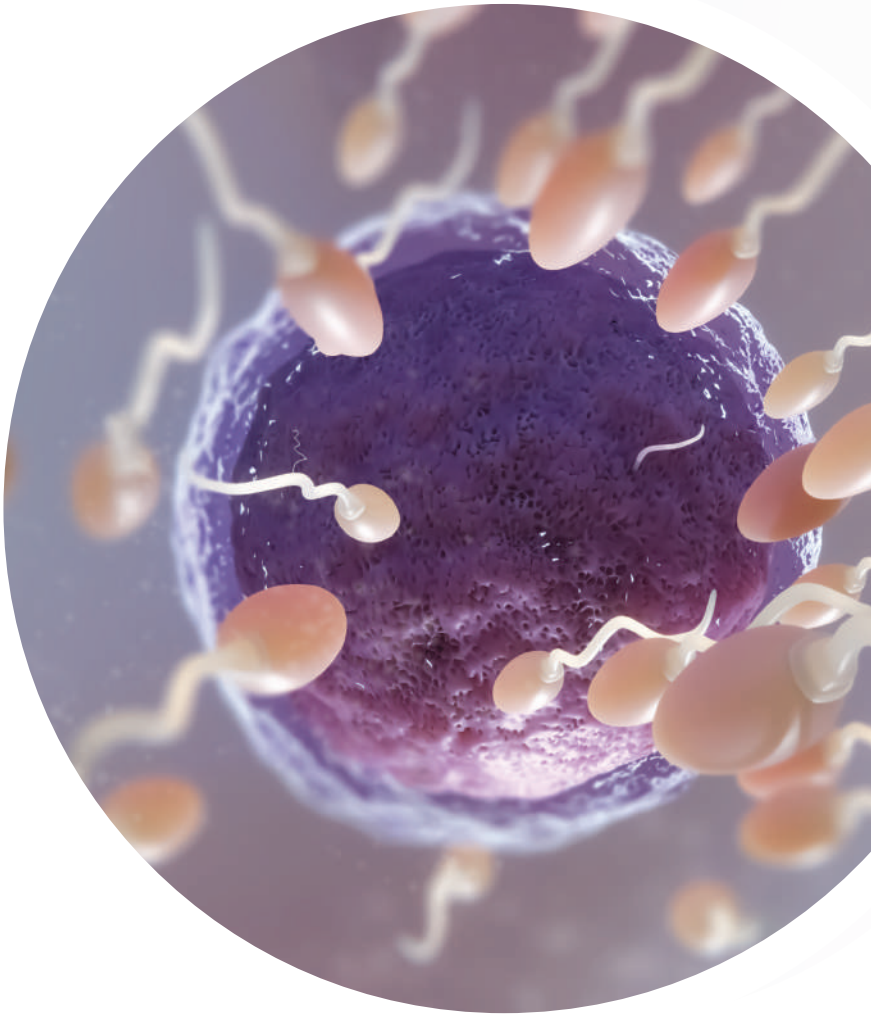
Indian Fertility Society

Volume 3 (May, 2025)

NEXUS

Sperm Function Test



Editor
Dr. Rupali Bassi Goyal
M: +91 9818331760
E: rupalibassi@hotmail.com

Dr Pankaj Talwar
President - IFS

It is with pleasure and great privilege we introduce our first Nexus bulletin titled "Intra Uterine Insemination". The Nexus series are being updated to bridge the gap between ART Clinicians and Embryologists keeping in mind the latest recommendations ans guidelines. The series will cover topics pertaining to quality control, basic IVF techniques and laboratory protocols.

My heartfelt congratulations to Dr Rupali Goyal, and the entire editorial team and very best wishes for the future.

Dr Shweta Mittal
Secretary General - IFS

Indian Fertility Society feels proud and congratulates the Editorial team for relaunch of Nexus-E-bulletin. The bulletin aims to enlighten and broaden the spectra of knowledge in the field of Embryology and Andrology. This is an attempt to train and educate the budding embryologist and infertility specialists from basics, in step with the latest guidelines and recommendations. The content of the E-Bulletin will disseminate scientific knowledge, Ethical practices along with latest research and development across the globe.

Dr. Rupali Bassi Goyal
Editor

Dr Nymphaea Walecha
Joint Editor

Dr Garima Kapoor
Guest Editor

Sunita Chauhan
Author

INDEX

Q1. 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. 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.

Importance of sperm function tests

1. **Identifies male factor infertility:** Sperm function tests help diagnose male factor infertility, which accounts for approximately 30-40% of infertility cases.
2. **Guides treatment options:** Test results inform treatment decisions, such as intrauterine insemination (IUI), in vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI).
3. **Predicts IVF Success:** Sperm function tests can predict the likelihood of success with IVF.
4. **Monitors Treatment Efficacy:** Repeat testing can assess the effectiveness of treatments aimed at improving sperm function.
5. **Provides insights into underlying conditions:** Abnormal test results may indicate underlying medical conditions.

Sperm function tests detect function of a certain part of spermatozoon 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.

Q2. What are the main indications for performing advanced sperm function test?

The patient with normal semen parameters may not be requiring any specialized tests (except in cases of RPL, UI or suspected DNA damage).

Indications for advanced sperm function tests:

- **Identifies male factor infertility:**
  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.
- **Unexplained infertility:**
  When couples experience unexplained infertility, advanced sperm function tests can help identify underlying sperm function issues that may be contributing to infertility.
- **Recurrent pregnancy loss:**
  Advanced sperm function tests may be indicated in cases of recurrent pregnancy loss to rule out sperm-related issues.
- **Failed fertilization in IVF/ICSI:**
  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.

- **Suspected sperm DNA damage:**
  Advanced sperm function tests, such as sperm DNA fragmentation testing, may be indicated when sperm DNA damage is suspected.

Sperm function tests detect function of a certain part of spermatozoon 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.

Q3. What are the extended and advanced examinations in male infertility?

The hallmark of the evolution of the male remains the diagnostic semen analysis. Male infertility is often due to insufficient sperm production, abnormal sperm morphology, impaired sperm motility or combinations.
To gain deeper insights into the biological basis of male factor infertility, a battery of functional tests has been developed aimed at assessing the competence of human spermatozoa to fulfill the fundamental processes essential to conception. The commonly accepted standard for defining the normal semen analysis in the criteria defined by the World Health Organization (WHO).

Extended examinations:

- **Indices of multiple sperm defects**
- **Sperm DNA fragmentation**
- **Genetics and genomic test**
- **Test related to immunology**
- **Assessment of immature germ cell in the ejaculate**
- **Testing for antibody coating of spermatozoa**
- **Biochemical assay for accessary sex gland function**
- **Assessment of sequence of equations**

Advanced examination:

- **Seminal oxidative stress and reactive oxygen species**
- **Assessment of the acrosome reaction**
- **Assessment of sperm chromatin**
- **Trans membrane ion flux and transport in sperm**
- **Computer –aided sperm analysis**

Q4. What is the Teratozoospermia index (TZI)?

Teratozoospermia index (TZI) is a predictor of fertilization potential. The Teratozoospermia Index (TZI) is a quantitative measure used to assess the morphology of sperm cells. It evaluates the average number of abnormalities per abnormal sperm. TZI provides a more detailed understanding of sperm morphology, which is essential for evaluating male fertility. A higher TZI value indicates more severe Teratozoospermia (abnormal sperm morphology).

It's calculation:

This is done by recording the number of normal and abnormal sperms. **TZI** has a maximum of four defects per abnormal spermatozoon: one each for head, midpiece and principal piece, and one for excess residual cytoplasm. 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.

It's relevance:

TZI is used in conjunction with other semen parameters, such as sperm concentration, motility, and morphology, to assess the overall quality of the semen sample. Helps in clinical evaluations in order to provide a comprehensive understanding of male reproductive health.

Abnormal TZI values may indicate:

- Teratozoospermia
- Male infertility
- Increased risk of failed fertilization or miscarriage

Q5. What is the importance and indication for doing DNA fragmentation test?

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 spermatogenesis, 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 etiology 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, gonad toxins, 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.

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)

Q6. What are the different methods to detect sperm DNA fragmentation index?

DNA fragmentation index (DFI) is a measurement of the percentage of sperm with damage DNA. It is assessed using variety of methods.

Methods to detect SDF

- **Comet assay**

The comet assay is based on the principle of permeabilization and electrophoretic migration of cleaved fragments of DNA. In the comet assay, DNA damage is quantified by measuring the displacement between the genetic material of the nucleus "comet head" and the resulting tail. The tail lengths are used as an index for the damage. Also, the tail moment—the product of the tail length and intensity (fraction of total DNA in the tails)—has been used as a measuring parameter. The chromosome comet assay is a new application that detects DNA damage by generating comets in sub-nuclear units, such as the chromosome, based on the chromosome isolation protocols currently used for whole-chromosome mounting in electron microscopy. It has not been used with sperm cells thus far.

Interpretation: Scoring of the first 50 randomly selected comets is done in each slide using appropriate comet software. However, comets with overlapping tails are not counted. Comets with no heads should be considered as sperm containing 100% DNA damage.

Advantages and limitations: The comet assay is a well-standardized, simple, versatile, sensitive, and rapid assay that correlates significantly with the TUNEL assay and SCSA. It can assess DNA damage qualitatively as well as quantitatively with low intra-assay variation. Two-tailed comet assay can discriminate between single- and double-stranded DNA breaks. Because it is based on fluorescence microscopy, the assay requires an experienced observer to analyse the slidesand interpret the results.

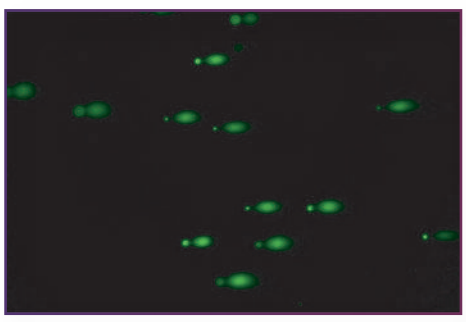


Fig 1: Comet assay of sperm: extensive DNA fragmentation, bright stain, small green spots, large comet tail

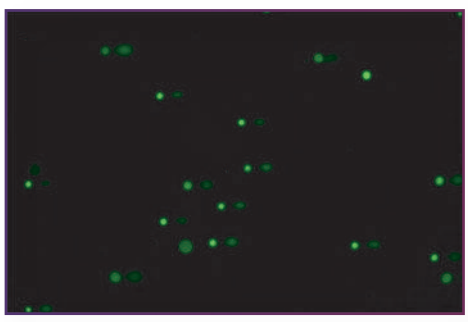


Fig 2: Comet assay of sperm: normal sperms with less DNA fragmentation, lacking visible damage stain

- **TUNEL assay**

This single-step staining method labels DNA breaks with fluorescein isothiocyanate (FITC)-dUTP followed by flow cytometric analysis. TUNEL utilizes a template-independent DNA polymerase called TdT, which non-preferentially adds deoxyribonucleotides to 3′-hydroxyl (OH) single- and double-stranded DNA. dUTP is the substrate that is added by the TdT enzyme to the free 3′-OH break-ends of DNA.

Interpretation:

- By flow cytometry using a blue light 488 nm laser. Measure green fluorescence of FITC-(or Alexa Fluor 488)-anti-Br-dU Ab at 530/20 nm. Measure red fluorescence of PI at > 600 nm.
- By flouresence microscopy, score a minimum of 500 spermatozoa per sample under 40× objective with an epifluorescence microscope (excitation between 460 and 490 nm and an emission > 515 nm). First count the number of spermatozoa per field stained with PI (red). Count the number of cells emitting green fluorescence (TUNEL-positive) in the same field. Then Calculate the percentage of TUNEL-positive cells

Advantages and limitations: It is relatively expensive and time and labour consuming. Also, a number of factors can significantly affect assay results, including the type and concentration of fixative, fixed sample storage time, the fluorochrome used to label DNA breaks, and the method used to analyse flow cytometric data.

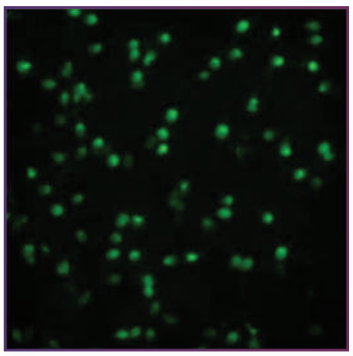


Fig 3: TUNNEL assay of sperm: positive control, sperm exhibiting DNA fragmentation

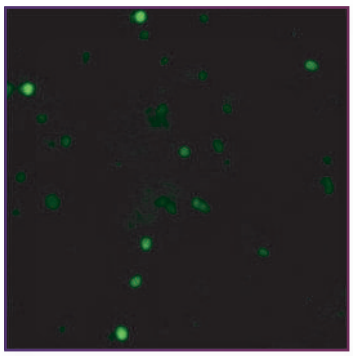


Fig 4: TUNNEL assay of sperm: baseline level damage (normal DFI)

- **SCD test (Halosperm® assay)**

The SCD test produces sperm nucleoids consisting of a central or core and peripheral halo caused by release of DNA loops, signifying the absence of DNA fragmentation. When sperm are treated with an acid solution prior to lysis buffer, a complete absence or a minimal halo is produced in spermatozoa with fragmented DNA. A distinct halo is seen in spermatozoa with intact DNA integrity. When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinized nuclei (nucleoids) show extended halos of DNA dispersion, which can be observed either by bright field microscopy or fluorescence microscopy. The presence of DNA breaks promotes the expansion of the halo of the nucleoid.

Interpretation: Assess the sample by light microscopy using a 100× immersion oil objective. The study of a minimum of 500 spermatozoa per sample is recommended. Sperms are observed for the halo formation. Sperms are categorized into four categories based on the halo formation- large halo, medium halo, small halo, no halo and degraded. Large halo and medium halo are sperm without DNA fragmentation, whereas small halo, no halo and degraded sperms are the ones possessing DNA fragmentation, accordingly percentages are calculated and reported as DFI.

Advantages and limitations: The SCD test is simple, fast, and reproducible. The currently available protocol is suitable for bright field microscopy as it significantly reduces equipment cost. This is the only test allowing assessment of SDF and chromosomal aneuploidy by FISH in the same cell. Oxidative DNA damage also can be simultaneously determined in the same sperm cell by combining the SCD test and incubation with an 8-oxoguanine DNA probe.

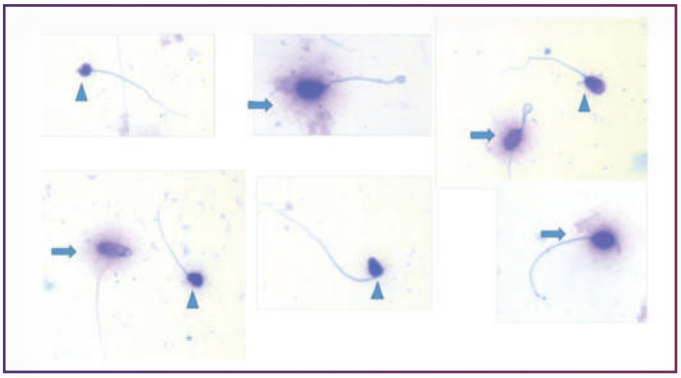


Fig 5: Arrowed figures: intact sperm DNA **Arrowheads figures:** fragmented sperm DNA

- **AO assay**

Acridine orange AO is a dye that intercalates with DNA or RNA and fluoresces to emit different colours, making it easy to differentiate cellular organelles. The binding that occurs is the property of electrostatic interactions between acridine molecules and base pairs of nucleic acid. It measures the susceptibility of sperm nuclear DNA to acid induced denaturation in situ by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The fluorochrome AO intercalates into double stranded DNA as a monomer and binds to single-stranded DNA as an aggregate. The monomeric AO bound to native DNA fluoresces green, whereas the aggregated AO on relaxed or denatured DNA fluoresces red.

Interpretation: For the analysis of the data, the sperm population is selected by applying a gate in the forward light scatter/side light scatter region. In a subsequent step, a green double-stranded DNA versus red single-stranded DNA fluorescence cytogram is used to gate-in only the AO-positive cells and subtract from the analysis cell debris and apoptotic bodies that lack genetic material. Consecutively, the shift of green to red fluorescence – or DFI – is calculated in each AO-positive sperm using the formula:

DNA fragmentation index (DFI) = (red fluorescence)/(red fluorescence + green fluorescence)

Advantages and limitations: The AO assay is a biologically stable measure of sperm DNA quality. This technique is highly reproducible. Limitations include inter-observer variability in case of fluorescence microscopic analysis and expensive instrumentation for flow cytometric analysis.

Other tests:

In situ NT assay: The NT assay is a modified version of the TUNEL assay; it quantifies the incorporation of biotinylated dUTP at single-strand DNA breaks in a reaction that is catalysed by the template-dependent (unlike TUNEL) enzyme DNA polymerase I.

Sperm chromatin structure assay: The SCSA measures in situ DNA susceptibility to the acid-induced conformational helix–coil transition by AO fluorescence staining.

TB staining: Toluidine Blue (TB), has high binding affinity for phosphate residues of sperm DNA in immature nuclei and provides a metachromatic shift from light blue to a purple–violet colour.

CMA3 Assay: CMA3 is a guanine–cytosine-specific fluorochrome that reveals poorly packaged chromatin in spermatozoa and is the indirect measure of protamine deficiency in sperm DNA.

AAB staining: Aniline blue is an acidic dye that has more binding affinity with the proteins in decondensed or loose chromatin due to the residual histones. AAB staining differentiates between lysine-rich histones and arginine/cysteine-rich protamines.

DBD FISH assay: The DBD–FISH is a technique that can detect DNA breaks in single cells, not only in the whole genome but also in specific sequences of DNA.

Measurement of 8-OHdG: This assay measures levels of 8-OHdG, which is a by-product of oxidative DNA damage, in spermatozoa.

Q7. How to assess round cells in semen sample?

Round cells are a type of cell found in semen, which can be either immature germ cells or leukocytes (white blood cells). They can be of spermatogenic origin or non-spermatogenic origin.

Classification of round cells:

1. **Leukocytes:** These are white blood cells that can be present in semen due to inflammation, infection, or immune. Leukocytes include granule-containing neutrophils, eosinophils and basophils, along with lymphocytes (T and B cells) and monocytes (precursors of macrophages), all which play a vital role in the body's immune system.
2. **Immature germ cells:** These are cells that are present in semen due to incomplete maturation of sperm cells. Immature germ cells can be further classified into different types, such as spermatogonia, spermatocytes, and spermatids.

Identification of Round Cells:

There are several other techniques for quantifying the leukocyte population in semen. As peroxidase-positive granulocytes are the predominant form of leukocytes in semen, routine assay of peroxidase activity is useful as an initial screening technique , Examine the chamber grid by grid with phase contrast optics at ×200 or ×400 magnification, Count at least 200 peroxidase-positive cells in each replicate, to achieve an acceptably low sampling error. Peroxidase-positive cells are stained brown, while peroxidase-negative cells are unstained .

Round cells can be identified using rapid romanowsky stain, papinicolau stain, photomicrography and phase contract microscopy. These techniques can help identify leukocytes and immature germ cells based on their nuclear and cytoplasmic characteristics. The WHO manual sixth edition for semen quotes that if the round cells are more than 1 × 106mL, they should be differentiated to see for leucocytes.

Clinical Significance The presence of round cells in semen can be an indicator of various conditions, including:

1. **Inflammation:** The presence of leukocytes in semen can indicate inflammation or infection in the reproductive tract.
2. **Immune responses:** The presence of leukocytes in semen can indicate immune responses against sperm cells or other cells in the reproductive tract.
3. **Spermatogenic dysfunction:** The presence of immature germ cells in semen can indicate spermatogenic dysfunction or incomplete maturation of sperm cells.

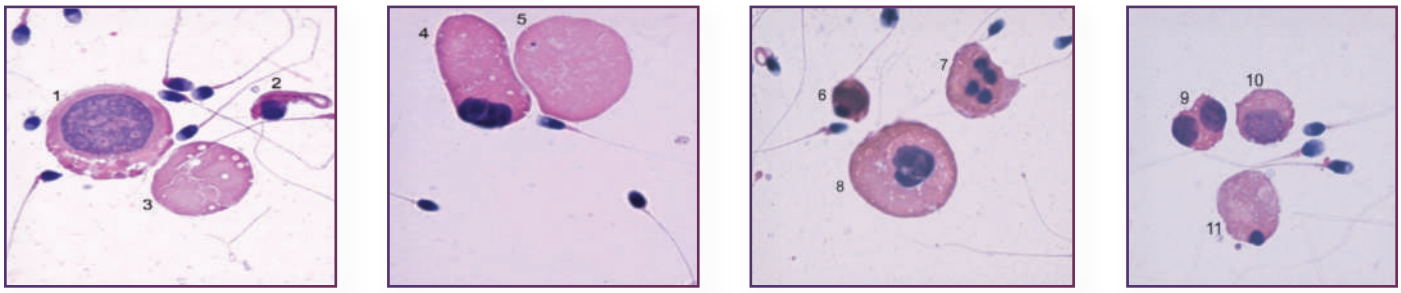


Fig 6: (papnicolou stained slides)

1- macrophage
2- abnormal spermatozoon
3,5- cytoplasm
4,9- dividing spermatid
6- not classifiable
7,11- degenerating spermatid
8- degenerating spermatid?
10- spermatocyte

Q8. What are the characteristics of morphologically normal sperm?

According to strict accepted criteria considering normal spermatozoan are:

Head morphology

Sperm head is the foremost part of the sperm cell. It contains acrosome and nucleus.

Head Shape

Sperm head is considered to be 3–5 µm in length and 2–3 µm in width with perfect oval shape.

1. Smooth: The head should have a smooth surface, without any noticeable irregularities.
2. Regularly contoured: The head should have a regular, even shape.
3. Generally oval: The head should be approximately oval in shape.

Acrosomal Region

1. Well-defined: The acrosomal region should be clearly visible and well-defined.
2. 40-70% of head area: The acrosomal region should occupy 40-70% of the total head area.
3. No large vacuoles: There should be no large vacuoles present in the acrosomal region.
4. Not more than two small vacuoles: There should be no more than two small vacuoles present in the acrosomal region.
5. Vacuoles should not occupy more than one fifth of the sperm head: Any small vacuoles

Post-Acrosomal Region

1. No vacuoles: There should be no vacuoles present in the post-acrosomal region.

Midpiece Morphology

The midpiece is the middle section of a sperm cell, connecting the head to the tail. It 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.
It plays a crucial role in providing energy for the sperm's movement through the mitochondria that wrap around the axoneme.

1. Slender and regular shape: The midpiece should be slender and have a regular shape, with no noticeable irregularities or distortions.
2. Length approximately the same as the sperm head: The length of the midpiece should be approximately the same as the length of the sperm head.
3. Major axis aligned with the sperm head's major axis: The major axis of the midpiece should be aligned with the major axis of the sperm head, indicating proper orientation and alignment of the sperm cell's structures.

Principal Piece Morphology

The principal piece is the longest section of the sperm tail, extending from the midpiece to the endpiece. It is at least 45 µ in length.

1. Uniform calibre: The principal piece should have a uniform diameter along its entire length.
2. Thinner than the midpiece: The principal piece should be thinner than the midpiece.
3. Approximately 45 µm long: The principal piece should be approximately 45 µm long, which is about 10 times the length of the sperm head.
4. Looped or coiled: The principal piece may be looped back on itself, but there should be no sharp angulation or kinking that would indicate a broken flagellum.

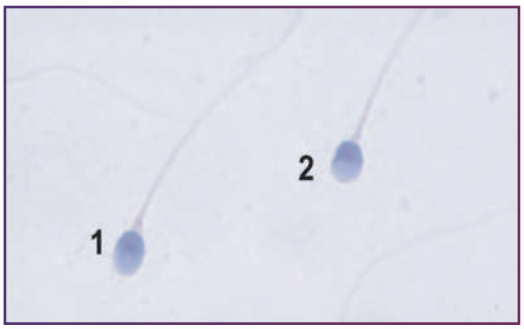


Fig 7:
1. Normal sperm
2. Abnormal sperm

Amorphous head shape

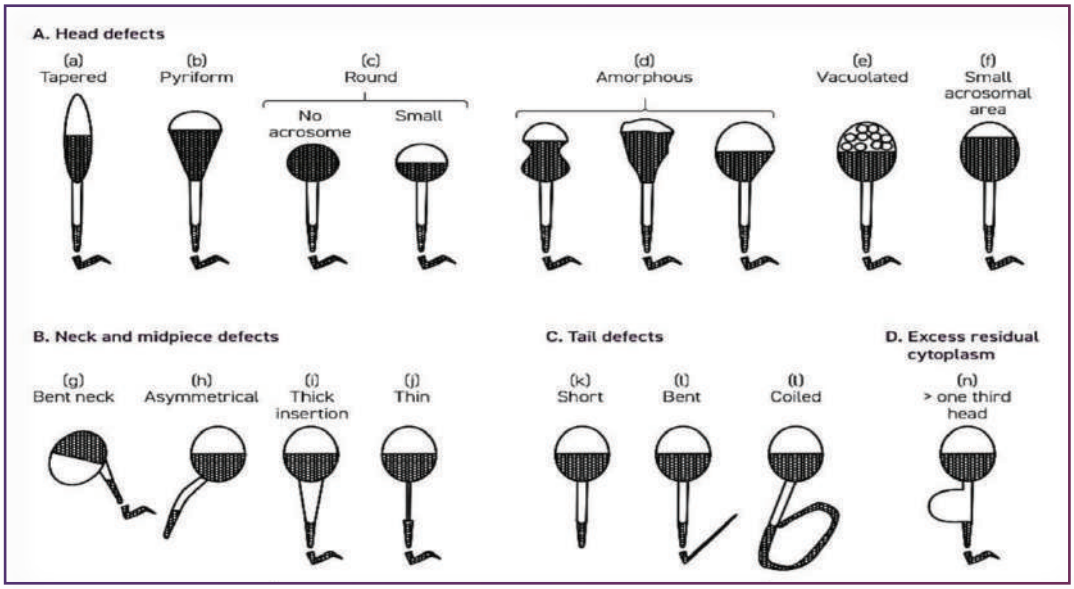


Fig 8: Sperm abnormalities

Q9. Discuss the methods to assess the morphology of sperms.

The morphology is evaluated by using air dried smears prepared from whole semen on glass slides and stained.

The sperm morphology can be assessed by staining methods:

Papinicolau staining:

Recommended method for sperm morphology assessment staining. Gives the best overall visibility of all regions of the human spermatozoon. The head is stained pale blue in the acrosomal region and dark blue in the post acrosomal region. It stains the acrosomal and post-acrosomal regions of the head, excess residual cytoplasm, the midpiece and the principal piece. The midpiece may show some red staining, and the tail is stained blue or reddish. Excess residual cytoplasm, usually located behind the head and around the midpiece, is usually stained green; if coloured reddish, it can indicate other abnormalities. The Papanicolaou stain also gives good staining of other cells. Slides stained using the Papanicolaou procedure if stored in the dark, they should be stable for months or years. Reagents used: Harris's haematoxylin, fixative, G–6 orange stain, EA–50 green stain, xylene.

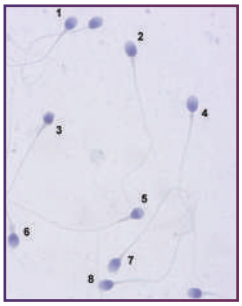


Fig 9:
Normal- 1, 6, 7
Abnormal- 2, 3, 4, 5

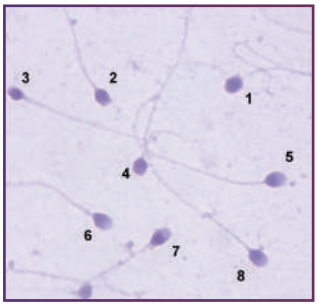


Fig 10:
Normal- 5
Abnormal- 1, 2, 3, 4, 6, 7, 8

Shor staining:

The Shorr stain provides similar percentages of normal forms as the Papanicolaou stain. Reagents used: Harris's haematoxylin, shorr solution, acetic ethanol, ammoniacal ethanol.

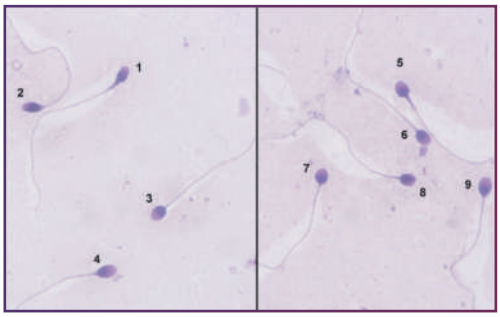


Fig 10:
Normal- 3, 6, 9
Abnormal- 1, 2, 4, 5, 7, 8

Rapid staining- diff quick:

Rapid staining methods can be useful when results are necessary the same day. Smears stained by rapid procedures have high background staining and may be of lower quality than those stained with Papanicolaou stain. More important, the sizes of the fixed and stained sperm heads differ in comparison with the Papanicolaou staining.
Reagents used: diff quick rapid kit- fixative, eosinophilic
xanthene, basophillic thiazine.

Preferable 200 spermatozoan are counted for various abnormalities and only those showing all parts normal are considered normal. The slides can be viewed unmounted or mounted, but mounted slides can be used in training and for IQC and intra-laboratory comparison. In addition, there is no risk of contamination of microscope objectives when slides are properly mounted.

Q10. 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, cortical reaction, sperm oocyte membrane fusion, decondensation of sperm nucleus and finally fusion of male and female pronucleus.

Acrosome reaction

The primary purpose of the acrosome reaction is to enable the sperm to penetrate the zona pellucida (ZP), the outer glycoprotein layer of the oocyte. The released enzymes, such as acrosin and hyaluronidase, break down the ZP, allowing the sperm to reach the oolemma, the plasma membrane of the oocyte. The acrosome reaction is triggered by the interaction between the sperm and the ZP.

Sperm-zona Pellucida interaction

Sperm bind to the ZP through specific receptors, such as ZP3, which recognize and bind to the sperm protein, acrosin. The binding of sperm to ZP induces the acrosome reaction, which releases enzymes that break down the ZP. The released enzymes, such as acrosin and hyaluronidase, break down the ZP, allowing the sperm to penetrate and reach the oolemma. After penetrating the ZP, the sperm bind to the oolemma through specific receptors, such as integrins.

Cortical reaction

The oocyte undergoes a cortical reaction, releasing cortical granules that alter the ZP and prevent polyspermy. The cortical reaction triggers a series of events that activate oocyte development and prepare the oocyte for embryonic development.

Oolemma binding and fusion

The sperm binds to the oocyte plasma membrane through specific receptors. Their membranes adhere to each other, facilitated by adhesion molecules. The sperm plasma membrane fuses with the oocyte plasma membrane, forming a fusion pore.

Decondensation of sperm nucleus

It is the first visible change in the fertilizing spermatozoon upon entry into the ooplasm and a prerequisite for pronuclear formation and syngamy. Decondensation of the sperm nucleus refers to the process by which the highly condensed sperm chromatin is relaxed and transformed into a more open, euchromatic structure.

Finally fusion of male and female pronucleus

Pronuclear fusion or karyogamy, is the process by which the genetic material from the sperm and oocyte combine to form a single nucleus. It combines the genetic material from the sperm and oocyte. Also restores the diploid number of chromosomes (46) and finally forms a single nucleus.
These crucial steps during sperm oocyte interaction ensure proper fertilization and the formation of a healthy zygote.

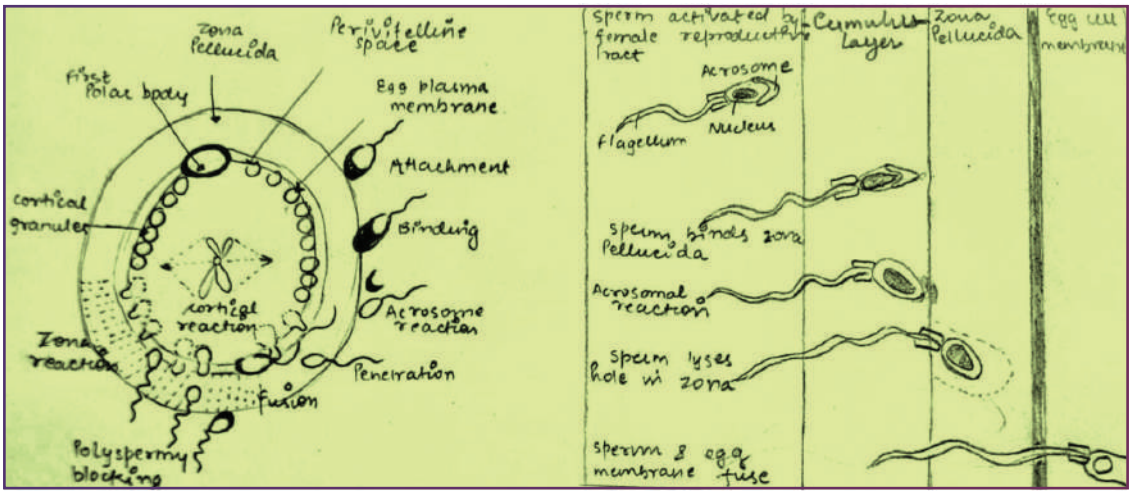


Fig 12: Sperm-oocyte interaction

Q11. What is 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

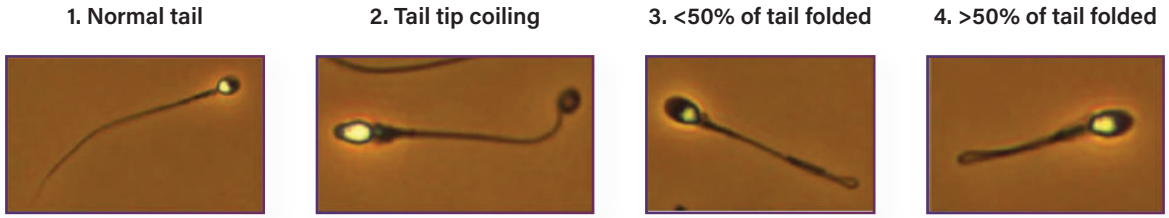


Fig 13: Tail coiling in HOST reacted sperms

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.

Q12. What is PICSI (Physiologically selected intracytoplasmic sperm injection)?

PICSI - physiologically selected intracytoplasmic sperm injection technique is based on the fact that the mature sperm head has a specific receptor that allows it to bind to hyaluronic acid (HA), the main component of the cumulus oophorous; this is in contrast to the immature spermatozoa. This technique is used to select sperm for ICSI, it involves placing the sperm in a dish with hyaluronic acid (HA,a natural compound found in the body. Selection is based on identifying sperm that can bind to hyaluronic acid. The bound sperm are considered mature and physiologically normal and are selected for fertilization. Spermatozoa that bind to HA have completed the spermatogenic process of remodeling the plasmatic membrane, cytoplasmic extrusion and nuclear maturity. Thus, they have a whole DNA and low frequency of aneuploidies and miscarriages.

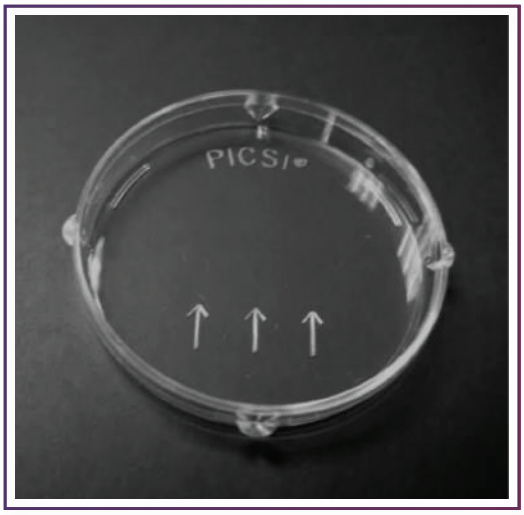


Fig 14: PICSI Dish

PICSI is indicated for the patients with:

- Previous failure or low fertilization rate even after ICSI.
- Poor embryo quality or their failure to develop (not related to poor egg quality).
- Repeated miscarriages.
- High fragmentation of sperm chromatin.

Q13. 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. Vitality test is not necessary when at least 40% of spermatozoa are motile. In samples with poor motility 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.

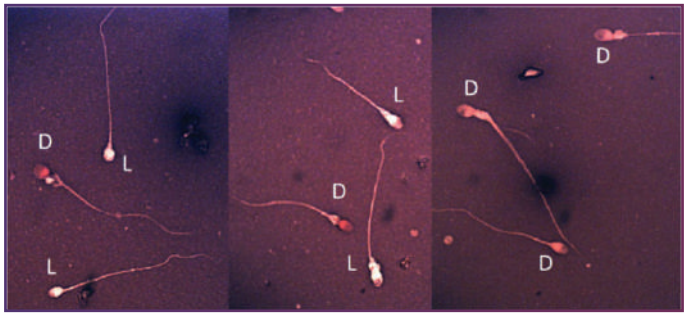


Fig 15: Eosin-Nigrosine staining

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.

Q14. 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 <1ml,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 concentration, 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.it must be remembered that as sample ages the fructose level will fall due to the utilization of fructose by spermatozoa.

Q15. 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. Anti-sperm - 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

Q16. What is MACS? What are the advantages of MACS.

Magnetic-Activated Cell Sorting (MACS) is an advanced technique that has been adapted for sperm sorting, in assisted reproductive technologies (ART).

Principle of MACS

MACS is employed to isolate and select sperm cells based on specific surface markers. Apoptotic spermatozoa contain externalized trans-located phosphatidylserine residues from the inner side of the plasma membrane to the outer side (early sign of apoptosis). Super-paramagnetic micro beads conjugated with annexin-V have a high affinity to phosphatidylserine but cannot pass through the intact sperm plasma membrane. The procedure involves labeling sperm cells with these beads. Annexin V binded sperms indicate compromised membrane integrity.

these beads. Annexin V binded sperms indicate compromised membrane integrity.

Procedure for Sperm Sorting Using MACS:

Fig 16: MACS

- **Labeling with Magnetic Beads:** Super paramagnetic beads coated with annexin V are added to the washed semen sample and mixture is incubated.
- **Separation:** The mixture is then loaded on top of separation column placed in a magnetic field. It is then allowed to pass through it. Apoptotic sperm cells bound to the magnetic beads are retained in the column, while unbound sperm cells (non-apoptotic) pass through.
- **Elution:** The column is removed from the magnetic field, the annexin V positive fraction is eluted from the column using annexin V binding buffer.

Advantages of MACS for Sperm Sorting:

1. **Enhanced Sperm Quality Selection:** The combination of MACS with DGC (density gradient centrifugation) yields a clean sperm population characterized by higher motility, viability, morphology, reduced apoptosis manifestations (including DNA fragmentation) and increased cryosurvival rates.
2. **Non-invasive and Gentle:** The process does not harm the sperm, maintaining their viability, and allows for the selection of sperm based on surface markers without the need for harsh chemical or mechanical methods.
3. **Improved Pregnancy Outcomes:** The selection of non apoptotic human spermatozoa after MACS
   - improves sperm fertilization potential
   - increases cleavage and pregnancy rates in oligoasthenozoospermic ART cases after ICSI
   - resulted in an ongoing pregnancy achieved with a clear reduction in the percentage of sperm DNA fragmentation
   - may be considered as a molecular preparation technique that complements conventional sperm preparation protocols (DGC) and may enhance ART success rates.

Q17. Describe the sperm kinematic measurements in computated semen analysis.

Straight-line velocity (VSL): Time average velocity of the sperm head along a straight line from its first position to its last position.
Curvilinear velocity (VCL): Time average velocity of the sperm head along its actual trajectory.
Average path velocity (VAP): Time average velocity of the sperm head along its average trajectory.
Linearity (LIN): Linearity of the curvilinear trajectory (VSL/VCL)
Wobble (WOB): Degree of oscillation of the actual sperm head trajectory around its average path (VAP/VCL).
Straightness (STR): Straightness of the average path (VSL/VAP)
Amplitude of lateral head (ALH): Amplitude of variations of the actual sperm head trajectory about its average trajectory displacement. The average trajectory is computed using a rectangular running average.

about its average trajectory displacement. The average trajectory is computed using a rectangular running average.
Riser displacement (RIS): Point-to-point distance of the actual sperm head trajectory to its average path. The average path is computed using an adaptive smoothing algorithm.
Beat-cross frequency (BCF): Time average rate at which the actual sperm trajectory crosses the average path trajectory.
Frequency of the fundamental (HAR): Fundamental frequency of the oscillation of the curvilinear trajectory around its average harmonic path. HAR is computed using the Fourier transformation.
Magnitude of the amplitude (MAG): Squared height of the HAR spectral peak. MAG is a measure of the peak to fundamental harmonic peak dispersion of the raw trajectory about its average path at the fundamental frequency.
Area of fundamental harmonic (VOL): Area under the fundamental harmonic peak in the magnitude spectrum. VOL is a harmonic measure of the power-bandwidth of the signal.
Specimen concentration (CON): Concentration of sperm cells in a sample in millions of sperm per mL of plasma or medium.
Percentage motility (MOT): Percentage of sperm cells in a suspension that are motile (in manual analysis, motility is defined by a moving flagellum; in computer-assisted semen analysis, motility is defined by a minimum VSL for each sperm).

Complete diagram of a Human Sperm Cell

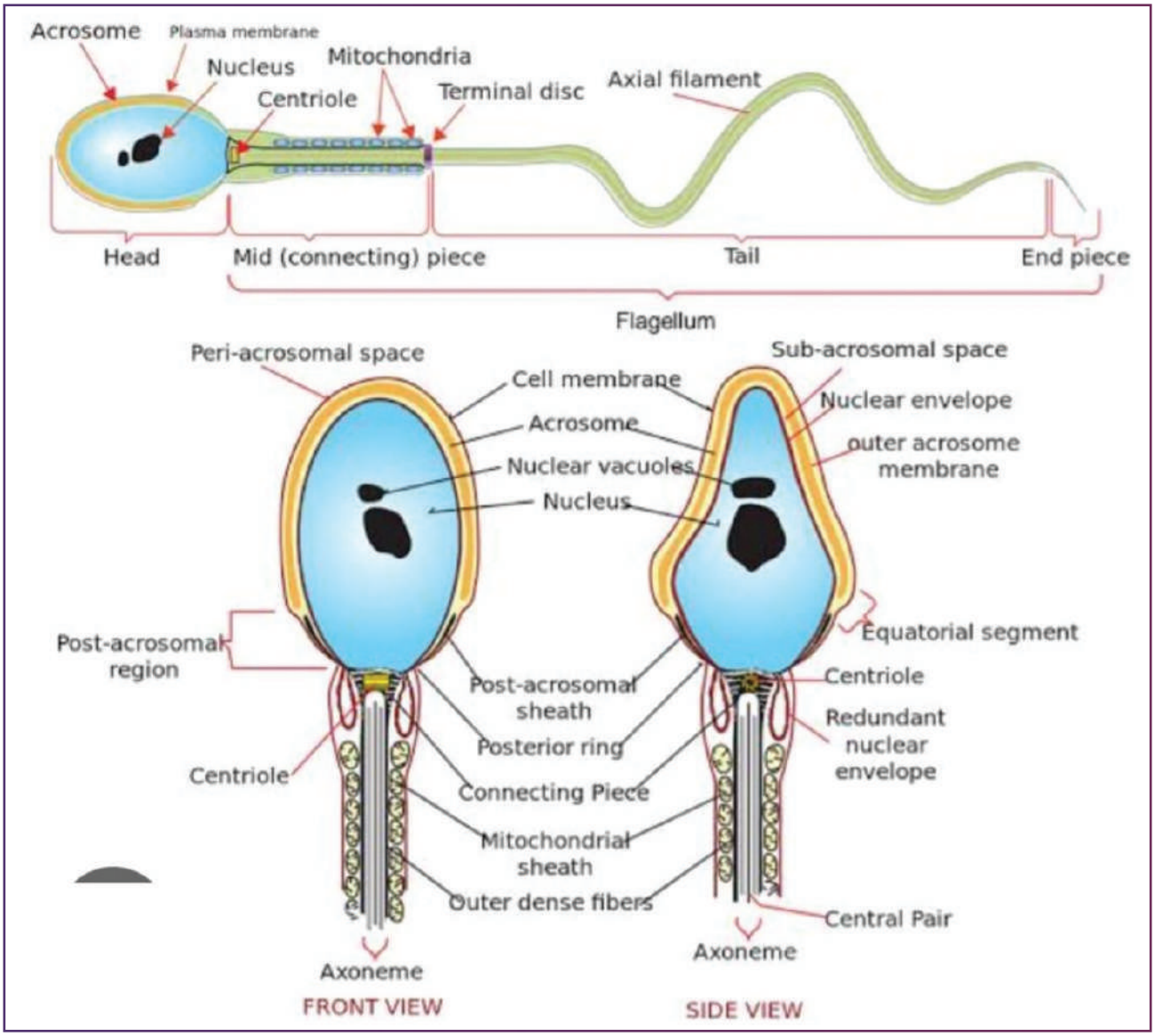


Fig 17: Sperm

Calculate the total number of abnormal spermatozoa

↓

Calculate Teratozoospermia Index (TZI)

TZI = Total abnormalities / Total abnormal spermatozoa

Interpret TZI results:
TZI < 1.6: Normal
TZI ≥ 1.8: Abnormal

Flowcharts:

A4: Teratozoospermia Index

Evaluate semen sample morphology

↓

Identify abnormal spermatozoa with defects in: head (H), midpiece (M), principal piece (P), excess residual cytoplasm (ERC)

↓

Count the number of abnormalities per abnormal spermatozoon

↓

Maximum of 4 defects per spermatozoon (H, M, P, ERC)

↓

vCalculate total abnormalities

↓

Calculate the total number of abnormal spermatozoa

↓

Calculate Teratozoospermia Index (TZI)

↓

TZI = Total abnormalities / Total abnormal spermatozoa

Interpret TZI results:
TZI < 1.6: Normal
TZI ≥ 1.8: Abnormal

A6: DNA Fragmentation Index Methods

COMET ASSAY

Slide preparation with NMP gel, place sperms mixed in LMP gel over NMP agrose gels slide

↓

Incubate slide in refrigerator followed by treatment
with lysis solution, lithium didiiodosalicyclate (LIS)

↓

Electrophoresis is carried out

↓

After electrohphoresis, stain with a stoichiometric DNA dye

↓

50 comets per slide are then analyzed

TUNNEL ASSAY

Aliquot of semen sample containing 2million - 10 million sperm to be washed twice with buffer and pellet is suspended in 500µl paraformaldehyde (fixation)

↓

Centrifuge in phosphate buffer and wash the pellet twice again

↓

Add buffer containing citrate and triton and incubate on
ice (permeabilzation), for stopping permeabilzation
centrifuge in phosphate buffer

↓

Sample is then divided into two aliquots – negative
control (TDT -) and test sample (TDT +) and an extra
aliquot (positive control) - incubated with micrococcal
nuclease or DNase I recombinant after permeablization

↓

All aliquots are centrifuged and resuspended

↓

Resuspension:
TDT - : buffer with labeled probe
TDT + and positive control: buffer with labeled probe
and TDT

↓

Incaubte for 1 hour

SCD

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 immediately onto 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 (acid DNA unwinding) 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)(stains) and leave to stain for 15-20 minutes

↓

Decant the stain and wash with distilled water

↓

Dry at RT

↓

Examine in Phase contrast microscope

A9: Sperm morphology staining methods

PAPINICOLOU STAINING

Fix slide in ethanol for at least 15 minutes

↓

Treat the slide in graded ethanol 80% for 30 seconds followed by 50% for 30 seconds

↓

Incubate the slide in purified water then stain the slide with Harris haematoxylin

↓

Incubate the slide in purified water, dip the slide in
acidic ethanol (4-8 dips), again place slide in purified water then wash under cold tap water

↓

Then treat the slide in increasing ethanol concenterations 50% (for 30 seconds), 80% (for 30
seconds), 95% (for 15 minutes)

↓

Now stain the slide in G-6 orange stain and treat
the slide in 95% ethanol three times for 30 seconds

↓

Stain the slide with EA-50 green stain followed by
treating in 95% ethanol for 30 seconds (2 times)
and 100% ethanol for 15 seconds (2 times)

↓

Mount the preparation either in ethanol-soluble
and ethanol-insoluble mountants.

SHOR STAINING

Fixation: immerse slides in acetic-ethanol or 75% (v/v) ethanol for 1 hour.

↓

Sequentially immerse the slides in:
running water 12–15 dips followed by
haematoxylin 1–2 minutes, running water 12–15 dips.

↓

Then dip the slide in ammoniacal ethanol followed
by running water 12–15 dips, 50% (v/v) ethanol 5 minutes.

↓

Expose the slide to shorr stain for 3–5 minutes

↓

Finally treat with increasing concentrations of ethanol 50% (v/v) ethanol 5 minutes, 75% (v/v) ethanol 5 minutes, 95% (v/v) ethanol 5 minutes.

↓

Observe the slide once slide dries.

RAPID STAINING: DIFF QUICK

Fixation: Immerse slides in triarylmethane fixative for 15 seconds or 95% methanol alone for 1 hour.

↓

Drain the excess solution by placing slides vertically on absorbent paper.

↓

Staining the fixed semen smear by sequentially immerse the slides in:
1. rapid stain solution 1 {buffered solution of Eosin Y (an anionic dye)} - 10 seconds
2. rapid stain solution 2 {buffered solution of thiazine dyes (cationic dyes) consisting of methylene blue and Azure A} - 5 seconds
3. running tap water: 10–15 dips to remove excess stain.

↓

Drain the excess solution at each step by placing slides vertically on absorbent paper.

A11: HOST (HYPO OSMOTIC SWELLING)

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)

↓

Evaluate at least 200 sperm and observe sperm tail for curling (swelling) or non-curling (straight)

↓

Commonly observed sperm swelling patterns- Tip swelling, hairpin swelling, shortened & thickened tail, partly or completely enveloped sperm tail

A13: Eosin Nigrosine vitaility staining:

EOSIN ALONE

5 µl of ejaculate is mixed with 5 µl of eosin solution on a microscope slide.

↓

Cover immediately with coverslip and leave for 30 seconds.

↓

vExamine the slide with negative phase contrast optics at ×200 or ×400 magnification.

↓

Tally the number of stained (dead) and unstained (vital) cells with the aid of a laboratory counter. Evaluate 200 spermatozoa, to achieve an acceptably low sampling error.

↓

Calculate the proportion of live cells.

↓

Report the percentage of vital spermatozoa to the nearest whole number.

EOSIN-NIGROSINE

cTake 50-µl aliquot of semen, mix with an equal volume of eosin–nigrosin suspension and wait for 30 seconds.

↓

Make a smear on a glass slide and allow it to dry in air.

↓

Examine immediately after drying or later after mounting with a permanent non-aqueous mounting medium.

↓

Examine the slide with brightfield optics at ×1000 magnification and oil immersion.

↓

Tally the number of stained (dead) or unstained (live) cells with the aid of a laboratory counter.

↓

Evaluate at least 200 spermatozoa, to achieve an acceptably low sampling error.

↓

Report the percentage of vital spermatozoa to the
nearest whole number.

A16: MACS

100 µLsperm sample is mixed with 100 µL of MACS microbeads and incubated at room temperature for 15 minutes

↓

The mixture is loaded on top of the separation column which is placed in the magnetic field. The column is rinsed with buffer.

↓

All the unlabeled (annexinV-negative) non-apoptotic spermatozoa pass through the column.

↓

The annexinV-positive (apoptotic) fraction is retained in the column.

↓

The column is removed from the magnetic field, and annexinV-positive fraction is eluted using the annexinV-binding buffer.

REFERENCES

Q1-Q4, Q7-Q9, Q11, Q13-Q15 : WHO Andrology Manual Sixth Edition 2021

Q5-Q6:
- WHO Andrology Manual Sixth Edition 2021
- Textbook of Assisted Reproductive Technqiues, Sixth edition, Volume 1, By David K. Gardner

Q10: Anifandis G, Messini C, Dafopoulos K, Sotiriou S, Messinis I. Molecular and cellular mechanisms of sperm-oocyte interactions opinions relative to in vitro fertilization (IVF). Int J Mol Sci. 2014 Jul 22;15(7):12972-97. doi: 10.3390/ijms150712972. PMID: 25054321; PMCID: PMC4139886.

Q12: Physiological ICSI (PICSI) vs. Conventional ICSI in Couples with Male Factor: A Systematic Review, Georgina et al. doi: 10.5935/1518-0557.20180027; Pacheco, A. Blanco, F. Bronet, M. Cruz, J. García-Fernández, and J. A. García-Velasco

Q16:
- WHO Andrology Manual Sixth Edition 2021
- https://www.google.com/url?sa=t&source=web&rct=j&opi=89978449&url=https://www.eshre.eu/-/media/sitecore files/SIGs/Embryology/Archive/Maribor/Magnus.pdf&ved=2ahUKEw-j_28b-9bqLAxWGs1YBHTapIsAQFnoECBwQAQ&usg=AOvVaw1vRL2ipUbP8w7UIThf4zwY

Q17: Textbook of Assisted Reproductive Technqiues, Sixth edition, Volume 1, By David K. Gardner