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5 Semen Preparation: Ejaculated Sperm for ICSI

INTRODUCTION

Prolonged exposure of sperm to seminal plasma results in a marked decline in both motility and viability. Sperm incubated in synthetic culture medium free of seminal plasma contamination show no such declines. It is essential, therefore, that spermatozoa for clinical procedures such as *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) be separated from the seminal environment as soon as possible after ejaculation.

The most common methods for sperm processing are the swim-up technique and the separation through a discontinuous colloidal density gradient. Alternatively, simple washing can be employed in cases of severely oligozoospermic ejaculates. The advantage of swim-up and density gradient are that they select the sperm population exhibiting better motility, in contrast to the non-selective concentration of spermatozoa obtained through a simple wash procedure.

Sperm processing by swim-up or density gradient eliminate immotile and dead spermatozoa, along with exfoliated cells, cellular debris, and amorphous material. Sperm preparation by swim-up removes seminal plasma and concentrates the most motile spermatozoa in a very small volume of sperm culture media. However, the sperm yield is low in cases of oligozoospermic ejaculates with low motility. For this reason, swim-up is preferred for normozoospermic specimens. Conversely, density gradient centrifugation is usually preferred to process ejaculates with low sperm number, motility, or morphology, as it allows the elimination of leukocytes and other microorganisms which are trapped in the gradient interphases. Density gradients can be altered to optimize sperm recovery by decreasing the gradient volume which limit the distance that the spermatozoa migrate, or by increasing the centrifugation time in cases of hyperviscous ejaculates.

MATERIALS, EQUIPMENT AND REAGENTS

Material and Equipment

- Sterile disposable serological pipettes (1, 2, 5 and 10 mL, e.g. cat. #4051 Costar or cat. #356543 Falcon, USA)
- Disposable polystyrene conical (swim-up) and round-bottom (density gradient) centrifuge tubes (sterile) with caps.

- Disposable transfer pipets (sterile)
- Pipettor 1 to 200 µL (Gilson, France) and Sterile tips (e.g. cat. #4804, Corning, USA)
- Pipetting device (e.g. Pipette-aid, Drummond Scientific, USA)
- 6 mL sterile centrifuge polystyrene tubes with caps (e.g. cat. #352003, Falcon, USA)
- Makler Chamber
- Fine point permanent marker pen (e.g. Sharpie, Sandford, USA)
- Laminar flow cabinet
- 37°C incubator
- Centrifuge (e.g. model 225; Fisher Scientific, USA).

Reagents

- HEPES-buffered Human Tubal Fluid (e.g. Modified HTF culture medium, cat. #90126, Irvine Scientific, USA)
- Human Serum Albumin (HSA, cat. #9988, Irvine Scientific, USA)
- Colloidal density gradient (e.g. Isolate®, cat. #99264, Irvine Scientific, USA) or Pure ception® , Sage Biopharma, Bedminster, NJ. Both are colloidal suspension of silica particles stabilized with covalently bonded hydrophilic silane supplied in HEPES
  1. Lower phase (90%)
  2. Upper phase (47%).

PROCEDURES

Double-density Gradient Centrifugation

Prepare Reagents

1. Bring all components of the gradient kit (upper and lower phase) and semen samples to 37°C, for 20 minutes, in the incubator.
2. Transfer 1 mL (volume of gradient may be reduced) of the lower phase colloidal gradient into a sterile conical bottom disposable centrifuge tube.
3. Layer 1 mL upper phase on top of the lower phase using a transfer pipet. Slowly dispense the upper phase lifting the pipet up the side of the tube as the level of the upper phase rises. A distinct line separating the two layers will be observed. This two-layer gradient is stable for up to two hours.
4. Label 15 mL centrifuge tube(s) with patient's name.

Analyze and Wash Specimen

Note: Sterile techniques should be used throughout specimen processing. Sperm processing should be performed inside a Laminar flow cabinet (e.g. Class II Bio-safety cabinet)

1. Semen specimen should be allowed to liquefy completely for 15 to 30 minutes in the 37°C incubator before processing.
2. Measure volume using a sterile 5 to 10 mL pipet.

3. Remove a drop of semen using sterile technique and do a count, motility and round cell count.
   Note: Perform a pre-wash analysis. While examining the specimen, pay particular attention to extraneous round cells, debris, and bacteria that may be present. If the number of round cells are >1 million/mL, perform Endtz test immediately. A positive Endtz test should be reported to the lab director immediately.
4. Gently place up to 2 mL of liquefied semen onto the upper phase. If volume is greater than 2 mL, it may be necessary to split the specimen into two tubes before processing.
5. Centrifuge for 20 minutes at 1600 rpm.
6. The supernatant should be removed with a sterile transfer pipette to the level directly below the second layer.
7. Using a transfer pipet, add 1.5 to 2 mL of HEPES-buffered sperm wash media (HTF) and resuspend pellet. Mix gently with pipet until sperm pellet is in suspension.
   Note: Buffered-medium (HEPES or similar) is to be used with atmospheric air 37°C incubators, and the tubes' caps should be tightly closed. If the 37°C incubator atmosphere is 5% (v/v) CO_2 in air, then the medium should be buffered with sodium bicarbonate or a similar buffer, and the tubes'caps should be loose to allow gas exchange. Adherence to these principles will ensure that the culture pH is compatible with sperm survival.
8. Centrifuge for seven minutes at 1600 rpm.
9. Again, remove supernatant from the centrifuge tube using a transfer pipet down to the pellet.
10. Resuspend the final pellet in a volume of 0.5 mL using a 1 mL sterile pipet with sperm wash media (HTF). Record the final volume. Do a routine post-wash semen analysis.

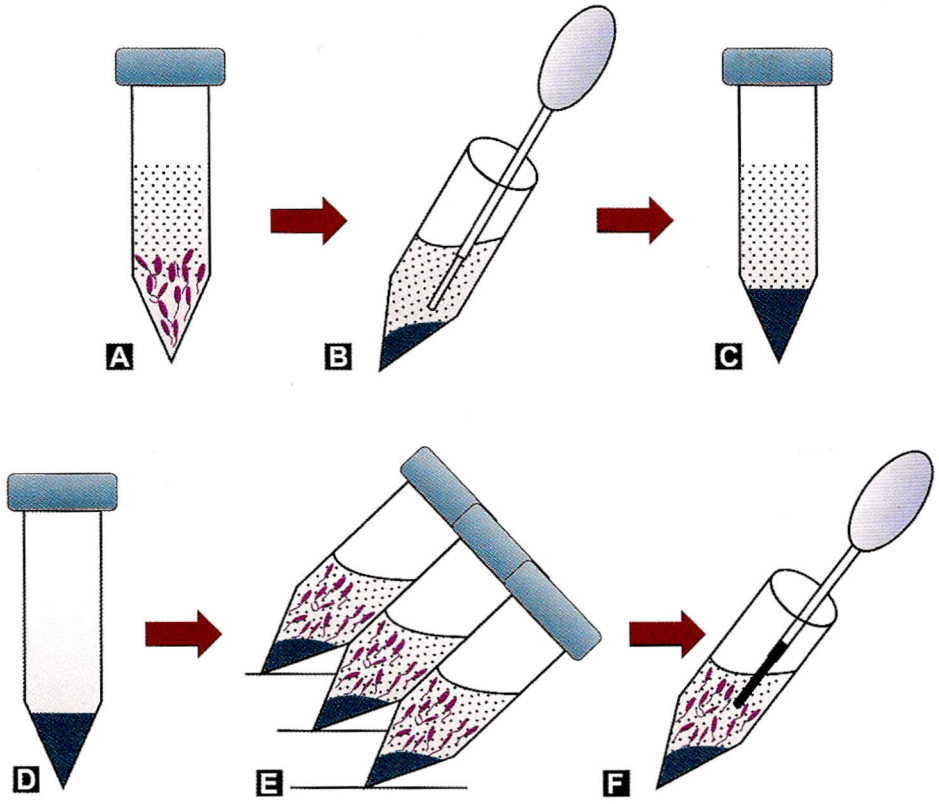
Swim-up (Figs 5.1A to F)

Spermatozoa may be selected by their ability to swim out of seminal plasma and into culture medium. The semen may be diluted and centrifuged prior to swim-up, although some authors argue against this step due to the risk of peroxidative damage to the sperm membranes. Alternatively, a direct swim-up of spermatozoa from semen can be used.

A. Prepare Reagents: Bring sperm wash media to 37°C for 20 minutes in the incubator.
B. Analyze and Wash Specimen as follows:
   Note: Sterile techniques should be used throughout specimen processing.

Swim-up from a Sperm Pellet

1. Specimen should be allowed to liquefy completely for 15 to 30 minutes in the 37°C incubator before processing.
2. Measure volume using a sterile 5 to 10 mL pipet.
   Note: Perform a pre-wash analysis. While examining the specimen, pay particular attention to extraneous round cells, debris, and bacteria that may be present. If the number of round cells are > 1 million/mL, perform Endtz test immediately. A positive Endtz test should be reported to the lab director immediately.



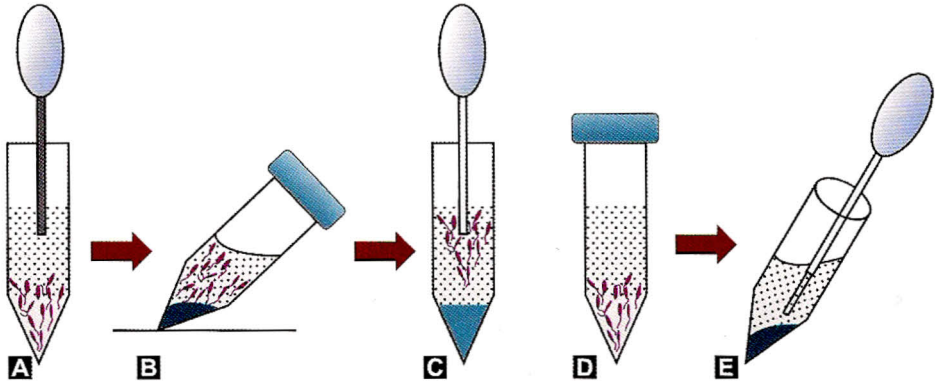
FIGURES 5.1A TO F: Swim-up from the centrifuged pellet

A. Transfer specimen from a plastic cup to a sterile 15 mL conical centrifuge tube. If specimen is >2 mL, split into two or more tubes.
   Gently mix the specimen with HEPES-buffered sperm wash media in a ratio of 1:4 by using a sterile pasteur pipet.
   Centrifuge the tubes at 1600 rpm for 10 minutes.
B. Carefully aspirate the supernatant without disturbing the pellet.
C. Resuspend the pellet in 3 mL of fresh HEPES-buffered sperm wash media (HTF). Centrifuge the tubes at 500 rpm for five minutes.
D. Discard the supernatant without disturbing the pellet and resuspend to a final volume of 600 μL of sperm medium supplemented with 5% HSA.
E. Divide the resuspended specimen in three equal aliquots of 200 μL. Underline each aliquot beneath 800 μL of protein-supplemented sperm medium in 15 mL centrifuge tubes. Incubate the tubes at 45° angle for one hour for sperm swim-up in vertical rack in a 37°C incubator.
F. After the incubation period, aspirate 600 to 700 μL supernatant from round bottom tube. Use a pasteur pipet, with the tip placed jut about the pellet surface. Pool supernatant from the two round bottom tubes into a single 15 mL conical centrifuge tube. Centrifuge the tube at 1600 rpm for seven minutes. Aspirate the supernatant from the top of the miniscus using a pasteur pipet. Resuspend the pellet in a volume of 0.5 mL protein-supplemented sperm wash media (HTF) using a 1 mL sterile pipet and keep at 37°C until ICSI. Record the final volume.

3. Transfer specimen from a plastic cup to a sterile 15 mL conical centrifuge tube. If specimen is >2 mL, split into two or more tubes.
4. Gently mix the specimen with HEPES-buffered sperm wash media in a ratio of 1:4 by using a sterile pasteur pipet.
5. Centrifuge the tubes at 1600 rpm for 10 minutes.
6. Carefully aspirate the supernatant without disturbing the pellet and resuspend the pellet in 3 mL of fresh HEPES-buffered sperm wash media (HTF).
7. Transfer the resuspended sample into two 15 mL sterile round bottom tubes using plastic pipets (1.5 mL in each).
8. Centrifuge the tubes at 500 rpm for 5 minutes.
9. Discard the supernatant without disturbing the pellet and resuspend to a final volume of 600 µL of sperm medium supplemented with 5% HSA.
10. Divide the resuspended specimen in three equal aliquots of 200 µL. Underline each aliquot beneath 800 µL of protein-supplemented sperm medium in 15 mL centrifuge tubes.
    Note: Buffered-medium (HEPES or similar) is to be used with atmospheric air 37°C incubators, and the tubes' caps should be tightly closed. If the 37°C incubator atmosphere is 5% (v/v) CO_2 in air, then the medium should be buffered with sodium bicarbonate or a similar buffer, and the tubes'caps should be loose to allow gas exchange. Adherence to these principles will ensure that the culture pH is compatible with sperm survival.
11. Incubate the tubes at 45° angle for one hour for sperm swim-up in vertical rack in a 37°C incubator.
12. After the incubation period, aspirate 600 to 700 µL supernatant from round bottom tube. Use a pasteur pipet, with the tip placed jut about the pellet surface.
13. Pool supernatant from the two round bottom tubes into a single 15 mL conical centrifuge tube. Centrifuge the tube at 1600 rpm for seven minutes.
14. Aspirate the supernatant from the top of the miniscus using a pasteur pipet.
15. Resuspend the pellet in a volume of 0.5 mL protein-supplemented sperm wash media (HTF) using a 1 mL sterile pipet and keep at 37°C until ICSI. Record the final volume.
16. Remove a small well-mixed aliquot (~0.1 mL) and make post-wash analysis.

Direct Swim-up (Figs 5.2A to E)

1. Place 1 mL of homogeneized and liquefied semen in a sterile 15 mL conical centrifuge tube, and gently layer 1.0 to 1.5 mL of protein-supplemented sperm medium over it. Alternatively, pipette the semen carefully under the supplemented culture medium.
2. Incline the tube at an angle of about 45°, to increase the surface area of the semen culture medium interface, and incubate for one hour at 37°C.
3. Gently return the tube to the upright position and remove the uppermost 1 mL of medium.
4. Dilute this with 1.5 to 2.0 mL of supplemented medium.
5. Centrifuge the tube at 1600 rpm for five minutes.
6. Aspirate the supernatant from the top of the miniscus using a pasteur pipet.
7. Resuspend the pellet in a volume of 0.5 mL protein-supplemented sperm wash media (HTF) using a 1 mL sterile pipet and keep at 37°C until ICSI. Record the final volume. Remove an aliquot for post-wash analysis.



FIGURES 5.2A TO E: Direct swim-up

A. Place 1 mL of homogeneized and liquefied semen in a sterile 15 mL conical centrifuge tube, and gently layer 1.0 to 1.5 mL of protein-supplemented sperm medium over it. Alternatively, pipette the semen carefully under the supplemented culture medium.
B. Incline the tube at an angle of about 45°, to increase the surface area of the semen culture medium interface, and incubate for one hour at 37°C.
C. Gently return the tube to the upright position and remove the uppermost 1 mL of medium.
D. Dilute this with 1.5–2.0 mL of supplemented medium and centrifuge the mixture at 1600 rpm for 5 minutes.
E. Aspirate the supernatant from the top of the meniscus using a pasteur pipet. Resuspend the pellet in a volume of 0.5 mL protein-supplemented sperm wash media (HTF) using a 1 mL sterile pipet and keep at 37°C until ICSI. Record the final volume. Remove an aliquot for post-wash analysis.

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