Both male and female infertility practices are now heavily dependent on assisted reproductive technologies (ART). In fact it is not an exaggeration to say that ART is continuing to shape the course of the fertility treatment in 21st century. ART is still an evolving field, and cutting edge technologies are continually emerging in the field. Numerous aspects of male gametes (spermatozoa) that have clinical significance are dealt with in ART laboratory. Assessment of fertility potential of spermatozoa is certainly the ultimate objective. ART laboratory also deals with another equally important aspect, utilization of spermatozoa in quality control testing, which monitor the quality of various ART procedures. Sperm fertility potential is assessed in multiple ways, collectively referred to as sperm function tests (SFT), while use of sperm in assessing quality of ART procedures is called sperm bioassay (SB). This chapter focuses on the role of SFT and SB in fertility treatment in the era of ART.

Reproductive success depends on the fulfillment of a series of functional prerequisites. Natural (in vivo) or forced (in vitro) union of the competent male and female gametes and their subsequent interactions are some of these prerequisites. Competencies of male and female gametes can be diagnosed by appropriate tests. In male infertility practices, fertility potential of males is primarily assessed by analyzing their ability to produce quality male gametes, spermatozoa. Quality sperm certifies male fertility potential. Semen analysis is the basic conventional method in evaluating such qualities in spermatozoa. In semen analysis, concentration, motility, viability and morphology of sperm are taken into account. Semen analysis being inexpensive, noninvasive and less technical remains to be the test of choice in male fertility evaluation. However, the test has its limitations. As scientific and technological advances are made, other tests are introduced not to supplant or replace semen analysis but to delve further into the specific causes of male infertility.

Tests beyond semen analysis that have been developed to understand the deeper insight of male infertility are comprised of semen culture, antisperm antibody, semen biochemical assay, sperm membrane integrity, acrosome reaction, hyperactivation and capacitation, viability, stress tolerance, zona binding, cervical mucus penetration, sperm maturity, chromosomal aneuploidy, chromosome micro deletion, sperm DNA fragmentation evaluation etc. Objective of the above mentioned tests are the same: to assess the fertilization potential of the sperm under investigation. In natural reproduction, spermatozoa are released into the vaginal reservoir from which they have to migrate through cervix, uterus and oviduct to meet oocyte for fertilization. Sperm have to bind and then penetrate through zona, cross oolema, and reach ooplasm to initiate the discharge of chromosomal content so that oocyte can prepare its complementary chromosome set by expulsion of excess ones via polar body extrusion. Competency of spermatozoa to accomplish these tasks are assessed in laboratory by a cohort of tests which fall under the category of sperm function tests, SFT.

On the other hand, male gamete has been an integral part of quality control for successful in vitro fertilization (IVF) since its inception. Edwards and Steptoe, the pioneers of human IVF, utilized human sperm assay (HSA) for testing the suitability of solutions and materials to be used in first successful IVF pregnancy in the world. Sperm assay allowed them to detect the factors that adversely affected the growth of human embryos in vitro thus offering an opportunity for optimizing embryo culture conditions. Subsequently, many other assays have been developed utilizing mouse zygote, embryos, hamster sperm, ovarian cells, cumulus cells etc in evaluating the quality of human embryo culture conditions. HSA, however, remains as one...
of the preferred quality control (QC) methods in fertility laboratories where it is routinely used as an in-house QC test and also as an externally administered proficiency test popularly called PT.1,4,8,9,19-23,26,32,52,55,61,64,69

Assisted reproductive technologies in general and IVF in particular have emerged to fulfill the treatment needs in infertility practices. In this chapter we elaborate on selected sperm function tests that have been developed to assess the fertility potential of human sperm, and sperm bioassays particularly HSA that have applications to in quality control of human ART.

### Sperm Function Tests (SFT)

Sperm function tests fall under the category of non-routine tests because of their highly selective uses. Unlike semen analysis which is applied to all infertile couples, the sperm function tests are chosen based on the specific needs of the male under investigation. Semen analysis, in most cases, is helpful in selecting a specific sperm function test or test series that may be essential for a subject. A large number of SFT have been developed and reported in literature. A select few of such tests of high clinical relevance are illustrated below.

#### Stress Tolerance

Idea of a sperm stress test was first proposed by Alvarez et al. in 1996.4 Concept behind this is that spermatozoa may face some physiological stress such as temperature, pH, or osmolarity fluctuations during the course of meeting their counterpart, oocyte. Sperm that handle such stress well can be considered better prepared to make fertilization happen either in vivo or in vitro. Spermatozoa derived from different individuals may exhibit different levels of stress tolerance. Stress tolerance values of sperm samples of previously proven fertile males can be used as a control in assessing the stress handling capabilities of sperm under investigation.

Alvarez et al and several others documented the poor stress tolerance of a sperm population having dual negative impacts. First, sperm failing in stress tolerance may also fail to penetrate the external barrier (zona pellucida) of egg due to their poor vigor. Secondly, sperm with inferior stress handling ability may lose motility and die. If such sperm accumulate in large quantities surrounding an oocyte, they may contribute to creating an unhealthy micro environment for the oocyte. In Alvarez’s proposed stress test, sperm are exposed briefly to elevated temperature and purportedly shows different subsets of sperm exhibiting motility of different grades. They proposed that relative abundance of sperm of different degrees of stress tolerance (as reflected in motility) in a sperm sample may have clinical significance.2,4,31,56,71

### Sperm Longevity

Fertile life of a spermatozoon can be measured by the duration of it’s motility. If spermatozoa prematurely lose motility, they also lose their natural fertilization potential since they cannot travel to meet oocyte. Therefore, how long sperm can sustain its motility is important to investigate. Some investigators think that longevity assessment may provide a valuable insight into the potential etiology of male infertility. Sperm longevity can be assessed using washed sperm maintained in culture. Some investigators have predicted higher fertilization potential of sperm exhibiting longer motility duration in culture. Unfortunately, no defined sperm longevity assessment assay has yet been established that could be amenable to routine use in a diagnostic andrology laboratory.31,39,53,54,56

#### Membrane Integrity

Integrity of sperm membrane assures the longevity of spermatozoa. Sperm possessing a weaker membrane may fail the task of fertilization by failing to reach oocyte due to motility loss owing to premature membrane integrity failure. Sperm membrane quality determines the osmoregulatory ability of spermatozoa. A simple test called hypo-osmotic swelling test (HOS-test), introduced by Jeyendran et al in 1984,45 to assess membrane integrity, got wide acceptance in human andrology laboratories. HOS-test in fact measures the sperm osmotic fragility. Live spermatozoa with a normal healthy membrane are able to withstand moderate hypoosmotic stress and swell upon exposure to hypoosmotic solution. Dead sperm, on other hand, whose plasma membranes are no longer intact, do not swell. Instead, they let solution pass through. In addition, dead sperm that still retain intact membranes as well as senescent spermatozoa with poor osmoregulatory capacity show uncontrolled swelling that rapidly results in rupture of over distended plasma membrane.6,45,56

World health organization (WHO) included HOS-test as a sperm function test in its laboratory manual for examination of human semen. This is most likely due to its procedural simplicity. A hypo-osmotic swelling solution is prepared for HOS-test by dissolving 0.735 g sodium citrate and fructose in 1 liter of distilled water. According to WHO (4th edition), HOS test is considered to be normal for a semen sample if more than 60% of spermatozoa undergo tail swelling indicating an intact membrane. If less than
50% of spermatozoa show tail swelling, the semen specimen is considered abnormal.\textsuperscript{2,4,5,6,7,23,73}

**Acrosome Reaction**

For successful invasion of zona pellucida of oocyte, acrosome of sperm head must be functional. Functional site of physiological acrosome reaction is the zona pellucida. It is understood that after binding with zona, sperm releases acrosomal enzyme which helps penetration through zona. Acrosomal morphology is considered to be related to successful or unsuccessful invasion of sperm through the zona pellucida. In the assessment of acrosome reaction, acrosome dysfunction is identified. In this assessment, presence of outer acrosomal membrane, acrosomal contents, and inner acrosomal membrane are evaluated by employing various staining methods using a light or fluorescence microscope. Flow cytometry is also used to examine acrosome. In flow cytometry, fluorescent-labelled lectins and antibodies are applied to visualize different components of the acrosome. Calcium ionophores or progesterone are used for evaluating the competence of spermatozoa to initiate acrosome reactions. Acrosome assessment is specially recommended in cases of abnormal head morphology.\textsuperscript{2,4,5,6,7,23,73}

**Sperm-Cervical Mucus Interaction**

Interactions between sperm and cervical mucus have been studied and reported under different title headings. Postcoital test, sperm-mucus interaction, mucus penetration test and in vitro sperm-mucus interaction are some notable examples. Sperm-cervical mucus interaction tests have also been designated by individual researcher’s name such as Kremer Test, Kurzrok-Miller Test. Cervical mucus is considered as the doorway which the sperm have to pass through to get access into internal reproductive tract. Cervical mucus is a hydrogel, that by controlling its viscosity, can regulate the entrance of sperm into reproductive tract. In other words cervical mucus can be hostile to sperm at times while favorable to them at others. Similarly, some sperm population may be better fit to pass through mucus compared to others. Therefore, in sperm-cervical mucus interaction studies, ability of sperm to penetrate cervical mucus is assessed in order to predict whether the sperm population under investigation has potential power to pass through the reproductive gate (cervix). Cervical mucus works as a biological gate of reproductive tract by providing favorable receptivity to sperm penetration at or near ovulation while interfering with entry at other times in each menstrual period.\textsuperscript{2,5,6,7,23,73}

Cervical mucus is a heterogenous secretion which contains more than 90% water, a large portion of which is bound with the matrix of mucin. Two ovarian hormones regulate secretion and constituents of cervical mucus. Estradiol (estrogens) stimulates mucus production while progesterone (progestogens) inhibits. Therefore, in a menstrual cycle, the amount and consistency of cervical mucus varies with the different phases (follicular, ovulatory, luteal) of the cycle.\textsuperscript{23,48,65,70}

In sperm-mucus interaction studies, the relative ability of different sperm samples passing the cervical mucus are assessed as possible cause(s) of infertility. The purpose of post coital test (PCT) is to determine the number of spermatozoa in cervical mucus, as well as their survival and behavior during the hours after coitus. In a test like PCT, cervical mucus is examined 2 to 8 hours after intercourse. American Society of Reproductive Medicine (ASRM) specifically recommends PCT for males having hyper-viscous semen, low volume semen, and unexplained infertility.\textsuperscript{2,4,5,6,7,23,73}

In *in vitro* cervical mucus penetration assay, a detailed assessment of sperm-cervical mucus interaction may be undertaken. When performing such tests, use of fresh semen no older than one hour post ejaculation is recommended. Mortimer\textsuperscript{56} suggested that semen samples are liquefied 30 minutes post-ejaculation, which is an ideal standard starting time. According to WHO,\textsuperscript{72,73} when there are difficulties in obtaining human cervical mucus, bovine estrus mucus provides a suitable alternative. One of the advantages of the in vitro interaction assay is that it allows detection of possible presence of antisperm antibody in either semen or cervical mucus. It should be pointed out that since antisperm antibodies in cervical mucus are locally secreted they may not be easily detected by analyzing female serum. When a positive antisperm antibody test is obtained using husband’s semen and wife’s mucus, a crossover test using donor semen and donor mucus is required to confirm whether the antibodies are in semen or in cervical mucus.\textsuperscript{30,58,72}

Cervical mucus is collected from excervical and endocervical canal. It can be aspirated with a tuberculin syringe (of course without needle), pipette, polyethylene tube, catheter or specially designed forceps. It is preferable to conduct the test with fresh mucus. Cervical mucus can be preserved in a refrigerator for a period of up to five days but the issue of dehydration should be taken into consideration. Use of frozen-thawed mucus is highly discouraged.

In evaluating cervical environment, mucus is assessed for its volume, consistency, ferning, spinnbarkeit, cellularity and pH. A grading system of mucus quality was first proposed by Insler et al in 1972\textsuperscript{72,73} considering five
parameters. Later on mucus cellularity was added to grading system.

**Sperm-Zona Pellucida Binding and Penetration**

Once spermatozoa manage to reach the vicinity of oocyte, they need to have ability to successfully interact with zona pellucida of oocyte so that they can penetrate into ooplasm for forming pronucleus. This particular aspect of the spermatozoa’s potential is evaluated by studying sperm-zona pellucida binding and penetration. Binding of sperm to zona pellucida leads to initiation of acrosome reaction, release of lytic acrosomal components and penetration through the zona matrix. Sperm fuses with outer sheath of zona and releases acrosomal enzyme which makes it’s penetration easier through zona pellucida. There are several ways, reported in literature, to assess the ability of sperm penetrating zona. Considering the significance of species specificity issue, direct assessment of interactions between human spermatozoa and human zona pellucida is essential. For sperm-zona binding tests (ZBT), human oocytes from pathological specimen, discarded ovaries or spare oocytes from IVF cycles can be used. However, proper consent and institutional approval must be obtained. Complete failure of binding sperm to the zona of a particular oocyte may indicate abnormality either of sperm or of oocyte.

Incorporation of an adequate control is essential for a valid ZBT. In some ZBT like hemizona assay (HZA), zona is divided into equal halves and exposure of each half is made to equal concentration of test (patient) and control (fertile) sperm. Alternatively, patient and control sperm populations can be labeled with dye (fluorochromes) and then mixed for testing in binding to the same intact zona. In this case, a few or no sperm of patient bound to zona compared to binding of control sperm usually indicates a sperm defect in the patient.

There will always be practical problem of getting fresh human zona pellucida. To circumvent this difficulty, some have come forward with idea of cryopreserving or storing zona in salt solution. Literatures show that zona stored in such ways preserve the functional capacity in binding spermatozoa. Some laboratories may have access to spare uninseminated oocytes (with proper consent and institutional approval) but others may not have such opportunity. To overcome this obstacle, obtaining zona from ovarian tissue either during gynecological surgery or postmortem can be an option but still requires proper approval.

**Sperm Maturity**

The concepts of sperm maturity in fertilization and subsequent developmental consequences have been emphasized by many investigators. Developmentally immature sperm are incompetent in taking part in fertilization though they are able to meet oocyte. Even fertilization by immature sperm occurs; it may result in poor embryonic development, decreased implantation, lower pregnancy rates, and recurrent pregnancy losses. According to a Yale group of investigators, mature and immature sperm are different with respect to morphological and morphometric attributes, creatine kinase, HspA2 level, chromosomal aneuploides, DNA degradation, zona pellucida binding properties and more. Studies show that during spermiogenesis, sperm plasma membrane undergoes a maturation-related transformation which facilitates formation of sites for zona pellucida binding. Studies have further revealed relationship between diminished sperm maturity and chromosomal aneuploides particularly disomies.

Fearing the consequences of using sperm with diminished maturity, methods have been developed to sort out mature sperm. Hyaluronan binding assay (HBA) is one of such method of selecting mature sperm over immature ones. The principle of this assay is that mature, not immature, sperm bind hyaluronan. Thus the mature ones can be separated from others using such a binding assay. Sperm binding hyaluronan are also able to bind zona pellucida as evidenced in hemizona assay. Further, the notion that sperm binding to hyaluronan via plasma membrane HA receptors represents unequivocal evidence of completed spermiogenic maturation.

**Sperm Chromatin**

Sperm that appear morphologically normal and exhibiting progressive motility cannot be taken as granted that they will not carry structural and functional defects in their chromatin. Abnormal as well as normal spermatozoa may have incompletely packed DNA with persistent endogenous nicks, failed replacement of histones by protamines or protamine deficiency. Apparently normal looking sperm that have chromatin deficiencies are incompetent in taking part in fertilization and subsequent development though they are able to meet the oocyte. Therefore, some researchers felt it necessary to evaluate the status of chromatin of sperm in infertile men. In normal fertile sperm, chromatin is a tightly packed structure because of disulfide cross linkages between protamines that allow
compaction of nuclear materials, and thus protection from stress. Defect in chromatin can be multifactorial. There can be protamine deficiency which may affect packaging and compaction during spermiogenesis ultimately resulting in defective chromatin which is unable to protect DNA. Thus DNA becomes vulnerable to multiple stress factors and damage in the form of DNA fragmentation occurs.

Over the past 30 years, various methods have been developed to assess sperm chromatin. Currently, there are five established methods which evaluate the integrity of sperm chromatin. These are sperm chromatin structure assay (SCSA), TUNEL assay, COMET assay, acridine orange test (AOT) and chromatin dispersion test (SCD). SCSA assay utilizes flow cytometry. In SCSA assay, normal intact DNA and fragmented DNA fluoresces green and red, respectively, thus detecting the degree of damage in DNA. TUNEL assay detects both single and double-stranded DNA breaks by labeling the free 3’-OH terminus. COMET assay involves embedding spermatozoa in agarose and evaluating DNA migration in comet tails. AOT is a microscopic procedure based on the same principle as the SCSA which utilizes flow cytometry. SCD test is based on the principle that sperm with fragmented DNA fail to produce characteristic halo that is observed in sperm possessing normal intact undamaged DNA. Whatever test is used, the normality of sperm chromatin and DNA may be essential for some patients to reveal the possible hidden causes of their reproductive failure, and its preventive measure.

Sperm chromosome micro deletion is also of concern. Chromosome micro deletions have been found both in sperm of fertile and infertile subjects, however, its prevalence is high in the infertile population. Commercial kits have been developed to detect chromosome micro deletion in human sperm.

Human sperm assay (HSA) one of the earliest sperm bioassay (SB) has been an integral part of human fertility laboratories for many obvious reasons. First, the assay method is user friendly requiring less technical skill and equipment. Animal models like mouse embryo assay (MEA) and hamster sperm motility assay (HSMAs), the alternate of HSA, may be commercially available but are expensive and labor intensive. Most importantly, when human sperm is used, no species differences have to be taken into account in interpreting and validating the outcome of the test. HSA has therefore been a convenient in house QC test in ART laboratories. Since the first report of successful human IVF, various modifications have been introduced to bring improvements in IVF techniques, and HSA played a role in such improvements. Therefore, HSA was not only used by the IVF pioneers, but it has also maintained a permanent footage in IVF laboratories to this day.

External PT providers for ART laboratories like American Association of Bioanalysts (AAB) and College of American Pathologists (CAP) also take advantage of human sperm. These PT providers developed proficiency tests using human sperm assay.

Cost and labor required for a bioassay are directly related to assay time. Longer the assay duration, more expensive the assay becomes. AAB, the largest PT provider for ART laboratories, particularly in the United States, set 48 hours assay time for HSA in toxicity testing of embryo culture media. In our recent study, we were able to show that in AAB sponsored HSA, what is concluded by 48 hours assay can also be concluded from that of 24 hours. In this study we also justified the optimum assay time. Loss of motility in any culture, which is even completely free of harmful elements (toxicants), is expected to occur as time progresses. This natural phenomenon of sperm motility loss in culture may overshadow real toxicant induced motility loss, producing erroneous results if the assay is extended beyond time that is actually required.

In conventional human sperm bioassay including AAB sponsored assay, changes in motility over time is monitored. In our study we showed that the mode of change in motility grade (motility quality) in adulterated media is uniquely different from that of control media. The onset of difference in motility quality between adulterated and control media can be identified earlier than the complete motility loss as it is logical that any harmful agent will affect motility quality first before motility gets completely lost. Therefore, inclusion of motility quality evaluation in HSA will increase its sensitivity and thus will help in identifying the difference earlier.
Uniquely, human sperm can remain in culture for a lengthy time. However, this should not be the reason for choosing longer assay times. Assay time should always be the minimum time required to detect the difference between control and experimental one. Impact of primary target determinants may be obscured by other unwanted variables if assay is prolonged. Assay time of a bioassay may not be a fixed one since it will vary depending on concentration and nature of toxicant present in the sample, and also depends on assay procedure applied. However, it is important to determine the assay time before the assay is performed.\textsuperscript{1,8,9,17–19,21,29,31–34,41,52–54,61–64,69}

**METHODOLOGIES**

Sperm function tests and bioassays that have been described above have well established protocols published in peer reviewed journals. Many of these protocols have become topics in andrology text books and also in andrology laboratory manuals. Some investigators have considered these as non-routine tests while others call them as research procedures, indicating that these tests are applied as needed. For convenience of the readers, following list of references has been prepared which particularly emphasized the laboratory protocols (methodologies). It is our anticipation that interested readers will have easy access to sperm function test and sperm assay protocols consulting these articles.

Agarwal et al,\textsuperscript{2,3} World Health Organization,\textsuperscript{72,73} The American Society of Andrology,\textsuperscript{48} Jeyendran et al,\textsuperscript{45} Alveraz et al,\textsuperscript{1} Lars et al,\textsuperscript{58} Nieschlag et al,\textsuperscript{40} Bjomdhall et al,\textsuperscript{10} Mortimer,\textsuperscript{55,56} Huszar et al,\textsuperscript{52,43} Chohan et al,\textsuperscript{16} Evenson and Wilson,\textsuperscript{27,28} Carrell et al,\textsuperscript{13,14} Rogers,\textsuperscript{65} Gardner et al,\textsuperscript{32} Nijs et al,\textsuperscript{61} DeJonge et al,\textsuperscript{21} Bavister and Andrews,\textsuperscript{8,9} Van den et al,\textsuperscript{62} Claassens et al,\textsuperscript{17} Critchlow et al,\textsuperscript{19} Miller et al,\textsuperscript{52} Franco et al,\textsuperscript{13} Hossain et al,\textsuperscript{37–41} Morimoto et al,\textsuperscript{54} Quinn et al,\textsuperscript{63} American Association of Bioanalysts,\textsuperscript{5} Hinsch et al,\textsuperscript{15} Monsour et al,\textsuperscript{25} Hong et al,\textsuperscript{36} Jequier,\textsuperscript{44} Lars et al,\textsuperscript{48} Nieschlag et al.\textsuperscript{60}

**IMPACT OF ART ON SPERM FUNCTION TEST AND SPERM BIOASSAY**

There is no doubt that assisted reproductive technology (ART) is shaping the course of fertility treatment modality. It can also be said that assisted reproductive technologies are flourishing to fulfill the growing treatment necessities in conquering infertility. In the era of ART, surgical intervention is hardly applied in infertility treatment. The importance of other conventional treatment approaches is also diminishing. In conventional treatment, diagnosis to identify the cause(s) of infertility is the first preferred approach. Once the cause is identified then attempt to cure that specific cause(s) is applied with the anticipation that the couple can conceive on their own.

However, in the era of ART, conventional approaches treating infertility are rapidly getting abandoned. In modern approach, identification and fixing of fertility obstacles are intentionally bypassed since the goal (pregnancy) can be achieved with the application of ART without fixing the obstacles. As a result, the importance of evaluation of male fertility potential is diminishing and thus diminishing the applications of sperm function tests. If a healthy pregnancy can be achieved by employing in vitro insemination (IVF or ICSI) then why bother diagnosing fertility potential which is costly and also time consuming. With the present proven success of IVF and ICSI, all involved in infertility treatment, patients as well as physicians, are losing interest in evaluating sperm fertility potential using sperm function test. Thus, on one hand, ART gave birth to sperm function tests, and on other hand, the same ART is also restricting its application by introducing new ART techniques. However, all patients may not be able to afford to get the benefit of cutting edge fertility technologies, or do not want to adopt such third party intervention in family building. For this section of patient population, application of sperm function test is still a viable option.\textsuperscript{2,3,12,33,40,67,75,76}

On the other hand, importance of sperm bioassay remains unaffected or in many cases, increased with the advancement of ART. In 1980s when the technology driven fertility treatments first started, success rate of such procedures was disappointing and frustrating compared to today’s success. Today’s higher success rate has been possible by improving the quality of the ART procedures. Sperm bioassay played a crucial role in bringing such quality in ART procedures. Human sperm have been utilized in checking quality of the media and materials used in ART procedures. Sperm bioassay and also other bioassays are thus an essential component of a successful human in vitro fertilization. It can thus be re-emphasized that human sperm bioassay was an integral part of IVF since its inception and remains that way today. As the importance of ART rises, the utilization of sperm bioassay also rises since sperm bioassay is now one of the preferred in-house QC test in ART laboratories worldwide. Thus, for the existing ART and ART developed in the future, the application of sperm bioassay will be there since quality control in ART is a routine task which requires bioassays.\textsuperscript{41,67}
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