Pre-freezing sperm preparation does not impair thawed spermatozoa binding to the zona pellucida

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The present study was conducted to assess the fertilizing potential of frozen–thawed spermatozoa, which were cryopreserved after separation on a Percoll gradient, or washed out of seminal plasma. For this purpose, binding to the zona pellucida and other characteristics of the treated sperm cells were compared with those of cryopreserved spermatozoa from the same original sample which were not manipulated before freezing. Semen specimens were obtained from 80 candidates for sperm donation. Percoll-treated sperm samples compared with the sibling, unprocessed controls had significantly higher values of sperm motility characteristics and percent of cells with normal morphology after freezing and thawing. Sperm binding ability to the zona pellucida was not statistically different (109 ± 8.1% and 94 ± 6.7% in unprocessed and Percoll-treated samples respectively). Sperm specimens processed by washing had significantly higher values for motility characteristics than untreated sibling samples, but no differences were found between the treated and untreated samples for morphology and binding to the zona pellucida (hemizona index of 75 ± 7.0% and 76 ± 6.7% in unprocessed and washed samples respectively). These findings suggest that, judged by the binding assay, the aforementioned pre-freezing separation processes have no adverse effect upon the fertilizing potential of the thawed sperm cells. These procedures make it possible to optimize the progressive motile sperm cell concentration of the frozen specimen, which facilitates the storage of samples with good quality, even when the features of the original semen are sub-optimal.

Key words: cryopreservation/hemizona assay/spermatozoa preparation

Introduction

Cryopreservation of human semen is a routine technique for storing donor or husband semen prior to artificial insemination or assisted reproduction techniques. Semen may be frozen before cancer therapy, vasectomy, or as a method that makes the required delay possible to ensure human immune deficiency virus-free sperm specimens prior to insemination of a recipient.

Frequently, particularly in men with a malignant disease, the quality of the semen sample for cryopreservation is poor in terms of concentration, motility and morphology (Botchan et al., 1997a, 1997b). Under such circumstances, the prospect for an acceptable quality of the frozen–thawed specimen is low. In view of the possibility that sperm separation after thawing may be impaired (Graczykowski and Siegel, 1991), it was suggested that the sample be treated prior to the freezing procedure, in order to select and enrich the specimen with higher-quality sperm cells (Barthelemay et al., 1990). However, the contribution of the treatment before freezing is not obvious because sperm manipulation may damage the cells and reduce their cryo-freezability and fertility potential.

This study was, therefore, conducted to assess the fertility potential of frozen–thawed sperm cells which had been cryopreserved after separation on Percoll gradients, or washed out of seminal plasma in comparison with that of the traditional method. This was evaluated using conventional sperm parameters combined with the zona pellucida binding assay, which was found to be an important predictor of fertilizing potential (Liu and Baker, 1992; Gamzu et al., 1994).

Materials and methods

Semen collection

Semen was obtained from 80 candidates for sperm donation. Each ejaculate was allowed to liquefy for at least 30 min at 37°C. A sample was taken to evaluate sperm concentration (by Makler chamber), percentage of motile spermatozoa and the degree of motility, graded from 1 (poor) to 4 (excellent, forward-moving spermatozoa). Normal morphology was assessed by strict criteria (Menkveld and Kruger, 1995). Each semen specimen was divided into two fractions: the first was cryopreserved without processing; and the second was manipulated, either by Percoll gradient (n = 52) or by washing out of seminal plasma (n = 28) before cryopreservation. The sperm washing method was adopted when permission to use Percoll was withdrawn.

Percoll separation procedure

The column 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 on the Percoll gradient and centrifuged for 20 min at 300 g. The sperm pellet was collected and re-suspended in 1 ml of human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 1% human serum albumin (Kamapharm Human Albumin; Kamada, Kibbutz Beit Kama, Israel). After centrifugation, the pellet was resuspended in 0.3 ml of HTF medium.
Washing procedure
This was performed by diluting 1 ml semen with HTF medium (1:2 v/v) and centrifuging at 300 g for 10 min to separate the spermatozoa from seminal plasma. The pellet was then suspended in 1 ml HTF medium, centrifuged once more, and resuspended in 0.3 ml of HTF medium.

Freezing procedure
The specimen was carefully diluted by the addition of droplets in equal volume to the freezing medium test yolk buffer (Irvine Scientific, Santa Ana, CA, USA). After dilution, the mixture was equilibrated for 15 min at room temperature, then sealed in 0.5 ml straws (L.M.V., Paris, France) and cooled in a semi-programmable freezer (Nicool, LM-10; Air Liquid, Paris, France). The straws were cooled from room temperature to –6°C, 1.7°C/min, then to –100°C, 5°C/min. The straws were then transferred directly to liquid nitrogen (–196°C) for storage (Yavetz et al., 1991).

Thawing procedure
The straws were allowed to thaw at 37°C for 5 min and assessed immediately for sperm parameters and binding capacity by the hemizona assay (HZA).

Hemizona assay: Thawed samples were washed twice with HTF medium and the pellet was overlaid with 0.5 ml medium and incubated at 37°C for 1 h to facilitate swim-up separation. After separation, the samples were diluted with the medium to achieve a motile sperm concentration of 500,000 cells/ml. The control sample for all the HZA performed in this study consisted of a cryopreserved pool of 12 ejaculates from different fertile donors, selected by virtue of good freezability. The pooled sample (31 ml), with 124×10⁶ spermatozoa/ml and 51% motile spermatozoa, was diluted 1:1 with freezing medium and transferred into 0.5-ml straws for freezing. The straws contained 55×10⁶ spermatozoa/ml and 40% motile sperm cells after thawing. Before the HZA was performed, a straw was thawed and the sperm cells were washed and prepared by swim-up separation as described previously.

Unfertilized oocytes from failed cycles were stored in a salt solution, as described elsewhere (Yanagimachi et al., 1979). On the day of the assay, the oocytes were removed from the salt solution and rinsed three times in HTF medium. Leitz micromanipulators (Leica, Wetzl, 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 hemizonas was counted to enable subtraction when the hemizona index (HZI) was calculated. The matching hemizonas were separately co-incubated in 50-µ1 droplets containing spermatozoa derived from the tested sample, and from the control sample with a motile sperm concentration of 0.5×10⁶/ml. The hemizona and spermatozoa were co-incubated for 4 h at 37°C. After the co-incubation period, the hemizonas were pipetted vigorously to dislodge all loosely attached sperm cells. The same two technicians counted the number of tightly bound sperm cells. Two oocytes were used for each assay. The results of the HZA were expressed by the HZI, which was calculated by dividing the number of those tested by the number of control sperm cells attached to the hemizonas. The final HZI, expressed as a percentage, was the average of the two hemizona indices. The intra- and inter-assay coefficients of variation were 8% and 14% respectively.

Statistical evaluation
The results are given as mean ± SE. A significant difference was defined using Student’s t-test and paired t-test, as appropriate.

Results
Sperm characteristics before cryopreservation were in the normal range for both groups, i.e. Percoll-treated (n = 52) and washed (n = 28) specimens. As was expected, no significant difference was found between the groups, which were established arbitrarily (Table I).

After thawing, no significant differences were found in sperm characteristics between the two groups of unprocessed semen, which served as controls for the Percoll gradient and washing procedures, except for the HZI which was higher in the Percoll-treated group (P = 0.03). The values of 109.1 ± 8.05% and 74.5 ± 7.01% (for Percoll gradient and washed groups respectively) were different, although both groups were treated in the same manner. However, sperm selection on the Percoll gradient yielded significantly higher values than the washed specimens for all parameters (P < 0.001 for concentration of progressive motile spermatozoa, per cent of motility and degree of motility; P < 0.01 for normal morphology) except for the HZI that was not affected by the treatment (P = 0.1, Table II). In the Percoll-treated group, the recovery of progressive motile spermatozoa was 55% higher than the washed samples (P < 0.01; Table II).

After freezing–thawing procedures, there was a significant increase in the concentration of cells with progressive motility, per cent and degree of motility and per cent of cells with normal morphology in the Percoll-treated samples, when compared with that of the sibling, unprocessed controls. No significant difference was seen in the HZI (Table II). In the washed sperm group, the processed specimens had significantly higher concentrations of progressive motile spermatozoa and per cent and degree of motility than the sibling, untreated samples. No differences were found in the percentage of normal morphology and the HZI between the treated and untreated samples (Table II).

Discussion
Cryopreservation of human semen is extremely important to the field of male infertility. However, there is dissension regarding the best cryopreservation protocol for human semen (Cohen et al., 1981; Mahadevan and Trounson, 1984a; Prins and Weidel, 1986). Many stages along the freezing process may impair the post-thawing sperm cell quality. The processes evaluated in the present study involved the removal of the seminal plasma, sperm separation and the addition of the cryoprotective medium. Previous studies confirmed the decrease in the number of sperm cells with intact plasma membrane (swollen or disrupted), after the addition of cryoprotective medium (Mahadevan and Trounson, 1984b). It appeared that the cryoprotective medium had a major deleterious effect on spermatozoa in the entire cryopreservation process (Barthelemy et al., 1990).

Another study failed to establish any significant difference when the dilution with the cryoprotective medium was carried out with the seminal plasma, or after its removal. Likewise, removal of seminal plasma, which requires centrifugation, had no striking effect on the status of the acrosome. On the contrary, less acrosomal injury was observed when the spermatozoa were...
Washed (n observed by Kobayashi that of unselected spermatozoa. The same phenomenon was motility after the freezing–thawing process, compared with selected by SpermPrep columns showed a slower decline of Statistically different from the corresponding unprocessed semen values (a results of each procedure are shown in comparison with the post-thaw unprocessed specimen 116 samples. However, it should be borne in mind that, in poor Percoll-treated samples when compared with that of the washed high recovery of progressive motile spermatozoa in the and Gagnon, 1992). These phenomena may be the basis for capacity of the frozen spermatozoa.

As far as the separation technique before freezing was concerned, Zavos et al. (1991) demonstrated that spermatozoa selected by SpermPrep columns showed a slower decline of motility after the freezing–thawing process, compared with that of unselected spermatozoa. The same phenomenon was observed by Kobayashi et al. (1991) who used a Percoll gradient. Percoll-selected spermatozoa before freezing retained greater longevity than raw samples (Sharma and Agarwal, 1996). Notwithstanding, spermatozoa selected by swim-up were as susceptible to the stress caused by freezing and thawing as unselected spermatozoa in the original semen sample (Perez-Sanchez et al., 1994).

It has been suggested that sperm processing alone can damage spermatozoa because of reactive oxygen species (ROS) production (Aitken and Clarkson, 1988). In this regard, Percoll-gradient centrifugation selected spermatozoa with a better functional competence than swim-up from a washed pellet, due to enhanced protection against peroxidative damage caused by the centrifugation process (Griveau and Lannou, 1994). Moreover, sperm washing by repeated centrifugation and resuspension increased the level of ROS by 20–50-fold, when compared with that of the original sample, but centrifugation without removing the seminal plasma reduced the levels of ROS, suggesting a protective role of seminal plasma (Iwasaki and Gagnon, 1992). These phenomena may be the basis for the high recovery of progressive motile spermatozoa in the Percoll-treated samples when compared with that of the washed samples. However, it should be borne in mind that, in poor specimens, the selective feature of the Percoll gradient decreases the yield of the number of progressive motile spermatozoa (Ng et al., 1992). Despite these data, the present study demonstrated that, with regard to zona pellucida binding, no difference was found between the two sperm preparation techniques, both of which did not change the zona pellucida binding ability when compared with that of unprocessed specimens.

Although it is acceptable that freezing and thawing are associated with a decrease in sperm motility and viability, several functional tests, such as the bovine mucus penetration, zona-free hamster egg penetration, hypo-osmotic swelling test and triple-stain technique for acrosome reaction, demonstrated that frozen spermatozoa maintained their fertilizability (Yoshida et al., 1990). In this regard, we reported previously that spermatozoa manipulated before freezing and thawing as unselected spermatozoa in the original semen sample (Perez-Sanchez et al., 1994). Statistically different from the corresponding unprocessed semen values (aP < 0.001, bP < 0.01 respectively).

Table I. Sperm characteristics before preparation and cryopreservation of the two groups (Percoll-treated and washed sperm samples)

| Treatment method | Volume (ml) | Concentration (× 10⁶/ml) | Motility (%) 1h | Degree of motility (1–4) 1h | Motility (%) 4h | Degree of motility (1–4) 4h | Normal morphology (%) | a Morphology is based on strict criteria. n = number of specimens.
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<tbody>
<tr>
<td>Percoll (n = 52)</td>
<td>3.4 ± 0.13</td>
<td>113.9 ± 5.15</td>
<td>51.9 ± 0.44</td>
<td>3.1 ± 0.02</td>
<td>36.9 ± 0.90</td>
<td>2.5 ± 0.04</td>
<td>15.5 ± 0.42</td>
</tr>
<tr>
<td>Washed (n = 28)</td>
<td>3.2 ± 0.25</td>
<td>110.9 ± 6.89</td>
<td>53.3 ± 0.54</td>
<td>3.0 ± 0.02</td>
<td>38.1 ± 1.48</td>
<td>2.4 ± 0.07</td>
<td>14.4 ± 0.50</td>
</tr>
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Table II. Post-thaw parameters of spermatozoa cryopreserved after separation on Percoll gradient (group 1) or washed out of seminal plasma (group 2). The results of each procedure are shown in comparison with the post-thaw unprocessed specimen

<table>
<thead>
<tr>
<th>Group size</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 1</th>
<th>Group 2</th>
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<tbody>
<tr>
<td>Progressive motile sperm concentration (× 10⁶/ml)</td>
<td>6.6 ± 0.50</td>
<td>18.5 ± 1.60</td>
<td>5.4 ± 0.49</td>
<td>10.1 ± 0.65</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>30.5 ± 1.28</td>
<td>44.8 ± 1.05</td>
<td>29.0 ± 1.29</td>
<td>36.2 ± 1.12</td>
</tr>
<tr>
<td>Degree of motility (1–4)</td>
<td>2.9 ± 0.03</td>
<td>3.1 ± 0.02</td>
<td>2.9 ± 0.02</td>
<td>3.0 ± 0.00</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>15.5 ± 0.44</td>
<td>16.9 ± 0.49</td>
<td>14.3 ± 0.49</td>
<td>14.5 ± 0.48</td>
</tr>
<tr>
<td>HZI (%)</td>
<td>109.1 ± 8.05</td>
<td>94.7 ± 6.66</td>
<td>74.5 ± 7.01</td>
<td>75.7 ± 6.70</td>
</tr>
<tr>
<td>Yield of progressive motile spermatozoa (%) a</td>
<td>21.6 ± 1.41</td>
<td>17.7 ± 1.39</td>
<td>19.6 ± 2.27</td>
<td>11.4 ± 1.19</td>
</tr>
</tbody>
</table>

Results are mean ± SE.

References


Received on March 26, 1998; accepted on September 24, 1998

Binding potency in pretreated thawed spermatozoa