Comparison of Three Sperm Preparation Media

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ABSTRACT:
Objective-In assisted reproduction, spermatozoa must be effectively separated from seminal plasma to undergo capacitation, a prerequisite for fertilization. Percoll was recently withdrawn from the American market because of safety concerns. The purpose of this study was to evaluate the quality of sperm separated by two different sperm washing media, and compare these results to sperm quality after separation on a Percoll (PerWash™) gradient.

Materials and Methods-Semen samples from 10 normozoospermic men were compared after separation on three media: Perwash™, a Percoll-type medium (Conception Technologies, La Jolla, CA), ISolate™ (Irvine Scientific, Santa Ana, CA), and SpermFertil™ (Embryotech Laboratories, Wilmington, MA). Semen characteristics examined were: sperm count, percentage motility, curvilinear velocity, lateral head displacement, percentage recovery of motile sperm, viability, hypo-osmotic swelling, and penetration in bovine cervical mucus. Sperm morphology was scored using World Health Organization and Kruger's strict criteria. The motility of the sperm was examined at one-hour intervals for three hours to determine which method produced samples with the longest period of motility.

Results-Total motile sperm count, motility, curvilinear velocity, and percentage of normal morphological forms as determined by the WHO method were significantly lower in specimens prepared by Sperm Fertil than in Isolate or Perwash specimens (P < .05). Tail abnormalities were significantly more frequent in specimens prepared by Sperm Fertil than in Isolate and Perwash specimens (P < .001). Percentage recovery of motile sperm was significantly higher in Isolate and Perwash specimens than in Sperm Fertil specimens (P < .05). Semen characteristics were similar in specimens prepared with Isolate and with Perwash. At all time intervals, sperm motility was higher in Isolate and Perwash specimens than in Sperm Fertil specimens (P < .001).

Conclusion-Sperm samples separated with a Sperm Fertil column have poorer sperm quality in several respects than samples prepared with Isolate or Perwash. This finding and the similarity between Isolate and Percoll procedures suggests that Isolate is a good alternative to Percoll-type media to prepare sperm for assisted reproduction. Int J Fertil 44(3):163-167, 1999

KEY WORDS: spermatozoa, Percoll, Isolate, Sperm Fertil, motility, hypo-osmotic swelling, viability

INTRODUCTION
The Need For Effective Sperm preparation methods has increased with the increased use of assisted reproductive techniques. Many methods have been developed to select spermatozoa, including self-migration, swim-up, centrifugation through albumin, Ficoll, Nycodenz, sephadex, glass wool, and sperm-prep columns [1-5].

This paper was presented at the 23rd Annual Meeting of the American Society of Andrology, Long Beach, California, March 26-29, 1998 (Abstract #110).
The recent withdrawal of the most widely used product, Percoll (Perwash™, Pharmacia, Upsala, Sweden), from the clinical market in late 1996 because of safety concerns has left clinicians searching for an effective substitute. Percoll, a sterile colloidal solution of silica particles covered by polyvinylpyrrolidone, produced high yields of motile sperm, did not appear to harm the cells, and was associated with good results in assisted reproduction (1,2,6-8]. However, Pharmacia took Percoll off the market after stating that unspecified toxins in the product might contaminate gametes or embryos during assisted reproduction. Although a recent study using mouse embryo bioassay found no evidence of endotoxin contamination in Percoll [9], the product remains unavailable for use. In addition, the U.S. Food and Drug Administration never certified Percoll for in vivo human use. Therefore, there is an urgent need to find sperm preparation methods than can provide samples of highly motile, morphologically normal sperm with minimal levels of contaminants.

In this study, we tested the quality of sperm separated by two different sperm washing media (Isolate™ and Sperm Fertil™), and compared these results to sperm quality after separation on a Percoll "Perwash" gradient. Isolate, is a sterile colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in a HEPES-buffered human tubal fluid medium. It is a two-layer gradient system like Percoll, which separates motile sperm from the seminal plasma by density centrifugation. Sperm Fertil, on the other hand, is a glass wool filtration column which separates motile spermatozoa by the filtration effect of the glass wool fibers. Sperm samples prepared by the three methods were tested in terms of motility, function, and morphology.

MATERIALS AND METHODS

Sperm Preparation

Semen samples from normal healthy donors (n = 10) were collected in sterile specimen jars by masturbation after 48 to 72 hours of sexual abstinence. The criteria for donor selection was based on the World Health Organization (WHO) guidelines [10]. Specimens were allowed to liquefy at 37°C for 30 minutes before semen analysis and sperm preparation.

Preparation and Use of Sperm Separation Media

Perwash (Enhance-S, Conception Technologies, La Jolla, CA), and Isolate (Irvine Scientific, Santa Ana, CA) gradients were prepared according to the manufacturers' instructions. The gradient consisted of a 47% upper phase and a 90% lower phase reconstituted with HEPES-buffered Ham's F-10 medium from a 100% stock solution. After equilibration at 37°C in an incubator for 20 minutes, 2 mL of the lower phase was transferred into a sterile 15-mL conical plastic disposable centrifuge tube and 2 mL of the upper phase was layered on top of it. The Sperm Fertil (Embryotech Laboratories, Wilmington, MA) glass wool column required no preparation.

Liquefied semen (0.5 to 1.0 mL) was layered on the upper phase and centrifuged at 500 g for 20 minutes at room temperature. The pellet was resuspended in 2 to 3 mL of human tubal fluid (HTF) medium and centrifuged again at 500 g for 7 minutes. The supernatant was removed and the final sperm pellet resuspended in HTF. Following the sperm washing, each sample was immediately (0 min) evaluated for sperm motion characteristics, sperm concentration (millions/mL), total motile sperm (millions), percentage of motile spermatozoa recovered, morphology, and functional characteristics (sperm viability, hypoosmotic swelling test, and bovine cervical mucus penetration test). Portions of each of the ten sperm samples were processed by the three techniques under investigation.

Analysis of Sperm Motility

Sperm samples were analyzed with the help of a computer-assisted motion analyzer (Cell-Trak, Model VP 110, Santa Rosa, CA). For each measurement, a 5-µL aliquot was loaded on a counting chamber (Micro Cell slide, Conception Technologies, La Jolla, CA) and analyzed for percentage motility, curvilinear velocity (total distance traveled by a given spermatozoon divided by the total time elapsed), linearity (departure of sperm track from a
straight line), and amplitude of lateral head displacement (mean width of sperm head oscillation). These sperm motion characteristics were measured immediately after sample preparation. In addition, percentage of motile sperm was measured in samples incubated for 60, 120, and 180 minutes. Overall motility was calculated by taking an average of the three motility measurements.

**Sperm Viability and Morphology**

The percentage of viable sperm in each aliquot was assessed after sperm separation. Each sample was mixed with an equal amount of 0.05% eosin-Y nigrosin to improve contrast. Dead sperm appear pink; unstained sperm were counted as viable. In each sample, two counts of 100 sperm were completed and the two figures averaged for each sample. For morphological evaluation, seminal smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL). Sperm morphology was assessed by World Health Organization and Kruger's strict criteria [10,11]. These sperm viability and morphology measurements were made immediately after sperm preparation.

**Hypo-osmotic Swelling**

One milliliter of hypo-osmotic solution (150 mOsm/L; 0.025 mM sodium citrate and 0.075 mM D[-] fructose) was added to 0.1 mL sperm suspension obtained after each sperm separation. At least 200 spermatozoa per sample were examined after incubation at 37°C for 60 minutes using a phase-contrast microscope (Model BH 2, Olympus, Tokyo, Japan), and the percentage of sperm with intact membranes was calculated [12].

**Bovine Cervical Mucus Penetration**

The bovine cervical mucus penetration test was performed using the Penetrak kit (Serono Diagnostics, Allentown, PA). For each sample, two capillary tubes were thawed at room temperature for 30 minutes and snapped at the red score mark above the mucus meniscus. The cut end was placed in a plastic beaker containing 200 µL of sperm suspension and left at room temperature for 90 minutes. The capillary tubes were then placed on a calibrated slide and examined by phase-contrast microscopy. The distance (in millimeters) covered by the vanguard sperm was measured.

**Statistical Analysis**

A two-way analysis of variance (ANOVA) was used to compare the three sperm separation methods using a SAS statistical software package, version 6.12 (SAS Institute, Cary, NC). Tukey's multiple range test was used to perform multiple comparisons when analysis of variance indicated significant differences among the different groups. A P value of 0.05 was considered statistically significant.

**RESULTS**

Specimens separated on Isolate and on Perwash had similar sperm motion characteristics, except that motility was slightly lower in the Isolate samples. All sperm motion characteristics except linearity and amplitude of lateral head displacement were significantly lower in specimens prepared by Sperm Fertil than in specimens prepared with either Isolate or Perwash. An "overall motility" measure calculated from all time periods showed that the Sperm Fertil sample had a significantly smaller percentage of total motile sperm at all incubation periods (Table I; Figure 1).

The three separation methods produced samples with similar viability, hypo-osmotic swelling, and ability to penetrate bovine cervical mucus (Table II). However, Sperm Fertil samples had significantly more sperm with tail abnormalities and significantly fewer sperm with normal morphology under WHO criteria.
### TABLE I
Sperm motion and counts in samples separated with three preparation media.*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Perwash&lt;sup&gt;TM&lt;/sup&gt;</th>
<th>Isolate&lt;sup&gt;TM&lt;/sup&gt;</th>
<th>P Value</th>
<th>Sperm Fertil</th>
<th>P Value&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>P Value&lt;sup&gt;¶&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (10&lt;sup&gt;6&lt;/sup&gt; mL)</td>
<td>26.47 ± 10.18</td>
<td>26.54 ± 10.18</td>
<td>1</td>
<td>22.14 ± 10.18</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Total motile sperm (10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>24.34 ± 7.19</td>
<td>24.64 ± 7.19</td>
<td>0.97</td>
<td>18.05 ± 7.19</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Sperm recovery (%)</td>
<td>39.56 ± 3.25</td>
<td>37.07 ± 3.25</td>
<td>0.85</td>
<td>25.68 ± 3.25</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Curvilinear velocity (µm/sec)</td>
<td>67.81 ± 2.14</td>
<td>65.05 ± 2.13</td>
<td>0.8</td>
<td>56.23 ± 2.14</td>
<td>0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>Linearity (%)</td>
<td>60.60 ± 1.69</td>
<td>60.60 ± 1.69</td>
<td>1</td>
<td>58.30 ± 1.69</td>
<td>0.77</td>
<td>0.77</td>
</tr>
<tr>
<td>Amplitude of lateral head displacement (µm/sec)</td>
<td>4.62 ± 0.17</td>
<td>4.46 ± 0.17</td>
<td>0.91</td>
<td>4.73 ± 0.17</td>
<td>0.97</td>
<td>0.67</td>
</tr>
<tr>
<td>Overall motility</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>84.30 ± 2.04</td>
<td>83.70 ± 2.04</td>
<td>1</td>
<td>73.90 ± 2.04</td>
<td>0.006</td>
<td>0.01</td>
</tr>
<tr>
<td>60 min</td>
<td>81.40 ± 1.96</td>
<td>78.10 ± 1.96</td>
<td>0.48</td>
<td>71.70 ± 1.96</td>
<td>0.007</td>
<td>0.08</td>
</tr>
<tr>
<td>120 min</td>
<td>80.00 ± 2.24</td>
<td>76.40 ± 2.24</td>
<td>0.51</td>
<td>69.10 ± 2.24</td>
<td>0.008</td>
<td>0.08</td>
</tr>
<tr>
<td>180 min</td>
<td>77.10 ± 1.87</td>
<td>70.50 ± 1.87</td>
<td>0.06</td>
<td>65.70 ± 1.87</td>
<td>0.001</td>
<td>0.19</td>
</tr>
<tr>
<td>Overall motility</td>
<td>79.50 ± 1.07</td>
<td>75.00 ± 1.07</td>
<td>0.01</td>
<td>68.83 ± 1.07</td>
<td>0.0001</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*Pairwise comparisons (three pairs) of all three media using Tukey’s procedure for multiple comparisons

† Comparison between Perwash and Isolate

‡ Comparison between Perwash and Sperm Fertil

¶ Comparison between Isolate and Sperm Fertil

Values are mean ± S.E.; P < .05 was considered significant.

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### TABLE II
Sperm function and morphology in samples separated with three preparation media.*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Perwash&lt;sup&gt;TM&lt;/sup&gt;</th>
<th>Isolate&lt;sup&gt;TM&lt;/sup&gt;</th>
<th>P Value</th>
<th>Sperm Fertil</th>
<th>P Value&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>P Value&lt;sup&gt;¶&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail abnormalities (%)</td>
<td>7.20 ± 1.37</td>
<td>10.60 ± 1.37</td>
<td>0.31</td>
<td>19.60 ± 1.37</td>
<td>0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>WHO:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal forms (%)</td>
<td>41.20 ± 1.91</td>
<td>40.80 ± 1.91</td>
<td>1</td>
<td>31.40 ± 1.91</td>
<td>0.006</td>
<td>0.009</td>
</tr>
<tr>
<td>Amorphous forms (%)</td>
<td>49.20 ± 1.77</td>
<td>47.20 ± 1.77</td>
<td>0.85</td>
<td>47.50 ± 1.77</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>35.60 ± 3.93</td>
<td>31.40 ± 3.93</td>
<td>0.87</td>
<td>42.20 ± 3.93</td>
<td>0.64</td>
<td>0.23</td>
</tr>
<tr>
<td>BCMP (mm)</td>
<td>32.40 ± 2.26</td>
<td>37.00 ± 2.26</td>
<td>0.49</td>
<td>33.00 ± 2.26</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>HOS ( % )</td>
<td>93.60 ± 1.79</td>
<td>92.00 ± 1.79</td>
<td>0.92</td>
<td>96.20 ± 1.79</td>
<td>0.74</td>
<td>0.36</td>
</tr>
<tr>
<td>Sperm morphology</td>
<td>13.10 ± 1.11</td>
<td>13.20 ± 1.11</td>
<td>1</td>
<td>9.50 ± 1.11</td>
<td>0.13</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Pairwise comparisons of all three media using Tukey’s procedure for multiple comparisons

BCMP = Bovine cervical mucus penetration; HOS = Hypo-osmotic swelling; WHO = World Health Organization

† Comparison between Perwash and Isolate

‡ Comparison between Perwash and Sperm Fertil

¶ Comparison between Isolate and Sperm Fertil

Values are mean ± S.E.; P < .05 was considered significant.

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**FIG. 1.** Effect of incubation time on the percentage of motile sperm in samples separated on three preparation media.
DISCUSSION

Poor sperm quality is a common problem in male infertility. Good sperm preparation techniques can improve sperm quality by selecting high percentages of motile and morphologically normal spermatozoa.

The removal of Percoll from the clinical market has given impetus to other companies to develop superior products for sperm separation. Our results show that Perwash (Percoll) produced the highest quality sperm samples, and that Isolate sperm samples were nearly as high in quality by most measures. Isolate would be a good alternative to Percoll-type media in assisted reproduction. Similar results were also obtained by two recent studies [13,14], which compared the sperm characteristics of specimens prepared using Percoll and Isolate and found that Isolate was an acceptable substitute for Percoll. However, Sperm Fertil, a glass-wool separation column, produced sperm samples that were lower in quality by several measures. Sperm Fertil samples had lower total motile sperm counts, percentage of motile sperm, curvilinear velocity, percentage of normal forms, and percentage recovery of motile sperm. Sperm Fertil samples also had greater numbers of sperm with tail abnormalities.

In conclusion, our study demonstrates that sperm separation by Isolate is a good alternative to Percoll-type media in the preparation of sperm for assisted reproduction.

REFERENCES