Second Issue
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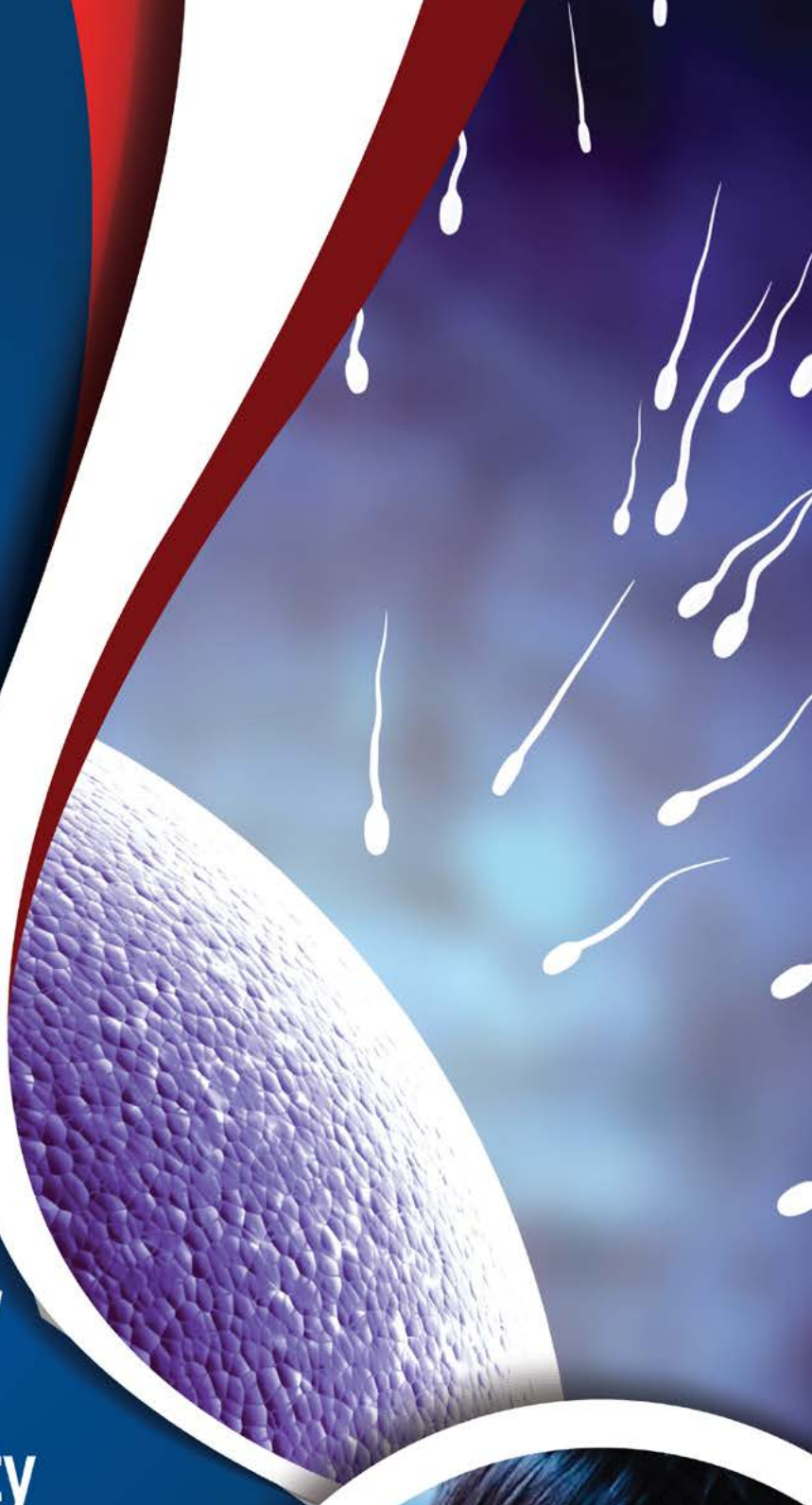


NEXUS

Indian Fertility Society & Origio, India Initiative

SEMEN ANALYSIS : Nuts & Bolts

www.indianfertilitysociety.org

It is a great privilege and pleasure to write this message for the second e-bulletin of IFS-Nexus. First bulletin titled “IUI : Nuts and Bolts” was appreciated by all and has turned out to be a ready reckoner for the beginers and experienced ART clinicians. The idea of Nexus has been initiated to bridge the gap between ART Clinicians and Embryologists. It aims to enhance the awareness about quality control, basic IVF techniques and lab protocols within the IVF community. On behalf of the Indian Fertility Society I sincerely thank “ORIGIO India Private Ltd” to partner with us in this great academic endeavor. Our third edition would be titled “Sperm Function Test : Demystified” and I am sure would educate all of us further.

My heartiest congratulations to Dr Pankaj Talwar, ORIGIO and the entire team and very best wishes fort the future.

Dr. Sohani Verma
President- IFS

Indian fertility Society feels proud and congratulates the editors on the launch of second edition of Nexus e-Bulletin: A journal to enlighten us and broaden the spectra of knowledge in Embryology & Andrology. We take it as our duty and responsibility to train and educate our budding embryologist and infertility specialists right from the basics and this bulletin is a step forward in this direction. It would not only help to disseminate scientific & ethical content but also constantly update everyone with new researches and developments across the globe.

Dr. K.D. Nayar
General Secretary-IFS

At the very onset the editorial team would like to thank all of you for positively appreciating our 1st bulletin titled “IUI - Nuts and Bolts”. Team nexus sincerely hopes to bring out such teaching material for you every month.

Such bulletins are call of the day and immensely bridges the gap between our friends in the peripheries and all those who does not have access to correct information.

Our present edition is focussed on simplifying the process of semen analysis and i am sure it will immensely benefit the readers. ART services require complex interaction between the clinical directors, embryologist and technicians for smooth functioning of the establishment. Unfortunately at the present 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.

This bulletin has been named **NEXUS - which means building bridges**. The primary aim is to bridge knowledge gap between the team members and shorten the learning curve. Keeping these thoughts in mind IFS has decided to bring out monthly bulletin on issues which are common to all the team members. 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 “Sperm Function Test :Demystified”.

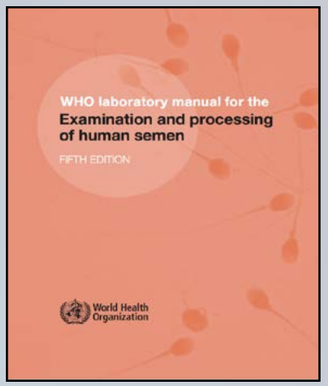
We would formally like to thank my friend Dr Ashish Fauzdar PhD, Scientist I (Embryology) at ESI-PGIMSR Hospital, Basaidarapur, New Delhi who has un-relentlessly worked towards bringing out this issue from conception to end.

We would also like to place on record our sincerest 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.

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

Lower reference limits (5th percentiles and their 95% CIs) for semen characteristics	
Parameters	**Lower reference limit (range)**
Semen volume (mL)	1.5 (1.4-1.7)
Total sperm number (10^6/ejaculate)	39 (33-46)
Sperm concentration (10^6/mL)	15 (12-16)
Total motility (PR + NP)	40 (38-42)
Progressive motility (PR, %)	32 (31-34)
Vitality (live spermatozoa, %)	58 (55-63)
Sperm morphology (normal forms, %)	4 (3.0-4.0)
Round cell concentration	Less than $1x10^6$ per ml
CIs=confidence intervals; NP=non-progressive; PR=progressive	



Frequently Asked Questions (FAQs)

1. What all things do we require for performing semen analysis in Andrology Laboratory?

Disposables Required: Centrifuge tubes, Transfer pipettes, glass slides, glass cover slips, Semen Collection Container (Disposables should be ART Grade & gamma sterilized) **(Fig. 1a)**.

Equipments Required: Dry heat bath & warming plate. Bright field phase contrast microscope with 10X, 20X, 40X, 100X objectives, Laminar flow hood & Sperm counting chambers. Dry heat Incubator & Centrifuge machine are optional **(Fig. 1b)**.

Figure 1a

Figure 1a: Gamma sterilized disposables required for processing semen samples including centrifuge tubes, transfer pipettes etc.

Figure 1b

Figure 1b: Dry heat bath & warming plate is required for semen analysis.

2. How to collect the Semen sample?

The sample should be collected in a private room near the laboratory, in order to limit the exposure of the semen to fluctuations in temperature and to control the time between collection and analysis. The sample should be obtained by masturbation and ejaculated into a sterile clean wide-mouthed container.

3. When to do semen analysis and the recommeded abstinence interval for collecting samples?

The samples with abnormal semen parameters (low count and motility) in previous report will be given final opinion after doing repeat examination done after minimum of two months. There is no absolute requirement for doing repeat analysis in samples with normal semen parameters.

The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence.

4. How to select the semen collection containers for semen analysis?

The semen collection container has to be tested for toxicity of the plastic and sterility before collecting patient sample in them. This is done by placing one half of the semen sample (high concentration, good motility) in a container that is known to be of non toxic material for semen sample (control). The other half of the sample is transferred in the new container being tested. The sperm motility is assessed at hourly intervals in replicate at room temperature or at 37°C for 4 hours. If there are no differences in motility between control and test then the test container can be considered non-toxic to spermatozoa and suitable for semen collection.

A semen collection container has to be clean, sterile and free of any microorganism for being suitable for semen analysis ***(Fig 4)***.

Figure 4

Figure 4: A wide mouthed flat bottom sterile non toxic semen sample collection container.

5. How to counsel the male partner for collection of semen sample?

- The man should be given clear oral/written instructions for the collection of semen sample.
- It should be emphasized to patient that the semen sample needs to be complete i.e. all the ejaculate has to be collected including the first, sperm-rich portion.
- The man should inform or report any loss of any fraction or spillage of the sample accidently.
- Sample should be collected in the labeled container with the man's name and identification number and the date and time of collection.

6. How to avoid mixing of semen samples in the laboratory?

The sample containers should be labeled with minimum of 2 patient identifiers that include patient name and unique identification number to avoid mixing of semen samples. An ejaculated semen sample obtained by masturbation has to be collected in separately labeled container preferably with both husband and the wife name.

7. Who should perform semen analysis in the laboratory?

Semen Analysis has to be performed by Embryologist/Andrologist who is trained and experienced in processing samples as per methodology described in 5th Edition of WHO manual.

8. What are the essential examinations to be carried out while performing semen analysis?

The essential macroscopic and microscopic examinations that should be done in the liquefied sample after 15-30 minutes of sample collection are:

- **Macroscopic Examination:** Liquefaction, Semen viscosity, Appearance of ejaculate, Semen volume, Semen pH
- **Microscopic Investigation:** Sperm number, Sperm motility, Sperm vitality, Sperm morphology

9. Describe the time frame for performing tests for semen analysis?

Ejaculated Semen Sample

Within first 5 minutes

Place the sample at the room temperature or at 37°C in an incubator

Between 30-60 minutes

- Appearance and Colour of the Ejaculate
- Semen Liquefaction, Semen Volume, Semen pH
- Sperm Motility & Sperm concentration, Sperm vitality test by wet preparation
- Sperm morphology smears
- Assessing peroxidase- positive cells (if round cells are present)
- Biochemical markers

Within 3 hours

Microbiological/culture studies

After 4 hours

Sperm Morphology (by Fixing, Staining)

Later on same day (or subsequent day if sample is frozen)

Assess accessory gland markers, performing indirect immunobead test

10. How to calculate the Semen volume?

The volume of the ejaculate is mainly contributed by seminal vesicles and prostate gland with small amount of fluids from the bulbourethral glands and epididymides. Precise measurement of volume is essential for semen evaluation as it included total number of spermatozoa and non-sperm cells.

The volume is best measured by weighing the sample in the vessel as per the following steps:

- Collect the sample in a pre-weighed, clean, disposable semen collection container.
- Weigh the collection container with semen (sample) and subtract the weight of the container to calculate the mass (weight) of semen sample
- Calculate the volume from the sample weight, by assuming the density of semen to be 1 g/ml (range 1.04-1.10g/ml); [Formula: Volume= Mass/Density]

Note:

- Empty specimen containers may have different weights, so each container has to be weighed separately.
- Measuring the volume of the ejaculate by aspirating the sample from the specimen container into a pipette or a syringe, or decanting it into a measuring cylinder, is not recommended. The coating of semen sample on the above disposables will lead to inacurrate estimation of the exact volume.

11. What inferences can be drawn if we have low volume sample?

The most of the semen ejaculate volume is contributed by secretions from seminal vesicles and prostate (>90%) gland. If there is low semen volume (≤1.5 ml) one should first rule out the possibility of spillage of semen sample during collection and then confirm the results by repeat semen analysis ***(Fig. 11)***.

After confirming the low semen volume then one should suspect the other physiological reasons for some kind of abnormality or obstruction as highlighted below.

- Seminal vesicles are abnormal or obstructed
- Ejaculatory ducts are obstructed
- Ejaculatory dysfunction (failure of emission or retrograde ejaculation)
- Absent Fructose and acidic pH are suggestive of ejaculatory and obsrtuction or seminal vesicle patholgy.

Figure 11a, b

Figure 11: Liquefied semen sample with low (≤1.5ml) semen volume.

12. What inferences can be drawn from increased volume sample?

The increased semen volume could be due to infection in seminal vesicles and prostate gland (prostatitis). The infection could result from presence of leukocytes and inflammatory substances in the accesory glands and male genital tract.

13. Define Liquefaction and Viscosity and elaborate its Clinical relevance?

Semen Parameters	Liquefaction	Viscosity
Definition	Conversion of ejaculated semisolid coagulated semen sample into completely **homogeneous watery liquid** by proteolytic action of enzymes released from prostate gland ***(Fig.13:a-b).***	Viscosity is **homogenous stickiness and consistency** and describes the unique quality of semen sample to stick to itself.
Clinical Relevance	Delayed liquefaction signifies the decreased proteolytic activity of prostrate enzymes. Thus It **interferes** in the both **macroscopic evaluation** and **microscopic investigation** of semen analysis	High viscosity can **interferes** with **semen analysis** and determination of **sperm motility, sperm concentration.** **It will also interfere** in detection of antibody-coated spermatozoa and measurement of biochemical markers

Figure 13a

Figure 13a,b: A viscous semen sample with homogenous stickiness and consistency.

Figure 13b

14. How would you handle the delayed liquefaction of semen sample?

Semen samples sometime can have delayed liquefaction or may not liquefy making semen evaluation difficult. In these situations, additional pretreatments are proposed including mechanical mixing and enzymatic digestion to liquefy the sample. This can be done by any of the method to promote liquefaction as summarized below.

- Addition of physiological medium (e.g. Sperm Preparation Media, Dulbecco's phosphate-buffered saline) to an equal volume of semen sample followed by repeated pipetting.
- Mechanically in-homogeneity can be reduced by repeated (6–10 times) gentle passage through a blunt gauge 18 (internal diameter 0.84 mm) needle attached to a syringe.
- Enzymatic Digestion by Bromelain proteolytic enzyme (10IU/ml) prepared in Dulbecco's phosphate-buffered saline) by mixing equal volume of semen sample in 1:2 ratio.

15. What are the indications for perfoming advanced sperm function test?

The patient with normal semen parameters may not be requiring any specialized tests. But in many cases of fertilization failures, sample with low sperm count and poor motility may require a battery of sperm function tests to evaluate different sperms parameters. Sperm function tests provide useful insights to deduce the exact reason for fertilization failure and also guide infertility experts to adopt best possible individualized treatment based on sperm function assay results.

Various sperm function tests have been proposed and further endorsed by different researchers in addition to the routine evaluation of fertility. Some of the commonly advised sperm function tests are as follows:

- Hyalauronon binding assay (HBA)
- DNA fragmentation index (DFI)
- Vitality staining
- Reactive Oxygen Species (ROS)
- Hypo-Osmotic Swelling (HOS) Test
- Semen Fructose detection

16. What is the role of Bromelain enzyme in semen analysis?

Occasionally semen samples may not liquefy (delayed liquefaction) during 40-60 minutes observation period. This makes semen analysis difficult. Pretreatment with bromelain, a proteolytic enzyme may help to promote liquefaction.

Bromelain (10 IU/ml): It is commercially available as ready to use solution. It is used by diluting the semen sample with equal volume of bromelain in 1:2 ratio and incubating the suspension at 37°C for 10 minutes ***(Fig 16)***

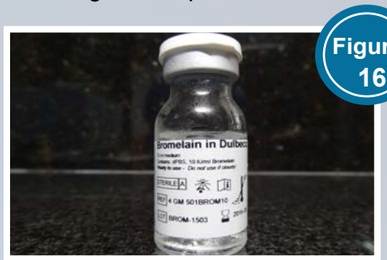


Figure 16: Bromelain helps to promote liquefaction. (Product Information: Bromelain10IU/ml, in dPBS, Cat no. 501 BROM10, Manufactured by Gynemed Medizinprodukete, GmBH & Co, Germany, Distributed by Astec India Pvt Ltd).

17. What is the role of microscopy in semen analysis?

Semen analysis is the first-tier screening test through routine microscopy and remains the cornerstone for the investigation of male infertility. It provides useful information about the sperm counts (millions/ml), sperm motility, viability or infection (pyospermia) of male reproductive tract.

For the semen evaluations, a phase-contrast microscope is recommended. The microscope, with at least a 50-watt light source, should preferably be binocular (have two eyepieces 10X), have a phase condenser and port for the camera attachment ***(Fig 17a)***.

Microscope should also include following accessories for Semen Analysis

- Objective with ×10 (yellow), ×20 (green), ×40 (blue) are required for assessment of motility, vitality, and counting of spermatozoa and non-sperm cells ***(Fig 17b)***.
- ×100 (white) oil-immersion bright field objective (for assessment of morphology and vitality)
- ×40 negative-phase objective (optional; for eosin vitality test)

Figure 17a: A phase-contrast microscope is recommended for assessing a semen sample.

Figure 17b: Positive phase objective ×10, ×20 and ×40 attached to the phase-contrast microscope for general assessment, motility, vitality, and counting of spermatozoa and non-sperm cells.

18. How would you handle the increased viscosity of semen sample?

Semen viscosity can be evaluated by introducing a glass rod into the sample and observing the length of the thread that forms upon withdrawal of the rod.

The viscosity is recorded as abnormal (or increased) when the thread exceeds 2 cm and this can be reduced by doing additional pretreatments

- Addition equal volume of physiological medium.
- Mechanical mixing by repeated gentle passage through broad gauge pipettes
- Enzymatic digestion by Bromelain.

19. How to calculate sperm concentration utilizing various sperm counting chambers?

The recommended method of calculating sperm concentration is through use of available sperm counting chambers. These are used for increased level of accuracy, precision for counting spermatozoa. The sperm counting chambers for semen analysis are recommended in WHO Manual 5th edition and include glass cover slip chamber, haemocytometer, Cell-VU, and Makler® chamber as described below.

- **Laboratory Slide and Glass Cover Slip**: The counting chamber using glass cover slip is the simplest method of calculating concentration of spermatozoa through wet preparations with chamber depth of approximately 20 µm deep. The volume of 10 µl of semen sample on a clean glass slide with a 22 mm × 22 mm coverslip (area 484 mm^2) provides a chamber of depth of 20.7 µm. The depth of a preparation (D, µm) is obtained by dividing the volume of the sample (V,µl = mm^3) by the area over which it is spread (A, mm^2): D = V/A. ***(Fig. 19a-1,a-2)***.

Figure 19a-1

Figure 19a-b: The laboratory glass slide with 22x22 mm cover slip counting chamber for calculating concentration of spermatozoa through wet preparations providing the chamber depth of approximately 20 µm deep.

Figure 19a-2

- **Leja Counting Chambers:** The sperm counting method involves the use of a dual-chamber glass slide **and** 2 pieces of 0.5-mm thick coverslip containing a laser-etched grid on the reverse side. The grid area is 1 mm x 1 mm and is divided into 100 smaller squares, each of which is 0.1 mm x 0.1 mm. A 4 µL volume of semen or sperm suspension is loaded into the left and right chambers and the number of sperm counted based on those sperm which touched the upper and left sides instead of the lower and right sides of each square of a counting chamber. This chamber has a reported depth of 20 µm ***(Fig. 19b).***
- **Makler® Chamber**: The Makler® Chamber was designed specifically for the determination of sperm concentration and motility in undiluted semen. The grid area in the center of the coverslip is 1 mm × 1 mm and is divided into 100 smaller squares, each of which is 0.1 mm × 0.1 mm. A 5 µL volume of semen or sperm suspension is loaded into the chamber and the number of sperm counted as indicated above for the Cell-VU method. The reported depth of Makler® Chamber is 0.01 mm ***(Fig. 19c)***.
- **Haemocytometer**: The improved Neubauer haemocytometer has two separate counting chambers, each with 3 mm x 3 mm pattern of gridlines etched on the glass surface. It uses a special thick coverslip (thickness 0.44 mm), which lies over the grids supported by glass pillars 0.1 mm above the chamber floor. Each counting area is divided into nine 1 mm × 1 mm grids. The haemocytometer provides 100 µm chamber depth as recommended.

Figure 19b: Leja ® slides are high quality disposable counting chambers for semen analysis with chamber depth of 20 µm for increased level of accuracy and precision (Manufactured by Leja Luzernestraat,10 2153 GN Nieuw-Vennep, The Netherlands)

Figure 19b

Figure 19c

Figure 19c: MAKLER® Counting Chamber for Rapid Sperm Analysis for counting spermatozoa with chamber depth of 0.01 mm. (Manufacture by SEFI MEDICAL INSTRUMENTS LTD. Distributed By: Irvine Scientific 2511 Daimler St. Santa Ana, CA 92705)

20. What is Agglutination and Aggregation. Elaborate their clinical relevance?

Semen Parameter	Agglutination	Aggregation
Definition	Agglutination specifically refers to adherence of **motile spermatozoa** to each other through their head-to-head, tail-to-tail or in a mixed way.	Aggregation is the adherence of **immotile spermatozoa** to each other or motile spermatozoa to mucus strands, non-sperm cells (e.g. epithelial) or debris.
Clinical Relevance	Abnormal agglutination is suggestive of immunological cause of infertility, further testing of anti sperm antibodies required It can severely impact sperm motility and concentration,	Abnormal aggregation may interfere in semen analysis parameter

Note: One should always remember that motile spermatozoa stuck to the round cells or debris or immotile spermatozoa is aggregation and should not be scored as agglutination.

21. What is the importance of documentation in semen analysis?

Continuous monitoring and improvement through documentation techniques is an important part of Quality Assurance (QA) and should be elaborately described in laboratory Quality Manual. Documentation technique is an important exercise to detect and correct the problems and document to subsequent prevent the problems in future as part of corrective and preventive action (CAPA).

The QA programme should necessary include but not limited to following documents as a ready reference and assess to laboratory personnel's:

- Standard operating procedures (SOPs)
- Forms and Documents for temperature monitoring
- Instrument records of calibration and preventive maintenance of instruments
- Referral notes, Laboratory worksheet
- Report formats, Reference Ranges
- Information leaflets to clients and referring clinicians.

22. What are the external and internal quality controls as applied in semen analysis?

External quality control (EQC)	• It's voluntary participation of laboratory performing semen analysis for getting samples as a part of EQC. • Quality tests performed by an external body makes comparisons between different laboratories for several procedures. • EQC is useful for detecting systematic variation and assessing accuracy of results
Internal quality control (IQC)	• IQC materials for semen parameters (sperm concentration, morphology and motility) are included as a part of regular workload and monitor the semen analysis outcome. • Internal quality control (IQC) monitors precision and indicates, through results outside the control limits, when the assay may be faulty, it also ensures that results are both accurate and precise. • It's important to have successful internal quality control results of semen analysis before releasing patient results in clinical setting.

23. When and how to do Sperm vitality testing?

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.

- The dye exclusion method 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.
- Hypo-osmotic swelling test (HOS) presumes that only cells (sperms) with intact membranes (live cells) will swell in hypotonic solutions.

Vitality testing using eosin-nigrosin dye uses nigrosin that provides dark background and increase the contrast between the background and sperm heads, which make them easier to distinguish. Eosin-nigrosin smear when observed in brightfield will reveal dead (membrane damaged), spermatozoan as red or dark pink, whereas spermatozoa with white heads or light pink heads are considered alive (membrane-intact) ***(Fig. 23).***

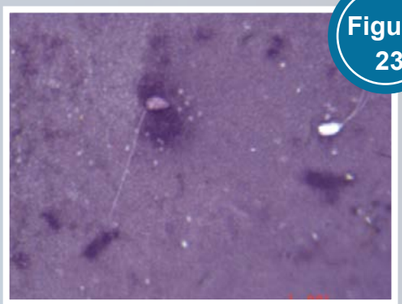


Figure 23: Eosin-nigrosin smear observed in brightfield microscopy. Spermatozoan with red or dark pink heads are considered as dead, whereas spermatozoa with white heads or light pink heads are considered alive. Nigrosin provides dark back ground

24. When are the indications to carry out 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. The morphology staining should be done in all cases.

Morphological defects have also been associated with increased DNA fragmentation, an increased incidence of structural chromosomal aberrations, immature chromatin and aneuploidy. Emphasis is therefore 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 ***(Fig. 24).***

Figure 24: Commercially available slides for assessing sperm morphology (Product Information: Easy Morpho kit, Manufacture by Cryo Lab International, Distributed by SAR Healthline Pvt Ltd, SAR House, Parayanchery, Calicut, India)

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

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. Assessing the proportion of sperm with receptors can then be used to decide which treatment is best for your patient.***(Fig. 25a).***

The mature spermatozoa that have developed receptors for HA can bind and can be viewed under the microscope. The bound sperms are differentiated from unbound sperm by their beating tails with heads and make no progressive movement**. (Normal value ≥ 65%)** ***(Fig. 25b)***

Figure 25a: HBA® Slide provides an answer to the proportion of mature binding spermatozoa (Cat.No BCT-HBA-10 HBA® slide, Manufactured by Biocoat, Inc, Distributed by ORIGIO a/s Knardrupvej 2, 2760 Måløv, Denmark)

Figure 25b: Mature bound sperms on HBA® slides with normal morphology, integrity and aneuploidy with rigorous tail movement with no forward motion of head.

26. 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 PICSI during ICSI ***(Fig. 26a-b)***.

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 patients diagnosed with low HBA® scores (≤65% binding ratio).

Figure 26a

Figure 26a: PICSI® Dish that binds mature sperm with high Hyaluranon binding ability. (Cat.No BCT-PICSI-20 20 PICSI® Dish, Manufactured by Biocoat, Inc, Distributed by ORIGIO a/s Knardrupvej 2, 2760 Måløv, Denmark

Figure 26b

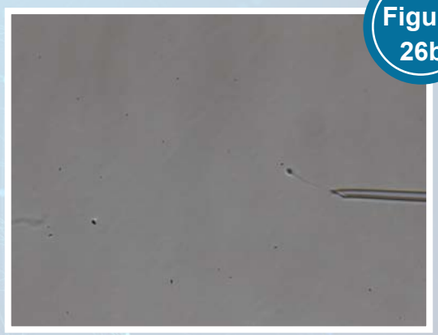


Figure 26b: PICSI procedure entails picking up of Mature bound sperm from PICSI® Dish

27. What is the importance of carrying out Reactive Oxygen Species (ROS) and technique?

Production of ROS is of concern because of potential pathological effects as described below:

- Spermatozoa like any other cell 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 may be estimated in whole ejaculate using various chemiluminance methods or by semi-quantitative assays using oxidative indicators ***(Fig. 27).***

Figure 27

Figure 27: A commercially available semi-quantitative assay kit for estimation of oxidative indicators ROS in whole ejaculate using chemiluminance methods

28. What is the importance of DNA fragmentation and its techniques?

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.

In the recent years, there has been a developing global interest among the reproductive biologist that sperm nuclear integrity should be assessed using SCSA, sperm chromatin dispersion (SCD) TUNEL or the comet assay ***(Fig. 28)***.

Figure 28

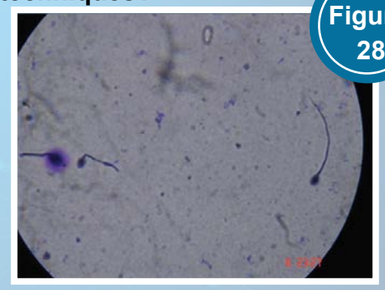


Figure 28: DNA fragmentation test, cells showing halo around the nucleus are good sperms

29. How to handle low sperm count?

Figure 29

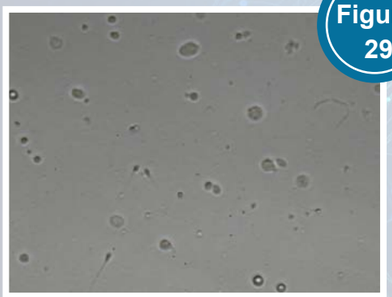


Figure 29: Semen sample from severe oligozoospermic patients with 0-4 spermatozoa per HPF in initial wet preparation.

When the sperm count is less than 10 million , we are faced with therapeutic dilemma. Following steps will help us design an algorithm.

- **Presence of WBC**. If present do semen culture and give appropriate antibiotics.
- If facing a case of idiopathic oligospermia, treat with antioxidants after ruling out infection.ROS estimation,DNA fragmentation assay may help.
- Never comment on single semen analysis in case of a low sperm count. Repeat the test after 4 weeks to confirm.

Therapeutic guidelines-

- Sperm count less than 5 million-Advise ICSI
- Sperm count between 10-15 million -Advise IVF or Insemination or IUI
- Sperm count between 5-10 million -IUI or IVF or ICSI may be carried out .

30. What is Azoospermia?

Azoospermia is defined as complete absence of spermatozoa in the ejaculate and in the sediment of a centrifuged semen sample ***(Fig. 30)***. This is further differentiated as :

- **Obstructive Azoospermia (OA)**: Absence of spermatozoa and spermatogenetic cells in semen and post-ejaculate urine due to obstruction of the genital tract with reduced or normal semen volume and normal endocrinal parameters.
- **Non Obstructive Azoopermia (NOA)**: Absense of sperms in the ejaculate with normal semen volume with or without altered FSH, LH, Testosterone values.

Examine the centrifuged samples to detect spermatozoa: When no spermatozoa are observed in wet preparation, the sample can be centrifuged to determine if any spermatozoa are present in the pellet. In presence of spermatozoa in either replicate report cryptozoospermia else report the sample as azoospermia **(Centrifugation speed-3000g for 15 minutes).**

Figure 30

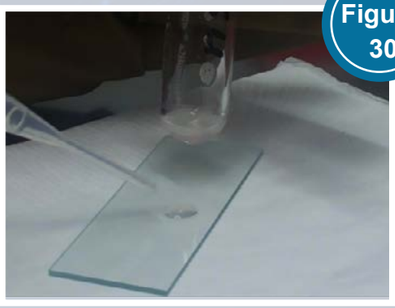


Figure 30: Diagnosis of azoospermia by observing spermatozoa in the sediment of a centrifuged sample.

31. How to distinguish dead sperms from immotile sperms?

It's important to do hypo-osmotic swelling test (HOST) when there are all dead sperms in semen sample. The basic principle of HOS test is the ability of live spermatozoa to withstand moderate hypo-osmotic stress. Sperm membrane plays an important functional role during fertilization process as can be evaluated by the HOS test (HOST). Dead spermatozoa whose membranes are no longer intact do not swell in hypotonic media.

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 ***(Fig. 31a).*** One can also use commercially available HEPES buffered reagent 'SpermMobil' to provide motility and differentiate dead sperms from immotile live sperms **(Fig. 31b)**. All these sperms that demonstrate swelling on HOS and that gain motility are selected can be taken for Intra cytoplasmic Sperm Injection (ICSI).

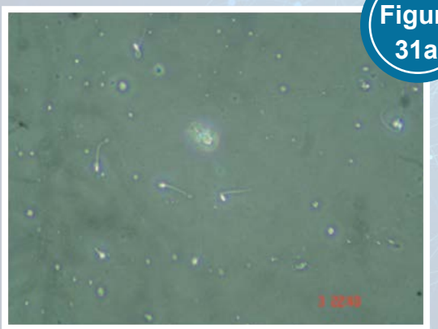


Figure 31a: Healthy spermatozoon showing swelling and curling of the tail region.

Figure 31b: commercially available HEPES buffered reagent 'SpermMobil' to provide motility and differentiate dead sperms from immotile live sperms.

32. How to handle potentially infectious semen sample?

Human body fluids, such as semen, are potentially infectious and should be handled and disposed of with special care. For the andrology laboratory, the most important infectious microorganisms that may be found in semen are HIV and hepatitis B and C viruses (HBV and HCV).

Laboratory personnel should treat all biological samples as potentially infectious and should use appropriate following caution in handling them.

- All laboratory personnel who work with human samples should be immunized against hepatitis B.
- Pipetting by mouth should not be permitted. Mechanical pipetting devices should always be used for the manipulation of liquids.
- All laboratory staff should wear a laboratory coat or disposable gown in the laboratory and remove it upon leaving.
- Laboratory personnel should wear disposable gloves (rubber, latex or vinyl, with or without powder), especially when handling fresh or frozen semen or seminal plasma.
- Staff should take precautions to prevent accidental wounds from sharp instruments that may be contaminated with semen, and avoid contact of semen with open skin, cuts, abrasions or lesions.
- Infectious sample has to be stored in separate liquid nitrogen containers to avoid contamination to other samples.

33. What are different methodologies to assess leucocyte in semen sample

Leukocytes in semen sample can impair sperm motility and DNA integrity through an oxidative attack. Excessive numbers of leukocytes in the ejaculate (leukocytospermia, pyospermia) may be associated with infection and poor sperm quality.

- **Microscopy**: Standard bright field microscopy under high power feild (HPF; x100) can differentiate different types (leuckocytes as macrophasge, monocytes, neutrophils) from round spermatozoa and thus can easily rule out presence of infection in the semen sample.
- **Peroxidase Test**: Leukocyte population in semen stained with cellular peroxidase using ortho-toluidine. This test identifies polymorphonuclear leucocytes that peroxidase positive from multi-nucleated spermatids, which are peroxidase-free. The total number of peroxidase positive cells in the ejaculate may reflect the severity of an inflammatory condition.
- **CD45 staining**: All class of human leukocytes express specific antigen (CD45) that can be detected with an appropriate monoclonal antibody through immunocytochemical staining. This procedure allows detection of different types of leukocytes (CD45-positive cells), such as macrophasge, monocytes, neutrophils, B-cells or T-cells. This technique is expensive and more time consuming but is more sensitive and useful than granulocyte peroxidase in distinguishing leukocytes and germ cells.

34. How to handle rare sperms?

A poor sperm production generally present with finding of 'rare sperms' during semen analysis. This condition can be either due to prior surgery, infection or from blockage of efferent ductules ***(Fig.34)***.

Clinical Management of 'Rare Sperm' sample:

- Confirm the findings with repeat semen analysis. If the report is abnormal, do at-least two analysis to confirm the findings (four weeks apart).
- Semen Culture to rule out infection. If the semen sample is infected, treat with antibiotic course. Repeat the semen analysis & culture to re-evaluate.
- Do appropriate DNA fragmentation (DFI) test, sperm chromatin dispersion (halo) studies, reactive oxygen species (ROS) and vitality studies. Based on results of sperm function test start on antioxidant therapy for 3-6 months and revaluate before planning ICSI.

Figure 34: sample from severe oligozoospermic patients with occasional sperms or "Rare Sperms".

35. How do you asses semen sample with Retrograde Ejaculation?

In retrogragrade ejaculation semen passes into the bladder at ejaculation and results in aspermia with no apparent ejaculate. This can be confirmed by examining post-ejaculatory urine for the presence of spermatozoa ***(Fig. 35)***.

If pharmacological treatment is not possible or not successful, spermatozoa may be retrieved from the urine. Alkalinization of the urine by ingestion of sodium bicarbonate, will increase the chance that any spermatozoa passing into the urine retain its motility and vitality.

Steps for extracting spermatozoa from men with retrograde ejaculation:

- Male partner is given course of oral alkalinisers for two days for alkalization of urine
- The 200-300 ml of sperm preperation media (pre-equilibrated at 37°C) is instilled into the urinary bladder through folly's catheter.
- Male partner is advised to mastrubate and later urinate in a semen collection jar. Urine sample is collected in multiple 15 ml sterile tubes and then centrifuged at 1500 rpm for 10 minutes. Supernatant is discarded and pellets are pooled in a fresh tube. Five ml fresh sperm preparation media is added to the tubes and mixed well. The suspension is centrifuged at the speed of 1500 rpm for 5 minutes. Supernatant is discarded and pellet layered with 2 ml of sperm preperation media and the tube is kept at an incline for 15 minutes. Swim up is then collected and used for IVF or IUI.

Figure 35.

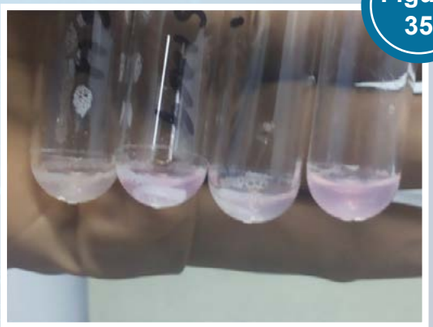


Figure 35: Sperm retrieval and sample preparation from patient with 'Retrograde' ejaculation

36. What is the corelation between RCF and RPM?

The force to which spermatozoa are subjected during centrifugation (relative centrifugal force, RCF) depends on the speed of rotation (N, revolutions per minute, r.p.m.) and the distance from the centre of the rotor to the point at which the force is to be measured (usually the bottom of the centrifuge tube) (radius, R, cm).[Formula RCF (g): $1.118 \times 10^{-5} \times R \times N^2$ (rpm) OR alternatively G-force = 0.000001118 x R x RPM²] ***(Fig.36)***

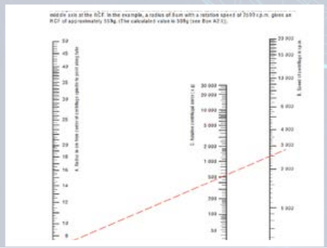


Figure 36

37. What are the personal precautions to be taken while doing semen analysis?

Laboratory personnel must take following precautions while doing semen analysis

- All laboratory staff should wear a laboratory coat or disposable gown
- Wear disposable gloves while handling fresh or frozen semen or seminal plasma
- Personnel should wash their hands regularly, especially before leaving the laboratory, after handling specimens and removing gowns and gloves.
- Surgical masks should be worn by all staff performing procedures that could potentially create aerosols or droplets, e.g. vortexing and centrifuging of open containers.
- The last drops of semen specimens should not be forcibly expelled from pipettes, because this can cause droplets or aerosols to form.

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