Frequency of disordered zona pellucida (ZP)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa–ZP binding

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Results of zona pellucida (ZP)-induced acrosome reaction (AR) are reported for 186 normospermic men with unexplained infertility and compared with 34 normal fertile men and 54 patients with disordered ZP-induced AR (DZPIAR) diagnosed after failure of standard IVF. For each ZP-induced AR test, four oocytes that failed to fertilize in IVF were incubated for 2 h with 2 × 10^6/ml motile spermatozoa. Spermatozoa tightly bound to the ZP were recovered by aspirating the oocytes with a pipette and the AR assessed using pisum sativum agglutinin labelled with fluorescein. The standard deviation of the difference was 5.2% for repeated tests for ZP-induced AR on different ejaculates from 54 men. The ranges for the ZP-induced AR were 3–98% for normospermic infertile men, 24–95% for fertile men and 0–16% for DZPIAR patients. In the normospermic group, there was a significant correlation between ZP-induced AR and sperm concentration (Spearman \( r = 0.238, P < 0.001 \)). Using ZP-induced AR \( \leq 16\% \) as the threshold for diagnosis of DZPIAR, the frequency of this condition in normospermic infertile men would be 25%. Thus DZPIAR is common with normospermic idiopathic infertility and this condition should be diagnosed before assisted reproductive technology since it requires intracytoplasmic sperm injection.

Key words: disordered ZP-induced AR/male infertility/semen analysis

Introduction

During the process of normal human fertilization a spermatozoon must bind to the zona pellucida (ZP), undergo the acrosome reaction (AR), penetrate the ZP and fuse with the oolemma (Yanagimachi, 1994). Many sperm characteristics including motility and morphology are important for normal spermatozoa–oocyte interaction. In particular, the human acrosome appears to be essential for initiation of spermatozoa–ZP interaction. Spermatozoa such as round-headed acrosome-less spermatozoa are not capable of binding to the ZP (von Bernhardi et al., 1990; Bourne et al., 1995). Acrosome morphology has been considered one of the most important features for assessment of sperm morphology (Jeulin et al., 1986; Liu and Baker, 1992a,b, 1994a; Ombelet et al., 1995; Menkveld et al., 1996). Morphologically abnormal spermatozoa with a small or abnormally shaped acrosome are less able to bind to the ZP (Liu and Baker, 1992a; Ombelet et al., 1995; Menkveld et al., 1996; Garrett et al., 1997). Similarly, spermatozoa that have spontaneously acrosome reacted in the medium are less capable of binding to the ZP and subsequently fertilizing oocytes in vitro (Fenichel et al., 1991; Tahahashi et al., 1992; ESHRE Andrology Special Interest Group, 1996).

In humans the physiological AR occurs on the surface of the ZP, induced by binding to the ZP glycoproteins (Cross et al., 1988; Tesarik, 1989; Liu and Baker, 1994a; Yanagimachi, 1994; Wassarman, 1999). The human ZP is a very efficient inducer of the AR (Cross et al., 1988; Coddington et al., 1990; Liu and Baker, 1994a, 1996a,b). There is a strong relationship between the ZP-induced AR and sperm concentration (Spearman \( r = 0.238, P < 0.001 \)). Using ZP-induced AR \( \leq 16\% \) as the threshold for diagnosis of DZPIAR, the frequency of this condition in normospermic infertile men would be 25%. Thus DZPIAR is common with normospermic idiopathic infertility and this condition should be diagnosed before assisted reproductive technology since it requires intracytoplasmic sperm injection.
to the ZP. DZPIAR patients achieve high fertilization and pregnancy rates with intracytoplasmic sperm injection (ICSI) (Liu et al., 1997). Therefore it is very important to diagnose DZPIAR before commencing assisted reproduction treatment to avoid failure of fertilization with standard IVF. In this study we have investigated the frequency of low ZP-induced AR in a large number of infertile men with normal semen samples and normal spermatozoa–ZP binding.

Materials and methods

Subjects

Semen samples from infertile men with normal semen analysis (n = 186) were tested for ZP-induced AR. The patients were seen between 1992 and 1999 and had normal sperm morphology >15% (n = 140), or 5–15% (n = 46). The patients with <15% normal morphology were only included if they had normal spermatozoa–ZP binding. Patients with defective spermatozoa–ZP binding (see below) or normal morphology <5% were excluded.

Also reported are results for 54 patients diagnosed with DZPIAR because of persistent low (<25%) or zero fertilization rates in two or more cycles with standard IVF treatments, and of 34 fertile men who were used as paired controls for the tests on the DZPIAR patients. The DZPIAR and control groups included patients reported previously (Liu and Baker, 1994a; Liu et al., 1997).

Human oocytes

Oocytes which showed no evidence of two pronuclei or cleavage at 48–60 h after insemination in a clinical IVF programme were used for the ZP-induced AR tests. If the oocyte had spermatozoa bound to the ZP from the IVF insemination, these were removed by aspiration using a glass micropipette with an inner diameter (120 µm) slightly smaller than the oocyte diameter (Liu and Baker, 1994a,b, 1996a). Most of the oocytes were obtained from patients with partial failure of fertilization and >50% of these unfertilized oocytes had spermatozoa penetrating the ZP from the IVF insemination. However, we have shown previously that oocytes with spermatozoa in the ZP have a similar ability for subsequent spermatozoa–ZP binding and ZP-induced AR as those without sperm penetration (Liu and Baker, 1996a). Some of the oocytes were stored in 1 mol/l ammonium sulphate at 4°C (Yanagimachi et al., 1979; Liu and Baker, 1992a). The salt-stored oocytes were washed in HTF medium with four changes of the medium at least 4 h before the spermatozoa–ZP interaction tests. The AR inducing activity is similar between fresh and salt-stored oocytes (Liu and Baker, 1996a).

All patients signed consent forms permitting use of their unfertilized oocytes for research. The Royal Women’s Hospital Research and Ethics Committees approved the project.

Semen analysis

Semen samples were obtained by masturbation after 2–5 days abstinence. All sperm tests were performed after liquefaction of the semen within 2 h. Sperm concentration and motility in semen were determined using standard methods (World Health Organization, 1992). Linear velocity (VSL) was assessed in 137 samples by Hamilton–Thorn Motility Analyzer as described previously (Liu et al., 1991).

Sperm morphology was assessed on smears prepared from semen after washing with 0.9% sodium chloride. Morphology slides were stained with the Shorr method after the smears were fixed in 90% ethanol for 30 min. Sperm morphology was assessed following the World Health Organization (1992) criteria for the silhouette plus internal staining characteristics with the acrosomal region being clearly seen, regular in shape and occupying at least half of the sperm head (Liu and Baker, 1992b). The percentage of spermatozoa with normal morphology was determined by assessing 200 spermatozoa from more than 10 individual fields under oil immersion with magnification of ×1000 under bright-field illumination (Jeulin et al., 1986; Liu and Baker, 1992b). These modified criteria are similar to the Kruger ‘strict’ criteria (Kruger et al., 1988) and normal fertile men should have normal sperm morphology >15% in semen. Between 5 and 15% normal morphology is of uncertain significance, with some samples in this range having good spermatozoa–ZP binding and fertilization in vitro and in vivo.

Sperm preparation

Motile spermatozoa were selected by a swim-up technique as follows: 0.3 ml of semen was carefully added to the bottom of a test tube (12×75 mm) containing 0.7 ml human tubal fluid (HTF; Irvine Scientific, Irvine, CA, USA), supplemented with 10% heat inactivated human serum. Care was taken to avoid disturbing the interface between the semen and the medium. After incubation for 1 h, 0.5 ml of the top layer of the medium containing motile spermatozoa was aspirated. The motile sperm suspension was then centrifuged at 800 g for 5 min, the supernatant removed and the sperm pellet washed again with 2 ml fresh HTF by centrifugation at 800 g for 5 min. The washed sperm pellet was resuspended with sperm supplemented HTF to a sperm concentration of 2×10⁶/ml for subsequent experiments.

Human ZP-induced acrosome reaction

For the ZP-induced AR, four oocytes were incubated with 2×10⁶ motile spermatozoa in 1 ml of medium for 2 h at 37°C in 5% CO₂ in air. For DZPIAR patients, spermatozoa from fertile donors were also used in paired control experiments using the same batch of the oocytes. After 2 h incubation, the oocytes were transferred to phosphate buffered saline, pH 7.4, containing 2 mg/ml bovine serum albumin and washed by repeated aspiration with a glass pipette (inside diameter ~250 µm) to dislodge spermatozoa loosely adhering to the surface of the ZP. Because a high concentration of spermatozoa was used in the insemination medium (20 times more than standard IVF insemination), the number of spermatozoa bound tightly to the ZP was >100/ZP for >90% of the oocytes. Semen samples with low spermatozoa–ZP binding (all four oocytes with <30/ZP) were not included in the present study because there were not enough spermatozoa on the ZP for accurate assessment of the acrosome reaction. Also, defective spermatozoa–ZP binding is usually a clear indication of a severe sperm abnormality (Liu and Baker, 1992b).

All spermatozoa bound to the surface of the ZP were then removed by repeated vigorous aspiration with a narrow gauge pipette with an inner diameter (~120 µm) slightly smaller than the oocyte (Figure 1; Liu and Baker, 1993, 1994a). This was performed on a glass slide with about 5 µl phosphate buffered saline (PBS) containing 0.2% BSA and the removed ZP-bound spermatozoa were smeared in a limited area (about 16 µm) which was marked with a glass pen to help find the spermatozoa under the microscope for acrosome assessment. This pipetting procedure does not affect sperm motility, morphology and acrosome status (Liu and Baker, 1994a).

Assessment of acrosome status

The acrosome status of spermatozoa was determined with fluorescein labelled pism sativum agglutinin (PSA; Sigma Co., St Louis, MO, USA) with a modification of the method of Cross et al. (1986). Sperm smears were fixed in 95% ethanol for 30 min after drying in air and then stained in 25 µg/ml PSA in PBS for at least 2 h. The slide was washed and mounted with distilled water and 200
Disordered zona pellucida-induced

Figure 1. Diagram illustrating the removal of spermatozoa bound to the surface of the zona pellucida (ZP) by aspiration with a glass pipette with diameter slightly smaller than the diameter of the oocyte for assessment of acrosomal status.

Figure 2. Agreement between the results of two ZP-induced acrosome reaction (AR) tests on spermatozoa in two separate ejaculates from the same man (n = 54). One of the points represents three subjects and three of the points represent two subjects with coincident results. The solid line shows the mean difference (bias 1.1%) and the dashed lines ±2 SD (10.5%) of the mean.

Figure 3. Comparison of ZP-induced AR results between normospermic infertile men (idiopathic, n = 186), fertile men (normal, n = 34) and DZPIAR patients (n = 54). There were significant differences (P < 0.001) between normal fertile men (mean 53%), DZPIAR patients (mean 6.5%) and normospermic men (mean 33%).

The spermatozoa were counted with a fluorescence microscope and oil immersion at a magnification of ×400. When more than half the head of a spermatozoa was brightly and uniformly fluorescing, the acrosome was considered to be intact. Sperm with a fluorescing band at the equatorial segment or no staining of the head (a rare pattern) were considered to be acrosome reacted. Only motile spermatozoa are capable of binding to the ZP, therefore spermatozoa removed from the ZP were alive and motile. These spermatozoa were then assessed for the AR and were unlikely to contain dead spermatozoa.

Statistical analysis
The significance of differences in mean ZP-induced AR between fertile men and DZPIAR patients was examined by t-test. Correlations between ZP-induced AR and other sperm characteristics in normospermic infertile men were examined by Spearman tests. Agreement between test results of the ZP-induced AR from two different ejaculates in the same men was examined by plotting the difference between the results against the mean. The differences were used to calculate the bias and standard deviation. The Kolmogorov–Smirnov test was used to examine the fit of the ZP-induced AR results to a log normal distribution.

Results

Variability of ZP-induced AR between ejaculates in the same men
To determine the variability of the ZP-induced AR in different ejaculates within the same man, 54 men had two tests for the ZP-induced AR within 2 to 10 weeks. There was a highly significant correlation between the two results (Spearman r = 0.761, P < 0.001). The mean difference between the results was 1.1% with standard deviation 5.2% (Figure 2).

ZP-induced AR in normal fertile men, DZPIAR patients and normospermic infertile men
In the 34 normal fertile men, the ZP-induced AR averaged 53% and ranged from 23–98%. In 54 patients with DZPIAR it averaged 6.5% and ranged from 0 to 16% (Figure 3). In the 186 normospermic infertile men tested, there was a wide range of ZP-induced AR results from 3–98%. Overall, 54% of the normospermic infertile men had ZP-induced AR between 20–60%, while 33% of the men had <20% and 13% had >60% (Figure 3).

The distribution of the ZP-induced AR results in the normal, DZPIAR and normospermic infertile men is skewed, with a long tail to the higher values. After logarithmic transformation, the combined results of all three groups departs significantly from a normal distribution (P = 0.009, Kolmogorov–Smirnov test). Removal of results <17% makes this test not significant (>16%, P = 0.843) but it is significant when results <11% are removed (>10%, P = 0.027), suggesting a separate population with results below approximately 12–16%.

Correlation between ZP-induced AR and other sperm characteristics
Semen analysis results for the normospermic infertile men are shown in Table I. Sperm concentration in semen was
significantly correlated with the ZP-induced AR (Spearman \( r = 0.243, P < 0.01 \)). All other sperm characteristics such as motility and morphology were not significantly correlated with the ZP-induced AR. There were significant correlations between percentage normal morphology and sperm concentration ( \( r = 0.232, P < 0.01 \)), and between motility ( \( r = 0.193, P < 0.01 \)) and velocity ( \( r = 0.232, P < 0.01 \)).

**Frequency of DZPIAR in normospermic men**

In this study a threshold of ZP-induced AR ≤16% was chosen for calculation of the frequency of DZPIAR. This value was based on previously reported results for the normal fertile men and DZPIAR patients, and the finding that patients with ≤16% ZP-induced AR had <20% of ZP penetrated in IVF (Liu and Baker, 1994a, 1996b). Of the normospermic infertile men 25% (47 of 186) had ZP-induced AR ≤16% and 13% had ZP-induced AR ≤10%. The frequency of patients with ZP-induced AR ≤16% was similar for those with sperm normal morphology ≥15% (24%, \( n = 140 \)), and <15% (28%, \( n = 46 \)).

**Discussion**

For the 54 men who had a second semen sample tested within 2–10 weeks, the standard deviation of the difference between the two results was 5.2%, indicating reasonable consistency in the ZP-induced AR test. This variability in repeated results is contributed to by counting errors in the assessment of acrosome status on groups of spermatozoa recovered after binding to the ZP. Because of this variability, low and marginal results should be confirmed with a second test.

Previous reports on patients with DZPIAR diagnosed after failure of standard IVF showed a clear separation between the patients with ZP-induced AR <17% and controls >22% (Figure 3) (Liu and Baker, 1994a; Liu et al., 1997). However in the 186 men with idiopathic infertility reported here, there are intermediate results. Only by combining the data from all three groups is there confirmation of departure from a single population, with the cut being in the region of 12–16%. Our previous study showed that normospermic infertile men with <10% ZP-induced AR had zero ZP penetration and those with ≤16% ZP-induced AR had <20% of the ZP penetrated in IVF (Liu and Baker, 1996b). Esterhuizen et al. (2001) also found reduced AR induced by solubilized ZP in patients with poor standard IVF results (Esterhuizen et al., 2001). It is therefore likely that fertility may be significantly reduced when the ZP-induced AR is below 10–20%, but the lower limit of normal requires more data for precise definition. In the meantime we use a working value of 16% since this is the highest value found in men diagnosed with DZPIAR after failure of standard IVF.

The finding of a significant correlation between ZP-induced AR and sperm concentration with about the same strength as the relationship between sperm concentration and motility is intriguing. This suggests that men with reduced sperm output have a reduced ZP-induced AR, indicating a possible non-specific association between defective AR and impaired spermato genesis. Abnormalities of the sperm plasma membrane which might reduce fluidity and impair the AR have been described with oligozoospermia (Ladha 1998). Thus it is possible there may be more than one form of DZPIAR. For example there are DZPIAR patients with normal semen and low ZP-induced AR as the only abnormality, and others with combined semen abnormalities and low ZP-induced AR. In the past we have not tested patients with other than mildly abnormal semen because the spermatozoa–ZP binding is usually reduced with moderate to severe abnormalities. We have tested some men with normal morphology ≤5% but normal spermatozoa–ZP binding and ~25% had a ZP-induced AR <17%.

In current clinical practice, treatment of patients by either standard IVF or ICSI in the first cycle is usually decided on semen analysis results. Standard IVF requires good sperm function, particularly spermatozoa–ZP binding and penetration which are essential for fertilization. With ICSI, several sperm functions are superfluous for fertilization, particularly those associated with spermatozoa–ZP interaction. Couples with severe sperm defects such as severe oligozoospermia, asthenozoospermia and teratozoospermia can be identified by routine semen analysis and ICSI is recommended for the first treatment. On the other hand, couples with unexplained infertility with normal semen analysis are usually treated with standard IVF. However, the present study shows it is possible that up to 25% may have low ZP-induced AR and are at risk of zero or very low fertilization rates in standard IVF. We have also found a similar frequency of low ZP-induced AR in pre-IVF patients with unexplained infertility (unpublished data). Although these patients can be treated with ICSI in the second cycle, there is a high cost to the patients both financially and emotionally. Failed attempts can also reduce confidence in future treatments. Therefore, screening for defects of spermatozoa–ZP interaction would minimize the risk of failure of fertilization in patients with unexplained infertility.

The past, complete failure of fertilization occurred in 15–20% of IVF treatments before ICSI was available. Today about 10% of patients have zero or low fertilization rates with standard IVF. Although oocyte immaturity or abnormalities can contribute to failure of fertilization, sperm defects are regarded as the most frequent contributors for incidents where most or all oocytes fail to fertilize (Liu and Baker, 2000). Sperm defects of motility and morphology or sperm antibodies are the main causes of reduced spermatozoa–ZP binding (Liu and Baker, 1992a, 1999b; Franken et al., 1993). Other infertile men can have defects of spermatozoa–ZP penetration (Overstreet and Hembree, 1976; Overstreet et al., 1980). Liu and Baker, 1994b). Our previous study on a large number of patients with

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**Table I. Results of sperm tests in normospermic infertile men**

<table>
<thead>
<tr>
<th>Test</th>
<th>( n )</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (10^9/ml)</td>
<td>186</td>
<td>119 (20–386)</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>186</td>
<td>61 (40–88)</td>
</tr>
<tr>
<td>Velocity (VSL, µm/s)</td>
<td>137</td>
<td>42 (21–58)</td>
</tr>
<tr>
<td>Normal sperm morphology (%)</td>
<td>186</td>
<td>25 (5–54)</td>
</tr>
<tr>
<td>ZP-induced AR (%)</td>
<td>186</td>
<td>33 (3–98)</td>
</tr>
</tbody>
</table>

ZP = zona pellucida; AR = acrosome reaction.

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failure of IVF showed that >70% had defective spermatozoa–ZP binding and 45% had no spermatozoa penetrating the ZP of any of the inseminated oocytes that had failed to fertilize. Over half these patients had normal standard semen analysis results (Liu and Baker, 2000). This further supports the contention that defective spermatozoa–ZP binding and penetration are major factors contributing to low fertilization with standard IVF.

The present study suggests that DZPIAR may be common in infertile men with normal semen analyses. These men are diagnosed as having unexplained infertility unless a test for ZP-induced AR is performed (Liu and Baker, 1994a). While DZPIAR patients have severe infertility and markedly reduced standard IVF results they achieve good fertilization and pregnancy rates with ICSI (Liu et al., 1997). In our ICSI programme the live birth, implantation and pregnancy rates are higher for DZPIAR patients than in patients with other types of sperm abnormalities such as asthenozoospermia and teratozoospermia. This may be because spermatozoa from DZPIAR patients have a single specific defect that interferes with ZP penetration and is simply bypassed by ICSI. The wives of these men are also likely to be fertile because of the severity of the male infertility with DZPIAR.

Other methods for testing the AR have been reported. The ionophore A23187 induced AR may predict spermatozoa fertilizing ability in vitro (Cummins et al., 1991; Yovich et al., 1994) and we found a significant correlation between the A23187 induced AR and IVF rate in patients with teratozoospermia Liu and Baker, 1998). However, in normospermic men, the A23187 induced AR was not correlated with either the ZP-induced AR or spermatozoa–ZP penetration (Liu and Baker, 1996b). Therefore, A23187 induced AR may not reflect the ability of spermatozoa to undergo the physiological AR induced by the ZP, and tests using the A23187 induced AR will not predict DZPIAR. Calvo et al. (1989) reported that the follicular fluid induced-AR distinguished a subgroup of men with unexplained infertility not detected by routine semen analysis. Although others showed that progesterone-induced AR is predictive of fertility in men with unexplained infertility, it is unknown whether progesterone-induced AR test results are useful for diagnosing DZPIAR.

Solubilized human ZP can be used to induce the AR (Esterhuizen et al., 2001). We showed that the results are significantly correlated with those of the ZP-induced AR test used for the present studies. However, we found that solubilized ZP in a concentration of 4ZP/µl was significantly less efficient in inducing the AR than were intact ZP (Liu and Baker, 1996a). It is possible that the conformational structure or the concentration of ZP proteins may affect the AR inducing activity. In addition, spermatozoa which bind to the ZP are a select group which may be more likely to acrosome react on the ZP than those in the whole sperm population within an insemination suspension exposed to solubilized ZP. We favour using intact ZP for testing the physiological AR of human spermatozoa. Also, patients with low ZP-binding can be identified with this test.

In our IVF clinic over 95% of patients are willing to donate their unfertilized oocytes for research or clinical tests and thus there were enough oocytes which failed to fertilize available for this study. Most oocytes that fail to fertilize in vitro can be used for testing ZP-induced AR. Immature oocytes (germinal vesicle oocytes) are useful for the test. However, degenerate and morphologically abnormal oocytes are not used. It is important to use a group of oocytes (optimally four) rather than a single oocyte for each test because of the variability in the quality of individual oocytes. When the oocytes are obtained from either the same or different IVF patients and used either fresh or after salt storing, results for each test and control sample are reasonably consistent, with differences between ZP-induced AR from different batches of four oocytes within ±15% (Liu and Baker, 1996a). This was confirmed in the present study where 54 men had more than one sample tested within 2–10 weeks. IVF clinics should therefore be encouraged to use unfertilized oocytes for screening DZPIAR in pre-IVF patients with unexplained infertility. Nevertheless, the assessment of the ZP-induced AR is still difficult because of the limited supply of oocytes and it is not practical in clinics without an assisted reproduction programme. In the future, recombinant human ZP or other alternative tests not requiring native human ZP have great potential for routine testing the ZP-induced AR (van Duin et al., 1994; Brewis et al., 1996; Whitmarsh et al., 1996).

In summary, testing the ZP-induced acrosome reaction using oocytes that failed to fertilize in IVF is useful for diagnosing low ZP-induced AR in men with normal semen analyses. The ZP-induced AR test provides reasonably consistent results between different ejaculates from the same man. For clinical application it is recommended that a second test is used to confirm the diagnosis of DZPIAR when results are low or marginal in the first test. The frequency of DZPIAR may be as high as 25% in normospermic men with idiopathic infertility and such patients should be assigned for ICSI rather than standard IVF.

**Acknowledgements**

We thank Mingli Liu for technical assistance, all the scientists in both Royal Women’s Hospital and Melbourne IVF Laboratories for collecting the oocytes and all staff in the Andrology Laboratory for collecting sperm samples. This study was supported by the Royal Women’s Hospital Research Committee.

**References**


Received on October 27, 2000; accepted on March 12, 2001