Selection of Spermatozoa

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Abstract

The isolation of mammalian spermatozoa from the surrounding seminal fluid is a crucial practice commonly applied in assisted reproductive technology applications. The selection of sperm isolation protocol is critically important for investigators, for clinicians preparing sperm samples to be used in reproductive biotechnologies, in veterinary andrology laboratories, and in animal husbandry. Considering the growing importance of the sperm selection techniques, this chapter deals with established sperm selection techniques, viz., simple washing of spermatozoa, swim-up, and discontinuous density-gradient protocol. In addition as a corollary to these protocols, techniques to recover spermatozoa from the epididymis or testicular tissues have been provided alongside.

Keywords

Migration • Filtration • Swim-up • Sephadex filtration • Colloid centrifugation

Contents

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2.1 Introduction

Ejaculated semen contains abnormal and senescent sperm, which exert detrimental effects on others, thus reducing fertility of physiologically normal cells [1, 2]. Under natural mating conditions, spermatozoa have to pass through cervical mucus, which acts a natural and effective physical barrier to nonmotile cells, affording clear advantages for disease reduction, genetic control, and economical production of young ones through differential selection of normal and motile spermatozoa. This advantage is lost in ART such as intracytoplasmic sperm injection (ICSI), intrauterine insemination (IUI), in vitro fertilization (IVF), and even artificial insemination (AI); in later case, seminal plasma becomes ineffective because of several folds dilution. Therefore, spermatozoa selection procedures capable of providing benefits of providing this physical barrier are required to remove dead and damaged spermatozoa found either in normal ejaculates or in frozen-thaw samples. It becomes imperative to select a protocol for separation of seminal plasma from spermatozoa to yield a final preparation sans non-germ cells and dead spermatozoa, and free from debris, but containing a high number of morphologically normal and motile cells, for a successful outcome from various assisted reproductive technologies (ARTs). Such ARTs are commonly employed in human and sometimes in animal andrology laboratories. Though seminal plasma components help spermatozoa penetrate cervical mucus [3], some of it (e.g., zinc and prostaglandins) are obstacles to successful fertilization when techniques of ART allow bypassing natural barriers, such as ICSI, AI, IUI, or IVF. In such laboratories, seminal plasma may need to be separated from sperm cells for various reasons, such as diagnostic tests to assess sperm function, recovery of most fertile sperm for therapeutic purposes, and ART.

Sperm selection protocols should ensure the recovering of an adequate spermatozoa without detrimental effects on viability, morphology, or any other quality characteristic in order to be useful for assisted reproductive technologies. This chapter deals with techniques to recover spermatozoa from the epididymis or testicular tissues and important protocols of sperm selections, viz., simple washing of spermatozoa, swim-up, and discontinuous density-gradient protocol. Though nano-purification of spermatozoa is a current procedure discussed in semen processing laboratories, it is still not fine-tuned to deserve a mention in this book of protocols. The chapter explains important considerations to select a protocol in a varied situation and their relative merits as well.

Investigators must bear in mind that to perform sperm-function tests, it is crucial that the seminal plasma is separated from the sperm within 1 h of collection. This limits any detrimental effect to sperm cells from deteriorating products of non-sperm cells. However, it is pertinent to note that sperm preparation techniques described in this chapter are not completely efficient in removing contaminating and infectious agents from semen.

2.2 Selecting Procedure for Sperm Separation

The nature of the each semen sample dictates the choice of sperm isolation technique for any ART program (see Table 2.1, [4]). Since the different procedures may yield variable quality of spermatozoa, the functional capacity of the prepared sperm cells determines the suitability of the procedure for various ART assays (described elsewhere in this treatise). The choice of the procedure selected determines the efficiency of a technique. The efficiency of a sperm selection technique is usually expressed in terms of recovery of the total number of motile spermatozoa, the absolute number, or in the form of recovery of morphologically normal and motile spermatozoa [5].

Table 2.1 Selection of sperm separation technique for different conditions

Protocol	Conditions	References
Dilution and centrifugation	Normozoospermic specimens for IUI	Boomsma et al. [6]
Density-gradient centrifugation	Specimens with one or more abnormalities, severe oligozoospermia, teratozoospermia, or asthenozoospermia	Morshedi et al. [7]
Glass wool columns	Specimens with one or more abnormalities, viscous or frozen semen	Johnson et al. [8]
Direct swim-up	Specimens with one or more abnormalities and in normozoospermic	Morshedi et al. [7]

2.3 Comparison of Sperm Selection Protocols

There are a number of sperm selection techniques available for separating viable cells from undesirable ones, cryoprotective agents, seminal plasma, and several other detrimental factors. The techniques include the simple sperm washing, the Percoll density-gradient centrifugation technique, direct swim-up, and glass wool filtration. Most of these spermatozoa selection procedures have been validated with human spermatozoa [9, 10], whereas some of these techniques have also been adapted for use with bovine spermatozoa [11, 12].

Selection of spermatozoa by filtration through a Sephadex column [13] and separation by density-gradient centrifugation in Percoll [14] have permitted improvements in the quality of bovine semen. However, in cases of poor semen quality [8], high viscosity [15], or cryopreserved ejaculates [16], the technique of filtration through glass wool has proved to be comparatively advantageous [17]. For the best selection of good-quality spermatozoa, and almost complete separation from other cell types and debris, the technique of choice remains discontinuous density gradients. This technique can also be tailored to optimize handling of specific properties of individual semen samples, i.e., by limiting the distance that the spermatozoa migrate and maximizing total motile sperm recovery, reducing the total volume of gradient material, or by increasing the centrifugation time for high-viscosity specimens. It is advisable that each laboratory determines the optimum centrifugation time (t) and centrifugal force (g) required to form a manageable sperm pellet. In samples containing extremely low number of sperm, modification of g or t may be necessary in order to increase the chances of recovering the greater number of spermatozoa. Such modifications to recommended g or t must be rigorously tested prior to implementation in any protocol.

Investigators have reported varied levels of contamination with seminal components in the final sperm preparation following swim-up and density-gradient centrifugation procedures [18]. Björndahl et al. [19] demonstrated time-dependent diffusion of zinc from seminal plasma into the overlaying swim-up medium by using the zinc from prostatic secretion as a marker. Following swim-up preparations, the final zinc concentration in sperm aliquot was higher than that after density-gradient preparation (Table 2.2).

2.4 Sperm Selection Protocols

General Principles

There are numerous protocols employed for selection of spermatozoa from a given semen sample. Since final harvest of spermatozoa is low from many such protocols, we have described only three simple sperm preparation protocols for sperm selection, viz., simple sperm washing, direct swim-up, and density-gradient separation.

For all the procedures listed above, it is advisable to use the culture medium containing a protein-supplemented balanced salt solution with a buffer suitable for the environmental conditions under which the sample will be processed. For ARTs, such as IUI, ICSI, IVF, AI, or gamete intrafallopian transfer (GIFT), it is necessary that the

Table 2.2 Comparative merits and demerits of sperm selection protocols

Protocol	Advantages	Disadvantages
Dilution and centrifugation	Provides highest yield of spermatozoa [5], fast, and easy	Not suitable for sample containing abnormal spermatozoa
Direct swim-up	Preferred method for separating out motile spermatozoa from low count [20], suitable for IVF and ICSI	Lower recovery of motile spermatozoa (<20%), [21], produces different levels of contamination
Density-gradient centrifugation	Greater number of motile sperm recovery, (>20%) [21], standardization is easier than the swim-up technique, more consistent results [5], suitable for IVF and ICSI procedures	Produces different levels of contamination
Glass wool column	Suitable for viscous, poor-quality, and frozen-thawed semen [18]	Low sperm harvest

IVF in vitro fertilization, *ICSI* intracytoplasmic sperm injection

highly purified bovine serum albumin (BSA) is free from bacterial, viral, and prion contamination. Commercial albumins that specifically produced ART are available. In conditions where the incubator contains only atmospheric air and the temperature is 37 °C, it is advisable to add the sperm preparation media with HEPES or a similar buffer, keeping the and the caps of the tubes tightly closed. On the other hand, if the incubator atmosphere is 5% (v/v) CO_2 in air and the temperature is 37 °C, buffer the medium with sodium bicarbonate or a similar one, and keep the caps of the test tubes loose to allow gas exchange. This is required so that the pH of the medium is compatible with sperm survival. Decision on appropriateness of the buffered medium depends upon the final disposition of the processed spermatozoa. For example, evaluating sperm-function tests requires a medium that supports sperm capacitation and therefore generally contains sodium bicarbonate (25 mM). Ensure that semen samples are collected in a sterile manner [5]. During sperm preparatory techniques for therapeutics, prevention of contamination by the use of sterile techniques and materials is mandatory.

2.4.1 Simple Washing of Spermatozoa

The procedure is adequate if semen samples are of good quality [5]. It is the most common application for preparing spermatozoa for IUI.

Materials

Ham's F-10 medium, bovine serum albumin (highly purified, BSA), HEPES salt

Ham's F-10 Medium

7.4 g of sodium chloride (NaCl)
1.2 g of sodium bicarbonate ($NaHCO_3$)
0.285 g of potassium chloride (KCl)
0.154 g of sodium monosodium phosphate (Na_2HPO_4)
0.153 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)
0.083 g of potassium dihydrogen phosphate (KH_2PO_4)
0.044 g of calcium chloride dihydrate ($CaCl_2.2H_2O$)
1.1g of D-glucose
750 mL of DW
Adjust the pH to 7.4 with 1 M sodium hydroxide (NaOH)
Dilute to 1000 mL DW

Note

(a) For incubation in air, add 20 mM HEPES (Na salt: 5.21 g/L) and reduce $NaHCO_3$ to 0.366 g/L.
(b) For density gradients, prepare a 10× concentrated stock solution by increasing the weights of the compounds tenfold, except for the bicarbonate.
(c) After preparing the gradient, supplement 100 mL with 0.12 g $NaHCO_3$.

BSA Supplement
300 mg BSA
1.5 mg of sodium pyruvate
0.18 mL of sodium lactate (60%, v/v syrup)
100 mg of sodium bicarbonate
50 mL Ham's F-10 medium

Serum Supplement
4 mL heat-inactivated (56 °C for 20 min) test animal's serum
1.5 mg of sodium pyruvate
0.18 mL of sodium lactate (60%, v/v syrup)
100 mg of sodium bicarbonate
46 mL Ham's F-10 medium

Procedure

(a) Gently but thoroughly mix the semen sample.
(b) Dilute the entire semen sample 1 + 1 with BSA-supplemented medium to promote removal of seminal plasma.
(c) Transfer the diluted suspension into multiple 3 mL centrifuge tubes.
(d) Centrifuge at 300–500 *g* for 5–10 min.
(e) Carefully aspirate and discard the supernatants.
(f) Resuspend the combined sperm pellets in 1 mL of BSA-supplemented medium by gentle pipetting.
(g) Centrifuge again at 300–500 *g* for 3–5 min.
(h) Carefully aspirate and discard the supernatant.
(i) By gentle pipetting, resuspend the sperm pellet in a volume of serum-supplemented medium appropriate for final disposition (IUI).
(j) Determine the final concentration and motility before use in ART.

Points to Ponder
Reduce the number of washings for removal of seminal plasma by increasing the tube volume as well as using still less. In such cases, increase the *g* and *t* to ensure formation of complete sperm pellet (e.g., 500–600 *g* for 8–10 min).

2.4.2 Sperm Swim-Up Technique

2.4.2.1 Direct Swim-Up I

Direct swim-up technique is useful for various investigations as well as for therapeutics. In the "swim-up" technique, sperm motility is utilized to allow them to swim out of seminal plasma and into the specific culture medium. In the direct swim-up technique, the culture media are either layered over the liquefied semen or vice versa. Following a time lag, motile spermatozoa swim out into the layered culture medium [5].

Materials
All materials as described in the above procedure may be used in this protocol.

Procedure

(a) Gently but thoroughly mix the semen sample.
(b) Take a sterile 15 mL conical centrifuge tube and place 1 mL of semen.
(c) Gently layer 1.2 mL of supplemented medium over it.
(d) Alternatively, pipette the semen carefully under the supplemented culture medium.
(e) Incline the tube at an angle of about 45°, to increase the surface area of the semen–culture medium interface.
(f) Incubate for 1 h at 37 °C.
(g) Gently return the tube to the upright position and remove the uppermost 1 mL of medium (contains highly motile sperm cells).
(h) Dilute the above collected medium with 1.5–2.0 mL of supplemented medium.
(i) Centrifuge at 300–500 g for 5 min.
(j) Discard the supernatant.
(k) Resuspend the sperm pellet in 0.5 mL of supplemented medium for assessment of SQP.
(l) The final specimen may be used directly for therapeutic or research purposes.

Points to Ponder

(a) It is advisable not to dilute or centrifuge the semen sample before subjecting them to swim-up to avoid peroxidative damage to the sperm membranes [22].

(b) Assess sperm quality parameters, e.g., concentration, total motility, and progressive motility before use.

2.4.2.2 Direct Swim-Up II

A slightly modified protocol for much greater concentration of sperm recovery [23] is described below. This sperm selection procedure is simple, rapid, and of high yield, the equipment needed is minimal, and a high fertility rate is obtained with the selected sample [24].

Materials

Sucrose, sodium chloride (NaCl), sodium lactate, HEPES, potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), glucose, magnesium sulfate ($MgSO_4$), sodium pyruvate, potassium hydrogen phosphate (K_2HPO_4), dextran, bovine serum albumin

Swim-Up Media (SM, pH 6.5)

200 mM sucrose
50 mM sodium chloride
18.6 mM sodium lactate
21 mM HEPES
10 mM potassium chloride
4 mM sodium bicarbonate
2.7 mM calcium chloride
2.8 mM glucose
0.4 mM magnesium sulfate
0.3 mM sodium pyruvate
0.3 mM potassium hydrogen phosphate
Dilute to 100 mL DW

Swim-Up Media (Working, SM-W)

10 mg bovine serum albumin
2 mL swim-up media

Dextran Solution (DS)

30 mg dextran
1 mL swim-up media

Procedure

(a) Take a round-bottom, 12–15-mm diameter tube.
(b) Pour 0.5 mL of fresh semen into the tube and gently layer 0.5 mL dextran solution.
(c) Over the above solution, gently layer 1.5 mL of SM-W solution.
(d) Keep the tube at 37 °C in a vertical position for 15 min.
(e) After 15 min, carefully remove 0.75 mL of the top media layer containing the sperm.
(f) Add 0.75 mL of the fresh SM-W solution.
(g) Repeat the incubation sequence three times so as to obtain four supernatants.
(h) Discard the first supernatant as it may contain contaminants from the seminal plasma.
(i) The last three supernatants are combined to get a final solution of 2.25 mL of suspension.
(j) Mark this final suspension as swim-up-selected sample.
(k) Determine the sperm quality parameters before use.

Points to Ponder

Always pre-warm the tubes and all solutions at 37 °C before use.

2.4.3 Discontinuous Density Gradients

Principle

In this technique, centrifugation of seminal plasma over density gradients (silane-coated colloidal silica) is carried out: this achieves sperm separation by their density. Additionally, a soft pellet formation at the bottom of the tube is further helped by active swim of motile spermatozoa through the gradient material.

A simple two-step discontinuous density-gradient preparation (with a 40% and 80% (v/v), top and bottom layer, respectively) method is most widely applied. This particular technique usually results in a fraction of highly motile sperm; moreover, resulting fraction is mostly free from contaminating leukocytes, debris, degenerating germ, and non-germ cells [5].

For semen processing, several commercial products (e.g., Percoll™) are available for making density gradients. Researchers must follow manufacturers' recommendations before using

such products, and any departure from procedural recommendations of the manufacturer should be based on evidence.

Materials
Ham's F-10 medium, bovine serum albumin (highly purified, BSA), HEPES salt, BSA supplement, serum supplement (as above), Percoll

Isotonic Density-Gradient Medium
10 mL Ham's F-10 medium (10x)
90 mL density-gradient medium
300 mg of BSA
3 mg sodium pyruvate
0.37 mL sodium lactate (60%, v/v syrup)
200 mg sodium bicarbonate

Gradient 80% (v/v)
10 mL BSA-supplemented medium
40 mL isotonic gradient medium

Gradient 80% (v/v)
30 mL BSA-supplemented medium
20 mL isotonic gradient medium

Procedure

(a) Pour 1 mL of 80% (v/v) density-gradient medium in a 15 mL cryo-centrifuge.
(b) Gently layer 1 mL of 40% (v/v) density-gradient medium over in the above tube.
(c) Mix the semen sample well.
(d) Place 1 mL of semen above the density-gradient media.
(e) Centrifuge at 300–400 *g* for 15–30 min.
(f) Remove most of the supernatant from the sperm pellet.
(g) Resuspend the sperm pellet in 5 mL of BSA-supplemented medium by gentle pipetting.
(h) Centrifuge at 200 *g* for 4–10 min.
(i) Repeat the washing procedure (steps g and h above).
(j) Resuspend the final pellet in supplemented medium.
(k) Determine the concentration, viability, and motility.

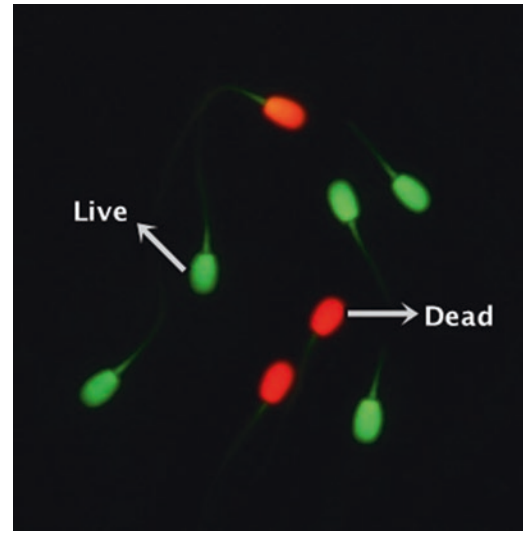

Fig 2.1 Microphotograph shows viability staining of spermatozoa using CFDA/PI fluorescent stain. Because of the intact membrane, live cells fluorescence green, whereas morbid cells allow entry of PI and turn red

Observations
Viability of the spermatozoa can be determined by any of the several assays available, namely, CFDA/PI (carboxyfluorescein diacetate/propidium iodide, Fig 2.1), H33258, or eosin-nigrosin staining. Assays for viability, motility, and concentration are given in the respective chapters.

Points to Ponder

(a) High relative molecular mass components having inherently low osmolality are part of most density-gradient media. It is for this reason that these should usually be prepared in medium that is isoosmotic with female reproductive tract fluids. This is useful when sperm cells are to be used for artificial insemination purposes.
(b) Although isotonic density-gradient media are generally referred to as 100, 80, and 40% (v/v), they are actually 90, 72, and 36% (v/v), respectively.
(c) Use more than one tube per semen sample, if necessary.
(d) Gently pipette to remove contaminated seminal plasma containing cellular debris.
(e) In some species, very high "g" is required to achieve desired results.

2.4.4 Glass Wool Filtration Column

Selection of spermatozoa using glass wool filtration column is described below [17].

Materials
Glass wool microfiber, Brackett and Oliphant (BO) medium, bovine serum albumin (BSA)

BO Supplemented Medium
5 mM caffeine sodium benzoate
50 μg heparin
50 mg BSA
Dilute to 5 mL BO medium

Procedure

Semen Preparation

(a) Empty content of two semen straws (0.5 mL) in a 1.5 mL cryovial.
(b) Thaw in a water bath at 37 °C for 1 min.

Sperm Selection

(a) Take a 2 mL disposable sterile syringe and remove the plunger.
(b) Gently place 25 mg of pre-cleaned glass wool microfiber at a depth of 1 cm in the above syringe.
(c) Vertically suspend the column and rinse repeatedly with BO supplemented medium.
(d) Insert the rinsed column nozzle in the collection tube place in a water bath at 37 °C.
(e) Gently layer 1 mL of thawed semen in filtration tube 37 °C for 5 to 10 min.
(f) Wash the recovered sample in 6 mL BO medium by centrifugation at 300 x g for 5 min.
(g) Adjust the sperm concentration to 1 million/mL in BO supplemented medium.
(h) Evaluate sperm quality parameters.

Points to Ponder

(a) Repeated rinsing with BO supplemented medium is required to remove any loose wool fibers prior to filtration.
(b) For IVF assay, use 100 μL of final sperm suspension.

2.5 Preparing Testicular and Epididymal Spermatozoa

In some of the experiments, spermatozoa sans seminal plasma are required, for example, in studies involving effect of seminal proteins on spermatozoa or in clinical cases involving obstructive azoospermia. Moreover, in ART where testicular spermatozoa are difficult to get by, harvesting of comparatively greater numbers of sperm is possible for therapeutic purposes, e.g., IUI. In such cases, testicular tissue and the epididymal spermatozoa are recovered and utilized, collection of which needs special preparation. In order to isolate the elongated spermatids bound with seminiferous tubule ("testicular spermatozoa"), employing mechanical or enzymatic procedures is recommended (Table 2.3).

Testicular Harvest of Spermatozoa
There are two procedures employed to harvest testicular spermatozoa for ART procedures, especially intracytoplasmic sperm injection (ICSI) [5].

Enzymatic

(a) Incubate the recovered testicular tissue with collagenase (e.g., 0.8 mg of *Clostridium histolyticum*, type 1A per mL of medium) for 1.5–2 h at 37 °C.
(b) Vortex at 30 min interval.
(c) Centrifuge at 100 *g* for 10 min and then ascertain quality of the pellet.

Table 2.3 Merits of epididymal vs testicular spermatozoa

Procedure	Advantages	Disadvantages	Remarks
Epididymal aspirations	Minimal contamination from RBC and non-germ cells, selection of motile sperm relatively straightforward	In some species, it is difficult to locate the epididymis	Concentrate sperm using density-gradient centrifugation, sperm wash for low number of sperm
Testicular sperm	Invariably contamination from RBC and non-germ cells, used for ICSI procedures	Additional steps are needed to isolate a clean preparation of spermatozoa; sperm numbers are low with poor motility	Retrieved by open biopsy (with or without microdissection) or by percutaneous needle biopsy

ICSI intracytoplasmic sperm injection

Mechanical

(a) Macerate the recovered testicular tissue suspended in culture medium with glass pestle until fine slurry of dissociated tissue is produced.
(b) Alternatively, use fine needles (attached to disposable tuberculin syringes) bent parallel to the base of the culture dish, and strip the cells from the seminiferous tubules.

2.6 Processing Sperm Suspensions for ICSI

Processing of the sperm suspension obtained by either of the two methods described above requires careful processing as detailed below [5].

Materials
Culture medium, mineral oil, polyvinylpyrrolidone

Polyvinylpyrrolidone Solution (10%)
100 g polyvinylpyrrolidone
1000 mL culture medium

Procedure

(a) Wash the specimens obtained by adding 1.5 mL of culture medium.
(b) Centrifuge at 300 *g* for 8–10 min.
(c) Remove the supernatant and resuspend the pellet in 0.5 mL of fresh culture medium.
(d) Estimate the motility and number of spermatozoa in the pellet.
(e) Place a 5–10 μL droplet of culture medium in a culture dish.
(f) Cover it with mineral oil (pre-equilibrated with CO_2).
(g) Introduce 5–10 μL of the sperm suspension into the culture medium.
(h) Carefully aspirate the motile spermatozoa found at the interface between the culture medium and oil with an ICSI pipette.
(i) Transfer them to a droplet of viscous solution, e.g., polyvinylpyrrolidone

Points to Ponder

Resuspend specimens with a low sperm number in a lower volume of medium.
Serum-supplemented Ham's F-10 medium can be used as a culture medium.

2.7 Background Information

It is crucial for an investigator to not only select a suitable protocol for separation of viable and fertile spermatozoa but also to organize his/her experimentation in such a way to achieve repeatable and acceptable results. Since such experimentations are not routinely used and are often one-off experiments, a mock carryout will be better before arrangements are made for actual experimentations.

We have suggested a possible spread of the experiments involving selection of the spermatozoa followed by their application in ICSI or other ARTs (Table 2.4).

Once sperm selection protocol is decided and aliquot containing live sperm cells is obtained, evaluation of the quality of the final sample is mandatory to measure success of the protocol and further processing.

Experiments need to be designed in such a way to optimally utilize the resources, namely, chemicals, fluorescent dyes, space, and time, for example, evaluation of multiple parameters (apoptosis and DNA fragmentations using fluorescent probes H33258 and in situ cell death detection kit, fluorescein, Roche Diagnostics, Indianapolis, Ind., respectively). In this procedure, spermatozoa fluorescence green with TUNEL technique, whereas H33258 probe imparts blue fluorescence (Fig. 2.2) to the spermatozoa at a different wavelength.

Table 2.4 Suggested spread out of experiments

Sl	Experiments	
	Session I	Session II
1	Sperm selection procedure	Cell concentration
2	Viability	Phosphatidylserine inversion
3	Motility	TUNEL assay (DNA integrity)
4	Mitochondrial functionality	IVF assay

TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling, *IVF* in vitro fertilization

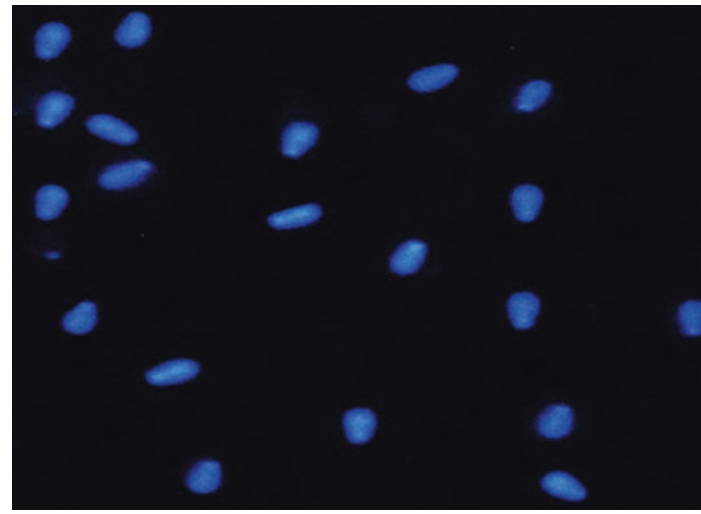

Fig 2.2 Microphotograph shows blue/cyan fluorescent spermatozoa after staining with H33258 fluorescent probe (excitation max., 346 nm; emission max., 460 nm). For color palette of other fluorescent probes, a different excitation/emission wavelength would be required and hence is visualized in the same field after switching the spectrum

Literature Cited

1. Saake RG, White JM (eds) (1972) Semen quality tests and their relationship to fertility. In: Proceedings of the 4th National Association of Animal Breeders, Technical Conference; April 18–20. National Association of Animal Breeders, Columbia, pp 22–27
2. Rocaa J, Parrillaa I, Gila MA, Cuelloa C, Martineza EA, Rodriguez-Martinez H (2016) Non-viable sperm in the ejaculate: lethal escorts for contemporary viable sperm. Anim Reprod Sci 169:24–31
3. Overstreet JW, Gould JE, Katz DF, Hanson FW (1980) In-vitro capacitation of human spermatozoa after passage through a column of cervical mucus. Fertil Steril 34:604–606
4. Canale D, Giorgi PM, Gasperini M, Pucci E, Barletta D, Gasperi M, Martino E (1994) Inter- and intra-individual variability of sperm morphology after selection with three different techniques: layering, swimup from pellet and Percoll. J Endocrinol Invest 17:729–732
5. WHO laboratory manual for the examination and processing of human semen. Cooper TG (ed) 5th edn. World Health Organization 2010, Switzerland
6. Boomsma CM, Heineman MJ, Cohlen BJ, Farquhar C (2007) Semen preparation techniques for intrauterine insemination. Cochrane Database Syst Rev 17(4):CD004507
7. Morshedi DHE, Taylor S, Oehninger S (2003) Efficacy and pregnancy outcome of two methods of semen preparation for intrauterine insemination: a prospective randomized study. Fertil Steril 79(Suppl. 3):1625–1632
8. Johnson DE, Confino E, Jeyendran RS (1996) Glass wool column fi ltration versus mini-Percoll gradient for processing poor quality semen samples. Fertil Steril 66:459–462
9. Bollendorf A, Check JH, Katsoff D, Lurie D (1994) Comparison of direct swim-up, mini-Percoll, and Sephadex G10 separation procedures. Arch Androl 32:157–162
10. Siegel MS, Haynie LB (1994) Effect of human sperm capacitation treatments on the penetration of freshly obtained and zona-free frozen hamster oocytes. Arch Androl 32:5–11
11. Lessley BA, Garner DL (1983) Isolation of motile spermatozoa by density gradient centrifugation in Percoll. Gamete Res 7:49–61
12. Anzar M, Graham EF (1996) Role of sperm motility and acrosome integrity in the filtration of bovine semen. Theriogenology 45:513–520
13. Januskauskas A, Lukoseviciute K, Nagy S, Johannisson A, Rodriguez-Martinez H (2005) Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation. Theriogenology 63:160–178
14. Saeki K, Hoshi M, Leibfried-Rutledge ML, First NL (1991) In vitro fertilization and development of bovine oocytes matured in serum-free medium. Biol Reprod 44:256–260
15. Sakkas D, Manicardi G, Bizzaro D, Bianchi PG (2003) Possible consequences of performing intracytoplasmic sperm injection (ICSI) with sperm possessing nuclear DNA damage. Hum Fertil (Camb) 3:26–30
16. Coetzee K, Erasmus EL, Kruger TF, Menkveld R, Lombard CJ (1994) Glass wool filter preparation of cryopreserved spermatozoa. Andrologia 26:33–34
17. Engel S, Weber H, Petzoldt R, Seidl B, Wiehe W, Sperl J (2001) An improved method of sperm selection by glass wool filtration. Andrologia 33:223–230
18. Lee H-L, Kim S-H, Ji D-B, Kim Y-J (2009) A comparative study of Sephadex, glass wool and Percoll separation techniques on sperm quality and IVF results for cryopreserved bovine semen. J Vet Sci 10(3):249–255
19. Björndahl L, Barratt CL (2005) Semen analysis: setting standards for the measurement of sperm numbers. J Androl 26:11
20. Mortimer D (1994) Laboratory standards in routine clinical andrology. Reprod Med Rev 3:97–111
21. Ng FL, Liu DY, Baker HW (1992) Comparison of Percoll, mini-Percoll and swim-up methods for sperm preparation from abnormal semen samples. Hum Reprod 7:261–266
22. Aitken RJ, Clarkson JS (1988) Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 9:367–376
23. García-López N, Ollero M, Muiño-Blanco T, Cebrián-Pérez JA (1996) A dextran swim-up procedure for the separation of highly motile and viable ram spermatozoa from seminal plasma. Theriogenology 46:141–151
24. Muiño-Blanco T, Cebrián I, Casaol A, Pérez-Pé R, José A, Cebrián-Pérez (2004) Understanding the sperm separation principles used in assisted reproductive technology as a turning point for biomedical students. In: Méndez-Vilas A (ed) Microscopy: advances in scientific research and education. FORMATEX, Extremadura

Key References

25. WHO laboratory manual, 5th edn 2010. See above [5] For excellent explanation of collection of sperm from epididymis and testis and sperm selection technique
26. Hae-Lee Lee and others (2009). See above [18] For excellent presentation of comparison of various protocols for sperm selection using Sephadex, glass wool and Percoll separation techniques on sperm quality and IVF results for frozen-thawed bovine semen