

## REVIEW

# Sperm preparation for assisted reproduction

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In ejaculated semen, there are factors, decapacitation factors, that can diminish the occurrence of acrosome reaction and hence reduce or even totally inhibit fertilizing capacity of the sperm.<sup>(1-3)</sup> Semen contains a variety of other constituents, including dead cells, lytic enzymes, and microorganism<sup>(4)</sup> some of which are likely to have the adverse effect on the gametes, the embryo, and/or the reproductive tract. The ejaculated semen also contains spasmogen that can cause smooth muscle contraction after intrauterine insemination (IUI).<sup>(5)</sup>

The spermatozoa must, therefore, be separated from the seminal plasma in order to proceed to the stage of capacitation followed by hyperactivation and the acrosome reaction which enables oocyte binding and fertilization. It has been shown that longer exposures to seminal environment in vitro can permanently diminish the fertilizing capacity of spermatozoa.<sup>(6)</sup> Therefore, it is essential that spermatozoa must be separated from the seminal plasma quickly and efficiently.<sup>(3)</sup>

Separation of human spermatozoa from seminal plasma is a principle requirement not only for assisted reproduction such as in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), IUI but also for assessment of the acrosome reaction<sup>(7)</sup> and in vitro tests of sperm fertilizing ability such as the zona-free hamster egg penetration test (HEPT) and hemizona binding test.<sup>(8)</sup>

The ideal sperm preparation technique would: 1) be simple, rapid and inexpensive; 2) recover the highest number of motile sperm with normal function and morphology; 3) result in no damage or physiologic alteration of the separated sperm; 4) remove dead sperm and other cells, including microorganisms; 5) remove toxic and bioactive substances; 6) process large volumes of semen; and 7) allow the final volume of the sperm suspension to be controlled. However, these criteria may differ in their importance depend on the application.<sup>(9)</sup>

In assisted reproduction, sperm preparation techniques were developed for IVF where the infertility problem was originally due to tubal factor. Edwards et al.<sup>(10)</sup> described a simple one-step washing procedure in the first report of the fertilization of a human oocyte in vitro, and a two-step washing procedure for the first clinical IVF success.<sup>(11)</sup> It is logical that, since the most functionally normal sperm is the one which is motile, attempts were made to let sperm migrate from the washed sperm pellet and be separated. Besides the earlier work of Drevious,<sup>(12)</sup> there was a report in 1981 about the development of a swim-up migration from the washed sperm pellet to separate the motile fraction of sperm.<sup>(13)</sup> The method gave desirable results for fertilization in vitro, and was also widely used for artificial insemination. However, as male factor and idiopathic infertility were included into the assisted

reproduction programs, the technique was found to be inappropriate for many cases and often associated with failed fertilization.<sup>(14,15)</sup> A variety of other sperm preparation techniques have improved the prognosis for such cases including the use of discontinuous Percoll gradients and sedimentation techniques which reduce or exclude centrifugation force when the spermatozoa are considered to be fragile.

Further specialized sperm preparation techniques were developed for specific conditions such as the presence of antispermatozoal antibodies, microsurgical epididymal sperm aspiration (MESA), testicular sperm extraction (TESE), micromanipulation, and microinsemination.

### Culture media for human spermatozoa

Many culture media are used experimentally and clinically for human spermatozoa. As regards the basic composition of these media, it includes anions e.g.,  $\text{HCO}_3^-$  and  $\text{Cl}^-$ ; cations e.g.,  $\text{Na}^+$  and  $\text{Ca}^{2+}$ ; energy sources e.g., glucose, pyruvate, lactate; and phenol red as a pH indicator.<sup>(16)</sup> Proteins, especially albumin, are used to support motility and are necessary for the capacitation.<sup>(17)</sup> In some laboratory testing, albumin can be used to prevent spermatozoa "sticking to glass phenomenon". Human serum albumin (HAS) should be included at  $\geq 10$  mg/ml although the physiological concentration in the oviduct during the periovulatory period is about 30 mg/ml.<sup>(18)</sup> The commonly used media include: Biggers, Whitten and Whittingham (BWW)<sup>(19)</sup>; Earle's balanced salts (EBS),<sup>(20)</sup> Ham's F-10,<sup>(21)</sup> human tubal fluid (HTF),<sup>(22)</sup> Lopata's medium,<sup>(23)</sup> Menezo's B and B media,<sup>(24)</sup> modified Krebs - Ringer - bicarbonate (KRB),<sup>(25)</sup> synthetic tubal fluid (STF),<sup>(26)</sup> and Whittingham's T<sub>6</sub> modified Tyrode's solution.<sup>(27)</sup>

### Method of sperm preparation

In general, there are four basic approaches for separating sperm from semen, including: 1) Sperm dilution and washing; 2) Sperm migration; 3) Selective washing procedures; 4) Adherence and filtration methods.

### Sperm dilution and washing

Simple dilution of semen with a relatively large volume (usually 5 to 10 times) of a medium e.g., Ham's F-10, and separation of the spermatozoa by centrifugation is the simplest method for washing of spermatozoa. Repeated centrifugation of the spermatozoa resuspended from the pellet (usually two or three times) is used to ensure adequate removal of contaminating seminal plasma. This procedure has a disadvantage that all the spermatozoa, including the dead and abnormal sperm, present in the original semen still remain in the final sperm preparation. The presence of these nonfunctional gametes may inhibit capacitation and increase the risk of developing antisperm antibodies if inseminated into the uterine cavity.<sup>(28)</sup>

For this preparation technique, the centrifugation should not be too forceful and recommended not to be greater than 200x g. Specimens should be largely free of cells and debris.<sup>(29)</sup>

### Sperm migration

Under in vivo conditions, human spermatozoa must be separated from the semen which contains decapacitation factors by active migration into favourable fertile cervical mucous. By this phenomenon, sperm separation techniques using a self-migration characteristic are widely used. These include the classical "swim-up" from liquefied semen into an overlay of culture medium, and "swim-down" into one or more layers of culture medium.

### Swim-up techniques

Principally, this is a self-migratory system which a physiological medium is layered upon semen. To increase the interface area, the semen aliquots must be placed in a series of round-bottomed tubes and the tubes have to be incubated at an angle of 20 to 45 degree to the horizontal plane. Incubation has to be at 37°C for an appropriate period, usually 30 to 90 minutes. Variations on the swim-up techniques have been proposed, including: the swim-up from washed sperm pellet, or washed and resuspended pellet, swim-up from raw semen; swim-up into a medium

containing highly purified sodium hyaluronate (sperm select system) and the migration-sedimentation approach.

### 1. Swim-up from washed sperm pellet

This procedure typically involves two-to three-fold dilutions with culture medium, followed by repeated centrifugation.<sup>(9)</sup> The produced pellets of spermatozoa are then overlaid with a 100-400ml of culture medium. After incubation, to allow the spermatozoa to "swim-out" from the pellets, the supernatants are harvested and the sperm concentration is adjusted.

This technique assumes that spermatozoa swimming from the pellets are active and normal. The proportion of motile spermatozoa, velocity, amplitude, linearity, and beat frequency are improved if swim-up is fairly brief; otherwise abnormal spermatozoa also swim up.<sup>(30,31)</sup> After swim-up processing, some spermatozoa display hyperactivation, the frequency of bacterial contamination is reduced, and improved pregnancy rates are obtained even with poor quality semen.<sup>(32)</sup> Nevertheless, perturbation and centrifugation performed during the process should be done gently, since abnormal spermatozoa may otherwise release reactive oxygen species that can damage other normal spermatozoa.<sup>(33)</sup>

### 2. Swim-up from washed and resuspended pellet

Swim-up from a washed pellet have been criticized due to the centrifugation process may pack the normal spermatozoa with all the deleterious factors, including reactive oxygen species from leukocytes and defective spermatozoa, into a tight pellet. This result in irreversible damage to the normal spermatozoa and impairment of their fertilizing ability.<sup>(33)</sup> To overcome this problem, centrifugation force must not exceed 200x g and the loose pellets are fully resuspended in 500 ml of a medium from which they migrate into an overlayer of sperm preparation medium.<sup>(34)</sup>

Sometimes, the lower sperm suspension is made more viscous, to stabilize the interface, by adding a high

concentration of albumin or a substance such as Ficoll.

### 3. Swim-up from raw semen

Aitken and Clarkson<sup>(33)</sup> demonstrated that separation of motile spermatozoa prior to centrifugation yield sperm suspensions of highest quality. Swim-up from raw semen gave better motility and sperm-oocyte fusion than swim-up from washed pellet.<sup>(33)</sup> The most recent edition of the WHO laboratory manual<sup>(20)</sup> advises a preliminary swim up of overlaid semen to harvest a relatively clean motile fraction before the washing-centrifugation steps.

Practically, aliquots of semen are placed in a series of tubes underneath layers of a culture medium. The tubes may be prepared either by gently layering the culture medium over liquefied semen or by the reverse procedure which provides a cleaner interface zone. After incubation, most of the upper layer (usually 75% to 80% of culture medium layer) is harvested from each tube and combined. This combined preparation is then centrifuged at 500x g for 5 to 10 minutes and resuspended into a fresh culture medium at the desired concentration of motile spermatozoa.<sup>(33,35-37)</sup>

### 4. Sperm select system

The sperm select system (Select Medical Systems, Williston, VT, USA) employs a highly purified preparation of sodium hyaluronate, molecular weight of 3000 KDa, with 1 mg/ml final concentration in culture medium. Swim-up from semen into sodium hyaluronate provided a higher percentage of motile spermatozoa compared with the swim-up from a washed pellet method. The effect of hyaluronic acid is due to a direct action on sperm metabolism or contractile properties rather than to preservation of sperm membrane integrity.<sup>(38)</sup>

In practice,<sup>(39)</sup> 0.75 ml sperm wash medium is mixed with one vial of sperm select medium. After incubating the mixture at 37°C in 5% CO<sub>2</sub> for 30 minutes, 0.5 ml of the equilibrated mixture is transferred gently to each of 4 test tubes containing 0.25 ml of liquefied semen. After incubation for 60 minutes, the top layer which contains the motile sperm fraction from

each tube is removed and combined in a single tube. The concentration and motility were then calculated for insemination in the routine manner.

## 5. Migration – Sedimentation techniques

This type of approach combines swim-up from semen with gravitational settling of spermatozoa from the upper medium layer. Without centrifugation step, true sedimentation and migration techniques was applied in Tea-Jondet glass tubes which compose of a small tube creating a well within a larger tube<sup>(40)</sup>(Figure 1). The tube is incubated for 60 to 180 minutes at 37°C. The samples from central well can be aspirated intermittently over 2 to 3 hours until sufficient spermatozoa are recovered. This method are particularly suited to high debris, oligo/asthenozoospermic cases. Spermatozoa recovered by this method are highly motile, and the normal morphology is improved.<sup>(41,42)</sup> However, the number of spermatozoa obtain after migration-sedimentation technique was lower than minipercoll technique.<sup>(41)</sup> Moreover, there was an evidence that spermatozoa from swim-up technique survive longer than that from migration-sedimentation technique and give higher fertilization rate in in vitro fertilization.<sup>(42)</sup>

### Swim-down (Albumin column filtration)

Originally, this technique has been described for separation of the Y chromosome-bearing sperm.<sup>(43)</sup> However, albumin columns were found to increase motile sperm percentage and to reduce the percentage of abnormal forms and the amount of debris.<sup>(43,44)</sup> The capacitation can also occur in vitro by using culture media containing albumin, however, this role of albumin is not well understood.<sup>(45)</sup>

As regards sex selection, it has been reported that albumin columns filtration can increase significantly the proportion of Y chromosome-bearing sperm.<sup>(46)</sup> This depend on the premise that Y bearing spermatozoa have a greater ability to penetrate the interface between the layers of a discontinuous gradient.<sup>(43,47)</sup> However, other studies reported a significantly lower Y bearing sperm concentration using this technique.<sup>(48,49)</sup> Principally, the technique involves

layering of washed sperm on the top of a vertical discontinuous gradient of BSA<sup>(43)</sup> or HAs.<sup>(44,47)</sup> Fractionation results from the ability of motile sperm to orient and propel themselves downward by gravity through a handicap of increased viscosity at a rate faster than sedimentation of non-motile sperm and debris which will remain on the top layer. To facilitate the maximum separation and simultaneously to achieve the highest recovery of motile sperm, either one- or two-layer(s) serum albumin columns are used. One layer column is preserved for the oligozoospermic specimens, particularly for those with significantly decreased motility. The isolation column contain 1 ml of 7.5% or 10% serum albumin on top with the sperm suspension. By one-layer, the separation may not be complete and some abnormal sperm or seminal debris may be found in the final isolated fraction.<sup>(47,50)</sup> In the cases that sperm concentration is within normal range, a two-layers column is recommended to make a highly motile and cleaner final semen fraction. However, this technique permits the recovery of motile sperm only 10-15 % from the original specimen.<sup>(47)</sup> This two-layer column compose of 1 ml of 7.5 % serum albumin layered in the column over 0.5 ml of 17.5% serum albumin.<sup>(47)</sup>

### Selective washing techniques

The major disadvantage of the swim-up and albumin gradient technique is that because they rely totally on the vigorous motility of the spermatozoa and they are not readily applicable to cases of male infertile involving severe asthenozoospermia or oligozoospermia. The solution to this problem is to use isotonic continuous<sup>(51)</sup> or discontinuous gradient techniques.<sup>(52,53)</sup> These techniques are based on the use of density gradient centrifugation to fractionate the subpopulations of spermatozoa depending on their specific gravity. Although Percoll (Kabi Pharmacia, Uppsala, Sweden) is by far the most widely used gradient material, there is a great interest in trying to establish methods using other gradient medium instead. This is due to an occasional endotoxin contamination of some Percoll batches and it is only licensed for use in vitro. In addition, an inflammatory response may be induced by the

intrauterine insemination of specimen contaminated with Percoll.

## Percoll gradients

Percoll gradients are prepared from particles of colloidal silica coated with polyvinylpyrrolidone (PVP). This coating with polymers is necessary because silica particles are cytotoxic. With colloidal property Percoll gradient facilitate banding of intact cells and particles at a position close to their true buoyant densities in the gradient. Instead of forming a packed pellet, spermatozoa will be accumulated within the gradient at their apparent buoyant density which can be easily removed and resuspended. Therefore, mechanical stresses, due to tightly packed sperm pellet and resuspension of the pellet, which are implicated as causes of sperm damage in washing procedures can be avoided.<sup>(33)</sup>

Two gradient layers : 95 %, 47.5%<sup>(54)</sup> or 80%, 40%<sup>(20)</sup> and three gradient layer : 95%, 70%, 50%<sup>(39)</sup> isotonic Percoll can be used on moderate oligo/asthenozoospermic cases particularly where there is increased abnormal sperm, high viscosity semen, and high leukocytes or debris content. The speed of centrifugation should never be over 200 x g to avoid pulling down the abnormal, immotile sperm and debris. The Percoll methods can be adjusted depend on the quality of the original semen. The variable methods include<sup>(55)</sup>:

Volume of gradient layers : In general 1-ml layer is used for normal semen parameters according to WHO criteria, a 0.5-ml layer is for mild to moderate male factor, and 0.3-ml layer (mini-Percoll) is used for severe male factor.

Direct layering of raw seminal fluid on the Percoll gradient or wash before layering : Depending on sperm concentration and semen volume, washing is used only for moderate to severe oligozoospermia. To make an appropriate sample for layering on the gradient, washing is used to concentrate either a high volume specimen or a very low concentration specimen to a small volume before layering.

Length of centrifugation time : The time can vary from 7 to 45 minutes. However, the shortest

possible centrifugation should be employed. Normal motile sperm migrate first and abnormal motile sperm are pulled down as further centrifugation then finally nonmotile sperm. Some other cells and debris may be pulled down by excessive centrifugation.

In general, the centrifugation time is 10 to 20 minutes for normal sperm samples, 10 to 30 minutes for mild to moderate oligozoospermic cases, and up to 45 minutes for severe oligozoospermic cases. Other factors that affect the centrifugation time are extremely viscous samples and samples with less than 20% motility which require a longer centrifugation time. The sample with low percentage of normal morphology should be centrifuged for shorter time than usual due to the normal sperm will be recovered first.

After Percoll centrifugation, the recovered sperm must be washed twice to remove any residual Percoll. However, the final sample may still contain a small quantities of Percoll particles. There is a great concern over the possible dangers of using the preparation for IUI. This is because the silica particle of Percoll is an established tissue irritant. Recently, Percoll was disapproved for isolating cells which will be re-introduced into humans for any clinical purposes and its use must be limited for research.

## Alternative gradient materials

To avoid adverse effect from insemination the sperm preparation contaminated with Percoll and to improve the yield of normal, motile spermatozoa obtained from oligozoospermic and asthenozoospermic samples, new gradient materials have been developed.

## Nycodenz

Nycodenz (Nyegaard & Co., Oslo, Norway) is an iodinated organic molecule, N,N $\phi$ -bis-2, 3-dihydroxypropyl-5- [N- (2, 3 - dihydroxypropyl) acetamido] -2, 4, 6-triiodoisophthalamide, dissolved in a Tris buffer. It is a biologically inert and nontoxic medium which was used for gradient fractionation of biologic specimens. This medium has been used as a x-ray contrast medium, iohexol or Omni-paque, which commonly employed in angiography.<sup>(56)</sup> There is an

evidence that spermatozoa recovered from Nycodenz gradient maintain the motility longer than that from Percoll gradient.<sup>(57)</sup> The sperm penetration assay results obtained from Nycodenz gradient are comparable to Percoll gradient and better than washed or swim-up technique.<sup>(58)</sup>

A stock solution of Nycodenz is prepared by diluting 9 ml of isotonic Nycodenz with 1 ml of Ham's F-10 medium supplemented with 1 mg/ml HSA. Either a two-layer (40%,80%) gradient, 4 ml each, or a four-layer (100%,65%,50%, and 35%), 2 ml each, is prepared by diluting the stock solution with Ham's F-10 medium. Diluted semen (1 ml) is layered on the top of the gradient and centrifuged at 300 x g for 20 minutes. For the two-layer gradient, the upper layer is removed and the pellet is washed once with Ham's F-10 medium. For the four-layer gradient, 100% and 65% layer are combined and centrifuged at 300 x g for 10 minutes. The pellet is washed once with Ham's F-10.

There is a new iso-osmolar nonionic dimeric hydrophilic contrast media, iodixanol, which expected to produce lower adverse events than other nondimeric contrast media. When iodixanol was compared to Percoll gradient centrifugation with semen from the same ejaculate, there was no significant difference between methods with regard to motility yield, normal sperm morphology, and sperm survival.<sup>(59)</sup>

## Isolate

Isolate (Irvine Scientific, CA, USA) is a sterile isoosmotic colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in a HEPES-buffered human tubal fluid. As a two-layers gradient system, Isolate is used for the separation of human spermatozoa from seminal plasma by density centrifugation.

Isolate is available as a working solution and supplied as 2 bottles, 50 ml each. One contains the upper layer and another contains the lower layer where the densities are different.

This new gradient material needs more investigation although there are evidences that it gives a comparable results to Percoll and enhance hemizona binding. Furthermore, it was proved to have neither

irritation nor cytotoxic effects.<sup>(60)</sup>

## Adherence and filtration methods

Adherence and filtration methods are based upon the "sticking-to-glass" phenomenon. It is known that dead and moribund spermatozoa are extremely sticky to the glass surface.<sup>(3)</sup> Glass filtration improves sperm quality by removing the majority of debris as well as agglutinated and dead spermatozoa from semen samples.<sup>(61)</sup> Principally, semen is placed on the top of a column of loosely packed glass wool fibers or small glass beads and allowed to flow slowly and collected in a glass test tube.<sup>(62)</sup>

## Glass wool fiber columns

Borosilicate glass (pyrex) has been found superior to the common soda lime glass wool because of absence of impurities, more strength and resistance that reduce its breakage into small particles. For preparing glass wool, approximately 40-50 mg of borosilicate glass fibers is used to pack the column either tightly at the bottom or loosely along the length of a disposable 5 inches Pasteur pipette.<sup>(62)</sup> A liquefied semen sample of up to 2.5 ml is placed on the top of the column and allowed to flow through it by gravity force. The debris and non-motile sperm will be trapped within the mesh as the sample flows through the network of fibers. Almost complete recovery of motile sperm can be achieved in the collected effluence. About 98% of dead sperm are found to adhere to the fibers as evidenced by supravital staining.<sup>(62,63)</sup> Additionally, it has been shown that glass wool filtration can improve sperm quality even in oligo- and/or asthenospermic cases.<sup>(64,65)</sup>

Artificial insemination of a sample prepared by this technique must be aware due to the danger of glass wool fragments in the final sperm population. Other disadvantages include decreasing of the volume of semen, sperm loss between 33% and 45%, and lastly, ultrastructural damage to the sperm plasma membrane and acrosome in some spermatozoa.<sup>(63,66)</sup>

## Glass bead column

This technique has been shown to select

motile human spermatozoa efficiently with high yields.<sup>(67)</sup> One advantage of glass bead over glass wool is its relatively large size which lessen the possibility of breakage into small pieces. However, using glass bead column has not been widely accepted for assisted reproduction.

### Sephadex gel filtration column

Sephadex gel filtration column has been available as a commercial sperm separation kit, namely SpermPrep. There is another sephadex preparation, which used a different bead size and less sephadex, called SpermPrep II. Both Percoll and SpermPrep II improve the percentage of better quality sperm.<sup>(68)</sup> However, there is a recently report that sephadex column were toxic to mouse gametes, embryos and human spermatozoa.<sup>(69)</sup> Practical use of this method should, therefore, be more investigated.

### Transmembrane migration

This method is basically used for testing the effect of drugs on the motility of sperm.<sup>(70)</sup> A motile sperm population is separated by traveling into culture medium across a nucleopore membrane filter. By this method, Agarwal et al<sup>(71)</sup> reported a significant increase of sperm motility and less leukocytes contamination. However, the yields are still very low.

### Clinical application

Various methods of sperm preparation have been developed aims to separate high number of motile sperm from non-motile, dead sperm, cellular debris, and seminal fluid. Which preparation method is suitable and should be chosen depend on the patient and the required final yield. For IUI, 5-20 million motile spermatozoa in 100-500 ml of final volume is needed,<sup>(72-74)</sup> whereas IVF or GIVF requires only 20,000 to 100,000 motile spermatozoa per oocyte.<sup>(75)</sup> A semen analysis for initial parameters, therefore, is essential to determine how to prepare any sample.

In IUI, the total motile sperm from post-preparation is highly correlated with the success rate.<sup>(73,76)</sup> In a recent study, pregnancy can be achieved by IUI even

in severely oligozoospermic specimens.<sup>(77)</sup> However, Ombelet et al,<sup>(78)</sup> found that in subgroup of inseminating motile count less than  $1 \times 10^6$ , no pregnancies occurred if the morphology score was less than 4%. To get enough motile sperm in final preparation, conventional swim-up technique is widely used for normal semen specimens while discontinuous Percoll gradient technique can be applied in both normal and subnormal sperm samples.<sup>(32)</sup>

In the sperm preparation for IVF and GIFT, swim-up and Percoll gradient techniques are also successfully used to recover the recommended number of sperm per ovum. Discontinuous gradient of Percoll has been proved to improve the capacity for in-vitro fertilization of spermatozoa from men with oligozoospermia or asthenozoospermia<sup>(79)</sup> and improve the population of spermatozoa with more level of nuclear maturity.<sup>(80)</sup> However, in cases of severe oligoasthenozoospermia, the motile spermatozoa recovery are very poor or even no recovery by using swim-up or Percoll gradient technique. In these particular cases, mini-Percoll gradient technique which reduce the volumn of Percoll layers can be used to allow a better migration of spermatozoa. Other preparation techniques were also applied, such as Nycodenz gradient and glass wool column filtration, however, these were not widely used in clinical practice.

In intracytoplasmic sperm injection (ICSI), the Percoll gradient centrifugation has been used for the preparation of ejaculated semen or epididymal spermatozoa.<sup>(81)</sup> One the other hand, the effluent and the minced tissue from testicular biopsy may be washed by simple centrifugation (300 g for 5 min).<sup>(82)</sup> However, recent report has shown that Percoll gradient centrifugation step may be omitted in the sperm preparation procedure for ICSI without any adverse effect on fertilization and embryo cleavage.<sup>(83)</sup> Therefore, the sperm preparation for ICSI may need only removal of seminal fluid because the spermatozoa that have normal motility and morphology will only be directly selected at the time of injection.

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