Comparison of Single- and Two-Layer Percoll Separation for Selection of Motile Spermatozoa

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ABSTRACT: Objective—Sperm recovery using a single-layer Percoll procedure is significantly better than using the swim-up technique for infertile men and patients with normal sperm characteristics, however, in normal men results have been contradictory. Some studies have shown further improvement in semen quality with multiple layers. Therefore, this study compared the effect of single-layer and two-layer Percoll procedures on sperm characteristics of normozoospermic men. Methods—Semen specimens from 10 normal donors were processed by layering 1 mL of the liquefied ejaculate on a single layer of 80% Percoll or on a two-layer (47% and 90%) Percoll gradient. Computer-assisted semen analysis was done to examine total motile sperm, percentage of recovery of motile cells, percent motility, curvilinear velocity, linearity, and amplitude of lateral head displacement. Each specimen was evaluated by the hypo-osmotic swelling (HOS) test, bovine cervical mucus penetration test, viability (eosin-nigrosin stain), and sperm morphology (WHO and Kruger's strict criteria). Results—Specimens processed with the two-layer Percoll procedure had significantly better recovery of spermatozoa, and significantly better percentage motility, linearity, amplitude of lateral head displacement, percentage tail swelling, and percentage viability than those separated on single-layer Percoll. Results for sperm morphology using WHO and Kruger's criteria were similar between the two methods (P = 0.92 for both sets of criteria). Conclusions—in normozoospermic men, the two-layer Percoll separation procedure significantly improves semen characteristics compared with separation on a single layer. Int J Fertil 42(6):412–417, 1997

KEY WORDS: spermatozoa, Percoll separation, viability, hypoosmotic swelling, morphology

INTRODUCTION

With the increased use of assisted reproductive techniques, the need for suitable and effective sperm preparation methods has gained significance. Separating spermatozoa from the seminal plasma is important to permit capacitation, a fundamental requirement for successful assisted reproductive technology. Separation requires the efficient removal of seminal plasma, plus contaminants (dead cells, immature germ cells, white blood cells, and bacteria) that may inhibit fertilization. Further, recovering a large number of motile sperm is critical for achieving pregnancy. Different methods have been developed for selecting spermatozoa, including self-migration, swim-up, centrifugation through albumin, Ficoll, Nycodenz, sephadex, glass wool, and sperm-prep columns [1–7].

Percoll is a sterile colloidal solution of silica particles covered by polyvinylpyrrolidone. It does not harm the cells and appears to be clinically useful when applied in assisted reproductive procedures [8–10]. Recently, the use of Percoll gradient in the
separation of human spermatozoa for assisted reproductive procedures has raised some safety concerns. Percoll manufactured by Pharmacia [Uppsala, Sweden] is not certified for in vivo use in humans and is not tested by the company or the U.S. Food and Drug Administration for the presence of suspected endotoxins. However, a recent study found no evidence of endotoxin contamination in Percoll by mouse embryo biosays [11].

Percoll can result in recovery of motile spermatozoa free of debris in the lower layer, which contains 85% to 100% Percoll. However, several variations of this technique have been reported [1,12–15]. Some studies have used different number of gradients, volumes, and Percoll concentrations in an attempt to determine the best combination [9,10,16]. The Percoll gradient technique has been described as being ideal for cryopreserved samples, retrograde ejaculate samples, epididymal and testicular aspirates, and for patients with poor semen quality [16–20]. A single-layer Percoll approach has been reported to improve recovery and sperm motion characteristics [21]. The concept of evaluating density gradient centrifugation versus single density is relevant, as it might simplify the processing of sperm. And, if true, using a single-layer procedure would be ideal, because it would be cost effective and much simpler technically than the gradient method.

The purpose of our study was to compare two different methods of sperm preparation, using specimens from normal men, by employing a single-layer (80%) Percoll sperm preparation method and also a two-layer Percoll gradient to examine the sperm motion and functional characteristics, morphology, and the percentage of sperm recovered.

**MATERIALS AND METHODS**

**Sperm Preparation**

Semens 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 were based on the World Health Organization guidelines [22]. Specimens were allowed to liquefy at 37°C for 30 minutes before the sperm count and other motion characteristics were evaluated.

**Preparation of Percoll Gradients**

For single-layer separation, 80% Percoll was made from 100% Percoll with HEPES-buffered Ham’s F-10 medium. For two-layer separation, Percoll gradient consisted of a 47% Percoll upper phase and a 90% lower phase made from 100% Percoll with HEPES-buffered Ham’s F-10 medium. 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. Using a transfer tube, 2 mL of the upper phase was layered; the interface between the layers was clearly visible. Liquified semen (0.5–1.0 mL) was gently placed on the 80% solution in the case of the single-layer, and on the upper phase in two-layer. All tubes were centrifuged at 500 x g for 20 minutes at room temperature. The pellet was resuspended in 2–3 mL of human tubal fluid (HTF) medium and centrifuged at 500 x g for 7 minutes. The supernatant was removed and the final sperm pellet resuspended in HTF. Motion characteristics and sperm motility were then evaluated at 0 time and after 60 minutes of incubation at 37°C.

**Analysis of Sperm Motility**

Sperm samples were analyzed on 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 (MicroCell slide, Conception Technologies, La Jolla, CA) and analyzed for percent motility, straight-line velocity (straight-line distance from the beginning of the sperm track divided by time), curvilinear velocity [total distance traveled by a given spermatozoon divided by total time elapsed], amplitude of lateral head displacement (mean width of sperm head oscillation), average path velocity, and linearity (departure of sperm track from a straight line).

**Hypo-Osmotic Swelling Determination**

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 Percoll separation. After incubation at 37°C for 60 minutes, a minimum of 200 spermatozoa per sample were examined using phase-contrast microscopy (Olympus, Model BH 2,
Tokyo, Japan), and the percentage of sperm with intact membranes was calculated [23].

**Sperm Viability and Morphology**

The percentage of viable sperm in each aliquot was assessed after Percoll separation. Each sample was mixed with an equal amount of 0.05% eosin-Y nigrosin to improve contrast. A total of 100 spermatozoa in duplicate from each sample were counted. The dead sperm appear pink; sperm without the dye were counted as viable. Sperm morphology was assessed by World Health Organization [22] and Kruger's strict criteria [24,25]. Sperm smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL).

**Bovine Cervical Mucus Penetration Test**

The bovine cervical mucus penetration test was performed using the Penetrak kit (Serono Diagnostics, Allentown, PA). Capillary tubes in duplicate were thawed at room temperature for 30 minutes and snipped 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 a room temperature for 90 minutes. The capillary tubes were then placed on a calibrated slide and examined using phase-contrast microscopy. The distance (in millimeters) covered by the vanguard sperm was measured.

**Statistical Analysis**

The paired Student's t test was used to analyze the data, using the SigmaStat (Jandel Corp., San Rafael, CA, 1992). A P value of 0.05 was considered as statistically significant.

**RESULTS**

Comparisons between the single-layer and two-layer Percoll-separated spermatozoa are shown in Table I. Total motile sperm values were comparable between the two separation methods. Compared to the single-layer separation, sperm recovery was significantly greater in the two-layer separation \(P = 0.04\). The percent motility also was significantly higher after the two-layer separation \(P = 0.002\); however, sperm motility after 60 minutes was comparable between the two methods \(P = 0.77\). Significant differences were seen in linearity \(P = 0.02\) and amplitude of lateral head displacement \(P = 0.01\).

Spermatozoa separated on the two-layer Percoll gradient had significantly better hypo-osmotic swelling \(P = 0.006\) and viability \(P = 0.02\) compared with specimens separated on a single layer (Table II). Sperm morphology values scored by either WHO or Kruger's criteria did not differ between the two sperm separation methods.

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparison of sperm motion characteristics of semen specimens separated by a single- vs. a two-layer Percoll method.</strong></td>
</tr>
<tr>
<td><strong>Semen Characteristic</strong></td>
</tr>
<tr>
<td>Total motile sperm (\times 10^6)</td>
</tr>
<tr>
<td>Sperm recovery (%)</td>
</tr>
<tr>
<td>Motility</td>
</tr>
<tr>
<td>0 minutes (%)</td>
</tr>
<tr>
<td>after 60 minutes (%)</td>
</tr>
<tr>
<td>VCL (µm/sec)</td>
</tr>
<tr>
<td>LIN (%)</td>
</tr>
<tr>
<td>ALH (µm)</td>
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</tbody>
</table>

VCL = curvilinear velocity; LIN = linearity; ALH = amplitude of lateral head displacement. Values are mean ± standard deviation. Statistical significance is \(P < .05\).
TABLE II
Comparison of sperm quality of specimens separated by a single-
vS. a two-layer Percoll method.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Single-layer Percoll</th>
<th>Two-layer Percoll</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS [%]</td>
<td>88.6 ± 7.8</td>
<td>91.6 ± 7.4</td>
<td>.006</td>
</tr>
<tr>
<td>Viability [%]</td>
<td>36.8 ± 13.7</td>
<td>41.8 ± 13.7</td>
<td>.02</td>
</tr>
<tr>
<td>BCMP [mm]</td>
<td>43.9 ± 15.0</td>
<td>46.1 ± 14.3</td>
<td>.53</td>
</tr>
<tr>
<td>Morphology [%]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO criteria</td>
<td>38.0 ± 11.1</td>
<td>37.3 ± 9.5</td>
<td>.92</td>
</tr>
<tr>
<td>Kruger’s strict criteria</td>
<td>13.5 ± 4.0</td>
<td>13.6 ± 5.0</td>
<td>.92</td>
</tr>
</tbody>
</table>

HOS = hyposmotic swelling; BCMP = bovine cervical mucus penetration.
Values are mean ± standard deviation. Statistical significance is P < .05.

DISCUSSION

Poor sperm quality in male-factor infertility limits the treatment options currently available. Sperm preparation techniques are aimed at removing capacitation factors to improve sperm motility and recovery of morphologically normal spermatozoa. Percoll separation by density gradient centrifugation is an alternative to the widely used methods based on self-migration of spermatozoa. The Percoll gradient both improves sperm motility and morphology and permits recovery of more spermatozoa [9,10,16,26]. The mechanism by which Percoll acts as a selecting and, possibly, a protecting medium is not known. The mechanism could involve reducing membrane damage, because it removes the white blood cells and abnormal spermatozoa, both of which generate reactive oxygen species, or it may involve eliminating factors in washed specimens that inhibit some step in the fertilization process [27]. Although in our study, separation with a single- or a two-layered Percoll procedure elicited virtually the same proportion of morphologically normal spermatozoa, the Percoll gradient technique has been reported to separate morphologically better sperm, which could lead to improved fertilization rates [26]. Improvement in sperm morphology has been reported after Percoll separation when compared with other separation methods, such as the swim-up method. However, as in our study, other investigators found no improvement in sperm morphology after Percoll separation [12,28]. This discrepancy could be due to differences in the Percoll procedures used, the heterogeneous nature of sperm function, and the morphological classification used.

Density gradient centrifugation successfully selects motile spermatozoa [29]. Various combinations of concentrations, volumes, and gradients have been reported to increase the proportion of sperm with progressive motility, and linearity [4]. A higher recovery of motile sperm was seen when the number of gradients was reduced. A two-layer Percoll separation, however, provided superior results with oligozoospermic and asthenozoospermic specimens [4].

A simplified single-layer Percoll technique compared with the swim-up method, has been reported using male factor infertility patients and men with normal semen characteristics [21]. This is the only study which reports improvement in semen characteristics—but only when compared with the swim-up technique. There are no reports that have explicitly compared single-layer Percoll with two or more Percoll layers.

A single-layer (90%) Percoll procedure was used successfully to separate epididymal sperm retrieved from an obstructed epididymis [14]. Semen characteristics also improved in both normal and male factor infertility patients when using a single-layer Percoll (MonoPercoll) technique [21]. However, others found contradictory results when analyzing normal semen specimens [7]. In our study, spermatozoa separated on a single-layer of Percoll did not show better semen characteristics than those separated on a two-layer Percoll gradient.
Earlier, we [20] reported improved sperm characteristics in cryopreserved specimens separated on a two-layer Percoll gradient. The greater percentage of sperm with tail swelling and viability obtained in our present study with a two-layer Percoll procedure is important, since the hypo-osmotic swelling test can discriminate between fertile and infertile semen samples [30]. Indeed, improved fertilization rates are observed with spermatozoa separated on two or more layers of Percoll [16,26].

In conclusion, our study confirms that a two-layer Percoll separation of semen specimens is superior to a single-layer separation, providing improved sperm motion characteristics, hypo-osmotic swelling, and viability. It would be interesting to see comparisons of the two techniques for subfertile men with abnormal semen analyses. Further studies may help determine if other sperm characteristics, such as the acrosome reaction and actual fertilizing ability, are also improved after separation on a two-layer Percoll gradient.

REFERENCES

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