USEFULNESS OF THE ACROBEAD TEST IN EVALUATING HUMAN ACROSOME FUNCTION IN FRESH AND CRYOPRESERVED SPERM

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ABSTRACT

Purpose: Assays for the acrosome reaction are usually cumbersome and lack reproducibility. Accurate determination of acrosomal status is important in patients diagnosed with male infertility before proceeding with intrauterine insemination or in vitro fertilization. We determined the optimum capacitation time and acrosomal status of fresh semen specimens in normal fertile men with the Acrobead† test, and whether the assay could be used to evaluate cryopreserved semen specimens.

Materials and Methods: Semen samples from 13 normal donors were divided, with half of the fresh ejaculate used for the Acrobead test and half cryopreserved for a minimum of 24 hours in liquid nitrogen before testing. Fresh and frozen specimens were prepared with the swim-up technique. Sperm concentration was adjusted to 4, 2, 1 and 0.5 × 10⁹/ml in 4 wells of a 96-well tissue culture plate. Ten μl polycrylamide beads (1.5 × 10⁹/ml) coated with anti-CD46 monoclonal antibodies (MH61 beads) were added to each well. The attachment of beads with acrosome reacted spermatozoa was scored after 0, 1, 3, 6 and 24 hours of incubation. Results were graded on a scale of 0 (no bead binding to the sperm) to 4 (complete attachment to the beads). Specimens with scores of at least 2 were considered normal.

Results: A score of at least 2 was noted in 3 of 13 fresh specimens (15.3%) at 1, 9 (69.2%) at 3, 11 (84.6%) at 6 and 13 (100%) after 24 hours. However, a significantly greater number of frozen specimens (8 of 13, or 62%) had a score of 2 or more at 1 hour of incubation and 100% bead attachment to sperm occurred at 3 hours.

Conclusions: Our results indicate that in fresh semen specimens an incubation period of 6 to 24 hours can be used to screen individuals who present with normal sperm characteristics but have slow acrosome reactions. Early acrosome reaction observed in cryopreserved specimens indicates that these spermatozoa have membrane damage and leakage of acrosome contents as a result of the freeze-thaw process. The Acrobead assay is a simple and objective test that can be used at a clinical andrology laboratory to evaluate the acrosomal status of fresh but not frozen human spermatozoa.

KEY WORDS: spermatozoa, cryopreservation, acrosome, sperm motility

Assessing the fertilizing capacity of spermatozoa is important in assisted reproductive procedures to select appropriate treatments for infertility. The fertilizing capacity depends on several factors, such as sperm motility, capacitation and the acrosomal reaction. Fusion of the sperm plasma and outer acrosomal membranes, followed by vesiculation and exocytosis of the inner acrosomal membrane occur as a consequence of capacitation and result in the release of acrosomal contents. This sequence of events is called the acrosome reaction, and must occur before the spermatozoa can penetrate the oocyte and cause fertilization.1-2 The acrosome reaction test and sperm penetration assay with hamster eggs are widely used to assess sperm and egg interaction. The sperm penetration assay assesses the ability of the acrosome reacted spermatozoa to penetrate and initiate fusion with the oocyte. An impaired acrosome reaction may account for poor sperm penetration assay results.

Unlike in most laboratory mammals, direct assessment of acrosomal loss is not feasible in human spermatozoa with phase contrast microscopy due to the small size of the human acrosome. Electron microscopy is of limited use because it is laborious and time-consuming. Techniques used to evaluate the acrosome reaction include triple-stain,3 the chlortetracycline technique4 and fluorescein conjugated lectins5 or antihuman abortion.6,7 The main disadvantage of the fluorescence techniques is that they cannot be used readily at a diagnostic laboratory, since they involve elaborate preparation of smears that must be stained with appropriate conjugated fluorochromes. Furthermore, the smears must be air dried and examined visually, which results in a subjective analysis. These steps are labor-intensive and not suitable for routine testing. In a clinical setting the ideal method requires minimum sperm preparation time and an accurate interpretation of the acrosome reaction. Also, if the results can be interpreted accurately in cryopreserved semen specimens, they could be frozen at routine semen analysis, thus avoiding the need for the patient to return specifically for acrosome evaluation. Additionally, the laboratory technologist would be able to run several specimens simultaneously, making the assay cost-effective. A monoclonal antisperm antibody test (Acrobead assay) has been developed recently to measure the extent of the acrosome reaction in fresh human semen.8-10 The results of this test correlate strongly with in vitro fertilization outcome.9 We evaluated the acrosomal status of semen specimens in normal men using the Acrobead assay. We also determined the time course and extent of the acrosome reaction in fresh and cryopreserved specimens.

MATERIALS AND METHODS

Sperm preparation. Semen samples were obtained after 48 hours of sexual abstinence from 13 healthy donors selected...
from a large pool of men after they were thoroughly tested and had met various criteria, that is a volume of 2.0 ml or more, sperm concentration 20 ×10⁶/ml or more, total sperm count 40 ×10⁶ or more, motility 50% or more forward progression, 30% or more morphologically normal forms and white blood cell concentration of less than 1 ×10⁶/ml. The semen samples were allowed to liquefy at 37°C before the sperm count and other motion characteristics were evaluated on a computer-assisted motion analyzer before freezing and after thawing. For each measurement a 5 μl aliquot of the sample was loaded on a counting slide and analyzed. Each ejaculate was divided into 2 aliquots, 1 of which was processed fresh and 1 was cryopreserved.

Cryopreservation of semen samples. All semen specimens were cryopreserved within 45 to 60 minutes after ejaculation and 13 were processed by a previously described vapor-freezing technique. In brief, semen specimens were diluted with an equal volume of TEST-yolk-buffer freezing medium, placed in sterile vials and cryopreserved by slow freezing at −20°C for 8 minutes followed by immersion in liquid nitrogen vapor (~78°C) for 2 hours and submersion in liquid nitrogen at −196°C. After storage for a minimum of 24 hours the samples were thawed at room temperature for 5 minutes and then for 20 minutes at 37°C in an incubator under 5% carbon dioxide.

Swim-up procedure. Semen specimens were analyzed after liquefaction. The ejaculate was diluted with Biggers, Whitten and Wittmack (BMW) medium containing 0.3% human serum albumin at a 1:2 ratio at 37°C. The samples were centrifuged at 300 × gravity for 7 minutes and, after removal of the supernatant, resuspended in BMW medium and centrifuged again for 5 minutes. A highly motile sperm population was selected by processing the sample with the swim-up technique. Briefly, the pellet was resuspended in 200 to 300 μl BMW medium (0.3% human serum albumin). Two to 3 round bottom tubes containing 0.7 ml BMW medium (0.3% human serum albumin) were then prepared for the swim-up method. Sperm suspension (100 μl) was gently layered underneath the medium. The test tubes were kept at a 45-degree angle for 60 minutes at 37°C in a 5% carbon dioxide incubator to allow the most motile sperm to swim up into the medium. After incubation the medium, which contained highly motile sperm, was aspirated and centrifuged at 300 × gravity for 7 minutes at room temperature. The supernatant was discarded and resuspended in 0.5 ml. capacitation medium (BMW medium containing 3.5% human serum albumin) and the sperm concentration was adjusted to 4 ×10⁹/ml.

Cryopreserved samples were thawed at 37°C for 20 minutes and centrifuged twice at 300 × gravity for 7 minutes, and the pellet was resuspended in capacitation medium. Sperm suspension was layered directly under the medium without washing and prepared for swim-up as described to provide a sperm concentration of 4 ×10⁹/ml.

Acroead assay. A 96-well tissue culture plate was used for the assay with 4 wells used for a given specimen. Then 100 μl of the capacitating medium (BMW medium with 3.5% human serum albumin) were placed in wells 2, 3, 4, and 300 μl sperm suspension (4 ×10⁶ sperm per ml) were added to well 1. Serial dilution was done in wells 2-4. Then a 100 μl aliquot was removed from well 1 and transferred to well 2, and the contents were mixed. Similarly, 100 μl aliquots were transferred and mixed in wells 3 and 4. To keep the volume constant in all wells a 100 μl aliquot from well 4 was discarded, which provided serial dilutions of 1:x, 1:2, 1:4, 1:8, and final sperm concentrations of 4, 2.0, 1.0 and 0.5 ×10⁶/ml.

Polyacrylamide beads coated with monoclonal antibody (MH61) were prepared for the assay by adding 5 μl of the stock bead suspension (3 ×10⁷ beads per ml) to 95 μl BMW buffer (containing 0.3% human serum albumin) to provide a bead concentration of 1.5 ×10⁹/ml. Using a vortex mixer a 10 μl suspension of the beads (15,000 beads) was added to each well. The contents were gently mixed in the reverse order from wells 4 to 1. The tissue culture plate was placed in an incubator at 37°C in an atmosphere of 5% carbon dioxide. Attachment of beads to the sperm was noted at 0, 1, 3, 6 and 24 hours of incubation (capacitation period) by observing each of the 4 wells under an inverted phase contrast microscope (×200 magnification).

Evaluation of acrosomal status. To assess the degree of attachment of the beads to the sperm at the end of each given incubation period the tissue culture plate was removed from the incubator and placed under a phase contrast microscope (×200 magnification) and each well was scored for bead-sperm attachment. Each well was observed in 5 different fields (fig. 1). The criteria for scoring each well were the observations of bead and sperm attachment in 5 random fields, that is center, left, right, top and bottom. Scoring was modified to make the assay objective by actually counting the free beads (no sperm bound) to the total number of beads present in a given field of observation. The results for all 5 fields were combined to calculate percentage binding. A score of 1 was assigned if binding was greater than 90%. 0.5 (intermediate score) if binding was greater than 50% but less than 90%, 0 if less than 50% binding was observed. Cumulative results for each well were reported on a scale of 0 to 4 after 24 hours.

Statistical analysis. Data were analyzed for statistical differences in the Acroead score between fresh and frozen specimens. A paired Student t test was used to compare the Acroead scores in fresh and frozen samples at each time period using computer software. The results of fresh and frozen specimens at different intervals were also analyzed by McNemar’s test. A value of p ≤0.05 was considered significant.

RESULTS

An increasing number of spermatozoa are acrosomially re-acted with increasing periods of capacitation, releasing the acrosomal contents (CD46-like antigens), which in turn increases their attachment to the beads coated with anti-CD46 monoclonal antibodies. Negative and positive fields are shown in figure 2. The distribution of Acroead score showed negative agglutination in all fresh specimens at 0 hour. The number of specimens with higher scores increased as the capacitation period increased. After 1 hour of capacitation 11 of 13 specimens (84.6%) had scores of less than 2, and 2 (15.3%) had a minimum score of 2. At 3 hours 9 of 13 specimens (69.2%) had a minimum score of 2 and 30.8% had a score of greater than 3. At 6 hours 11 (84.6%) and 3 (23.1%) of 13 specimens demonstrated scores of greater than 2 and 3, respectively, and 1 had a score of 4. After 24 hours of capacitation all 13 specimens had undergone the acrosome reaction (score of greater than 2), 11 (84.6%) had a score of greater than 3 and 8 (61.5%) had a score of 4. The mean scores at different capacitation periods are given in table 1.

Compared to fresh specimens, a minimum score of 2 was noted in 8 of 13 frozen specimens (61.5%) at 1 hour of capacitation and in all specimens by 3 hours. At 24 hours 12 of 13 specimens (92.3%) had a score of 4 and 1 had a score of 3 (fig. 3). The concordance among scores was analyzed in fresh and frozen specimens (table 2 and fig. 3). No significant correlation was noted between Acroead scores and semen characteristics (count, motility, curvilinear velocity, straight line velocity, average path velocity, linearity and amplitude of lateral head displacement) after 24 hours of incubation.

DISCUSSION

In recent years treatment of infertility has focused on the male factor, and it has become increasingly clear that stan-
EVALUATION OF ACROSOME REACTION BY ACROBEAD TEST

Semen ejaculate
Washed sperm
Swim-up 37°C, 5% CO₂, 1 hr
Sperm concentration 4 X 10⁶ sperm/mL
96-Well tissue culture plate

200 µL 4 X 10⁶ sperm/mL
4 X 10⁶ sperm/mL
100 µL buffer
100 µL 100 µL 100 µL sperm sperm sperm sperm
Discard 100 µL

Dilution X 1 X 2 X 4 X 8
Add 1.5 X 10⁴ MH-61 beads (15,000 beads)
10 µL aliquot

Sperm concentration in each well
X 10⁵ sperm
Incubate at 37°C in 5% CO₂
Assess the degree of agglutination (clustering)

Single well under phase contrast (X 200)

Fig. 1. Protocol for Acrobead assay and scoring of specimen. CO₂, carbon dioxide

Fig. 2. Negative (A) and positive (B) bead sperm attachment (arrows)

dard semen analysis has limited predictive value for fertilization. When spermatozoa fail to fertilize oocytes in vitro the cause is difficult to determine but a disturbance in the acrosome reaction is a possible cause of decreased fertility potential. Thus, evaluation of the acrosome reaction is important for the diagnosis and further treatment of male infertility, characterizing capacitation status, diagnosing male subfertility and assessing sperm damage after cryopreservation. Furthermore, knowing which men with idiopathic infertility have acrosomal insufficiency will help clinicians
TABLE 1. Acrohead scores in 13 fresh and frozen semen specimens at different periods of capacitation

<table>
<thead>
<tr>
<th>Capacitation (hrs.)</th>
<th>Mean Score ± SD*</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Specimen</td>
<td>Frozen Specimen</td>
</tr>
<tr>
<td>0</td>
<td>0.04 ± 0.14</td>
<td>0.34</td>
</tr>
<tr>
<td>1</td>
<td>2.4 ± 1.60</td>
<td>0.01†</td>
</tr>
<tr>
<td>3</td>
<td>2.9 ± 0.74</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>3.7 ± 0.39</td>
<td>0.06</td>
</tr>
<tr>
<td>24</td>
<td>3.2 ± 0.28</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Graded on a scale of 0 (no bead sperm attachment) to 4 (complete attachment of bead and sperm).
† Significant, paired Student’s t test.

Figure 3. Acrohead score in fresh (□) and frozen (■) semen specimens after capacitation. Values are mean plus or minus standard deviation. Asterisk indicates significant value (p < 0.05).

TABLE 2. Distribution of Acrohead scores in 13 fresh and frozen sperm specimens

<table>
<thead>
<tr>
<th>Capacitation (hrs.)</th>
<th>0-2</th>
<th>More than 2</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Specimen</td>
<td>Frozen Specimen</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0.01*</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0.27</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0.58</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>1</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Significant by McNemar’s test.

choose appropriate treatment. The use of in vitro fertilization in these cases, without first evaluating the acrosome reaction, usually results in poor outcome.9,10 Other assisted reproductive procedures, such as intracytoplasmic sperm injection, can provide better results in these men.10,11-13 Also, characterization of the acrosome reaction may allow use of the less expensive intruterine insemination method because the longer capacitation period required may improve fertilization outcome in certain patients.

Although the sperm penetration assay, which uses zona free hamster oocytes, provides useful information to clinicians,13,19-22 its results are difficult to reproduce, there is a high false-negative rate and the assay is laborious to perform routinely at a clinical laboratory. A significant number of men with abnormal sperm penetration assay results and even some with negative results achieve pregnancy. False-positive rates of 20 to 40% (pregnancy rates) and false-negative rates of 5 to 30% have been reported with the sperm penetration assay using in vivo conception as the end point, compared to 20 to 30 and 0 to 100%, respectively, using in vitro fertilization as the end point.23-24 Sperm penetration assay results correlated poorly with in vitro fertilization particularly in male factor cases. Two long-term prospective studies on the sperm penetration assay have recently reported opposite findings,25,24 further emphasizing the controversial nature of this assay. These studies cautioned clinicians not to be unduly optimistic after a positive sperm penetration assay and that negative results should not be used to eliminate men from consideration for in vitro fertilization. The second generation sperm penetration assays are based on refrigeration of spermatozoa containing egg yolk phospholipids making the spermatozoa highly labile. On restoring the sperm to room temperature, the elevated intracellular calcium ions trigger capacitation. Although these protocols are said to provide more accurate information,25 their cumbersome nature limits the number of laboratories that use them for testing in patients. The hemizona assay, using intact human oocytes or bisected human zona pellucida, is used to evaluate the fertilizing capacity of the sperm.

However, it cannot be used for routine investigation because of the difficulty in obtaining human oocytes for testing. The Acrohead test is a new assay that evaluates the fertilizing capacity of the human spermatozoa without the need for human or other mammalian ova. This test has good reproducibility and is a good predictor of in vitro fertilization failure.9

The Acrohead agglutination test is based on the fact that human spermatozoa express the CD46 antigen after the acrosome reaction has occurred, suggesting that these spermatozoa have fertilizing capacity. The acrosome reaction occurred in 9 of our 13 fresh specimens after a capacitation period of 3 hours and in all specimens by 24 hours. Thus, a capacitation period of 6 to 24 hours could be used to identify individuals with normal sperm characteristics but a possible acrosome abnormality. Evidence of the acrosome reaction before 6 hours or no evidence of the reaction by 24 hours would indicate that the patient had an abnormality. Higher scores observed in frozen specimens after only 1 hour of capacitation indicate that freezing and thawing cause membrane stress, loss of membrane phospholipids and leakage of acrosomal contents.23 Our results have shown a significant increase in the spontaneous acrosome reaction after cryopreservation (unpublished observations) compared to fresh specimens, and suggest that there is an increase in sperm membrane damage and leakage of acrosomal contents during freezing and thawing. Sperm membrane and acrosome damage as a result of cryopreservation has also been demonstrated by others.26-29

Decrease in the longevity and functional capacity of the frozen and thawed spermatozoa has also been attributed to increased lipid peroxidation and decreased superoxide dismutase levels.28 A premature capacitation and acrosome reactions have been proposed as the primary effect of cryopreservation,28 which may be responsible for the high scores noted in our frozen specimens.

The results of the Acrohead assay were compared with a fluorescein isothiocyanate staining technique using pism sativum and peanut agglutinin in fresh and frozen specimens. The fluorescein isothiocyanate staining test scores of acrosome reacted sperm were considered as a gold standard. The Acrohead test results in normal donor specimens showed a high positive correlation with the percentage of acrosomally reacted sperm stained by the fluorescein isothiocyanate method (unpublished data).

Good reproducibility has also been reported between the percentage of acrosome reacted spermatozoa as determined by fluorescein isothiocyanate conjugated pism sativum agglutinin staining and the Acrohead score, and the scores
correlated positively with in vitro fertilization results. Clinically, the Acrobeat test is important particularly in patients with a negative (score of less than 2) Acrobeat test even after 24 hours of capacitation. These patients may be advised to undergo intracytoplasmic sperm injection rather than traditional in vitro fertilization.

CONCLUSIONS

The Acrobeat test is simple and inexpensive, and unlike the sperm penetration assay it does not require mammalian ova. The test can be performed routinely in infertile men undergoing urological treatment to evaluate the fertilizing capacity of fresh but not frozen human spermatozoa.

Jar-Chi Lee performed the statistical analysis and Karen Seifarth provided technical assistance.

REFERENCES