COMPARISON OF DIRECT SWIM-UP, MINI-PERCOLL, AND SEPHADEX G10 SEPARATION PROCEDURES

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A comparison was made of three sperm separation techniques—direct swim-up, mini-Percoll, and Sephadex G-10—on semen parameters, including count, % motility, forward progression, and hypo-osmotic swelling (HOS) test scores. Overall, the best quality sperm (less debris, best % and quality motility, and best HOS scores) were noted with the direct swim-up procedure. Mini-Percoll resulted in the highest count, but the worst HOS score. Sephadex G-10 resulted in quality of semen specimen almost as good as the direct swim-up procedure, but did allow some noncellular debris and bacteria to filter through the column.

Keywords semen separation techniques, swim-up, mini-Percoll, Sephadex G-10

The separation of spermatozoa from seminal plasma is necessary for in vitro fertilization (IVF) or intrauterine insemination (IUI). The separation process is needed to inhibit the introduction of harmful prostaglandins, leukocytes, or bacteria into the uterine cavity and allows capacitation by removing decapacitation factors [17]. Certain separation procedures involve sperm that are apparently normal, e.g., performing IUI for cervical factor [4]. However, in other instances the procedure is performed for IVF or timed IUI because of an apparent male factor problem. Some separation procedures produce a specimen that has good quality, as evidenced by motility, morphology, and absence of leukocytes and debris, but at the sacrifice of sperm concentration.

Alternatively, sperm suspensions prepared by repeated centrifugation and washing will retain noncellular debris, epithelial cells, nonmotile and nonviable spermatozoa, leukocytes, and bacteria. Improvement of a washed specimen as described above can be achieved by allowing the sperm from the centrifuged pellet to swim up into the overlying medium. However, there are data to suggest that the initial centrifugation process may release, from nonviable sperm

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and/or leukocytes, an excess generation of reactive oxygen species (ROS), which may have an adverse effect on the better quality sperm [1]. Thus, theoretically, a potentially superior separated sperm specimen might be achieved by carefully layering culture fluid over unprocessed semen, as in the direct swim-up process [3, 19, 20].

Another popular method of sperm separation is the discontinuous Percoll gradient method [3, 14, 17]. Percoll medium consists of colloidal silica particles coated with polyvinylpyrrolidone. The final product is relatively free of leukocytes, bacteria, and other seminal debris [2, 13, 18]; a highly motile sperm fraction is usually found in the region between 85 and 100%. Though this procedure does require a centrifugation step first, which might release harmful oxidants, the subsequent separation of the better sperm from contaminating cells or abnormal spermatozoa should also minimize peroxidative damage of sperm membranes by free oxygen species [1, 15, 21]. One problem with the Percoll gradient technique is that there is a low rate of sperm recovery, especially if an oligospermic specimen is used. A mini-Percoll method of sperm preparation has been designed to help produce a greater yield of sperm, in comparison to resuspension methods, while removing seminal debris [23].

Sephadex gel filtration is a less frequently used method of separation. The original description used a larger size bead (Sephadex G-50) [25]. Reducing the size of the beads (Sephadex G-15) resulted in a specimen with improved motility compared to the larger beads (marketed as SpermPrep II) [5]. Recently an even smaller bead size (Sephadex G-10) was used to separate sperm prior to cryopreservation and the thawed specimen was superior to cryopreserved sperm first separated by Percoll discontinuous gradient technique [12].

Most andrologists and clinicians agree that the standard semen analysis does not distinguish a fertile from subfertile male. One study found that when evaluating infertile couples where a female factor is identified, males with motile densities (MD) $< 2.5 \times 10^6$/mL achieved a lower pregnancy rate (PR) than males with higher levels within 6 months of the female factor being corrected [6]. Furthermore, in a similar group of infertile couples, neither sperm morphology by either standard or strict morphology method was able to clearly separate fertile from subfertile males [7, 8]. The problem with MDs and morphology is not merely that "subnormal" levels are not good predictors of the subfertile males, but also not being able to establish with confidence that at least a normal MD and morphology will select the fertile male. In fact, one study, using a therapeutic donor insemination (TDI) probe, found high PRs in females, who had all infertility factors corrected but failed to conceive, despite exposure to the male partner's sperm for at least eight cycles, when switching to TDI, even when the male partner seemingly had normal semen parameters [9].

The hypoosmotic swelling (HOS) test is a simple, easily reproducible test that evaluates the functional integrity of the sperm membrane [16]. In contrast to sperm count and percent motility, this test is very stable over time [24]. When less than 50% of the sperm show characteristic swelling of sperm tails there is a correlation with very poor PRs, even when all other semen parameters are normal [10]. It is suggested that separation with Sephadex G-50 results in semen specimens with inferior HOS test scores compared to conventional swim-up or Percoll discontinuous gradient techniques [11]. However, when the Sephadex bead size was reduced (G-15), a superior HOS score was found compared to Percoll or Sephadex G-50 [22]. Sephadex G-15 also produced an equal quality specimen similar to Percoll in sperm concentration and motility; unfortunately, there were debris and other cells in the final Sephadex G-15 product.

In the present study three methods of sperm separation were evaluated based on final sperm concentration, percent and pattern of motility, and the HOS test. The "gold standard" chosen for normospermic samples is usually a modified swim-up procedure without centrifugation. The mini-Percoll technique was used for the second separation procedure, but was modified by decreasing the centrifugation time; the hypothesis was that the mini-Percoll would recover the same amount of motile sperm in less time. The Sephadex technique was used for the third separation procedure, but because there was still debris with G-15 beads, the smaller bead size of Sephadex G-10 was evaluated.

MATERIALS AND METHODS

Experimental Design and Statistical Analysis. Ten semen specimens were initially evaluated for sperm count, % motility, debris, quality of motility (A, B, C, D grading), and hypoosmotic swelling (HOS) test. The HOS test was performed by mixing 0.1 mL liquefied semen with 1.0 mL hypoosmotic reagent and incubating at 37°C for 30 min. After incubation the sperm were microscopically evaluated for % swelling. The specimens were divided into three equal aliquots and then separated by the three aforementioned procedures. The same semen parameters were repeated on the separated washed sperm.

Data were analyzed using Friedman analysis of variance with multiple comparisons. This nonparametric test was used since it did not require the data to be normally distributed.

Reagents. To simplify the sperm washing procedures, the media used for all three techniques was modified human tubal fluid (HTF)–Hepes (Irvine Scientific) with 0.5% human serum albumin fraction V (Irvine Scientific). Isotonic Percoll was made using one part modified HTF–Hepes 10× (Irvine Scientific) and nine parts Percoll (Sigma). Then, 0.5% human serum albumin fraction V was added to the Percoll mixture. Osmolality was lowered to 275–285 using a 1:10 dilution of cell culture water (Irvine Scientific) and Percoll.

Direct Swim-up Separation. The direct swim-up procedure was described by Aitken and Clarkson [1] and modified by Mortimer (personal communication). The technique involves underlaying 0.2 mL liquefied semen below 0.5 mL media in 12 × 75-mm tubes. The tubes are placed in a 37°C incubator for 1 h. After incubation, the top 0.25–0.3 mL was removed.

Mini-Percoll Separation. Liquefied semen was diluted 1:2 with modified HTF–Hepes supplemented with 0.5% human serum albumin and then centrifuged at 200*g* for 10 min. Pellets were resuspended in 0.3 mL medium and layered on a discontinuous Percoll gradient consisting of 0.3 mL each 90, 60, and 45% of isotonic Percoll. The 15-mL centrifuge tubes were then centrifuged at 300*g* for 15 min. The top semen layer was discarded. Two-thirds of the bottom 90% layer was then removed and washed one time. This procedure was modified from that of Ord et al. [23] as follows: Isotonic Percoll was made with modified HTF-Hepes 10×, instead of Ham's F-10 10×. Sperm diluting media was the same. Centrifuge time was cut from 30–45 min to 15 min to allow % motility to be at its highest, since sperm may become nonmotile after an additional 15–30 min centrifugation.

Sephadex G-10 Separation. Sephadex columns were made using 2-mL nontoxic syringes with plungers removed. A small amount of glass wool was placed at the bottom of the syringe. Hydrated Sephadex G-10-120 was then added to a 3-cm height. The syringes were placed in 15-mL centrifuge tubes and spun at 300*g* for 1 min. The columns were then placed in new centrifuge tubes, the semen was diluted 1:1 with media, and 250-μL aliquots were placed on top of the columns and centrifuged at 300*g* for 1 min. The columns were then discarded and the washed sperm were pooled.

RESULTS

The initial count, % motility, forward progression, motile density, and HOS scores of the specimens measured initially and after sperm preparation are presented in Table 1. The semen parameters following Sephadex G-10 were not significantly different from the direct swim-up method. The percentages of total motile sperm recovered were also similar: 35.5% for swim-up as compared to 30.3% for Sephadex G-10. Compared to the initial specimen, these two washes increased motility, but only the swim-up significantly increased HOS scores. The mini-Percoll technique recovered 55% of total motile sperm, but had lower motility and HOS scores than the direct swim-up and Sephadex G-10 preparations. The final HOS scores dropped from the initial scores in 7 out of the 10 samples. The mini-Percoll specimen had a lower motile density than the initial specimen, but all other parameters were comparable.

DISCUSSION

The direct swim-up technique resulted in the highest percentage of motile sperm with the best quality motility and the highest HOS score. Thus, for males with high sperm counts, but with borderline HOS scores or only a fair quality of motility, direct swim-up, without centrifugation, seems to be more appropriate than either mini-Percoll or Sephadex G-10. The percentage and quality of motility and percentage with HOS swelling were also improved significantly over the initial specimen with Sephadex G-10 separation; however, the count was not much higher than the direct swim-up procedure and, unfortunately, the final samples contained seminal debris. The Sephadex technique is easy to use, but unless it can be demonstrated that there is no adverse clinical effect of the increase in debris, this method cannot be recommended as a first-line procedure.

The Percoll discontinuous gradient procedure resulted in a specimen free from debris, with increased sperm quality. We previously demonstrated a 40% recovery of motile sperm compared to the initial specimen [11]. The modified mini-Percoll technique produced a debris-free specimen with over 55% recovery of motile sperm, but HOS scores following mini-Percoll

TABLE 1 Initial Count, Percent Motility, Quality of Motility, HOS, and Results of Separation Techniques

Parameter	Initial	Direct Swim-up	Mini-Percoll	Sephadex G-10
Count (million/mL)	66 ± 56	13.9 ± 14.9[c]	44 ± 49	18.2 ± 19.3[c]
Motility (%)	58 ± 18.6	82 ± 23[c]	54 ± 29[a,b]	76 ± 23[c]
Progressive forward motion				
Quality A (%)	28 ± 12.3	55 ± 25	40 ± 28	50 ± 33
Quality B (%)	67 ± 12.0	42 ± 27	40 ± 19.4	50 ± 33
Quality C (%)	6.0 ± 6.6	3.0 ± 6.7	17.0 ± 25	0.0 ± 0.0
Motile density (million/mL)	34 ± 32	12.1 ± 14.3[c]	19.4 ± 26[c]	13.5 ± 16.5[c]
HOS (%)	61 ± 19.4	83 ± 12.9[c]	60 ± 24[a,b]	73 ± 19.4
Motile sperm recovered (%)		36 ± 32	55 ± 45	30 ± 20

[a] $p < .05$ comparing swim-up to mini-Percoll.
[b] $p < .05$ comparing Sephadex to mini-Percoll.
[c] $p < .05$ comparing initial to sperm preparation.

were lower than those obtained from the other two procedures. Mini-Percoll was the only one of the three procedures that did not improve the HOS score: Seven of the ten specimens had lower HOS scores than the initial specimen. In two earlier studies, we found that the conventional Percoll technique did not improve HOS scores in one study and improved them by only 15% in the second study [5, 11]. Thus, these data are consistent with the previous conclusions by Ord et al. [23] that this technique may be especially good for oligozoospermic specimens. In one study of men with male factor problems there were no real differences in specimens following Percoll or mini-Percoll separation, but both resulted in higher MDs than the swim-up procedure [22]; however, % acrosome reaction and forward progressive of motility was better with swim-up. This study involved washing by centrifugation prior to sperm separation.

It would appear that the direct swim-up technique may be the better sperm separation procedure to use for IVF preparation, especially when the initial sperm count is good and the diagnosis is for unexplained infertility or cervical factor; this would be especially true if the initial HOS score is low. Mini-Percoll would seem to be a good choice for oligospermic counts. One caveat, however, would be that if the final HOS score falls below 50%, perhaps a direct swim-up procedure can be performed following the mini-Percoll separated sperm. A good laboratory should not use one separation technique exclusively, but should change the technique according to the specimen.

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