

Human sperm selection by glass wool filtration and two-layer, discontinuous Percoll gradient centrifugation*†

Johann Rhemrev‡§

Rajasingam S. Jeyendran, D.V.M., Ph.D.‡||

Jan P. W. Vermeiden, Ph.D.§

Lourens J. D. Zaneveld, D.V.M., Ph.D.‡¶

Rush University, Rush-Presbyterian-St. Luke's Medical Center, and Institute for Reproductive Medicine, Chicago, Illinois, and Vrije Universiteit, Amsterdam, The Netherlands

Glass wool filtration and two-layer, discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation resulted in an average recovery of 50% to 70% of the progressively motile and about 50% of the hypoosmotic swelling (HOS)-positive spermatozoa. Glass wool filtration tended to be more successful than Percoll centrifugation when the ejaculates were asthenozoospermic or produced a suspect/abnormal HOS test. After selection, the acrosin activity increased approximately two- to threefold, but no significant improvement in the percentage of normal sperm forms occurred. Experiments with mixtures of untreated and frozen-thawed ejaculates confirmed that glass wool filtration is more effective in removing nonmotile and HOS-negative spermatozoa than the two-layer Percoll centrifugation technique when the percentage of these types of spermatozoa in the ejaculate is high. The simplicity of these techniques and the good recovery of apparently viable spermatozoa makes these methods more desirable than other, more complicated techniques or procedures that yield a lower recovery of motile spermatozoa. *Fertil Steril* 51:685, 1989

Under physiologic conditions, cervical mucus blocks the transport of nonmotile and poorly mo-

tile spermatozoa. In order to imitate these conditions, many in vitro fertilization (IVF) clinics partially separate the motile from the immotile spermatozoa. The most widely used selection procedure is called the "rise" or "swim-up" technique, in which the spermatozoa that are able to swim into an overlying medium are collected.^{1,2} Alternately, motile spermatozoa can be separated over an albumin column.^{3,4} Although a high percentage of the spermatozoa obtained by these methods are motile, recovery of the motile spermatozoa from the ejaculate is small, usually 15% or less. A sperm selection procedure that generally results in a higher recovery of motile spermatozoa is centrifugation over a Percoll (Pharmacia, Uppsala, Sweden) gradient.⁵⁻¹⁴ However, most of the Percoll centrifugation techniques are somewhat complicated, employing either a continuous gradient or multiple, discontinuous layers of different density, which makes them less desirable from a clinical standpoint. Simple, two-layer discontinuous Percoll gra-

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‡ Department of Obstetrics and Gynecology and Department of Biochemistry, Rush University, Rush-Presbyterian-St. Luke's Medical Center.

§ Department of Obstetrics and Gynecology, Vrije Universiteit.

|| Institute for Reproductive Medicine.

¶ Reprint requests: Lourens J. D. Zaneveld, D.V.M., Ph.D., Obstetrics-Gynecology Research, Rush-Presbyterian-St. Luke's Medical Center, 1653 West Congress Parkway, Chicago, Illinois 60612-3864.

dient centrifugation techniques also have been proposed,^{11,14} but these have not been evaluated as yet in detail. Another simple method of selecting motile spermatozoa is filtration through glass wool¹⁵⁻¹⁹ or glass beads.²⁰ Good yields of motile spermatozoa were reported by one of the glass wool techniques^{18,19} that are higher than those obtained by a "swim-up" procedure.²¹

It was the present objective to evaluate the two-layer, discontinuous, Percoll gradient centrifugation technique and glass wool filtration in more detail and to compare their efficacy. A number of different sperm parameters were studied including sperm number, motility, progressive motility, the reaction in hypoosmotic medium (hypoosmotic swelling [HOS] test), morphology, and acrosin activity. It was concluded that both the Percoll gradient centrifugation technique and glass wool filtration produce a high yield of viable spermatozoa. Both methods are equally as good in removing non-viable spermatozoa from normozoospermic ejaculates, but glass wool filtration appears to be more effective when the ejaculate is asthenozoospermic or produces a low HOS test. A brief communication on this subject has been published previously.²²

MATERIALS AND METHODS

Semen Collection and Analysis

Ejaculates were obtained by self-masturbation from apparently normal men without a history of infertility and from patients with possible fertility problems. Analyses of sperm concentration, sperm count, percent sperm motility, the percent of spermatozoa with forward progression, and the ability of the spermatozoa to swell under hypoosmotic conditions (HOS test) were performed as described previously.²³ The acrosin activity of the spermatozoa was assessed by clinical assay.²⁴

The ejaculates were allowed to liquefy completely and the sperm properties were determined. Subsequently, the ejaculate was divided into two fractions. One of the fractions was subjected to selection by glass wool filtration and the other by Percoll gradient centrifugation. The starting ejaculate volume that was used for both techniques was always 0.5 ml so that the yields could be compared after the selection procedures. Because of the limited number of spermatozoa, not all of the sperm characteristics could be determined after selection. For this reason, separate series of experiments

were performed during which different sperm characteristics were studied.

Percoll Centrifugation

The two-layer Percoll centrifugation technique employed was similar to that reported by Vermeiden et al.¹⁴ Stock isotonic Percoll was prepared by adding 9 ml Percoll to 1 milliliter of a solution consisting of 49.6 gm Ham's F-10 (Irvine Scientific, Santa Ana, CA), 13.17 gm NaHCO₃, 627 mg Na-lactate, and 37.5 mg penicillin and 37.5 mg streptomycin in 0.5 l glass-distilled H₂O. The Ham's F-10 solution that was used in all other cases consisted of 4.96 Ham's F-10, 1.317 gm NaCHO₃, 62.7 mg Na-lactate, 7 mg CaCl₂, 37.5 mg penicillin, and 37.5 mg streptomycin in 0.5 l glass-distilled H₂O, pH 7.8. The bottom fraction of the Percoll gradient (81% Percoll) consisted of 9 ml of the Percoll stock solution mixed with 1 ml of Ham's F-10 solution. The upper fraction (36% Percoll) consisted of 4 ml of the Percoll stock solution mixed with 6 ml of Ham's F-10 solution. To prepare the system for the selection of spermatozoa, 1 ml of the bottom fraction was placed in a 15 ml plastic, conical centrifuge tube (Becton Dickinson, Rutherford, NJ) and 1.0 ml of the upper fraction was carefully layered over the bottom fraction.

Subsequently, 0.5 ml of a well-mixed, completely liquefied ejaculate was layered over the upper fraction and the tube was centrifuged at $750 \times g$ for 15 minutes. Both supernatant fractions were removed until 0.3 ml of the Percoll and sperm pellet remained in the bottom of the tube. The sperm pellet was resuspended in 1 ml Ham's F-10 solution containing 3.5% human serum albumin (HSA; fraction V, No A-1653; Sigma Chemical Co., St. Louis, MO). The mixture was recentrifuged at $200 \times g$ for 5 minutes, the supernatant discarded, the pellet resuspended in 1 ml Ham's F-10 solution containing 3.5% HSA and centrifuged at $200 \times g$ for 5 minutes. The supernatant solution again was discarded and the sperm pellet was resuspended in 1 ml Ham's F-10 solution containing 3.5% HSA.

Glass Wool Filtration

Glass wool filtration was performed by a modification of the technique by Jeyendran et al.¹⁸ as described in more detail by Van der Ven et al.²¹ The tip was removed from a 1 ml Becton Dickinson syringe and 15 mg of glass wool (microfiber code 112; Mannville Co, Denver, CO) was placed into the barrel of the syringe using a forceps and was gently

Table 1 Sperm Characteristics Before and After Selection Using Ejaculates with a Sperm Motility of $\geq 40\%$ ^a

Characteristic	Before treatment ^b	After treatment	
		Percoll	Glass wool
Number ($\times 10^6$)	36.4 \pm 6.3	15.5 \pm 2.6	16.0 \pm 2.5
Motility (%)	57.2 \pm 2.3	72.1 \pm 2.5	78.7 \pm 2.0
Number of motile sperm ($\times 10^6$)	23.0 \pm 5.2	11.9 \pm 2.3	12.5 \pm 1.9
Progressive motility (%) ^c	43.4 \pm 2.6	56.0 \pm 3.0	63.8 \pm 2.2
Number of progressively motile sperm ($\times 10^6$)	18.4 \pm 4.7	9.7 \pm 2.1	10.5 \pm 1.8
HOS-positive sperm (%)	62.7 \pm 2.6	74.8 \pm 2.1	78.7 \pm 2.3
Number of HOS-positive sperm ($\times 10^6$)	25.5 \pm 5.9	12.8 \pm 3.1	13.4 \pm 2.8

^a See text for experimental detail. Mean \pm SEM. N = 26 except for the HOS test (n = 17).

^b In 0.5 ml ejaculate.

^c Percent of all spermatozoa.

pushed further to a depth of 6.0 mm from the bottom of the syringe. The filter was rinsed twice with 2 ml Ham's F-10 solution containing 3.5% HSA or until no glass wool fibers were present in the eluates based on light microscopic observations at 200 \times . A portion (0.5 ml) of the well-mixed ejaculate was diluted with 0.5 ml Ham's F-10 solution containing 3.5% HSA. The diluted semen was centrifuged at 1,000 $\times g$ for 10 minutes, the supernatant solution was removed, and the sperm pellet was resuspended in 0.25 ml Ham's F-10 solution containing 3.5% HSA. The sperm mixture was placed over the wet glass wool column and allowed to filter. Subsequently, 0.75 ml of Ham's F-10 containing 3.5% HSA was added to rinse the column and to recover trapped, viable spermatozoa. The entire filtrate was collected (1 ml).

Statistical Analysis

The mean and standard error (SEM) were calculated for all of the variables studied. The data were subjected to analysis of variance and, where appropriate, to Newman-Keuls significant difference test. Values were considered significantly different for each other when $P < 0.05$.

RESULTS

Initially, the motility and membrane integrity of the spermatozoa were studied. In the first series of

experiments, ejaculates with normal sperm motility ($\geq 40\%$) were employed. Both selection methods produced highly motile sperm populations with good forward progression (Table 1). Averages of 52% to 54% of the progressively motile spermatozoa were recovered from the ejaculate. The recovery of HOS-positive spermatozoa from the original ejaculate averaged 50% to 53%. Filtration over glass wool tended to give somewhat better results than Percoll gradient centrifugation, but the differences were statistically insignificant.

In the second series of experiments, ejaculates were used that possessed a sperm motility of less than 40%, i.e., were asthenozoospermic (Table 2). Although both techniques improved the percent motility and forward progression, the sperm population obtained after filtration over glass wool possessed significantly ($P < 0.05$) higher percentages of motile and forwardly progressive spermatozoa than that obtained after Percoll gradient centrifugation. An average of 56% of the progressively motile spermatozoa were recovered after Percoll gradient centrifugation and an average of 71% after glass wool filtration.

The percentage of HOS-positive spermatozoa was approximately the same whether the asthenozoospermic ejaculates were treated by glass wool filtration or Percoll centrifugation (Table 2). Because sperm motility and their reaction in the HOS

Table 2 Sperm Characteristics Before and After Selection Using Ejaculates with a Sperm Motility of $< 40\%$ ^a

Characteristic	Before treatment ^b	After treatment	
		Percoll	Glass wool
Number ($\times 10^6$)	18.9 \pm 4.7	6.5 \pm 1.7	6.0 \pm 2.2
Motility (%)	31.4 \pm 2.4	57.1 \pm 3.9*	73.6 \pm 3.2*
Number of motile sperm ($\times 10^6$)	6.2 \pm 1.8	3.8 \pm 1.1	4.1 \pm 1.3
Progressive motility (%) ^c	21.0 \pm 3.7	36.0 \pm 4.4**	57.0 \pm 3.9**
Number of progressively motile sperm ($\times 10^6$)	4.5 \pm 1.5	2.5 \pm 0.8	3.2 \pm 1.1
HOS-positive sperm (%)	55.8 \pm 5.7	73.6 \pm 5.4	72.7 \pm 2.0
Number of HOS-positive sperm ($\times 10^6$)	9.1 \pm 2.5	4.4 \pm 1.2	3.6 \pm 1.1

^a See text for experimental detail. Mean \pm SEM. N = 11 except for the HOS test (n = 9). Values with a common superscript (* or **) are significantly different from each other ($P < 0.05$).

^b In 0.5 ml ejaculate.

^c Percent of all spermatozoa.

Table 3 Sperm Motility (%) Before and After Selection of Mixed Semen Samples Consisting of Untreated and Frozen-Thawed Ejaculates^a

Percent frozen-thawed semen	Before treatment	After treatment	
		Percoll	Glass wool
0	55.0 ± 3.4	75.0 ± 2.7	77.8 ± 4.2
25	42.3 ± 0.5	71.3 ± 3.1	83.3 ± 6.9
50	33.0 ± 1.7	56.3 ± 4.0	85.5 ± 3.6
75	20.3 ± 0.7	46.0 ± 4.6	89.0 ± 4.0
100	0	0	0

^a See text for experimental detail. The values represent mean ± SEM (n = 4).

test are not necessarily related, the HOS test data obtained in the first two series of experiments were reexamined by separating the ejaculates according to their HOS test results instead of sperm motility. Ejaculates were considered normal if they contained 60% or more HOS-positive spermatozoa and suspect or abnormal if they contained less than 60% HOS-positive spermatozoa. The HOS-normal ejaculates (n = 13) averaged $56 \pm 15 \times 10^6$ HOS-positive spermatozoa (71 ± 2% of ejaculate total) and the HOS-suspect/abnormal ejaculates (n = 13) averaged $24 \pm 5 \times 10^6$ HOS-positive spermatozoa (50 ± 2% of ejaculate total). Glass wool filtration yielded, respectively, $26 \pm 7 \times 10^6$ and $20 \pm 8 \times 10^6$ HOS-positive spermatozoa from the normal and suspect/abnormal ejaculates and Percoll centrifugation, respectively, $23 \pm 7 \times 10^6$ and $16 \pm 5 \times 10^6$. Therefore, more HOS-positive spermatozoa tended to be recovered after glass wool filtration than after Percoll gradient centrifugation, particularly when the original ejaculates were suspect/abnormal.

In a third series of experiments, the acrosin activity of the spermatozoa was determined before and after the selection of 11 ejaculates. Before selection, the acrosin activity was $30.9 \pm 3.6 \mu\text{IU}/10^6$ spermatozoa, ranging from 8 to $50 \mu\text{IU}/10^6$ spermatozoa. After Percoll gradient centrifugation, the acrosin activity was $86.6 \pm 11.1 \mu\text{IU}/10^6$ spermatozoa, ranging from 35 to $144 \mu\text{IU}/10^6$ spermatozoa, and after glass wool filtration $77.6 \pm 9.4 \mu\text{IU}/10^6$ spermatozoa, ranging from 35 to $139 \mu\text{IU}/10^6$ spermatozoa. The acrosin values after the two selection procedures were significantly higher ($P < 0.05$) than the starting material, but did not differ from each other.

In a fourth series of experiments, the morphology of the spermatozoa was studied before and af-

ter selection of 12 ejaculates. Before selection, the mean ± SEM of normally shaped spermatozoa was $62.1 \pm 2.2\%$, with a range of 46% to 71%. After selection by Percoll and glass wool, these values were, respectively, $68.3 \pm 3.7\%$ (range, 45% to 85%) and $65.1 \pm 2.0\%$ (range, 55% to 79%). The values before and after selection were not significantly different from each other.

The second series of experiments indicated that glass wool filtration may be more effective in separating motile spermatozoa than Percoll gradient centrifugation when the ejaculate is asthenozoospermic. To investigate this in more detail, a final series of experiments was performed with different mixtures of untreated and freeze-immobilized spermatozoa. Ejaculates were pooled and placed in the freezer at -15°C for 24 hours and slowly thawed to room temperature. This procedure was repeated twice and resulted in the death of 94% to 100% of the spermatozoa, as was verified by the vital stain procedure. Subsequently, ejaculates from the same donor were obtained and mixtures of the untreated and frozen-thawed semen samples were prepared so that the final mixtures either consisted entirely of the untreated semen sample, 75% untreated and 25% treated, 50% untreated and 50% treated, 25% untreated and 75% untreated, or entirely of the treated sample. The motility and HOS test values of these samples were assessed before and after subjecting them to the selection procedures. The results are summarized in Tables 3 and 4. The percentage of motile and HOS-positive spermatozoa remained essentially identical after glass wool filtration independent of the concentration of frozen-thawed spermatozoa in the original sample until essentially no motile or HOS-positive spermatozoa were present in the sample, i.e., when the entire sample consisted of frozen-

Table 4 HOS-Positive Spermatozoa (%) Before and After Selection of Mixed Semen Samples Consisting of Untreated and Frozen-Thawed Ejaculates^a

Percent frozen-thawed semen	Before treatment	After treatment	
		Percoll	Glass wool
0	54.8 ± 4.6	70.0 ± 3.5	71.5 ± 2.4
25	51.5 ± 3.1	68.0 ± 5.3	71.8 ± 6.1
50	38.0 ± 2.4	53.0 ± 5.7	71.0 ± 3.4
75	23.3 ± 7.1	33.0 ± 7.8	68.0 ± 6.4
100	6.8 ± 0.8	3.5 ± 0.9	0

^a See text for experimental detail. Mean ± SEM (n = 4).

thawed spermatozoa. By contrast, Percoll centrifugation yielded steadily decreasing percentages of motile and HOS-positive spermatozoa as the percentage of frozen-thawed spermatozoa in the original sample increased. The decrease became statistically significant ($P < 0.05$) when 50% of the semen mixture or more consisted of frozen-thawed semen.

DISCUSSION

The mechanisms whereby two-layer, discontinuous Percoll gradient centrifugation and glass wool filtration select viable spermatozoa is poorly understood. It is likely that glass wool filtration removes nonviable spermatozoa by two mechanisms: (1) spermatozoa with abnormal membranes adhere to the glass wool; and (2) nonmotile spermatozoa are less likely to pass through the glass wool column. In regard to Percoll, it has been suggested⁶ that centrifugation causes the sperm head (the heaviest part of the cell) to be directed centrifugally so that all spermatozoa align parallel to the centrifugal force. Subsequently, those spermatozoa that are progressively motile will move more readily towards the bottom of the centrifuge tube than the nonmotile or poorly motile spermatozoa.

The present results show that these two relatively simple methods produce good yields of motile and HOS-positive spermatozoa. On the average, about 50% to 70% of the progressively motile spermatozoa and about 50% of the HOS-positive spermatozoa were recovered by these techniques. The percent sperm motility obtained by the present methods also was high, averaging about 70% to 80%, except in the case of Percoll centrifugation of asthenozoospermic ejaculates. Although it has been reported that fractions with a higher percentage of sperm motility can be obtained by more complicated Percoll gradient or other procedures,^{2,6-13} the yield of motile spermatozoa obtained by these techniques, i.e., the recovery from the original ejaculate, generally is so low that this methodology is less desirable clinically, particularly in the case of oligozoospermic ejaculates.

As could have been expected, ejaculates with below normal percent sperm motility or percent HOS-positive spermatozoa showed a greater improvement in these characteristics after treatment than normal ejaculates. Neither glass wool filtration nor two-layer, discontinuous Percoll gradient centrifugation caused a significant improvement in the percentage of normal sperm forms. However,

both methods caused a significant increase in spermatozoa with high acrosin levels. Based on these properties, it can be concluded that both methods yield sperm populations that are more viable and probably more fertile per cell basis than the original ejaculates. Both techniques already have been employed with success for IVF.^{14,19,21}

Glass wool filtration appears to be more effective in removing nonmotile and HOS-negative spermatozoa than the two-layer Percoll centrifugation technique, particularly when the ejaculate contains a high percentage of these types of spermatozoa. This was further exemplified by preparing mixtures of untreated and frozen-thawed spermatozoa. As the percentage of frozen-thawed spermatozoa increased, the percent of motile and HOS-positive spermatozoa of the fractions recovered after Percoll centrifugation decreased. By contrast, no such decreases were seen when the mixtures were subject to glass wool filtration, indicating that the glass wool filter retained its capacity to remove inactive spermatozoa even when the percentage of such spermatozoa became high. Thus, glass wool filtration appears to be preferable to two-layer Percoll centrifugation when the ejaculate possesses a low percentage of motile or HOS-positive spermatozoa. Another advantage of the glass wool filtration technique is that it tends to break up viscous ejaculates.^{15,16} Thus, in contrast to other selection techniques, glass wool filtration can be used with poorly liquefying ejaculates.

The glass wool filtration technique has been criticized for causing ultrastructural damage to spermatozoa.¹⁷ However, of the five ejaculates studied by these investigators, only one showed significant membrane damage. In contradiction to the electron microscopic observations, the spermatozoa obtained by glass wool filtration of this ejaculate survived cryopreservation equally as well or better than those obtained from the other ejaculates. In addition, the studies by these investigators showed that all glass wool filtered samples survived storage at 22°C for 12 hours better than the spermatozoa in the original ejaculates. It is unlikely that such sperm survival would have occurred when their membranes were damaged. Another study did not find ultrastructural changes after glass wool filtration.¹⁹ The present results also argue against the occurrence of significant damage to the spermatozoa since glass wool filtration resulted in sperm populations with a high percentage of forwardly progressive and membrane-intact (HOS-positive) spermatozoa. Furthermore, sperm populations ob-

tained by glass wool filtration effectively penetrate zona-free hamster eggs²⁵ and fertilize human eggs in vitro.^{19,21} Therefore, no good evidence exists that glass wool filtration leads to significant functional changes of the spermatozoa.

Care has to be taken in the preparation of the glass wool columns so that the glass wool is not too tightly or too loosely packed. The correct type of glass wool must be used. Company specifications are often poor, so that each batch of glass wool has to be tested first for its ability to separate spermatozoa before it can be used clinically. Also, some glass wool fibers may appear in the filtrate even after the column has been rinsed extensively. This presents no problems for IVF, since the eggs are removed after incubation with the spermatozoa but the presence of fibers may be of concern when the spermatozoa are used for artificial insemination. Therefore, before using glass wool filtered spermatozoa for artificial insemination, it is important to assure the absence of glass wool fibers by light microscopic observations.

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