Acrosome Reaction Measurement

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1 Introduction

The peanut-agglutinin assay is used to assess the acrosomal status of sperm. This assay is an indicator of semen quality as male infertility may be caused by spermatozoa lacking intact acrosomes at ejaculation [1–3]. Acrosomal status after induction of the acrosome reaction can be assessed by microscopy or flow cytometry with fluorescently labeled lectins, such as *Pisum sativum* (pea agglutinin) or *Arachis hypogaea* (peanut lectin), or monoclonal antibodies against the acrosome antigen CD46 [4–7]. Acrosome reaction is an exocytotic process that occurs after the spermatozoa bind to the zona pellucida and must take place before the spermatozoon can penetrate the oocyte layers and fertilize the oocyte. A very small percentage of spermatozoa undergo spontaneous acrosome reaction (<5 %). In many patients premature acrosome reaction may occur, and these spermatozoa lose the ability to fertilize as the acrosomal enzymes are released even in the absence of the egg in its vicinity. After capacitation, the spermatozoon is ready to undergo acrosome reaction [4–7]. Competence of capacitated spermatozoa can be tested by using calcium ionophore.

2 Specimen Collection

1. The physician instructs the patient on proper collection technique (for details, see Routine Semen Analysis protocol).
2. If collection is on site, the technologist instructs the patient again and shows the collection room. Patients can bring the sample from home if they reach the lab within 60 min.
3. The patient collects the specimen into a sterile container and brings it to the laboratory at the appointed time.

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3 Reagents

A. Components of the sperm separation Cell isolation medium
B. Lower phase (80 %)
C. Upper phase (40 %)
D. Sperm washing media (modified HTF with 5.0 mg/mL human albumin)
E. Dulbecco's phosphate-buffered saline (PBS)
F. *Pisum sativum* agglutinin (PSA) labeled with fluorescein isothiocyanate (FITC)
G. Calcium ionophore A23187
H. Hoechst 33258
I. Fluorescent mounting medium
J. Cold ethanol 95 % (v/v)
K. Disposable polystyrene conical centrifuge tubes (sterile) with caps
L. Sterile graduated serological pipettes
M. Eppendorf pipettes and tips (5–50 µL)
N. Disposable transfer pipettes (sterile)
O. Sterile graduated serological pipettes (2 and 5 mL)
P. Colored Eppendorf tubes
Q. Sperm counting chamber slides 20 µm depth
R. Frosted microscope slides
S. Cover slips
T. Aqueous mounting media
U. Immersion oil (low fluorescence)

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4 Equipment

A. Centrifuge
B. Light microscope
C. Fluorescent microscope equipped with Ploemopak epifluorescence module and mercury ultraviolet lamp
D. 37 °C incubator

5 Reagent Preparation

5.1 PSA-FITC Stain

i. Stock PSA solution (5 mg/mL): Aliquot 10 µL of stock solution and store at −20 °C.

Working PSA solution (100 µg/mL):
Thaw an aliquot of 10 µL stain and add 490 µL of PBS and store at 4 °C. This solution is stable for up to 4 weeks.
Note: This must be performed in indirect light.

5.2 Hoechst 33258 Stain

Stock Hoechst 33258 solution (1 mg/mL):
i. Weigh 5 mg of Hoechst in 5 mL of PBS. Prepare 10 µL aliquots in a colored Eppendorf tubes. Store at −20 °C.
ii. Working Hoechst 33258 solution (2 µg/mL): Thaw an aliquot of 10 µL stain and add 990 µL of PBS. Vortex and take 100 µL of the solution and add 400 µL of PBS.
Note: This must be performed in indirect light.

5.3 Calcium Ionophore (Free Acid, FW 523.6)

Preparation of stock solution: (5 mmol/L stock solution):
i. Dissolve 1 mg of A23187 in 380 µL of DMSO.
ii. Aliquot 50 µL of the above solution and add 450 µL PBS to give a 500 µM solution.
iii. Aliquot 50 µL into Eppendorf tubes and store at −20 °C.
Note: This must be performed in indirect light.

6 Specimen Preparation

A. The semen sample is allowed to undergo liquefaction in the 37 °C incubator for 20 min.
B. Record the patient name, clinic number, period of sexual abstinence, date and time of specimen collection, and age of specimen when the semen analysis is performed.
C. Record the initial physical characteristics such as volume, pH, color, etc.
D. Load 5 µL of the well-mixed semen onto a sperm counting chamber.
E. Perform semen analysis for sperm concentration and motility manually.

7 Sperm Preparation by Density Gradient

A. Label one 15 mL centrifuge tube(s) with the patient's name, clinic number, wash media, and date.
B. Label a 2 mL conical beaker for post-wash analysis.
C. Remove a warmed tube of sperm wash media (HTF) from the 37 °C incubator and label with the patient's name and color code labeling tape.
D. Gently place up to 3 mL of liquefied semen onto the upper phase (leaving approximately 0.1 mL in original container for a prewash analysis). If volume is greater than 3 mL, it may be necessary to split the specimen into two tubes before processing.
E. Centrifuge for 20 min at 1600 rpm.
F. Note: Occasionally samples that do not liquefy properly and remain too viscous will be encountered. In such cases, viscosity treatment system may be used.
G. While specimen is in centrifuge, perform routine semen analysis.
H. The supernatant should be removed with a sterile transfer pipette to the level directly below the second layer (see Fig. 19.1).
I. Using a transfer pipette, add 2 mL of sperm wash media (HTF) and resuspend the pellet. Mix gently with pipette until sperm pellet is in suspension.
J. Centrifuge for 7 min at 1600 rpm.
K. Again, remove supernatant from the centrifuge tube using a transfer pipette down to the pellet.
L. Resuspend the final pellet in a volume of 1.0 mL HTF. Record the final volume and sperm concentration. Adjust the sperm concentration to $2–5 \times 10^6$ sperm/mL.

Fig. 19.1 Removal of supernatant HTF with sterile transfer pipette [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

8 Sperm Capacitation

A. Divide the above sample into two aliquots of 500 µL each and label as Test and Control. Centrifuge the sample and discard the supernatant. To the pellet add 500 µL of HTF medium containing 3 % albumin (30 mg/mL). Capacitate the sample in a 5 % CO_2 atmosphere for 3 h at 37 °C.

9 Acrosome Reaction after Ionophore Challenge (ARIC) Test

A. Thaw one aliquot of calcium ionophore A23187 solution (500 µM/L). To the test sample, add 10 µL of the ionophore (10 µM/L). To the control tube, add 10 µL dimethyl sulfoxide (10 %, vol./vol. DMSO:PBS) solution to serve as a control. Incubate the tubes for 30 min at 37 °C.
Note: This step must be performed in indirect light.

10 Viability Testing with Hoechst Stain

A. After incubation, centrifuge the test and control tubes at 1600 rpm for 7 min. Discard the supernatant. To the pellet add 100 µL of PBS and 100 µL of Hoechst working solution (2 mg/mL). Incubate the samples for 10 min in the dark.
B. Centrifuge the tubes at 1600 rpm for 7 min. Discard the supernatant and resuspend the pellet in 100 µL of PBS to remove excess stain. Centrifuge the tubes at 1600 rpm for 7 min. Resuspend the pellet in 100 µL PBS.
Note: This step must be performed in indirect light.

11 Assessment of Acrosome Status

A. Smear a 10 µL aliquot of the above solution on a frosted antibody microscope slide and allowed to dry in the dark at room temperature.
B. Immerse air-dried slides in a Coplin jar with 95 % ice-cold ethanol for 30 min. to permeabilize the sperm membranes.
C. To the air-dried smears, add 10 µL of the FITC-PSA and incubate for 15 min in the dark.
D. Wash gently in PBS to remove excess label by rinsing 10–15 times. Allow the slides to air dry.

12 Staining with FITC-PSA

A. Pipette 10 µL of FITC-PSA solution from the working FITC-PSA aliquot on each of the marked circles of a frosted slide. Gently spread the drop using the pipette tip to insure the solution covers the entire surface area of the spot.
B. After 15 min, fill a small beaker with distilled water. Dip each slide into the distilled water and give 20–25 dips. Let slides dry. Once the slides are dry, they are ready to be observed and counted under the fluorescent microscope.

13 Observing the Acrosome Reaction

A. Turn on the epifluorescence microscope (Leitz dialux, Germany) equipped with Ploemopak epi-illumination module and mercury ultraviolet lamp. Filter cube I.2 is used to observe FITC-PSA staining which fluoresces

"apple-green" and cube A.2 for Hoechst 33258, which fluoresces a bright medium blue.

B. Examine the same spermatozoa by interchanging the two filters.

C. Count a total of 200 spermatozoa per sample at 100× magnification. Scoring must be completed within 48 h of staining.

14 Staining Patterns Showing Sperm Viability and Acrosome Status

A. Viable spermatozoa: Hoechst 33258 stain is excluded from viable cells (live spermatozoa). The sperm head shows a pale-blue fluorescence.

B. Dead spermatozoa: Hoechst 33258 stains the nuclei of damaged cells (dead spermatozoa), which show a bright blue-white fluorescence.

Intact acrosome: Acrosomal region of the sperm head exhibits a uniform apple-green fluorescence.

C. Reacted acrosome: Only the equatorial segment of the acrosome is stained.

Score a total of 200 viable spermatozoa per sample. Calculate the percentage of intact and acrosome-reacted spermatozoa.

D. Assess the percentage of acrosome-reacted spermatozoa in the test samples (Induced AR %) and control samples (spontaneous AR%).

E. Count acrosome reaction in viable sperm only (Fig. 19.2).

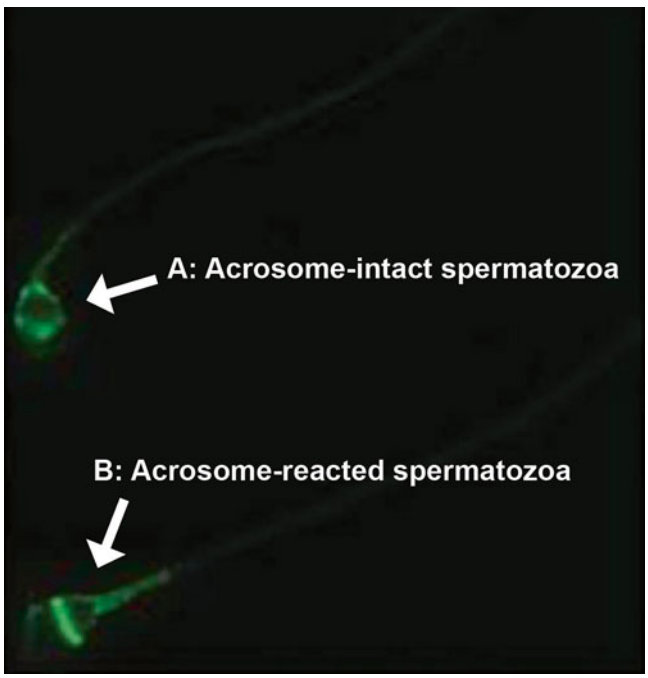

Fig. 19.2 Spermatozoa showing spontaneous and induced acrosome reaction. AI, acrosome-induced reaction; AR, acrosome reacted [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

15 Results

15.1 Calculation of ARIC Score

ARIC score: (percentage of induced acrosome reaction) minus (percentage of spontaneous AR) × 100

Normal values

ARIC% = 15 %

Abnormal sperm function: ARIC% 10–15 %

>20 % spontaneous AR suggests occurrence of premature AR

16 Quality Control

For quality control a sample of known AR should be included with each run [8–11].

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