The use of hemizona assay in the evaluation of the optimal sperm preparation technique

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The objective of the study was to evaluate the benefit of different sperm preparation methods by using the hemizona assay. A total of 58 men admitted to the male infertility clinic for evaluation were tested by routine semen analysis and hemizona assay. Five different techniques (swim-up, TEST-yolk buffer, Percoll, pentoxifylline and progesterone) were used for preparation of sperm suspensions. The effect of these treatments on the sperm-binding capacity using the hemizona assay was assessed. The routine swim-up preparation was used as the reference method. Of the four preparation methods, only the TEST-yolk buffer and pentoxifylline exhibited an overall statistically significant improvement in sperm-binding capacity in comparison with the swim-up preparation method (P = 0.01 and 0.001 respectively). Following preparation with Percoll and progesterone there was no change in the mean value of binding capacity, compared with swim-up. However, examination of the effect of the four treatments on each specimen individually yielded a diversity in the response, e.g. having the capability to enhance, damage or be ineffective in sperm binding capacity. The results support the conclusion that in-vitro sperm preparation methods can affect sperm binding to the zona pellucida. Since there is a diversity in the response of sperm samples to different treatments, the hemizona assay can be used in selecting the optimal sperm preparation method prior to its use for assisted reproductive techniques. This is advocated mainly for the ‘male factor’ group.

Key words: hemizona assay/sperm preparation

Introduction

In recent years there has been a rapid development of methods for the improvement of sperm quality, e.g. increasing the percentage of sperm cells with progressive motility and good morphology (Berger et al., 1985; Mortimer, 1991). However, these methods have not always been associated with concomitant improvement in fertilizing capacity (Tournaye et al., 1993). In fact, processing of spermatozoa may be deleterious to the sperm cells. Aitken and Clarkson (1987) have shown that processing of spermatozoa using centrifugation may decrease fertilizing capacity.

It is difficult to determine which sperm preparation method should be used. The lack of standardization, and the heterogeneity of semen makes this decision even more difficult. Since semen analysis does not appear to accurately predict sperm fertilizing capacity (Barlow et al., 1991), it seems unlikely that this analysis will help to define the proper sperm preparation method.

Thus, the use of more sophisticated techniques for selecting an appropriate sperm treatment method, such as bioassay of sperm functions, is crucial to the evaluation of spermatozoon/oocyte interaction. The hemizona assay may be the most suitable method for detection of defects in sperm function that influence fertilization. The hemizona assay was developed as an internally controlled, homologous bioassay of spermatozoon–zona pellucida binding, with a high predictive value for fertilization outcome (Franken et al., 1993). Tight binding of spermatozoa to the zona pellucida is considered to be a prerequisite for subsequent penetration and fertilization (Bedford, 1977), and was shown to be correlated with penetration of the zona pellucida (Bedford and Kim, 1993) and with fertilization rates (Liu and Baker, 1994a,b).

The hemizona assay allows evaluation of the effect of different sperm preparation methods on sperm binding. It may thus facilitate the use of sperm preparation methods that are specifically matched to the requirement of an individual sperm sample.

Thus, the aim of the present study was to evaluate the effect of different sperm preparation methods on sperm binding capacity assessed by the hemizona assay.

Materials and methods

Patient selection

A total of 58 men admitted to the infertility clinic were evaluated by the hemizona assay. No exclusions were made in regard to the cause of infertility. However, most (90%) were classified as male factor infertility, while the rest were evaluated because of unexplained infertility.

Semen preparation

The patients delivered semen by masturbation. After liquefaction, sperm concentration and motility were measured using the Makler chamber and morphology was evaluated by strict criteria (Menkveld et al., 1990). The specimen was then divided into aliquots for different sperm preparation methods.
Experimental design

Each patient was required to provide ejaculates (mean number ± SE, 2.1 ± 0.12) with about 7 days in between each, for completion of diagnosis. The mean number of different preparation methods applied to each patient was 3.6 ± 0.13, and these were randomly performed in any given specimen. During this study, by observing the poor ability of progesterone to increase sperm-binding capacity, we decided to exclude progesterone from the remaining samples. This resulted in a relatively small number of samples (n = 24) being treated with progesterone.

Swim-up and progesterone

Semen was washed twice with medium pre-incubated with 5% CO₂ [Ham’s F-10 (Flow Laboratories, Irvine, Scotland) + 1% human serum albumin], and the pellet overlaid with 0.5 ml of medium and incubated at 37°C (in the absence of 5% CO₂) for 1 h to enable swim-up separation. After separation the samples were diluted with medium or progesterone (1 μg/ml of Proluton depot; Schering-AG, Berlin-Agis, Germany) to achieve a motile sperm concentration of 0.5×10⁶/ml.

TEST-yolk buffer treatment

Sperm samples were washed twice with Ham’s F-10 medium and the pellet was overlaid with 0.5 ml of TEST-yolk buffer (Irving Scientific, Santa Ana, CA, USA). No attempt was made to standardize sperm concentration during TEST-yolk buffer incubation. After 2 h incubation at room temperature, samples were washed twice, and the pellet was overlaid with 0.5 ml of medium and incubated at 37°C for 1 h to enable swim-up separation. The samples were diluted with medium to achieve a final motile sperm concentration of 0.5×10⁶/ml.

Percoll gradient

A two-layer discontinuous Percoll gradient was prepared by layering 1 ml aliquots of 40% upper and 80% lower isotonic Percoll (Pharmacia, Uppsala, Sweden). Up to 1 ml of the ejaculate was then layered onto the Percoll gradient and centrifuged for 20 min at 300 g. The sperm pellet was collected and resuspended in 1 ml of Ham’s F-10 medium. After another centrifugation the pellet was overlaid with 0.5 ml of Ham’s F-10 to allow swim-up separation.

Pentoxifylline

Semen was washed twice with Ham’s F-10 medium and the pellet mixed with 3 mM pentoxifylline (Sigma, St Louis, MO, USA) dissolved in Ham’s F-10. After 30 min incubation at room temperature, samples were washed twice with medium and the pellet was overlaid with 0.5 ml of medium and incubated at 37°C for 1 h to enable swim-up separation.

Control semen

A cryopreserved pool of six ejaculates from different donors served as a control sample for all the hemizona assays performed in this study (Gamzu et al., 1992).

Hemizona assay

Oocytes that failed to fertilize after 48 h in the in-vitro fertilization (IVF) programme were obtained and stored in a salt solution, following protocols described elsewhere (Yanagimachi et al., 1979). On the day of the assay, the required number of oocytes was removed from the salt solution and rinsed three times in Ham’s F-10 medium. Leitz micro-manipulators (Leica, Wetzlar, Germany) were used for cutting the oocytes as described previously (Burkman et al., 1988; Gamzu et al., 1992). The number of sperm cells that could not be removed from the matching hemizonae were counted to enable subtraction when calculation of the hemizona index was done. The matching hemizonae were separately coincubated in 50 μl droplets containing spermatozoa derived from the tested sample, and from a control sample with a motile spermatozoa concentration of 0.5×10⁶/ml. The hemizonae and spermatozoa were coincubated for 4 h at 37°C. Following the coincubation period, the hemizonae were vigorously pipetted to dislodge all loosely attached sperm cells. The number of tightly bound sperm cells was counted by the same two technicians using a phase-contrast microscope.

Two oocytes were used for each assay. The results of the hemizona assay were expressed by the hemizona index which was calculated by dividing the number of patient sperm cells by the number of control sperm cells attached to the hemizona. The final hemizona index (expressed as a percentage) was the average of the two hemizona indices. The intra- and interassay coefficients of variation were 8% and 14% respectively.

Statistical evaluation

The results are given as mean ± SE. A significant effect of a given treatment was defined using the paired t-test adjusted by Bonferroni (Fleiss, 1986). The differences between the improved and damaged subgroups of each treatment were defined by McNemar’s test (Bishop et al., 1975).

Results

A total of 58 men were studied. All semen samples studied (n = 58) were prepared by swim-up, which served as the reference method and baseline hemizona index. Sperm samples were prepared with TEST-yolk buffer (n = 51), Percoll (n = 48), pentoxifylline (n = 31), and progesterone (n = 24).

The hemizona index using swim-up preparation, as well as the hemizona index of each preparation method, is given in Table I. No difference was found between hemizona indices obtained by swim-up in the four groups. In comparison with the hemizona index obtained by swim-up separation only, TEST-yolk buffer and pentoxifylline preparation exhibited a statistically improved hemizona index (P = 0.01 and 0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Hemizona index</th>
<th>Hemizona index treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST-yolk buffer</td>
<td>51</td>
<td>25 ± 3.7</td>
<td>34 ± 4.4*</td>
</tr>
<tr>
<td>Percoll</td>
<td>48</td>
<td>24 ± 3.5</td>
<td>29 ± 4.9</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>31</td>
<td>22 ± 4.4</td>
<td>35 ± 5.7**</td>
</tr>
<tr>
<td>Progesterone</td>
<td>24</td>
<td>34 ± 7.5</td>
<td>24 ± 5.1</td>
</tr>
</tbody>
</table>

*,**Statistically different from swim-up value (P < 0.01, < 0.001 respectively).
The recent development of various sperm preparation techniques provides the clinician with new options for improvement of sperm function. These techniques include, among others, Percoll gradient, pentoxifylline, TEST-yolk buffer and progesterone treatments. The first two are currently the most popular, and are routinely used by many laboratories. Their use is supported by various studies showing the benefit of these treatments for sperm function (Berger et al., 1985; Mortimer, 1991). However, one should bear in mind that sperm preparation techniques may be harmful to certain semen samples, e.g. by damaging sperm integrity and producing free radicals (Barlow et al., 1991), or by affecting sperm binding to the zona pellucida.

Our results concur with these arguments. Although the treatments were shown to have a positive effect on sperm binding capacity in certain cases, they also were shown to have a damaging effect on sperm-binding capacity in some other specimens. Overall, only TEST-yolk buffer and pentoxifylline showed a statistically significant improvement in the hemizona index. Of the studied preparation methods, Percoll was shown to have the highest apparent potential to damage sperm-binding capacity. This may be associated with the repeated centrifugation that accompanies Percoll gradient separation. This manipulation results in higher numbers of reactive oxygen species forming in the sperm pellet, causing peroxidation of sperm plasma membrane phospholipids and increased superoxide production (Aitken and Clarkson, 1988). On the other hand the high effectiveness of pentoxifylline preparation, which exhibited a markedly improved binding capacity with a minimal apparent potential to damage sperm binding, is noteworthy.

Therefore, considering the lack of lucid indication for the use of these sperm preparation techniques, it is suggested that their effect on sperm function should be tested by the hemizona assay. A significant improvement should be determined statistically in regard to the intra-assay variation, or by clinically using the threshold hemizona index. The proper value for expressing the intra-assay variation and hemizona index threshold that discriminates between fertile and infertile zones should be determined by each laboratory. In this manner the optimal preparation method for each semen sample may be chosen, thereby improving treatment results. This is especially recommended when a tested sperm specimen exhibits an abnormal hemizona index, or after poor fertilization rates in an IVF programme.

The evidence presented in this study introduces a novel approach to the treatment of male factor infertility. Subfertile semen samples, which are often described as oligoteratoasthenozoospermic, are not to be considered as a homogeneous group in regard to aetiology and treatment. The pathogenesis of this condition varies, and each semen sample must be considered as a distinct entity. Therefore the methods for evaluation and treatment should be adjusted to each sample after intense analysis, and the indiscriminate use of a single sperm preparation method is to be avoided.

References
Aitken, R.J. and Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive

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### Table II. The effect of sperm preparation methods on the fertile status of the sperm specimens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Improved (%)</th>
<th>Unchanged (%)</th>
<th>Damaged (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST-yolk buffer</td>
<td>37 (73)*</td>
<td>0 (0)</td>
<td>14 (27)</td>
</tr>
<tr>
<td>Percoll</td>
<td>22 (46)</td>
<td>8 (16)</td>
<td>18 (38)</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>21 (68)*</td>
<td>5 (16)</td>
<td>16 (56)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>12 (50)</td>
<td>6 (25)</td>
<td>6 (25)</td>
</tr>
</tbody>
</table>

*Improved = increase of >16% in the hemizona index following the treatment compared with swim-up.

### Table III. The effect of sperm preparation methods on the fertility status of the sperm specimen, as judged by a threshold hemizona index of 23%

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Improved (%)</th>
<th>Unchanged (%)</th>
<th>Damaged (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST-yolk buffer</td>
<td>7 (14)*</td>
<td>41 (80)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Percoll</td>
<td>5 (10)</td>
<td>36 (75)</td>
<td>7 (15)</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>9 (29)*</td>
<td>17 (53)</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3 (13)</td>
<td>21 (87)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Improved = increase in the hemizona index over the 23% threshold following the treatment compared with swim-up.

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Of sperm function. These techniques include, among others, Percoll gradient, pentoxifylline, TEST-yolk buffer and progesterone treatments. The first two are currently the most popular, and are routinely used by many laboratories. Their use is supported by various studies showing the benefit of these treatments for sperm function (Berger et al., 1985; Mortimer, 1991). However, one should bear in mind that sperm preparation techniques may be harmful to certain semen samples, e.g. by damaging sperm integrity and producing free radicals (Barlow et al., 1991), or by affecting sperm binding to the zona pellucida.

Discussion
The recent development of various sperm preparation techniques provides the clinician with new options for improvement respectively). The other preparation methods, Percoll and progesterone, did not result in an overall improved sperm-binding capacity.

The results were further analysed by inspecting the effect of each treatment on the sperm specimens individually. A change in the hemizona index greater than twice the intra-assay coefficient of variation (which is above the interassay coefficient of variation) following the treatment was considered significant. Accordingly, the samples treated were defined as improved, unchanged and those that were damaged following each preparation method (Table II). Again only TEST-yolk buffer and pentoxifylline were shown to have a significant effect on improvement of hemizona index. The samples were further classified using a threshold hemizona index of 23%, which was shown to be the cut-off point between fertile (hemizona index ≥23%) and infertile (hemizona index <23%) sperm samples in our laboratory (Gamzu et al., 1994). The purpose of this analysis was to give the clinical significance of each treatment (Table III). The analysis confirmed that both TEST-yolk buffer and pentoxifylline significantly improved sperm binding. Variables of the raw semen had no influence on the response to each treatment (data not shown).

<table>
<thead>
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*Improved = increase of >16% in the hemizona index following the treatment compared with swim-up.

*Unchanged = change of <16% in the hemizona index following the treatment compared with swim-up.

*Damaged = decrease of >16% in the hemizona index following the treatment compared with swim-up.

**Statistically significant (P < 0.05).**

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