Sperm Function Tests

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Manmohan Misro

Learning Objectives

- Routine semen analysis
- Rationale behind sperm function tests
- Laboratory tests of sperm function
- Modified protocols of specialized tests

13.1 Introduction

The penultimate goal of the male gamete is to release the paternal genetic material closest to the female counterpart leading to a successful completion of the physiological process called fertilization. Thus, from the stages of formation in the testis till its final union with the oocyte in the oviduct, the main function of the male gamete is much alike a vector protecting the genetic material by all means and delivering the same at the target site. This is being achieved by a variety of meaningful modifications in the sperm morphology or characteristics during different stages of its production in the testes and maturation in the epididymis, finally ending up with its fusion with the oocyte after completing its sojourn in the female reproductive tract. At each stage, the sperm has to undergo modifications, both topographically and biochemically. Any minor or major deviations in the process during such modifications render the sperm incompetent for fertilization or result in the production of a functionally defective sperm. In assisted reproduction procedure, such as

M. Misro, PhD
Reproductive Biomedicine, National Institute of Health and Family Welfare,
New Delhi, India
e-mail: mm_misro@yahoo.com

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intracytoplasmic sperm injection (ICSI), many such necessary physiological steps are bypassed since the sperm is injected directly into the fully matured oocyte triggering nuclear fusion and fertilization. However, under normal physiological circumstances, all such stages with specific modifications are very much relevant to sperm function. Deficiencies in sperm characteristics are directly associated with sperm underperformance as sperm factor contributes a significant percentage (>70%) of all forms of male infertility.

Infertility in male is a complex problem affecting approximately 5% of eligible couples. Conventionally, male infertility is diagnosed following a simple evaluation of semen parameters like sperm count, motility and morphology. Additional tests on germ cell cytology and other semen biochemical tests mostly provide information on genital tract patency, accessory gland secretory efficiency or problems related to ejaculations or emissions. But, all such tests reveal very little about the functional competency of sperm, and their physiological significance changes with the passage of sperm from male to female reproductive tract. Starting from its precursor cell, the round spermatid, till its subsequent maturation into a full-fledged specialized sperm, there are subtle alterations not only in shape but also in chromatin chemistry which makes the later resistant to varied physiological fluctuations and exigencies. Such a process does not always ensure uniformity leading to the production of only "good" sperm in an ejaculate. As long as the proportion of defective creation remains small, it is mostly taken care of by sheer number of sperm as millions are being produced. However, subtle changes in sperm function in an ejaculate become apparent only when the defective population exceeds a particular threshold. The physiological significance of all such site-specific modifications and the appropriate test protocol for examining its associated function in the full-fledged mature sperm under in vitro conditions merit consideration since the same cannot be investigated and tested in vivo. However, sperm function testing has to be done in addition to routine analysis of sperm which cannot be dispensed with. Therefore, before we discuss the sperm function and associated testing protocols in detail, it is prudent to briefly analyse the laboratory methodology that is routinely followed under male infertility investigations.

13.2 Routine Analysis of Semen

It is an accepted fact that initial laboratory investigation concerning male fertility should start from the basic semen analysis, the method of which along with standard references has now been updated in the recent World Health Organization (WHO) manual. However, conventional semen analysis, as such, does not provide any details on sperm function. Instead, the results provide some basic but crucial information on the functional status of the germinal epithelium, epididymis and accessory sex glands which is directly linked to the physiological capacity and function of these organs. Besides the assessment of sperm concentration, the results of this analysis further yield full details on two important sperm characteristics, sperm

motility and morphology, which have direct implications on fertilizing ability of sperm. Usually, two consecutive semen analyses 15 days apart from the same subject are carried out to categorize a semen sample as normal or subnormal (WHO 2010).

13.2.1 Assessment of Sperm Concentration

Concentration of sperm in the ejaculate is determined under a microscope using Neubauer's haemocytometer. Sperm concentration is determined on the basis of sperm counted per unit volume of semen utilized, multiplied by number of times it is diluted for examination. Total sperm count in the ejaculate is subsequently obtained after multiplying sperm concentration with semen volume (WHO 2010).

13.2.2 Assessment of Sperm Motility

There has been a little updating in the categorization of sperm motility in the new WHO manual. Earlier, forward progressive motility used to be classified under two heads, fast forward and slow forward. Individuals often found this method difficult to delineate these two categories without bias (Cooper and Yeung 2006). Now, sperm motility has been graded as progressive, nonprogressive or immotile as described below:

- Progressively motile (PR) – refers to those sperm which are moving fast, linearly or in large circles
- Nonprogressively motile (NP) – refers to those sperm which are static in spite of depicting active or docile flagellar movement
- Immotile (IM) – those sperm which are static and show no flagellar movement

Accordingly, PR and NP sperm are considered to calculate total sperm motility (WHO 2010).

13.2.3 Assessment of Sperm Morphology

Recent WHO guidelines utilize strict criteria given by Kruger for the assessment of sperm morphology. Kruger's criteria have been described in the chapter, "The Sperm". Using this criterion morphologically, normal sperm (3–5%) have been successfully used in in vitro fertilization, intrauterine insemination and spontaneous pregnancies (Coetzee et al. 1998; Van Waart et al. 2001; Van der Merwe et al. 2005). Accordingly, values lower than the above cut-off are classified as subnormal. The

Table 13.1 Reference values for semen parameters, as published in consecutive WHO manuals (Esteves et al. 2011)

Semen parameters	WHO, 1992	WHO, 1999	WHO, 2010[1]
Volume	≥2 ml	≥2 mL	1.5 mL
Sperm concentration/mL	≥20 × 10^6/mL	≥20 × 10^6/mL	15 × 10^6/mL
Total sperm count	≥40 × 10^6	≥40 × 10^6	39 × 10^6
Total motility (% motile sperm)	≥50%	≥50%	40%
Progressive motility[2]	≥25% (grade a)	≥25% (grade a)	32% (grade a + b)
Vitality (% alive)	≥75%	≥75%	58%
Morphology	≥30%[3]	14%[4]	4%[5]
Leukocyte count	<1.0 × 10^6/mL	<1.0 × 10^6/mL	<1.0 × 10^6/mL

WHO World Health Organization
[1]Lower reference limit obtained from the lower fifth centile value
[2]Grade a = rapid progressive motility (0.25 mm/s), grade b = slow/sluggish progressive motility (5–25 mm/s), normal = 50% motility (grades a + b) or 25% rapid progressive motility (grade a) within 60 min of ejaculation
[3]Arbitrary value
[4]No actual value given, but multicentric studies refer >14% (strict criteria) for in vitro fertilization (IVF)
[5]Normal shaped sperm according to Tygerberg (Kruger) strict criteria

cut-off is obtained from the data collected from volunteers of eight countries on the basis of their female partners achieving normal pregnancy in ≤12 months. The same data was then utilized to generate reference distributions of all other semen parameters. Table 13.1 provides the information of all the reference ranges in comparison to WHO guidelines of 1992, 1999 and 2010 (Esteves et al. 2011). Based on the lower reference limits given by WHO (2010), different nomenclatures has been used for the semen quality (see Table 13.2).

13.3 Rationale for Sperm Function Testing

Since basic infertility investigations in approximately 20% of couples yield normal semen parameters, etiologically they are classified as idiopathic. Other additional tests are, therefore, necessary to determine specific functional disorders associated with sperm abnormalities difficult to be detected in conventional semen analysis. Besides, sperm factor as a result of subnormal sperm function has been reported to be an indicator for couples with recurrent pregnancy loss (Gopalkrishnan et al. 2000; Saxena et al. 2008). The use of sperm from sperm banks further necessitates the use of such testing protocols for quality control before they are utilized in procedures associated with assisted reproduction for the treatment of infertility.

Sperm function test, ideally, should reveal the following crucial clinical information:

Table 13.2 Nomenclature used to indicate quality of semen parameters

Nomenclature	Total sperm count	Progressive motility (%)	Normal morphology (%)	Remarks
Normozoospermia	≥39 × 10^6 per ejaculate	≥32	≥4	
Oligozoospermia	<39 × 10^6 per ejaculate	–	–	
Asthenozoospermia	–	<32	–	
Teratozoospermia	–	–	<4	
Oligoasthenozoospermia	<39 × 10^6 per ejaculate	<32	–	
Oligoteratozoospermia	<39 × 10^6 per ejaculate	–	<4	
Asthenoteratozoospermia	–	<32	<4	
Oligoasthenoterato-zoospermia	<39 × 10^6 per ejaculate	<32	<4	
Azoospermia	No sperm	–	–	
Cryptozoospermia	Very low	–	–	Sperm few in number, identified only after centrifugation
Necrozoospermia	–	<32	–	High % of dead and immotile sperm
Aspermia	No semen			
Haemospermia (haematospermia)	Presence of erythrocytes in the ejaculate			
Leucospermia (leucocytospermia, pyospermia)	Presence of leukocytes in the ejaculate >1.0 × 10^6/ml			

(i) Able to detect a specific sperm dysfunction (Muller 2000)
(ii) Able to predict fertilization and pregnancy rates
(iii) Able to suggest appropriate therapeutic treatment for the same

Irrespective of requirement of specialized infrastructure and expertise, procedures of sperm function tests have now been recognized as essential and separately grouped under research procedures in the new WHO manual 2010 (Lamb 2010). However, more initiatives are needed to develop these tests in the form of routine protocols which can be performed along with regular semen analysis. Some of the related developments along with recently developed test protocols are described in the subsequent sections.

13.4 Tests of Sperm Function

13.4.1 Acrosome and Its Function

Under normal physiological circumstances, activation of acrosomal matrix to release its contents follows capacitation; both the events occur in the female reproductive tract and are considered prerequisites for successful fertilization. Only the acrosome-reacted sperm are able to release the acrosomal contents, aiding in penetrating the cumulus mass and zona pellucida (ZP). In fact, it is the sperm-zona binding that triggers the acrosome reaction resulting in release of the proteolytic enzymes designed to facilitate both ZP penetration and remodelling of the sperm surface in preparation for oocyte fusion (Aitken 2006). Though the clinical application of the acrosome reaction bioassay has been questioned by many, calcium ionophore-induced acrosome reaction testing has been found to predict the fertilizing potential of human sperm in vitro (Cummins et al. 1991).

Acrosome reaction has been reported to be induced in vitro through biochemical agents such as calcium ionophore A23187 and progesterone (Oehninger et al. 1994; Yovich et al. 1994; Perry et al. 1995). It opens up the possibility of monitoring acrosome reaction in vitro with the help of these agents. However, the test protocols based on this are complex that utilize several specific antibodies to different acrosomal components (Kallajoki et al. 1986; Sanchez et al. 1991).

There is another way of analysing acrosome function by assessing the acrosome status of the sperm. This is done by incubating sperm with fluorescent lectins that inherently bind to outer acrosomal membrane (*Arachis hypogaea* agglutinin) or to acrosomal contents (plasma sativum agglutinin). After binding with probe, the sperm depicts a fluorescent acrosomal cap or a band around its equatorial segment when acrosome reaction occurs (Aitken 2006).

Acrosome reaction in sperm can also be induced by binding through ZP (Menkveld et al. 1991; Liu and Baker 1992a, b). It is strongly supported by the fact that sperm binding to zona is directly correlated with normal morphology of acrosome in sperm (Liu and Baker 1988; Menkveld et al. 1996). The capacity of sperm acrosome to react in the presence of solubilized ZP has now been developed as a therapeutic tool using either the partner's own sperm or those available from other subjects (Franken et al. 1997). The procedure utilizes stripping of zona from oocytes by aspirating vigorously using a pipette with 90 μm inner diameter and incubating the same with sperm for inducing acrosome reaction. This is otherwise known as zona-induced acrosome reaction (ZIAR) test and has been recognized as an indicator of sperm dysfunction among men presenting normal semen parameters. The finding of such a study on in vitro fertilization outcome of 35 couples has been described in Table 13.3 (Franken et al. 2007).

Further simplification of test protocol on acrosome status of sperm comes from the assay of gelatin digestion following the release of acrosomal contents of sperm (Gopalkrishnan 1995). Though the assay has a wider acceptability, it suffers from the fact that its completion time is very long, about two and half hours, and sometimes fails to be reproducible even with experienced technical hands. The protocol

Table 13.3 Semen parameters versus acrosome response against the outcome of in vitro fertilization (IVF) of 35 couples as per results of zona-induced acrosome reaction (ZIAR) test

ZIAR	Mean sperm concentration (10^6 cells/ml)	Morphology (% normal)	Motility (%)	HZI	ZIAR	No. of oocytes	Fertilized group retrieved
<15%	55.8 ± 16	12.9 ± 4	54.5 ± 13	53 ± 13	3.7 ± 3[a]	8.9 ± 6	10.3 ± 21[c]
(*n* = 20)	(22–100)	(8–19)	(40–70)	(40–70)	(0–8)	(2–22)	(0–60)
>15%	66.4 ± 24	12.6 ± 3	64.7 ± 11	67 ± 12	22.2 ± 3[b]	8.2 ± 4	85.2 ± 11[d]
(*n* = 15)	(22–100)	(7–18)	(51–80)	(49–88)	(16–27)	(4–18)	(60–100)

Fisher's exact *t*-test: a vs b, $P = 0.001$; c vs d, $P = 0.001$

was further updated in the form of a kit which at present is being developed in the form of a commercial kit to be available in the Indian market soon. The protocol as described in the original protocol and the modified patented version are described below (Gopalkrishnan 1995; Misro and Chaki 2005a, b, c).

13.4.1.1 Test for Acrosome Status and Function (Gopalkrishnan 1995)

Time requirement: 2 h 40 min

The acrosome of sperm contains a number of proteases, which play a crucial role in the penetration of sperm through outer investments of oocyte.

The basis of this method is that acrosomal enzymes once released will dissolve protein (e.g. gelatin) through which light can pass, leading to the observation of halo structure around the sperm head when examined under a microscope. The protocol is described below.

Preparation of reagents:

Preparation of gelatin slides:

- Prepare 5% gelatin in distilled water (dist. H_2O) at 50°C.
- Take 40 µl of gelatin solution (warm) and spread on pre-cleaned slide and keep the gelatin-coated slides horizontally for 24 h at 4°C.
- Fix the gelatin slides in 0.05% glutaraldehyde in phosphate buffer saline (PBS) for 2 min.
- Wash the fixed gelatin slides twice in dist. H_2O.
- Gelatin slides are stored at 4°C vertically. The slides can be used up to 4 weeks.

Phosphate-buffered saline (*PBS*):
Sodium chloride (NaCl) = 0.2 gm
Disodium phosphate (Na_2HPO_4) = 0.14 gm
Potassium dihydrogen phosphate (KH_2PO_4) = 0.0023 gm
Dist. H_2O = 100 ml
PBS-glutaraldehyde solution:
25% glutaraldehyde = 0.2 ml
PBS = 100 ml

PBS-D-glucose (pH 7.8) solution:
PBS = 100 ml
D-Glucose =3 gms

Protocol:

- Dilute the semen sample 1:20 with PBS-D-glucose.
- Equilibrate for 30 min at 37°C.
- Bring the gelatin-coated slides to room temperature.
- Take 20 µl of diluted semen and gently smear on the gelatin slide.
- Keep for 5–10 min at room temperature to remove excess moisture.
- Incubate for 2 h at 37°C with wet filter paper on the sides in a petri dish.
- Observe the slide under microscope (400×).
- Count the sperm with halos against those without halos and calculate the percentage.

Normal: >50% sperm with halos.

Significance: This test evaluates the functional status of sperm acrosome and is a good indicator of sperm's ability to penetrate the oocyte investments.

Clinical relevance: Loss of acrosome, absence of acrosome or irregular acrosome may indicate the possibility of low or nil fertilization.

13.4.1.2 Lab Test Kit for Acrosome Status and Function (Misro and Chaki 2005a)

Time required: 40 min

Reagents provided in the kit: Gelatin-coated slides (black colour) and acrosome reaction solution (blue colour). Both are stable at room temperature.

Apparatus required: Any light microscope, an incubator maintained at 50°C, a micropipette and a moisture chamber (moisture chamber can be made using petri dish and blotting paper).

Protocol:

- Take 500 µl acrosome reaction solution in any small tube.
- Add 50 or100 µl of liquefied semen sample.
- Incubate at room temperature for 5 min.
- Make a smear smoothly on coated slide.
- Air-dry excess liquid on the slide. Do not do overdrying.
- Place the slide at 50°C in a moisture chamber and incubate for 30 min. Moisture chamber should be prewarmed at 50°C.
- Let it air-dry and observe under microscope.

Count the percentage of sperm with halos surroundings their heads. Normal acrosome status if >50% sperm with halos surrounding their head are found (see Fig. 13.1).

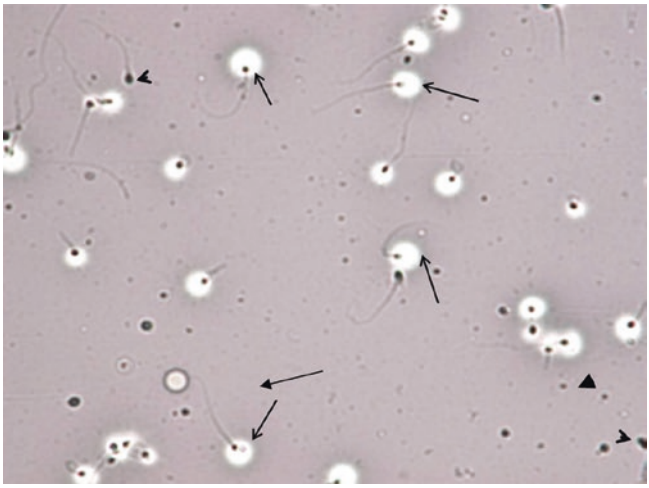

Fig. 13.1 Acrosome status: *Arrows* indicate sperm with halo (acrosome positive), and arrow heads indicate sperm without halo (acrosome negative)

13.4.2 Test for Sperm Plasma Membrane Integrity

Hypoosmotic swelling (HOS) of the sperm tail demonstrates the functional integrity of the sperm plasma membrane. The rationale of the test is based on the assumption that an undamaged sperm tail membrane permits the free flow of fluid into the cell under hypoosmotic conditions. The resultant swelling is manifested in the form of various types of sperm tail coiling that can easily be observed under light microscopy. The effect of hypoosmotic challenge is identical in all mammalian sperm (Drevius and Eriksson 1966). The hypoosmotic solution most commonly used for the purpose is composed of sodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$; 0.735 g) and fructose (1.351 g) in 100 mL of dist. H_2O, with an osmolarity of 150 mOsm/litre. The same has been used in human sperm to test the plasma membrane integrity (Jeyendran et al. 1984, 1992; WHO 1992). Despite divergent degrees of correlation reported between the HOS test and different sperm evaluation parameters, the test has been recognized as the predictor of human sperm fertility (Okada et al. 1990). Though it is a very simple and sensitive test, it has never been routinely practised due to its lengthy duration of approximately 60 min, as per the WHO protocol. The protocol has been appropriately modified further and has been developed in the form of a laboratory kit (Misro and Chaki 2005a, b, c). The detailed procedure of the original as well the modified version is included here for better handling and reproducibility.

Reagents required: Hypoosmotic solution

Dissolve 0.735 g sodium citrate $Na_3C_6H_5O_7.2H_2O$ and 1.351 g fructose in 100 ml dist. H_2O. Store aliquots of this solution frozen at −20°C. Thaw and mix well before use.

Method (Jeyendran et al. 1984):

Time required: *60 min*

- Warm 1 ml HOS solution in a closed Eppendorf tube at 37°C for about 5 min.
- Add 0.1 ml liquefied semen to the solution and mix gently.
- Keep at 37°C for 100 min, but not longer than 120 min.
- Examine the sperm cells with phase-contrast microscope. Swelling of sperm is identified as changes in the shape of the tail as shown in Fig. 13.2. Repeat twice the score of swollen sperm in a total of 100 sperm, and calculate the mean percentage.

Modified method (Misro and Chaki 2005a, b, c):
Time required: *10 min*
Reagents provided: HOS solution (patented).
Storage condition: Room temperature.
Apparatus required: Any ordinary microscope and micropipette (10–100 µl).

Protocol:

- Take 500 µl of HOS solution in a small tube.
- Add 50 or 100 µl (depending on high/low sperm count) of liquefied semen sample to it.
- Mix gently and incubate at room temperature for 5 min.
- At the end of the incubation time, add 50 µl of colour stop solution and mix gently.
- Place a small drop of the mixture on a clean glass slide and cover it with cover slip.
- Observe it under a microscope and count the percentage of sperm with coiled tail (see Fig. 13.2). If the sample shows bent tail before the test, reduce the number after the test to get the actual result.

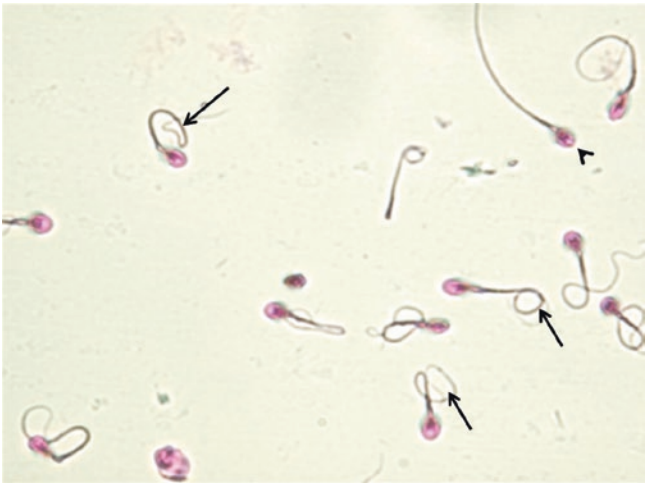

Fig. 13.2 The *arrows* indicate sperm positively responding to HOS test, whereas the arrow head indicates abnormal sperm (normal range, ≥60% with coiled tail)

13.4.3 Nuclear Chromatin Decondensation Test

Sperm chromatin is highly condensed, and the process of condensation starts right from the round spermatid stage at the level of the testis where they are formed. This is facilitated by the replacement of histone protein with protamine in the haploid chromatin network. However, the process of nuclear chromatin condensation continues during the transit of sperm from the testis and is completed during epididymal maturation. Since the condensation process involves millions of sperm and is indirectly dependent on the association of protamines in the sperm DNA, it is likely that all the sperm might not be condensed to the extent required. If a significant percentage of sperm is either too condensed or loosely condensed, this may reflect in their functional ability during fertilization. This is supported by the fact that during normal fertilization process following oocyte penetration, sperm chromatin decondenses, swells and forms the pronucleus prior to the fusion with oocyte nuclei. Thus, sperm chromatin decondensation is an essential prerequisite for successful fertilization, and the way this is tested in vitro using a semen sample is described below. The procedure as reported in the literature is again time consuming (Gopalkrishnan 1995). Therefore, a modified method to facilitate quick assessment of chromatin decondensation was developed, and the test can be completed within 10 min (Misro and Chaki 2005a, b, c). Both the procedures are described below for comparison and usability.

Method (Gopalkrishnan 1995):

Time required: *40 min*

1. Mix sperm in 0.1 ml semen and 0.5 ml insemination medium, and then wash with 10 ml 0.9% sodium chloride and centrifuged at 2000 rpm for 10 min.
2. Suspended pellet in 0.1 ml 50 mmol/L sodium borate buffer (pH 9.0) containing 2 mmol/L DTT prepared daily.
3. Incubate suspension at room temperature (18–20°C) for 30 min.
4. Add 0.1 ml of 1% (w/v) SDS in sodium borate buffer and mix the suspension gently and incubate at room temperature for 2 min.
5. Stop the reaction and fix sperm by addition of 0.2 ml of 2.5% glutaraldehyde in sodium borate buffer.
6. Mix suspension (15 µl) with 5 µl of 0.8% rose bengal in 0.9% sodium chloride on a glass slide and cover with a square (22x22 mm) coverslip and examine under a phase-contrast microscope.

Method (Drevius and Eriksson 1966; Jeyendran et al. 1984; Misro and Chaki 2005c):

Reagents provided in the kit: One sachet containing chemical ingredients and a stop solution (patented).

Time required: *10 min*

Storage condition: Once prepared, the solution is stable for 3–4 days in a refrigerator at 4°C.

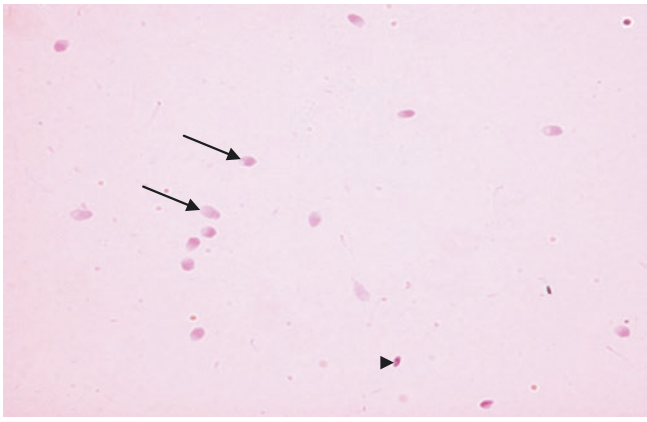

Fig. 13.3 Nuclear chromatin decondensation test. *Arrows* showing positive whereas the arrowhead showing negative NCD test (normal range, ≥ 70% with enlarged heads)

Apparatus required: A small centrifuge, microscope, micropipette and incubator maintained at 50°C.

Protocol:

Dissolve the whole content of a sachet in 10 ml dist. H_2O to make NCD (nuclear chromatin decondensation) solution.

- Take 500 µl of NCD solution (prewarmed at 50°C) in a small tube.
- Add 50–100 µl (depending on high/low sperm count) of liquefied semen sample to it. Mix gently and incubate at 50°C temperature for 5 min.
- Stop the reaction by adding 100 µl of stop solution.
- Place a small drop on a clean glass slide and cover with coverslip.
- Observe under the microscope and count % of decondensed (enlarged heads) sperm (see Fig. 13.3).

Besides the three easy-to-do methods of sperm function tests as described above, there are a few other tests that are very much specialized and require similar infrastructure and expertise to perform. In the following sections, therefore, their significance and usefulness are discussed without describing the method as such in detail.

13.4.4 Zona-Free Hamster Penetration Assay

The assay is based on the observation that following removal of the zona, the hamster egg becomes promiscuous and allows sperm from several species including humans to penetrate (Yanagimachi et al. 1976). This comprehensive bioassay examines the ability of human sperm to capacitate, to undergo the acrosome reaction and to undergo nuclear decondensation identical to the initial steps of fertilization after penetrating the egg. Though the test has gone several modifications since its development, its predictive value still remains to be controversial. A significantly higher

correlation to in vitro fertilization outcome combined with low false positives and unacceptable levels of false negatives has been reported (Perreault and Rogers 1982; Aitken et al. 1991). The test is no longer commonly followed today because of the fact that it is labour intensive, technically demanding and extremely difficult to standardize.

13.4.5 Hemizona/Zona Pellucid (ZP) Binding Assay

ZP binding assay is based on the physiological observation that sperm-zona interactions are crucial leading to subsequent steps of fertilization. ZP is an acellular glycoprotein that surrounds the oocyte and serves as the site for sperm-egg recognition and induction of acrosome reaction. The glycoprotein ZP3 is reported to mediate such recognition and activations of sperm, while another glycoprotein ZP2 facilitates the binding of the acrosome-reacted sperm to the zona matrix. The assay developed for the purpose outlined the bioassay conditions and oocyte retrieval procedures followed in the hemizona assay and competitive intact ZP binding test (Burkman et al. 1988; Liu and Baker 1992a, b). Both bioassays provide functional homologues to physiological steps of sperm binding to ZP and provide a meaningful and distinct comparison between infertile versus fertile semen samples. Though the test has the advantage of providing information on sperm-zona interaction and associated defects, it is no longer widely offered. The reason behind this is that zona for the test is usually collected from excess, unutilized eggs or those eggs which did not fertilize. Moreover, eggs are to be bisected which require micromanipulation skills. The bisected ova are then incubated with sperm samples and examined following a period of incubation. Novel biomaterials may provide an alternate standard matrix to detect sperm-zona binding dysfunction in future and have the potential to develop as new laboratory protocols (Lamb 2010).

13.4.6 Test for Sperm DNA Damage

It is recognized that apparently sperm with normal morphology in an infertile semen sample may also have some DNA fragmentation which cannot be detected in routine examinations (Avendano et al. 2009). Sperm DNA fragmentation assessment in vitro has been increasingly recognized as a tool to assess the fertilizing potential combined with better diagnostic and prognostic prospects than the conventional assessment of parameters like sperm morphology, concentration and motility. When used along with assisted reproductive techniques, it has the risk of ending with early pregnancy loss leading to a low rate of carry home live births. Under normal circumstances, relatively small but not the large DNA damages are repaired during pre- and postreplication processes. Fertilization with such defective sperm carrying DNA damages may result in pregnancy loss or birth of offsprings with major or minor congenital malformations (Samsi et al. 2008). However, many andrologists still are not sure whether or not sperm DNA assessment should be routinely used in

the male infertility evaluation though a variety of tests are available for the purpose. Since tests for whole sperm chromatin are already discussed earlier, tests which are typically developed to assess sperm DNA fragmentation defects are briefly described below, though these tests are seldom performed unless and until specifically recommended.

13.4.6.1 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is a diagnostic technique to identify DNA strand breaks. It incorporates biotinylated deoxyuridine (dUTP) to 3'OH at DNA strand breaks, both single and double. Therefore, the intensity of signal increases with the increase in DNA strand breaks. During the development protocol, biotinylated dUTP is utilized to label the 3'ends of the fragmented DNA using recombinant deoxynucleotidyl transferase (TdT) enzyme. Sperm with normal DNA have capped telomeres at the 3'OH end. Thus, they do not fluoresce under a fluorescence microscope. But, those with DNA strand breaks having multiple 3'OH ends provide bright fluorescence (Lopes et al. 1998). Alternative use of TUNEL includes enzymatic detection of fragmented DNA. This is generally carried out using the enzyme horseradish peroxidase labelled with peroxidase streptavidin (streptavidin-HRP) which is in turn linked to biotinylated nucleotides. Hydrogen peroxide is used as the substrate and diaminobenzidine (DAB) as the chromogen, and the detection of the strand break is determined following the development of a deep brown stain.

The advantages of TUNEL assay are that it can detect both single- and double-strand breaks and provides useful information on the extent of DNA damage of sperm in a semen sample. It is, however, mostly a qualitative assay in the sense that the extent of DNA stand breaks in a single sperm cannot be quantified.

13.4.6.2 Comet Assay

The comet assay is also known as single gel electrophoresis assay, first described by Ostling and Johnson in 1984. It was later used with microgels which were utilized for electrophoresis under highly alkaline conditions (Singh et al. 1988). Alkaline conditions help in the uncoiling of DNA where the free ends migrate towards anode under the applied electric field. Following staining with acridine orange, the sperm head containing the DNA appears "comet"-like comprising a distinct head and tail. While the head represents intact DNA, the tail consists of strands of degraded DNA. In this way, both single- and double-strand breaks are easily analysed. This method was developed to measure low levels of strand breaks. Since then, a number of advancements have greatly increased the flexibility and utility of this technique for detecting various forms of DNA damage (e.g. single- and double-strand breaks, oxidative DNA base damage and DNA-DNA/DNA-protein/DNA-drug cross linking) and DNA repair in virtually any eukaryotic cell. The assay works on the principle that smaller fragmented DNAs migrate at a faster rate to anode as compared to the non-fragmented, intact DNAs. The protocol can be carried out both in neutral and acidic pH conditions.

To describe the method briefly, a suspension of sperm is mixed with low-melting agarose and layered onto a precoated agarose slide. The slide containing sperm is incubated inside a Coplin jar containing high salt concentration to release the DNA following removal of cellular proteins. The DNA then unwinds under alkaline conditions, and the alkali-labile sites are considered as single-strand breaks in the liberated DNA. Treatment with nucleases under controlled conditions is sometimes considered to experimentally induce strand breaks in the DNA for maintaining a parallel internal control. The slide containing sperm is then placed within an electrophoretic chamber and electrophoresed under alkaline (pH > 13) conditions that forces broken DNA strands to migrate close to anode. The released sperm DNA is stained with ethidium bromide and scored. The assay is very helpful to study various forms of DNA damage by altering steps of lysis and electrophoresis under controlled laboratory conditions. Though the method has acquired scientific validity, it requires sophisticated laboratory set-up. Therefore, it is not widely practised in andrology laboratories unless specifically recommended.

13.4.7 Test for Assessment of Sperm Oxidative Stress

Oxidative stress impairs all the functional capacities of sperm. Oxidative stress represents the imbalance between pro- and antioxidants present in any cellular system. Oxidative stress arises when pro-oxidants like reactive oxygen species (ROS), a class of free radicals, are generated in excess than what the cellular system can neutralize through antioxidants that normally limit the damaging effects. Free radicals are also generated in the normal course of oxidative metabolism. These free radicals are useful to some extent in the normal physiological processes like sperm capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion. However, generated in excess, they induce pathological effects leading to loss of function, apoptosis and cell death (see Fig. 13.4; Kothari et al. 2010).

It is imperative to find out the source of ROS generation. The external source of ROS is mostly represented by the leucocytes that are present in almost every ejaculate, and the internal source is represented by sperm themselves (Wolff 1995). It is reported that extrinsic ROS generated by leucocytes predominately affect the sperm plasma membrane leading to reduced motility (Henkel et al. 2005). In contrast, intrinsic ROS induces DNA damage (Franken and Oehninger 2012).

Estimation of sperm ROS can be easily measured with the help of a chemo-luminometer. Multiple factors affect chemiluminescent reactions. These include the concentration of reaction mixture, sample volume, temperature control and background luminescence. A brief procedure of measurement of sperm ROS using a chemo-luminometer is described below (Agarwal et al. 2008):

1. Centrifuge semen samples at 300 × g for 5 min, and remove the seminal plasma.
2. Suspend the sperm pellet in 3 mL of Dulbecco's PBS solution and wash again at 300 × g for 5 min.

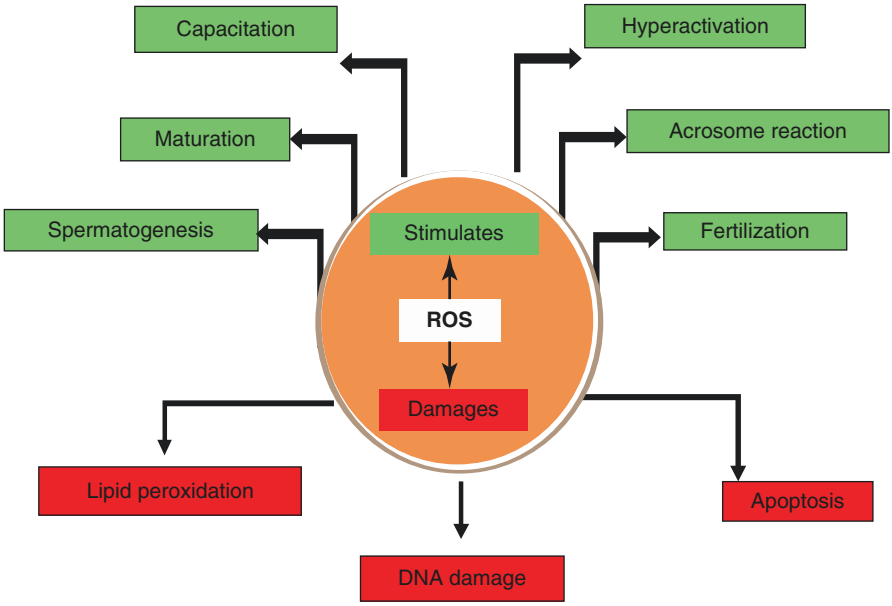

Fig. 13.4 Physiological roles of reactive oxygen species (ROS) include maturation, capacitation, hyperactivation, spermatogenesis, acrosome reaction and fertilization

3. The sperm concentration is adjusted to 20 × 106/mL before ROS measurement. Measure ROS by a chemiluminescence assay using 5 μL of luminol (5 mM, 5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma Chemical Company, St. Louis, MO, USA).
4. Measure chemiluminescence in the integration mode using chemo-luminometer at 37°C for 15 min after adding luminol. ROS production is expressed as counted photons per minute (cpm)/20 × 10^6 sperm.

Several independent studies have also demonstrated that chemiluminescent assays of redox activity reflect the fertilizing potential of human sperm both in vivo and in vitro (Zorn et al. 2003; Said et al. 2004). However, in spite of their potential as diagnostic tools, these are only very limitedly utilized.

Key Questions

- What is the rationale of doing sperm function tests in the laboratory?
- Write the lower reference limit of various semen parameters updated by WHO in 2010.
- What is the principle of comet assay?
- Describe the test to be used for analysing sperm nuclear chromatin condensation.
- Write the principles of the tests used to examine acrosome function.

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