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The Sperm Penetration Assay for the Assessment of Fertilization Capacity

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Summary

The sperm penetration assay, or zona-free hamster oocyte penetration assay is utilized to measure the ability of sperm to undergo capacitation, fuse with the egg membrane and decondense the sperm head within the cytoplasm of the oocyte resulting in the formation of the male pronucleus. The test is scored by calculation the percentage of ova that are penetrated or the average number of sperm penetrations per ovum. It has been used to identify those couples who will have a high likelihood of success with in vitro fertilization.

Keywords

male infertility; sperm; zona pellucida; gamete interaction; sperm-oocyte penetration

1. INTRODUCTION

Infertility is a difficult and stressful condition for both the patients and the treating physicians as well. The failure to conceive within 1 year occurs in about 15% of couples(1), and about 50% of problems related to conception are either caused entirely by the male or is a combined problem with the male and his female partner. Male infertility continues to be a clinical challenge of increasing significance. The routine semen analysis remains the cornerstone in the evaluation of male factor infertility, however the diagnostic capabilities currently available to assist in formulating a diagnosis have rapidly evolved to include specialized functional tests.

Even men with normal semen parameters may not be fertile necessitating the development of tests designed to measure sperm functions such as cervical mucus penetration, capacitation, sperm-zona pellucida binding, sperm-ova binding, the acrosome reaction sperm penetration, and decondensation.

The sperm penetration assay was first developed in the 1970s. In 1976, Yanagimachi and colleagues observed that upon removal of the zona pellucida of hamster ova, the eggs were “promiscuous” and allowed penetration by sperm of other species(2). This test measures the ability of sperm to undergo capacitation, fuse with the egg membrane and decondense the sperm head resulting in the formation of the male pronucleus. Although a number of studies

showed that this test was a useful predictor of fertilization in conventional *in vitro* fertilization (IVF), the test was plagued by false negatives and assay variability. Johnson, et al.,(3, 4) improved this assay by altering the conditions to enhance sperm penetration rates by orders of magnitude and by devising a control method to ensure assay precision and accuracy. A positive score on this test was highly predictive of a positive outcome in IVF(5, 6). Unfortunately, the test rarely is ordered today despite providing useful information. A modification of this assay used in combination with intra-cytoplasmic sperm injection (ICSI) into hamster oocytes accurately identified male factor patients who failed to fertilize after IVF/ICSI due to a defect in sperm head decondensation(7).

2. MATERIALS

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Equipment

1. Incubator
2. Hemocytometer counting chamber
3. Phase contrast microscope
4. Microscope slides with cover slips
5. Pasteur pipette 9 ½ “
6. Centrifuge
7. Glass test tube 12 × 75 mm
8. Graduated tube 15 mL
9. Micro-test tube 0.4 mL (Biorad #233-9502)
10. 26 gauge 3/8” needle
11. Culture dish 35 × 10 mm (Falcon, #1008)
12. Dissecting microscope
13. Mounting micropipette
14. CO₂ chamber

2.2 Reagents

1. Sterile water
2. Sterile saline
3. Modified Biggers-Whitten-Whittingham (MBWW) media
4. TEST-yolk buffer (TYB)
5. 37 IU Human chorionic gonadotropin (hCG)
6. 0.5 mg/ mL Trypsin (ICN Pharmaceutical, #103140)
7. 0.5 mL Hyaluronidase
8. Mineral Oil (Squibb)
9. 8% Glycerol

10. 30 IU Pregnant mare's serum (PMS): 0.3 mL of 100 IU/ mL of MS dissolved in sterile saline

2.3 Sperm Preparation

1. Semen sample from patient
2. Semen sample from control (frozen or fresh)

2.4 Ova Preparation

1. Female Hamster: Between 7 to 12 weeks old

3. METHODS

Carry out all procedures at room temperature unless otherwise specified.

3.1 Sperm Preparation

Quality control is maintained where a fertile control, either frozen or fresh, is processed along with the patient sample. (See Note 1)

1. The ejaculate is collected in a wide-mouth container, provided by the physician, and allowed to liquefy for 30 minutes at 37°C.
2. The specimen is transferred to a 15 mL graduated tube to measure the volume.
3. Using a 50µL pipette, a small drop of semen is applied onto a pre-warmed (37°C) microscope slide and covered with a #1 coverslip.
4. 50µL of semen is aliquotted directly in 0.95 mL sterile H₂O (1:20 dilution) in a 12 × 75 mm glass test tube. The diluted semen is then mixed by agitation, and a small amount is loaded into a hemocytometer counting chamber and set aside.
5. Sperm quality is assessed by viewing the slide of undiluted semen on a 37°C warmed stage at 400x magnification under phase-contrast.
6. The sperm should be counted once they have settle to the same focal plane on the hemocytometer counting grid.
7. Multiply the final count by the percent motility and multiply this product by the volume to yield the total number of motile cells per specimen. (sperm count $\times 10^6$ cells/mL] $\times$ % motility $\times$ volume= total number of motile cells).
8. The semen is then diluted 1:1 with room-temperature TYB and mixed with the aid of a pasteur pipette. Allow solution to cool gradually. (See note 2)
9. Remove the samples from the refrigerator at 8:00 AM on the day of the assay. A visible sperm pellet should be present at the bottom of the tube (Samples that are collected on Monday are processed on Wednesday).
10. Aspirate the TYB seminal plasma supernatant down to the top of the visible pellet.

¹*Frozen control:* A vial from the frozen control pool is retrieved from liquid nitrogen and submerged into the refrigerated container holding the patient's samples for 14 to 20 hours at 4°C. At 8:00 AM on the day of the assay, 6 mL of warm MBWW is added to the frozen control. From this step onwards the control is processed the same way as the patient samples.

Fresh control: A fresh control (fresh ejaculate from a pregnancy-proven donor) is run with every assay and is process exactly like the patient samples.

²It is important to cool the mixture gradually. Immerse the semen/ TYB mixtures in a container of water at room temperature, and place this apparatus in the refrigerator. Not more than 2 hours should elapse from the time the sample is collected to the start of the cooling process in the refrigerator.

11. Immediately add 6 mL of MBWW warmed to 37°C, and thoroughly resuspend the pellet using a transfer pipette. (See note 3)
12. Centrifuge all tubes for 10 minutes at $600 \times g$.
13. Aspirate the supernatant and resuspend the pellet in 3 mL of MBWW.
14. Divide the resuspension equally into 6 tubes (12×75 mm) and cap.
15. Centrifuge for 5 minutes at $600 \times g$ and place the tubes at 37°C heat block for 60-90 minutes to allow motile sperm to swim up into the clear supernatant.
16. Using a Pasteur pipette, aspirate the supernatant from each set of tubes, taking care not to disturb the sperm pellets, and pool the supernatants into another 15 mL graduated tube. Centrifuge at $600 \times g$.
17. Determine the sperm concentration using a hemocytometer. Continue counting the sperm until the count reaches 100. Often it is necessary to dilute a 50 mL aliquot into a 200 mL aliquot of distilled water due to low sperm concentrations. (See notes 4 and 5)

* If less than 250,000 motile sperm are recovered in the swim-up, a micro-assay is performed as follows:

1. Place 0.2 mL MBWW (37°C) in a 0.4 mL micro-test tube
2. Add the sperm suspension, and centrifuge at $600 \times g$ for 5 minutes.
3. After centrifugation, place the micro-test tube on a 37°C heater block to await placement of ova into the sperm.

3.2 Ova Preparation

1. At 9:00 AM on day 1 of the assay period each hamster is stimulated with 30 IU of PMS injected intra-peritoneally. (See note 6)
2. A 37 IU dose of hCG is given at 3:00 PM on day 3 in an identical manner and volume. (See note 7)
3. At 8:00 AM on the morning of the 4th day, the hamsters are sacrificed in an atmosphere saturated with CO₂.
4. The oviducts are excised and placed in the first of three ice-cold saline washes after which the oviducts are singularly transferred to a 35×10 mm culture dish containing iced saline on a dissecting microscope.
5. At 40x magnification, the bulbous section of the duct is punctured with a 26 gauge needle to free the cumulus mass enclosing the ova into the cold saline.
6. Once the entire mass has been teased free it is transferred by a Pasteur pipette to a collection tube containing 0.5 mL ice-cold hyaluronidase. (See note 8)

³The preparation of semen samples from the removal from the refrigerator on the day of the assay to when the warm MBWW is warmed should be performed in rapid sequence on individual samples to assure the same temperature shock to all of the samples. This "thermal shock" is an important step for maximizing penetration rates.

⁴When using the hemocytometer to count the sperm, if the count of 100 should fall in the middle of a square, finish counting that square. Record the number of sperm counted over the total number of squares on the worksheet. For example: If 109 sperm were counted in three squares, this value would be recorded as 109/3 on the worksheet.

⁵The final swim-up volume is calculated as follows: Rec volume $\times$ (# of sperm/ #squares $\times$ 100) = Final volume

⁶The intra-peritoneal injection is made substerially with downward angulation, using a syringe fitted with a 26 gauge 3/8" needle.

⁷Both PMS and hCG injection solutions can be aliquotted and stored in a 20°C freezer for up to 1 year. It is important to thaw these solutions above room temperature and to use them within 30 minutes to maximize ova yield. The assay requires that one hamster be injected for every two patients schedule.

7. The cumuli are transferred to a culture dish containing one drop of room-temperature MBWW. This done at the time of harvest of the swim-up sperm in order to synchronize the ova and sperm preparations.
8. By stirring and allowing the ova to settle, the suspended cumulus cells left in the supernatant and most of the buffer are removed with the Pasteur pipette. (See note 9)
9. The ova can now be transferred using a pasteur pipette with an inner born twice the diameter of the zona-intact ova. By skillful application of suction and pressure, ova can be harvested quickly without any remaining cellular debris.
10. The zona pellucida and the first polar body are removed by placing the ova in a 200 μ L droplet of MBWW containing 0.5mg/ mL trypsin for ~ 2 minutes.
11. The ova are then washed by passage through three consecutive drops of fresh MBWW prior to incubation with sperm. (See note 10)

3.3 Sperm and Ova Incubation

To minimize inter-assay variation in SPA methodology, the period of time after thermal shocking the sperm to the time the sperm and ova is incubated is held between 2 and 2 ½ hours.

1. Centrifuge all the “swim-up” samples for 5 minutes at $600 \times g$.
2. Immediately aspirate down to 0.1 mL (or lower when indicated), and then resuspend the sperm to the desired volume with MBWW.
3. Put two 50 μ L droplets in a small culture dish and overlay with 37°C silicone oil.
4. Eight ova are placed in each twin 50 μ L droplets containing 250,000 100% motile sperm.
5. Place culture dishes in tissue culture incubator at 37°C for 2 ½ to 3 hours.
6. When incubation is complete, the excess sperm bound to the outer ovum membrane must be removed to provide an unobstructed view of those sperm that have penetrated.

* If samples with low motile sperm recovery are placed in micro-test tubes, add ova for incubation in the following manner:

1. Carefully draw no more than 10 ova into the micropipette, and place the tip just under the surface of the MBWW in the tube. Allow the ova to gently “fall” into the liquid and settle to the bottom of the tube. Be careful not to disrupt the sperm pellet in the bottom of the tube.
2. The tube is then placed on the 37°C heat block, where it is then incubated for 3 hours prior to mounting the ova.

⁸The cumuli are collected and kept on ice until it is time to process them for incubation with the sperm.

⁹It is necessary to remove the smaller cumulus cells, as they are enzymatically detached from the ova, to prevent interference with the subsequent trypsinization step.

¹⁰It is also important to process the ova (up to 500) within 20 minutes from the time the cumulus mass is warmed at room temperature with MBWW. The penetration quality of fresh ova is preserved if the ova are kept at a low temperature for up to 24 hours. For this reason the oviducts and cumulus masses are stored in iced buffer until needed. This also prevents ova from animals sacrificed first from having a lower penetration rate than those harvested later.

3.4 Mounting Ova

1. Use a mounting micropipette with an inner bore the same diameter as the zona-free ova to pass through three individual drops of buffer. (See note 11)
2. The coverslips are prepared just prior to use by placing a very small dot of paraffin-vaseline (1:1) mixture on all corners.
3. Retrieve the ova so that a sufficient volume of buffer is drawn up the micropipette after the last ovum.
4. When the ova are placed on the slide, a small droplet will be formed before the ova are expelled, which will prevent the fragile ova from rupturing.
5. The final droplet containing ova should be 1-2 μ L and quickly covered with the prepared slip.
6. Gently press down on one of the corners, so that the droplet will spread and gradually draw the coverslip down, expelling air. This flattens the penetrated ova into one or two focal planes when viewed for scoring.
7. Slides are scored immediately on mounting.

3.5 Scoring Slides

1. Scoring must be performed using a phase-contrast microscope at 400x magnification.
2. The number of penetrated sperm in each ovum is recorded (See note 12).
3. The sperm capacitation index (SCI) is simply the average number of penetrations per ovum for all ova counted.

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¹¹Using the mounting pipette with an inner bore the same diameter as the zona-free ova creates forces in the micropipette tip that shear off the bound sperm. The ova are kept in buffer, while a standard slide is cleaned with ethanol.

¹²A penetrated sperm is indicated by the presence of a swollen head associated with a tail. Using this assay, sperm penetrations are numerous, so that the clear areas of the heads will often merge together and may become indistinguishable. In this case, it is necessary to count tails without heads. It is very rare that an unpenetrated (normal) sperm bound to the outside of the ova membrane will separate head from tail.

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1. .
FIGURE of OVA with penetrated swollen sperm heads.