Human sperm bound to the zona pellucida have normal nuclear chromatin as assessed by acridine orange fluorescence

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BACKGROUND: The aim of this study was to determine if only sperm with double stranded DNA could bind to the human zona pellucida (ZP). METHODS: Sperm samples from 124 infertile men with a range of semen abnormalities were studied. Oocytes that had failed to fertilize in IVF or ICSI were used for the sperm-ZP binding test. A group of four oocytes were incubated for 2 h with $2 \times 10^6$/ml motile sperm selected by colloidal silica gradient centrifugation (CSGC). After assessing the number of sperm bound per ZP, all sperm bound to the surface of the ZP of four oocytes were dislodged and placed on a glass slide. The double (green fluorescence) or single stranded (denatured, red fluorescence) DNA of sperm in semen, motile sperm selected by CSGC and ZP-bound sperm, was assessed by acridine orange (AO) fluorescence. RESULTS: The percentage of sperm with green fluorescence was significantly correlated with normal sperm morphology in semen and after CSGC preparation. The proportion of sperm with green fluorescence was significantly higher in motile sperm selected by CSGC than ejaculated sperm. There were very few sperm (average <8%) with red fluorescence bound to the ZP, even in men who had very high (>70%) proportions of sperm with red fluorescence in their semen. CONCLUSION: Sperm binding to human ZP is highly selective for double stranded DNA. Sperm with single stranded or denatured DNA bind less or do not bind at all to the ZP, probably because of defects of motility and, more especially, morphology.

Keywords: Male infertility; sperm chromatin DNA normality; sperm-ZP interaction

Introduction

The normality of sperm nuclear chromatin DNA plays a critical role in human fertilization and subsequent embryonic development. In recent times, there have been many reports on the relationship between fertilization and pregnancy rates in clinical assisted reproductive techniques (ART) and sperm DNA normalities detected by various methods including acridine orange (AO) fluorescence staining, assessed either by microscopy or flow cytometry (sperm chromatin structure assay, SCISA), terminal deoxynucleotidyl transferase-mediated d UDp nick-end labelling (TUNEL) or electrophoresis (comet assays). The AO fluorescence stain distinguishes sperm with either double (green fluorescence) or single (denatured, red fluorescence) stranded DNA. There are reports of relationships between abnormal sperm DNA detected by AO and adverse outcomes of both IVF and ICSI with lower implantation rates, higher pregnancy loss and even suggestions there may be effects in offspring (Lopes et al., 1998; Evenson et al., 1999; Larson et al., 2000; Larson-Cook et al., 2003; Morris, 2002; Aitken et al., 2003, 2004; Virro et al., 2004; Evenson and Wixon, 2006). However, a number of studies showed a significant relationship between abnormal sperm DNA assessed in semen with fertilization and pregnancy rates only in conventional IVF (Liu and Baker, 1992a; Tomlinson et al., 2001; Apedaile et al., 2004; Bungum et al., 2004; Henkel et al., 2004; Huang et al., 2005; Zini et al., 2005). Others found weak or no significant relationship between sperm DNA test results and outcome of either IVF or ICSI (Angelopoulos et al., 1998; Gandini et al., 2004; Payne et al., 2004; Zini et al., 2005; Boe-Hansen et al., 2006). Therefore, clinical value of tests for sperm DNA normalities is still inconclusive. One of the major problems is that the tests of sperm DNA normalities are usually assessed in semen which contains a large number of immotile, non-viable or degenerated sperm. Therefore, most sperm with abnormal DNA detected in semen largely reflect population of non-viable or dead sperm with denatured DNA. However, these non-viable or immotile sperm are not involved in the process of fertilization either
in vivo or in IVF. Thus, the test of DNA normalities should be performed in motile sperm population, which may provide more useful information about the sperm fertilizing ability. Usually, the results of normal sperm DNA in semen detected by the AO stain are highly significantly correlated with sperm motility, viability and normal morphology (Claassens et al., 1992; Giwercman et al., 2003; Apedaile et al., 2004; Wyrobek et al., 2006). Therefore, it is necessary to show a possible added value to routine semen analysis of tests of sperm DNA normalities. This can be done by using multivariate logistic regression modelling or by studying results of tests performed only on the motile sperm population since only these can fertilize.

Previous studies showed clearly that motile sperm selected by either swim-up or density gradient centrifugation had significantly lower abnormal DNA than the sperm in the semen. (Angelopoulou et al., 1998; Spano et al., 1999; Sakkas et al., 2000; Tomlinson et al., 2001; Giwercman et al., 2003). Therefore, this may be why there is no relationship between abnormal sperm DNA assessed in semen and fertilization and pregnancy rate in ICSI since only motile sperm are used for ICSI (Gandini et al., 2004, Zini et al., 2005). Although some researchers speculate that human oocytes are capable of repairing damaged DNA of sperm injected by ICSI, there is so far no direct evidence to show that injection of a sperm with damaged or denatured DNA into oocytes by ICSI can produce normal fertilization and pregnancy (Gandini et al., 2004; Zini et al., 2005).

During the processes of human fertilization in vivo or in conventional IVF, sperm must be capable of binding to the zona pellucida (ZP), undergoing the acrosome reaction, and penetrating the ZP and finally fusing with the oolemma (Yanagimachi et al., 1994, Wassarman, 1999). In fertile men, only a very small proportion of motile sperm (average of 14%) is capable of binding to the ZP in vitro when unlimited ZP receptors are provided (Liu et al., 2003). There is very strong relationship between normal morphology of sperm in the insemination medium and the proportion of sperm bound to the ZP (Liu and Baker, 1992a, 1994; Liu et al., 2003). Therefore, the human ZP selectively binds sperm with normal morphology, and particularly, shape and relative size of the acrosome region are important (Menkveld et al., 1991, 1996; Liu and Baker, 1992b, 1994; Garrett et al., 1997). Morphologically abnormal sperm such as round-headed sperm lacking the acrosome (a rare cause of male sterility) are not capable of binding to the ZP or oolemma of the ZP-free human oocytes (Bourne et al., 1995). However, most commonly teratozoospermic infertile men have a mixture of various types of abnormal morphology which also significantly reduces sperm-ZP binding capacity (Liu and Baker, 1992a). This evidence suggests that the ZP of human oocytes has the capacity to selectively bind normal functional sperm (Menkveld et al., 1991; Liu and Baker, 1992b, 1994).

In the current literature, there is no report of the capacity of sperm with normal (double stranded) or abnormal (denatured or single stranded) DNA to bind to the human ZP. In this study, sperm samples from 124 infertile men who had variable semen analysis and sperm-ZP binding capacity were studied to determine if sperm binding to the human ZP is selective for double stranded DNA. Normalities of sperm DNA between semen, motile sperm selected by colloidal silica gradient centrifugation (CSGC) and bound to the human ZP were compared by AO fluorescence.

Materials and Methods

Subjects and semen analysis

Semen samples were obtained by masturbation after 2–5 days abstinence from 124 infertile men with an average age of 36 (range 25–53) years old who attended our infertility clinics in the Royal Women’s Hospital and Melbourne IVF, between January and October 2006. Routine semen analysis was performed after liquefaction of the semen within 1 h according to WHO manual (World Health Organization, 1999). Both total motility (WHO criteria grades a + b + c) and progressive motility (a + b) were assessed manually by counting 200 sperm. Viability of sperm was assessed by Eosin Y exclusion.

Morphology of sperm in both semen and CSGC preparation was assessed on smears prepared by washing of sperm with 10 ml 0.9% sodium chloride (World Health Organization, 1999). Morphology slides were stained with the Shorr method after the smears were fixed in 90% ethanol for 30 min (Liu and Baker, 1992a; World Health Organization, 1999). Washing sperm to remove seminal plasma or protein in medium decreases background staining and produces clearer images of sperm. The percentage of normal sperm morphology was assessed according to strict 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 CSGC (PureSperm, Nidacon International AB, Molndal, Sweden) with two layers of 1 ml 40% and 1 ml 80% PureSperm. The pellet of motile sperm obtained from CSGC was washed once with 1 ml of human tubal liquid (HTF) supplemented with 10% human serum. The washed sperm pellet was resuspended with serum supplemented HTF to a sperm concentration of 2 × 10⁶/ml for the ZP-interaction test.

Human oocytes

Oocytes which showed no evidence of two pronuclei or cleavage at 48–60 h after insemination in IVF, or after injection in ICSI, or immature oocytes not injected in ICSI were used for the sperm-ZP binding test. 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, 2004). Oocytes with >10 sperm penetrating the ZP, degenerated, activated or morphologically abnormal oocytes were not used. Oocytes were pooled from several patients and 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 the use of their unfertilized oocytes or sperm samples for research. The Royal Women’s Hospital Research and Ethics Committees approved the project.

AO fluorescence for sperm chromatin DNA

The normality of sperm chromatin DNA was assessed by the AO (Sigma Chemical Co, St Louis, MO, USA) fluorescence method described by Tejada et al. (1984). Briefly, after air-drying the sperm smears prepared from semen, CSGC and ZP-bound, the smears were fixed in Carnoy’s solution (three parts of methanol and one part of...
glacial acetic acid) for at least 3 h or overnight at 4°C. After fixation, the slides were allowed air-dry for a few minutes before staining with AO solution. The AO staining solution was prepared daily as follows: 10 ml of 1% AO stock solution in distilled water was added to a mixture of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M Na2HPO47H2O, pH 2.5. The 1% AO stock solution was stored in the dark at 4°C for 4 weeks. The fixed and air-dried sperm smear was stained in AO working solution for 5 min and then the slide was gently rinsed and mounted with distilled water. The percentage of sperm with normal DNA was determined by randomly scoring 200 sperm under a fluorescence microscope (Dialux 20, Leitz, Wetzlar, Germany) with ×400 magnification and excitation of 450–490 nm. Sperm with normal (double-stranded) DNA fluoresce green, and those with denatured or single stranded DNA fluoresce red or yellow.

In order to obtain consistent results from this procedure, it is important to fix sperm smear on the same day of preparation and stain on the next day. Storage of either fixed and non-fixed smears for later staining could affect the results. To reduce variation of the intensity of fluorescence, each stained slide should be read immediately after removal from the staining solution following washing.

**Sperm-ZP binding**

For each sperm sample, motile sperm (2 × 10⁶) in 1 ml of medium were incubated with a group of 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. The total number of sperm bound to all four oocytes was then counted. Under these experimental conditions, with the high concentration of sperm in the insemination medium (20 times more than standard IVF insemination), the number of sperm bound tightly to the ZP is usually greater than 100/ZP with sperm from fertile or normospermic men. Sperm samples with an average ≥40 sperm/ZP were considered to have normal sperm-ZP binding (Liu and Baker, 2004).

**Assessment of nuclear chromatin DNA normality of sperm bound to the ZP**

After counting the number of sperm bound to the ZP, 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) slightly smaller than the oocyte (Liu and Baker, 2004). 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 very small area (~16 mm²), which was marked on the back of the slides with a glass pen to help find the sperm under the microscope. After drying in air, the smears were fixed overnight at 4°C and then stained with the AO solution as described earlier. For men with normal sperm-ZP binding, 200 ZP-bound sperm were scored for each slide. For those with abnormal sperm-ZP binding (<40 sperm bound/ZP), all ZP-bound sperm were scored for each slide.

**Statistical analysis**

The significance of correlations between results of semen analysis and DNA normality of sperm in semen, CSGC and bound to the surface of the ZP was examined by Spearman tests. Differences of AO fluorescence of sperm in semen, CSGC and bound to the ZP were examined by non-parametric Friedman and Wilcoxon tests.

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**Results**

In preliminary experiments, to determine whether the ZP of immature oocytes was different from that of mature oocytes (unfertilized) for binding sperm with normal green fluorescence, duplicate sperm-ZP binding tests were performed using the same sperm incubated with either four mature or four immature (germinal vesicle and metaphase I) oocytes. Sperm samples from five men were tested. There was a similar proportion of ZP-bound sperm with normal green fluorescence (mean ± SD, 95.2 ± 3.7 versus 95.8 ± 3.3, t = 1.177, P < 0.05) between mature and immature oocytes. Thus, both mature and immature oocytes obtained from IVF/ICSI were used in this study.

There was a wide range for all the sperm test results including the AO test and sperm-ZP binding (Table 1). In all 124 men studied, 14 of them had low sperm-ZP binding (average <40 sperm bound/ZP); six men had an average of 45 (range 30–75) ZP-bound sperm assessed by the AO test and eight men had no AO test performed because too few sperm bound to the ZP (Table 2). A total of <100 sperm assessed per sample corresponds to high statistical counting error in the AO scores. Thus, the 14 men with low sperm-ZP binding were excluded from the main statistical analysis but presented separately in Table 2.

Both normal sperm morphology and green fluorescence (double stranded DNA) sperm were significantly higher in motile sperm selected by CSGC than sperm in semen. There was a significantly higher proportion of sperm with green fluorescence in both CSGC (average 67%) and the ZP-bound sperm (92%) than in semen (50%, Table 4 and Fig. 1). Similar results were also observed in six men with low sperm-ZP binding (Table 2).

On average only a few (8%) of the ZP-bound sperm had red fluorescence (single stranded DNA). With higher proportions of abnormal DNA sperm in semen, for example, <30% (mean 23% with range 12–30%) double stranded DNA, still average 83% (range 60–99%) of ZP-bound sperm had normal green fluorescence (Fig. 1). Although there was no significant difference in the number of sperm bound/ZP between men with ≤30% (n = 22, mean ± SD, 76 ± 33 sperm bound/ZP) and >30% (n = 102, 83 ± 30 sperm bound/ZP) of sperm with green fluorescence in semen, the proportion

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**Table 1:** Summary of sperm tests results

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>Volume of ejaculate (ml)</td>
<td>124</td>
<td>34 ± 1.6</td>
<td>0.6–9.2</td>
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<tr>
<td>Sperm count (10⁶/ml)</td>
<td>124</td>
<td>76 ± 66</td>
<td>2–333</td>
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<tr>
<td>Total motility (%)</td>
<td>124</td>
<td>49 ± 11</td>
<td>20–70</td>
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<tr>
<td>Progressive motility (%)</td>
<td>124</td>
<td>36 ± 12</td>
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<tr>
<td>Live (%)</td>
<td>124</td>
<td>78 ± 8</td>
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</tr>
<tr>
<td>Normal sperm morphology (% semen)</td>
<td>124</td>
<td>7 ± 7</td>
<td>0–33</td>
</tr>
<tr>
<td>Normal sperm morphology (% CSGC)</td>
<td>124</td>
<td>13 ± 10</td>
<td>0–60</td>
</tr>
<tr>
<td>Green sperm (% semen)</td>
<td>124</td>
<td>50 ± 19</td>
<td>11–87</td>
</tr>
<tr>
<td>Green sperm (% CSGC)</td>
<td>124</td>
<td>67 ± 18</td>
<td>22–98</td>
</tr>
<tr>
<td>Green sperm (% ZP-bound)*</td>
<td>116</td>
<td>92 ± 9</td>
<td>60–100</td>
</tr>
<tr>
<td>Number of sperm bound/ZP</td>
<td>124</td>
<td>82 ± 16</td>
<td>2–100</td>
</tr>
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</table>

*The AO test was not performed in eight patients who had a few sperm bound to the ZP.
of sperm with green fluorescence in semen was significantly correlated with the number of sperm bound to the ZP ($n = 124$, Spearman rho $= 0.242$, $P < 0.01$).

The proportions of sperm with green fluorescence were highly correlated between semen, CSGC and ZP-bound (Fig. 2). Over 80% of the samples studied had >90% of ZP-bound sperm with double stranded DNA. There were highly significant correlations between green fluorescence and normal sperm morphology and progressive motility in semen (Fig. 3). Similarly, normal sperm morphology in CSGC was also highly correlated with green fluorescence sperm in CSGC (Spearman $r = 0.505$, $P < 0.001$). Normal sperm morphology in both semen (Spearman rho $= 0.407$, $P < 0.001$) and CSGC (Spearman rho $= 0.486$, $P < 0.001$) was highly significant correlated with the number of sperm bound to the ZP.

**Table 2:** The AO results for 14 men with low sperm-ZP binding (<40 sperm bound/ZP)

<table>
<thead>
<tr>
<th>Patients number</th>
<th>Number of sperm bound/ZP</th>
<th>Green sperm (%)a</th>
<th>Semen</th>
<th>CSGC</th>
<th>ZP-bound</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>55</td>
<td>81</td>
<td>98</td>
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<tr>
<td>2</td>
<td>10</td>
<td>87</td>
<td>89</td>
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<td>82</td>
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<td>58</td>
<td>69</td>
<td>98</td>
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<td>2</td>
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<td>58</td>
<td>69</td>
<td>ND</td>
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<tr>
<td>14</td>
<td>2</td>
<td>33</td>
<td>51</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

**a**Two hundred sperm assessed per sample for semen or CSGC but only average 45 (range 30–75) sperm of ZP-bound sperm (from four ZPs) assessed in six men or ZP-bound sperm was not determined (ND) in eight men.

![Figure 1](image)

**Figure 1:** Comparison of the proportion of sperm with normal AO fluorescence in semen, CSGC (PureSperm) and bound to the ZP in men with normal sperm-ZP binding (A, $n = 110$) or in subgroup of the men with $\leq 30\%$ normal chromatin DNA of sperm in semen (B, $n = 20$). Each line represents an individual subject. There are significant increases in normal AO fluorescence from semen to CSGC and CSGC to ZP bound for both A and B ($P < 0.01$).

![Figure 2](image)

**Figure 2:** Correlation of normal AO fluorescence of sperm between: semen and CSGC (PureSperm, A, Spearman rho $= 0.746$, $P < 0.001$), semen and ZP-bound (B, Spearman rho $= 0.482$, $P < 0.001$) and PureSperm and ZP-bound sperm (C, Spearman rho $= 0.529$, $P < 0.001$).
Figure 3: Correlation between normal AO fluorescence and normal sperm morphology in semen (A, Spearman rho = 0.555, P < 0.001) and progressive motility (B, Spearman rho = 0.331, P < 0.01).

The proportion of sperm with normal AO green sperm in semen was significantly correlated with the number of sperm bound to the ZP. This indicates sperm morphological defects are associated with sperm chromatin abnormalities, which may reflect sperm immaturity or abnormal spermatogenesis. This reinforces why sperm morphology is such a significant factor related to fertilization rate in conventional IVF (Kruger et al., 1988; Liu and Baker, 1992a). Our previous study also found a high frequency of defective sperm-ZP interaction in men with severe teratozoospermic semen (Liu and Baker, 2004). The proportion of sperm with normal morphology was significantly related to the number of sperm bound to the ZP.

In conventional IVF, defective sperm-ZP binding or penetration is the most frequent cause of failure of fertilization (Liu and Baker, 2000). Our previous study found that the green AO fluorescence of sperm in semen was significantly correlated with fertilization rate in IVF, but it was less significant than sperm-ZP binding and normal morphology in multivariate logistic regression analysis (Liu and Baker, 1992a). Although the clinical value of the AO test of sperm in semen for prediction of ART outcome is still contradictory in current literature, the AO test, particularly with the microscopy method, is very simple to perform and addition of this test to routine semen analysis may provide additional information about the sperm fertilizing ability. For clinical application, patients with severe sperm defects such as strict normal morphology (<5% or AO green fluorescence sperm <30%), or defective sperm-ZP binding or penetration, should be treated directly with ICSI since these patients have high risk of low or zero fertilization rate with IVF. However, for the many patients without severe sperm defects, the optimal treatment for the first cycle is difficult to determine. Usually, they are initially assigned to IVF and if this fails, are then treated by ICSI. In ICSI, a single motile sperm without gross abnormal morphology is selected for injection. This selection may also eliminate sperm with abnormal DNA since sperm DNA normality is highly correlated with both motility and morphology. This could explain why there is no relationship between sperm DNA test results and fertilization or pregnancy rates in ICSI (Gandini et al., 2004; Payne et al., 2004; Zini et al., 2005; Boe-Hansen et al., 2006).

In conclusion, we show here that sperm with single stranded or denatured DNA generally do not bind to the ZP. Thus it could be concluded that the sperm-ZP binding process plays an important role not only in selection of sperm with normal motility and morphology but also normal chromatin DNA. However, as the proportion of sperm with normal chromatin DNA is highly correlated with both progressive motility and morphology, the mechanism of the selection against sperm with abnormal chromatin DNA is likely related to the high selectivity of sperm-ZP binding process for normally shaped motile sperm. Currently used assessments of sperm DNA normalities in semen may not accurately reflect chromatin DNA status of the motile sperm in the population, since...
there are also variable proportions of immotile, non-viable and degenerate, sperm, which will contribute most of the abnormal DNA test results.

Acknowledgments

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