa was then removed and placed in a plastic tube (Falcon 2058; Becton Dickinson) for the ZIAR. This fraction was then washed once at 400 g for 10 min with DPBS and the pellet was resuspended in 100 µl DPBS, whilst the remaining volume was used for insemination purposes in the IVF laboratory.

ZIAR test

Prior to the study, technician and sperm specimen variation was recorded, by establishing intra- and interassay/technician coefficients of variations (CV) for PSA–FITC (Pisum sativum agglutinin–fluorescein isothiocyanate) staining during ZIAR testing. Intra-assay and technician variation was determined by evaluating 100 cells on five different microscopic fields (total 500 cells) from the same semen specimen by each of three technicians. Inters assay and technician variation was accomplished by evaluating staining by counting 100 cells on slides prepared from five different sperm specimens by the same sperm donor. CVs for both intra- and interassay and intra- and inter-technician values were calculated by dividing the mean with SD×100% for each observation. The inter- and intra-assay as well as inter- and intra-technician CV was <15 and <10% among the slides and three technicians respectively. Results were discarded in cases where the CV for assay and technician exceeded 15 or 10%.

A total of 20 µl of the zonae solution and 20 µl prepared spermatozoa (5×10⁶ spermatozoa/ml) were mixed in a microplate (Greiner Lab and Scientific Equipment, North Riding, Republic of South Africa). The mixture was aspirated into a Hamilton Pipette Tip® (Hamilton, Separations, Republic of South Africa) using a 1.0 ml sterile, non-pyrogenic latex free syringe (Becton Dickinson). The tip containing the spermatozoa–zona solution was incubated in a Falcon dish (Falcon 3003®; Becton Dickinson) for 60 min at 37°C and 5% CO₂. After the incubation period the spermatozoa were expelled onto the glass slide and coded ‘test’ spermatozoa, while ‘control’ spermatozoa were aspirated from the DPBS resuspended sample described above. Pilot studies have indicated that NaOH neutralized acid Tyrode’s solution had similar effect on the AR compared with that recorded with DPBS, namely 13.7% ± 2 for neutralized acid Tyrode’s compared with 14.6% ± 3 for DPBS (P > 0.5). We therefore used DPBS to resuspend the control spermatozoa in all forthcoming experiments. Slides were air-dried and fixed in 100% ethanol for 24 h. Previous experiments in our laboratory indicated that prolonged exposure of spermatozoa to ethanol during the fixation period increased the sensitivity of the PSA–FITC staining. This allowed us to record AR mediated by lower ZP concentrations (0.3 ZP/µl) generally reported in the literature, i.e. 2 ZP/µl (Liu and Baker, 1996b). Thereafter the spermatozoa were stained for 2 h at room temperature with 30 µg/ml PSA labelled with FITC (L-0770; Sigma- Aldrich). Finally, slides were washed in DPBS and mounted with DPBS. A minimum of 100 spermatozoa were counted under a Nikon fluorescent microscope (Labophot 2; Nikon; IMP; Johannesburg, Republic of South Africa), Filter Ex 465–495 with ×400 magnification. The following staining patterns were evaluated as acrosome reacted spermatozoa: (i) patchy staining on acrosomal surface, (ii) distinct staining in the equatorial region occurring as an equatorial bar and (iii) no staining observed over entire sperm head. Since we used only swim-up sperm samples, the motility was in all cases >80%. AR data present the findings recorded for live spermatozoa. Spermatozoa with patchy FITC–PSA staining were classified as a population of spermatozoa where the AR was initiated and all were classified as acrosome reacted. The slides were evaluated on the day of oocyte aspiration prior to the IVF outcome in order to ensure that the AR results were recorded blindly by the technicians. The zona induced AR was calculated as the difference between zona induced AR minus the spontaneous (unstimulated) AR results (A minus B, Table I).

Statistical analyses

The diagnostic accuracy of the ZIAR results was illustrated with the interactive dot diagram, while the positive and negative predictive values were recorded with the receiver operating characteristics (ROC) curve (Altman and Bland, 1994; Schoonjans et al., 1995).

Results

The sperm parameters and AR (mean ± SD) obtained from fertile sperm donors are depicted in Table I. All the AR data were obtained by ZP from donated IVF metaphase II oocytes, which, compared with zonae from prophase I oocytes, had not only an enhanced acrosome inducing capacity, but also exhibited increased sperm–zona binding potential (Oehninger et al., 1992; Franken et al., 1994). The range for ZP induced AR, namely 18–25% in Table I, was within the range for metaphase II oocytes. However, the dose–response curve for the induction of the AR that we have established (Franken et al., 2000) was recorded using post-mortem retrieved prophase I oocytes. We routinely use the prophase I dose–response curve
Table I. Semen sperm parameters and acrosome reaction results of 10 fertile (14 samples) patient population (mean ± SD and range)

<table>
<thead>
<tr>
<th>Stimulated AR (test) (0.25 ZP/µl)</th>
<th>Unstimulated AR (control) A–B</th>
<th>% ZIAR AR (%) B</th>
<th>Sperm parameters (10⁶ cells/ml)</th>
<th>Motility (%)</th>
<th>Morphology (%) normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>36.6 ± 3.2 (29–41)</td>
<td>14.8 ± 2.7 (11–20)</td>
<td>21.3 ± 2.6 (18–25)</td>
<td>58.8 ± 12 (45–85)</td>
<td>60.0 ± 4</td>
<td>17.7 ± 3</td>
</tr>
</tbody>
</table>

AR = acrosome reaction; ZIAR = zona pellucida induced acrosome reaction.

Table II. Semen sperm parameters, acrosome reaction and IVF results of 35 couples attending assisted reproductive programme (mean ± SD, range)

<table>
<thead>
<tr>
<th>Semen sperm parameters</th>
<th>Stimulated AR (test) (0.25 ZP/µl)</th>
<th>Unstimulated AR (control) A–B</th>
<th>ZIAR (%) A–B</th>
<th>IVF results of metaphase II oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>sperm conc. (10⁶ cells/ml)</td>
<td>60.2 ± 4 (22–100)</td>
<td>12.7 ± 0.6 (7–18)</td>
<td>58.8 ± 2 (40–80)</td>
<td>no. oocytes retrieved 0.6</td>
</tr>
<tr>
<td>morphology (% normal)</td>
<td>27.0 ± 2 (7–49)</td>
<td>15.4 ± 1 (5–46)</td>
<td>12.8 ± 2 (1–27)</td>
<td>no. fertilized 95.4</td>
</tr>
<tr>
<td>motility (%)</td>
<td>8.5 ± 5.4 (2–22)</td>
<td>3.3 ± 0.6 (0–10)</td>
<td>3.0 ± 0.5 (0–10)</td>
<td>no. divided 95.4</td>
</tr>
</tbody>
</table>

AR = acrosome reaction; ZIAR = zona pellucida induced acrosome reaction.

![Figure 1](image1.png)

**Figure 1.** The interactive dot diagram of zona pellucida induced acrosome reaction (ZIAR) results and IVF rates reported for metaphase II oocytes.

As reference (0.3 ZP/µl: positive control) in all experiments related to ZP mediated AR. The semen parameters, ZIAR as well as the IVF results of the 35 couples are depicted in Table II.

The correlation coefficient between the ZIAR and fertilization rates was $r = 0.95$ ($P = 0.0001$). The interactive dot diagram (Figure 1) illustrates the diagnostic power of ZIAR. In order to evaluate the clinical importance of the ZIAR data that was recorded for the 35 couples, a ZIAR cut-off value of 15% was chosen for the group. This value was chosen based on the following: (i) shifting the cut-off points for ZIAR (from 7–19%) during the ROC analyses the 15% level revealed optimum sensitivity and specificity for morphology (i.e. 60 and 68% respectively) and 100 and 90% respectively for fertilization; (ii) furthermore, the mean ZIAR value recorded for the group was 12.8 ± 2%, which was rounded off to 15% when using 1 × SD above the mean; and (iii) there was a clear cut in the data at 15%.

The IVF rates were calculated as the total fertilization rate (total number of pre-ovulatory oocytes fertilized/total number of pre-ovulatory oocytes inseminated) at 88.0 ± 19% (mean ± SD). The minimum total fertilization rate (mean minus 2 × SD) that could be considered normal in our IVF programme was therefore 50%. Total fertilization values <50% were considered to be abnormal for the IVF laboratory (Franken et al., 1993). From the ROC analyses, the optimum sensitivity and specificity was calculated at 50% for the best separation between the two groups, ZIAR >15% and ZIAR <15%.

A cut-off value for the fertilization rates of 50% was therefore chosen for the optimum separation between the two groups ZIAR >15% and ZIAR <15%. The corresponding sensitivity and specificity was 93 and 100% respectively. Using the interactive dot diagram as guide we then divided the couples according to the ZIAR test outcome. The mean (±SD) sperm parameters, acrosome response of the ZIAR groups >15% and 68% respectively. These results were expected since normal and G-pattern morphology was an inclusive criterion for the study.

**ROC curve analyses**

The discriminating power of the ZIAR to identify fertilization failure among the 35 couples was calculated with ROC analyses. The power of the ZIAR (>15% and <15% acrosome reacted) of a given sperm sample to predict the fertilization is represented in Figure 3. The area under the ROC curve, for
Table III. Semen sperm parameters, acrosome response and IVF outcome of couples divided according to the ZIAR test results

<table>
<thead>
<tr>
<th>ZIAR group</th>
<th>Semen sperm parameters</th>
<th>Stimulated AR (test) (0.25 ZP/µl)</th>
<th>Unstimulated AR (control) AR (%)</th>
<th>ZIAR (%)</th>
<th>IVF results of metaphase II oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sperm conc. (10⁶ cells/ml)</td>
<td>morphology (normal)</td>
<td>motility (%)</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>&lt;15%</td>
<td>55.8 ± 16</td>
<td>12.9 ± 4</td>
<td>54.5 ± 13</td>
<td>21.6 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.9 ± 13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>n = 20</td>
<td>(22–100)</td>
<td>(8–19)</td>
<td>(40–70)</td>
<td>(8–35)</td>
<td>(6–40)</td>
</tr>
<tr>
<td>&gt;15%</td>
<td>66.4 ± 24</td>
<td>12.6 ± 3</td>
<td>64.7 ± 11</td>
<td>34.8 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2 ± 5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Unpaired t-test: a versus b: \( P < 0.001 \); c versus d: \( P > 0.05 \); e versus f: \( P < 0.001 \); g versus h: \( P < 0.001 \); i versus j: \( P < 0.001 \); k versus m: \( P < 0.001 \). AR = acrosome reaction; ZIAR = zona pellucida induced acrosome reaction.

Figure 2. The interactive dot diagram of zona pellucida induced acrosome reaction (ZIAR) results and spermatozoa morphology. The ZIAR data of 99%, illustrated the predictive power of the test. An area under the curve of 0.99 implied that a randomly selected individual with ZIAR result of <15% would have fertilization failure in 99% of cases. The confidence interval (0.89–1.00) does not include 0.5, implying that ZIAR results have the ability to distinguish between fertilization rates of >50% and <50%.

Rescued ICSI

The oocytes (\( n = 146 \)) of 16 couples were used during a rescued ICSI procedure after fertilization failure was reported in the IVF laboratory. The results of the ZIAR test, as well as the fertilization data during IVF and rescued ICSI, are presented in Table IV. The fertilization rate achieved during rescued ICSI was 39.8 ± 28%. Retrospectively, the ZIAR test results, i.e. 3.3 ± 2%, were indicative of fertilization failure among these couples. During the following cycle, two couples from this group were prospectively referred to the ICSI programme on account of their ZIAR data as well as the IVF fertilization history. During this cycle, the retrieved oocytes were randomly allocated to undergo either IVF or ICSI treatment. A total of 21 and 12 oocytes were retrieved from these patients, of which 11 and six respectively were allocated to the IVF programme. The rest of the oocytes, namely 10 and six respectively received ICSI treatment. Complete fertilization failure was again reported for both patients during IVF, while 7/10 (70%) and 4/6 (66%) fertilization rates were reported during the ICSI treatment.

Discussion

Substantial information concerning the mammalian sperm acrosome reaction (AR) and its relevance during fertilization processes has accumulated during the past few years (Bielfeld et al., 1994; Liu and Baker 1994, 1996a; Brucker and Lipford, 1995; Franken et al., 1996, 1997). The data include information on the mechanism of the AR the role in the fertilization process, the characterization of AR abnormalities compromising fertility, and methods whereby these abnormalities can be diagnosed and treated (Liu and Baker, 1996a,b). Recent studies have evaluated the nature of the receptors involved in the response and the way AR-inducing signals are transduced from the receptors to the membrane fusion effectors responsible for the ensuing exocytotic reaction (Luconi et al., 1998). Progesterone and zona pellucida glycoprotein (ZP3) have been identified as natural oocyte-associated AR-inducing ligands, and their sequential action has been shown to underlie the physiological AR (Melendrez et al., 1994; Roldan et al., 1994; Franken et al., 1997).
The ARIC test identifies two types of AR pathology, namely, ‘AR insufficiency’ (Tesarik and Mendoza, 1993) and ‘AR prematurity’ (Tesarik and Mendoza, 1995). AR insufficiency describes cases in which the difference in frequency of AR between ionophore-treated and untreated aliquots of a capacitated sperm population is <15%, while AR prematurity defines cases in which the frequency of spontaneous AR is >20% (Tesarik and Mendoza, 1995). Both of the mentioned pathologies can occur in the same patient. AR insufficiency as revealed by the ARIC test can only reflect downstream anomalies of the calcium influx in the signal transduction cascade responsible for AR induction (Tesarik, 1996).

During the present study we defined normal AR in cases where the ZIAR was significantly correlated with IVF and ZIAR outcome was >15%. Insufficient AR was identified in cases where ZIAR was <15% and typically associated with fertilization failure. The percentage of spermatozoa among the group of men showing an insufficient AR also had an elevated spontaneous AR of 17.9%. These findings, i.e. patients with normal conventional semen analyses, failure to fertilize with standard IVF and low ZIAR test results, may have a similar condition to that described by Liu and Baker (1994). They reported on 10 couples with long-standing infertility presenting reduced frequency of the AR of spermatozoa bound to the ZP, concluding that the possibility of the existence of a zona bound sperm population with a disordered AR causing impaired fertility (Liu and Baker, 1994). In close agreement with our findings, Liu and Baker (1997) concluded that patients with disordered ZIAR can be treated with ICSI. Patients with a long history of idiopathic infertility or complete fertilization failure should be tested for this condition using spermatozoa–zona pellucida interaction tests. Reduced ZIAR patients should be directed to ICSI rather than to standard IVF.

Tesarik and Mendoza (1995) described a ‘premature’ AR which might be an indication of dysfunctional capacitation processes leading to a ‘premature’ AR manifesting in an increased number of spontaneous acrosome reacted cells. We were unable to identify a ‘premature AR’ group in our study since the ZIAR <15% group did not differ statistically between stimulated and spontaneous AR, i.e. 21.6 ± 11% and 17.9 ± 13% (P > 0.05, unpaired t-test). There was also no difference between normal controls (14.8 ± 2.7, Table I) and those patients with low ZIAR (17.9 ± 13, Table III).

It is important to note that among the ZIAR >15% group, the percentage of normal spermatozoa was reported to be 12.9 ± 4% (95% confidence interval, 11.2–14.6%). The reported fertilization rates among men with 5–14% normal spermatozoa and in those cases where no corrective measurements were taken (increase insemination concentration) were 64% (Kruger et al., 1986). The ZIAR test therefore identified a sperm population that has normal sperm parameters, with dysfunctional AR to solubilized human ZP. ZIAR test results can be clinically used to distinguish between couples that will benefit from IVF treatment, but moreover seem to be an indicator for ICSI therapy. The 16 couples with complete fertilization failure during IVF of 146 metaphase II oocytes, and who were treated with a rescued ICSI procedure (Table IV), had a decreased ZIAR of 3.3 ± 2% (mean ± SD). ZIAR results in these cases could possibly be seen as a powerful indicator of fertilization failure. On account of the ZIAR results, the metaphase II oocytes of two couples from rescued ICSI group, who did not achieve success during the first treatment period, were randomly divided and used for IVF or ICSI treatment during the following cycle. Both couples had failed fertilization during IVF, whereas 70 and 66% fertilization rates respectively for patient 1 and 2 were recorded during ICSI.

The fundamental utility of the ZIAR test stems from its being a functional bioassay of sperm performance in relation to the human ZP. Together with tight binding to the ZP, the ZIAR potential of a sperm population can be seen as a requisite for IVF. The ZIAR results provide useful discrimination between men capable of achieving fertilization in vitro versus those who are unlikely to be successful. When a larger number of patients have been tested in comparable prospective studies, the ZIAR test may become a valuable tool in the diagnostic scheme of the consulting clinician, since the outcome of the test underlines the most effective clinical option, namely IVF or ICSI.

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