

Chapter 2

Methods of Sperm Vitality Assessment

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Abstract

Sperm vitality is a reflection of the proportion of live, membrane-intact spermatozoa determined by either dye exclusion or osmoregulatory capacity under hypo-osmotic conditions. In this chapter we address the two most common methods of sperm vitality assessment: eosin–nigrosin staining and the hypo-osmotic swelling test, both utilized in clinical Andrology laboratories.

Key words: Sperm vitality, Eosin–nigrosin staining, Hypo-osmotic swelling

1. Introduction

Vitality is a reflection of the proportion of live spermatozoa determined by the evaluation of cellular and/or membrane integrity. When the percentage of immotile spermatozoa exceeds 40%, it becomes clinically important to verify the proportion of live spermatozoa (1). This assessment can differentiate between necrozoospermia and total absence of motility, indicative of structural defects in the flagellum (2). Accurate motility evaluation can also be verified by sperm vitality tests, as the percentage of immotile spermatozoa should not be higher than the percentage of dead spermatozoa (3).

Most assessments of sperm vitality are based on the ability of the cell membrane to exclude dyes from entering the spermatozoa and permeate into its nucleus. Vital stains are suitable for bright field microscopy (eosin, eosin–nigrosin, trypan blue) or fluorescence microscopy; some fluorochromes can be used for flow-cytometric assessment (propidium iodine, hoechst, ethidium homodimer-1, or Yo-Pro-1) (1, 4).

Sperm vitality can also be assessed by measurement of osmoregulatory capacity under hypo-osmotic conditions (150 mOsm/L), an indication of functional sperm membrane

integrity (5). The hypo-osmotic swelling (HOS) test is based on the principle that a living spermatozoon maintains an osmotic gradient by controlled swelling, which results in curling of its tail under hypo-osmotic conditions, whereas a dead spermatozoon exhibits uncontrolled swelling to the degree of membrane rupture, resulting in tail straightening (3). The HOS test identifies live spermatozoa without killing them, allowing utilization of these spermatozoa for therapeutic procedures, such as intracytoplasmic sperm injection (ICSI). However, the test has limitations, in that it is known that some live spermatozoa can have a nonfunctional membrane (6).

While several techniques and dyes are available for sperm vitality evaluation, in this chapter we describe dye exclusion with eosin–nigrosin staining and HOS test, both recommended by the WHO for use in clinical Andrology laboratories (1).

2. Materials

2.1. *Eosin–Nigrosin Staining*

1. Eosin–nigrosin stain: Dissolve 0.67 g eosin Y (color index 45380) and 0.9 g of sodium chloride in 100 mL of distilled water in a glass beaker placed on a stirring hot plate (see Note 1). Heat gently and add 10.0 g nigrosin (color index 50420) and dissolve it before bringing the stain to a boil. As soon as boiling is observed, remove the beaker from the hot plate and allow it to cool to room temperature. Strain using filter paper, seal, and store at 4 °C in a dark glass bottle (see Note 2).
2. Phosphate-Buffered Saline (PBS): Dissolve one packet of Sigma PBS, pH 7.4 powder in 1,000 mL of distilled water to obtain a 0.01 M PBS solution (see Note 3). Store at room temperature for up to 6 months.
3. Microscope with bright field optics and $\times 100$ oil immersion objective.
4. Stirring hot plate.
5. Microscope slides, $25 \times 75 \times 1$ mm.
6. Coverslips, 22×50 mm, #1 thickness.
7. Disposable Pasteur pipettes.
8. Microtubes, 1.5 mL.
9. Immersion oil.
10. Mounting medium.
11. Laboratory counter.
12. Laboratory timer.

2.2. Hypo-osmotic Swelling Test

1. Hypo-osmotic solution: Dissolve 1.375 g D-fructose and 0.75 g of sodium citrate dihydrate in 100 mL of distilled water. Store at 4 °C for up to 6 months. Alternatively the solution can be aliquoted to 1 mL and kept frozen at -20°C for up to 1 year.
2. Microscope with phase contrast optics and ×40 objective.
3. Laboratory hot plate.
4. Microscope slides, 25 × 75 × 1 mm.
5. Coverslips, 22 × 22 mm, #1 thickness.
6. Disposable Pasteur pipettes.
7. Microtubes, 1.5 mL.
8. Laboratory counter.
9. Laboratory timer.

3. Methods

Assess sperm vitality at least 30 min after sample collection to allow for liquefaction of the sample but no longer than 1 h after ejaculation. Mix sample well by swirling before taking aliquots for both types of vitality evaluation. Prior to performing vitality assessment obtain information regarding quality of the semen sample such as sperm concentration and motility. In case of oligozoospermia, when sperm concentrations are below the lower reference limit, the sample can be concentrated by centrifugation (see Note 4).

3.1. Eosin–Nigrosin Staining

1. Add 50 µL of eosin–nigrosin stain to 50 µL liquefied semen in a microtube, and mix well (see Note 5).
2. After 30 s, transfer one drop of the mixture onto a microscope slide and smear (see Note 6).
3. Allow the slide to air-dry.
4. Place one drop of mounting medium and cover with a 22 × 50 mm coverslip (see Note 7).
5. Examine the slide under ×100 oil immersion with a bright field microscope (see Note 8). Live spermatozoa are left unstained (membrane-intact) and dead spermatozoa stain pink or red (membrane-damaged) (see Note 9) (Fig. 1).
6. Evaluate at least 200 spermatozoa, tally the numbers of live and dead spermatozoa using a laboratory counter, and calculate the percentage of live spermatozoa (see Note 10).
7. The lower reference limit for vitality assessed by eosin–nigrosin stain (membrane-intact spermatozoa) is 58% (5th percentile, 95% CI 55–63) (1).

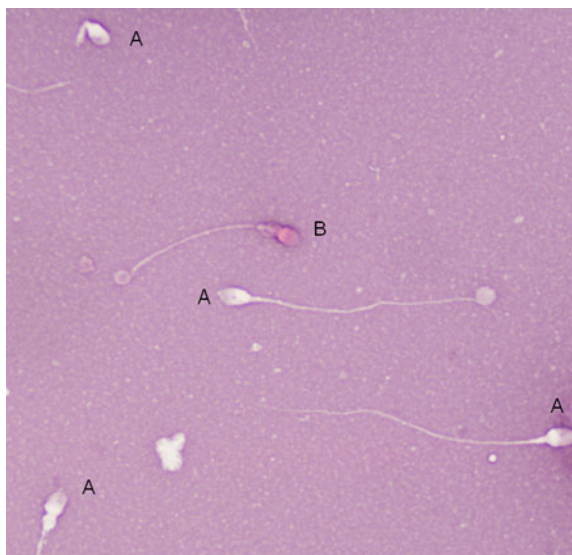


Fig. 1. Eosin–nigrosin staining: Live spermatozoa are unstained (A); dead spermatozoa are stained pink or red (B).

3.2. Hypo-osmotic Swelling Test

1. Place a 1 mL aliquot of hypo-osmotic solution into a 1.5 mL microtube, and warm it up to 37°C.
2. Add 0.1 mL of liquefied, well-mixed semen (see Note 11).
3. Incubate the tube at 37 °C for 30 min (see Note 12).
4. Mix the contents of the tube with a Pasteur pipette.
5. Place one drop of this mixture onto a microscope slide and cover with a 22 × 22 mm coverslip.
6. Allow spermatozoa to settle for 1 min.
7. Examine the slide with ×40 objective utilizing a phase-contrast microscope. Live spermatozoa show controlled swelling visualized by the curling of their tail (membrane-intact) and dead spermatozoa have straight, non-coiled tails (membrane damaged) (see Notes 13 and 14) (Fig. 2).
8. Calculate the HOS test score by evaluating at least 200 spermatozoa. Tally the numbers of live (swollen) and dead (straight tail) spermatozoa using a laboratory counter and calculate the percentage of vital spermatozoa (see Note 15).
9. The lower reference limit for vitality assessed by the HOS test (membrane-intact spermatozoa) is 58% (5th percentile, 95% CI 55–63) (1).

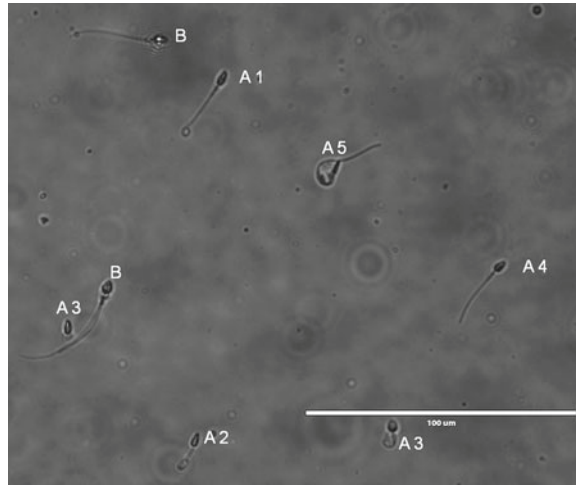


Fig. 2. HOS test: Live spermatozoa show swelling with various changes of the tail region (A1–A5): tail tip enlargement (A1), different degrees of tail curling (A2, A3), shortening and thickening of the tail (A4), partial tail swelling (A5). Dead spermatozoa have a straight tail (B).

4. Notes

1. Use a magnetic stir bar to dissolve ingredients for eosin-nigrosin staining.
2. Warm the stain to room temperature before use.
3. PBS can be used if the specimen is very viscous. Combine sample 1:2 with 0.01 M PBS solution, and mix well with a Pasteur pipette by drawing it in and out of the pipette. Spin the sample down by centrifugation at $300\times g$ for 10 min. Remove the seminal plasma, and resuspend the pellet with PBS, and then proceed to follow the protocol as for a sample with normal viscosity. If viscosity remains, repeat the process with a second PBS wash.
4. In cases of severe oligozoospermia (sperm concentration less than five million/mL), spin the sample down by centrifugation at $300\times g$ for 10 min. Remove the seminal plasma, resuspend the pellet in PBS, and then perform the viability testing on a drop from the re-suspended sample. After concentrating sperm follow the protocol as for a sample with a normal sperm concentration. In some cases of severe oligozoospermia, several slides are necessary to prepare for evaluation of a sufficient number of spermatozoa.
5. Use a disposable Pasteur pipette to mix the sample and stain. Avoid making air bubbles.

6. Avoid making the smear too thick, as the background could be too dark to visualize the spermatozoa.
7. Coverslipping using mounting medium is optional and required only for long-term storage of slides. Depending on the mounting medium used, it may take up to several days for the coverslip to be permanently glued to the slide. While slides can be evaluated shortly after coverslipping, wait for mounting medium to dry completely before long-term storage. The slides can be stored indefinitely at room temperature.
8. The nigrosin stain provides a dark purple background that makes it easier to visualize unstained spermatozoa.
9. Spermatozoa with staining restricted to the neck region but an unstained head area are referred to as “leaky necks” and are considered alive.
10. If the percentage of live cells counted is less than the sperm motility, reexamine the sample for motility and perform the stain again.
11. In cases of mild oligozoospermia, an additional 0.1–0.3 mL of semen can be added to the hypo-osmotic solution to increase sperm concentration. In cases of severe oligozoospermia, see Note 4.
12. Swelling of spermatozoa with intact membranes in hypo-osmotic solution will start within 5 min; however the shapes will stabilize after 30 min of the incubation (7). If spermatozoa are going to be utilized for a therapeutic procedure, such as ICSI, incubation should be limited to 5 min.
13. Live spermatozoa with poor osmoregularity, such as senescent spermatozoa, will show uncontrolled swelling similar to dead spermatozoa to the degree of membrane lysis and tail straightening (3).
14. Various types of tail changes can be seen as the evidence of spermatozoa swelling, including only tail tip enlargement, a hairpin curvature at the lower part of the tail, different degrees of tail curling, coiling or swelling, shortening and thickening of the tail, and total swelling of tail (Fig. 2).
15. Prior to the HOS test, neat (unprocessed) semen samples should be examined to determine if any spermatozoa have undergone spontaneous swelling or have spermatozoa with coiled tails. If any affected spermatozoa (tail defects or swollen) are detected, the neat sample should be scored in the same manner as a sample after incubation in hypo-osmotic solution. The number of affected spermatozoa in neat samples should be subtracted from the HOS test score in order to obtain an accurate assessment.

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