High frequency of defective sperm–zona pellucida interaction in oligozoospermic infertile men

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BACKGROUND: The ability of sperm to interact with the zona pellucida (ZP) plays a critical role during the process of human fertilization. The aim of this study is to determine frequency of defective sperm–ZP interaction in oligozoospermic infertile men.

METHODS: Sperm–ZP binding assays and the ZP-induced acrosome reaction (AR) were performed in 72 infertile men with a sperm concentration <20 × 10⁶/ml. Oocytes that had previously failed to fertilize in a clinical IVF programme were used for the tests. Motile sperm (2 × 10⁶/ml) selected by swim-up from each semen sample were incubated with four oocytes for 2 h. The number of sperm bound per ZP and the ZP-induced AR were assessed. Under these conditions, an average of <40 sperm bound/ZP was defined as low sperm–ZP binding and a ZP-induced AR <16% was defined as low ZP-induced AR.

RESULTS: In the 72 oligozoospermic men, 28% (20/72) had low sperm–ZP binding. Of those with normal sperm–ZP binding, 69% (36/52) had low ZP-induced AR. Overall, 78% (56/72) had either low ZP-binding or normal ZP binding but low ZP-induced AR. This means that only 22% (16/72) had both normal sperm–ZP binding and normal ZP-induced AR.

CONCLUSION: Oligozoospermic men have a very high frequency of defective sperm–ZP interaction, consistent with their low natural fertility or low fertilization rate in conventional IVF. Infertile couples with oligozoospermic semen should be treated by ICSI rather than by conventional IVF.

Key words: male infertility/oligozoospermia/sperm–zona pellucida interaction

Introduction

Sperm concentration in semen is correlated with human pregnancy rates (Baker, 2001). Traditionally, oligozoospermia is defined as a sperm concentration <20 × 10⁶/ml (World Health Organization, 1999). Although the low sperm number may be the main factor reducing the chance of natural conception, low motility and poor sperm morphology often accompany and accentuate the defect regardless of the cause of the oligozoospermia (Liu and Baker, 1992). Although many oligozoospermic men have enough motile sperm for them to be harvested by sperm preparation techniques for insemination of oocytes by standard IVF, previous experience of IVF treatment in couples with moderate-to-severe oligozoospermia (sperm count <5 × 10⁶/ml) was generally unsatisfactory. The fertilization rate was usually low, averaging ~35% although some pregnancies were achieved (Yovich et al., 1985; Baker et al., 1993). Natural pregnancies have also been reported in men with severe oligozoospermia (sperm concentration <2 × 10⁶/ml) (Bostofte et al., 1982; Burger and Baker, 1984; Sokol and Sparkes, 1987; World Health Organization Task Force, 1990). Therefore, other sperm defects rather than low sperm number alone may be critical for the severity of infertility in oligozoospermic men.

The capacity of sperm to interact with the oocyte during the process of fertilization is crucial and involves the sequential steps: sperm–ZP binding, the ZP-induced AR, sperm–ZP penetration and sperm–oolemma fusion (Yanagimachi, 1994; Wassarman, 1999). While abnormalities of either sperm or oocytes could lead to failure of fertilization, in conventional IVF, failure of fertilization of all or most of the oocytes collected from the one woman is usually caused by defective sperm–ZP binding and penetration associated mainly with abnormal sperm morphology and disordered ZP-induced AR (Liu and Baker, 2000; Liu et al., 2001). Oocyte immaturity affects normal fertilization but does not affect sperm–ZP binding and penetration in vitro (Overstreet et al., 1976; Lopata and Leung, 1988). Overstreet et al. (1976, 1980) originally reported that testing sperm–ZP interaction using immature oocytes recovered from surgically removed human ovaries provides an important indicator for defective sperm fertilizing ability that may not be revealed by routine semen analysis. More recent studies clearly show that tests for sperm–ZP
binding and the ZP-induced AR using unfertilized human oocytes obtained from clinical IVF provide useful markers for sperm fertilizing ability in vitro (Burkman et al., 1988; Liu et al., 1988; Oehninger et al., 1989; Liu et al., 2001).

Aitken et al. (1989) evaluated sperm function in oligozoospermic men using the calcium ionophore A23187-enhanced human sperm ZP-free hamster oocyte penetration assay and found that 88% of oligozoospermic men (n = 74) had <25% penetration and 58% had no penetration. This suggests that a high proportion of oligozoospermic men may have low calcium ionophore A23187-induced AR or other sperm defects preventing sperm–oolemma fusion, oocyte penetration and sperm head decondensation.

Using the human ZP-induced AR test, we showed a high frequency of defective sperm–ZP interactions in severely teratozoospermic infertile men (strict normal sperm morphology ≤5%, sperm count >20×10^6/ml and motility >30%) (Liu and Baker, 2003b). In this study we have investigated the frequency of defective sperm–ZP binding and the ZP-induced AR in 72 oligozoospermic (<20×10^6/ml) infertile men who had sufficient motile sperm harvested by swim-up for insemination of oocytes in vitro.

Materials and methods

Subjects

Semen samples were obtained from 72 oligozoospermic infertile men (sperm count >20×10^6/ml) who attended infertility clinics in both The Royal Women’s Hospital and Melbourne IVF between February 1996 to November 2002. The oligozoospermia was caused by untreated primary seminiferous tubule defects.

Human oocytes

Oocytes which showed no evidence of two pronuclei or cleavage at 48–60 h after insemination in the clinical IVF programme were used for the sperm–ZP interaction 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) slightly smaller than the oocyte diameter (Liu and Baker, 1996a). Most of the oocytes were obtained from patients with partial failure of fertilization in standard IVF and >50% of these unfertilized oocytes had one or a few sperm penetrating the ZP from the IVF insemination. We have shown previously that oocytes with <10 sperm penetrating the ZP have an ability for subsequent sperm–ZP binding and ZP-induced AR similar to those without sperm penetration (Liu and Baker, 1996a). Degenerate, activated or morphologically abnormal oocytes, as well as oocytes with >10 sperm penetrating the ZP were not used for the test. Oocytes were pooled from several patients, kept in the 5% CO₂ incubator and used within 3 days.

All patients signed consent forms permitting use of their unfertilized oocytes or sperm samples 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 1 h. Sperm concentration and motility in semen were determined using standard methods (World Health Organization, 1999). Semen samples with sperm counts <4×10^6/ml or total motility <20% were not used in this study since they provided insufficient motile sperm for performing the sperm–ZP interaction test. The leukocyte concentration in samples used was not determined using specific staining, but samples with high numbers (>2×10^6/ml) of round cells were excluded from the study.

Morphology of sperm was assessed on smears from both insemination medium (swim-up motile sperm) and semen, prepared by washing of sperm with 10 ml 0.9% sodium chloride. Morphology slides were stained with the Shorr method after fixing in 90% ethanol for 30 min (Jeulin et al., 1986; Liu and Baker, 1992; World Health Organization, 1999). For each slide, 200 sperm were scored from ≥10 individual fields using oil immersion with magnification of ×1000 under bright-field illumination. The percentage of normal sperm morphology was assessed according to strict criteria (Kruger et al., 1988; World Health Organization, 1999).

Sperm preparation

Motile sperm were selected by a swim-up technique. The sperm pellet obtained by centrifugation 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, USA) supplemented with 10% heat-inactivated human serum (ICN Biomedicals, 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 2×10^6/ml for subsequent experiments.

Sperm–ZP binding

For each sperm–ZP interaction test, motile sperm (2×10^6) 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 loosely adhering to the surface of the ZP. Because a high concentration of sperm was used in the insemination medium (20-fold higher than IVF insemination), the number of sperm from fertile men bound tightly to the ZP was >100/ oocyte (Liu and Baker, 1994; Liu et al., 2001). This experimental design allows enough ZP-bound sperm to be recovered for assessment of the AR.

Assessment of the human ZP-induced AR

All sperm bound to surface of the four ZP were then removed by repeated vigorous aspiration with a narrow gauge pipette with an inner diameter (~120 μm) slightly smaller than the oocyte (Liu and Baker, 1994, 1996a). This was performed on a glass slide with ~5 μl PBS containing 0.2% BSA and the removed ZP-bound sperm were smeared in a limited area (~16 mm²), which was marked on the back of the slide with a glass pen to help find the sperm under the microscope. This pipetting procedure for removing sperm from the surface of ZP does not affect sperm motility, morphology or acrosome status (Liu and Baker, 1996a).

The acrosome status of sperm removed from the ZP was determined with fluorescein-labelled Pisum Sativum agglutinin (PSA; Sigma Co., USA) using 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 ≥2 h at 4°C. The slide was washed and mounted with distilled water and the percentage of AR was determined by scoring 200 of the sperm removed from all four ZP

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per test, using a fluorescence microscope with oil immersion at a magnification of \( \times 400 \). When more than half the head of a sperm 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 (Figure 1).

### Thresholds for low sperm–ZP binding and low ZP-induced AR

A threshold of \( \leq 40 \) sperm bound/ZP was used to define low sperm–ZP binding. This threshold for a sperm concentration of \( 2 \times 10^6 / \text{ml} \) in the insemination medium is equivalent to \( <2 \) sperm bound/ZP with the standard IVF insemination sperm concentration of \( 1 \times 10^7 / \text{ml} \) (Liu and Baker, 2000). The ZP-induced AR \( \leq 16\% \) was used for classification of low ZP-induced AR according to our previous reports that men with \( \leq 16\% \) ZP-induced AR have very low (<30\%) sperm–ZP penetration and fertilization rates with standard IVF (Liu and Baker, 1996b, 2003b).

### Statistical analysis

The significance of correlations between sperm–ZP binding, ZP-induced AR and other sperm characteristics were examined by non-parametric Spearman’s \( \rho \) and Spearman’s correlation coefficient and its \( P \) value are reported. \( \chi^2 \)-Test was used for comparison of the proportions of men with low (\( \leq 16\% \)) ZP-induced AR and non-parametric Wilcoxon’s rank sum test for comparison of mean results of ZP-induced AR between oligozoospermic, normozoospermic or teratozoospermic men from previous studies (Liu et al., 2001; Liu and Baker, 2003a).

### Results

#### Sperm tests results

Table I shows mean and ranges for all sperm test and sperm–oocyte interaction test results. The average number of sperm bound was \( 70/ZP \) and 20 men had an average of \( \leq 40 \) sperm bound/ZP. The mean ZP-induced AR for the 52 men with normal sperm–ZP binding was 15\% and ranged from 1 to 57\% (Figure 2).

![Figure 1](http://humrep.oxfordjournals.org/)

**Figure 1.** An example for *Pisum sativum* agglutinin–fluorescein isothiocyanate staining of the acrosome of sperm removed from the surface of zona pellucida. There were five acrosome intact (AI) and four acrosome-reacted (AR) sperm. The photograph was taken with \( \times 400 \) magnification.

#### Frequency of defective sperm–ZP binding and the ZP-induced AR

As described above, a threshold of \( \leq 40 \) sperm bound/ZP was used to define low sperm–ZP binding and the ZP-induced AR \( \leq 16\% \) was used for classification of low ZP-induced AR. Of the 72 oligozoospermic men, 28\% (20/72) had low sperm–ZP binding. Of the 52 men with normal sperm–ZP binding, 69\%
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Correlation between sperm tests
The percentage of sperm with progressive motility in semen was significantly correlated with number of sperm bound/ZP (ρ = 0.274, P < 0.05). Sperm concentration and all other semen analysis results did not correlate with either sperm–ZP binding or the ZP-induced AR. There was a significant correlation between sperm count and motility (ρ = 0.295, P < 0.05), and between normal sperm morphology and both total motility (ρ = 0.951, P < 0.01) and progressive motility (ρ = 0.973, P < 0.01).

Eleven men had two ZP-induced AR tests performed on sperm from two separate ejaculates within 2–10 weeks, with similar results obtained (mean ± SD 11.6 ± 7.2%, range 5–30% versus 11.2 ± 6%, range 4–25%, mean difference ± SD 0.23 ± 4.9%).

Discussion
The ZP-induced AR is highly correlated with sperm–ZP penetration and fertilization rate in conventional IVF in patients with normal semen analysis (Liu and Baker, 1996b, 2003b; Esterhuizen et al., 2001; Bastiaan et al., 2003). Patients with unexplained infertility with ZP-induced AR <16% have average fertilization rates of <30% with conventional IVF (Liu and Baker, 2003b). Therefore, the ZP-induced AR is a useful clinical test for sperm fertilizing ability and for selection between conventional IVF and ICSI for treatment of patients.

The present study shows that >28% of oligozoospermic men had low sperm–ZP binding and 69% of the remainder with normal ZP binding had low ZP-induced AR. Overall, defective sperm–ZP binding or ZP-induced AR was present in 78% of oligozoospermic men, indicating that a high frequency of defective sperm–ZP interaction contributes to the infertility in oligozoospermia. Oligozoospermic men with defective sperm–ZP interaction should be treated by ICSI to avoid low or zero fertilization rates in conventional IVF. On the other hand, ~22% of oligozoospermic infertile men had normal sperm–ZP binding and normal ZP-induced AR. These men may have better chances of producing fertilization either in vivo or with conventional IVF.

The frequency of low ZP-induced AR (69%) in this group of oligozoospermic men who could be tested is much higher than in normozoospermic (25–29%) and severely teratozoospermic (48%) infertile men (Liu et al., 2001; Liu and Baker, 2003a,b). It is interesting that >64% of severely teratozoospermic men had low ZP-induced AR if they also had sperm counts between 20 and <60 X 10⁶/ml (Liu and Baker, 2003a). It is likely that these high frequencies of defective sperm–ZP interaction are related to the degree of disordered spermatogenesis. It has been reported that abnormalities of sperm plasma membrane may reduce fluidity and affect the AR in oligozoospermic men (Ladha, 1998). Further study is required to determine the cause for low ZP-induced AR in men with low sperm production.

Others have reported that the ability of sperm to undergo the calcium ionophore A23877- induced AR may also predict sperm fertilizing ability in vitro (Cummins et al., 1991; Yovich et al., 1994). However, we found A23187-induced AR correlates with fertilization rate of IVF only in patients with teratozoospermia (strict normal sperm morphology <15%; Liu and Baker 1998). Also A23187-induced AR does not correlate with the ZP-induced AR or sperm–ZP penetration in normozoospermic men (Liu and Baker, 1996a,b). Therefore, the A23187-induced AR may predict other sperm dysfunction but not defective ZP-induced AR.

Similarly, the AR induced by progesterone or human follicular fluid may also predict sperm fertilizing ability in men with unexplained infertility (Calvo et al., 1989; Krausz et al., 1995, 1996). However, these progesterone-induced AR tests usually involve long periods (18–20 h) of preincubation of sperm which are inconvenient for routine testing. Also sperm lose the ability to bind to the ZP after 18 h preincubation (Singer et al., 1985). We have been unable to study relationship between the ZP-induced AR and the AR induced by progesterone and human follicular fluid since we have not been able to induce the AR with either agent.

Human sperm binding to the ZP is highly species specific and human sperm do not bind to the ZP of most other mammalian oocytes (Bedford, 1977; Liu et al., 1991). Human oocytes are essential for clinical testing of the sperm–ZP interaction. In routine clinical IVF, ~20–30% of oocytes inseminated fail to fertilize and these unfertilized oocytes provide valuable biological material for testing sperm function for other infertile patients before they commence assisted reproduction treatment. Most oocytes that fail to fertilize and immature (germinal vesicle) oocytes unsuitable for ICSI are useful for the test. However, degenerate, spontaneous activated and morphologically abnormal oocytes and those with large numbers of sperm (>10) penetrating the ZP 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. Previous tests showed that ~10–20% of unfertilized oocytes obtained from clinical IVF do not bind sperm from fertile men (Liu et al., 1988). However, it is rare for all oocytes recovered from one woman to fail to bind sperm (Liu and Baker, 2000). Solubilized human ZP can also be used for induction of the AR and a microaassay has been reported (Franken et al., 2000; Bastiaan et al., 2003). Nevertheless, access to human oocytes for routine tests of sperm oocyte interaction is still limited. At the moment, no chemical or biological material can substitute for native human ZP in this test. Recombinant human ZP still remains unavailable despite some claims of the production of biologically active material (van Duin et al., 1994; Brewis et al., 1996; Whitmarsh et al., 1996; Dong et al., 2001).

This study indicates that the infertility of oligozoospermic men occurs not only because of the low sperm numbers but also because of the high frequency of defective sperm–ZP inter-
action. Importantly, sperm from most (78%) of the oligozoospermic men have either low sperm–ZP binding or low ZP-induced AR, both of which are likely to cause failure of sperm–ZP penetration and fertilization. However, 22% have near normal sperm–oocyte interaction and may be more likely to conceive naturally or with conventional IVF. For clinical management of patients, sperm–ZP interaction tests are useful for distinguishing between these two groups so that effective treatments can be chosen. Practically, if sperm–ZP interaction tests are not available, patients with oligozoospermia should be treated with ICSI since ~78% of them have either defective sperm–ZP binding or low ZP-induced AR. This will minimize the risk of fertilization failure with conventional IVF. The underlying biochemical and molecular causes of defective sperm–ZP interaction in oligozoospermic men require further study.

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