Tohoku J. Exp. Med., 1993, **169**, 225-231

Effects of Percoll Discontinuous Density Gradients vs SpermPrep™II* vs Sephadex G-50 Gel Filtration on Semen Parameters.

JEROME H. CHECK, PANAYIOTIS M. ZAVOS*, DIANE KATSOFF and DANIEL KIEFER

*The University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School at Camden, Cooper Hospital/University Medical Center, Department of OB/GYN, Division of Reproductive Endocrinology and Infertility, Camden, New Jersey, 08103, USA, and *University of Kentucky, Andrology Institute of Lexington and Central Baptist Hospital, Lexington, Kentucky, 40502, USA*

CHECK, J.H., ZAVOS, P.M., KATSOFF, D., KIEFER, D. *Effects of Percoll Discontinous Density Gradients vs SpermPrep™II* vs Sephadex G-50 Gel Filtration on Semen Parameters.* Tohoku J. Exp. Med., 1993, **169** (3), 225-231 —— There are various methods of separating sperm from seminal plasma for subsequent intrauterine insemination (IUI) and for in vitro fertilization (IVF). The purpose of the present study was to assess and compare semen parameters following Sephadex, Percoll and SpermPrep™II separation techniques. The SpermPrep™ II is also a Sephadex preparation but uses a different bead size, less Sephadex and is a quicker method. The specimens ($n=16$) were initially evaluated for count, (C ; $\times 10^6$/ml) % motility (MO), grade of motility (GR ; %), and HOS test scores. Each specimen was then divided into 3 equal aliquots and prepared as follows : 1) layered onto a modified Percoll discontinuous density gradient column ; 2) processed using SpermPrep™II ; and 3) filtered through a Sephadex gel filtration column (G-50). The results show all 3 treatments yielded lower ($p<0.01$) counts as compared to control values but no differences were noted among them. Percoll and SpermPrep™II increased MO ($p<0.01$) where Sephadex reduced it ($p=$NS). Percoll and SpermPrep™II improved the % of best quality sperm but not Sephadex with SpermPrep™II being higher than Percoll. There were increases ($p<0.05$) in HOS values in all experimental treatments with SpermPrep™II being the best. However, this study did not show as many males with HOS scores below 50% as noted in other studies. Finally, Percoll and SpermPrep™II seem equally effective methods for producing high quality sperm for IUI or IVF although SpermPrep™II is quite faster. ———— SpermPrep™II ; Sephadex ; Percoll dis-

Received January 12, 1992 ; revision accepted for publication February 5, 1993.

Address for correspondence : J.H. Check, M.D., The University of Medicine and Dentistry of New Jersey, Camden, New Jersey, 08103, USA. Presented at the American Society of Andrology Annual Meeting, Bethesda, Maryland, USA, March 28-30, 1992.

*SpermPrep™II, ZBL, Inc., Lexington, Kentucky, USA.

continuous density gradient; hypoosmotic swelling test; semen filtration

Human seminal plasma, which is the nongamete portion of the ejaculate, is composed of cells, cellular particles and debris, and fluids from the testis, excretory ducts, and accessory sex glands of the male. The total ejaculate, however, in addition to the seminal plasma, contains the gametes that are a mixture of motile, nonmotile, and perhaps agglutinated spermatozoa.

Currently, spermatozoa used for either intrauterine insemination (IUI) or in vitro fertilization insemination (IVF) could yield higher rates of conception if these spermatozoa are selected on the basis of their motility, progressive motility, and morphological characteristics (McClure et al. 1989). Furthermore, removal of all debris and background, as well as antifertility factors present in the seminal plasma may also increase the fertilizing ability of these spermatozoa (Russell and Rogers 1987). A number of semen manipulative techniques are currently available to remove the undesirable spermatozoa, debris, and other factors and to increase sperm quality before performance of the various assisted reproductive techniques. These various manipulative techniques include the most popular, the swim-up or sperm-rise method (Russell and Rogers 1987). Less popular methods include Ficoll centrifugation (Kaneko et al. 1980), Percoll density gradient (McClure et al. 1989; Pickering et al. 1989), and Sephadex or glass-wool-fiber filtration (Quinlivan et al. 1982; Katayama et al. 1989) and the swim-down or sedimentation-type methods (Dmowski et al. 1979). It should be emphasized however, that many of these manipulative techniques often increase sperm quality at the expense of numbers of recovered spermatozoa, which may not be advantageous, especially for patients with seminal deficiencies such as oligozoospermia or asthenozoospermia. Also these techniques are very tedious and time consuming. Recently, a new technique (SpermPrep™II; ZBL, Inc., Lexington, KY, USA) has been introduced which yields a significantly higher level of sperm recovery and is quite rapid and reproducible (Zavos and Centola 1990a; Centola and Zavos 1991). Because of these advantages, the SpermPrep™II technique could have significant effects in the manner that seminal specimens are prepared and improved prior to their use in any artificial (noncoital) reproduction procedures.

In this study, we evaluated and compared among each other three sperm separation methods that are rapid and efficient in the recovery of motile sperm, ie., Percoll gradient separation vs SpermPrep™II vs Sephadex G-50 gel filtration.

Materials and Methods

Semen collection and evaluation

Ejaculates were collected from sixteen semen donors of known fertility. Ejaculates were collected via masturbation with 3-4 days of abstinence each time. After semen samples were produced and completely liquefied (within 15-30 min), each specimen was evaluated according to standard procedures recommended by the World Health Organiza-

tion (WHO) with a phase-contrast microscope (World Health Organization 1987). Semen measures included volume, sperm count per milliliter, percentage sperm motility, grade of sperm motility (Zavos and Cohen 1980), sperm morphologic features and the hypoosmotic swelling test (HOS ; Van der Ven et al. 1986). All seminal parameters were evaluated by the same technician.

Semen preparation

After semen evaluation, each sample was divided into three aliquots and treated as follows : 1) processed via a modified Percoll discontinuous density gradient column ; 2) filtered via SpermPrep™II method and ; 3) filtered through a Sephadex G-50 gel filtration column. Data for the various treatments applied were compared to each other using one way analysis of variance followed by either the Student's *t*-test or the paired Student's *t*-test where appropriate.

Modified Percoll density

The discontinuous, four-layer Percoll gradient technique (Suarez et al. 1986), modified for human semen (Check et al. 1992) was used to separate one aliquot of semen from each specimen. In brief, an isotonic, 90% (v/v) solution of Percoll (Sigma Chemical Co., St. Louis, MO, USA) was prepared by adding 10X DPBS (Irving Scientific, Irvine, CA, USA) buffer. This solution was diluted with modified Human Tubal Fluid (HTF) medium, (modified with 0.05% Human Serum Albumin Fraction V), to produce 50%, 60%, 70% and 80% (v/v) Percoll solutions (Suarez et al. 1986). Using a 15 ml disposable centrifuge tube, 1 ml of each solution was carefully layered one over another, starting with 80% at the bottom, then a layer of 70%, 60% and then 50% on the top. The semen was diluted 1 : 1 (v/v) with modified HTF, layered (up to 2 ml) onto the top of the discontinuous gradients and was centrifuged for 20 min at 300 × g. The 80% fraction (0.75 ml) that contained the sperm pellet near the bottom was collected with a disposable pipette, transferred to a fresh tube with 1 ml of fresh modified HTF medium and recentrifuged for 5 min at 300 × g. The final sperm pellet was reconstituted up to a final volume of 0.5 ml in fresh buffer and evaluated accordingly.

SpermPrep™II filtration procedure

The SpermPrep™II was used very similarly as previously described for the SpermPrep™ technology (Zavos and Cohen 1980 ; Siiteri et al. 1988 ; Zavos and Centola 1990b ; Zavos 1991) with some simplified modifications and according to the manufacturer's specifications (ZBL, Inc.). Modified Ham's F-10 was used for all steps during the SpermPrep™II filtration process (Centola and Zavos 1991). SpermPrep™II comes with an extension funnel which was attached to the column during the preparation steps. It should be emphasized also, that proper standard laboratory techniques were employed in our laboratory throughout the filtration process. Those techniques included complete sterility and maintenance of all semen diluents, the SpermPrep™II filter and all other materials within a temperature range of 30-35°C.

Sephadex G-50 filtration

Glass filtration columns (0.9 cm × 23 cm), filled halfway with Sephadex G-50 Fine (Sigma) were hydrated by adding 20 ml of Hepes Buffered Hank's Solution (HBHS) and simultaneously allowing it to run through the column. Semen was diluted 1 : 1 with HBHS and centrifuged at 500 × g for 10 min and the sperm pellet was resuspended to 1 ml with fresh HBHS. The washed sperm aliquot was added to the last 1 ml above the gel beads in the column. A 10 ml volume of HBHS was added on top of the column-semen to allow the sperm to flow out of the column for 10-15 min. The recovered semen aliquot was centrifuged at 500 × g for 10 min and the sperm pellet was resuspended in 0.5 ml of fresh buffer and evaluated accordingly.

Results

The semen parameters before and after the three separation procedures were employed are shown in Table 1. The mean motile sperm densities (MSD : sperm count $\times 10^6 \times$ motility %) were $35.4 \pm 24.9 \times 10^6$/ml for the initial specimen (means $\pm$ S.D.). These values were significantly reduced ($p < 0.01$) to 12.2 ± 9.4 for Percoll, 10.1 ± 8.4 for SpermPrep™II, and 9.3 ± 11.1 for Sephadex G-50. No differences were noted among the three treatments ($p > 0.05$).

There was a significant increase in percentage of grade A sperm ($p < 0.01$) for SpermPrep™II compared to either Percoll or Sephadex G-50; Percoll was significantly higher ($p < 0.01$) than Sephadex G-50. Percoll and SpermPrep™II, but not Sephadex G-50, were significantly higher than the Initial specimen ($p < 0.01$). There were significant reductions ($p < 0.01$) in MSD values between the initial concentration and following the application of all three treatments. However, no differences were noted in MSD values recovered ($p > 0.05$) among the three treatments applied.

All three techniques showed significant improvements in the HOS test results as compared to the initial specimen ($p < 0.01$). The highest HOS test score of 84.9% in SpermPrep™II was significantly higher ($p < 0.01$) than scores obtained either via Percoll or Sephadex G-50 (76.5% vs. 71.9%, respectively). The percent motility was significantly increased when the Percoll and SpermPrep™II methods were applied as compared to the initial specimen ($p < 0.01$). Although differences in the percent motility were lower with Sephadex G-50 (40.2%) than the initial values (50.3%), these differences were not significant ($p > 0.05$).

Discussion

The objective of the current study was to evaluate the qualitative and quantitative characteristics of spermatozoa recovered following semen separation

Table 1. *Comparison of Percoll Discontinuous Density Gradient, SpermPrep™II Filtration Method and Sephadex G-50 Filtration on Semen Parameters Recovered (means ± S.D.)*

Treatments	Sperm parameters considered ($n = 16$)			
	Count ($\times 10^6$)	Motility (%)	Grade (%)	HOS* (%)
Initial (control)	70.3 ± 46.9^a	50.3 ± 13.5^a	31.8 ± 15.8^a	66.3 ± 8.9^a
Percoll	19.3 ± 14.8^b	64.5 ± 12.3^b	43.4 ± 16.8^b	76.5 ± 12.2^b
SpermPrep™II	13.7 ± 11.2^b	73.8 ± 13.1^b	58.4 ± 13.82^c	84.9 ± 10.1^b
Sephadex G-50	25.7 ± 29.8^b	40.2 ± 23.7^a	33.7 ± 14.6^a	71.9 ± 13.4^a

*HOS, Hypoosmotic swelling test.

[a,b,c]Means followed by the same superscript within each column are not different ($p < 0.05$).

via the Percoll gradient, SpermPrep™II filtration and Sephadex G-50 filtration methods. The rationale for comparing the SpermPrep™II and Sephadex G-50 was to compare two methods that utilize two different sizes of Sephadex beads as a means for filtering human spermatozoa, with the Sephadex G-50 method utilizing the largest bead size of the two methods. It should be emphasized, however, that no matter what technique may be employed in separating spermatozoa for assisted reproductive technology (ART) purposes, the selected or recovered spermatozoa must possess a variety of qualities and adequate numbers in order to allow proper fertilization and the onset of pregnancy to take place.

A significant improvement in the percentage of motile sperm was noted by using the SpermPrep™II method as compared to the Sephadex G-50 method. The MSD values were similar in all three procedures ($p > 0.05$). Also, the highest HOS test scores were achieved via the use of the SpermPrep™II method. Previous observations showed that the Sephadex G-50 method yielded lower HOS scores when compared to Percoll (Check et al. 1992). At this time, there is no data to support the concept that higher pregnancy rates are associated with specimens with higher HOS test scores, but, only that specimens with HOS test scores of $<50\%$ may yield lower pregnancy rates (Siiteri et al. 1988).

The noted qualitative sperm improvements obtained via the SpermPrep™II method (Table 1) are consistent with previous observations (Zavos and Centola 1990b, Centola and Zavos 1991; Zavos 1991, 1992). Also, the data presented in this study indicated that the SpermPrep™II filtration method yielded spermatozoa of similar qualitative characteristics as those recovered via the Percoll method ($p > 0.05$). However, the SpermPrep™II filtration method was less time consuming than the Percoll procedure. The filtration procedure was completed in 20 to 25 min, whereas the Percoll procedure took approximately 1.0 hr or more to complete. This is, of course, of great clinical importance because the SpermPrep™II filtration process can save precious time for the technical personnel, the clinician, and the patient. Equally important, the time savings might significantly reduce the detrimental effects on sperm viability and subsequent fertilization especially with deficient and/or frozen-thawed specimens.

In conclusion, since no significant differences were noted between the Percoll discontinuous gradient separation and the SpermPrep™II filtration column method, further comparative studies will be needed to determine which technique is more effective toward achieving higher pregnancy rates. The SpermPrep™II method, however, is easier and faster to perform than the Percoll method and allows the recovery of large numbers of high quality spermatozoa. The SpermPrep™II method, along with the increased filtration efficiency, and the important time savings that it provides, demonstrates that this new technique is the method of choice for selecting motile, morphologically normal spermatozoa from fresh or frozen-thawed human semen for use in the various assisted reproductive technologies including intrauterine insemination.

References

1) Centola, G.M. & Zavos, P.M. (1991) Qualitative/quantitative improvements in post-thaw human semen using SpermPrep™. *J. Assisted Reproductive Technology-Andrology.*, **2**, 335-239.
2) Check, J.H., Katsoff, D., Kozak, J. & Lurie, D. (1992) Effect of swim-up, Percoll and Sephadex sperm separation methods on the hypoosmotic swelling test. *Hum. Reprod.*, **7**, 109-111.
3) Dmowski, W.P., Gaynor, L., Rao, R., Lawrence, M. & Scommegna, A. (1979) Use of albumin gradients for X and Y sperm separation and clinical experience with male sex preselection. *Fertil. Steril.*, **31**, 52-57.
4) Kaneko, S., Moriwaki, C. & Sato, H. (1980) Development of multiple exposure photography method for analysis of sperm motility and preparation of washed sperm with Ficoll density gradient. *Jpn. J. Fertil. Steril.*, **25**, 491-493.
5) Katayama, K.P., Stehlik, E. & Jeyendran, R.S. (1989) In vitro fertilization outcome : glass wool-filtered sperm versus swim-up sperm. *Fertil. Steril.*, **52**, 670-672.
6) McClure, R.D., Nunes, L. & Tom, R. (1989) Semen manipulations improved sperm recovery and function with a two-layer Percoll gradients. *Fertil. Steril.*, **51**, 874-877.
7) Pickering, S.J., Fleming, T.P., Braude, P.R., Bolton, V.N. & Gresham, G.A.G. (1989) Are human spermatozoa separated on a Percoll density gradient safe for therapeutic use ? *Fertil. Steril.*, **51**, 1024-1029.
8) Quinlivan, W.L.G., Preciado, K., Long, T.L. & Sullivan, H. (1982) Separation of human X and Y spermatozoa by albumin gradients and Sephadex chromatography. *Fertil. Steril.*, **37**, 104-107.
9) Russell, L. D. & Rogers, B.J. (1987) Improvement in the quality and fertilization potential of a human sperm population using the rise technique. *J. Androl.*, **8**, 25-33.
10) Siiteri, J.E., Dandekar, P. & Meizel, S. (1988) Human sperm acrosome reaction-initiating activity associated with the human cumulus oophorus and mural granulosa cells. *J. Exp. Zool.*, **246**, 71-80.
11) Suarez, S.S., Wolf, D.P. & Meizel, S. (1986) Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res.*, **14**, 107-121.
12) Van der Ven, H.H., Heyendran, R.S., Al-Hasani, S., Perez-Pelaez, M., Dietrich, K. & Zaneveld, L.J.D. (1986) Correlation between human sperm swelling in hypoosmotic medium (HOS) test and in vitro fertilization. *J. Androl.*, **7**, 190-196.
13) World Health Organization (1987) WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge, The Press Syndicate of the University of Cambridge, pp. 1-13.
14) Zavos, P.M. (1991) Selection de spermatozoides viables a partit d'echantillons de spermes humains congeles-decongeles : comparaison de la methode du "swim-up" et d'une nouvelle methode de filtration : le SpermPrep™. *Contraception-fertilite-sexualite*, **19**, 293-297.
15) Zavos, P.M. (1992) Preparation of human frozen-thawed seminal specimens using the SpermPrep™II filtration method : Improvements over the conventional swim-up method. *Fertil. Steril.*, **57**, 1326-1330.
16) Zavos, P.M. & Cohen, M.R. (1980) Bovine mucus penetration test : An assay for fresh and cryopreserved human spermatozoa. *Fertil. Steril.*, **34**, 175-176.
17) Zavos, P.M. & Centola, G.M. (1990a) Qualitative and quantitative improvements in human spermatozoa recovered via the swim-up and a new semen filtration method. *Infertility*, **13**, 25-34.
18) Zavos, P.M. & Centola, G.M. (1990b) Ameliorations seminales des spermatozoides utilises pour la fecondation artificielle : Comparaison entre la methode de "nage

ascendante" et une nouvelle methode de filtration du sperme sur colonne. *Contraception fertilite-sexualite*, **18**, 943-948.