ANDROLOGY

A New Method for the Purification of Human Motile Spermatozoa Applying Density-Gradient Centrifugation: Polysucrose Media Compared to Percoll Media

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Purpose: A newly developed method for the isolation of human motile spermatozoa using density-gradient centrifugation was compared with the traditionally used Percoll technique.

Method: Sperm samples were divided into two equal aliquots, which were purified with either the traditionally performed Percoll technique or a new alternative based on polysucrose/Optiprep media. For each sample the isolation was performed during the same run of the centrifuge.

Results: The average recovery of progressively motile spermatozoa with the polysucrose/Optiprep method was significantly higher ($48 \pm 7\%$) than with the Percoll method ($38 \pm 6\%$) ($n = 18$). The average percentage of motile spermatozoa and the motility score were similar in the purified preparations.

Conclusions: The new polysucrose/Optiprep-based density-gradient centrifugation technique for the isolation of motile human spermatozoa is as good as the traditionally used Percoll method and may replace it in connection with assisted reproduction techniques.

KEY WORDS: sperm purification; assisted reproduction techniques; in vitro fertilization; density-gradient centrifugation.

INTRODUCTION

The isolation of motile spermatozoa with fertilizing ability based on discontinuous density-gradient centrif-

ugation techniques is widely recognized as some of the most useful methods in connection with assisted reproduction techniques (ART)(1). The method based on Percoll media has gained widespread use and has been employed successfully for the past decade (2,3). As a result, probably a hundred thousand or more children have been born after the fertilizing spermatozoa have been in contact with the Percoll media. However, the manufacturer, Pharmacia/Upjohn, has now withdrawn Percoll media for the use of sperm purification, because they did not find the product safe to use in connection with ART. Because there is no obvious candidate to replace the Percoll media, there is a need to develop alternatives to the Percoll media.

The Percoll media possess a number of advantages that paved the way for their widespread use: (i) the most motile spermatozoa are isolated at a high yield using an easy-to-perform laboratory procedure; and (ii) motile spermatozoa are quickly removed from the seminal plasma, somatic cells, and dead and incorrectly formed spermatozoa. Motile spermatozoa are thereby protected from the potential stress of reactive oxygen species, which have been shown to reduce the fertilizing capacity (4,5). However, the Percoll media also possess some disadvantages: (i) silica-based polymers have a tendency to precipitate, resulting in copurification with the spermatozoa; and (ii) the endotoxin content is very high.

The aim of this study was to develop a sperm purification technique in which the advantages of the Percoll media are maintained while the disadvantages are omitted. We tested two substances, polysucrose and Optiprep, which are approved for human in vivo use,

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to form two density media, which in a similar way to the Percoll media, can be used to purify progressively motile spermatozoa in connection with ART.

MATERIALS AND METHODS

Semen samples were obtained from 18 healthy men attending our clinic for infertility treatment. Six of the samples were oligozoospermic according to World Health Organization classification, with a total sperm count of less than $20 \times 10^6/\text{ml}$, and 12 were normospermatic, with a total concentration of more than 20×10^6 sperm cells per ml. Each semen sample was divided into two equal aliquots, which were used for simultaneous semen purification with the Percoll media and the polysucrose media, respectively. The semen sample was placed on top of the Percoll or polysucrose gradient and treated as described below.

Preparation of Percoll Media

The Percoll media were prepared as described by Yding Andersen and Jørgensen (6). Briefly, Percoll (Pharmacia/Upjohn, Uppsala, Sweden) was diluted 1:10 with Earle's balanced salt solution (EBSS) $\times 10$ (Gibco, Flow Laboratories, Roskilde, Denmark) and supplemented with 100 IU/ml penicillin (Leo, Copenhagen, Denmark) and 1.8 g/L NaHCO_3 (Sigma, Bie & Berntsen, Denmark) adjusted to 280 mosmol/kg, and designated "100% Percoll." The day before use the 100% Percoll was equilibrated with 5% CO_2 and 95% air for 8–10 min and stored at 4°C under an atmosphere of 5% CO_2 and 95% air until the next day.

On the day of use the Percoll medium was brought to room temperature and centrifuged (10 min at 1000g) to sediment any aggregates of silica particles.

Preparation of Polysucrose Media

A medium with a density of approximately 1.05 g/ml was prepared by making a sperm washing solution (Medicult, Copenhagen, Denmark), 12% (w/v) with polysucrose-400 (Nycomed, Oslo, Norway). Osmolality was adjusted to 330 mosmol/kg by the addition of EBSS $\times 10$. This medium was designated the *light polysucrose medium* (LPM).

Another medium, with a density of approximately 1.09 g/ml, was prepared by the addition of 10% (v/v) Optiprep (Nycomed) to the LPM. Osmolality was adjusted to 350 mosmol/kg with EBSS $\times 10$. This

medium was designated the *heavy polysucrose medium* (HPM).

Both media were sterile filtered before use.

Semen Purification

Percoll purification of spermatozoa was performed by mixing the 100% Percoll medium with EBSS supplemented with 10 mg/ml HSA (Earle's medium) to form 55% (v/v) (1.08 ± 0.002 g/ml) and 80% (v/v) (1.10 ± 0.002 g/ml) Percoll medium. The gradient was formed placing the semen sample on top of 2.0 ml of 55% Percoll medium underlaid with 2.0 ml of 80% Percoll medium.

Polysucrose purification of spermatozoa was performed by placing the semen sample on top of 2.0 ml LPM underlaid with 2.0 ml HPM.

The two gradients were centrifuged at 300g for 25 min during the same run. After centrifugation, the pellet was collected after removal of the overlaying phases. The spermatozoa were washed two times with 2 ml of Earle's medium and centrifuged at 300g for 10 min. The two purified preparations of spermatozoa were evaluated for the concentration of progressively motile and nonmotile spermatozoa plus motility (0–4) in a blinded fashion by an experienced embryologist using a Makler counting chamber and compared to the semen sample. The recovery of motile spermatozoa was calculated as the percentage of the number of motile spermatozoa in the purified preparation compared to the number in the raw semen used for purification.

The volume of semen sample used for each purification gradient varied from 0.2 to 1 ml. Sperm samples were used only after informed consent by the couple in accordance with the Helsinki Declaration.

Sperm Survival Test

After a parallel sperm purification, the pellets were washed as described above. Following evaluation of the purified sperm samples, the atmosphere in the tubes was exchanged with 5% $\text{CO}_2/95\%$ air to assure the optimal pH, and the tubes, with tight lids, were left at room temperature for 24 hr, after which the samples were evaluated again.

Measurement of Levels of Endotoxins

Levels of endotoxins were measured with a commercially available kit (Limulus Amebocyte Lysate

Table I. Isolation of Human Spermatozoa by Density-Gradient Centrifugation: Comparison Between Percoll and Polysucrose Media^a

	Semen	Percoll purified	Polysucrose purified
No. sperm ($\times 10^6$)/ml			
Motile	27 $\pm$ 8 (1–150)	7.8 $\pm$ 1.6 (0.4–22)*	10.6 $\pm$ 2.2 (0.3–35)*
Nonmotile	28 $\pm$ 10 (0.1–150)	7.1 $\pm$ 2.5 (0.04–32)	8.9 $\pm$ 3.2 (0–50)
% motile	54 $\pm$ 5 (20–91)	64 $\pm$ 6 (23–95)	65 $\pm$ 7 (17–100)
Motility (0–4)	2.9 $\pm$ 0.1 (1.5–3.5)	3.1 $\pm$ 0.1 (2–3.5)	3.2 $\pm$ 0.1 (2.5–4)
Recovery of motile sperm (%)	100	38 $\pm$ 6 (10–88)**	48 $\pm$ 7 (12–99)**

^a Mean $\pm$ SE (range) of 18 individual semen samples.

* $P = 0.057$ comparing the Percoll and the polysucrose groups.

** Percoll purified are significantly lower than polysucrose purified ($P < 0.02$). None of the other groups differ significantly.

Coatest; Endosafe, Charleston, SC) according to the manufacturer's instructions.

Statistical Analysis

Comparison between the two types of sperm purification procedures was done by Student's *t* test for paired observations.

RESULTS

The average numbers of motile and nonmotile spermatozoa, percentage motile spermatozoa, motility score, and recovery of progressively motile spermatozoa after purification are shown in Table I together with data for the semen samples. The polysucrose/Optiprep method facilitates a higher recovery of progressively motile spermatozoa (based on the percentage), however, the actual values of total motile sperm recovered are not statistically different ($P = 0.057$). The average number of nonmotile sperm, percentage motile, and motility score are similar between the two groups.

Table II presents data for the 6 oligozoospermogenic and 12 normospermogenic samples separately.

As expected, the yield of progressively motile spermatozoa is lower in the oligozoospermogenic samples compared to the normospermogenic samples, but for each of the two types of samples the picture is similar to the overall result as shown in Table I.

On seven occasions spermatozoa purified by each of the two techniques were used to fertilize oocytes, which were retrieved from the spouse during the course of in vitro fertilization (IVF) treatment. From each woman half of the oocytes were inseminated with spermatozoa from the polysucrose- and the Percoll-purified spermatozoa, respectively. The average frequencies at which the oocytes fertilized and cleaved were identical between the two types of sperm purification. Likewise, the number of blastomers in preembryos and the quality score were also comparable. Three of the IVF-treated women became pregnant after replacement of the morphologically best-looking preembryos, irrespective of which type of sperm purification was used.

Sperm survival tests were performed on five sperm samples. In each case, there were no differences in sperm survival and the other parameters evaluated between the two types of sperm purification.

Table II. Isolation of Human Motile Spermatozoa by Density-Gradient Centrifugation: Results for Oligozoospermogenic and Normospermogenic Samples^a

	Percoll purified		Polysucrose purified	
	Semen $<20 \times 10^6$ /ml	Semen $>20 \times 10^6$ /ml	Semen $<20 \times 10^6$ /ml	Semen $>20 \times 10^6$ /ml
No. sperm samples	6	12	6	12
No. sperm ($\times 10^6$)/ml				
Motile	1.3 $\pm$ 0.5	10.6 $\pm$ 2.0	2.0 $\pm$ 1.0	14.1 $\pm$ 2.9
Nonmotile	0.9 $\pm$ 0.3	9.9 $\pm$ 3.4	1.3 $\pm$ 0.6	12.3 $\pm$ 4.3
% motile	65 $\pm$ 9	61 $\pm$ 7	67 $\pm$ 10	59 $\pm$ 8
Motility (0–4)	3.0 $\pm$ 0.2	3.1 $\pm$ 0.1	3.2 $\pm$ 0.1	3.1 $\pm$ 0.2
Recovery of motile sperm (%)	24 $\pm$ 7	42 $\pm$ 8	32 $\pm$ 13	52 $\pm$ 8

^a Values are means $\pm$ SE.

Whereas levels of endotoxins in the 100% Percoll medium exceeded 6000 pg/ml, levels in the two types of polysucrose media were measured to 84 pg/ml.

DISCUSSION

This study demonstrates that polysucrose-based density media can replace Percoll-based media for the purification of human motile spermatozoa in connection with ART. The two types of media are identical with regard to the average recovery of percentage motile spermatozoa, and the polysucrose-based media have a significantly higher recovery of progressively motile spermatozoa compared to the Percoll method. In practical terms the two methods are also identical: both use a two-layer gradient, and the isolation procedures are similar. Polysucrose and Optiprep are both completely dissolved in the media, and the washing procedure is likely to remove them from the spermatozoa. The possibility of crystal formation in the Percoll media, which will copurify with the spermatozoa, poses a potential risk for the deposit of Percoll crystals in the uterus or in the oocyte. If this occurs, it is likely to reduce the possibilities for conception. Polysucrose and Optiprep are both available in pure forms approved for human *in vivo* use and contain endotoxins at a level similar to that of the culture media used for ART, which is at least two orders of magnitude lower than the amount found in the Percoll media. Our Percoll media over the years have consistently contained high levels of endotoxins. The other chemicals used to prepare the Percoll media (i.e., EBBS $\times$ 10, NaHCO_3 , and penicillin) are also used to prepare our usual culture media, which very seldom contain measurable endotoxin levels, showing that the high levels of endotoxin originate in the Percoll medium itself.

Therefore, the polysucrose-based purification is comparable to the Percoll method in the main outcome measures and, in addition, avoids some of the disadvantages of the Percoll method.

The silica-based particles in the Percoll media are responsible for the increased density. The particles are too big to enter cells and are left outside. Therefore, the Percoll media do not alter the density of the cells and separation with Percoll media is dependent solely on the density of the cells. A replacement for Percoll should preferentially fulfill the same requirements, and substances that may enter the cells should be avoided. Substances that enter the cells may cause an increased density of unwanted spermatozoa (i.e., dead or abnormal) and somatic cells, which may lead to coprecipita-

tion of impurities in the pellet together with the motile spermatozoa.

Sucrose is well-known for its inability to cross membranes. This characteristic of sucrose is used in most cryopreservation programs, where preembryos are exposed to high concentrations of sucrose (i.e., molar) to decrease the intracellular water content. Polysucrose, which is a nontoxic inert polymer of sucrose with a molecular weight of approximately 400 kDa, seems to possess the same properties as the monomer. These physicochemical properties are likely to qualify polysucrose as a good replacement for Percoll.

Another important point with the polysucrose-based method is the adjustment of the osmolality. The osmolality of seminal plasma is higher than that of the circulation and reaches 360 to 380 mosmol/kg (7,8). The Percoll medium is usually adjusted to an osmolality of 280 mosmol/kg, comparable to that of the circulation. However, since the Percoll particles are too big to enter the cells, weak hypoosmotic swelling does not interfere with the purification of spermatozoa. In contrast to the Percoll particles, the polysucrose is completely dissolved and hypoosmotic swelling may force the polysucrose to enter the cells, with a resulting unwanted increase in density. Therefore, to avoid hypoosmotic swelling of the cells in the seminal plasma, the osmolality of the polysucrose media is kept in a range similar to that of seminal plasma. After the purification of the motile spermatozoa the succeeding washing procedure ensures an osmolality of approximately 280 mosmol/kg, similar to that of the culture or the insemination media. The light polysucrose layer (the upper layer) has a density of approximately 1.05 g/ml, which is sufficient to separate spermatozoa from the seminal plasma, somatic cells, debris, and dead spermatozoa. Further addition of polysucrose to the media will not result in a solution with a sufficient density to serve as the bottom layer. The addition of Optiprep, which is a low molecular weight iodinated substance developed as an X-ray contrast medium, together with the polysucrose increases the density of the bottom layer, causing separation of the spermatozoa according to density. The substance in Optiprep that raises the density of the media is a dimer of the substance present in the former Nycoprep, which has been used successfully for sperm purification (9–11). Furthermore, one study found an increased long-term retention of sperm motility of Nycoprep-purified spermatozoa compared to Percoll-purified spermatozoa (12). The present study did not demonstrate such a difference, but a more detailed study of the

polysucrose/Optiprep method is needed to disclose a possible difference with the present method.

Polysucrose has also been used to purify motile spermatozoa in connection with ART. A polysucrose-based modification of the swim-up procedure gave a significantly improved fertilization and cleavage rate compared to the conventional swim-up procedure (13,14). These results compare favorably with ours, where we, in a few cases, have used polysucrose-purified spermatozoa for fertilization, with a resulting fertilization rate similar to that for Percoll-purified spermatozoa. A prospective randomized study is now planned to evaluate the efficacy of this new polysucrose-based sperm purification procedure.

Purification of spermatozoa with high rates of recovery and motility using polysucrose and Optiprep has clinical application not only for IVF but also for intra-uterine insemination. This study demonstrates that even sperm samples with a low number of spermatozoa show a good recovery, and especially, oligoasthenozoospermic samples may be improved to increase the potential of fertilization by polysucrose purification.

In conclusion, the polysucrose-based sperm purification procedure may replace the traditionally used Percoll method. The two techniques show similar yields and motility parameters, and in addition, the polysucrose technique shows much lower levels of potentially harmful endotoxins. Both procedures use an identical density-gradient centrifugation technique. Investigations are proceeding in an attempt to refine further the polysucrose/Optiprep purification procedure and to evaluate the efficacy of this procedure on semen samples from patients with spermatozoal autoantibodies and on cryopreserved semen. Studies to evaluate the efficacy of this procedure in a clinical setting are also under way.

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