Frequency of defective sperm–zona pellucida interaction in severely teratozoospermic infertile men

De Yi Liu1 and H.W.Gordon Baker

University of Melbourne Department of Obstetrics and Gynaecology, Reproductive Services, Royal Women’s Hospital and Melbourne IVF, Victoria 3035, Australia

1To whom correspondence should be addressed at: E-mail: dyl@unimelb.edu.au

BACKGROUND: The frequency of defective sperm–zona pellucida (ZP) interaction in teratozoospermic infertile men was investigated. METHODS: Sperm–ZP binding and the ZP-induced acrosome reaction (ZPIAR) were performed in 125 infertile men with <5% of their sperm with normal morphology (strict criteria), but with a sperm count >20×10⁶/ml and total motility >30% in semen. Oocytes that failed to fertilize in clinical IVF were used for the tests. Four oocytes were incubated for 2 h with 2×10⁶/ml motile sperm selected by swim-up. The number of sperm bound per ZP and ZPIAR were assessed. Under these conditions, an average <40 sperm bound per ZP was defined as poor sperm–ZP binding, and a ZPIAR <16% was defined as low ZPIAR. RESULTS: Among 125 teratozoospermic men, 31% (39/125) had poor sperm–ZP binding. Of those without poor ZP binding, 48% (41/86) had low ZPIAR. Some 64% (28/44) with sperm counts between 20 and 60×10⁶/ml had low ZPIAR. Only 36% (45/125) had normal sperm–ZP binding and ZPIAR. CONCLUSIONS: Defective sperm–ZP interaction was present in 64% of teratozoospermic infertile men: 31% had defective sperm-ZP binding, and 33% low ZPIAR. The frequency of low ZPIAR was higher in men with sperm counts between 20–60×10⁶/ml.

Key words: male infertility/sperm–ZP binding/teratozoospermia/ZP-induced acrosome reaction

Introduction

Sperm morphology is an important sperm characteristic for the prediction of sperm fertilizing ability with standard IVF. Using strict criteria for morphology assessment, fertile men should have a normal sperm morphology in ≥15% of the ejaculate (Kruger et al., 1988; World Health Organization, 1992). Men with a normal sperm morphology of ≤5% are considered to have severe teratozoospermia, and this is common in subfertile man either combined with oligozoospermia or asthenozoospermia, or as an isolated defect (Baker, 2001). Although some patients with severe teratozoospermia may be able to produce natural pregnancies, most need assisted reproductive techniques (ART) in order to father children. Before ICSI became available, patients with severe teratozoospermia were often treated with standard IVF, but the results were generally poor such that the average fertilization rate was <35% (Baker et al., 1993). Failure of fertilization in vitro in these patients is usually due to the inability of sperm to bind to or penetrate the zona pellucida (ZP) (Liu and Baker, 1992a,b). Today, patients with severe teratozoospermia can be treated successfully with ICSI, thereby allowing the fertilizing sperm to bypass the ZP. However, the mechanism of failure of fertilization and infertility with teratozoospermia is poorly understood, especially as some men with this condition still have normal fertilization rates in IVF or natural conceptions.
(Jeulin et al., 1986; Liu and Baker, 1992a,b; Ombelet et al., 1995; Menkveld et al., 1996).

In humans, sperm require a normal intact acrosome for binding to the ZP, and the physiological AR occurs on the surface of the ZP after tight binding to the ZP (Cross et al., 1988; Tesarik, 1989; Yanagimachi, 1994; Liu and Baker, 1996a,b; Wassarman, 1999). The human ZP is a very efficient inducer of the AR, and the ZP-induced AR is highly correlated with sperm–ZP penetration in vitro (Cross et al., 1988; Coddington et al., 1990; Liu and Baker, 1994, 1996a,b). Although disordered ZP-induced AR (DZPIAR) was discovered in patients with unexplained failure of fertilization with standard IVF (Liu and Baker, 1994), more recently it has been found in prospective studies that 25–30% of men with unexplained infertility, normal semen analysis and normal sperm–ZP binding have low ZPIAR (Liu et al., 2001; Liu and Baker, 2003). In the present study, the frequency of defective sperm–ZP binding and the ZP-induced AR in infertile men with isolated teratozoospermia was investigated.

Materials and methods

Male subjects

Semen samples were obtained from 125 infertile men with severe teratozoospermia (strict normal morphology ≤ 5% (Kruger et al., 1988; World Health Organization, 1992), sperm count > 20 × 10⁶/ml and progressive motility > 30%, and were studied for sperm–ZP interaction, sperm–ZP binding and, if > 40 sperm per ZP, also ZP-induced AR. All the men attended for infertility treatment between January, 1995 and June, 2002. Men with normal semen analysis, oligozoospermia or asthenozoospermia were excluded from the study.

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 sperm bound to the ZP from the IVF insemination, these were removed by aspiration using a fine glass pipette with an inner diameter (120 μm) that was slightly smaller than the oocyte diameter (Liu and Baker, 1994, 1996a). Most of the oocytes were obtained from patients with partial failure of fertilization in standard IVF, and more than 50% of these unfertilized oocytes had one or a few sperm penetrating the ZP from the IVF insemination. It has been shown previously that oocytes with less than 10 sperm penetrating the ZP have a similar ability for subsequent sperm–ZP binding and ZP-induced AR as those with no sperm penetration (Liu and Baker, 1996a). Oocytes with more than 10 sperm penetrating the ZP were not used. Likewise, degenerate, activated or morphologically abnormal oocytes were not used for the sperm–ZP interaction test. Oocytes were pooled from several patients and either used for the test on the same day or kept in the incubator and used within next 2–3 days.

All patients signed consent forms permitting use of their unfertilized oocytes or sperm samples for research. The study was approved by The Royal Women’s Hospital Research and Ethics Committees.

Semen analysis

Semen samples were obtained by masturbation after 2–5 days abstinence. All sperm tests were performed after liquefaction of the semen within 1 h. Sperm concentration and motility in semen were determined using standard methods (World Health Organization, 1992).

Morphology of sperm in both semen and insemination medium (swim-up motile sperm) were assessed on smears prepared by washing of sperm with 10 ml 0.9% sodium chloride (World Health Organization, 1992). Morphology slides were stained with the Shorr method after the smears were fixed in 90% ethanol for 30 min (Jeulin et al., 1986; Liu and Baker, 1992b). Washing sperm to remove seminal plasma or protein decreases background staining and produces clearer images of sperm. The percentage of normal sperm morphology was assessed according to strict (Tygerberg) criteria (Kruger et al., 1988). For each sperm sample, 200 spermatozoa were scored from at least 10 individual fields using oil immersion with magnification of ×1000 under bright-field illumination.

Sperm preparation

Motile sperm 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 (ICN Biomedicals, Irvine, CA, USA). 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 sperm was aspirated. The motile sperm suspension was then centrifuged at 1000 g for 5 min, the supernatant removed and the sperm pellet washed again with 1 ml fresh HTF by centrifugation at 1000 g for 5 min. The washed sperm pellet was resuspended with serum supplemented HTF to a sperm concentration of 2 × 10⁶/ml for the sperm–ZP interaction test.

Sperm–ZP binding

For the sperm–ZP interaction tests, motile sperm (2 × 10⁶) in 1 ml of medium were incubated with four oocytes for 2 h at 37°C in 5% CO₂ in air. After incubation, the oocytes were transferred to phosphate-buffered saline (PBS), pH 7.4, containing 2 mg/ml bovine serum albumin (BSA) and washed by repeated aspiration with a glass pipette (inside diameter ~250 μm) to dislodge sperm that were loosely adherent to the surface of the ZP. Sperm binding to all four oocytes was assessed. Under these experimental conditions, with the high concentration of sperm in the insemination medium (20-fold more than standard IVF insemination), the number of sperm bound tightly to the ZP was more than 100 per ZP with sperm from fertile or normospermic men. For this study, sperm samples with an average of no more than 40 sperm per ZP were considered to have poor sperm–ZP binding, and no further testing was carried out (Liu and Baker, 2000).

ZP-induced AR

For sperm samples without poor ZP-binding, all sperm bound to surface of the four ZPs were removed by repeated vigorous aspiration with a narrow-gauge pipette with an inner diameter (~120 μm) which was slightly smaller than the oocyte (Liu and Baker, 1994, 1996a). This was carried out on a glass slide with ~5 μl PBS containing 0.2% BSA, and the removed ZP-bound sperm were smeared over a limited area (~16 mm²), which was marked with a glass-pen on the back of the slides to help locate the sperm under the microscope. This pipetting procedure for removing sperm from the surface of ZP does not affect sperm motility, morphology and acrosome status (Liu and Baker, 1996a).

The acrosome status of sperm removed from the ZP was determined with fluorescence-labelised Pismus sativum agglutinin (PSA; Sigma Co., St Louis, MO, USA), using a modification of a previously published method (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.
were examined using non-parametric (Wilcoxon rank sum) tests. The ZP-induced AR in men with low and high sperm concentrations was analysed after log transformation. Differences of related to sperm±ZP binding or the ZP-induced AR. Sperm determination which of sperm characteristics was the most significantly Spearman tests. Multiple regression analysis was also used to relationship of sperm characteristics to either sperm–ZP binding or the ZP-induced AR was also analysed using multiple regression. Only sperm motility was significantly related to sperm–ZP binding (P<0.01) in 125 patients. Only sperm concentration in semen was significantly related to the ZP-induced AR in 86 patients with more than 40 sperm bound per ZP (P<0.01).

Frequency of defective sperm–ZP binding and low ZPIAR with teratozoospermia

In the present study, a threshold average number of sperm bound per ZP of ≤40 was used as a threshold for poor sperm–ZP binding. A ZP-induced AR of ≤16% was used for classification of low ZPIAR according to previous reports (Liu and Baker, 1994; Liu et al., 2001). Among infertile men with isolated teratozoospermia, 31% (39/125) had poor sperm–ZP binding; among the other 86 men, 48% (41/86) had low ZPIAR. Overall, only 36% (45/125) had normal ZP-binding and normal ZP-induced AR. In other words, 64% had either poor sperm–ZP binding or low ZPIAR.

As shown above, the sperm count was significantly correlated with the ZP-induced AR, and men with sperm counts between 20–60×10⁶/ml had significantly lower mean (±SD) ZP-induced AR than those with sperm counts >60×10⁶/ml (17 ± 15 versus 29 ± 22; Wilcoxon test, z = 3.218, P<0.001). The frequency of low ZPIAR was 64% in men who had both serve teratozoospermia (normal morphology ≤5%) and low normal sperm counts (20–60×10⁶ /ml). In contrast, the frequency of low ZPIAR was 31% (13/42) in men with sperm counts >60×10⁶/ml.

Discussion

The results of the present study show that 31% of infertile men with isolated teratozoospermia had poor sperm–ZP binding and 33% had normal ZP-binding, but low ZPIAR. Therefore, defective sperm–ZP interaction could explain the infertility in

### Table 1. Results of sperm tests in 125 teratozoospermic infertile men

<table>
<thead>
<tr>
<th>Test</th>
<th>n</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (10⁶/ml)</td>
<td>125</td>
<td>76 (20–329)</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>125</td>
<td>54 (33–73)</td>
</tr>
<tr>
<td>Live (%)</td>
<td>125</td>
<td>76 (54–93)</td>
</tr>
<tr>
<td>Normal sperm morphology (%), semen</td>
<td>125</td>
<td>3.2 (0–5)</td>
</tr>
<tr>
<td>Normal sperm morphology (%), swim-up</td>
<td>125</td>
<td>6.5 (0–15)</td>
</tr>
<tr>
<td>No sperm-bound/ZP</td>
<td>125</td>
<td>62 (1–100)</td>
</tr>
<tr>
<td>ZP-induced AR (%)</td>
<td>86</td>
<td>22.5 (1–90)</td>
</tr>
</tbody>
</table>

*Test only carried out in subjects with mean sperm binding scores >40 per ZP.

AR = acrosome reaction; ZP = zona pellucida.
64% of these men. However, 36% of the men had both normal sperm–ZP binding and normal ZP-induced AR, and would presumably have a reasonable chance of achieving fertilization either *in vivo* or *in vitro*; alternatively, there may be other factors causing their infertility. This heterogeneity may explain why some groups have not shown a clear relationship between sperm morphology and fertilization rates with standard IVF (Alper et al., 1985; Cohen et al., 1985). In addition, sperm–ZP interaction tests may be useful to distinguish between these two groups, after which adequate treatments can be applied individually. The group with defective sperm–ZP interaction (either low ZP-binding or low ZPIAR) should be treated with ICSI, as sperm from these men have little chance of either binding to or penetrating the ZP. On the other hand, those with normal sperm–ZP binding and a normal ZP-induced AR may be encouraged to continue to try for natural conception or undergo standard IVF if no other cause of infertility is apparent.

In the present study, only 31% of the severely teratozoospermic men had poor sperm–ZP binding. This low proportion might be due to the high sperm concentration used for insemination (about 20-fold higher than used with standard IVF). Previously, the threshold of no more than 40 sperm bound per ZP with a sperm concentration of \(2 \times 10^6/\text{ml}\) was seen to be equivalent to no more than two sperm bound per ZP with a sperm concentration in the insemination medium of \(1 \times 10^5/\text{ml}\) (as used with oocytes in standard IVF; Liu and Baker, 2000). Others have also shown that increasing the number of sperm for insemination in teratozoospermic men will increase the number of sperm bound to the ZP (Oehninger et al., 1988).

In the present study, it was found that 48% of teratozoospermic men without severely impaired sperm–ZP binding had ZP-induced AR \(\leq 16\%\), which is classified as low ZPIAR. This frequency of low ZPIAR with teratozoospermia is much higher than in men with unexplained infertility with normal semen analysis (~25–30%; Liu et al., 2001; Liu and Baker, 2003). When the sperm count in semen was taken into account, over 64% of severe teratozoospermic men had low ZPIAR if they also had sperm counts between 20 and \(\leq 60 \times 10^6/\text{ml}\) (men with sperm counts \(<20 \times 10^6/\text{ml}\) were excluded from this study). In contrast, with teratozoospermia and a sperm count \(>60 \times 10^6/\text{ml}\), the frequency of low ZPIAR was 31%, and this was similar to the value found in normozoospermic infertile men (Liu et al., 2001; Liu and Baker, 2003). Therefore, sperm count could be an important additional indicator for predicting which infertile men with isolated teratozoospermia are more likely to have impaired sperm–ZP interaction. This finding may be very useful clinically as many IVF/ICSI clinics do not perform sperm–ZP interaction tests routinely. The ZPIAR is highly correlated with sperm–ZP penetration and fertilization rates *in vitro* in normozoospermic infertile men (Liu and Baker, 1996b; Esterhuizen et al., 2001; Liu and Baker, 2003). The results of a previous study showed that normozoospermic infertile men with <10% ZPIAR had zero sperm–ZP penetration, while in those men with <16% ZPIAR, fewer than 20% of the ZP were penetrated *in vitro* (Liu and Baker, 1996b). Therefore, patients with both normal sperm morphology of \(<=5\%\) plus sperm count \(<60 \times 10^6/\text{ml}\) should be recommended for ICSI because of the high frequency of low ZPIAR and the high risk of poor fertilization rates with standard IVF.

The finding of a significant correlation between ZP-induced AR and sperm concentration was consistent with previous studies in men with normal semen analysis and unexplained infertility (Liu et al., 2001; Liu and Baker, 2003). This may suggest that men with sperm concentrations within the lower region of the conventional normal range (\(=60 \times 10^6/\text{ml}\)) may have a degree of impaired spermatogenesis causing sperm dysfunction, particularly in the ability to interact with oocytes. However, further study is needed to determine the underlying cause of the high frequency of low ZPIAR with sperm counts in the range of \(20–60 \times 10^6/\text{ml}\). This may also indicate that a standard lower reference limit of \(20 \times 10^6/\text{ml}\) is too low.

Calcium ionophore A23187-induced AR may predict sperm fertilizing ability *in vitro* (Cummins et al., 1991; Yovich et al.,...
either in vivo with isolated teratozoospermia. One-third of severe teratozoospermic semen, A23187-induced AR was not correlated with either the ZP-induced AR or sperm–ZP penetration (Liu and Baker, 1996b). Therefore, A23187-induced AR may not reflect the ability of sperm to undergo the physiological AR as induced by the ZP, and tests using A23187-induced AR will not predict ZPIAR. It has been reported (Calvo et al., 1989) that follicular fluid induced-AR distinguished a subgroup of men with unexplained infertility who were not detected by routine semen analysis. Although others showed that progesterone-induced AR is predictive of fertility in men with unexplained infertility, it is not known whether progesterone-induced AR test results are related to ZPIAR (Krausz et al., 1996).

In clinical IVF, about 20–30% of inseminated oocytes fail to fertilize, and these are valuable for testing sperm function for other clinical patients before they commence ART treatment. Most oocytes that fail to fertilize in vitro can be used for testing ZP-induced AR; immature oocytes (germinal vesicle oocytes) may also be used in this test. However, degenerate, spontaneously activated and morphologically abnormal oocytes and those with more than 10 sperm penetrated into the ZP are not used. It is important to use a group of oocytes (optimally four) rather than a single oocyte for each test, due to variability in the quality of individual oocytes. Differences between ZP-induced AR from different batches of four oocytes or two sperm samples in the same male are <15% (Liu and Baker, 1996a; Liu et al., 2001). However, in practical terms, routine testing for ZPIAR is difficult and is usually limited to a small proportion of patients because insufficient human oocytes are available. In future, the development of recombinant human ZP or other alternative tests that do not require native human ZP will have great potential for routine testing for the ZP-induced AR (van Duin et al., 1994; Brewis et al., 1996; Whitmarsh et al., 1996; Dong et al., 2001).

The results of the present study provide a more detailed explanation of why teratozoospermia is associated with infertility and poor results with standard IVF. The sperm–ZP interaction tests using unfertilized oocytes from IVF may be useful for distinguishing severe and less severely infertile men with isolated teratozoospermia. One-third of severe teratozoospermic men may still have a chance to achieve fertilization either in vivo or in vitro with standard IVF, but two-thirds should be treated by ICSI from the outset.

Acknowledgements

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

References


Submitted on November 5, 2002; accepted on December 18, 2002