

# 4

## Evaluation of sperm

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### Introduction

Abnormalities in sperm production or function, alone or in combination with other factors, account for 35–50% of all cases of infertility. Although a battery of tests and treatments have been described and continue to be used in the evaluation of female infertility, the male has been essentially neglected. It would appear that the majority of programs offering advanced reproductive technologies (ART) employ only a cursory evaluation of the male – rarely extending beyond semen analysis and antisperm antibody detection. Several factors certainly account for this disparity. First, most practitioners of ART are gynecologists or gynecologic subspecialists who have little formal training in the evaluation of the infertile or subfertile male. Secondly, the urologists, who perhaps theoretically should have taken the lead in this area, have devoted little of their literature or research budgets to the evaluation of the infertile male. Thirdly, and perhaps most important, is the inescapable fact that sperm function testing remains a very controversial area of research. Many tests have been described, yet few have been extensively evaluated in a proper scientific manner. Those that have continue to be weighed down by persistent criticisms of poor sensitivity or specificity, a lack of standardization of methodology, suboptimal study design, problems with outcome assessment, and the lack of long-term follow-up. Although many of these same criticisms could also be leveled against most diagnostic algorithms for female infertility, in that arena, the tests continue to prevail over their critics. Fourth, like female infertility, male infertility is certainly multifactorial. It is improbable that one sperm function test will prove to be a panacea, owing to the multiple steps involved in fertilization. In addition to arriving at the site of fertilization, sperm must undergo capacitation and the acrosome reaction; they must penetrate the cumulus, bind to the zona pellucida, penetrate through the zona, fuse with the oolemma, activate the oocyte, undergo nuclear decondensation, form the male pronucleus, and then fuse with the

female pronucleus. Finally, with the advent and rapid continued development of microassisted fertilization, sperm function testing has assumed a role of even lesser importance. As fertilization and pregnancy rates improve with procedures such as intracytoplasmic sperm injection (ICSI), more and more logical questions are being asked about the proper role for sperm function testing. This chapter reviews the most commonly employed techniques for sperm evaluation, and examines the issues surrounding their utility in the modern ART program.

### Patient history

A thorough history of the infertile couple at the time of the initial consultation will frequently reveal conditions that could affect semen quality. Some of the important factors to consider are:

1. Reproductive history, including previous pregnancies with this and other partners.
2. Sexual interaction of the couple, including frequency and timing of intercourse as well as the duration of their attempt to become pregnant.
3. Past medical and surgical history: specific attention should be paid to sexually transmitted diseases, prostatitis, or epididymitis, as well as scrotal trauma or surgery – including varicocele repair, vasectomy, inguinal herniorrhaphy, and vasovasostomy.
4. Exposure to medication, drugs, and toxins, including occupational and leisure activities, either in the past or in the present.<sup>1</sup>

### Semen analysis

The hallmark of the evaluation of the male remains the semen analysis. It is well known that the inpatient variability of semen specimens from fertile men can vary significantly over time.<sup>2</sup> This decreases the diagnostic information that can be obtained from a single analysis, often necessitating additional analyses. What is also apparent from literature analyzing

**Table 4.1 World Health Organization (WHO), normal values for semen analysis**

Parameter	Normal values
Liquefaction	Complete within 60 minutes at room temperature
Appearance	Homogeneous, gray, and opalescent
Consistency	Leaves a pipette as discrete droplets
Volume	≥ 2 ml
pH	≥ 7.2
Concentration	20 million sperm/ml semen or more
Total number	40 million sperm per ejaculate or more
Motility	50% or more with forward progression, or 25% or more with rapid progression within 60 minutes of collection
Morphology <sup>†</sup>	30% or more with normal forms; 15% or more with normal forms*
Vitality <sup>†</sup>	75% or more; 50% or more
Leukocytes <sup>†</sup>	Fewer than 1 million/ml; fewer than 1 million/ml
Immunobead test <sup>†</sup>	Fewer than 20% with adherent particles; fewer than 50% motile sperm with adherent particles
MAR test <sup>†</sup>	Fewer than 10% with adherent particles; fewer than 50% motile sperm with adherent particles

\*Kruger strict morphology.

<sup>†</sup>First value from 3rd edn; second value from 4th edn.

MAR, mixed agglutination reaction.

samples from 'infertile' patients is that the deficiencies revealed may not be sufficient to prevent pregnancy from occurring: rather, they may simply lower the probability of pregnancy, resulting in so-called 'subfertility.' Clearly, the overall prognosis for a successful pregnancy is dependent on the complex combination of variables in semen quality coupled with the multiple factors inherent in the female reproductive system that must each function flawlessly to enable a pregnancy to occur. The commonly accepted standard for defining the normal semen analysis are the criteria defined by the World Health Organization (WHO). These parameters are listed in Table 4.1.

### Collection of the specimen

When the semen analysis is scheduled, instructions should be given to the couple to ensure collection of an optimum semen sample. Written instructions are useful, especially if the patient is collecting the specimen outside of the clinical setting. During the initial infertility evaluation, a semen specimen should be obtained following a 2–7 day abstinence from sexual activity.<sup>2</sup> A shorter period of time may adversely affect the semen volume and sperm concentration, although it may enhance sperm motility. A longer period of abstinence may reduce the sperm motility. In light of the natural variability in semen quality that all men exhibit, the initial semen collection may not accurately reflect a typical ejaculate for that patient. A second collection, with a 2–7 day abstinence period, can eliminate the tension associated with the initial semen collection, as well as provide a second specimen from which a typical set of semen parameters can be determined. This second collection may also

be used to determine the optimal abstinence period for this particular patient.

Masturbation is the preferred method of collection. The use of lubricants is discouraged since most are spermicidal. However, some mineral oils and a few water-based lubricants are acceptable. Since masturbation may present significant difficulty for some men, either in the clinic or at home, an alternative method of collection must be available. The use of certain silastic condoms (seminal collection devices) during intercourse may be an acceptable second choice. Interrupted intercourse should not be considered, as this method tends to lose the sperm-rich initial few drops of semen while transferring many bacteria to the specimen container.<sup>2,3</sup>

### Care of the specimen

Appropriate care of the ejaculate between collection and examination is important. Specimens should be collected only in approved, sterile, plastic, disposable cups. Washed containers may contain soap or residue from previous contents, which can kill or contaminate the sperm. Delivery of the semen to the laboratory should occur within 60 minutes of collection, and the specimen should be kept at room temperature during transport. These recommendations are designed to maintain optimal sperm motility through the time of analysis.

### Container labeling

The information recorded on the specimen container label should include the husband's name as well as a unique identifying number. Typically, a social-security number, birth date, or a clinic-assigned patient

number is used. Other helpful information recorded on the label should include the date and time of collection and the number of days since the last ejaculation. When the specimen is received from the patient, it is important to confirm that the information provided on the label is complete and accurate.

## Examination of the specimen

### Liquefaction and viscosity

When the semen sample arrives in the laboratory, it is checked for liquefaction and viscosity. Although similar, these factors are distinct from each other.<sup>4,5</sup> Liquefaction is a natural change in the consistency of semen from a semi-liquid to a liquid. Before this process is completed, sperm are contained in a gel-like matrix that prevents their homogeneous distribution. Aliquots taken from this uneven distribution of sperm for the purpose of determining concentration, motility, or morphology may not be truly representative of the specimen as a whole. As liquefaction occurs over 15–30 minutes, sperm are released and distributed throughout the semen. Incomplete liquefaction may adversely affect the semen analysis by preventing this even distribution. The coagulum that characterizes freshly ejaculated semen results from secretions from the seminal vesicles. The liquefaction of this coagulum is the result of enzymatic secretions from the prostate. Watery semen, in the absence of a coagulum, may indicate the absence of the ejaculatory duct or seminal vesicles. Inadequate liquefaction, in the presence of a coagulum, may indicate a deficiency of prostatic enzymes.<sup>6,7</sup>

Viscosity refers to the liquefied specimen's tendency to form drops from the tip of a pipette. If drops form and fall freely, the specimen has a normal viscosity. If drops will not form or the semen cannot be easily drawn up into a pipette, viscosity is high. Highly viscous semen may also prevent the homogeneous distribution of sperm. Treatment with an enzyme, such as chymotrypsin,<sup>8</sup> or aspiration through an 18-gauge needle, may improve the distribution of sperm before an aliquot is removed for counting. Any addition of medium containing enzymes should be recorded, as this affects the actual sperm concentration. The new volume must be factored in when calculating the total sperm count.

### Semen volume

Semen volume is best measured with a serological pipette that is graduated to 0.1 ml. This volume is recorded and later multiplied by the sperm concentration in order to obtain the total count. A normal seminal volume before dilution is considered to be >2 ml.<sup>2</sup>

### Sperm concentration

A variety of counting chambers are available for determining sperm concentration. Those more commonly

used include the hemocytometer, the Makler counting chamber, and the MicroCell. Regardless of the type of chamber used, an aliquot from a homogeneous, mixed semen sample is placed onto a room temperature chamber. The chamber is covered with a glass coverslip, which allows the sperm to distribute evenly in a very thin layer. Sperm within a grid are counted, and a calculation is made according to the formula for the type of chamber used. Accuracy is improved by including a greater number of rows or squares in the count. Sperm counts should be performed immediately after loading semen onto the chamber. Waiting until the heat from the microscope light increases the speed of the sperm may inaccurately enhance the count.

As indicated earlier, a particular patient's sperm count may vary significantly from one ejaculate to another. This observation holds true for both fertile and infertile males, further complicating the definition of a normal range for sperm concentration. Demographic studies employing historic controls were used to define a sperm concentration of <20 million/ml as abnormal.<sup>9,10</sup> Although several investigators observed that significantly fewer pregnancies occurred when men had sperm counts <20 million/ml, the prognosis for pregnancy did not increase proportionately to the sperm concentration above this threshold.

### Sperm motility

Sperm motility may be affected by many factors, including:

- the patient's age and general health
- the length of time since the last ejaculation
- the patient's exposure to outside influences such as excessive heat or toxins
- the method of collection
- the length of time and adequacy of handling from collection to analysis.

When the aliquot of semen is placed on the room temperature counting chamber, the count and motility should be determined immediately. As previously stated, this will prevent the influence of the heat from the microscope light source from influencing the results. If a chamber with a grid is used to count the sperm, the motility can be determined at the same time as the concentration by using a multiple-click cell counter to tally motile and nonmotile sperm and then totaling these numbers to arrive at the true sperm concentration. The accuracy improves as more sperm are counted. If a wet-mount slide is used to determine motility, more than one area of the slide should be used, and each count should include at least 100 sperm.

Prior to examining the specimen for motility, the slide or counting chamber should be examined for signs of sperm clumping. Sperm clumping to other

sperm, head to head, head to tail, or tail to tail, may indicate the presence of sperm antibodies in the semen. This should not be confused with clumping of sperm to other cellular debris in the semen, which is not associated with the presence of antibodies. In either case, sperm clumping may affect the accuracy of both the sperm count and the motility.<sup>2,3</sup>

Motility is one of the most important prerequisites for achieving fertilization and pregnancy. The head of the sperm must be delivered a great distance in vivo through the barriers of the reproductive tract to the site of the egg. Sperm must have sufficient motility in order to penetrate both the layers of coronal cells and the zona pellucida before fusing with the egg's cell membrane (oolemma). An exact threshold level of motility required to accomplish fertilization and pregnancy, however, has never been described.<sup>9</sup> This may be due to variables in the equipment and techniques used in assessing motility.

### Progression

Whereas sperm motility represents the quantitative parameter of sperm movement expressed as a percentage, sperm progression represents the quality of sperm movement expressed on a subjective scale. A typical scale, such as the one below, attempts to depict the type of movement exhibited by most of the sperm visualized on a chamber grid. With the advent of successful microassisted fertilization, scales such as this have assumed more limited utility. Nevertheless, for those laboratories that quantify motility, a score of 0 means no motility, 1 means motility with vibratory motion without forward progression, 2 means motility with slow, erratic forward progression, 3 means motility with relatively straightforward motion, and 4 means motility with rapid forward progression.<sup>3</sup>

### Sperm vitality

When a motility evaluation yields a low proportion of moving sperm (less than 50%), a vitality stain may be beneficial. This is a method used to distinguish non-motile sperm that are living from those that are dead. This technique will be discussed later in the sperm function section.

### Additional cell types

While observing sperm in a counting chamber or on a slide, additional cell types may also be seen. These include endothelial cells from the urethra, epithelial cells from the skin, immature sperm cells, and white blood cells. The most common and significant of these cell types is referred to collectively as 'round cells.' These include immature sperm cells and white blood cells.

In order to distinguish between them, an aliquot of semen can be placed in a thin layer on a slide and

air-dried. The cells are fixed to the slide and stained using a Wright–Giemsa or Bryan–Leishman stain. When viewed under 400× or 1000×, cell types may be differentiated primarily by their nuclear morphology. Immature sperm have one to three round nuclei within a common cytoplasm. Polymorphonuclear leukocytes may also be multinucleate, but the staining method will typically reveal characteristic nuclear bridges between their irregularly shaped nuclei.<sup>2</sup> A peroxidase stain may be used to identify granulocytes and to differentiate them from the immature sperm. The presence of greater than 1 million white blood cells per ml of semen may indicate an infection in the urethra or accessory glands, which provide the majority of the seminal plasma. Such infections could contribute to infertility.<sup>1,2</sup> As such, these samples must be cultured so that the offending organism can be identified and appropriate treatment can be instituted.

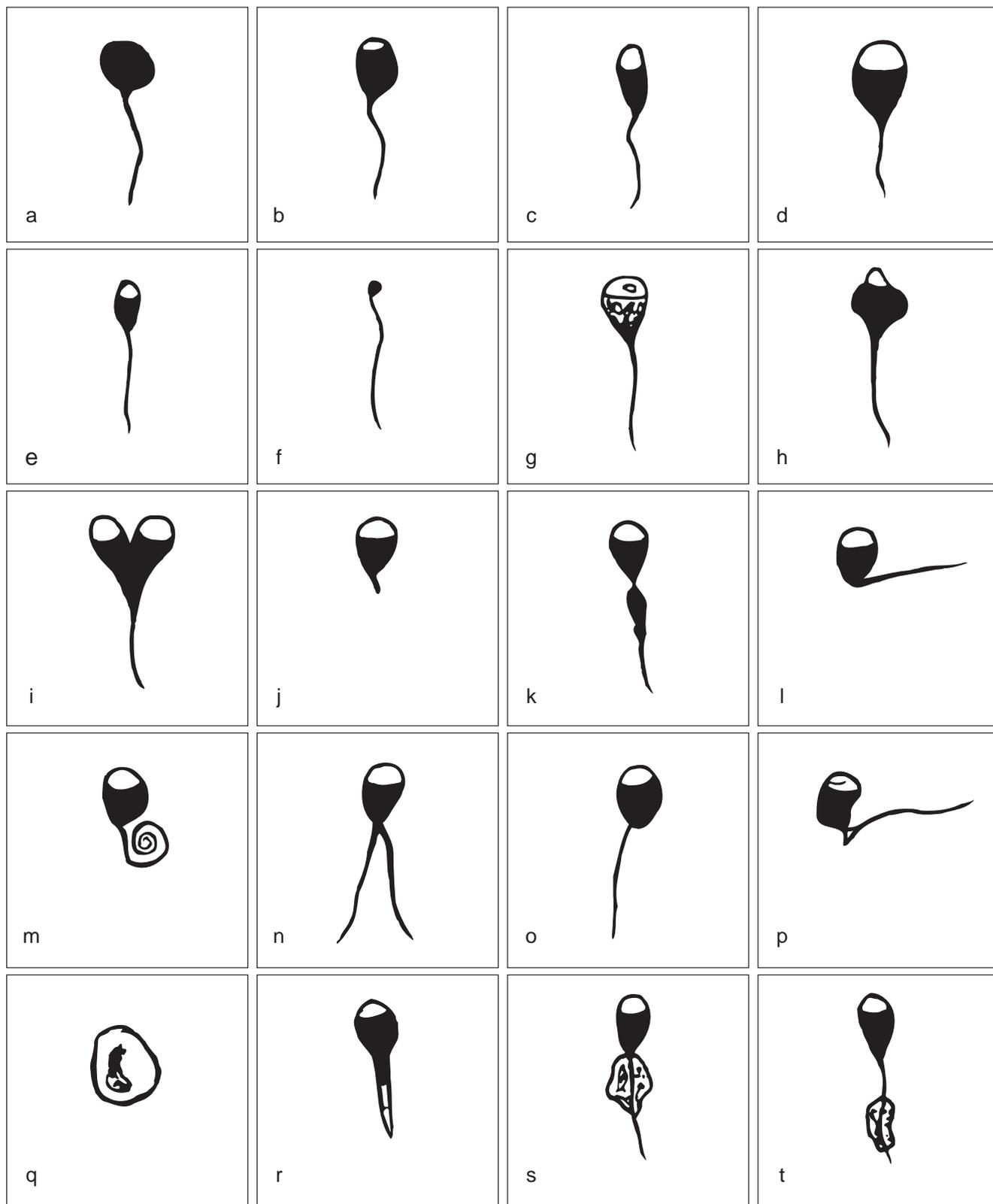
### Sperm morphology

Sperm morphology can be assessed in several ways. The most common classification systems are the 3rd edn WHO standard and the 4th edn WHO standard that incorporates Kruger strict criteria (Fig 4.1). The third edition WHO method requires either a wet-slide preparation or a fixed, stained slide. A 10–20 ml drop of semen is placed on a slide. After placing a coverslip over the specimen, morphology may be determined at 400× by phase contrast microscopy. Alternatively, the drop of semen may be mixed with an equal volume of fixative plus stain (typically Papanicolaou or a Diff-Quik kit) prior to placing it on the slide. At least 100 sperm must be counted at 400× or 1000× with phase contrast or bright field microscopy. WHO criteria for assessing normal forms include the following:

- *Head* – oval and smooth heads are normal; round, pyriform, pin, double, and amorphous heads are all abnormal.
- *Mid-piece* – a normal midpiece is straight and slightly thicker than the tail.
- *Tail* – single, unbroken, straight tails, without kinks or coils are normal.

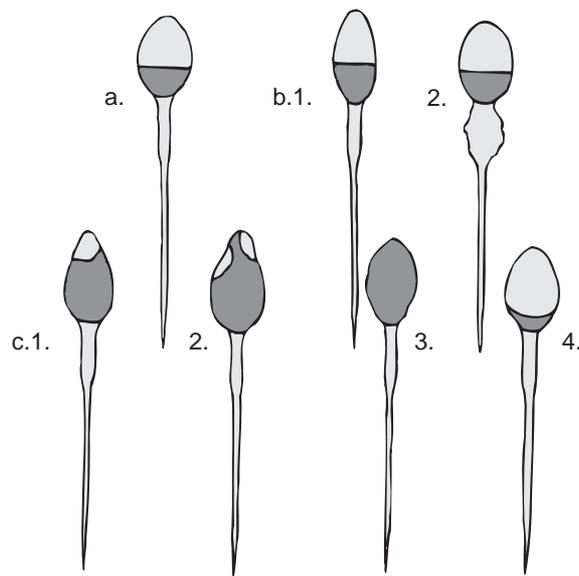
A normal semen analysis should contain at least 30% normal sperm using WHO 3rd edn criteria.

In order to employ Kruger strict criteria, sperm morphology is evaluated by placing 5 µl of liquefied semen on a slide, making a thin smear, and air-drying it at room temperature. The slide is then fixed and stained (typically with a Papanicolaou stain or a Diff-Quik kit). Slides are read using bright field microscopy under 1000× or higher magnification. At least 200 sperm should be counted for an accurate evaluation. The Kruger criteria for assessing normal forms include the following (Fig 4.2):<sup>12,13</sup>



**Fig 4.1** Different types of sperm malformations. Reproduced from reference 11.

a, Round head/no acrosome; b, Small acrosome; c, Elongated head; d, Megalo head; e, Small head; f, Pinhead; g, Vacuolated head; h, Amorphous head; i, Bicephalic; j, Loose head; k, Amorphous head; l, Broken neck; m, Coiled tail; n, Double tail; o, Abaxial tail attachment; p, Multiple defects; q, Immature germ cell; r, Elongated spermatid; s, Proximal cytoplasmic droplet; t, Distal cytoplasmic droplet.



**Fig 4.2** Diagrammatic representation of quick-stained spermatozoa.

a, Normal form; b.1, Slightly amorphous head; b.2, Neck defect; c.1, and 2, Abnormally small acrosome; c.3, No acrosome; c.4, Acrosome >70% of sperm head. Reproduced from reference 12.

- *Head* – smooth; oval configuration; length, 5–6  $\mu\text{m}$  diameter, 2.5–3.5  $\mu\text{m}$ ; acrosome, must constitute 40–70% of the sperm head.
- *Mid-piece* – slender, axially attached; <1  $\mu\text{m}$  in width and approximately 1.5 $\times$  head length; no cytoplasmic droplets >50% of the size of the sperm head.
- *Tail* – single, unbroken, straight, without kinks or coils approximately 45  $\mu\text{m}$  in length.

As described by Kruger et al, sperm forms that are not clearly normal should be considered abnormal. The presence of  $\geq 15\%$  normal sperm morphology should be interpreted as a normal result. Normal morphology of 4–14% should be considered to be borderline, and normal morphology <4% is abnormal.<sup>12,13</sup>

Normal sperm morphology has been reported to be directly related to fertilization potential. This may be due to the abnormal sperm's inability to deliver normal genetic material to the cytoplasm of the egg. From video recordings, it appears that abnormal sperm are more likely to have diminished, aberrant, or absent motility. This reduced or unusual motility may result from hydrodynamic inefficiency due to the head shape, abnormalities in the tail structure which prevent normal motion, and/or deficiencies in energy production necessary for motility.<sup>14,15</sup> In addition to compromised motility, abnormal sperm do not appear to bind to the zona of the egg as well as do normal sperm. This has been demonstrated in studies employing the hemizona binding assay.<sup>16</sup>

In vitro fertilization (IVF) has helped further to elucidate the role that normal sperm morphology plays in the fertilization process and in pregnancy. Both methods of determining normal sperm morphology,

the 3rd edn WHO method and the Kruger strict method, have been used to predict a patient's fertility. Several studies have concluded that the Kruger method of strict morphology determination shows the most consistent prediction of fertilization in vitro following conventional insemination.<sup>1,17,18</sup> This method of assessing normal sperm morphology, because of its precise, nonsubjective nature, establishes a threshold below which abnormal morphology becomes a contributing factor in infertility.

## Computer-assisted semen analysis

Computer-assisted semen analysis (CASA) was initially developed to improve the accuracy of manual semen analysis. Its goal is to establish a standardized, objective, reproducible test for sperm concentration, motility, and morphology. The technique also attempts, for the first time, to actually characterize sperm movement. The automated sperm movement measurements – known as kinematics – include straight-line velocity, curvilinear velocity, and mean angular displacement (Table 4.2). The use of CASA requires specialized equipment, including a phase contrast microscope, video camera, video recorder, video monitor, computer, and printer.

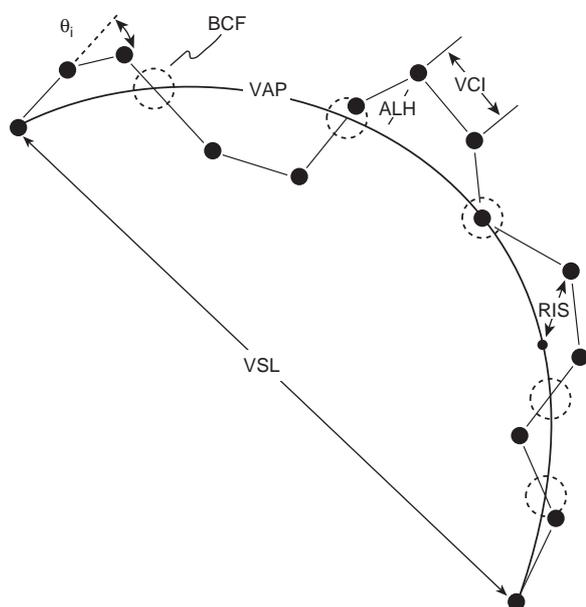
To perform CASA, sperm are placed on either a Makler or a MicroCell chamber and they are then viewed under a microscope. The video camera records the moving images of the sperm cells and the computer digitizes them. The digitized images consist of pixels whose changing locations are recorded frame by frame. Thirty to 200 frames per minute are produced. The changing locations of each sperm are recorded and their trajectories are computed (Fig 4.3).<sup>19</sup> In this manner, hyperactive motion can also be detected and recorded. Hyperactive sperm exhibit a whip-like, thrashing movement, which is thought to be associated with sperm that are removed from seminal plasma and ready to fertilize the oocytes.<sup>19,20</sup>

Persistent questions about the validity and reproducibility of results have kept CASA from becoming a standard procedure in the andrology laboratory. The accuracy of sperm concentration appears to be diminished in the presence of either severe oligospermia or excessive numbers of sperm. In cases of oligospermia, counts may be overestimated due to the machine counting debris as sperm. High concentrations of sperm may be underestimated in the presence of clumping. High sperm concentrations can also cause overestimations in counting due to the manner in which the software handles collisions between motile sperm and nonmotile sperm. In these cases, diluting the sample may improve the accuracy of the count.<sup>20,21</sup> Sperm concentration also appears to be closely related to the type of counting chamber employed. Similar to the challenges reported with manual counting, sperm counts may vary whether using a Makler or a MicroCell.

**Table 4.2 Kinematic measurements in computer-assisted semen analysis (CASA)**

Symbol	Name	Definition
VSL	Straight-line velocity	Time average velocity of the sperm head along a straight line from its first position to its last position
VCL	Curvilinear velocity	Time average velocity of the sperm head along its actual trajectory
VAP	Average path velocity	Time average velocity of the sperm head along its average trajectory
LIN	Linearity	Linearity of the curvilinear trajectory (VSL/VCL)
WOB	Wobble	Degree of oscillation of the actual sperm-head trajectory around its average path (VAP/VCL)
STR	Straightness	Straightness of the average path (VSL/VAP)
ALH	Amplitude of lateral head displacement	Amplitude of variations of the actual sperm-head trajectory about its average trajectory (the average trajectory is computed using a rectangular running average)
RIS	Riser displacement	Point to point distance of the actual sperm-head trajectory to its average path (the average path is computed using an adaptive smoothing algorithm)
BCF	Beat-cross frequency	Time average rate at which the actual sperm trajectory crosses the average path trajectory
HAR	Frequency of the fundamental harmonic	Fundamental frequency of the oscillation of the curvilinear trajectory around its average path (HAR is computed using the Fourier transformation)
MAG	Magnitude of the fundamental harmonic	Amplitude squared height of the HAR spectral peak (MAG is a measure of the peak to peak dispersion of the raw trajectory about its average path at the fundamental frequency)
VOL	Area of fundamental harmonic	Area under the fundamental harmonic peak in the magnitude spectrum (VOL is a harmonic measure of the power-bandwidth of the signal)
CON	Specimen concentration	Concentration of sperm cells in a sample in millions of sperm per milliliter of plasma or medium
MOT	Percentage motility	Percentage of sperm cells in a suspension that are motile (in manual analysis, motility is defined by a moving flagellum; in CASA, motility is defined by a minimum VSL for each sperm)

Reproduced from reference 19.



**Fig 4.3** Examples of kinematic measurements involved in a single sperm tracing (see Table 4.2 for explanation of acronyms). Reproduced from reference 19.

Sperm motion parameters identified by CASA have been assessed by several investigators for their ability to predict fertilization potential. Certain types of motion have been determined to be important in achieving specific actions related to fertilization, such as cervical mucus penetration and zona binding. However, the overall value of CASA for predicting pregnancy is still

the subject of much debate. In summary, persistent questions about results and their interpretation continue to limit the routine use of CASA. As reproducibility improves over all ranges of sperm concentration, CASA may become the standard for semen analysis. The use of fluorescent DNA staining with CASA may also improve its reliability. In addition, as the kinematics of sperm motion becomes better understood, CASA may play an integral role in determining the optimal method of assisted reproductive technology that should be utilized for specific types of male factor patients.

### Sperm antibodies

Because mature spermatozoa are formed after puberty, they can be recognized as foreign protein by the male immune system. In the testicle, the sperm are protected from circulating immunoglobulins by the tight junctions of the Sertoli cells. As long as the sperm are contained within the lumen of the male reproductive tract, they are sequestered from the immune system, and no antibodies form to their surface antigens. If there is a breach in this so-called 'blood:testis barrier,' an immune response may be initiated. The most common causes of a breach in the reproductive tract, which could initiate antibody formation, include vasectomy, varicocele repair, testicular biopsy, torsion, trauma, and infection.<sup>22,23</sup> Once formed, antibodies are secreted into the fluids of the accessory glands, specifically the prostate and seminal vesicles. At the time of ejaculation, the fluids from these glands

contribute most of the volume to the seminal plasma. These antibodies can then come into contact with the sperm and may cause them to clump. In women, the atraumatic introduction of sperm into the reproductive tract as a result of intercourse or artificial insemination does not appear to be a factor in the production of sperm antibodies. However, events that induce trauma, or introduce sperm to the mucous membranes outside of the reproductive tract, can induce antibody formation. Proposed examples of such events include trauma to the vaginal mucosa during intercourse or the deposition of sperm into the gastrointestinal tract by way of oral or anal intercourse.<sup>23</sup> There are several tests currently employed for detecting the presence of sperm antibodies. The two most common are the mixed agglutination reaction and the immunobead binding test.

#### **The mixed agglutination reaction (MAR)**

This test is performed by mixing semen, immunoglobulin G (IgG)- or IgA-coated latex beads or red blood cells, and IgG or IgA antiserum on a microscope slide. The slides are incubated and observed at 400 $\times$ . At least 200 sperm are counted. If antibodies are present, the sperm will form clumps with the coated latex beads or coated red blood cells. If antibodies are absent, the sperm will swim freely. The level of antibody concentration considered to be clinically relevant must be established by each center conducting the test. The WHO considers a level of binding of  $\geq 50\%$  to be clinically significant. This test is used only for detection of direct antibodies in men, and is not specific for the location of bead attachment to the sperm.

#### **The immunobead binding test**

This test is performed by combining IgG- or IgA-coated latex beads and washed sperm on a slide. The sperm must be removed from the seminal plasma by washing the sample with media plus bovine serum albumin (BSA). The presence of human protein on the surface of the sperm interferes with the binding of the immunobeads to the sperm, and thus may mask a positive result.

After washing, the sperm are placed on a slide with IgG- or IgA-coated latex beads and read at 200 $\times$  or 400 $\times$ . If antibodies are present, the small beads will attach directly to the sperm. This test provides potentially greater information than the mixed agglutination reaction, as results consider the number of sperm bound by beads, the type of antigen involved in binding, and the specific location where the bead is bound to the sperm. If antibodies are absent, the beads will not attach. This test can be used for the detection of direct antibodies in men. However, unlike the MAR test, it may also be used to detect antibodies produced in a woman's serum, follicular fluid, or cervical mucus by incubating these bodily fluids with washed sperm that have previously tested negative for antibodies.

To perform an indirect test, known direct antibody-negative sperm are washed free of seminal plasma and resuspended in a small volume of media plus BSA. They are incubated for 1 hour at 37°C with the bodily fluid to be tested. The sperm are then washed free of the bodily fluid, resuspended in media plus BSA, and mixed on a slide with IgG- or IgA-coated latex beads. The test is interpreted by noting the percentage and location of bead attachment. The 3rd edn WHO standard considers a level of binding of  $\geq 20\%$  to represent a positive test, whereas the 4th edn WHO standard considers a level of  $\geq 50\%$  to be positive. Clinical significance is commonly considered to be a level of binding of  $\geq 50\%$ .<sup>9,24</sup> The clinical value of antisperm antibody testing is predicated on the observation that the presence of a significant concentration of antibodies may impair fertilization. It has been reported that antibody-positive sperm may have difficulty penetrating cervical mucus. Although, in these cases, intrauterine insemination (IUI) or IVF may improve the prognosis for fertilization, antibody levels  $>80\%$ , coupled with subpar concentration, motility, or morphology, may necessitate the addition of ICSI in order to achieve the highest percentage of fertilization.<sup>25</sup> As suggested by the literature, andrology laboratories may do a significantly better job of preparing sperm if they are aware of the presence of antibodies. Specifically, it has been demonstrated that the use of increased concentrations of protein in the media used for sperm preparation will reduce the adverse effect of antisperm antibodies on sperm motility. In summary, antisperm antibodies have been demonstrated to be a contributing factor in infertility. While their presence alone may not be sufficient to prevent pregnancy, their detection should encourage the andrologist to pursue additional appropriate action.

### **Sperm vitality**

An intact plasma membrane is an integral component of, and possibly a biologic/diagnostic indicator for, sperm viability. The underlying principle is that viable sperm contain intact plasma membranes that prevent the passage of certain stains, whereas nonviable sperm have defects within their membranes that allow for staining of the sperm. Several so-called vital stains have been employed for this purpose. They include eosin Y, trypan blue, and/or nigrosin.<sup>26</sup> When viewed with either bright field or phase contrast microscopy, these stains allow for the differentiation of viable, nonmotile sperm from dead sperm. This procedure may therefore play a significant role in determining the percentage of immotile sperm that are viable and available for ICSI. Unfortunately, however, dyes such as eosin Y are specific DNA probes that may have toxic effects if they enter a viable sperm or oocyte, which precludes the use of these sperm for ICSI or insemination. Flow cytometry has also been utilized for the determination of sperm viability. Like vital staining, flow cytometry is based on the principle that an intact plasma membrane will prevent the passage of

nucleic acid-specific stains. Some techniques, such as the one described by Noiles et al, employ dual staining, which can differentiate between an intact membrane and a damaged membrane.<sup>27</sup> There are no studies that prospectively evaluate sperm viability staining as a predictor of ART outcome.

## Hypo-osmotic swelling test

Another means of assessing the sperm plasma membrane is the hypo-osmotic swelling test (HOST). This assay is predicated upon the observation that all living cells are permeable to water, although to different degrees. The human sperm membrane has one of the highest hydraulic conductivity coefficients (2.4  $\mu\text{l}/\text{min}/\text{atm}$  at 22°C) of any mammalian cell.<sup>28</sup>

As originally described, the HOST involves placing a sperm specimen into hypotonic conditions of approximately 150 mosmol.<sup>29</sup> This environment, while not sufficiently hypotonic to cause cell lysis, will cause swelling of the sperm cells. As the tail wells, the fibers curl, and this change can be detected by phase contrast microscopy, differential interference contrast (DIC), or Hoffman optics. The normal range for a positive test is typically considered to be a score  $\geq 60\%$ , i.e. 60% of the cells demonstrate curling of the tails. A negative test is defined as  $< 50\%$  curling.<sup>30</sup> This test generated a significant amount of initial interest, and several investigators compared it to the sperm penetration assay (SPA) as an *in vitro* surrogate for fertilization, reporting good correlation.<sup>31,32</sup> More recently, the test has been employed as a predictor of ART outcome, with conflicting results. Although one group reported a favorable correlation, another found no predictive value for the test.<sup>33,34</sup> It has also been suggested that, owing to sperm morphology changes in response to the test, the HOST may facilitate an embryologist's ability to select sperm appropriate for injection. In our program at the Texas Fertility Center, we use the HOST to identify sperm suitable for use in ICSI cases where all sperm are nonmotile. In summary, the HOST currently lacks sufficient critical evaluation to determine its true role in the assessment and/or treatment of the infertile male.

## Assays of the sperm acrosome

The acrosome is an intracellular organelle, similar to a lysosome, which forms a cap-like structure over the apical portion of the sperm nucleus.<sup>35</sup> The acrosome contains multiple hydrolytic enzymes, including hyaluronidase, neuraminidase, proacrosin, phospholipase, and acid phosphatase, which, when released, are thought to facilitate sperm passage through the cumulus mass, and possibly the zona pellucida as well (Fig 4.4). Once sperm undergo capacitation, they are capable of an acrosome reaction. This reaction is apparently triggered by fusion of the sperm plasma membrane with the outer acrosomal membrane at

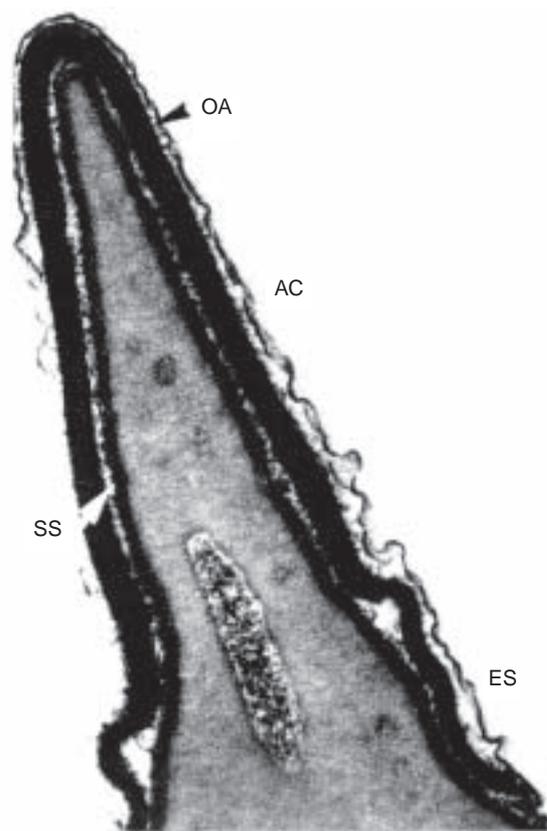
multiple sites, leading to diffusion of the acrosomal enzymes into the extracellular space. This results in dissolution of the plasma membrane and acrosome, leaving the inner acrosomal membrane exposed over the head of the sperm (Fig 4.5).

Although electron microscopy has produced many elegant pictures of acrosome-intact and acrosome-reacted sperm, it is not always possible to know if sperm that fail to exhibit an acrosome have truly acrosome-reacted, or could possibly be dead. In addition, electron microscopy is not a technique available to all andrologists. This has led to the necessity for the development of biochemical markers for the acrosome reaction. Throughout the 1970s and 1980s, multiple biochemical tests were described using a variety of lectins, antibodies, and stains. Although they apparently correlated well with electron microscopy, the tests were still time-consuming and difficult to perform.<sup>36,37</sup>

Contemporary assays for the determination of acrosomal status employ fluorescent plant lectins or monoclonal antibodies, which can be detected much more easily with fluorescence microscopy.<sup>38,39</sup> These assays may prove to be of value if they can truly identify males who manifest deficiencies in their ability to undergo the acrosome reaction. Hypothetically, such patients may need to have their sperm specially preincubated – such as with follicular fluid or calcium ionophore – prior to insemination if they fail to acrosome-react on their own. Conversely, this test may help to identify a small subpopulation of males who prematurely acrosome-react. Several studies have reported an association between ejaculated sperm with low percentages of acrosome-intact sperm and poor subsequent fertilization.<sup>40</sup> These areas certainly await additional study.

## Other biochemical tests

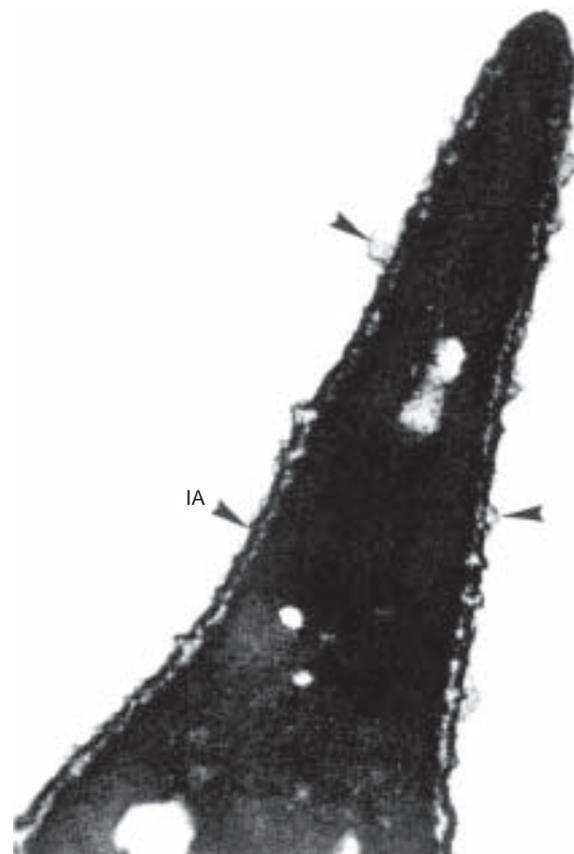
As noted above, one of the predominant enzymes present in the acrosome is proacrosin. The enzymatic action of acrosin is not necessarily correlated to the presence of an intact acrosome; therefore, assays for the presence of acrosin have been described.<sup>41</sup> Acrosin activity has been reported to be greater in fertile males than in infertile males;<sup>42</sup> however, there are no prospective evaluations correlating acrosin activity to fertilization rates in ART patients. Like all other tissues that require energy synthesis and transport, spermatozoa contain measurable levels of creatinine phosphokinase (CPK). Two isomers, CK-M and CK-B, have been described, and differences have been noted in these levels in semen specimens from fertile and infertile males. Specifically, CK-M levels exceed CK-B levels in normospermic males, while CK-B levels are greater in spermatozoa from oligospermic males.<sup>43</sup> In this same study, researchers found that semen samples in which CK-M/CK-B ratios exceeded 10% exhibited higher fertilization rates in IVF than specimens with lower ratios. Few other studies have addressed this topic.



**Fig 4.4** Sperm head with intact acrosome. OA, outer acrosomal membrane; AC, acrosomal cap; ES, equatorial segment; SS, subacrosomal space; Reproduced from reference 11.

### Sperm penetration assay

The sperm penetration assay or hamster egg penetration assay (HEPA) was initially described by Yanagimachi et al in 1976.<sup>44</sup> It measures the ability of sperm to undergo capacitation and the acrosome reaction, penetrate the oolemma, and then decondense. In this test, oocytes from the golden hamster are first treated in order to remove the zona pellucida. As one of the functions of the zona is to confer species specificity, its presence would preclude performance of this test. However, zona removal obviously prohibits the HEPA from being able to assess sperm for the presence of zona receptors. Following zona removal, human sperm are incubated for 48 hours with the hamster oocytes, and the number of penetrations with nuclear decondensation are calculated. As originally described, it was hoped that the test would correlate with the ability of human sperm to fertilize human oocytes *in vitro*. Although the test was designed to assess the ability of sperm to fuse to the oolemma, it also indirectly assesses sperm capacitation, the acrosome reaction, and the ability of the sperm to be incorporated into the ooplasm. Unfortunately, however, intrinsic in the design of the test is its inability to assess the sperm's ability to bind to – and penetrate



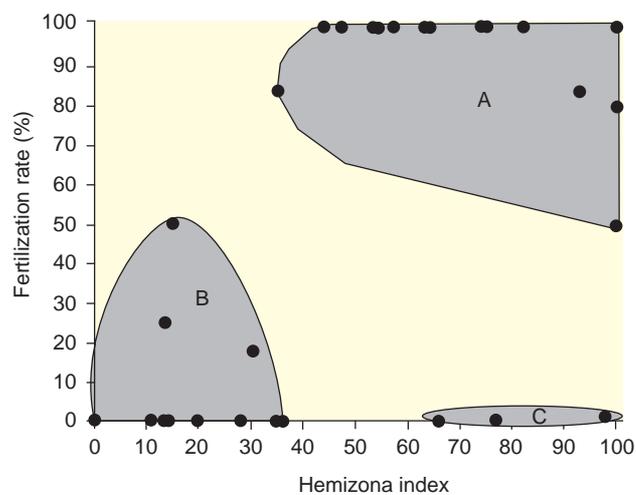
**Fig 4.5** Acrosome-reacted sperm. IA, inner acrosomal membrane; Reproduced from reference 11.

through – the zona pellucida. This factor continues to be one of the major criticisms that plague this test.

Throughout the 1980s, multiple modifications of the SPA were published. These included modifications of the techniques for sperm preparation prior to the performance of the assay, such as inducing the acrosome reaction or incubation with TEST yolk buffer, changes in the protocol methodology itself, and modifications of the scoring system.<sup>45,46</sup> Published reports demonstrated widely varying conclusions, such as the finding that the SPA could identify anywhere from 0 to 78% of men whose sperm would fail to fertilize oocytes in ART procedures.<sup>47</sup> Most criticisms of the SPA literature center on poor standardization of the assay, poor reproducibility of the test, and lack of a standard normal range. Although some reports suggest a correlation between the SPA and fertility, neither a large literature review<sup>47</sup> nor a prospective long-term (5-year) follow-up study demonstrate such a correlation.<sup>48</sup> In light of these considerations, support for this test has gradually waned.

### Hemizona assay

Over the past several years, a growing body of research has demonstrated a significant correlation between tests of sperm:zona pellucida binding and subsequent fertilization in ART. This led the European Society for



**Fig 4.6** Cluster analysis of hemizona assay index and fertilization rate. A, good fertilization; B, poor fertilization; C, false-positive hemizona assay index. Reproduced from reference 52.

Human Reproduction and Embryology (ESHRE) Andrology Special Interest Group to recommend inclusion of such tests in the advanced evaluation of the male.<sup>49</sup> Like the SPA, the hemizona assay (HZA) employs sperm and nonviable oocytes in an *in vitro* assessment of fertilization.<sup>50</sup> In this test, however, both gametes are human in origin. As described, the HZA assesses the ability of sperm to undergo capacitation, acrosome react, and bind tightly to the zona. Classically, oocytes that failed to fertilize during an ART procedure are bisected, and then sperm from a proven fertile donor (500 000/ml) are added to one hemizona, while sperm from the subject male are added to the other hemizona. Following a 4-hour incubation, each hemizona is removed and pipetted in order to dislodge loosely attached sperm. A comparison or hemizona index (HZI) is then calculated by dividing the number of test sperm tightly bound to the hemizona by the number of control (fertile) sperm bound to the other hemizona:

$$\text{HZI} = \frac{\text{number of test sperm bound}}{\text{number of control sperm bound}} \times 100$$

This test assesses the ability of sperm to bind to the zona itself. Although expensive, labor-intensive, and difficult to perform, there are some data that suggest that the HZA may help to identify individuals with a poor prognosis for success with ART<sup>51,52</sup> (Fig 4.6). A more recent prospective study employing receiver operating characteristic (ROC) curve analysis has also suggested that HZA results may be used to predict subsequent fertilization in ART procedures with both high sensitivity and specificity.<sup>53</sup>

### Mannose binding assay

Another test has been developed in order to assess the ability of sperm to bind to the zona. This *in vitro* procedure is based on a series of observations that suggest



**Fig 4.7** Mannose-positive (brown) and mannose-negative (clear) sperm. Courtesy of Tammy Dey, Kaylen Silverberg.

that sperm:oocyte interaction involves the recognition by a sperm surface receptor of a specific complementary receptor on the surface of the zona pellucida. This zona receptor appears to be a glycoprotein, the predominant sugar moiety of which is mannose.<sup>54</sup> In an elegant series of experiments, Mori et al determined that sperm:zona binding could be curtailed by the addition of a series of sugars to the incubating media. Although many sugars impaired binding, the addition of mannose totally inhibited sperm:oocyte interaction.<sup>55</sup>

*In vitro* assays in which labeled probes of mannose conjugated to albumin are co-incubated with semen specimens allow for the differential staining of sperm (Fig 4.7). Those that bind the probe are thought to possess the sperm surface receptor for the mannose-rich zona glycoprotein. Several investigators, including our group, have subsequently demonstrated that sperm from fertile populations exhibit greater mannose binding than do sperm from infertile males.<sup>56–58</sup> This new area shows promise in the area of sperm function testing, but also invites further study.

### Assays of sperm DNA integrity

The most current area of investigation into sperm function involves the assessment of sperm DNA integrity. Sperm chromatin has been demonstrated to be packaged very differently than chromatin in somatic cells. Specifically, the DNA is organized in such a manner that it remains very compact and stable.<sup>59</sup> As there are many different ways in which either this organization or the sperm chromatin itself can be damaged, several different assays of sperm chromatin assessment have been developed.

There are two basic types of assays: direct assays, such as the 'Comet' and 'TUNEL' assays; indirect assays such as the sperm chromatin structure assay or 'SCSA'. The direct assays detect actual breakages in the DNA, while the indirect assays measure the relative proportions of single (abnormal) and double (normal) stranded

DNA within the sperm following acid treatment. Data from several studies suggest that infertile men have significantly greater amounts of DNA damage than fertile men.<sup>59-61</sup> There is also a suggestion that this finding is similarly present in the male partner of couples experiencing recurrent miscarriage. Despite these reports, at the present time, there is no conclusive correlation between the results of sperm DNA integrity testing and pregnancy rates achieved either naturally or with the advanced reproductive technologies. As such, the Practice Committee of the American Society for Reproductive Medicine has recently recommended that the routine testing of sperm DNA integrity should not be included in the evaluation of the infertile couple.<sup>62</sup>

## Conclusion

In summary, there have been many recent advances in the diagnostic evaluation of sperm and sperm function. Although many tests of sperm function have been described, there remains a lack of consensus as to both the role of testing and the identification of the appropriate test(s) to perform. Owing to the complicated nature of sperm function, it is improbable that a single test will emerge with sufficient sensitivity, specificity, and positive and negative predictive values required of a first-line diagnostic tool for all affected males. A more likely scenario will be similar to that in female infertility, where a battery of tests – each evaluating a specific function – are employed as needed.

In light of profound recent advances in gamete micromanipulation, a more germane issue might be the overall relevance of sperm function testing in the contemporary andrology laboratory. Although this issue is quite controversial, it is likely that sperm function testing will continue to play a role in the evaluation of the infertile male. Just as ART is not the treatment of choice for all infertile females, it is not likely that micromanipulation will become standard treatment for all infertile males.

The gold standard of sperm function remains the ability to fertilize an oocyte in vitro. Therefore, in order to continue to address the above questions, it is incumbent upon investigators to design appropriate prospective trials to assess these tests thoroughly. Those tests that demonstrate a statistically significant correlation with fertilization in vitro must then undergo additional evaluation in order to assess clinical significance if we hope to develop an appropriate diagnostic algorithm.

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