SPERM PREPARATION FOR INTRA-UTERINE INSEMINATION

Nicola J. Davies, MSc,
David C. Cumming, MB ChB, FRCSC,

Department of Obstetrics and Gynaecology,
University of Alberta

ABSTRACT

Controlled ovarian hyperstimulation and sperm injection or intra-uterine insemination (COH/SI) have become important forms of treatment for unexplained and male factor infertility. An extensive literature on sperm preparation has developed (more than 2,000 papers), and the recent withdrawal of indications for human use of Percoll makes it necessary to re-examine the methods available which have been proven effective. In this paper, we have reviewed methods of sperm preparation and their applicability to intra-uterine insemination.

RÉSUMÉ

L'hyperstimulation ovarienne contrôlée et l'injection de sperme, ou insémination intra-utérine (HOC/IS), sont maintenant des formes de traitement importantes pour l'infertilité non expliquée ou attribuable à l'homme. Comme un nombre considérable de publications sur la préparation des spermatozoïdes ont été publiées (plus de 2000 articles) et qu'on vient de déclarer Percoll inutilisable pour les humains, il nous faut maintenant ré-examiner les procédés qui ont prouvé leur efficacité. Cet article passe en revue les méthodes actuelles de préparation du sperme et évalue leur possibilité d'utilisation pour l'insémination intra-utérine.

J SOC OBSTET GYNAECOL CAN 1999;21(14):1294-1303

KEY WORDS

Intrauterine insemination, sperm preparation.

Received on May 7th, 1999. Revised and accepted on June 29th, 1999.

INTRODUCTION

Preparation of sperm by removing the liquid component of semen and resuspending spermatozoa in an artificial medium has always been an essential step in *in vitro* fertilization (IVF). Although not subjected to randomized controlled trials, worthwhile increases in pregnancy rates have also been reported using prepared or "washed" sperm for intra-uterine insemination (IUI) of women who have been treated with clomiphene or gonadotrophins.[1,2] Intrauterine insemination has, therefore, become an accepted treatment for male factor infertility and for unexplained infertility. Although the need to separate sperm from seminal plasma for IUI has been questioned,[3] a range of separation techniques of variable complexity are used for both IVF and IUI.

Techniques for the preparation of sperm include a simple wash with centrifugation and mechanical resuspension,[2] Percoll gradients,[4] swim-up procedures,[5] albumin gradients,[6] glass wool filtration,[7] Sephadex columns,[8] Ficoll columns[9] and migration sedimentation.[10] The methods share the common goal of recovery of sperm from seminal fluid with the highest possible yield, producing a specimen of high count and motility that is free of seminal proteins and microbial contamination, without introducing iatrogenic effects that may diminish sperm motility, viability and, ultimately, fertilizing potential. The goals of sperm preparation for IVF and IUI differ. While lower numbers of sperm may be acceptable at the cost of higher quality for IVF, in the case of sperm washing for IUI, it is considered necessary to have a suspension containing as many high quality sperm as possible.

Sperm preparation techniques are not problem-free. Concerns have focused on the possibility of mechanical damage to sperm from centrifugation and resuspension, leukocyte and bacterial contamination and contamination of the sperm sample with elements from the preparation medium. Centrifugation-induced mechanical damage in particular may lead to low recovery of motile sperm, may damage sperm integrity and increase levels of reactive oxygen species (ROS), particularly in specimens with poor semen characteristics.[11]

Investigators examining sperm washing methods have frequently focused on the intermediate measure of *in vitro* sperm quality without addressing the question of improved pregnancy rates and outcome. Pregnancy rates are variable among centres, methods, diagnoses and concomitant forms of ovarian stimulation. Randomized controlled trials involving all of the various factors are difficult to organize because of the numbers required to satisfy each variable. Even when relatively simple methods are being compared, very large numbers of subjects are needed.[2] Such large multicentre studies clearly show that the methods are effective, and encourage those centres with poor pregnancy results to re-examine their techniques.

Sperm washing using centrifugation and mechanical resuspension is the simplest of techniques and probably the most commonly used for IUI.[2] For euspermic males, it produces a reasonable sample which, coupled even with the use of clomiphene citrate, produces in our centre a pregnancy rate consistently in the seven to eight percent per cycle over six cycles, as originally reported a decade ago.[3] While the method is simple and fast, there is concern that it causes mechanical damage. Other techniques may need to be considered.

SPERM SEPARATION USING PERCOLL AND OTHER DENSITY GRADIENTS

Several density gradients have been investigated over the last 12 years, but Percoll (Pharmacia-Upjohn, Sweden) was by far the most commonly used until it was recently withdrawn for human use following an adverse report from the Food and Drug Administration in the USA. A wide variety of Percoll-based gradients have been described for use as both continuous[12] and discontinuous[13] gradients. Probably the most practical Percoll gradient procedure was developed by Dravland and Mortimer.[14]

Percoll gradients have been described as being ideal for cryopreserved samples, retrograde ejaculate samples, epididymal and testicular aspirates and for use in preparing samples from patients with poor semen quality. The method is said to select predominantly motile sperm cells with normal morphology which are free from contamination by other seminal constituents. Advantages of Percoll processing over simple washing and swim-up methods have been offered by some[15-18] but by no means all[19-21] investigators. Some reports have dealt with the deleterious effects of Percoll upon the ultrastructural integrity of human sperm[19] as well as upon longevity as measured by motility maintenance.[22] But in a recent study, Percoll produced significantly greater numbers of specimens with

normal sperm morphology and higher absolute quantitative improvement than did swim-up techniques.[23]

Sperm had fully condensed chromatin and a clear appearance because of the absence of coating envelopes or a high level of surface fructose residues, in contrast with non-capacitated sperm. Although swim-up yielded a higher percentage of oval and motile sperm than Percoll, the Percoll-recovered sperm had a significantly higher percentage of acrosome intact sperm. In male factor cycles, when Percoll was compared to swim-up, Percoll extracted a higher total number of sperm, increased the concentration of motile sperm and enhanced the recovery rate of motile, morphologically normal sperm.[23]

Suggesting that normal Percoll gradients are not very effective in the treatment of oligoasthenozoospermia, Ord and co-workers[24] developed a density gradient consisting of a reduced volume of a discontinuous Percoll gradient for application in severe male factor infertility (mini-Percoll). The authors reported significantly higher *in vitro* fertilization rates for this specific group of patients. Percoll and mini-Percoll have been compared with the swim-up technique for preparing sperm; the results have been inconsistent. In one study, Percoll and mini-Percoll produced similar results, with a mean recovery of sperm with progressive motility significantly higher than that achieved with swim-up.[18] However, swim-up resulted in the recovery of sperm with a higher mean motility, velocity and percentage of normal forms. The authors concluded that swim-up was superior to Percoll and mini-Percoll. In another study, mini-Percoll produced specimens with significantly greater normal sperm morphology, morphology improvement, motility, hypo-osmotic swelling and survival than direct swim-up and SpermPrep filtration.[25] The data here suggested that mini-Percoll was superior to swim-up or SpermPrep.

Although Percoll has been used extensively and has been widely accepted as the method of choice for the preparation of sperm for intra-uterine insemination, concerns have arisen about its safety. Percoll is a colloidal suspension of silica particles coated with polyvinylpyrrolidine (PVP). Silica is a recognized chronic irritant to human tissues.[26] It has been postulated that the introduction of silica particles into the peritoneal cavity during intra-uterine insemination might stimulate an inflammatory response similar to that observed from some silica-based surgical glove powders. However, sperm are washed and centrifuged twice following isolation on a Percoll density gradient and most, if not all of the silica particles, are removed, thus reducing the chances of an inflammatory response.

Percoll preparation methods have been used for some time by IUI programmes with no untoward effects.[27] Damage to sperm and to embryos has been postulated but has not been reported, with many studies describing normal pregnancies leading to normal infants. Percoll has been reported to be safe with mouse and human sperm, with mice oocytes and with mouse IVF, embryo development and viability.[28,29] There was no evidence of endotoxin contamination in Percoll when using a mouse embryo bioassay as a test.[30] It has also been suggested that the PVP coating on these particles might cause damage. There is as yet no indication of an increased risk of severe chromosomal aberrations or major congenital malformations after the birth of children from intracytoplasmic sperm injection (ICSI) using sperm prepared from Percoll.[31] The concerns of possible damage, however, resulted in Percoll not being approved for human use by the US Food and Drug Administration and the recent withdrawal of Percoll from all clinical applications. This resulted in assisted reproductive technology (ART) laboratories having to find alternative sperm preparation methods. Other density gradient preparations have been investigated, including Nycodenz (Nyegaard & Co, Oslo, Norway), Iodixanol, Accudenz and PureSperm (Nidacon Int., Sweden). Despite its withdrawal from clinical practice, Percoll remains the laboratory gold standard to which other methods are compared.

Gellert-Mortimer and colleagues[22] first investigated the potential of an alternative density gradient medium to Percoll. Nycodenz consists of N,N-bis-2,3-dihydroxypropyl-5-(N-[2,3-dihydroxypropyl] acetamido) 2,4,6-triiodoiso-pthalamide dissolved in Tris buffer. Both continuous and discontinuous Nycodenz gradients have been evaluated but a four-layer discontinuous gradient was found to produce populations of highly motile sperm, with better yields and survival than swim-up and Percoll, from oligozoospermic and asthenozoospermic semen samples.[22] This technique clearly has great potential in the preparation of motile sperm from poor quality semen, and warrants further investigation for IUI.

The undesirable formation of a dense pellet during density gradient centrifugation can be avoided if the density of the bottom-most gradient layer exceeds the density

of human sperm. Under these conditions, sperm will migrate to the interface above this layer during centrifugation. Due to the inherent physical and chemical properties of Percoll, Ficoll and Nycodenz, isotonic gradients with densities greater than human sperm cannot be achieved. However, a dimeric form of Iohexol called Iodixanol is suitable for this purpose. Iodixanol is non-ionic and consists of two aromatic rings, each substituted with three iodine atoms.[32] Sperm appeared to tolerate Iodixanol density gradient centrifugation well and exhibited a recovery of motile, morphologically normal sperm, with a sperm survival during a 24-hour period similar to that produced with Percoll. Although there appears to be no major advantage to the use of Iodixanol over Percoll with respect to sperm yield or survival, there is an advantage with respect to patient safety if the final sperm preparation is to be used for therapeutic insemination. It has been tested extensively to determine its potential as a mutagen, antigen and reproductive toxin. The safety of Iodixanol has been established further through a series of clinical trials that have resulted in the approval of Iodixanol for human use. There have been no studies of its use in insemination.

Accudenz is a non-ionic medium based on a triiodonated molecule which produces sperm fractions with more extended motility than Percoll.[22,33] The density of the medium is high (2.1 g/ml), and water solubility is high because of the presence of a substituted ring linked to hydrophilic groups. Both Accudenz and Percoll gradients yielded sperm with increased motility compared to the initial semen, however, recovery and retention of motile sperm were more efficient with Accudenz. The high recovery of motile sperm in Accudenz did not occur at the expense of sperm maturity or function. There were similar penetration rates in the zona-free hamster oocyte penetration assay by sperm prepared with Percoll and Accudenz. Accudenz appears to be a good choice of sperm preparation medium, due to the lack of endotoxin formation, low rate of complications with internal human use and its approval for human use by the FDA. While use in intra-uterine insemination has been recommended,[33] it does not appear to have had much further investigation.

PureSperm is the registered trade name of a newly developed density gradient product designed for the separation of normal sperm cells by centrifugation of human ejaculate. It consists of an isotonic salt solution containing colloidal-silica particles which are firmly coated with saline. The material is not cytotoxic when used according to the standard procedures of ART. Because of the close similarity in formulation with a mineral rather than organic substance, it was anticipated that PureSperm would also show the same characteristics of high specific gravity with low viscosity effect. There were no significant differences between the sperm populations obtained using PureSperm and those using Percoll in either the percentage yield, progressively motile fraction, sperm kinetics or maximal hyperactivation levels.[34] Yields were rather variable, and some samples prepared using Percoll did show modified motility, including premature hyperactivation, immediately after preparation. PureSperm can be used as a direct replacement for Percoll. It allows the preparation of selected highly motile populations of human sperm which are functionally indistinguishable from those obtained using Percoll. The ability to use the same gradient technique and centrifugation conditions will make the transition to this new medium a simple process for ART laboratories. While use in IUI has been recommended, there do not appear to be any studies demonstrating its effectiveness.

SPERM SEPARATION USING SWIM-UP

Since 1969, when Edwards and his colleagues[35] first described a simple two-step washing procedure, swim-up from a washed pellet had been highly successful in producing sperm preparations for IVF. The method has been adapted for use in IUI, particularly for suboptimal specimens. A high percentage of morphologically normal motile sperm were recovered while other cells were absent. The method did produce reactive oxygen species having a deleterious effect on the sperm and yielding a low recovery rate, and it has been suggested that it should not be used in clinical practice.[35] It has been replaced by the swim-up from semen technique, in which the specimen is diluted with insemination medium and centrifuged twice. The resultant pellet containing the sperm is overlaid with medium. During a one-hour incubation, the motile fractions swim into the medium and are isolated. The method has been found to produce results similar to Percoll in a randomized prospective trial.[29]

Migration-sedimentation involves a swim-up technique with a gravitational settling of spermatozoa from the upper medium layer.[36] Sperm recovered by this method were reported to have an improved percentage of normal

morphology, according to strict criteria, over untreated semen.[37] Migration-sedimentation proved best when compared to swim-up at improving sperm motility and morphology in samples from both fertile and sub-fertile patients, but the yield was relatively low. The fertilization rate *in vitro* was significantly higher after swim-up than by migration-sedimentation. Sperm survival also declined after 24 hours and the method was not recommended for sperm preparation.[38] Migration-sedimentation remains an interesting method of sperm preparation, which may be useful for male factor samples to be used for ICSI where sperm survival is not a factor. It should not be used for IUI.

Sperm Select is a commercial product of a highly purified sterile and non-pyrogenic preparation of sodium hyaluronate (Pharmacia, Uppsala, Sweden) with an average molecular weight of 3,000,000 Da, used at a final concentration of one mg/mL in culture medium. Swim-up from semen into Sperm Select gave a significantly higher percentage of motile sperm than the traditional swim-up method.[39] It is not known if the improved sperm recovery resulted from the use of Sperm Select or from the use of a method which did not involve the initial formation of a pellet of unselected sperm. The increased viscosity of the medium will cause a high proportion of motile sperm to reach the upper portion of the solution contained in the preparation tube. However, numbers of sperm are relatively low,[40] making it somewhat less useful for IUI than more complex forms of ART.

FILTRATION METHODS

The use of the glass wool column separates sperm according to motility and the size of the sperm head[41] and has been reported to remove the majority of debris as well as agglutinated and dead sperm from semen samples.[42] Recovery of viable sperm appears significantly higher than swim-up procedures, and sperm preparations retain their ability to fertilize human oocytes *in vitro*. It also improves sperm quality, even in men with oligo-and/or asthenozoospermia.[43,44] There is evidence of a much higher percentage of normal chromatin condensed sperm with higher percentages of mature nuclei.[41]

Although glass wool filtration did result in higher sperm recovery rates, it did not have any effect on fertilization and pregnancy outcomes.[45] The outcome of IVF-ET following preparation with glass wool filtration in combination with swim-up showed better results than swim-up alone. It was, therefore, concluded that glass wool filtration alone did not produce better results but, in combination with swim-up, it resulted in significantly better sperm morphology and improved outcome of IVF-ET for fresh samples.[46] This technique has a possible application in clinical IVF but must be viewed with caution, as a report indicated the danger of glass wool fragments in the final sperm population inducing damage to the sperm plasma membrane and acrosome.[43] A commercially available glass wool filtration kit (Sperm Fertil, Mello Ltd., Exeter, UK) has been shown not to release glass wool fragments into the culture medium. Use of glass wool techniques have been limited in IUI but have been reported to compare well with other methods.[47]

The glass bead column filtration method, which was first described for the preparation of hamster sperm for *in vitro* capacitation, has been shown to select motile sperm efficiently from semen with higher yields than the swim-up technique.[48,49] Samples processed with glass bead columns provide motile forms but with less velocity and linearity than sperm recovered by the swim-up technique. Because of the large size of the beads, concerns about their possible carry-over into insemination medium were lessened. However, reports have shown that glass beads are still present in the filtrate. Using glass beads for effective sperm preparation even for assisted reproduction has not been widely accepted and there are no reports of its use.

A commercial sperm suspension kit based upon Sephadex beads (SpermPrep: ZBL, Inc., Lexington, KY) has become available. SpermPrep Medium is specifically formulated and highly recommended for use with SpermPrep filters. The medium is sterile, buffered with HEPES to maintain a pH within physiological levels, and supplemented with three percent bovine serum albumin. The SpermPrep filtration method entraps sperm with morphological abnormalities by isolating them in a filter matrix that consists of a physiologically inert polysaccharide derivative, resulting in recovery of a greater number of motile, morphologically normal sperm, than swim-up.[50,51] It has been reported to produce significantly higher yields of morphologically normal sperm than migration-sedimentation and swim-up.[52] Results of investigations for clinical application have been varied. Swim-up and Percoll methods produced no change in the functional integrity of the sperm, but SpermPrep resulted in a marked decrease.

There was also a higher proportion of abnormal semen after preparation with Sephadex than after the other preparation methods. However, the time required to harvest sperm through the SpermPrep column can be as short as 15 minutes of processing.[54] The SpermPrep column appears not to concentrate the motile sperm, which can be a problem with oligozoo-or asthenozoospermia ejaculates. The SpermPrep method has been reported to increase the number of sperm recovered in comparison to the wash swim-up technique in post-thaw human semen.[55] The overall quality of sperm using SpermPrep was reported to be similar to samples obtained using a commercially available Percoll gradient.[34] It is also claimed that SpermPrep may be beneficial in preparing sperm from infertile men with low acrosin profiles and is able to withhold single-stranded abnormal sperm.[55] In this study, this method presented the highest seminal plasma contamination in insemination media.

The Zavos Swim-up Column (ZSC) (Meditech, Canada), like SpermPrep, is a simple self-contained system for the recovery of high-quality progressively motile sperm from semen. The ZSC has been reported to be a fast, inexpensive and simple one-step standardized method. The ZSC washes and simultaneously selects sperm, thus eliminating the need for dilution and centrifugation. The ZSC column combines the swim-up/swim-down methods, yielding a greater number of high-quality, morphologically normal, motile sperm. Because the semen specimen is safely protected within the conical cavity inside the ZSC column prior to the addition of medium, the risks of mixing the medium with the specimen are eliminated. Thus, the possible contamination of the medium that contains the healthy sperm is totally reduced. The incubation period (15 minutes to one hour depending upon the procedure to be performed) causes motile and morphologically normal sperm to swim up and out of the cone, and down into medium. When the ZSC column is used in conjunction with SpermPrep medium, maximal retrieval of highly motile sperm can be expected. There appear to be no studies specifically for IUI. Using a similar technology, The Research Instruments Migration Sedimentation Chamber (RI Limited, UK) for sperm preparation is reported to recover a high percentage of vigorously motile, morphologically normal sperm, with improved quality. This chamber is described as separating free swimming sperm from sperm aggregated due to anti-sperm antibodies. Centrifugation is not required, resulting in no sperm tail damage, less induction of ROS and a reduction in operator time. Again controlled studies for IUI appear not to have been undertaken.

TRANSMEMBRANE MIGRATION METHODS

Transmembrane migration techniques make use of an apparatus in which motile sperm are separated from the culture medium by a nucleopore membrane filter. The filters have pores which are cylindrical and at right angles to the plane of the membrane.[56] The sperm, therefore, have straight channels to swim through, but the membranes have a very low transparency, and the yield of sperm recovered is very poor. Another approach to separating viable sperm by means of a membrane (L4 membrane) was undertaken using a membrane which had been developed for selective removal of leukocytes.[57,58] This may be of importance in patients with increased numbers of leukocytes in the ejaculate. The L4 membrane seems to be selective for sperm with normal membrane integrity and sperm producing low amounts of reactive oxygen species.[59] Semen analysis and the hamster egg penetration test showed improvement in sperm quality and fertilizing ability after filtration through the L4 membrane.[57] The penetration rate and index after filtration also increased over values for the swim-up method. Results suggest that sperm filtration through the L4 membrane provides results superior to those of the traditional swim-up. Due to the substantial saving in time associated with its use, the L4 membrane can be used during sperm processing. While it has been recommended for IUI, there have been no studies.

The importance of seminal leukocyte concentrations for sperm quality has been a matter of debate.[60,61] Methods which remove leukocytes, for example the L4 membrane or removal by paramagnetic bead treatment,[62] can enhance fertilizing potential. The addition of anti-oxidants like glutathione, hypotaurine, and such ROS-scavenging enzymes as catalase or superoxide dismutase to the sperm washing medium has also been reported to prevent the damage caused by centrifugation and maintain the motility of sperm in reconstituted leukocyte-contaminated semen by protecting them against excess ROS.[63-65] A commercial salt solution (Sperm-Fit, Ellios Biomedia) containing antioxidants can be used to treat sperm during

liquefaction and centrifugation, causing an increase in the recovery of sperm with good function. Incubation with Sperm-Fit produced a higher percentage of motility after Percoll preparation.[6]

DISCUSSION

The most commonly used methods of sperm preparation have been simple wash and resuspension, swim-up and Percoll gradient. It is difficult to find good evidence that there is a substantial and consistent difference between the methods. Following the recent withdrawal of Percoll from all clinical applications, it has become necessary to find alternative sperm preparation methods and to re-examine the older methods. Although the success of a sperm preparation method is often assessed by the yield of motile sperm, a choice of method also depends on its technical complexity, the materials and apparatus required and time costs. Estimated preparation and technologist times are appended in Table 1. Any exposure of sperm during preparation to factors that may cause iatrogenic sperm dysfunction must obviously be avoided. Consequently, methods involving centrifugal washing prior to the selection of motile sperm should, in theory at least, be avoided. In practice, it has not been shown that other methods are consistently superior in IUI.

Direct swim-up from semen or centrifugation and mechanical resuspension are the simplest ways to obtain motile sperm populations with normal semen samples. Obtaining satisfactory results for lower quality semen samples remains problematic. Clinical experience suggests that a reasonable pregnancy rate may be obtained with male infertility by the use of IUI[3] but randomized control studies are lacking. Extrapolation of laboratory results to the clinical situation or even of clinical IVF results to IUI without specific research is clearly inappropriate. Until useful data become available, the preferred method of sperm preparation will remain a matter of debate in both IVF and IUI. The choice of semen preparation technique does not rest on fertilization and pregnancy probabilities, but on practical implications and on examination of patients before their introduction into IUI programmes. It is difficult to determine which sperm preparation method should be used. The lack of standardization and the heterogeneity of semen make this decision even more difficult. No single separation technique has been shown to be consistently superior in all situations. Ideally, sperm specimens should be evaluated individually to determine the best separation method before cycles of treatment are begun. The technique which yields the most morphologically normal, motile sperm that survive in culture should be used. The ideal sperm preparation technique should, therefore, be rapid, simple and inexpensive, recover all motile sperm in the specimen, result in no damage or physiological alteration of the separated sperm, remove dead sperm and other cells and remove toxic substances. Further studies investigating all sperm preparation techniques need to be carried out to find the best method to produce consistently excellent results.

TABLE 1
PREPARATION AND TECHNOLOGIST TIMES INVOLVED IN SPERM PREPARATION METHODS

Method	Total time	Technologist time
Percoll	40 minutes	12 minutes
Iodixanol	54 minutes	12 minutes
Accudenz	40 minutes	12 minutes
Nycodenz	40 minutes	12 minutes
SpermPrep 11	54 minutes	13 minutes
Zavos Swim-up	15-60 minutes	5 minutes
Swim-up	90 minutes	10 minutes
Glass wool filtration	25 minutes	13 minutes
Centrifuge and resuspension	25 minutes	10 minutes
Transmembrane methods	20 minutes	5 minutes

REFERENCES

1. Van der Westerlaken LA, Naaktgeboren N, Helmerhorst FM. Evaluation of pregnancy rates after intrauterine insemination according to indication, age, and sperm parameters. J Assist Reprod Genet 1998;15:359-64.
2. Guzick DS, Carson SA, Coutifaris C, Overstreet JW, Factor-Litvak P, Steinkampf MP, Hill JA, Mastroianni L Jr, Buster JE, Nakajima SJ, Vogel DL, Canfield RE. Efficacy of superovulation and intrauterine insemination in the treatment of infertility. N Engl J Med 1999;340:177-83.
3. Cumming DC. Pregnancy rates following intrauterine insemination with washed or unwashed sperm. Fertil Steril 1988;49:735-6.
4. Gorus FK, Pipeleers DG. A rapid method for the fractionation of human spermatozoa according to their progressive motility. Fertil Steril 1981;35:662-5 .
5. Lopata A, Patullo MJ, Chang, A, James B. A method for collecting motile spermatozoa from human semen. Fertil Steril 1976;27:677-84.
6. Harris SJ, Milligan MP, Masson GM, Dennis KJ. Improved separation of motile sperm in asthenospermia and its application to artificial insemination homology. Fertil Steril 1981;36:219-21.

7. Paulson JD, Polakoski KL, Leto S. Further characterization of glass wool column filtration of human semen. Fertil Steril 1979;32:125-6.
8. Steeno O, Adimoelja A, Steeno J. Separation of X- and Y-bearing human spermatozoa with the Sephadex gel-filtration method. Andrology 1975;7:95-7.
9. Harris RAP. A highly efficient method for washing mammalian spermatozoa. J Reprod Fertil 1976;48:347-53.
10. Lucena E, Lucena C, Gomez M,Ortiz JA, Ruiz A, Arango A, Diaz C, Beuerman C. Recovery of motile sperm using the migration-sedimentation technique in an *in vitro* fertilisation-embryo transfer programme. Hum Reprod 1989;4:163-5.
11. Shekarriz M, DeWire DM, Thomas AJ Jr., Agarwal AA. A method of human semen centrifugation to minimise the iatrogenic sperm injuries caused by reactive oxygen species. Eur Urol 1995;28:31-5.
12. Bolton VN, Braude PR. Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self-generating density gradients. Arch Androl 1984;13:167-76.
13. Pousette A, Akerlof E, Rosenborg L, Fredricsson B. Increase in progressive motility and improved morphology of human spermatozoa following their migration through Percoll gradients. Int J Androl 1986;9:1-13.
14. Dravland JE, Mortimer D. A simple discontinuous Percoll gradient procedure for washing human sperm. IRCS Med Sci 1985;13:16-9.
15. Guerin JF, Mathieu C, Lornage J, Pinatel MC, Boulieu D. Improvement of survival and fertilising capacity of human spermatozoa in an IVF programme by selection on discontinous Percoll gradients. Hum Reprod 1989;4:798-804.
16. Punjabi U, Gerris J, van Bijlen J, Del Beke L, Gielis M, Buytaert P. Comparison between different pre-treatment techniques for sperm recovery prior to intrauterine insemination, GIFT or IVF. Hum Reprod 1990;5:75-83.
17. Serafini P, Blank W, Tran C, Mansourian M, Tan T, Batzofin J. Enhanced penetration of zona-free hamster ova by sperm prepared by Nycodenz and Percoll gradient centrifugation. Fertil Steril 1990;53:551-5.
18. Ng FL, Liu DY, Baker HW. Comparison of Percoll, mini-Percoll and swim-up methods for sperm preparation from abnormal semen samples. Hum Reprod 1992;7: 261-6.
19. Arcidiacono A, Walt H, Campana A, Balerna M. The use of Percoll gradients for the preparation of subpopulations of human spermatozoa. Int J Androl 1983; 6:433-45.
20. Bornman MS, Sevenster CBO, Milander C, Otto BS, Olivier I. The effect of semen processing on sperm morphology. Andrologia 1989;21:117-9.
21. Morales P, Vantman D, Barros C, Vigil P. Human spermatozoa selected by Percoll gradient or swim-up are equally capable of binding to the human zona pellucida and undergoing the acrosome reaction. Hum Reprod 1991;6:401-4.
22. Gellert-Mortimer S, Clarke GN, Baker HWG, Hyne RV, Johnston WIH. Evaluation of Nycodenz and Percoll density gradient for the selection of motile human spermatozoa. Fertil Steril 1988;49:335-41.
23. Prakash P, Leykin L, Chen Z, Toth T, Sayegh R, Schiff I, Isaacson K. Preparation by differential gradient centrifugation is better than swim-up in selecting sperm with normal morphology (strict criteria). Fertil Steril 1998;69:722-6.
24. Ord T, Patrizio P, Marello E, Balmaceda JP, Asch RH. Mini-Percoll: a new method of semen preparation for IVF in severe male factor infertility. Hum Reprod 1990;5:987-9.
25. Smith S, Hosid S, Scott L. Use of postseparation sperm parameters to determine the method of choice for sperm preparation for assisted reproductive technology. Fertil Steril 1995;63:591-7.
26. Bolton VN, Warren RE, Braude PR. Removal of bacterial contaminates from semen for in vitro fertilization or artificial insemination by the use of buoyant density centrifugation. Fertil Steril 1986;46:1128-32.
27. Pardo M, Barri PN, Bancells N, Coroleu B, Buxederas C, Pomerol JM Jr., Saater J. Spermatozoa selection in discontinuous Percoll gradients for use in artificial insemination. Fertil Steril 1988;49:505-9.
28. Scott L, Smith S, Hosid S. The effect of Percoll on mouse in vitro fertilization (IVF), zygote development and embryo viability. Abstracts of the 51st annual meeting of the American Society for Reproductive Medicine. Fertil Steril 1995;Suppl:0-132:564.
29. Carrell DT, Kuneck PH, Hatasaka HH, Jones KP, Campbell BF. A randomized, prospective analysis of five sperm preparation techniques before intrauterine insemination of husband sperm. Fertil Steril 1998;69:122-6.
30. Scott L, Smith S. Mouse in vitro fertilization, embryo development and viability and human sperm motility in substances used for human sperm preparation for assisted reproduction. Fertil Steril 1997;67:372-81.
31. Wisanto A, Bonduelle M, Camus M, Tournaye H, Magnus M, Liebaers I, Van Steirteghem A, Devroey P. Obstetric outcome of 904 pregnancies after intracytoplasmic sperm injection. Hum Reprod 1996;11 (Suppl 4):121-9.
32. Smith TT, Byers M, Kaftani D, Whitford W. The use of Iodixanol as a density gradient material for separating human sperm from semen. Arch Androl 1997;38:223-30.
33. Sbacia M, Sayme N, Grasso J, Vigue L, Huszar G. Sperm function and choice of preparation media: comparison of Percoll and Accudentz gradient centrifugation. Fertil Steril 1996;53:551-5.
34. Centola GM, Herko R, Andolina E, Weisensel S. Comparison of sperm separation methods: effect on recovery, motility, motion parameters, and hyperactivation. Fertil Steril 1998;70:1173-5.
35. Edwards RG, Bavister BD, Steptoe PC. Early stages of

fertilisation *in vitro* of human oocytes matured *in vitro*. Nature 1969;221:632-5.
36. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 1988;9:367-76.
37. Menkveld R, Stander FSH, Kotze TJ vW, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 1990;5:586-92.
38. Yavetz H, Hauser R, Homonnai ZT, Paz GF, Lessing JB, Amit A, Yogev I. Separation of sperm cells by sedimentation technique is not suitable for *in vitro* fertilization purposes. Andrologia 1996;28:3-6.
39. Huszar G, Willets M, Corrales M. Hyaluronic acid (SpermSelect) improves retention of sperm motility and velocity in normozoospermic and oligozoospermic specimens. Fertil Steril 1990;54:1127-34.
40. Zimmerman ER, Robertson KR, Kim H, Drobnis EZ, Nakajima ST. Semen preparation with the SpermSelect system versus a washing technique. Fertil Steril 1994;61:269-75.
41. Henkel RR, Franken DR, Lombard CJ, Schill WB. Selective capacity of glass wool filtration for the separation of human spermatozoa with condensed chromatin: a possible therapeutic modality for male factor cases? J Assist Reprod Genet 1994;11:395-400.
42. Paulson JD, Polakoski KL, Leto S. Further characterization of glass wool column filtration of human semen. Fertil Steril 1979;32:125-9.
43. Sherman JK, Paulson JD, Liu KC. Effect of glass wool filtration on ultrastructure of human spermatozoa. Fertil Steril 1982;36:643-7.
44. Van der Ven HH, Jjeyendran RS, Al-hassani S, Tunnerhoff A, Hoebbel K, Diedrich K, Kreb D, Oerez-Pelae M. Glass wool column filtration of human semen in relation to swim-up procedure and outcome of IVF. Hum Reprod 1988;3:85-7.
45. Chen SU, Ho HN, Chen HF, Chao KH, Wu MY, Chen CD, Huang SC, Lee TY, Yang YS. Combination of direct swim-up technique and discontinuous Percoll gradient centrifugation for sperm preparation of oligoasthenozoospermic samples. Arch Androl 1996;37:103-9.
46. Al-Hasani S, Alpustun S, Ludwig M, Diedrich K, Bauer O, KupkerW, Wolff A, Krebs D. The combination of two semen preparation techniques (glass wool filtration and swim-up) and their effect on the morphology of recovered spermatozoa and outcome of IVF-ET. Int J Androl 1996;19:55-60.
47. Weerasooriya TR, Goonewardene IM. Sperm preparation and in utero insemination in the management of subfertility. Ceylon Med J 1996;41:54-7.
48. Daya S, Gwatkin RBL, Bissessar H. Separation of motile human spermatozoa by means of a glass bead column. Gamete Res 1987;17:375-80.
49. Calamera JC, Quiros MC, Brugo S, Nicholson RF. Comparison between swim-up and glass bead column techniques for the separation of human spermatozoa. Andrologia 1991;23:259-61.
50. Zavos PM. Sperm preparation substances—toxic? [letter] Fertil Steril 1997;68:569-70.
51. Zavos PM. Preparation of human frozen-thawed seminal specimens using SpermPrep filtration method: improvements over the conventional swim-up method. Fertil Steril 1992;57(6):326-30.
52. Sofikitis N, Miyagawa I. The SpermPrep filtration column selectively entraps single-stranded DNA spermatozoa. Jpn J Fertil Steril 1992;37:384-7.
53. Check JH, Katsoff D, Kozak J, Lurie D. Effect of swim-up, Percoll and Sephadex sperm preparation methods on the hypo-osmotic swelling test. Hum Reprod 1992;7: 109-11.
54. Gabriel LK, Vawda AI. Preparation of human sperm for assisted conception: a comparative study. Arch Androl 1993;30:1-6.
55. Centola GM, Herko R, Andolina E, Weisensel S. Comparison of sperm separation methods: effect on motility, recovery, motion parameters and hyperactivity. Fertil Steril 1998;70:1153-7.
56. Chijioke PC, Crocker PR, Gilliam M, Owens MD, Pearson RM. Importance of filter structure for the transmembrane migration studies of sperm motility. Hum Reprod 1988;3:241-4.
57. Agarwal A, Ikemoto I, Loughlin KR. Levels of reactive oxygen species before and after sperm preparation of swim-up and L4 filtration. Arch Androl 1994:32:169-74.
58. Agarwal A, Manglona A, Loughlin KR. Improvement in semen quality and sperm fertilizing ability after filtration through the L4 membrane: comparison of results with swim-up technique. J Urol 1992:147:1539-41.
59. Agarwal A, Manglona A, Loughlin KR. Filtration of spermatozoa through L4 membrane: a new method. Fertil Steril 1991;56:1162-5.
60. Tomlinson MJ, Barratt CLR, Cooke ID. Prospective study of leukocytes and leukocyte subpopulations in semen suggests that they are not a cause of male infertility. Fertil Steril 1993;60:1069-75.
61. Aitken RJ, West K, Buckingham W. Leukocyte infiltration into the human ejaculate and its association with semen quality, oxidative stress and sperm function. J Androl 1994;15:343-52.
62. Aitken RJ, Buckingham DW, West K, Brindle J. On the use of paramagnetic beads and ferrofluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. Am J Reprod Immunol 1996;35:541-51.
63. Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. Leukocytospermia is associated with poor semen quality. Fertil Steril 1990;53:528-36.
64. Baker HWG, Brindle J, Irvine DS. Protective effect of antioxidants on the impairment of sperm motility by

activated polymorphonuclear leukocytes. Fertil Steril 1996;65:411-9.
65. Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J Reprod Fertil 1995:103:17-26.
66. Parinaud J, Le Lannou D, Vieitez G, Griveau JF, Milhet P, Richoilley G. Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit) following ejaculation. Hum Reprod 1997;12:2434-6.