Volume 3 (December 2016)

INDIAN FERTILITY SOCIETY

origio

a CooperSurgical Company

NEXUS

Indian Fertility Society & ORIGIO India Initiative

Sperm Function Tests: Demystified

It is a great privilege and pleasure to write this message for the third E-bulletin of IFS-Nexus. The idea of Nexus has been initiated to bridge the gap between ART Clinicians and Embryologists.

Time has finally come to shift the focus to andrology and sperm function test having exhausted majority of our resources in field of embryology and related avenues.

I am sure that the research and scientific management of males will go a long way in improving the fertility outcome at our ART centres.

Amalgamation of urology and andrology is need of the day and needs better deciphering and understanding of the underlying events.

On behalf of the Indian Fertility Society, I sincerely thank “ORIGIO India Private Ltd” for participating with us in this academic endeavor.

Dr. Sohani Verma
President-IFS

This is an honor for me to write best wishes message for this very special Nexus E-bulletin on “Sperm Function test”.

First and second bulletins titled “IUI: Nuts and Bolts” and Semen Analysis: Simplified was appreciated by all and have turned out to be ready reckoner for the readers.

Sperm health is the key word in fertility management and all sincere efforts should be made to simplify the science.

Indian fertility Society feels proud and congratulates the editors on the launch of third edition of Nexus e-Bulletin. It would not only help to disseminate scientific & ethical content but also constantly update everyone with new researches and developments across the world.

I wish the editorial team best of luck in this endeavour

Dr. K.D. Nayar
Secretary General-IFS

At the very onset the editorial team would like to thank all of you for positively appreciating 1st and 2nd E-bulletins of Nexus. Team 'Nexus' sincerely hopes to bring out such teaching material for you regularly. The bulletin has been named NEXUS - which means building bridges.

Such bulletins are call of the day and enormously bridge the gap between the existing knowledge and recent advances. Our present edition is focused on simplifying the process of semen function test and covers all essential details with nice algorithms. Part 1 deal with commonly asked questions and part 2 with bench side work. I am sure it will immensely benefit you all.

ART services require composite communication between the clinicians, embryologist and technicians for smooth functioning of the ART center. Ill-advisedly at the present-day moment we don't have many qualified reproductive laboratory support staff and our young clinicians are also new to this branch having setup the centres with sheer enthusiasm without much of formal training.

Our motto is "knowledge empowers" and we sincerely hope that you would enjoy reading this write up. Feel free to communicate with us at any point of time and contribute critically. Your comments would be published in the next bulletin, which is titled "Semen cryopreservation".

We would also like to place on record our truthful thanks to Origio India limited who are helping us in publication of this bulletin and off course I promise that there is no conflict of interest at any level.

Wish you happy reading and yes don't forget to file this issue.

I would formally like to thank my friend Dr Ashish Fauzdar PhD, Scientist I (Embryology) at ESI-PGIMSR Hospital, Basaidarapur, New Delhi. He has un-relentlessly worked towards bringing out this issue from conception to end.

Prof (Dr) Pankaj Talwar
Joint Secretary-IFS
Editor NEXUS

Nexus Vol: 3, Part-1

Sperm Function Tests (SFTs): Demystified(Concepts)

1. What is the importance of sperm function tests in infertility treatment?

The basic semen analysis has limited predictive value for pregnancy, in couples trying to achieve through natural conception and in couples undergoing advanced Assisted Reproductive Technologies (ART).

It is often realized that there is need for more extended sperm functional testing that helps in better understanding of sperm dysfunctions concealed during routine semen analysis. Ideally, the sequential analysis of sperm functions could assist clinicians in planning the therapeutic approach and predicting the outcomes of treatments such as Intra uterine insemination (IUI), In-vitro fertilization (IVF) and intra cytoplasmic sperm injection (ICSI).

Many couples without severe sperm defects can be treated by less invasive and in-expensive options provided the sperm function tests ensure normal fertilization without the use of ICSI.

2. What are the main indications for performing advanced sperm function test?

The patient with normal semen parameters may not be requiring any specialized tests. But in the cases of fertilization failures, sample with low sperm count and poor sperm motility may require sperm function tests to evaluate different semen parameters.

Sperm function tests provide useful insights to deduce the exact reason for fertilization failure and also guide infertility experts to adopt the best possible individualized treatment based on sperm function assay results. With absolute normal semen analysis parameters it may not be necessary to advise any specialized tests to the male. But in many cases of borderline parameters it becomes obligatory to do a battery of sperm function tests to evaluate different semen parameters.

These tests detect function of a certain part of spermatozoan and give insight on the events in fertilization of the oocyte. It is arduous to depend on a single group of tests for predicting fertility outcome as the fertility is dependent upon on sum total of all the functional parameters of the sperm and reliance on any one of them will be inappropriate.

3. What are the crucial steps during sperm-oocyte interaction?

The steps leading to sperm maturation and fertilization occur during passage of spermatozoa in the female genital tract. These include capacitation in the cervical mucus followed by **acrosome reaction, sperm zona pellucida interaction, sperm-oocyte membrane fusion, decondensation of sperm nucleus and finally fusion of male and female pronucleus.**

4. Define Capacitation?

Capacitation can be defined as time dependent, reversible changes that sperm undergo especially in the female genital tract, which lead to the ability of sperm to undergo acrosome reaction (AR). These changes include loss of extrinsic proteins, including acrosome stabilizing factors along with the loss of membrane cholesterol. These intricate steps results in sperms having more fluid and pliable membranes.

During capacitation sperm motility changes to the characteristic pattern, and induction of AR becomes possible. The rapid spiraling pattern of the sperm movement and subsequently hyperactivated sperm motility is commonly associated with sperm capacitation. Computer assisted semen analysis allows objective and repeatable quantification of sperm motility associated with sperm capacitation.

5. Elaborate on the importance of Acrosome reaction?

Sperm binding to the zona pellucida (ZP) triggers the release of hydrolyzing enzymes and is known as Acrosome Reaction (AR).

The acrosome reaction is an exocytotic process of spermatozoa and a prerequisite for fertilization. Only acrosome-reacted spermatozoa are able to pass through the ZP, bind the oocyte plasma membrane, and fuse with the oocyte. The physiological acrosome reaction occurs at the ZP after sperm binding.

The ZP-induced acrosome reaction can be assessed on spermatozoa removed from the surface of the ZP or exposed to disaggregated human ZP proteins.

These tests are limited by the restricted availability of human ZP's. Acrosomal status after induction of the acrosome reaction can be assessed by **microscopy, flow cytometry, or fluorescently labeled lectins**. The zona-induced acrosome reaction (ZIAR) is calculated as the difference between the ZIAR (stimulated) and the spontaneous (unstimulated) acrosome reaction results.

A clinical cutoff value of 15% is reported for the difference between ZIAR and spontaneous acrosome reaction. It is postulated that the failure to pass zona-binding assays, cases with poor acrosome reactivity to solubilized ZP should be referred for ICSI.

6. What is the role of Sperm penetration Assay (SPA) as sperm function test in predicting infertility?

This test was one of the first bioassays of sperm function that got developed. In this heterologous system, human sperm were subjected to capacitating conditions and incubated with hamster oocytes devoid of the zona pellucida enzymatically. The sperm penetration assay with zona-free hamster ova was widely used in the pre-ICSI days.

The SPA measures the spermatozoa's ability to undergo capacitation, acrosome reaction, fusion and penetration through the oolemma, and decondensation within the cytoplasm of hamster oocytes and was used to evaluate male fertility potential.

The conventional SPA depends on the occurrence of spontaneous acrosome reactions in populations of spermatozoa incubated for prolonged periods in vitro. The fusion of human spermatozoa to the hamster oocyte is functionally the same as that with the human vitelline membrane, since it is initiated by the plasma membrane overlying the equatorial segment of acrosome-reacted human spermatozoa.

As a prognosticator of IVF failure, the sensitivity varied from 0.00 to 0.78 and specificity ranged from 0.51 to 1.00 for diagnosing male factor infertility (Mao.*et.al*, 1988). The reproducibility of this assay and standardization of methods between laboratories is low as this procedure is less efficient and the biological process may involve different mechanism.

The studies have shown a poor clinical value of the SPA as a predictor of fertilization with good sensitivity but very high false-positive rates. There are concerns over the the validity and reproducibility of the SPA. This assay is considered as heterologous, time-consuming, and relatively expensive test should probably not be used to evaluate fertility potential (Oehniger S *et.al*, 2000.

7. What is the role of Sperm-zona pellucida interaction and discuss available methods for detection?

The zona plays a major role in controlling fertilization and it is the most potent and only physiological inducer of the acrosome reaction. To fertilize a human oocyte, sperm must recognize and tightly bind specific receptors on the zona, which are species specific. Zona binding and subsequent acrosome reaction induction is the precursor for final fertilization. Abnormal sperm zona pellucida interaction may be the major dysfunctional step in preventing fertilization.

IVF treatment and observation of IVF oocytes can give insight into the zona binding of the sperm. Two commonly available zona binding assays are **hemizona assay** and a **competitive intact zona binding assay**.

8. Elaborate on the available sperm-ZP binding assays?

The interaction between spermatozoa and the ZP is a critical event leading to fertilization and reflects multiple sperm functions (i.e., completion of capacitation as manifested by the ability to bind to the ZP and to undergo ligand-induced acrosome reaction).

The two most commonly used sperm–ZP binding tests are :

- **Hemizona assay (HZA)**
- **Competitive zona sperm binding**

Although both assays have different methodologies but they both use assessment of tight binding of sperm to the ZP as the primary endpoint. In an independent comparison in an internally controlled assay have demonstrated high predictive value for the outcome of fertilization in vitro.

The HZA is an internally controlled bioassay that evaluates the ZP binding potential of a sperm population. The HZA provides a functional homologous test for sperm binding to the ZP during which populations of fertile and infertile spermatozoa are compared within the same assay. The assay uses matching halves of a human ZP from an oocyte with no developmental potential (salt-stored or cadaveric).

The HZA is indicated in cases where repeated poor or no fertilization is recorded during IVF therapy or in the presence of moderate-severe

oligoasthenoteratozoospermia to determine clinical management. Furthermore, it has been demonstrated that oligozoospermic men have a very high frequency of defective sperm-ZP interaction. This is examined by sperm-zona binding assays and the ZP-induced acrosome reaction consistent with their low natural fertility or low fertilization rate in conventional IVF.

In the HZA, sperm from fertile men are used as a control and typically exhibit significantly higher binding capacity to hemizonae compared with sperm from infertile patients. Prospective clinical studies reported a cutoff Hemizona index (HZI) value of 35% as predictive of IVF outcome.

Of importance, reported clinical results for IUI therapy showed that an HZI <30 was associated with a significantly lower pregnancy rate compared with patients with HZI >30.

Consequently, results of this sperm function test are useful in counseling couples before allocating them into alternative therapeutic methods, that is, IUI versus ICSI. Patients with poor sperm-zona binding results should be referred to the ICSI laboratory.

This is especially true in developing countries where medical insurance and the financial restrictions of the patients are important.

9. What are the common available methods for detection of acrosome reaction?

A population of capacitated sperm can be stimulated for acrosome reaction and release acrosin in the culture medium.

Individual sperm that have their acrosome reacted may be detected by variety of methods including:

- Labeling with fluorescent lectins
- Monoclonal antibodies to specific proteins
- Histochemical staining binding with antibody-bound beads.
- Flow cytometry using specific fluorochrome staining.

a. *Acrosomal assay*

The sperm acrosome is located on the top of the sperm head. During acrosome reaction, the outer acrosomal membrane and the plasma membrane fuse and vesiculate to discharge the acrosomal contents.

One of the most important sperm membrane property related to maturation is ability to bind to hyaluronic acid. The study of hyaluronic acid bound sperm reveals that the sperms exhibit uniform morphology as well as nuclear maturation indicated by aniline blue staining.

Hyaluronic acid receptors on sperm head are important for inducing specific capacitation motility pattern as well as for zona binding. In vitro binding test is a very good indicator of sperm membrane receptor and is performed by binding to hyaluronic acid immobilized on plastic of glass surfaces or through commercially available HBA slides.

b. *Gelatin Assay*

Multiple enzymes are present in the sperm acrosome including several acid hydrolases commonly found in lysosomes. Acrosomal enzymes help the sperm to penetrate the cumulus mass and the zona pellucida. Gelatin film lysis is one such method, which detects acrosome proteolytic activity in terms of gelatin digestion in the area surrounding the sperm head.
The precoated gelatin film on the glass slide is lysed coming in contact with proteolytic enzymes leaching out of acrosome and allow light to pass through creating a halo around the sperm head which can be examined using a microscope. The sperm heads showing no halos are considered negative for acrosomal status and function.

10: What are the essential steps of fertilization during Sperm-oocyte interaction steps?

After sperm achieving zona penetration, the acromose-reacted and acrosome-spent sperm bind and fuse with the plasma membrane of the oocyte. Specific receptors are involved in this process. The fusion process initiates the release of the oocyte cortical granules, which result in alteration of zona to prevent further sperm binding and penetration.

Under the oocytes control the sperm is drawn into ooplasm where its nucleus decondenses and forms male pronucleus. Sperm-oocyte interaction through sperm penetration assay and IVF are difficult to interpret as they involve 2 living cells. It also depend on the sequence of events in these assays as only final step can be observed. If sperm penetration test and fertilization is successful, then it is evident that at least one sperm has performed all its required functions.

11. What are the available methods for detecting Sperm vitality (alive sperms)?

Sperm vitality should be determined in semen samples with ≤40% progressive motile spermatozoa. The sperm vitality testing will reveal the proportion of spermatozoa that are “alive.” The percentage of live spermatozoa is assessed by identifying sperms with an intact cell membrane, from dye exclusion or by hypotonic swelling sperm function test.

a The dye exclusion method: It is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-permeant stains with loss of osmo regulation which does not allow the dye to difuse out.

b Hypo-osmotic swelling (HOS) test: This presumes test that only sperms with intact membranes (live cells) will swell in hypotonic solutions.

12. Describe in details dye exclusion method for evaluating Sperm vitality staining and its clinical relevance?

Sperm membrane integrity (structural & functional) may compromise sperm fertilizing capacity (by affecting motility, capacitation, acrosome reaction & binding of sperm on Zona Pellucida).

The sperm vitality is reflected in the proportion of spermatozoa that are "alive." It is measured by assessing the ability of sperm plasma membrane to exclude extra-cellular substances like dyes. Sperm vitality should be determined in semen samples with <50% motile spermatozoa.

Vitality assessment also provides check on the accuracy of motility assessments; as the percentage of live spermatozoa should slightly exceed the total percentage of motile spermatozoa.

- **Plain eosin staining:** Assess vitality in wet smears for quick assessment at the same time of count and motility assessment.

- **Eosin-nigrosin staining:** is also used for assessing vitality by providing dark background, which makes it easier to assess the slides.

Spermatozoa that are white (unstained) are counted as live, and those showing any degree of pink or red are dead. Spermatozoa stained with this kit cannot be used for any further procedures.

The technique is based on the principle that dead cells will take up the eosin, and as a result stain pink. Eosin-nigrosin smear when observed in brightfield will reveal spermatozoan red or dark pink, dead (membrane damaged), whereas spermatozoa with white head or light pink heads are considered alive (membrane-intact).

The assessment can be carried out at any time and slides also can be preserved for future assessment and record. It's important to differentiate between live spermatozoa from dead spermatozoa for use in ICSI (low viability samples) through HOS test. ***(for more information on protocols for performing test refer to section P-II.1 (a-e) of Part-II).***

13. Describe Hypo-osmotic swelling (HOS) test and its clinical relevance?

Water permeability is an important physiological property of all cell membranes and spermatozoa's are no exception. Membranes allow selective transport of fluids and molecules through it.

Sperm membrane plays an important functional role during fertilization process as can be evaluated by the HOS test (HOST). Hypo-osmotic swelling test is based on the ability of live spermatozoa to withstand moderate hypo-osmotic stress with use of hypo-osmolar reagent. With moderate hypo-osmotic stress membranes swell and reach steady state where fluid passing into the cells and that pumped out by intact functional membrane equal quantity. The cells will swell to varying degrees at this stage but will not burst open. Dead spermatozoa whose membranes are no longer intact do not swell in hypotonic media.

The clinical value of HOST is being constantly evaluated in the literature. HOS reacted sperm can be semi-quantitatively and subjectively graded as grade A to G based on the amount of swelling and curling of the tails. Percentage of each grade can be scored and reported as a percentage after counting 200 sperms.

More than 60% HOS reacted sperms are considered as normal and abnormal if <50% show tail curling. Scores between 50% and 60% are considered intermediate. HOS can be generally used as an additional indicator of sperm vitality and can be used to diagnose spermatozoa with immotile cilia syndrome.

Presently with the correlation with HOS grading and DNA fragmentation status HOS remains only reliable indicator for selection of spermatozoon for selection in ICSI. ***(for more information on protocols for performing test refer to section P-II.2(a-d) of Part II).***

14. What are the indications for doing sperm morphology staining?

Human semen samples contain spermatozoa with different kinds of malformations. Defective spermatogenesis and some epididymal pathologies are commonly associated with an increased percentage of spermatozoa with abnormal shapes. Abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA.

Morphological defects have also been associated with increased DNA fragmentation, an increased incidence of structural chromosomal aberrations, immature chromatin and aneuploidy. The morphology staining

can be ideally done in all cases through commercially available ready to use kits including pre-stained slides and reagents.

During the evaluation emphasis is given to the form of the head, although the sperm tail (midpiece and principal piece) is also considered.

Sperm morphology is considered abnormal when normal morphological sperms are less than defined lower reference limits of ≤4% (5th centiles at 95% CI) as per WHO manual 5th edition.

15. What are the criteria for assessment of sperm morphology?

Variations of sperm morphology make an assessment of morphology difficult. By strict application of certain criteria of sperm morphology, relationship between the percentage of normal forms and various fertility pointers have been established The classification system evolved by Kruger et al. and subsequently accepted by WHO give very low threshold for accepting normal morphology and very few samples show more than 25% normal spermatozoa with most in the range of 4–30% in fertile population. Patients with <4% normal forms had a low fertilization rate of 7.6%. Those with normal morphology between 4% and 14% had a considerably enhanced fertilization rate of 63.9% (P < 0.0001). Spermatozoa with >14% normal forms fertilized within the normal range for the laboratory.

According to strict accepted criteria considering normal spermatozoan are:

- **Sperm head** is considered to be 3–5 µ in length and 2–3 µ in width with perfect oval shape.
- **Mid-piece** is about 1 µ in diameter with straight and regular outline and aligned to the longitudinal axis of the head with 7–8 µ in length.
- **The tail** is slender uncoiled and at least 45 µ in length.
- **Cytoplasmic droplet** is a tiny portion that is usually retained in the sperm flagellum. CDs have a role in motility development during sperm epidymal maturation.

Any sperm not meeting these criteria is considered abnormal in strict criteria. The following categories of Sperm defects should be noted for evaluation of sperm morphology:

- **Head defects:** Large or small, tapered, pyriform, round, amorphous, vacuolated (more than two vacuoles or >20% of the head area occupied by unstained vacuolar areas), vacuoles in the post-acrosomal region, small or large acrosomal areas (<40% or >70% of the head area), double heads, or any combination.
- **Neck and midpiece defects:** Asymmetrical insertion of the midpiece into the head, thick or irregular, sharply bent, abnormally thin, or any combination.
- **Principal piece defects:** Short, multiple, broken, smooth hairpin bends, sharply angulated bends, of irregular width, coiled, or any combination of these.
- **Excess residual cytoplasm (ERC):** It is associated with abnormal spermatozoa produced from a defective spermatogenic process. Spermatozoa characterized by large amounts of irregular stained cytoplasm, one third or more of the sperm head size, often associated with defective midpieces are abnormal.

The morphology is evaluated by using air dried smears prepared from whole semen on glass slides and stained using **Diff-Quick** or **PAP Method**. Both give satisfactory results. Preferable 200 spermatozoan are counted for various abnormalities and only those showing all parts normal are considered normal.

There are various commercially available sperm morphology stain kit that are rapid aid in evaluating sperm morphology. These help distinguish the different parts of the sperm cell (head, acrosome, equatorial region, midpiece, and tail) making it easier to differentiate between a normal and abnormal spermatozoa ***(for more information on protocols for performing test refer to section P-II.3 (a-f). of Part II).***

16. What is the relevance of Teratozoo-spermia index (TZI)?

Teratozoospermia index (TZI) is a predictor of fertilization potential. It is a multiple anomalies index where a total number of 100 sperms are counted. This is done by recording the number of normal and abnormal sperms. In this each abnormal sperm is seen for a maximal of 4 abnormalities. Total number of all separate abnormalities is then divided by total number of abnormal spermatozoa counted, which gives the TZI. The normal TZI is <1.6. If TZI is >1.8 ICSI is done irrespective of the count and motility.

17. What is the role of pH in sperm function test?

pH is defined as hydrogen ion concentration (reciprocal logarithmic expression of Hydrogen ion concentration) as a measure of alkalinity (pH > 7.0) & acidity (pH < 7.0).

Semen pH is primarily determined by the ratio between seminal vesicle alkaline secretion (pH 8.2 -8.6) & prostate acid secretion (pH 6.8 - 7.2). Therefore, semen pH is slightly alkaline (pH 7.6 - 8.6). pH is also time dependent from the moment of collection. According to

WHO laboratory Manual (2010), a reference value for semen pH is 7.2 or more; however for clinical purpose to facilitate interpretation & diagnosis, semen pH of <7.6 or >8.6 is considered abnormal.

The Abnormalities in semen pH may be due to various clinical or procedural factors: If the semen volume is low accompanied by higher pH (above 9.0) is often due to the pathology of the prostate gland (acute prostatitis, vaculities, bilateral epididymitis) whereas in case of chronic prostatitis, pH is generally less than 7.2. Low semen volume accompanied by low pH (below 7.2) is often due to a deficiency in seminal vesicle fluid.

The change is pH could be due to procedural factors with initial fraction loss during semen collection may result into higher pH (above 8.6) or due to incubation of semen for a long time, results in high pH (above 8.6) due to breakdown of amines & amides. The pH of seminal fluid is best measured using litmus paper with a pH range that lies between 6-10. The use of pH meters in pH measurement of semen is not recommended. ***(for more information on procedural steps for performing test refer to section P-II.4 (a-f). of Part II).***

18. What is the importance of evaluation of semen viscosity?

After ejaculation, semen normally coagulates into a gelatinous mass & then liquefies within 60 min at room temperature or 37°C. Proteins originating from seminal vesicle cause coagulation while proteases originating from prostate cause the coagulum to liquefy. Viscosity measures friction between various seminal fluid components as they slide by one another.

Usually viscosity & liquefaction time go hand in hand. i.e. if liquefaction time is more, then viscosity is increased. High viscosity combined with poor sperm motility can lead to a marked decrease in fertilization capacity due to problems with delivery i.e. the poor or total absence of sperm release into the cervical mucus. Therefore, its viscosity must be made normal before performing semen analysis & sperm function tests.

Viscosity can be evaluated by performing-

- **String Test**
- **Modified Pipette Method**

Viscosity is measured in centemeters (cm) by the length of 'Spinnbarkeit' or 'Threadiness'. Highly viscous sample should be treated with **Chymotrypsin** or **Bromelain**. Both treatments do not affect the sperm function tests ***(for more information on procedural steps for performing test refer to section P-II.5 (a-e) of Part II).***

19. What is the role of Hyaluronic Binding Assay (HBA) in Semen Analysis?

Hyaluronic acid binding by human spermatozoa indicates cellular maturity, viability, and spermatozoa with intact acrosomes. Only mature, motile spermatozoa bind to hyaluronan through specific receptors. This ability to bind to hyaluronan is not present in immature spermatozoa.

Furthermore, hyaluronan binding assay (HBA) represents a more convenient and reproducible laboratory test for identifying correct patient for ICSI. HBA results may assist clinicians in the therapeutic approach, that it, in assigning patients for either IVF or ICSI treatment (Cayli S *et.al*, 2004).

The use of hyaluronan-bound spermatozoa for an ICSI procedure has the advantage that mature spermatozoa, with high DNA integrity and low frequency of chromosomal aneuploidies, will be selected for injection. The HBA scores are also significantly associated with the fertilization rates and biochemical pregnancies. No correlation was recorded between HBA and the standard semen parameters.
Huszar *et. al,* described another sperm maturation marker has been identified as a testis-expressed protein HspA2. The HspA2 acts as a hyaluronic acid (HA) receptor during normal fertilization. It has been shown that HA-bound spermatozoa have increased developmental maturity, including enhanced chromatin integrity, normal morphology, and increased functional potential. Furthermore HA-bound sperm showed decreased aneuploidy and decreased active caspase-3.

The sperm-Hyaluronan Binding Assay is designed to provide a qualitative assessment of sperm quality, maturity, and fertilizing potential. Hyaluronan (Hyaluronic acid, HA) is a naturally occurring substance and is the major component of the cumulus oophorus matrix surrounding the human oocyte. The fully mature sperm that have completed spermatogenesis have developed receptors for HA. Immature sperm do not have developed HA receptors and do not bind. They express higher rates of DNA fragmentation, higher rates of aneuploidy, and lowered cytoplasmic maturity but can still show normal motility and morphology. HBA ® slide will distinguish between mature sperm that express hyaluronan receptors and those that so not express HA receptors (Yaga *et.al*, 2010).

Assessing the proportion of sperm with receptors can then be used to decide which treatment is best for your patient. The mature spermatozoa that have developed receptors for HA can bind and viewed in the microscope. The bound sperms are differentiated from unbound sperm by their beating tails with heads and make no progressive movement. (Normal value ≥ 65%).

20. What is 'PICSI' and its advantages?

"Physiologic" ICSI (PICSI): PICSI is performed in PICSI® dish that have hyaluronon coated wells. The hyaluronon binds with mature sperms with high DNA integrity. These bound sperms with continuous flickering of the tails are picked for PICISI during ICISI. There are studies that have found combination of the diagnostic abilities of the HBA® slide and the HA sperm selection abilities of the PICSI® dish has improved clinical pregnancy rate and significantly reduced pregnancy loss rate in ICSI patients diagnosed with low HBA® scores (≤65% binding ratio).

21. What is the role of Reactive Oxygen Species (ROS) as sperm function test?

Production of ROS is of concern because of potential pathological effects on spermatozoa like any other cell that constantly require O_2 for metabolism and produce deleterious metabolites such as ROS that can modify cell function and/or damage to endanger sperm survival. Spermatozoa are particularly susceptible to ROS-induced damage compared to other cells as they have relatively large quantities of polyunsaturated fatty acids in the membrane, and their cytoplasm contains a low concentration of scavenging enzymes.

ROS can be estimated in whole ejaculate using various **chemiluminance methods** or by **semi-quantitative assays** using oxidative indicators.

22. What is the importance of sperm DNA fragmentation?

The integrity of paternal genome delivered by the spermatozoon is of paramount importance in the initiation of viable pregnancy. The fragmented DNA delivered by the sperm is incompatible with normal embryonic development. DNA damage can occur due to apoptosis during spermatogenesis, DNA strand breaks during the remodeling of sperm chromatin during spermiogenesis, DNA fragmentation induced by ROS in the genital tract, or due to environmental toxins. Post testicular damage during passage through epididymis appears to play a major role in causing sperm DNA fragmentation.

The detection of proportion of sperms showing damage is important but few sperms, which show normal DNA may be sufficient for good pregnancy development. Predictive value is always uncertain and cannot have 100% negative predictive value.

Sperm chromatin and DNA tests measure defects in nuclear chromatin compaction and damage to DNA respectively though commercially available kits. The evaluation of sperm chromatin and DNA structure was initially undertaken to improve our understanding of spermatogenesis, sperm physiology, sensitivity to reproductive toxicants and reproductive biology. More recently, sperm chromatin and DNA tests have been used in the evaluation of the infertile man in the hope that these tests may provide a more accurate diagnosis than standard sperm parameters alone.

The conventional sperm parameters include sperm concentration, motility and morphology; they show a high degree of biological variability and are only fair measures of fertility potential. Sperm chromatin and DNA integrity tests have also been studied in the context of Assisted Reproductive Technologies (ARTs) to assess their ability to predict pregnancy outcome after assisted reproduction because conventional sperm parameters are poor predictors of ART outcomes.

The aetiology of human sperm DNA damage is probably multifactorial and may be caused by:

- **Primary or intrinsic defects**: Genetic or developmental abnormalities.
- **Secondary or extrinsic factors:** Causing testicular or post-testicular injury, gonadotoxins, hyperthermia, oxidants, endocrine abnormalities.

It has been suggested that protamine deficiency (with consequent aberrant chromatin remodeling), reactive oxygen species and abortive apoptosis may be responsible for sperm DNA damage.

The sperm DNA fragmentation assays have help in timely intervention from IUI to IVF to ICSI. There is lesser impact of sperm DNA damage has on early fertility checkpoints. In IVF and ICSI pregnancy loss, DNA damage has a moderate positive effect.

23. What are the indications for doing DNA fragmentation test?

The main indication for doing DNA fragmentation includes:

- Unexplained or persistent infertility
- Failure to conceive after 5-6 intrauterine insemination (IUI) cycles
- Low fertilization rates
- Poor embryo quality in IVF cycles
- Implantation failure after IVF
- Recurrent miscarriage
- Prolonged stay in an environment that exposes to reproductive toxins
- Abnormal semen analysis
- Advancing male age (>45 years)

24. What are the available methodologies for detecting sperm DNA fragmentation?

In the recent years, there has been a developing global interests among the reproductive biologist that sperm nuclear integrity and can be assessed using:

- Sperm Chromatin Structure Assay (SCSA)
- Sperm Chromatin Dispersion (SCD)
- Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-labelling (TUNEL)
- Comet Assay

a. *Sperm Chromatin Structure Assay (SCSA)*

This test is based on the differential staining of chromatin single and double strand breaks by acridine orange, SCSA allows the measurement of both the extent of sperm DNA fragmentation and sperm chromatin nuclear protein alterations, such as a lack of protamination. Typically, frozen sperm samples are thawed and exposed to a 30 seconds low-pH acid denaturation, immediately exposed to acridine orange and processed for flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA).

The SCSA uses acridine orange staining to label the double stranded DNA with green and the single stranded DNA with red. The proportion of these two emissions, with a previous acid-denaturing step, has widely been demonstrated to determine the percentage of DNA fragmentation. The percentage of spermatozoa with DNA fragmentation shows increased red fluorescence, unlike the non-fragmented population that shows a normal level of red fluorescence.

Moreover, SCSA provides also an additional parameter named high DNA stainability (HDS). This parameter is a measure of the percentage of immature spermatozoa within the semen sample, which can also be taken into account on the male infertility assessment. The reported values for SCSA threshold vary from 20 to 30% of SDF for predicting male infertility.

b. *Sperm Chromatin Dispersion (SCD)*

Sperm Chromatin Dispersion (SCD) test is based on their detection of sperm DNA fragmentation (SDF) on the denaturing capacity of the sperm chromatin. The integrity of sperm DNA is being recognized as an important factor for successful reproductive outcomes. Though several techniques exists to detect sperm DNA fragmentation, but SCD is a simple, reliable and reproducible technique.

The SCD test can be performed using the Halosperm kit (Halotech DNA; Madrid, Spain) or locally available kit (Cryo-Lab International, Chennai, India) following the manufacturer's instructions. Using SCD test DNA fragmentation can be accurately measure using conventional brightfeild microscopy.

The SCD test assesses the capacity of the sperm chromatin to form dispersion halos, and allows differentiating the non-fragmented spermatozoa (with halo) from the fragmented spermatozoa (without halo). Samples are stained with propidium iodide and 200 spermatozoa were assessed and classified as fragmented or nonfragmented spermatozoa using a fluorescence microscope (Olympus AX70, Olympus Optical Co., Hamburg, Germany) or brightfeild microscope respectively depending upon the protocol. The cut-off value of ~23% is described in the literature for predicting male infertility through SCD test. ***(for more information on protocols for performing test refer to section P-II.9 (a-f). of Part II).***

c. *Comet Assay*

The COMET assay (single-cell gel electrophoresis) is a simple method to assess sperm DNA integrity. Briefly, a mix of spermatozoa and low melting point agarose is spread on a two-well slide, and submitted to various treatments meant to induce DNA unwinding. The cells undergo an electrophoresis and, using a fluorescent dye. The DNA forms a structure resembling a comet; the head consists of intact DNA and the tail is made of broken DNA or strands with heterogeneous molecular weights. The intensity of the comet representing the proportion of DNA that has been broken off and the distance travelled by the comet, the relative sizes of those pieces of DNA.

The alkaline COMET assay gives a comprehensive measure of DNA damage as it reveals multiple DNA damage subtypes (i.e. single and double DNA strand breaks, and, at higher pH conditions, alkali-labile sites). The COMET assay involves the collection of data at the level of the single cell; therefore, very few cells are needed to complete the procedure, allowing the use of samples from men having very low sperm count. the alkaline The studies with Comet assay have showed a threshold value of 45.37% of DNA fragmentation for predicting male infertility (Ribas-Maynou *et.al.,* 2013).

d. *TUNEL*

The TUNEL (Terminal Deoxynucleotidyl Transferase-mediated dUTP nick end-labelling) assay is a common method to detect DNA breaks resulting from apoptotic cascades. Although spermatozoa cannot undergo apoptosis per se, as they do not have the machinery for new protein synthesis, an essential element of the apoptotic. This assay does accurately identify open 3′-OH ends in DNA. The assay relies on the incorporation and detection of fluorescent UTPs at both blunt and single 3′-OH ends.

The immunohistochemistry or flow cytometry are commonly used for the TUNEL assay but TUNEL assay had shown greater accuracy with FACS (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA), as more cells can be assayed. In this assay frozen sperm

samples are thawed, fixed, permeabilized and stained before being processed by flow cytometry. The TUNEL assay is considered a direct approach, because of the absence of cell lysis and DNA denaturation steps. This feature may impede its effectiveness because of limited access to 3′-OH nicks by highly compacted sperm DNA.

The commonly available commercial kit for TUNEL is Roche Applied Science In Situ Cell Death Detection Kit. The TUNEL assay showed a threshold value for male infertility of 20.05% for SDF (Ribas-Maynou *et.al.,* 2013).

25. What is the importance of nuclear chromatin de-condensation?

Sperm nucleus volume is 1/30th of the eukaryotic cell in spite of having just half the DNA quantity. This reduction of volume and orderly packaging of DNA requires very novel methods. DNA, which is packaged properly, can be studied by decondensation in vitro, induced by ethylenediaminetetraacetic acid (EDTA), glutathione (present in oocyte) or DTT.

Sperms with damaged DNA or not properly packaged DNA do not show full DNA decondensation. DNA breaks detected by the test may be in an insignificant area like exons or introns of the chromosome with no consequence on pregnancy development. The chromatin in spermatozoa is in a highly condensed state prior to fertilization.

The appropriate nuclear chromatin de-condensation and subsequent male pronucleus formation is essential for fertilization and normal zygote development. Highly condensed nuclear chromatin in spermatozoa is because of the formation of S-S cross links between its histone units. The cleavage of S-S link can be induced in vitro by sodium dodecyl sulfate and EDTA. Such induced decondensation is a predictor of the good fertilizing ability of spermatozoa.

The result of the test is expressed as the percent swollen cells or NCD reacted cells to total sperms. More than 70% sperms in normal samples show NCD positivity.

26. What is the role of Nuclear Protein Assay in sperm function test?

Mammalian sperm & DNA is the most tightly compacted eukaryotic DNA which is in sharp contrast to DNA structure in somatic cells nuclei. Compaction & organization help protect sperm chromatin during transport through the male & female reproductive tract. This also ensures delivery of the paternal genome in a form that allows developing embryo to accurately express genetic information.

Somatic cell nuclear DNA is wrapped around an octomer of histones & packaged in to solenoid structure. This type of packaging adds histones which increase the chromatin volume. During spermiogenesis, sperm chromatin undergoes a series of modifications in which histones are lost & replaced by protamines.

Protamines are approximately half the size of histones. They are highly basic sperm-specific nuclear proteins that are characterized by an arginine-rich core & cysteine residues. Protamines condense the DNA strands & form the basic packing unit of sperm chromatin called a 'Toroid'. Toroid structure confer a higher order of DNA packaging in sperm than that found in somatic cells. Humans express equal quantities of two protamines, protamine 1 & protamine 2. The mean P1/P2 ratio is approximately 1.
In human chromatin, ~85% of the histones are replaced by protamines. Approximately 15% of the histones are retained subsequently making chromatin less tightly compacted. During spermatogensis, first, somatic histones are replaced by testis-specific histone variants, which are replaced by transition proteins (Tp1a & Tp2) in a process involving extensive DNA rearrangement & remodeling.

During the elongating spermatid stage, the transition proteins are replaced in the condensing chromatin by protamines. Chromatin remodeling is facilitated by the coordinated loosening of the chromatin by histone hyperacetylation & by the DNA topoisomerase II (topo II), which produce temporary nicks in the sperm DNA to relieve torsional stress that results from super coiling. The same enzyme Topo II normally repairs these temporary nicks prior to completion of spermiogenesis & ejaculation.

However, if these nicks are not repaired, DNA fragmented sperm may be present in the ejaculate. Defects in the chromatin remodeling process result in the production of spermatozoa that are characterized by reduction in the efficiency of protamination, abnormal protamine 1 to protamine 2 ratio, & relatively high nucleohistone content.

Thereby, creating a state of vulnerability where spermatozoa DNA become increasingly susceptibly to oxidative damage. Abnormal Protamine P1/P2 ratio is associated with low sperm count, decreased sperm motility & morphology, diminished fertilization ability, & increased sperm chromatin damage.

Sperm nuclear protein assay (chromatin maturity) is done via aniline blue staining. Aniline blue stains persistent histones in the sperm nucleus ***(for more information on procedural steps for performing test refer to section P-II.10 (a-f). of Part II).***

27. What do you understand by Mitochondrial Activity Index (MAI) test and available methods for its estimation?

Sperms when released into seminiferous tubule lumen are immotile or at the most very feeble motile. This is due to immaturity of the plasma membrane of the testicular spermatozoa. This is true of testicular aspirated sperms also which do not show motility even after exposure to the culture medium. The energy required for this flagellar activity is derived from adenosine triphosphate (ATP) produced in the mitochondria concentrated in the midpiece of spermatozoa. The long passage of spermatozoa through female tract needs robust mitochondrial apparatus producing an optimal quantity of ATP.

The mitochondrial oxido-reductive enzyme apparatus can be tested using indicators like nitro blue tetrazolium, which produce blue insoluble pigment in and around mid-piece. Evaluation can be carried out in smears showing stained mid-piece from good sperms to poor staining of nonmotile or poor mitochondrial activity sperms. Indirect semi-quantitative tests also are developed, which can give indirect evidence of mitochondrial activity depending on the quantity of color developed. The test was found to have a significant correlation with the sperm motility parameters.

28. What are the important semen biochemical tests for evaluating an infertile male?

Seminal plasma is a complex secretion with various substances some of which are specific to the accessory gland and contribute to a fraction of ejaculate. Important secretions are from seminal vesicles and prostate glands. Each secretion has a characteristic marker to detect presence, absence, dysfunction or infection of specific glands. It is necessary to include more than one marker for each gland and to standardize time for collecting aliquot from well mixed complete ejaculate.

Any abnormal or unusual value must be reconfirmed before drawing any conclusions or taking therapeutic decisions. Common secretions of the male genital tract are as mentioned below:

- **Prostate gland:** Acid phosphatase, citric acid, Zinc and magnesium.
- **Seminal vesicles:** Fructose, Prostaglandins.
- **Epididymis:** L - Carnitine, Alpha-glucosidase Glycerophsophocholine.

The precise role of these specific substances contributing to sperm function is still obscure. Zinc has a probable role in protecting and stabilizing condensed sperm chromatin. Fructose is secreted by seminal vesicles and provides an energy source to spermatozoa for anaerobic metabolism. This is an important energy source for the sperm and exclusion of the seminal vesicular component from the ejaculate will result in almost completely immotile sperm. It is a marker for seminal vesicle function, and there levels in semen are androgen dependent. Fructose levels should be determined in any patient with azoospermia and especially in those whose ejaculate volume is <1 ml, suggesting seminal obstruction or atresia or ejaculatory tract duct obstruction. Disorders of the seminal vesicles and a subsequent reduction in the fructose concentration in semen will also result in a reduced motility of semen.

Another situation where fructose estimates are helpful is in men with polyzoospermia and low motility. Occasionally in men with very high sperm concentrations (more than 350 million sperm/ml), the sperm are immotile due to a relative deficiency of fructose. It's important for both qualitative and quantitative estimation of fructose while evaluating patients of male infertility through commercially available reagents. It must be remembered that as sample ages; the fructose level will fall due to the utilization of fructose by spermatozoa. ***(for more information on procedural steps for performing test refer to section P-II.6 (a-d) of Part II).***

29. What is the role of α-Glycosidase in semen analysis?

Poor quality semen may result from testicular production of abnormal sperm or from post testicular damage of sperm in the epididymis or the abnormal ejaculate from accessory gland. Male accessory sex organs made of epithelial / mesenchymal components, require 'Androgen' for proliferation & maintenance of their functions. The epididymis makes a significant contribution to the development of a fertile sperm, through four classical functions, sperm maturation, sperm transport, sperm concentration & sperm storage. α-Glycosidase represents as io-marker of epididymis as its major contribution is from epididymis.

The evaluation of α-Glycosidase is regarded as a reliable parameter of epididymal potency. Post-testicular masturbation of sperm taking place in the epididymis is now well recorded, makes a significant contribution to the development of fertile ejaculate. Differential diagnosis of azoospermia only the determination of the α-Glycosidase is useful. The reduction of α-Glycosidase in infertile male is to be attributed to reduced sperm count.

30. What is the clinical relevance of anti sperm antibodies in infertility treatment?

Spermatozoa are antigenic. The testis is an immunologically privileged site protected from access by either lymphocytes or macrophage. However, damage to the genital tract may allow sperm to come in

contact with immunologically competent cells, such as lymphocytes, & thus allow the generation of antibodies against sperm surface antigens. Sperm antibodies may interfere with spermatogenesis & sperm maturation in the male, & hinder sperm transport, cervical mucus penetration, capacitation, & fertilization in the female. Antisperm - antibodies may also hinder spermatozoon fertilizing capacity.

It is estimated that 5 – 10% of male infertility is caused by sperm autoimmunity & approximately 10–15% of women with unexplained infertility have circulating sperm antibodies.The diagnosis of **Anti-Sperm Antibody (ASA)** mediated infertility lacks a pathognomonic clinical picture.

However, an ASA should be suspected if, the semen analysis shows sperm agglutination or clumping (i.e. motile spermatozoa stick to each other head-to-head, tail-to-tail or in a mixed way) in the absence of clinical infection. Sperm antibodies can be present without sperm agglutination; equally, agglutination can be caused by factors others than sperm antibodies.

Following are the conditions for suspicion:

- History of Testicular Injury or Surgery
- Low sperm motility
- Increased round cells i.e. leucocytes (repeated genital infection)
- Poor results of Post Coital Test
- Sperm 'shaking' in sperm-cervical Mucus contact test
- Unexplained infertility
- Genetic predisposition

Anti-sperm Antibodies (ASAs) in semen belong almost exclusively to two immunoglobulin classes : IgA & IgG. IgM antibodies, because of their larger size, are rarely found in semen. IgA antibodies may have greater clinical importance than IgG antibodies.

ASA in infertile couples are detected in two ways:

1. **Direct Test -** detect antibodies on sperm membrane.
2. **Indirect Test-**detect antisperm antibodies in sperm-free fluids, i.e. Seminal Plasma, blood serum, solubilized cervical mucus.

Numbers of tests are available, but preferred test is **Mixed Antiglobulin Reaction (MAR)**. The advantage of MAR Test is that it can be applied directly to fresh, untreated semen samples.

The results can be obtained within few minutes & is ***quick, simple & repeatable (for more information on procedural steps for performing test refer to section P-II.11(a-f) of Part II).***

31. What is take home message on Sperm Function Test at present?

There is still a gap in understanding of the complex nature of molecular interactions between oocyte and sperm leading to fertilization. Exact chain of events needs further clarification, with identification of specific molecules and gamete function should become the basis of new diagnostic tests.

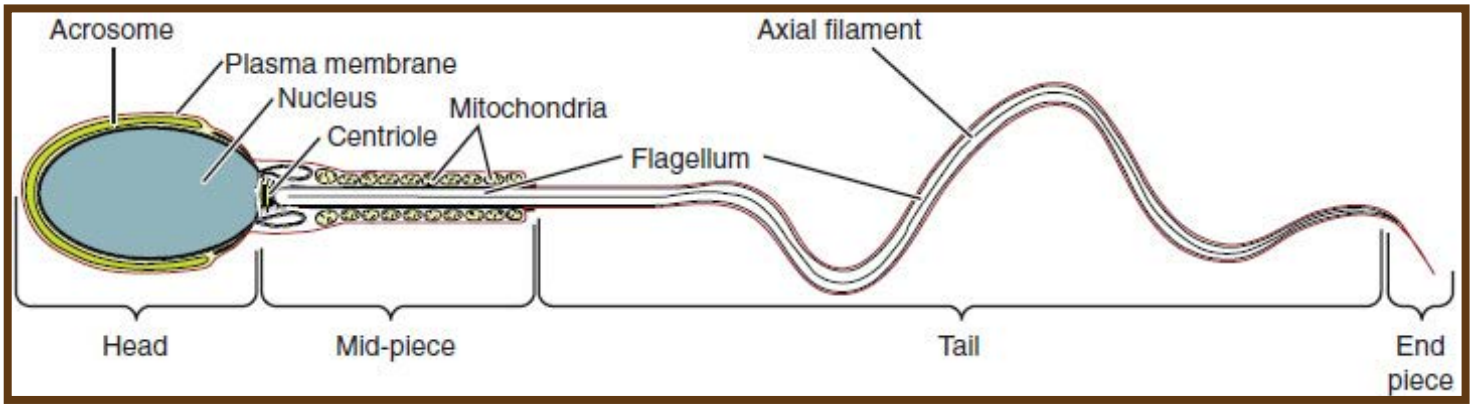
Since there is more than one sperm function involved, no single test of a single function will have perfect sensitivity. Therefore, batteries of tests are required for diagnosis. A major goal of new tests should be the identification of molecular defects in sperm function, allowing rapid development of biochemical tests, with the goal to specifically target therapies toward male subfertility. Tests with high sensitivity, high predictive value, and low false positive rate are desirable. Valid and more predictive tests need to be developed with strict validation and specificity.

Sperm function test should not be routine investigations as they are complex, expensive, not rigorously tested, do not always provide clinically useful information and typically do not affect treatment. The prediction of male fertility potential is probably an elusive goal owing to the multifactorial nature of conception. The present sperm functional assays are highly predictive of IVF results. However, the implementation of ICSI has basically eliminated the need for such tests.

It is our opinion that this does not represent a right approach. On the contrary, sperm functional tests have the potential to assist the clinician in the decision making process. If patients fail sperm functional testing, then that would eliminate the time, effort, and expense of couples undergoing lower complexity therapies such as IUI and direct them to ICSI without delay.

Points to be kept in mind when prescribing for Sperm Function Test:

- High-quality clinical data missing on sperm function test
- For a test to be useful, it must have strong predictive value for pregnancy outcome and have little overlap between fertile and infertile samples.
- Multicentric clinical trials essential for developing robust thresholds to integrate sperm function test especially DNA tests into routine clinical practice as they could predict ART clinical outcome.
- They may be useful for identifying a male factor contributing to unexplained infertility or for selecting therapy.
- There is a need for targeted sperm function testing, but to whom and which one(s) is unclear.
- New tools which are simple, cheap, reliable, repeatable, effective combined with more robust assessments urgently required.



NEXUS Volume:3 (Part-II)

CONTENTS

Section No.	Name of Section	Page No.
P.II.A	General Instructions	16
P.II.1	Sperm Vitality	16
P.II.2	HOS Test	17
P.II.3	Sperm Morphology Staining	17
P.II.4	Semen pH	18
P.II.5	Semen Viscosity	18
P.II.6	Semen Fructose (Qualitative)	19
P.II.7	Sperm Confirm	19
P.II.8	Sperm Leukocyte Detection	20
P.II.9	Sperm DNA fragmentation	20
P.II.10	Nuclear Protein Assay	21
P.II.11	Antisperm Antibody (IgA & IgG) MAR Test	22
P.II.B	Important Instructions	23
P.II.C	Acknowledgements	23

Nexus Vol: 3 (Part-II)

Sperm Function Tests (SFTs): Demystified(Protocols)

II.A GENERAL INSTRUCTIONS

II.A.i Sample Preparation Instruction

Semen sample is collected with:
- Abstinence period of 2-7days.
- Ideal collection through masturbation in sterile container.

Non-spermicidal polyurethane semen collection pouch can be used when required.
• Semen sample is allowed to liquefy and then well mixed for performing test.
• Ideally test is to be performed within 30 to 60 min of collection.

II.A.ii Special Instructions

- Hyperviscous semen sample should be processed to bring towards normal viscosity by use of treating with Bromelin or treatement with Chymotrpsin enzyme.
- Severe oligospermic semen sample (i.e. sample with Sperm Concentration less than 5 millions/mL) should be processed to concentrate the sperm concentration to around 8-10 millions/mL before performing the test.
- Frozen semen plasma must be thawed at 37°C before performing test.

II.A.iii Equipments Required for SFTs

Microscope
Pipette Set
Stopwatch
Microtip Box
Staining Tray
Glass Slide Tray
Biochemistry Analyzer
Controlled Temperature 37°C Dry bath
Slide Warmer
Semen Analysis Chamber
Glass Slide Stand
Centrifuge Machine
Stop-watch

II.A.iv Disposable Required for SFTs

Hand gloves
Non-spermicidal semen collection pouch
Pasteur Pipettes, Glass Slides
Microtubes / Storage Vials Microtubes / Storage Vials
Coplin Jar
Semen Collection Container
Microtip Box
Coverslips
Filter Papers
Test tube stand
Ethanol

P-II.1.Sperm Vitality

(Evaluation of sperm membrane structural integrity)

Bench time for test: 15-18 mins

II.1a. Reagent Required: Eosin - Nigrosin Solution

II.1b Quick View

Take 10 µL Liquefied Semen sample
↓
Add 50µL Eosin – Nigrosin
↓
Solution & mix well
↓
Wait 30 secs
↓
Take 10µL sample &prepare smear.
↓
Air dry
↓
Examine under Microscope 100X objective as soon as possible

II.1c Examination

• Examine smear under microscope with help of 100x lens.
• Examine at least 200 sperms & count the following: Unstained / White Sperm (Indicative of Live Sperm), Red / Dark Pink Sperm (Indicative of Dead Sperm)

II.1d Results

Total number of Sperms Evaluated: .....
Number of Unstained Sperm (Live): .....
Number of Stained Sperm (Dead): .....

II.1e Reference value

Normal: > 58%
Equivocal: > 55% & < 63%
Abnormal: < 55%

(As per fifth edition of WHO laboratory manual for examination and processing of human semen)

II.1f Reference Image

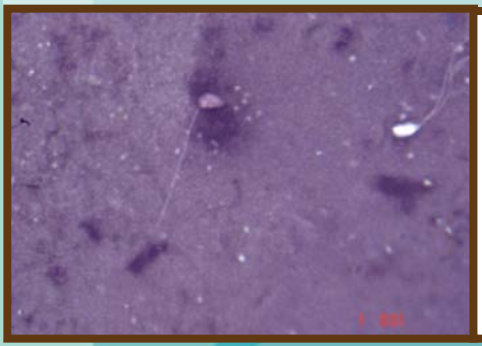


Figure II.1: *Eosin-nigrosin stain used for assessing sperm vitality with nigrosin providing dark background which makes it easier to differentiate stained (dead) and unstained (live) sperms*

P-II.2 HOS Test

(*Evaluation of sperm membrane structural & functional integrity*) **Bench time for test: 45 mins**

II.2a Quick View

Take 1mL HOS reagent
↓
Keep at 37°C for 5–10 min
↓
Add 0.1ml liquefied semen sample & mix
↓
Incubate at 37°C for 30 min
↓
After incubation place 10–15 µL on glass slide
↓
Put coverslip avoiding air bubbles
↓
Examine under Microscope with **40x** *magnification (Prefer Phase Contrast Microscope)*

II.2b Examination

• Evaluate at least 200 sperm and observe sperm tail for curling (swelling) or non-curling (straight)
• Commonly observed sperm swelling patterns (fig.2b):
- Tip swelling
- Hairpin swelling
- Shortened & thickened tail
- Partly or completely enveloped sperm tail

II.2c Results

- **No. of Sperm Evaluated :** .....
- **No. of HOS +ve Sperm :** .....
- **No. of HOS -ve Sperm :** .....

Normal: > 58%
Equivocal : > 55% & < 63%
Abnormal : < 55%

(As per fifth edition of WHO laboratory manual for examination and processing of Human Semen)

II.2d Reference Image

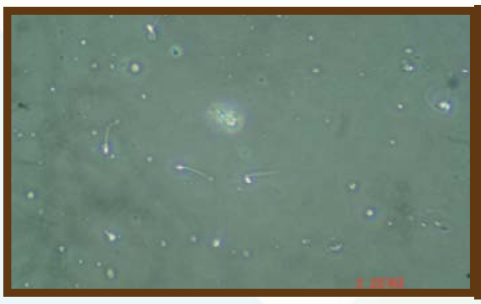


Figure2a: *Healthy spermatozoon showing swelling and curling of the tail region as positive for HOS test and no-viable sperms as negative*

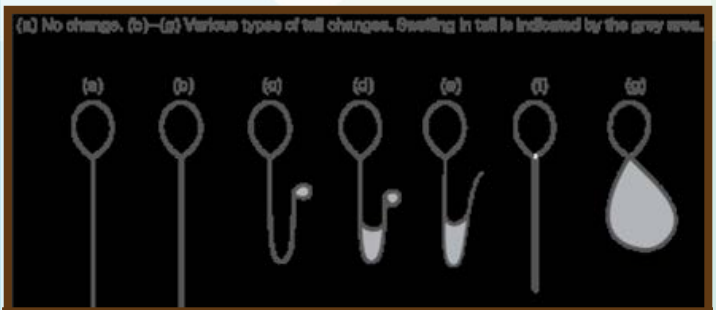


Figure 2b: *Schematic Representation of Fifth edition of WHO laboratory manual for examination and processing of Human Semen).*

P-II.3 Sperm Morphology Staining Method

Bench time for test: 20 mins

II.3a. Reagents

Red Stain A: Red Stain-50 ml in coplin jar
Blue Stain B: Blue Stain-50 ml in coplin jar
Fixative 10 ml in dropper bottle

II.3b Quick View

Take semen sample (5-10µl) for preparation of the smear
↓
Allow the smear to air dry
↓
Fix the smear with few drops of fixative
↓
Stain in stain A
↓
Then stain in stain B
↓
Allow the smear to air dry.
↓
Observe staining under a light microscope (100X) using oil immersion.

II.3c Examination

- Examine at least 200 sperm and count: Normal & Abnormal sperms with detailed abnormality (defects) pertaining to head, mid piece and tail.

II.3d. Results

Calculate the percentage of normal and abnormal sperm

Sperm Deformity Index = Total No. Of Defect / No. Of Sperm Evaluated

Teratozoospermic Index = Total No. Of Defect / Total No. Of Abnormal Sperms

No of sperm classified as normal (% Normal) = Total No of Normal sperms X 100 / Total No. Of Abnormal Sperms

II.3e. Reference value

Normal reference value / range: 4% (3%-4%)
Normal: >15%; G-pattern: >5-14%; P - pattern: <4%

(As per fifth edition of WHO laboratory manual for examination and processing of human semen)

II.3f. Reference Image

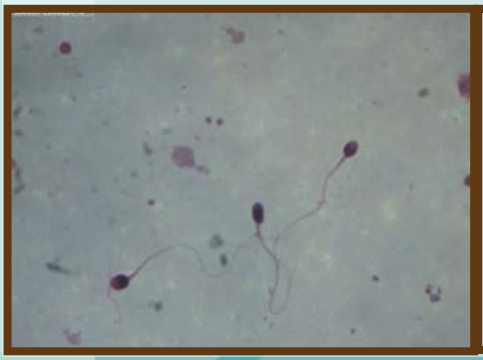


Figure.3: *Sperm morphology slides to differentiate between normal and abnormal sperms.*

P-II.4 Semen pH

(Evaluation of semen pH)

Bench time for test: 1min

II.4a. Requirement

pH strips (between **6-10**)

II.4b Quick View

pH Strip
↓
Put 10µL of Liquefied Semen on Yellow Circle
↓
After 45–50 secs
↓
Read the pH value by colour comparison

II.4c Examination

Observe color change after 45 – 50 secs. Compare color with adjacent chart provided on strip. The compared color match denotes the pH of semen sample.

II.4d Results

Semen pH : .....

II.4e: Reference Range

- **Normal reference value: > 7.2**

(As per fifth edition of WHO laboratory manual for examination and processing of human semen)

II.4f. Reference Image

Figure 4: *pH strip for measuring the semen sample pH through visual color change*

P-II.5 Semen Viscosity

(Reagent for hyper viscous semen)

Bench time for test : 25 mins

II.5a. Reagents

Chymotrypsin enzyme, Bromelin

II.5b. Quick View

Take viscous semen sample and measure volume
↓
Add 10µL/ml of Reagent - Chymotrypsin to semen sample at 37°C for 10-15 min
↓
Mix Well semen sample & perform String test
↓
If the sample is 'Hyperviscous" thread more than 2 cm in length
↓
Add 10 µL/mL of Reagent Bromelin to semen sample & mix well.
↓
Keep sample (well mixed) at 37°C for about 10-15 min (max 30 min).

II.5c. Examination

Perform string test on reagent added sample to recheck viscosity and note the length of the 'string'.

II.5d Result

- If length of string is less than 2 cm, viscosity of sample should be considered as normal.

II.5e: Reference Image

Figure 5: *A hyperviscous semen sample before semen analysis*

P-II.6: Fructose (Qualitative)

Biomarker of seminal vesicle activity

Bench time for test: 2 mins

II.6a. Reagent

Fructose Reagent

II.6b Quick View

Take 1mL Fructose Reagent
↓
Add 0.1 mL of semen sample & mix
↓
Boil mixture for 30-45 sec
↓
Observe Color change

II.6c. Results

Change in color from colorless to red color to be observed.

Colorless to Red tone: Fructose Present
No Color change: Fructose Absent

II.6d. Reference Image

Figure 6: *Semen fructose qualitative test showing labeled tubes test sample (T) tube with no color change in test result showing fructose negative.*

P-II.7.Sperm Confirm

(To confirm sperms in a smear)

Bench time for test: 10 mins

II.7a Reagents

Stain – I, Stain – II & Stain - III

II.7b Quick View

Pellet sample
↓
Take 10 µL form smear & dry it
↓
1mL Stain – I for 5 min
↓
Drain off Stain - I & Rinse in Distilled water
↓
1mL of Stain – II for 3-5 sec
↓
Drain off Stain - II & Rinse in Stain - III
↓
Dry the smear
↓
Examine

II.7c Examination

- Examine smear under the microscope with the help of 100x objective lens.
- If immature sperm are present in the smear, then they appear as stained red bodies.

II.7d Resuts

Semen Smear Examination (Centrifuged Sample)- Microscopic Examination of Stained Smear

- **Sperm Found**
- **No Sperm Found**

II.7e Reference Range

Number of sperm present as per the field:

1 +: Few sperm seen in smear
2 +: Presence of sperm more than 2-3 per microscopic field.
3+: Presence of sperm more than 5 per microscopic filed
4+: Presence of sperm is almost in each microscopic field.

(As per Fifth Edition of WHO Laboratory manual For Examination And Processing Of Human Semen.)

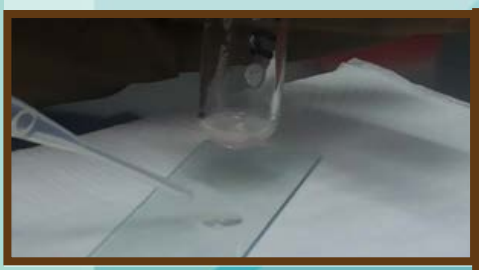


Figure 7: *Diagnosis of azoospermia by observing spermatozoa in the sediment of a centrifuged sample*

P-II.8. Sperm Leukocytes

(Detection of leukocytes by using peroxidase test)

Bench time for test: 40 mins

II.8a. Reagents

Reagent as provided with the kit- I

II.8b. Quick View

Measure the volume of semen in ml
↓
Add 0.9 ml Reagent I
↓
Add 100 µL of liquefied semen & mix well
↓
Add drop of Reagent - II & mix well
↓
Put micro tube at room temperature for 20–30 min
↓
Take 10 µL from this tube to 10 micron depth chamber
↓
Examine under microscope
using 20x objective lens (Preferably Phase Contrast)

II.8c. Examination

Examine under 20x objective lens in phase contrast microscope. Count atleast 10 squares in the given field of focus.

- Number of WBC in 10 squares.
- Number of brown colored WBC (peroxidase positive) in 10 squares.

II.8d. Results

- **Semen Volume :** ..... mL
  - **White Blood Cells:** ..... millions / mL
  - **White Blood Cells:** ..... millions / ejaculate
- **Peroxidase Positive:**
  White Blood Cells : ..... millions / mL
- **Peroxidase Negative:**
  White Blood Cells : ..... millions / ejaculate

II.8e. Normal Reference Range

Normal : < 1 million/mL
Equivocal : > 1 million/mL & < 2 million/mL
Abnormal : > 2 million/mL

(As per Fifth edition of WHO laboratory manual for examination and processing of Human Semen)

II.8f. Reference Image

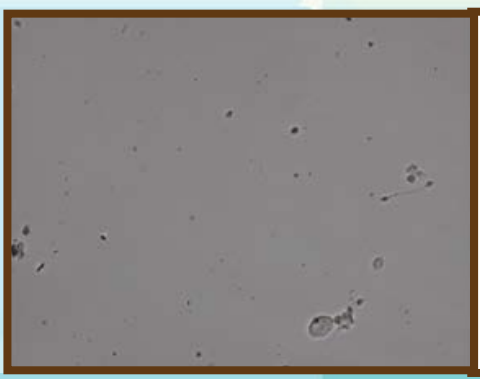


Figure 8: Presence of brown colored leukocytes indicating the infection in the semen sample

P-II.9 DNA Fragmentation

(Assay for assessing sperm DNA fragmentation)

Bench time for test: 90 mins

II.9a. Reagents

- Pre-coated slides, Agarose in tubes
- Solution A, Solution B, Solution C & Solution D

II.9b. Quick View

Liquefied semen sample(5-10 million/ml)
↓
Melt Agarose at 90°C for 5 min
↓
Transfer Agarose in warmer at 37°C for 5 min
↓
Add 25µl semen sample to agarose
↓
Place sperm suspension immediatelyonto pretreated slide and place cover slip
↓
Leave slide at 4°C for 5 min
↓
After 5 min remove cover slip
↓
Incubate the slide in Solution A for 7min
↓
Then incubate in Solution B (lysis) for 25 min
↓
Flood the slide with distilled water for 5 min
↓
Ethanol dehydration 70%, 90% &100% (2 min each), Leave for air dry at room temperature (RT)
↓
Flood the slide with solution C+ solution D (1:1) and leave to stain for 15-20 minutes
↓
Decant the stain and wash with distilled water
↓
Dry at RT
↓
Examine in Phase contrast microscope

II.9c. Examination

Sperm DNA fragmentation (SDF) index calculation (classify at least 300-500 sperm cells as follows)

- **Sperm without DNA fragmentation-** Big Halo & Medium Halo
- **Sperm with DNA fragmentation-** Small Halo, Without Halo, Without Halo and Degraded

Sperm Evaluation

- **No. of Sperm Evaluated:** .....
- **Sperms with Non-fragmented DNA:** .....
- **Sperms with Fragmented DNA:** .....
- **Degraded Sperms:** .....

II.9d. Result

SDF(%) index=

Number of Sperms with DNA fragmented X100
Total number of sperms counted

DNA Fragmentation Index (DFI) : .....

II.9e. Normal reference value / range for DFI Index

- **Normal:** DFI < 15%
- **Equivocal:** DFI > 15% & DFI < 25%
- **Abnormal:** DFI > 25%

II.9f. Reference Image

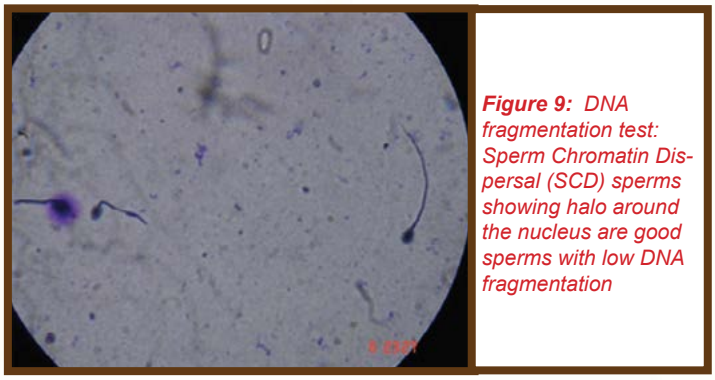


Figure 9: DNA fragmentation test: Sperm Chromatin Dispersal (SCD) sperms showing halo around the nucleus are good sperms with low DNA fragmentation

P-II.10. Nuclear Protein Assay

(sperm nuclear protein assay using "Aniline Blue" staining)

Bench time for test: 90 mins

II.10a. Reagents

- Fixative Solution
- Stain – I & Stain - II
- Normal Saline: 30 mL

II.10b. Quick View

Processed or neat semen sample
↓
Take 5 µL / 10 µL semen sample, prepare smear & dry
Add drop of Regent - II & mix well
↓
1 mL Fixative solution for 5 min
↓
Drain off Fixative Solution & Rinse in Distilled water
↓
1 mL Stain - I for 5 min
↓
Drain off Stain - I & Rinse in Distilled Water
↓
1 mL Stain - II for 2 min
↓
Drain off Stain - II & Rinse the in Distilled Water
↓
Dry the smear
↓
Examine under 100X objective

II.10c. Examination

- Examine the smear under the microscope with the help of 100x lens (count at least 200 sperms & count the following [Mostly in Post acrosomal region]

- Sperm head with blue stain (Indicates sperm with immature nuclear protein).
- Sperm head with red stain (Indicates sperm with mature nuclear protein).

II.10d. Results

- **No. Of Sperm evaluated :** .....
- **Sperm With Mature Nuclear protein :** .....
- **Sperm With Immature Nuclear protein :** .....

II.10e. Reference range

- **Normal:** Immature Nuclear Protein < 15%
- **Equivocal:** Immature Nuclear Protein > 15% & < 25%
- **Abnormal:** Immature Nuclear Protein > 25%

(As per Fifth edition of WHO laboratory manual forexamination and processing of Human Semen)

II.10f. Reference Image

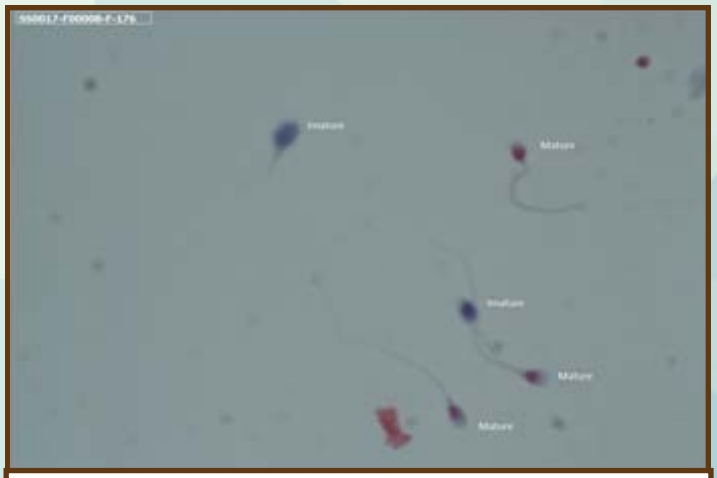


Figure 10: Nucelar Protein Assay: The assay differentiating between the mature (pink stained) and immature (blue stained) sperm.

P-II.11 Anti-sperm Antibody (IgA& IgG) MAR Test

(Mixed Antiglobulin Test)

Bench time for test: IgA & IgG

II.11a. Reagents

1. Latex particles coated with IgA)
2. Latex Particle coated with IgG
3. Antihuman IgG Antibody

II.11b. Quick View (IgA)

Take 5µL Liquefied semen
↓
Add 5µL Latex reagent & mix well
↓
Keep in Humid chamber for 3 min at 37^{0}C
↓
Examine after 3 min incubation
↓
Keep the same slide in
↓
Humid chamber for 7 min more at 37^{0}C
↓
Examine the same slide under Microscope after 10 min (3 + 7 = 10 min)

II.11c. Quick View (IgG)

Take 5µL Liquefied semen
↓
Add 5µL Latex reagent & mix well
↓
Add 5µL of Antihuman IgG Antibody & mix well
↓
Keep in Humid chamber for 3 min at 37^{0}C
↓
Examine after 3 min incubation
↓
Keep the same slide in
↓
Humid chamber for 7 min more at 37^{0}C
↓
Examine the same slide under Microscope after 10 min (3 + 7 = 10 min)

II.11d. Examination

- Examine the prepared slide from step 5 by using 40x objective lens preferably phase contrast.
- Examine 200 – 500 sperms & count the following :
  - Number of motile sperms.
  - Number of motile sperms attached to red colored latex particle.
  - Define grade & group of agglutination.

II.11e. Results

	3min	10min
No. of Motile Sperm		
Motile Sperm Attached To Red Colored Latex Particle		
Grade of sperm agglutination		
Group of sperm agglutination		

II.11f. Reference Image

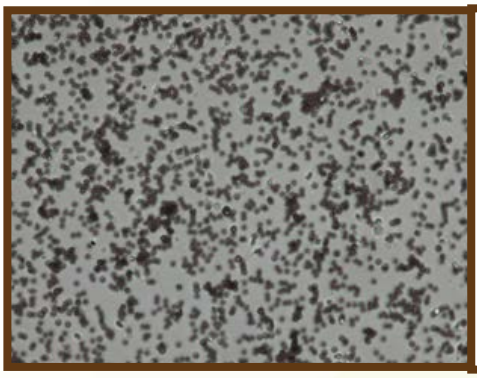


Figure 11a: *Anti-sperm Antibody (IgA) direct MAR Test: The semen sample with motile sperms bound to red colored latex particle showing agglutination.*

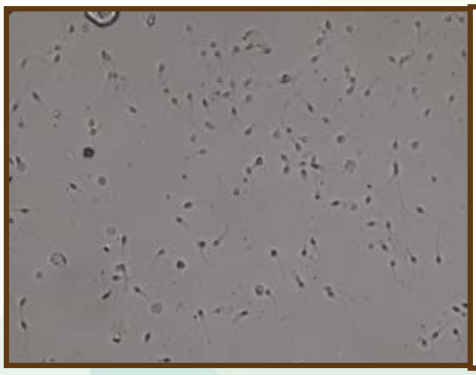


Figure 11b: *Anti-sperm Antibody (IgG) direct MAR Test: The semen sample with motile sperms unbound to green colored latex particle showing no agglutation.*

ACKNOWLEDGEMENTS

1. SAR Healthline Pvt Ltd, Calicut, India, Mob:+91-9958029696
2. Cryocell India Pvt.Ltd A-35, Nirman Vihar, Delh-110092i, India (Tel: +91-11-22016106 Email: info@cryocellindia.com)
3. Sperm Processor Pvt. Ltd. 6, Welcome Nagar, Garkheda, Aurangabad (MS) - 431005, India. Ph.: +91 240 6603800, Fax: +91 240 2341694, Email: info@spermprocessor.com)
4. Candore Biosciences, 1st floor Shashwat complex, Ahmendabad-380013, India; Mob:(+91 8460083121,7927683121); Email: info@candore.in)
5. ORIGIO India Private Limited, C-401, Delphi, Hiranandani Business Park, Powai, Mumbai 400 076, India, Phone No. : +91 22 4928 0030 I E-mail: mmagdum@origio.com

P-II.B. IMPORTANT INSTRUCTIONS

II.B.i. Storage Conditions

- The kit should be stored in dark at 2°C - 8°C after receiving.
- Bring all the reagents to room temperature before use.
- Once opened, store reagents in the fridge protected from light.
- Expiry date is printed on the outside of the box.

II.B.ii Limitations

- These sperm function test provides presumptive quantitative information of sperm.
- This parameter should be analyzed by a specialist.
- The result should be evaluated taking into account all clinical & laboratory findings related to the same sample

II.B.iii. Precautions

- All patient samples & reagents should be treated as potentially infectious & the user must wear protective gloves, eye protection & laboratory coats when performing the test.
- The kit should be discarded in a proper biohazard container after testing.
- Do not eat, drink or smoke in the area where specimens & kit reagents are handled.
- Do not use beyond the expiration date which appears on the package label.
- It is recommended to use of gloves & face mask.

II.B.iv. Safety & Environment

- Do not release the products used into the environment. Follow centre guidelines for the storage & disposable of toxic substances.
- Biological samples must be handled as potentially infectious

BIBLIOGRAPHY

1. Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human spermatozoa: creatine kinase, caspase-3 and Bclx expression in mature and diminished maturity sperm. Mol Hum Reprod 2004;10:365–72.
2. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human spermatozoa indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 2003;79(Suppl 3):1616–24.
3. Ribas-Maynou J, Garcia-Peir_o A, Abad C, Amengual MJ, Navarro J & Benet J. (2012a) Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology, 2013, 1, 715–722.
4. Mao C, Grimes DA. The sperm penetration assay: can it discriminate between fertile and infertile men? Am J Obstet Gynecol 1988;159: 279–86.
5. Oehninger S, Franken DR, Sayed EM, Barroso G, Kohm P. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta analysis. Hum Reprod Update 2000;6:1160–8.
6. Yagci et al. (2010) Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: An acridine orange fluorescence study. J Androl; 31:566-572.
7. Parmegiani L. et. al. (2010) Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Ferti Steril 93:598-604.

For queries and feedback
Dr (Prof) Pankaj Talwar
Editor Nexus
Jt. Secretary Indian Fertility Society
E: pankaj_1310@yahoo.co.in, M: +91 9810790063