Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception

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To evaluate and optimize the sperm separation efficiency of a novel silane-coated silica bead (Puresperm), serial studies were carried out to compare the various sperm parameters between: (i) three-layer (90%–70%–40%) Puresperm and three-layer (90%–70%–40%) conventional polyvinylpyrrolidone (PVP)-coated silica bead (Percoll) gradients; (ii) three-layer (90%–70%–40%) and two-layer (90%–45%) Puresperm gradients and separately the same for Percoll; and (iii) large (3.0 ml) and small (0.75 ml) semen loading volumes on three-layer Puresperm gradients. Normozoospermic semen samples were treated and analysed in 12 replicates for each experiment. Manual evaluation of concentration, percentage motility, percentage vitality, percentage normal morphology; computer-assisted semen analysis evaluation of concentration, percentage motility, grade of motility, motion characteristics (curvilinear velocity, linearity, amplitude of lateral head velocity, beat cross frequency, percentage hyperactivation); and yields from the initial semen samples were compared. Percoll was found to be superior to Puresperm in concentration, percentage motility, percentage vitality and yields after three-layer density gradient centrifugation. There were no significant differences in sperm parameters between two- and three-layer Percoll gradients, but three-layer Puresperm gradients behaved significantly better than two-layer gradients. Large semen volume loads on three-layer Puresperm gradients resulted in greater sperm concentrations, percentage motility, percentage vitality and percentage normal morphology, but small semen volume loads produced greater yields of good-quality spermatozoa. In the light of Percoll being withdrawn from the shelf for the use of assisted reproduction because of the presence of PVP, three-layer Puresperm gradients with large semen loading volumes appear to be an attractive alternative for sperm separation in medically assisted conception.
Key words: Percoll/Puresperm/PVP/silane/sperm separation

Introduction

Recently, medically assisted conception procedures such as intrauterine insemination (IUI), in-vitro fertilization (IVF) and other assisted reproductive techniques (ART) have been applied extensively to alleviate the problem of subfertility. Processing of semen is an integral part of all these treatments. Many laboratory procedures have been proposed to process semen for the purpose of enhancing its fertilizing ability. All these techniques must be able to produce a final sperm suspension that is free of seminal plasma, debris, microbial contamination, and with a high recovery of motile and morphologically normal spermatozoa (Miller *et al.*, 1996).

Since the advent of sperm separation into fractions (Gorus and Pipeleers, 1981), Percoll-based density gradients have been widely applied for sperm separation in various medically assisted conception procedures. Different kinds of such gradients have been described with varying results (Mortimer, 1994). Because of their simplicity, rapidity and excellent yields, they have become very popular for processing normal semen samples. Although there still exists some debate about their usefulness in subnormal semen samples, a wealth of data continues to be presented in the literature (Chen *et al.*, 1995, 1996). Meanwhile, some concerns about their limitations have received great attention recently. Retained Percoll particles after sperm washing may act as tissue irritants when IUI is carried out (Arora *et al.*, 1994). Possible deleterious effects of centrifugation of Percoll gradients on sperm longevity have also been raised (Gellert-Mortimer *et al.*, 1988). A serious clinical shortcoming arises from frequent contamination of the fractions by endotoxins (Scott and Smith, 1997).

Recently, several novel alternative products have been developed. These have been claimed to be endotoxin-free and have been introduced for IUI and ART programmes. Until now, there has been limited evaluation of these new products. One of the products, a silane-coated silica gel is available in the market as GRAD-50 from Scandinavian IVF Science AB, Gothenburg, Sweden, and as Puresperm from NidaCon Laboratories AB also from Gothenburg, Sweden. The former was shown to produce poor sperm survival and recovery of motile spermatozoa when compared with that of the conventional polyvinylpyrrolidone (PVP)-coated silica beads (Percoll; Pharmacia, Uppsala, Sweden) (Franceus *et al.*, 1995). Pure sperm, however, has not been evaluated adequately, although it is popular and used by several ART and andrology laboratories.

In this study, we comparatively evaluated a silane-coated silica preparation (Puresperm) with the conventionally used PVP-coated silica (Percoll) for efficiency of sperm separation for medically assisted conception procedures. The objectives of this study were: (i) to assess whether Puresperm was superior to Percoll in the form of a three-layered discontinuous density gradient (40%–70%–90%) with fixed centrifugation, column height and semen volume; (ii) to assess whether the standard three-layered gradients (both Percoll and Puresperm) could be simplified to two-layered gradients (45%–90%) with-

out compromising on their final efficiency; and (iii) to assess the influence of different loading volumes of semen on top of the three-layer Puresperm column on final efficiency.

Materials and methods

Source of semen and media

Semen samples were obtained by masturbation from adult males enrolled for routine diagnostic semen analysis. The samples were collected aseptically in sterile containers after 2 days of abstinence. A total of 48 normozoospermic semen samples from 48 different men were collected for the four experiments of this study with 12 different normozoospermic samples in 12 replicates allotted for each experiment. Routine semen assessment was carried out according to the World Health Organization guidelines (World Health Organization, 1992).

Isotonic 100% Percoll (Pharmacia, Uppsala, Sweden) was prepared by mixing 9 volumes of original stock Percoll with 1 volume of 10 times concentrated Earle's balanced salt solution (Sigma, Irvine, UK) supplemented with 10 mM HEPES buffer (Gibco, Grand Island, NY, USA) and 0.25 mM of sodium bicarbonate (Sigma, St. Louis, MO, USA). Silane-coated silica (Puresperm; NidaCon Laboratories AB, Gothenburg, Sweden) was purchased as a 100% isotonic solution ready for direct dilution. IVF-50 (Scandinavian IVF Science AB, Gothenburg, Sweden) was used for preparation of the different centrifugation gradients by dilution with 100% Percoll or Puresperm.

Experiment 1: comparison of three-layer Percoll and Puresperm gradients for sperm separation

On the day before use, the IVF-50 medium was equilibrated at 37°C in 5% CO_2 in air overnight. Percoll and Puresperm gradients (90, 70 and 40%) were prepared by dilution of the 100% stock solution with IVF-50 before each experiment. A 1.5 ml aliquot of 90% Percoll or Puresperm was first placed at the bottom of a 10-ml centrifuge tube (Falcon, Franklin Lakes, NJ, USA). A 1.5 ml volume of 70% Percoll or Puresperm was carefully layered over the 90% layer to create a gradient interface. Another 1.5 ml layer of 40% Percoll or Puresperm was then placed gently on top of the 70% layer to form the discontinuous Percoll or Puresperm column with three distinct density gradient layers. Aliquots of 1.5 ml of liquefied semen were then layered over each of the Percoll/Puresperm columns. All tubes were then centrifuged at 200 *g* for 20 min. After centrifugation, the seminal fluid, the 40 and 70% Percoll/Puresperm layers, and 1 ml of the 90% Percoll/Puresperm layer were removed using a sterile Pasteur pipette, leaving behind the sperm pellet in 0.5 ml of 90% Percoll/Puresperm. Using a new Pasteur pipette, the sperm pellet was resuspended in the 0.5 ml of Percoll/Puresperm, and the sperm suspensions were transferred to new 5-ml centrifuge tubes (Falcon). The sperm suspension was then resuspended in 4 ml of IVF-50 medium and the tubes centrifuged at 300 *g* for 10 min. After centrifugation, the upper layer (4 ml) was removed with a Pasteur pipette and the sperm pellet in a remaining 0.5 ml of medium was resuspended. From this suspension, a small aliquot of spermatozoa was removed for assays.

Experiment 2: comparison of two-layer and three-layer Percoll gradients for sperm separation

Three-layer Percoll gradients were prepared and spermatozoa were processed into a final 0.5 ml suspension as previously described. For two-layer gradients, 2.25 ml of 90% Percoll was placed at the bottom of a 10-ml centrifuge tube. Another layer of 2.25 ml of 45% Percoll was layered carefully over the 90% layer to create a gradient interface and to form the discontinuous Percoll column with two distinct

density gradient layers. Aliquots of 1.5 ml of liquefied semen were then layered over each of the Percoll columns. The centrifugation and washing procedures were the same as in Experiment 1. For the two-layer gradients, the semen and whole 45% layer and 1.75 ml of the 90% Percoll layer were removed, in the first instance, leaving the final 0.5 ml Percoll for resuspension and further processing as described previously in Experiment 1 for the three-layered gradients. The assays were the same.

Experiment 3: comparison of two-layer and three-layer Puresperm gradients for sperm separation

Three-layer Puresperm gradients were prepared as described in Experiment 1. For two-layer gradients, 2.25 ml of 90% Puresperm was placed at the bottom of a 10-ml centrifuge tube. A layer of 2.25 ml of 45% Puresperm was carefully layered over the 90% layer to create a gradient interface and to form the discontinuous Puresperm column with two distinct density gradient layers. The centrifugation and washing procedures were the same as previously described. For the two-layer gradients, the semen and whole 45% layer and 1.75 ml of the 90% Puresperm layer were removed, in the first instance, leaving the final 0.5 ml Puresperm for resuspension and further processing similar to Experiments 1 and 2. The assays were the same.

Experiment 4: comparison of different semen loading volumes on three-layer Puresperm gradients for sperm separation

Three-layer Puresperm gradients were prepared as in Experiment 1. A 3.0 ml aliquot of liquefied semen was then layered over one of the three-layer Puresperm columns and 0.75 ml of liquefied semen was layered over another three-layer Puresperm column. The two aliquots of semen stratified were derived from the same semen sample. The centrifugation, washing procedures and assays were the same as in Experiment 1.

Parameters evaluated and calculated

Complete sets of assays were performed for the original ejaculate and for the sperm suspension obtained after Percoll or Puresperm column treatment. The parameters evaluated included: (i) manual evaluation of sperm concentration and percentage motility by counting with a Makler chamber; (ii) computer automated semen analysis (CASA) of the concentration, percentage motility, grade of sperm motility and sperm movement characteristics using the Hamilton–Thorn sperm autoanalyser model 2030; (iii) evaluation of sperm vitality using the conventional eosin–nigrosin stained smear; and (iv) evaluation of sperm morphology using Kruger's strict criteria (Diff–Quik stain). The calculated parameters included counts and recovery rates (yields) from initial semen samples for morphologically normal spermatozoa, grade a motility spermatozoa, and live spermatozoa. Counts were calculated by multiplying percentages, concentrations and volumes of the final and initial samples. Recovery rates (yields) were calculated by dividing the retrieved counts (after treatment) by initial counts (before treatment).

Sperm movement characteristics that were analysed included curvilinear velocity (VCL) (grading A: rapid, >25 μm/s; B: medium, >10 μm/s, <25 μm/s; C: slow, <10 μm/s; D: static), linearity (LIN), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), and percentage hyperactivation motility fraction (HA). Automatic sorting for identification of hyperactivation fractions was achieved with the use of Burkman's criteria (Burkman, 1991). The system parameter settings for CASA are shown in Table I. Using the CASA system up to 200 spermatozoa were counted for each parameter.

A total of 200 spermatozoa were scored for morphology for each slide under oil immersion and ×1000 magnification. The strict

morphological criteria modified from guidelines set out by Kruger *et al.* (1986, 1988) and Hall *et al.* (1995) specific for the Diff–Quik staining technique were used (Kruger *et al.*, 1987).

Statistical analysis

Non-parametric analysis using Wilcoxon matched-pairs signed-ranks test was done by SPSS-PC+ program (SPSS Inc. Chicago, IL, USA). The difference was considered significant if the calculated two-tailed P-value was < 0.05.

Results

Experiment 1

The results of Experiment 1 are summarized in Tables II, III and IV. After centrifugation and washing the sperm pellet, Percoll significantly improved percentage motility from the original semen samples but Puresperm did not. However, grade A motility, VCL, ALH, BCF and the HA were significantly improved from the original semen samples for both Percoll and Puresperm. Percentage vitality and percentage normal forms were also significantly improved by both silica prepara-

tions. Interestingly, Percoll generated significantly greater sperm numbers and motile spermatozoa than Puresperm, while no significant changes were observed in percentage normal morphology between the two silica preparations.

It was clear that Percoll gradients recovered significantly greater normal sperm forms, grade A motility, combination of grade A + B motility and percentage vitality than those of Puresperm gradients.

Experiment 2

The results of Experiment 2 are also summarized in Tables II, III and IV. After treatment, both the two-layer and three-layer Percoll gradients significantly improved the percentage motility, percentage vitality and percentage normal forms when compared with those of the original semen samples. However, there were no significant differences in sperm numbers, motile spermatozoa, vitality and morphology between these two Percoll gradients. Grade A motility, VCL, ALH, BCF and HA were also significantly improved from the original semen samples for both Percoll gradients. There were no significant differences in sperm numbers, percentage motility, grading of motility and all motion characteristics between the two Percoll gradients. The three-layer Percoll gradients recovered no significant differences in normal sperm forms, grade A motility, combination of grade A + B motility and percentage vitality when compared with those of two-layer Percoll gradients.

Experiment 3

The results of Experiment 3 are also summarized in Tables II, III and IV. After treatment, both the three-layer and two-layer Puresperm gradients significantly improved the percentage motility, percentage vitality and percentage normal forms when compared with those of the original raw semen samples. However, three-layer Puresperm gradients produced significantly greater percentages of motile spermatozoa, vital spermatozoa and normal forms compared with those of the

Table I. System parameter settings used in Hamilton–Thorn computer automated sperm autoanalyser

Parameter	Setting
Frames at frame rate	7–20 at 25 Hz
Minimum contrast	8 units
Minimum size	4 unit is equivalent to 1× pixel
LO/HI size gates	0.7–1.6
LO/HI intensity gates	0.7–1.6
Non-motile head size	11 μm^2
Non-motile intensity	265 (50 Hz unit)
Medium VAP value	25 $\mu m/s$
Low VAP value	10 $\mu m/s$
Slow cell motile	Yes
Threshold STR	80%

VAP = path velocity; STR = straightness.

Table II. Visual comparisons of sperm characteristics using different separation columns after centrifugation and washing (mean ± SD)

Experiment	Column	Concentration (× 10^6/ml)	Motility (%)	Vitality (%)	Normal forms (%)
1	Semen	97.3 ± 48.7[a]	60.7 ± 9.7[d]	64.4 ± 8.3[g]	21.7 ± 7.8[j]
	3-layer P	50.6 ± 31.1[b]	89.5 ± 8.4[e]	89.7 ± 7.0[h]	50.5 ± 16.4[k]
	3-layer PS	18.0 ± 26.7[c]	74.3 ± 13.9[f]	81.7 ± 11.8[i]	48.9 ± 17.2[l]
2	Semen	114.08 ± 45.7[a]	48.5 ± 15.3[d]	56.3 ± 10.6[g]	31.6 ± 8.7[j]
	3-layer P	31.7 ± 14.3[b]	91.9 ± 4.5[e]	91.5 ± 2.9[h]	57.6 ± 8.1[k]
	2-layer P	27.5 ± 15.4[c]	89.5 ± 5.0[f]	89.4 ± 4.6[i]	57.0 ± 6.7[l]
3	Semen	79.5 ± 54.5[a]	56.7 ± 13.9[d]	64.1 ± 13.6[g]	17.5 ± 6.1[j]
	3-layer PS	19.0 ± 13.0[b]	85.5 ± 11.4[e]	88.3 ± 6.1[h]	41.1 ± 9.6[k]
	2-layer PS	15.8 ± 14.0[c]	77.5 ± 14.0[f]	83.5 ± 7.2[i]	38.4 ± 9.3[l]
4	Semen	120.0 ± 58.3[a]	47.9 ± 13.2[d]	54.8 ± 13.7[g]	28.5 ± 20.5[j]
	LV:3-layer PS	48.0 ± 50.3[b]	87.4 ± 11.9[e]	89.6 ± 7.4[h]	61.8 ± 13.0[k]
	SV:3-layer PS	20.6 ± 17.1[c]	81.3 ± 13.0[f]	84.6 ± 10.9[l]	58.9 ± 13.4[l]

P = Percoll; PS = Puresperm; LV = large volume; SV = small volume.
Experiment 1: [a,b]$P < 0.05$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [a,c]$P < 0.01$; [d,f]$P > 0.05$; [g,i]$P < 0.01$; [j,l]$P < 0.01$; [b,c]$P < 0.01$; [e,f]$P < 0.01$; [h,i]$P < 0.01$; [k,l]$P > 0.05$.
Experiment 2: [a,b]$P < 0.05$; [d,e]$P < 0.05$; [g,h]$P < 0.05$; [j,k]$P < 0.05$; [a,c]$P < 0.05$; [d,f]$P < 0.05$; [g,i]$P < 0.05$; [j,l]$P < 0.05$; [b,c]$P > 0.05$; [e,f]$P > 0.05$; [h,i]$P > 0.05$; [k,l]$P > 0.05$.
Experiment 3: [a,b]$P < 0.01$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [a,c]$P < 0.01$; [d,f]$P < 0.01$; [g,i]$P < 0.01$; [j,l]$P < 0.01$; [b,c]$P > 0.05$; [e,f]$P < 0.01$; [h,i]$P < 0.01$; [k,l]$P < 0.01$.
Experiment 4: [a,b]$P < 0.01$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [a,c]$P < 0.01$; [d,f]$P < 0.01$; [g,i]$P < 0.01$; [j,l]$P < 0.01$; [b,c]$P < 0.01$; [e,f]$P < 0.05$; [h,i]$P < 0.05$; [k,l]$P < 0.01$.

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Table III. Computer-automated semen analysis comparisons of sperm characteristics using different separation columns after centrifugation and washing (mean ± SD)

Expt	Column	Concentration (× 10⁶/ml)	Motility (%)	Grade of motility# (%)				Motion characteristics (%)				
				A	B	C	D	VCL (μm/s)	LIN (%)	ALH (μm)	BCF (Hz/s)	HA (%)
1	Semen	129.6 ± 48.9[a]	71.4 ± 8.9[d]	52.7 ± 11.2[g]	13.9 ± 5.3[j]	4.8 ± 4.1[m]	28.6 ± 8.9[p]	51 ± 9[s]	65 ± 8[v]	3.0 ± 0.8[y]	8.9 ± 1.7[ab]	2.0 ± 3.1[ae]
	3-layer P	69.9 ± 85.2[b]	88.6 ± 5.4[e]	80.0 ± 9.3[h]	7.2 ± 4.1[k]	1.3 ± 1.3[n]	11.4 ± 5.4[q]	76 ± 12[t]	66 ± 8[w]	4.6 ± 1.1[z]	12.6 ± 0.8[ac]	8.0 ± 8.5[af]
	3-layer PS	34.8 ± 66.8[c]	74.7 ± 13.6[f]	70.4 ± 13.3[i]	4.2 ± 2.8[l]	0.8 ± 1.0[o]	25.3 ± 13.5[r]	77 ± 8[u]	74 ± 7[x]	4.1 ± 0.8[aa]	12.9 ± 1.5[ad]	5.0 ± 5.4[ag]
2	Semen	115.4 ± 51.0[a]	54.7 ± 18.0[d]	39.3 ± 17.5[g]	12.2 ± 5.5[j]	5.5 ± 3.4[m]	43.0 ± 19.2[p]	48 ± 8[s]	69 ± 6[v]	2.3 ± 1.0[y]	8.2 ± 1.6[ab]	0.7 ± 0.7[ae]
	3-layer P	33.5 ± 17.2[b]	88.8 ± 4.1[e]	80.0 ± 8.5[h]	7.7 ± 4.3[k]	1.3 ± 1.5[n]	11.1 ± 4.2[q]	72 ± 11[t]	71 ± 8[w]	4.1 ± 1.1[z]	12.5 ± 0.9[ac]	4.2 ± 4.4[af]
	2-layer P	28.0 ± 14.0[c]	89.3 ± 5.1[f]	81.8 ± 6.9[i]	6.5 ± 3.1[l]	1.2 ± 0.6[o]	10.5 ± 5.2[r]	76 ± 10[u]	77 ± 7[x]	3.7 ± 0.8[aa]	11.9 ± 1.1[ad]	4.4 ± 5.1[ag]
3	Semen	77.0 ± 54.1[a]	62.5 ± 14.7[d]	47.1 ± 15.2[g]	11.6 ± 2.8[j]	3.8 ± 2.1[m]	37.5 ± 4.7[p]	50 ± 9[s]	73 ± 8[v]	2.5 ± 0.7[y]	9.1 ± 1.2[ab]	0.8 ± 1.2[ae]
	3-layer PS	18.2 ± 11.7[b]	88.8 ± 5.0[e]	82.4 ± 7.6[h]	5.8 ± 3.3[k]	0.6 ± 0.7[n]	11.2 ± 5.0[q]	67 ± 10[t]	76 ± 5[w]	3.4 ± 0.7[z]	12.1 ± 1.1[ac]	2.6 ± 3.2[af]
	2-layer PS	15.1 ± 13.5[c]	82.4 ± 8.3[f]	75.4 ± 11.4[i]	6.0 ± 4.1[l]	1.8 ± 2.9[o]	16.9 ± 7.9[r]	70 ± 8[u]	77 ± 7[x]	3.4 ± 0.8[aa]	12.0 ± 0.9[ad]	1.9 ± 2.5[ag]
4	Semen	114.4 ± 58.8[a]	53.7 ± 18.0[d]	39.3 ± 19.4[g]	13.1 ± 4.6[j]	4.7 ± 3.9[m]	42.9 ± 18.1[p]	48 ± 12[s]	71 ± 7[v]	2.4 ± 1[y]	7.6 ± 0.8[ab]	1.2 ± 2.2[ae]
	LV:3-layer PS	49.2 ± 53.1[b]	87.6 ± 4.7[e]	82.2 ± 7.0[h]	5.0 ± 3.2[k]	1.1 ± 0.9[n]	11.6 ± 4.6[q]	75 ± 13[t]	78 ± 7[w]	3.3 ± 1[z]	10.8 ± 1.2[ac]	3.5 ± 3.9[af]
	SV:3-layer PS	20.6 ± 17.1[c]	81.3 ± 13.0[f]	79.6 ± 9.2[i]	4.5 ± 2.5[l]	1.0 ± 0.9[o]	14.9 ± 7.9[r]	74 ± 14[u]	80 ± 4[x]	3.2 ± 1[aa]	10.8 ± 1.3[ad]	2.5 ± 3.4[ag]

VCL = curvilinear velocity; LIN = linearity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; HA = hyperactivation; PS = Puresperm; LV = large volume; SV = small volume.

#WHO grading: A = rapid (>25 μm/s), B = medium (>10 μm/s, <25 μm/s), C = slow (<10 μm/s), D = static.

Experiment 1: [a,b]$P < 0.05$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [m,n]$P < 0.05$; [p,q]$P < 0.01$; [s,t]$P < 0.01$; [v,w]$P > 0.05$; [y,z]$P < 0.01$; [ab,ac]$P < 0.01$; [ae,af]$P < 0.01$; [a,c]$P < 0.01$; [d,f]$P > 0.05$; [g,i]$P < 0.05$; [j,l]$P < 0.01$; [m,o]$P < 0.01$; [p,r]$P > 0.05$; [s,u]$P < 0.01$; [v,x]$P < 0.05$; [y,aa]$P < 0.01$; [ab,ad]$P < 0.01$; [ae,ag]$P < 0.05$; [b,c]$P < 0.01$; [e,f]$P < 0.01$; [h,i]$P < 0.05$; [k,l]$P > 0.05$; [n,o]$P > 0.05$; [q,r]$P < 0.01$; [t,u]$P > 0.05$; [w,x]$P < 0.05$; [z,aa]$P < 0.05$; [ac,ad]$P > 0.05$; [af,ag]$P > 0.05$.

Experiment 2: [a,b]$P < 0.05$; [d,e]$P < 0.05$; [g,h]$P < 0.05$; [j,k]$P < 0.05$; [m,n]$P < 0.05$; [p,q]$P < 0.05$; [s,t]$P < 0.05$; [v,w]$P > 0.05$; [y,z]$P < 0.05$; [ab,ac]$P < 0.05$; [ae,af]$P < 0.05$; [a,c]$P < 0.05$; [d,f]$P < 0.05$; [g,i]$P < 0.05$; [j,l]$P < 0.05$; [m,o]$P < 0.05$; [p,r]$P < 0.05$; [s,u]$P < 0.05$; [v,x]$P < 0.05$; [y,aa]$P < 0.05$; [ab,ad]$P < 0.05$; [ae,ag]$P < 0.05$; [b,c]$P < 0.05$; [e,f]$P < 0.05$; [h,i]$P = 0.05$; [k,l]$P > 0.05$; [n,o]$P > 0.05$; [q,r]$P > 0.05$; [t,u]$P > 0.05$; [w,x]$P > 0.05$; [z,aa]$P > 0.05$; [ac,ad]$P > 0.05$; [af,ag]$P > 0.05$.

Experiment 3: [a,b]$P < 0.01$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [m,n]$P < 0.01$; [p,q]$P < 0.01$; [s,t]$P < 0.01$; [v,w]$P > 0.05$; [y,z]$P < 0.01$; [ab,ac]$P < 0.01$; [ae,af]$P < 0.05$; [a,c]$P < 0.01$; [d,f]$P < 0.01$; [g,i]$P < 0.01$; [j,l]$P < 0.01$; [m,o]$P < 0.01$; [p,r]$P < 0.01$; [s,u]$P < 0.01$; [v,x]$P > 0.05$; [y,aa]$P < 0.05$; [ab,ad]$P < 0.05$; [ae,ag]$P > 0.05$ [b,c]$P > 0.05$; [e,f]$P < 0.01$; [h,i]$P < 0.05$; [k,l]$P > 0.05$; [n,o]$P > 0.05$; [q,r]$P < 0.01$; [t,u]$P > 0.05$; [w,x]$P > 0.05$; [z,aa]$P > 0.05$; [ac,ad]$P > 0.05$; [af,ag]$P > 0.05$.

Experiment 4: [a,b]$P < 0.01$; [d,e]$P < 0.01$; [g,h]$P < 0.01$; [j,k]$P < 0.01$; [m,n]$P < 0.01$; [p,q]$P < 0.01$; [s,t]$P < 0.01$; [v,w]$P < 0.05$; [y,z]$P < 0.01$; [ab,ac]$P < 0.01$; [ae,af]$P > 0.05$ [a,c]$P < 0.01$; [d,f]$P < 0.01$; [g,i]$P < 0.01$; [j,l]$P < 0.01$; [m,o]$P < 0.01$; [p,r]$P < 0.01$; [s,u]$P < 0.01$; [v,x]$P < 0.01$; [y,aa]$P < 0.05$; [ab,ad]$P < 0.01$; [ae,ag]$P > 0.05$ [b,c]$P < 0.01$; [e,f]$P > 0.05$; [h,i]$P > 0.05$; [k,l]$P > 0.05$; [n,o]$P > 0.05$; [q,r]$P > 0.05$; [t,u]$P > 0.05$; [w,x]$P > 0.05$; [z,aa]$P > 0.05$; [ac,ad]$P > 0.05$; [af,ag]$P > 0.05$.

Table IV. Comparisons of percentage sperm recovery between different separation columns after centrifugation and washing (mean ± SD)

Experiment	Column	Normal forms (%)	Grade A motility (%)	Grade A + B motility (%)	Vitality (%)
1	3-layer P	38 ± 29[a]	25 ± 17[c]	21 ± 14[e]	22 ± 16[g]
	3-layer PS	16 ± 23[b]	8 ± 11[d]	7 ± 10[f]	9 ± 12[h]
2	3-layer P	18 ± 8[a]	23 ± 13[c]	19 ± 10[e]	17 ± 8[g]
	2-layer P	15 ± 6[b]	20 ± 10[d]	16 ± 7[f]	14 ± 5[h]
3	3-layer PS	21 ± 13[a]	17 ± 12[c]	14 ± 9[e]	13 ± 8[g]
	2-layer PS	13 ± 6[b]	11 ± 7[d]	9 ± 5[f]	8 ± 4[h]
4	LV:3-layer PS	17 ± 9[a]	15 ± 8[c]	11 ± 7[e]	12 ± 8[g]
	SV:3-layer PS	26 ± 15[b]	24 ± 15[d]	18 ± 14[f]	18 ± 14[h]

P = Percoll; PS = Puresperm; LV = large volume; SV = small volume.
Experiment 1: [a,b]$P < 0.01$; [c,d]$P < 0.01$; [e,f]$P < 0.01$; [g,h]$P < 0.01$.
Experiment 2: [a,b]$P > 0.05$; [c,d]$P > 0.05$; [e,f]$P > 0.05$; [g,h]$P > 0.05$.
Experiment 3: [a,b]$P < 0.05$; [c,d]$P < 0.05$; [e,f]$P < 0.05$; [g,h]$P < 0.05$.
Experiment 4: [a,b]$P < 0.01$; [c,d]$P < 0.01$; [e,f]$P < 0.01$; [g,h]$P < 0.01$.

two-layer Puresperm gradients. Grade A motility, VCL, ALH, and BCF were also significantly improved from the original semen samples for both Puresperm gradients. There were no significant differences in sperm numbers, grade B and C motility, and all these motion characteristics between these two Puresperm gradients (Table III). However, three-layer Puresperm gradients recovered significantly greater normal sperm forms, grade A motility, combination of grade A + B motility and percentage vitality than two-layer Puresperm gradients.

Experiment 4

The results of Experiment 4 are summarized in Tables II, III and IV. After treatment, both the large- and small-loading volume Puresperm columns significantly improved the percentage motility, percentage vitality and percentage normal forms when compared with those of the original raw semen samples. Large-loading volume Puresperm gradients generated significantly greater numbers of spermatozoa, motile spermatozoa, vital spermatozoa and normal forms than the small-loading volume Puresperm gradients. Grade A motility, VCL, LIN,

ALH and BCF were also significantly improved from the original semen samples for both Puresperm gradients. The large-loading volume Puresperm gradients produced significantly greater numbers of spermatozoa, while there were no significant differences in percentage motility, grade of motility and motion characteristics between these two Puresperm gradients. However, small-loading volume Puresperm gradients recovered significantly greater yields in normal sperm forms, grade A motility, combination of grade A + B motility and percentage vitality than large-loading volume Puresperm gradients.

Discussion

The results of Experiment 1 demonstrated clearly that, of the parameters measured, Percoll separation could generate higher post-processed sperm concentrations, percentage motility, percentage grade A motility, and percentage vitality than Puresperm separation. Although the differences in sperm motion parameters and percentage normal morphology were non-significant, it appeared that Percoll was superior to Puresperm owing to higher yields of percentage normal forms and live spermatozoa and better percentage motility spermatozoa. In terms of morphology and motion characteristics of the separated spermatozoa, Puresperm was actually as good as Percoll. The major difference was in the recovery rates (yields).

The results in this study were different from those reported by Kossakowski et al. (1997), who demonstrated no differences in the yield of progressive motile spermatozoa. When comparing with their data, we have observed that the mean yield of progressive motile spermatozoa for Puresperm was much lower than that of Percoll with wider standard deviations (Percoll: 40 ± 23%; Puresperm: 34 ± 35%). All the other motion parameters they evaluated also showed no difference between these two density gradient media. The differences in results could be explained in part by the different methodology employed in the procedure, although both studies used the same materials. Unfortunately, it was not possible to compare the impact of these two columns on the percentage vitality and percentage normal morphology with their data because they did not evaluate them.

It is unclear as to why Puresperm produced lower yields of the processed spermatozoa. The average diameter of Percoll particles is ~ 17.2 nm. The size of the naked silica particles used in Puresperm is around 15 nm. We could assume that the density gradient particles used in Percoll are slightly bigger than those of Puresperm. The other physical characteristic that is different between these two products is the density. Percoll has a density of 1.13 ± 0.005 g/ml, while Puresperm has slightly higher and more variable density ranging from 1.10 to 1.25 g/ml. When Percoll gradients are made, initial dilution must be further carried out to make an iso-osmotic solution of 100% Percoll. The above factors may explain the lower recovery from the Puresperm columns.

From a clinical point of view, Puresperm might be better than Percoll because it contains lower endotoxin levels. Its use in sperm separation for ART has led to reduced fragmentation of embryos and higher implantation rates when compared with those of Percoll (Fong et al., 1998). It is therefore evident that either modification of the density structure of the Puresperm product or removal of the endotoxin content of the Percoll product may fulfil the requirement of generating superior spermatozoa without endotoxin contamination for the medically assisted conception procedures.

Although the number of Percoll gradients used for sperm separation could range from one layer (Shalika et al., 1995) to over 10 layers (Kaneko et al., 1983), the most commonly employed columns comprise two to three layers. However, there is a paucity of reported information on the impact of different gradients on the final composition of the sperm suspension that is obtained. Based on the sperm yields from different columns, Ruiz-Romero et al. (1995) reported that the four-layer was poorer than the two- or three-layer columns. Also smaller volume columns (0.5-ml layer) were better than larger volume (1-ml layer) columns. In the case of asthenozoospermic samples, the 0.5-ml/two-layer column was the most effective. In contrast, we compared 1.5-ml/three-layer columns and 2.25-ml/two-layer columns by recruiting many more parameters The results showed that two- and three-layer Percoll columns produced final suspensions with almost the same sperm characteristics. Comparable recovery rates of specific selected sperm groups were also obtained. The percentage of spermatozoa with normal morphology increased from 31.6% in the original semen samples to 57.0 to 57.6% (1.8- to 1.82-fold increase) in the final suspension. The values were quite close to the values of 27.3 to 53.8% in normal samples reported by other workers (Singer et al., 1995).

Optimization of the density gradients for producing spermatozoa with maximized fertilizing capacity using these separation techniques have been reported (Camenzind et al., 1991; Mortimer, 1994). The 1.5-ml/two-layer (81 to 40.5%) and 1.0-ml/two-layer (95 to 47.5%) columns were both preferred. The former was selected as the gradient of choice because of higher yields when compared with the latter (35.4 versus 30.0%). The results of the present study also demonstrated that the impact of density on the final yield superseded the volume of the column. This result was also comparable with our findings that Puresperm recovered less spermatozoa than Percoll, probably due to density differences.

Since there were no proper published studies demonstrating the optimal composition and protocols to be used for the novel silane-coated silica particle density gradient columns, the conventional processing conditions for Percoll were recommended by the respective manufacturers. The processing conditions may be different for the separation of spermatozoa using colloidal silane–silica gels. This may explain why lower numbers of selected spermatozoa were generated with Puresperm columns than with the Percoll columns given the same centrifugation conditions.

The results of Experiment 3 showed that a three-layer Puresperm column could produce a final suspension of spermatozoa with higher percentage motility, percentage vitality, percentage normal morphology, and recovery of specific selected spermatozoa than a two-layer Puresperm column. The percentage of spermatozoa with normal morphology increased from 17.5 in the original semen samples to 38.4 to 41.4%

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(2.19- to 2.35-fold increase) in the final suspension. There are no other available data to compare these results. The major differences in the density and other characteristics may probably explain why Puresperm demonstrated differences, while Percoll showed no significant differences in the two- and three-layer columns. The physical characteristics of Puresperm might allow selection of fewer sperm cells, which in turn is influenced more easily by the layering effect. It is possible that greater numbers of good sperm cells could pass easily through the upper layer and the interface of Percoll than Puresperm and, as such, the two-layer Percoll could produce similar numbers of good spermatozoa as the three-layer. The Puresperm columns and interface may possibly filter out some of the good spermatozoa through their respective columns and thus exert a better fractionating effect on the remaining spermatozoa recovered in the bottom layer.

The results in Experiment 4 showed clearly that, with a larger initial loading volume of semen, the concentration of the final sperm harvest could be greatly enhanced. In this study, with a fourfold difference in the loading volume, the difference in final concentration was more than twofold (2.3- to 2.8-fold). There were also increases in the harvest of percentage motility, percentage vitality and percentage normal sperm morphology. Thus, the large-loading volume method was superior. The reasons for this particular phenomenon may be attributed to the fact that larger loading volume semen samples contain a higher total number of better-quality spermatozoa that will be centrifuged or filtered into the bottom layer, so that it will take a longer time to overdrive the column system. More poor-quality spermatozoa then get a lower chance to be filtered through the column. However, with a lower total number of better-quality spermatozoa in the small-loading volume samples, the samples soon become overdriven by the filtration, with lower-quality spermatozoa still having higher chances of going down through the column following the better-quality spermatozoa.

It was concluded that the choice of a large- or small-loading volume depends on the clinical use of the recovered sperm suspension. If the aim is to obtain a final suspension of better-quality spermatozoa in terms of normal forms, motility and vitality rather than just increased numbers, such as for ART procedures, then large-loading volumes may be preferable. If the aim is to generate as many spermatozoa as possible irrespective of the quality, such as for IUI, then small-loading volumes may be preferable.

The results of this study demonstrated several benefits of Puresperm. Since the manufacturers have withdrawn the use of Percoll for human assisted reproductive techniques, the three-layer Puresperm gradient appears to be an attractive alternative for sperm separation in medically assisted conception procedures.

References

Arora, M., Carver Ward, J.A., Jaroudi, K.A. *et al.* (1994) Is Percoll safe for *in vivo* use? *Fertil. Steril.*, **61**, 979–981.

Burkman, L.J. (1991) Discrimination between nonhyperactivated and classical hyperactivated motility pattern in human spermatozoa using computerized analysis. *Fertil. Steril.*, **55**, 363–371.

Camenzind, A., Smylie, M. and Mortimer, D. (1991) Optimization of Percoll gradients for human spermatozoa. 7th World Congress on IVF and Assisted Procreation. *Hum. Reprod.*, **6** (suppl.), 291–292.

Chen, S.U., Ho, H.N., Chen, H.F. *et al.* (1995) Comparison between a two-layer discontinuous Percoll gradient and swim-up for sperm preparation on normal and abnormal semen samples. *J. Assist. Reprod. Genet.*, **12**, 698–703.

Chen, S.U., Ho, H.N., Chen, H.F. *et al.* (1996) Combination of direct swim-up technique and discontinuous Percoll gradient centrifugation for sperm preparation of oligoasthenozoospermic samples. *Arch. Androl.*, **37**, 103–109.

Fong, C.Y., Bongso, A., Ng, S.C. *et al. (*1998) Blastocyst transfer after enzymatic treatment of the zona pellucida: improving IVF and understanding implantation. *Hum. Reprod.*, **13**, 2926–2932.

Franceus, N., Janssens, R. and Carle, M. (1995) Comparison of three Percoll media with an endotoxin-free density gradient medium. *Hum. Reprod.*, **10** (abstract 032), 17.

Gellert-Mortimer, S.T., Clark, G.N., Baker, H.W.G. *et al.* (1988) Evaluation of Nycodenz and Percoll density gradients for the selection of motile human spermatozoa. *Fertil. Steril.*, **49**, 335–341.

Gorus, F.K. and Pipeleers, D.G. (1981) A rapid method for the fractionation of human spermatozoa according to their progressive motility. *Fertil. Steril.*, **35**, 662–665.

Hall, J.A., Fishel, S.B., Timson, J.A. *et al.* (1995) Human sperm morphology evaluation pre- and post-Percoll gradient centrifugation. *Hum. Reprod.*, **10**, 342–346.

Kaneko, S., Yamaguchi, J., Kobayashi, T. *et al.* (1983) Separation of human X- and Y-bearing sperm using Percoll density gradient centrifugation. *Fertil. Steril.*, **40**, 661–665.

Kossakowski, J., Morrison, L. and Mortimer, D. (1997) Evaluation of Puresperm gradients instead of Percoll for human spermatozoa. *Hum. Reprod.*, **12** (abstract book 1), 88–89.

Kruger, T.F., Menkveld, R., Stander, F.S.H. *et al.* (1986) Sperm morphological features as a prognostic factor in *in vitro* fertilization. *Fertil. Steril.*, **46**, 1118–1123.

Kruger, T.F., Ackerman, S.B., Simmons, K.F. *et al.* (1987) A quick, reliable staining technique for human sperm morphology. *Arch. Androl.*, **18**, 275–277.

Kruger, T.F., Swanson, R.J., Acosta, A.A. *et al.* (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil. Steril.*, **49**, 112–117.

Miller, K.F., Falcone, T. and Goldberg, J.M. (1996) Variation in recovery of motile sperm after preparation by a simple Percoll gradient technique. *J. Assist. Reprod. Genet.*, **13**, 485–488.

Mortimer, D. (1994) Sperm recovery techniques to maximize fertilizing capacity. *Reprod. Fertil. Dev.*, **6**, 25–31.

Ruiz-Romero, J., Antich, M. and Bassas, L. (1995) Choosing among different technical variations of Percoll centrifugation for sperm selection. *Andrologia*, **27**, 149–153.

Scott, L. and Smith, S. (1997) Mouse *in vitro* fertilization, embryo development and viability, and human sperm motility in substances used for human sperm preparation for assisted reproduction. *Fertil. Steril.*, **67**, 372–381.

Shalika, S., Dugan, K., Pelesh, D. *et al.* (1995) Mono-Percoll separation technique improves sperm recovery of normal and male factor specimens when compared with the swim-up technique. *Hum. Reprod.*, **10**, 3195–3197.

Singer, R., Fish, B., Levinsky, H. *et al.* (1995) Separation of human semen on Percoll gradients: effect on percentage of motile and morphologically normal sperm and proportion of acrosome reacted sperm. *Int. J. Fertil. Menopausal Stud.*, **40**, 161–166.

World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction.* World Health Organization, Cambridge University Press, Cambridge.