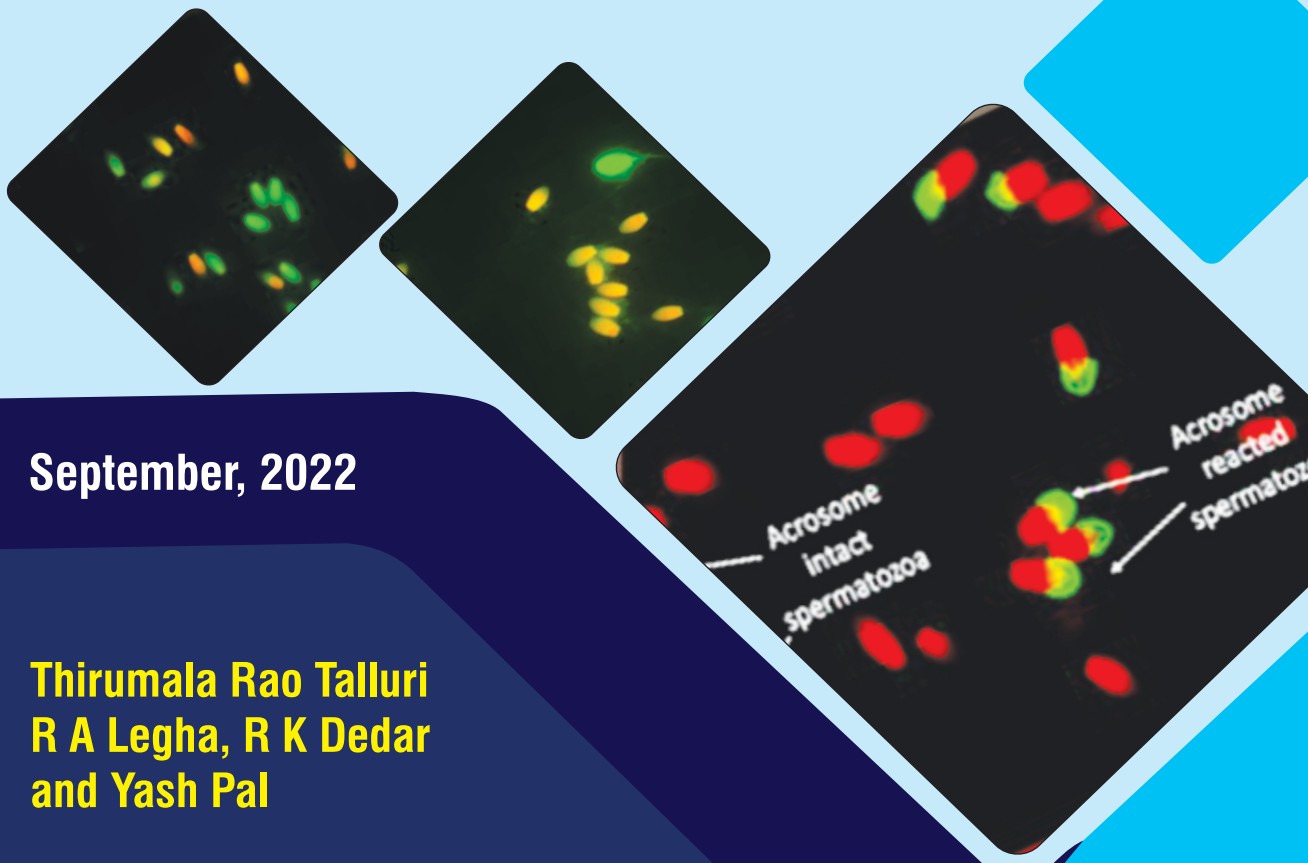
Technical Bulletin
on
STALLION SEMEN EVALUATION TECHNIQUES



September, 2022

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Published by : Director
ICAR-National Research Centre on Equines
Sirsa Road, Hisar-125001 (Haryana)

Year of Publication : September, 2022

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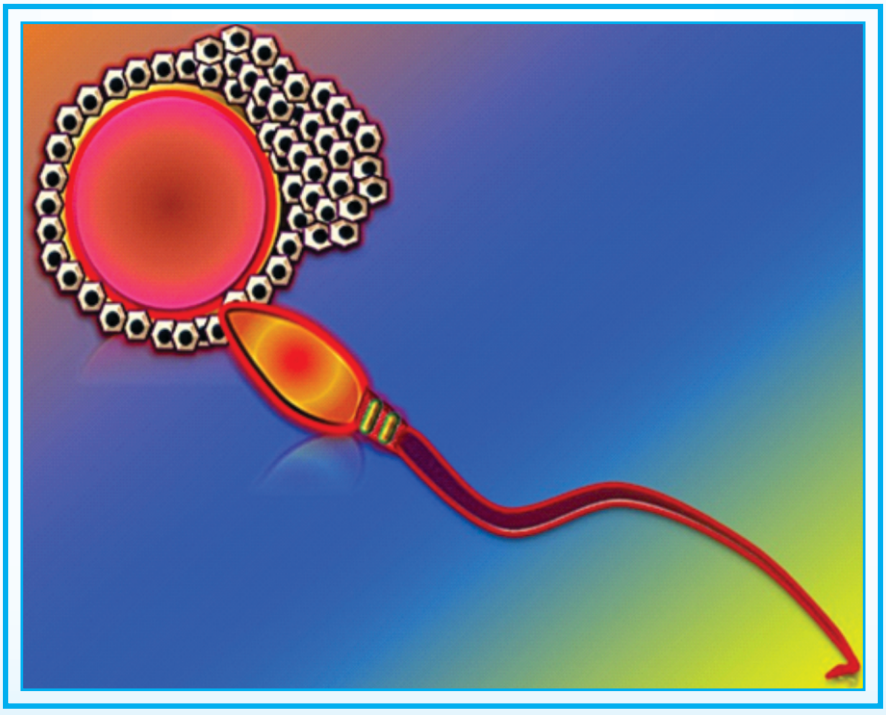
Designing and Setting : TR Talluri and Yash Pal

To be cited as : T R Talluri et al. "**Stallion semen evaluation techniques**". ICAR-NRCE Technical Bulletin, 2022, pp.1-40



Director's Foreword

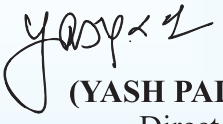
The equine breeding industry has increased exponentially in the last 30 years in different countries around the world, where the use of different assisted reproductive technologies (ARTs) such as artificial insemination (AI) with cooled or frozen semen, embryo transfer (ET) are now considered as a routine procedure for equine researchers and veterinarians. As compared to other domestic species such as bulls or boars, wherein the selection or culling criteria are almost based on their reproductive capacity, stallions are selected by their pedigree, conformation and/or athletic performance. Thus, there is a significant percentage of stallions whose semen does not satisfy the "high-quality" criteria used for other species, leading to an important proportion of studs with marginal semen quality with possible impaired fertility.

Though various factors determine the conception rate of females, the role of male assumes greater significance in AI programme, as a single ejaculate is being used for breeding many mares. In this context, selection of elite stallions having superior fertility with good quality semen production potential assumes greater importance. The recent understanding and advanced biotechnological tools have enhanced the scope of selecting highly fertile stallions for artificial insemination.

Moreover, while the use of semen technologies such as sperm cryopreservation or sex-sorting is highly developed in other species, their use in the equine breeding industry is relatively scarce. This is mainly related to several physiological characteristics of the equine spermatozoa, which makes it more susceptible to cellular and molecular damage during cooling, freezing, and sex-sorting. Hence, selection of right spermatozoa for insemination or breeding is the at most criteria.

Semen evaluation is an important component for the assessment of the stallion breeding potential, as well as for the diagnosis of subfertility / infertility cases. Furthermore, accurate estimation of the damage suffered by the sperm cell after cooling or freezing procedures is necessary for the development of newer procedures to maintain sperm integrity and function. This technical bulletin will be useful for academicians and to the researchers engaged in equine semen and the related techniques. This can be used as teaching and training material to learn the process and techniques of semen evaluation of horses and donkeys. This will serve as a reference material for future research work to study staining techniques to assess the semen quality. These evaluation techniques of stallion semen can be applied to select the best dose and batch of semen for the benefit of equine owners and for sustainment of the species.

It is a matter of pleasure to present the technical bulletin on 'Stallion semen evaluation techniques'. I congratulate the team of authors for bringing out this technical bulletin.



(YASH PAL)
Director
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Contents

IMPORTANCE OF SEMEN EVALUATION AND PARAMETERS TO BE ASSESSED

Breeding soundness examination in stallions involves a clinical and a spermatological examination (Götze 1942, Kenney et al. 1983, Merkt and Klug 1989, Hurtgen, 1992, Varner 2016). The examination aims to gather information concerning the health status (general, hereditary, genital), the ability of the stallion to mount a mare and the fertilization capacity of the stallion's semen. The stallion's ejaculate has to meet certain requirements of volume, color, sperm density, total sperm number, progressive motility, morphology, pH and plasma membrane integrity. In order to be classified as a fertile individual, the ejaculate has to contain at least 1×10^9 morphologically normal, progressively motile sperm in total (Kenney et al. 1983).

The equine breeding industry has increased exponentially in the last 30 years in different countries around the world, where the use of different assisted reproductive technologies (ARTs) such as artificial insemination (AI) with cooled or frozen semen, embryo transfer (ET) and more recently intracytoplasmic-sperm injection (ICSI) are now considered as a routine procedure for veterinarians (Hinrichs, 2018; Panarace et al., 2014). The possibility to spread genetics from superior sires worldwide has increased the popularity of these ARTs among breeders, owners and stud farms, which highlights the importance of correctly assessing the reproductive potential of stallions.

As compared to other domestic species such as bulls or boars, wherein the selection or culling criteria are almost based on their reproductive capacity, stallions are selected by their pedigree, conformation and/or athletic performance. Thus, there is a significant percentage of stallions whose semen does not satisfy the “high-quality” criteria used for other species, leading to an important proportion of studs with marginal semen quality with possible impaired fertility (Varner, 2016; Miro-Moran et al., 2013). Moreover, while the use of semen technologies such as sperm cryopreservation or sex-sorting is highly developed in other species, their use in the equine breeding industry is relatively scarce. This is mainly related to several physiological characteristics of the equine spermatozoa, which makes it more susceptible to cellular and molecular damage during cooling, freezing, and sex-sorting. Although the mechanisms related to those effects are beyond the scope of this chapter, the main effects of those techniques are related to osmotic and oxidative imbalances, which culminates on several alterations in cellular organelles such as plasma membrane, acrosome, mitochondria and DNA integrity, among others (Peña et al., 2015; Balao da Silva et al., 2016; Martín Munoz et al., 2015). It is therefore imperative to use a battery of tests for semen evaluation in order to correctly estimate semen quality and fertility of a given group of stallions.

Male fertility refers to the ability of the sperm to fertilise and activate the ovum and support early embryonic development. A good quality sperm is an important factor

for normal reproduction and contain genomic DNA for transfer of genetic information which contributes other molecules such as messenger RNA (mRNA), microRNAs (miRNA), proteins, and biomolecules that are vitally important for fertility. The most important part in predicting male fertility of domestic animals is evaluation of semen quality and to date a single semen evaluation test is not able to predict its quality or fertility. The good quality sperm has to have all its parts functional in the process of fertilization. Hence, a set of semen tests has to be carried out to assess appropriate sperm traits with low redundancy of assay results.

Semen analysis consists of a set of descriptive measurements of spermatozoa and seminal fluid parameters that helps to assess the semen quality. The lists of basic tests include

1. Quantitative parameters such as volume, concentration and total sperm number
2. Qualitative parameters such as mass activity, appearance and colour.

From the above basic parameters it is not possible to predict that the animal is fertile, therefore further laboratory tests are conducted to check the quality of the spermatozoa in detail.

The laboratory methods like membrane integrity, viability, hypo osmotic swelling, acrosomal integrity, mitochondrial activity, binding activity, DNA integrity by Fulgen's stain and DNA distribution by COMET assay and in vitro fertilization tests are used to evaluate semen quality (Selvaraju et al., 2009, 2010). Most recently, research is focused on profiling of seminal proteins and sperm membrane proteins and sperm transcripts profiling are focused to find the marker proteins of high fertile, low fertile and sub fertile animals.

Sperm motility is the foremost parameter assessed in semen to predict the fertility of the animal. The Computer assisted semen analysis is one of the advanced laboratory semen evaluation procedure done in the recent years in which the sperm velocity parameters can be assayed.

Evaluation of the morphology of the spermatozoa of each ejaculate of the animal is also very important, because the spermatozoa having a proper morphology is an indication of healthy sperm cell carrying proper nuclear shape with the intact DNA and acrosome. The morphology of the spermatozoa can be studied by staining the cells by rose Bengal stain and the DNA intactness is assayed by Fuelgen's staining.

The plasma membrane integrity is important for sperm metabolism including motility, capacitation, ova binding and acrosome reaction.The plasma membrane integrity can be assessed by Eosin-Nigrosine Staining. Not only the integrity of the

membrane, it is also important to check the proper function of the membrane by conducting functional membrane integrity by hypo-osmotic swelling test in which the spermatozoa will make an attempt to maintain the osmotic equilibrium.

Acrosomal integrity is the test performed to check the acrosome intactness, since the intact acrosome with the proper enzyme function is important for penetration of the sperm through zona pellucida. Intact acrosome assay can be done through Giemsa staining and enzyme quality is measured through acrosomal proteolytic assay using Gelatin-Substrate slide Technique.

Mitochondria are considered as energy production house since they supply energy to the normal cell metabolic functions. During cryopreservation there are more chances of damage to the mitochondrial membrane and leakage of mitochondrial enzymes to the extracellular fluid which may result in loss of proper function of the cell even though the sperm meets most of the criteria mentioned above. For assessing, mitochondrial membrane potential (MMP), JC-1{5,5`,6,6`-tetrachloro-1,1`,3,3`-teraethyl benzimidazolyl carbocyanineiodide} is used. If the mitochondrial membrane is not damaged even after cryopreservation this dye will penetrate through the functional membrane and binds with the mitochondrial enzyme and gives the fluorescence from which the percentage of the total fluorescence cells is considered for good quality semen. To check the mitochondrial enzymes function, a yellow tetrazole, 3[4,5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide dye (MTT) is used. This yellow dye is reduced to purple formazan by succinate dehydrogenase system present in the cells with healthy mitochondria. The higher the concentration of spermatozoa with high quality mitochondrial enzyme, the more is the amount of formazan formed. Hence these two are the potential methods to evaluate the good quality semen.

Fructose is a sugar present in the seminal plasma as a source of energy. The fructose in the semen extender is utilised during the period of systematic cryopreservation, thawing and until it reaches the female reproductive tract. If number of active cells is more, the utilization of fructose provided in the extender or in the seminal plasma will also be more. Therefore, fructolysis index is a strong assay to assess metabolically active spermatozoa in the semen, which is more important when the sperm reaches the female reproductive tract.

Even though sperm reaches the site of fertilization in the female reproductive tract successfully, defect in binding and penetration is the most common causes of fertility failure. To check the binding and penetration capacity, hemi-zona assay is done where zona pellucida is isolated from oocytes and divided in to two halves, one half is incubated with fertile sperm (positive control) and the other with test sperm. The ratio of fertile to test binding is measured, less than 30% is considered as abnormal.

GENERAL SEMINAL PARAMETERS TO BE ASSESSED AND THEIR IMPORTANCE

Sperm quality is the culmination of a variety of factors such as the total sperm number, sperm motility, sperm morphology, and sperm fertilizing ability. Presently in an assessment of the quality of a stallion's ejaculate, it is often based only on the volume, motility, and concentration of the sperm. These are relatively easy parameters to quantify. Morphologic assessment, which an analysis of the sperm shapes, is a more complex task that requires significant expertise. In many cases where the sperm have acceptable motility and the numbers are adequate the underlying problem often lies with the morphology of the sperm. Monitoring sperm morphology along with other parameters such as motility and sperm number allows a owner to identify seasonal patterns, or problems in spermatogenesis in breeding stallions.

Sperm is a complex structure with all its functional parts contributing to successful fertilisation of the ovum. Therefore, accurate assessment of semen quality should imply that the applied tests are able to recognize deviations in the sperm cells, including their internal structures, organelles and their functional status, so as to be correlated with their ultimate fertilizing potential. Semen quality assessment methods are very important in predicting the fertilizing ability of persevered spermatozoa and to improve animal reproductive technology.

Total Motility

Total motility of semen has been one of the most common parameters used for assessing semen quality due to its simplicity. The quality of the semen is graded based on the total motility of spermatozoa which can be visualised under the normal bright field microscope. The motility of fresh semen does not predict either the fertility or the freezability of sperm cells, although significant relationship between motility and conception rate were reported. The initial motility was significantly related with live sperm percent and preservability. A significant positive correlation was observed between the sperm motility and freezability.

Sperm concentration

The concentration of the spermatozoa varies with the growth (size and weight) and maturity of stallions, feeding regime, reproductive soundness and the size of testes. The concentration also differs among different age groups of stallions, at different seasons of the year and in different geographical locations. Further, the production of spermatozoa is a continuous process in sexually mature stallions. However during the process of ejaculation, spermatozoa are diluted with seminal plasma and only a fraction of sperm reserve is ejaculated. The sperm reserve in the ejaculate is also said to be dependent on the frequency and degree of excitement.

Sperm motility

The sperm motility is essential feature for travelling in the female reproductive tract and to reach the site of fertilization. The sperm motility is also essential for penetration through zona-pellucida. Though the motility is a good indicator of sperm viability, it has not always proved to be a good predictor of fertility.

Plasma membrane integrity

The plasma lemma (sperm outer membrane) integrity was one of the most common semen quality tests and these tests had shown positive correlation with non-return rate after AI in cattle. The vital stains such as eosin/ nigrosine and trypan-blue are used to evaluate plasma lemma integrity because a defective sperm plasma lemma and good quality sperm plasma lemma is easily differentiated on the basis of the permeability of these stains. The plasma lemma integrity and its proper function are essential for sperm metabolism including motility, capacitation, ova binding, acrosome reaction and hence assessment of membrane integrity throughout the sperm is important to determine sperm fertility.

The tests carried out to assess semen qualities such as volume, sperm-concentration, motility and plasma lemma integrity are essential to provide an overall estimate of the reproductive performance of a stallions, more particularly to establish the routines involved in extension and storage. However, these tests are not sufficient to predict semen fertility accurately.

Functional membrane integrity

Sperm membrane functional status is essential since an intact and functionally active membrane is required for metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte. When spermatozoa are suspended in a hypo-osmotic solution, water will enter the spermatozoa in an attempt to attain osmotic equilibrium. This increases the volume of the cell, thereby reducing the initial length of the flagellum, and the plasma membrane bulges. The influx of water only occurs in the tail region and creates different types of curls. The appearance of curl in the tail of a sperm is an indicator that water has been transported in a physiological manner into the cell to reach osmotic equilibrium which in turn indicates an intact flagellar membrane and live sperm cells.

The functional membrane integrity has been reported to have good correlation with sperm motility and fertility. Functional sperm tests such as acrosome integrity and HOST have been used for the objective evaluation of sperms and are highly correlated with fertility of stallions.

Acrosomal integrity

The acrosomal integrity is an essential feature for sperm binding to zona pellucid

and penetration through zona pellucida. The acrosomal intactness can be measured by giemsa staining. Studies are also indicating that not only the presence of acrosome cap, but also enzyme quality is important for successful penetration through cumulus cells, also binding and penetration through zona. The enzyme quality can be measured by acrosomal proteolytic assay or gelatinolysis test.

Mitochondrial membrane potential

Mitochondrial membrane potential has been widely used for characterization of cellular metabolism, viability, and apoptosis in various cells. So, mitochondrial function has been focused to assess semen quality by JC-1 stain. The mitochondrial sheath surrounding the mid piece of the sperm generates the energy that aids transit in the female reproductive tract, penetration and fertilization of the egg. Hence, assessment of the mitochondrial function than the standard viability tests has been considered more useful to assess semen quality.

The mitochondrial membrane potential had significantly large correlations with progressive forward motility, plasma lemma integrity and functional membrane integrity (Selvaraju et al., 2008).

DNA quality

The DNA fragmentation in spermatozoa reflects the integrity of the genetic material and analysing the quality of the genetic material is important as it is transmitted to the offspring. If the level of DNA fragmentation exceeds the DNA repair capacity of the oocyte may have serious consequences to the embryo viability and successful birth of the offspring. The commonly used techniques to assess sperm DNA integrity are the TUNEL,

Comet and sperm chromatin structure (SCSA) assays. The quality of the DNA, double stranded and single stranded can be measured by acridine orange (AO) staining. The spermatozoa with double stranded fluoresce green whereas the sperm with single strand fluoresce yellow to orange in colour. The ratio of green to orange colour indicates the nature of DNA quality. The fluorescence can be measured in either fluorescence microscope or flow cytometry (sperm chromatin structure assay). The fragmentation of DNA can be measured by TUNEL assay or comet assay. Different studies indicate that sperm DNA fragmentation, as measured by AO staining, TUNEL assay and Comet assays are highly valuable indicator of male fertility. However no strict cut-off values have been reported to select good quality semen.

Calcium influx

Capacitation is an important, but rather incompletely understood phenomenon that a spermatozoon undergoes before it can fertilize the oocyte. It primarily takes place in the oviduct (Samardzija et al., 2006) when various cellular changes such an increase in

membrane fluidity due to lipid modifications, an influx of calcium to the sperm perinuclear and neck regions and flagellum, the generation of controlled amounts of reactive oxygen species, as well as the phosphorylation of protein residues, occur in a concerted manner (De Jonge, 2005;Tulsiani et al., 2007). Calcium influx is one of the primary steps involved in the sperm capacitation process. The rise in intracellular calcium ultimately leads to the phosphorylation of tyrosine and serine residues from proteins regulating the signalling cascade.

Biochemical tests to evaluate the quality of semen

Biochemical components of the semen are secreted from testes, epididymis and accessory sex glands mainly seminal vesicles and prostate. The biochemical analysis of semen provides the information about the functional status of these organs.The markers include fructose (marker for seminal vesicles function), acid phosphatase and citrate (marker for prostate function), glycerophosphorylcholine and inositol (marker for epididymal duct), antioxidants and antioxidant enzymes (marker for excurrent duct system).

Biochemical components of seminal plasma (SP) are secreted from rete testis, epididymis and accessory sex glands of the male reproductive tract. Accessory sex glands known as seminal vesicle, prostate and bulbourethral glands contribute to most of the volume of the ejaculate and seminal vesicle secretion constitutes major portion of SP at ejaculation.

SP is a complex fluid that mediates the chemical function of the ejaculate such as activation and augmentation of motility by spermatozoa, buffering to provide the optimal osmotic and nutrient medium, prevention of premature stabilization of plasma membrane with capacitation inhibitors. Additionally, SP neutralizes the acidic pH of the female reproductive tract and has beneficial immunoregulatory role for survival of spermatozoa. Several factors in SP viz., proteins, cytokines, sex hormones and prostaglandins accompany the migration of spermatozoa to female reproductive tract as well as possess potential biological capabilities to protect spermatozoa from different pathogens.

The role of seminal plasma on sperm function is contradictory. This view has been contradicted by the advent of reproductive technologies with which it is possible to fertilize ova with washed spermatozoa and produce viable embryos resulting in live offspring without exposure of the female reproductive tract to SP. On the other hand, the use of extensively diluted or seminal plasma removed and preserved semen resulted in lower fertility rates than natural mating in livestock species, thus suggesting the inconsistent role of seminal plasma on fertility.

These evidences suggested that SP components might participate in key events related to sperm function, fertilization and embryo development in the female reproductive tract.

Lipid peroxidation levels

Lipid peroxidation level of semen/spermatozoa can be determined by measuring the malondialdehyde (MDA) production, using thiobarbituric acid (TBA). The spermatozoa motility and functional membrane integrity was reported to have negative correlation with lipid peroxidation levels. ROS has been shown to decrease spermatozoa motility by lipid peroxidation of plasma membrane. Addition of biomolecules such as IGF-I improved post-thaw spermatozoa motility by reducing lipid peroxidation levels (Selvaraju et al., 2009).

Superoxide dismutase (SOD)

SOD spontaneously dismutes O2- anion to form O2 and H2O2, while catalase converts H2O2 to O2 and H2O. SOD protects spermatozoa against spontaneous O2 toxicity and lipid peroxidation (LPO). SOD and catalase also remove O2 generated by NADPH-oxidase in neutrophils and may play an important role in decreasing LPO and protecting spermatozoa during genito-urinary inflammation. Seminal plasma gives some protection against this via its constituent antioxidants. However, this level of protection is perhaps not sufficient for long-term storage.

Hence, increased oxidative stress is encountered during the manipulation involved in reproductive techniques, or the antioxidants are removed along with the seminal plasma when the sperm are being prepared for cryopreservation.

The fertility rates of human sperm can be predicted by measuring the SOD activity which is thought to be the major factor protecting human sperm against LPO. SOD activity has positive correlation with sperm livability and hypoosmotic swelling test (HOST) response in buffaloes. Addition of SOD to bovine semen improved the capacitation status of a sample and addition of vitamin E (an antioxidant) protected the sperm membranes from peroxidation

Catalase activity (CAT)

Catalase is an antioxidant enzyme, naturally occurring in seminal plasma with a role in protecting sperm against LPO. Although the reports on this are contradictory, the catalase activity in seminal plasma had positive correlation with gross motility, livability and HOST positive response of stallions spermatozoa. A significantly increased post-thaw motility, membrane integrity, viability and acrosomal integrity were reported with addition of 50U/ml of catalase and 100U/ml ofSOD to stallions semen extender.

MTT assay

A yellow tetrazole, 3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide (MTT) is a water-soluble tetrazolium salt which converts to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by succinate

dehydrogenase system of the active mitochondria. The amount of formazan formed can be determined spectrophotometrically and thus, serves as an indicator of the active mitochondria in 30 million spermatozoa cells in the sample. MTT reduction rates were decreased significantly ($P < 0.001$) by increasing the portion of dead sperm cells and significantly ($P<0.001$) correlated with the results obtained by flow cytometer for sperm functional parameters (sperm viability, mitochondrial activity, acrosomal integrity yielding correlation coefficient of $r= 0.950$, $r=0.926$ and $r=0.959$ respectively) and concluded that MTT test is a reliable method for an objective evaluation of equine and bovine semen.

Fructolysis index

Fructose is the sugar present in the seminal plasma in reduced form, which is readily converted to lactic acid by spermatozoa, and acts as an important source of energy for the sperm cells. The rate of fructolysis represents an accurate and simple means of evaluation of semen. The fructolysis index (mg of fructose utilized by 10 spermatozoa in one hour at 37°C) in normal bull and ram semen is 1.4-2.0. On the other hand, semen with poor sperm motility gives much lower index values, while azoospermic and necrospermic semen were reported to be completely unable to metabolize fructose.

Other biochemical tests

Oxygen consumption rate: Apart from MBRT test, the oxygen consumption rate of semen samples could be used as a measure of potential fertilizing capacity of spermatozoa and found an oxygen uptake of 11.2 nmol O2 8 /mL/min/ 10 cell.

Millovanov's resistance test: Millovanov's resistance test showed ability of the spermatozoa to with stand 1% sodium chloride solution. The amount of one percent sodium chloride solution required to cease the motility reflects the resistance of spermatozoa.

Traditionally, assessment of sperm motility and morphology have been considered landmarks of stallion semen evaluation. The Manual for Clinical Assessment of Stallion Fertility published by the Society for Theriogenology (SFT) uses both tests to classify the fertility potential of a stallion. Based on the SFT guidelines, a stud must ejaculate a minimum of 1 billion of progressively motile-morphologically normal sperm to be considered as a satisfactory prospective breeder (Kenney et al., 1983). These two semen quality tests were included in the stallion breeding soundness examination by their easiness to be conducted under field conditions with limited equipment and were adapted from the SFT stallions breeding soundness examination guidelines.

Bacterial Load (FSD)	:	5000CFUs /ml
Hypo osmotic swelling test (HOST)	:	≥40%
Incubation /Thermo Resistance Test	:	Standard drop in motility by 10% after every 30 minutes
Acrosome Integrity (Fresh Semen)	:	≥70%
Percent Intact Acrosome (PIA)	:	≥65%
Sperm Concentration	:	500-600 million spermatozoa per dose(0.5ml) Medium straw)
Total Sperm Abnormality	:	≤20%
Fresh Semen	:	Minimum of $300x10^6$ progressively motile sperm Cells
Chilled Semen Diluted/Fresh Sperm Dose	:	Minimum of $300x10^6$ progressively motile sperm cells at the time of portioning. Insemination within 12 hours of collection Storage conditions maintained Progressively motile spermatozoa at the time of insemination no less than 35%
Diluted/Transported Sperm Dose	:	Minimum of $600x10^6$ progressively motile cells at sperm the time of portioning Maximum volume 40ml (1:2 dilution) Insemination 24-36 hrs after collection Storage conditions maintained Progressively motile spermatozoa at the time of insemination no less than 35%. All semen leaving the insemination centre has to be treated as diluted/transport semen
Frozen Semen		
Volume dose	:	Dependant on manufacturing process
Sperm dose	:	minimum of 35% progressively motile spermatozoa post thawing Minimum of $250x10^6$ progressively motile sperm cells per AI dose post-thawing A breeding dose for insemination of one mare has to contain a minimum of 3 straws

Table. SFT guidelines for equine spermatozoa

SEMEN COLLECTION FROM STALLIONS FOR SEMEN EVALUATION

The history of semen freezing goes back to discovery of Polge *et al*. (1949) that bull sperm cells can survive freezing (cryopreservation). Only a year later researchers successfully froze stallion spermatozoa, and in 1957, the first pregnancy from frozen stallion sperm was reported in Guelph, Ontario. For economic reasons most of the early research was focused on bull semen, but in the last 15 years, the successful and growing use of frozen semen in bulls encouraged many to carry out further research into cryopreservation of equine semen (Leopold Sybil, www.livinglegends.org.au). The utilization and importance of frozen semen is increasing in artificial insemination (AI) as horse breed registries are now allowing foals born through AI (Loomis, 2001) with frozen semen and due to advances in semen cryopreservation techniques. In addition, pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural mating (Samper *et al*., 1991). However, to improve reproductive efficiency when using cryopreserved semen, attention should be given to factors such as the stallion, the quality and handling of the semen, and age as well as reproductive history and management of the mare (Allen, 2005).

Artificial insemination (AI) was first started as early as in 14th century as per Arabic scriptures in the horse. First systematic exploitation of this technique took place with horses around the start of the 20th century. Although it was known by 1776 that cooling, semen held the spermatozoa in a dormant state and thereafter followed a huge interest in the physics of cold. Cryopreservation of spermatozoa remained an enigma until the discovery of glycerol. The cooled semen is very successful for short-term storage and use but long-term storage by this method is not possible. In order to realize many of the potential advantages of AI, long-term storage is necessary. This has become possible by freezing, a system that halts the metabolic processes of the spermatozoa, allowing indefinite storage without loss of fertility. The discovery of cryoprotectant properties of glycerol made cryopreservation of semen possible. As a result, the spermatozoa of many species can now be stored at -196°C in liquid nitrogen for long duration without losing its fertilizing ability. There are several advantages of semen cryopreservation which are listed as under

- Allows the long-term preservation of stallion semen.
- Enables the establishment of a semen bank.
- Enables introduction of superior germplasm of stallion even several years after death.
- Enables a major expansion in international trade.
- Plays important role for controlling the sexually transmitted diseases from stallion to mares.

Principle

Cryopreservation of semen is a technique where sperm cells being preserved are exposed to subzero temperature such as-196°C. This technique is based on principle to minimize biological activities of sperm cells to its least and thus production of negligible amount of waste products; by gradual cooling to subzero temperature. Media, which is used to dilute sperm cells, helps to supplement energy, nutrient requirement and resist change in its pH. To save sperm cells from adverse effect of subzero temperature some cryoprotectant/s alone or in combination are added in media used for semen dilution otherwise lead to sperm cell death. However, the sperm cells exposed to low subzero temperature often get damaged to approximately 50 percent during the process of freezing and thawing even after addition of cryoprotectant mainly due to intra and extra-cellular ice crystal formation, solution effect and cell dehydration. Initially the basic procedures for cryopreservation of bull spermatozoa were applied to stallion spermatozoa, but soon researchers realized that stallion spermatozoa are much more fragile and therefore more difficult to freeze and thaw than semen from other species. In the last 20 years, scientists from several countries have been working at developing and improving the techniques used to successfully freeze stallion spermatozoa. The use of cryoprotectant such as glycerol, first identified for use with bovine spermatozoa, prevents some of the damage. However, the success of cryopreservation depends upon many other factors, including interactions between cryoprotectant, extender, cooling rate, warming rate and packaging, as well as individual variation, which appear to be particularly significant in the stallion. Some loss in spermatozoa viability must be expected, to the processing procedures prior to freezing as well as the actual freezing process.

Cleaning and sterilization of plastic and glassware

For production of clean and bacteriological free semen, washing of the plastic and glassware used for the whole process of collection of semen and cryopreservation is to be sterilized properly. All the glassware and plasticware to be used should be dipped in laboratory detergent solution (2-3% solution in water) for hours and then cleaned thoroughly using soft brush to make article grease as well as stain free. Dipping in chromic acid solution for overnight removes cloudiness over glassware. The articles are then washed with tap water two-three times and finally rinsed with distilled water to remove deposit of any salt etc which appears on drying.

It is common practice to sterilize all the glassware and metallic ware in hot air oven at 180°C for 2 hours. Articles sensitive to heat damage should not be used for heat sterilization. All the rubber articles and AV should be autoclaved at 10 lb (4 kg) pressure and 115.6°C temperature for 20 minutes. Higher pressure would change shape of such

articles. Buffer solutions may also be autoclaved at 17 lb (7 kg) pressure and 121°C for 15-20 minutes. Solutions containing sugar should not be autoclaved as heat destroys the sugar. All vessels to be sterilized by autoclave should be loosely plugged/capped and should not be filled completely with solution. Immediately after autoclave, the articles should be kept in hot air oven at around 40°C for drying. Sterilized items should be kept in air tight cabinets or hot air oven. Straws, gauze etc may be sterilized using ultraviolet radiation in closed chamber.

Fig. Overall steps involved in collection and cryopreservation of equine semen.

Collection of egg yolk

Fresh eggs are first cleaned with cotton and wiped with 70% alcohol. Eggs are broken by slow striking from its narrow end with caution not to disturb yolk. Albumin is discarded and yolk is placed cautiously over sterilized piece of blotting paper to avoid rupture of yolk membrane. Syringe (20 ml) adaptor without needle is pierced through the yolk membrane and yolk is aspirated as much as possible by pulling plunger. Syringe is now emptied in suitable measuring cylinder, ready to mix with solution A and B. To collect 20ml of egg yolk, 4 to 6 eggs are enough.

Preparation of AV

The AV is prepared by assembling its parts and filling hot water within the space provided through nozzle with help of funnel. The optimum temperature of AV is

maintained 42 to 45°C and lubricated well with liquid paraffin or vaseline before use. Higher temperature of AV may cause irritation to stallion penis and cause damage to sperms. The pressure inside AV is such that the stallion is able to penetrate penis with ease. A pre-warmed graduated collection bottle covered with thermo-jacket is attached to one end with AV to accumulate an ejaculate and to prevent sperm from thermal shock. Ejaculation occurs into the lumen of the AV lined with a disposable plastic liner and is collected into graduated collection bottle. There are three major types of AV's such as Colorado, French (INRA) and Missouri model that satisfy these criteria in slightly different ways. Because of large size, comparatively heavier and vigorous thrust by the stallion at ejaculation, the AV used has a handle to hold it firmly.

Fig. Preparation of Artificial Vagina (AV)

Semen collection procedure

Semen is collected by means of artificial vagina (AV) over an estrus mare/jenny or dummy mare. In case dummy is not available and using an estrus female, her tail is bandaged; perineal area is washed and cleaned after proper restraining. The operator holding AV and the animal handlers should be alert and take necessary precautions to protect themselves from mare as well as stallion. Use of helmets during semen collection is an added precaution while stallion is mounting and dismounting. The penis of the jack/stallion is washed with luke warm water before mounting. Just after dismounting penis is given flush of mild betadine/antiseptic solution. Ejaculation is completed in 15 to 20 seconds marked by pulsation at the base of penis and flagging of tail.

Fig: Process of semen collection in stallions

Removal of gel and evaluation

Immediately after collection, remove of gel fraction by filtering through gauge or fit IMV gel filter in AV to get gel free semen. Small sample of gel free semen is evaluated for its quality i.e. initial motility, progressive motility, pH, sperm concentration, live-dead count and abnormality of sperms *etc*.

Fig. Gel Removal from the fresh semen

MACROSCOPIC AND CONVENTIONAL METHODS OF STALLION SEMEN EVALUATION

Basic assays for routine semen evaluation are divided into two parts: macroscopic and microscopic examination. In commercial semen production programmes, basic evaluation (volume, colour, consistency, density, concentration and initial progressive motility) of ejaculates should be completed in less than 8–10 min, all the while keeping them in water bath at 37 °C for optimum production of quality semen doses.

Macroscopic evaluation of semen quality parameters (SQP) of an ejaculate provides gross estimates of the reproductive performance of the stallion. It also helps in selection of samples for further microscopic examination. Macroscopic evaluation consists of certain combination of tests such as overall appearance (colour, consistency, density and odour) and volume performed with naked eyes under natural daylight within 10 min of semen collection. Once ejaculates have passed macroscopic evaluation, they are further subjected to microscopic evaluation before processing for cryopreservation. The odour, the osmotic pressure and the pH of the ejaculate are not routinely recorded during semen processing protocols.

Appearance and consistency: This is done by visual observation. A good sample should appear milky white in colour, evenly turbid and without clots. Abnormal ejaculates may be yellow (urine contamination, white blood cells) or pink (haemorrhage). Clots or flakes in the semen may be indicative of pus that comes from tubular tract or accessory glands. Consistency may range from watery to creamy depending upon the spermatozoa concentration within the sample. Creamy appearance of an ejaculate is indicative of concentrated while a watery ejaculate often has fewer sperm cells.

Consistency of semen varies from thick creamy to watery depending on the concentration of spermatozoa in the semen. Grades of consistency of an ejaculate are recorded from thick creamy to watery, considering water as control. The ejaculate should have a relatively uniform opaque appearance, whereas translucent sperm contains few sperm. Pathological conditions of the testis, epididymis or accessory sex glands affect the consistency of the ejaculates. Thin, watery and less milky semen indicates initial stages of epididymitis, whereas thick and viscous semen is indicative of catarrhal condition of accessory sex glands.

Volume: The volume (in millilitres) of gel-free semen is determined using a graduated cylinder. Gel-free semen volume can be measured using a pre-warmed graduated cylinder or graduated centrifuge tube. For a more precise measure of semen volume the weight of the semen can be determined. An accurate assessment of volume is critical for the determination of total number of spermatozoa in an ejaculate. Volume of the ejaculate may be increased following excessive teasing of a stallion to a mare in estrus prior to collection. However, the concentration of sperm per millilitre is reduced, and as a consequence the overall number of sperm in the ejaculate is not affected. Concentration

and volume are thus inversely related: the higher the concentration, the lower the volume, and vice versa.

The total ejaculate volume is recorded directly from the graduated collection bottle, soon after its collection. Gel free semen volume is recorded from another graduated bottle used to collect filtrate after filtering semen through sterilized thin gauze to retain gel fraction. The gauze with the trapped gel fraction is discarded. This process is bypassed if an in-line filter is placed in AV liner.

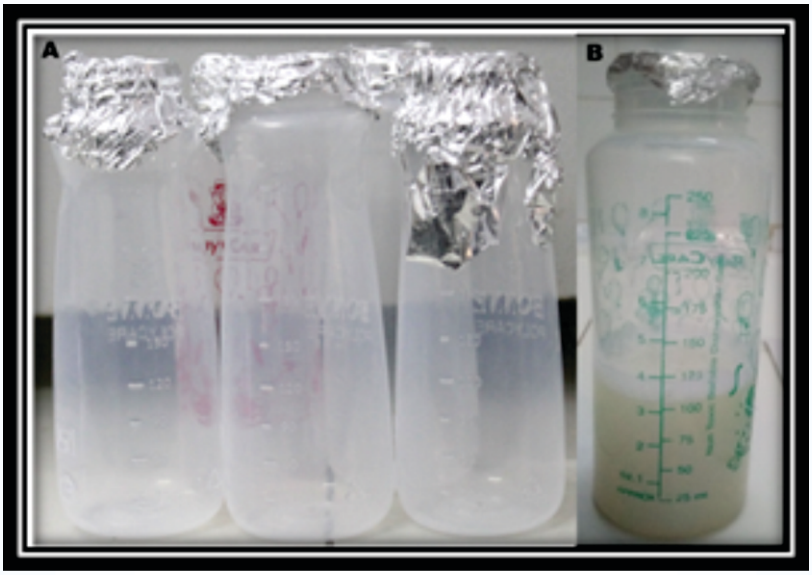


Fig. Semen collection bottles used for semen collection from stallions. A.sterilised empty bottles; B. semen collected in the bottle

A nylon filter with a pore size of 37 µm, minimize the loss of spermatozoa within the filter and retain the gel. Ejaculate volume of jack/stallion semen may vary between 30 and 250 ml. Variations in volume may be due to individual stallion variation and may be indicative of abnormalities, age, workload and length of teasing etc. Semen volume is usually less in young, small sized, excessively used males, an incomplete ejaculation and seminal vasculitis. Teasing of jack/stallion result in higher ejaculate volume.

Colour: Always judge colour of the ejaculate in natural daylight. Semen ejaculate of the stallions resemble whole-milk colour, depending on the sperm cell concentration and presence of non-germinal cells, with the variation of yellowish white to greyish white. The white opaqueness is entirely due to mass of sperm, whereas varying shades of yellow are due to presence of riboflavin, which fades upon exposure to light. Contamination of ejaculate occurs when either donating stallions are maintained unhygienically or recommended protocol is not followed at collections. Observation of any contaminant such as hair, dung, or dirt warrants immediate exclusion of the specimen from further processing. The presence of sediment containing spermatozoa at

the bottom of the collection tube is a normal feature if the ejaculate is left undisturbed for a few minutes.

pH: pH is a measure of the acidity or alkalinity of a solution and measurement of the pH of semen is part of a complete breeding soundness evaluation of a stallion. The pH of raw semen should be determined as soon as possible after collection. Incubation of a semen sample for a prolonged period of time may lead to a lower pH due to the accumulation of metabolic by-products, such as lactic acid. A pH meter should ideally be used to determine the pH of raw semen. Measurement with pH paper will be less precise. The normal pH of equine semen ranges from 7.2 to 7.7. The pH of normal stallion semen can be affected by season, frequency of ejaculation, and sperm concentration. Inflammation of the reproductive tract or contamination of the ejaculate with soap or urine can lead to an abnormally high pH.

Technique

pH Measurement

A sample of raw semen is collected for evaluation.

The pH meter should be standardized prior to use to obtain an accurate reading using the instructions that come with the meter

In horse stallion, a wider pH range (6.2 to 7.8) had been reported which is similar for jack though a close range of 7.35 to 7.7 is considered normal.

Osmolarity Measurement: Osmolality is the concentration of a solution in terms of osmoles of solute particles per kilogram of solvent. An osmole refers to the number of moles of ions that contribute to a solution's osmotic pressure (i.e., 1 mol/l of NaCl corresponds to an osmolarity of 2 osmol/l as the NaCl dissociates into individual Na^+ and Cl^- ions in solution). Osmolality can be measured in either raw semen or seminal plasma.

Procedure

A 20 µl sample of raw semen or seminal plasma is obtained and transferred into the bottom of a sample tube. The tube may be tapped lightly to eliminate air bubbles and to unite the fluid sample in the bottom of the tube.

The loaded sample tube is placed into the sample well of the osmometer.

The measuring head is lowered into the sample tube.

The osmolarity test is initiated.

The probe should be wiped clean after each test and rinsed with distilled water or alcohol.

The osmolality of equine seminal plasma varies considerably by stallion. There is no significant difference in osmolality between the first and second ejaculates or difference due to season. The osmolality of stallion semen has been reported to be 331–336 mOsm. An elevation in osmolality may be indicative of sample contamination with urine or water-soluble lubricants. Significant alterations in pH and/or osmolality may be associated with a reduction in sperm motility and potentially a reduction in fertility.

MICROSCOPIC AND ANALYSIS OF FUNCTIONAL SEMINAL PARAMETERS

Although the parameters mentioned in the earlier chapter provide abundant information, it is well accepted that these parameters give only crude information on the fertility of stallions. Prediction of male fertility could be improved if additional microscopic parameters based on functional characteristics of spermatozoa are evaluated which are further discussed as below.

Concentration: The determination of spermatozoal concentration is required for the accurate calculation of a breeding dose and subsequent dilution of semen for cooled semen transport. Sperm concentration should also be determined as part of a routine stallion breeding soundness examination. There are several methods used to determine spermatozoal concentration in semen samples including the use of a hemocytometer, spectrophotometer, flow cytometer, or fluorescent assay techniques. Concentration of spermatozoa per milliliter in the ejaculate may be manually counted using a hemocytometer as the hemocytometer is the least expensive method and gold standard method. The hemocytometer has two individual sides that are counted independently. Results from each side are then averaged to determine the total number of spermatozoa per milliliter.

Preparation of diluting fluid for sperm count

0.05 gm eosin Y (water soluble) and 1 gm sodium chloride aredissolved in 100ml distilled water.

Or,

1 ml of formalin is added in 100 ml normal saline solution to make 1% formal saline solution. In 100 ml formal saline 0.05 gm of eosin is added to get diluting fluid.

Manual method of dilution (1:100) using diluting fluid

The 1:100 dilution of semen with diluting fluid can be made using a unopette diluter or a hand dilution. The unopette is easier, but sometimes the cells will clump, so an accurate count cannot be made. Manual method of dilution is little time consuming, but the cells do not to clump which is good to get a more accurate sperm count.

Manual method

1. 1ml of semen sample is dissolved in 9 ml formal saline to make a 1:10 dilution.
2. 1 ml of this 1:10 dilution is added to 9 ml formal saline to make a 1:100 dilution.

Sperm count technique

The hemocytometer have two counting chambers each divide into 9 primary squares. The central square has triple lines around it. Inside the triple lines are 25 (5x5) secondary squares also bounded by triple lines. Within each of the 25 secondary squares are 16 (4x4) tertiary squares. To count the concentration of semen sample the hemocytometer is loaded with a 1:100 dilution of semen under cover slip placed over the chambers. The 1:100 dilutions can be made using a unopette diluter or a hand dilution. After the sample allowed to settle, all the sperm heads in the middle big square (the square with 25 secondary squares are counted. The number of sperm heads counted in a single chamber is multiplied by 10^6 to give the concentration of cells/cc.

Fig. Picture of Neubauer's haemocytometer

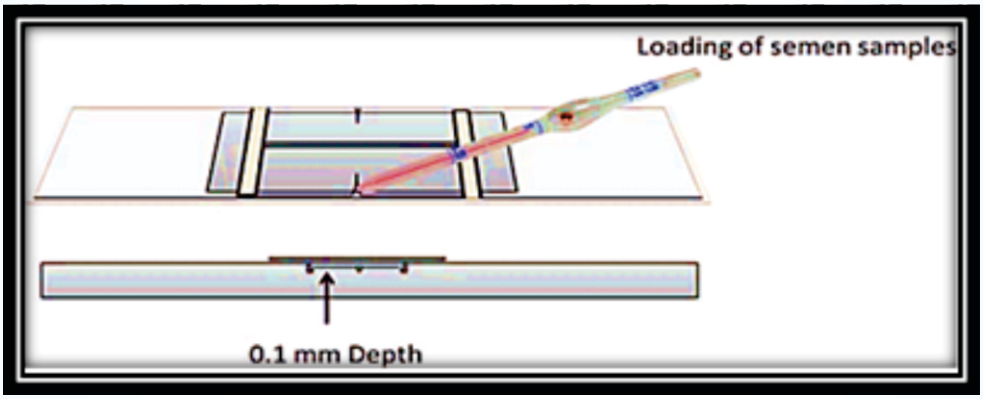
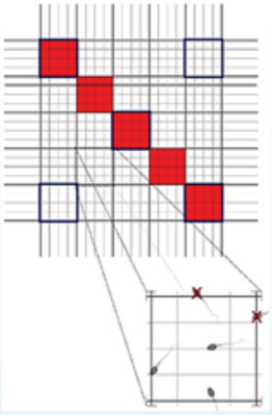


Fig. Picture depicting the procedure for loading of counting chambers in Neubauer's Haemocytometer

Sperm concentration (per ml) = $\frac{N \times D \times 4000 \times 1000}{n}$ Where, N=No. of Sperms counted
D=Dilution rate (here 100)
n=No. of tertiary squares counted



Total sperm numbers per ejaculate is obtained by multiplying the sperm concentration per ml with the volume of the ejaculate. Total numbers of progressively motile sperm can be calculated by multiplying with percent progressively motile cells.

Motility

An accurate evaluation of sperm motility is an important component of the BSE. The motility of raw, gel-free semen may be difficult to evaluate accurately since the concentration is often too high to examine individual cells, and sperm cells in raw semen tend to clump or agglutinate. Motility, the easiest and most certain way of determining the viability, is also the most desirable spermatozoa characteristics to decide the fate of the sample for further processing. It is expressed as either mass activity or individual progressive motility in fresh semen. In post-thaw semen, motility estimates are crucial criteria to discriminate between a good or bad batch of semen.

Fresh gel-free semen should be extended as soon as possible to preserve the viability of the spermatozoa and to permit observation of individual sperm motility characteristics. In addition, spermatozoa in a "raw" or undiluted semen sample are often highly concentrated and tend to clump or agglutinate, making an accurate estimate of motility in raw semen difficult or impossible. Motility evaluation of extended cooled semen should be performed after allowing a small aliquot of semen to warm to approximately 37°C for approximately 10 minutes. If the extended semen is not warmed properly, the motility values may be underestimated. It is important that all materials that come in contact with the semen are clean, dry, and warmed to approximately 37°C. Ideally, estimates of sperm motility should be made as soon as possible after semen collection.

Procedure

- Semen is obtained from the stallion and the volume and concentration are determined.
- A small volume of semen is diluted into warmed semen extender. Ideally, semen should be evaluated at a consistent concentration of 25 million sperm per milliliter. A convenient method to dilute semen to this concentration is to add 180 µl of raw semen to an appropriate volume of warm extender to yield a final concentration of 25 million sperm/ml.
- Alternatively a 1:20 (semen : extender) dilution can be prepared by adding 0.25 ml of semen to 4.75 ml of warmed extender.
- A small drop (6–10 µl) of extended semen is placed on a clean glass slide and covered with a clean coverslip. The quality of the examination is enhanced if the glass slides and coverslips are maintained at approximately 37°C using a slide warmer. Care should be taken to avoid trapping air bubbles in the semen when applying the coverslip.

- The slide is placed on the stage of a good quality light microscope, preferably a phase-contrast microscope equipped with a stage warmer. It is essential that the specimen be kept warm or the percentage of progressive sperm motility will be underestimated.
- Immediately after the slide is prepared, it is examined microscopically at a magnification of 200× to 400×. Five microscope fields should be examined and the results averaged.
- Generally, two motility estimates are determined when examining a semen sample:

– Total motility refers to the percentage of sperm that exhibit any type of movement, and includes sperm moving in a straight line, circling, and/or twitching.

– Progressive motility refers to sperm that exhibit forward motility across the microscopic field or are moving in a large circular path.

- Estimates of total and progressive motility should be evaluated from multiple visual fields and a representative average recorded.

Sperm motility estimates should be made quickly and efficiently after semen collection, especially if the microscope stage is not heated. In general, most fertile stallions should have a progressive sperm motility of at least 60%. Spermatozoa may normally swim in a large circular path due to an abaxial attachment of the midpiece to the sperm head.

Sperm motility assessment through CASA

Spermatozoa motility is a very important attribute most commonly exploited for discriminating between a good and a bad semen sample at the fresh as well as post-cryopreservation stage. Accurate measurement of motility provides crucial information about viability of spermatozoa as well.

Visual assessment of sperm motility is subjective, prone to bias, and may vary between examiners. Computer-assisted sperm analysis (CASA) systems have been developed to provide a more objective assessment of total and progressive motility Computer-assisted (or automated) sperm analysis or CASA is an objective way to evaluate spermatozoal motility. Modern CASA systems also advertise the ability to assess spermatozoal morphology, concentration, and viability. However, CASA units are not as accurate as traditional methods of morphology and concentration assessments.

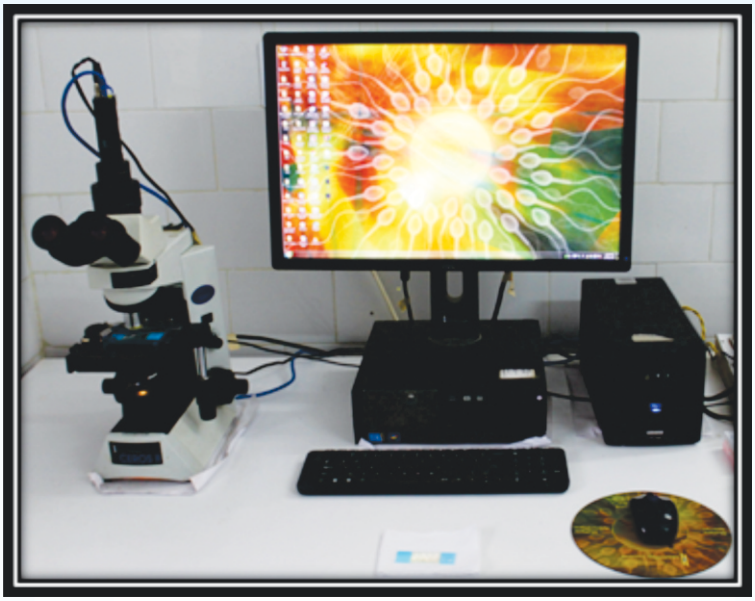


Fig. Computer assisted semen analyser

CASA employs the use of a microscope, a video camera, and computer software for the analysis. The system records multiple images of sperm movement (60 frames per second with 40 frames analyzed) to provide data that the computer system then uses to "connect the dots" resulting in a linear path of individual sperm movement. The dots marked on the computer screen are referred to as centroids and mark the location of the sperm head. In order for the computer to distinguish between debris and actual spermatozoa, certain gates or parameters must be pre-set in the software so that only cells of a certain size are evaluated and so that motility estimation may be performed based on velocity measurements. The gates are usually pre-set by the manufacturer for a particular species, but can be adjusted by the end user. A special microscope slide is recommended for use in the CASA system that has a predefined volume (10 or 20 μm depth chamber). The fixed volume is sufficient to allow free movement of spermatozoa, but limits the depth so that the optics can focus on a group of spermatozoa. Regular microscope slides and coverslips may be used, but this may result in uneven slide depth and more variation between samples as the depth of field varies. Depth of the chamber will influence results, so a notation should be made as to the size of chamber that was used in the analysis. Some systems accommodate fluorescent labeling of spermatozoa to avoid interference from debris in the sample, such as particulate matter in semen extenders. This makes analysis of frozen-thawed semen samples possible in samples with unclarified egg yolk extenders. Most equine skim milk-type semen extenders used for cooled semen transport

do not interfere with motility readings and may be used to dilute semen for CASA analysis.

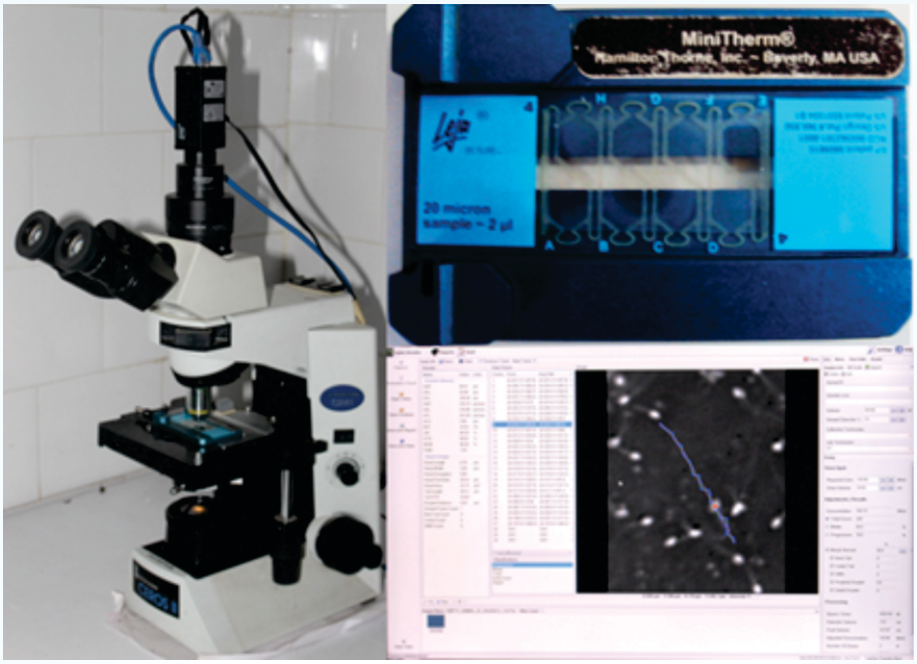


Fig. Microscope with thermostage and Leja slide in place for semen evaluation

Procedure

- Obtain a semen sample and filter to remove any gross debris and the gel fraction.
- Determine the spermatozoal concentration in the raw sample.
- Dilute the semen with primary semen extender to obtain a final concentration of 25–50 million spermatozoa/ml. The extender should be free of debris that could interfere with the sample measurement. Dilution is important in order to avoid contact between spermatozoa and cross-over artefacts in which one spermatozoon moves across the path of another spermatozoon, which could potentially confuse the software as to the true path of an individual spermatozoon.
- Mix the sample thoroughly just prior to sampling.
- Using a mechanical pipette and attached tip, load the chamber on the CASA microscope slide with the appropriate volume.
- Place the slide on a heated microscope stage (37°C) on the CASA machine.

Variations in temperature will have a significant effect on motility estimates so a heated microscope stage is a requirement for analysis.

- Orientate the slide on the stage so that the objective is located in the central region of the coverslip chamber. It is important to avoid the edges of the chamber where spermatozoa may not be as motile due to drying of the sample or interaction with the wall of the chamber.
- Ensure that there is no flowing movement to the liquid on the slide by observing the spermatozoal movement on the monitor. If all spermatozoa are moving the same direction independent of tail movement, the slide may have been overfilled. Either wait a few seconds to see if the sample stabilizes or make a new slide.
- Initiate the software to evaluate spermatozoal motility. Typically five or more separate fields are read to provide an average assessment of motility. Avoid regions of the slide with large amounts of debris. Also, avoid bias in the selection of fields in which sperm appear more or less motile.
- Each slide chamber is to be used only once and then discarded. However, the slide has two to eight chambers that may be used to independently assess multiple different semen samples. Only one chamber should be used per stallion. A permanent marker can be used to "X" out a used chamber to avoid accidental refilling.

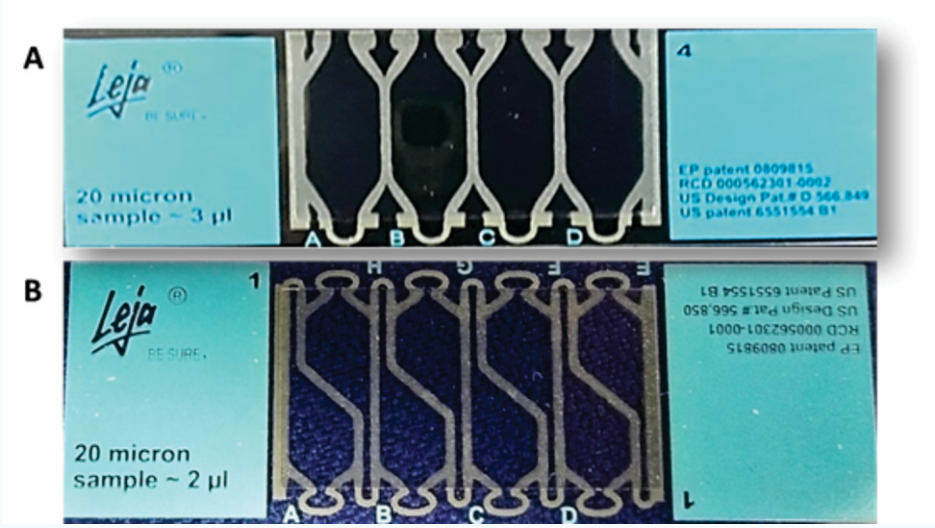


Fig. (A) Four-chambered and (B) Eight chambered Leja slides

Sl	Terminology and unit	What it means
1.	VCL, curvilinear velocity (μm/s)	Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope. It is a measure of a cell vigour.
2.	VSL, straight-line (rectilinear) velocity (μm/s)	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
3.	VAP[a], average path velocity (μm/s)	Time-averaged velocity of a sperm head along its average path
4.	ALH[b], amplitude of lateral head displacement (μm/s)	Magnitude of lateral displacement of a sperm head about its average path
5.	LIN, linearity	The linearity of a curvilinear path, VSL/VCL
6.	WOB, wobble	A measure of oscillation of the actual path about the average path, VAP/VCL
7.	STR, straightness	Linearity of the average path, VSL/VAP
8.	BCF, beat-cross frequency (Hz)	The average rate at which the curvilinear path crosses the average path
9.	MAD, mean angular displacement (degrees)	The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory

[a]VAP is computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; since these algorithms vary between instruments, therefore values may not be comparable among systems
[b]ALH is expressed as a maximum or an average of such displacements. Since different CASA instruments compute ALH using different algorithms, therefore values may not be comparable among systems

Fig. Terminology used in CASA for sperm kinematics

A minimum of 400 motile or 1,000 total spermatozoa should be counted. A number of measurements of spermatozoal motility are provided by the software. Total and progressive motility are the two parameters most commonly reported. Equine spermatozoa should have an average path velocity of >50 μm/s with a straightness of >75% to be considered progressively motile. Spermatozoa with an average path velocity <20 μm/s are considered non-motile. Sperm with an average path velocity between 50 and 20 μm/s are considered slow moving.

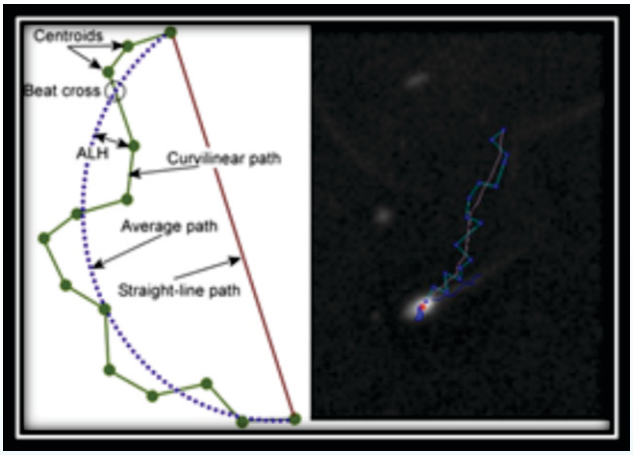


Fig. Kinematics of stallion spermatozoa

Sperm head size is identified by cells that range from 14 to 80 µm2. This variation allows sperm to change orientation without the software losing the cell. Motility is divided into immotile, local motile, or progressively motile. Immotile cells have an average orientation change of less than 9.5 µm. Locally motile cells have a DSL <6 µm. All others are considered progressively motile. Progressively motile cells are evaluated for classification as hyperactive, linear, or non-linear. Progressively motile cells that exhibit a VCL >80 and LIN <0.65 and ALHD >6.5 are categorized as hyperactive. Hyperactive cells display enhanced motility moving somewhat erratically. In some species this is considered a sign of capacitated spermatozoa. Progressively motile cells that exhibit a STR >0.9 and LIN >0.5 are categorized as linear. Progressively motile cells that exhibit a STR <0.9 and LIN <0.5 are categorized as non-linear.

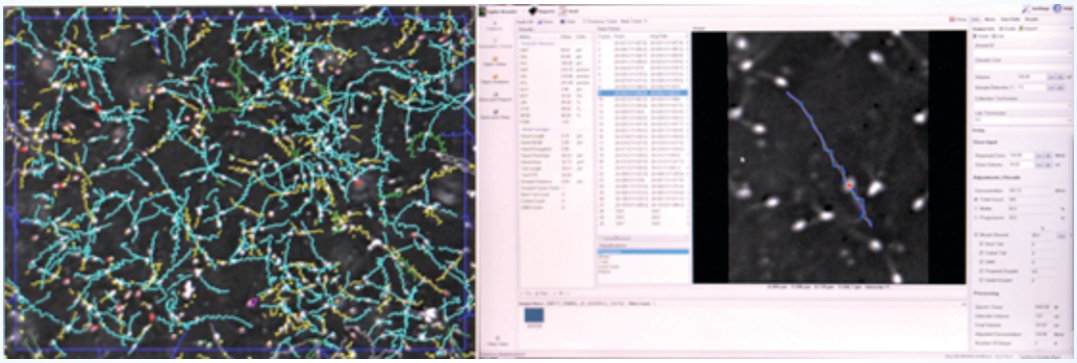


Fig. Kinematics of stallion spermatozoa

Algorithms used to calculate these indices may vary between CASA software resulting in potentially different results for the same semen sample when using different systems. In addition, variations in the settings for spermatozoal head size and others may also yield differing results. Thus, it is important to report all settings when publishing results.

Longevity

Prior to the beginning of each breeding season, the longevity of spermatozoa should be determined for breeding stallions. Storage may be at room temperature or at cooled semen shipment temperature and may be with or without a semen extender. For practical considerations the longevity testing should be done under the same conditions as those under which the semen would be normally used. Longevity of sperm motility is used as an indicator of ejaculate quality. Longevity can be determined by the evaluation of motion characteristics over time for raw and extended semen samples maintained ateither room temperature (20–25˚C) or cooled to refrigerator temperature (4–6∘C).

Raw Semen Longevity

Place semen in a 15 ml centrifuge tube in a rack at room temperature (20∘C, 68∘F). The tube should be covered with aluminium foil to prevent the admittance of light.

Interpretation for Raw Semen Longevity

Good sperm longevity: >10% progressive motility at 6 hours post-collection when held at room temperature.

Poor sperm longevity: <10% progressive motility at 3 hours post-collection when held at room temperature.

Morphology

Sperm morphology evaluation is an essential component of semen analysis and provides the clinician with valuable information for assessing the breeding soundness of a stallion and the potential fertility of individual semen samples. The most widespread (and dangerous) misconception regarding the evaluation of stallion sperm morphology is the assumption that morphology is not important or that it is less important than sperm motility. Sperm morphology greatly impacts fertility in all species studied, and it would not be expected to be any different in horses.

Proper evaluation of sperm morphology is a considerable technical challenge as the process is subjective and results are largely dependent on the proficiency and experience of the evaluator. As a consequence, significant differences in results can be observed among different clinicians. Sperm morphology can be determined after staining with an eosin-nigrosin stain, which has also been used as a "live-dead" stain. An alternative is to preserve semen in buffered formol-saline, prepare a wet mount slide, and examine the semen sample using a phase-contrast microscope.

Accepted values for semen analysis are: (i) a minimum of 60% progressively motile spermatozoa and 60% morphologically normal spermatozoa in each ejaculate collected; and (ii) a minimum of 1.0 billion progressively motile, morphologically normal spermatozoa in the second of two ejaculates collected 1 hour apart after sexual rest for 1 week at any time of the year. Based on the overall results of the reproductive evaluation, stallions are classified as satisfactory, questionable, or unsatisfactory breeding prospects.

There is a wide variation in sperm morphology among breeding stallions, but in general the average stallion has approximately 50% morphologically normal sperm. Sperm morphology can vary considerably during the breeding season and routine evaluations (every 2–4 weeks) should be performed to determine sperm morphology characteristics of a particular stallion. More than 30% sperm head defects, >25% proximal cytoplasmic droplets, or <40% normal sperm are reasons for concern.

Viability assessment

Morphologic evaluation of stallion semen is essential to any breeding soundness examination. The percentage of morphologically normal sperm is positively correlated with pregnancy rates in all species. Many methods have been developed to determine the quality of spermatozoa including sperm chromatin integrity, acrosomal reaction and capacitation, and special stains. A stain used routinely for semen evaluation across species is eosin-nigrosin, otherwise known as Hankcock's stain. The eosin component will penetrate through the plasma membrane of dead sperm cell and is excluded from live cells. Nigrosin provides background contrast to evaluate morphology and differentiate between live and dead sperm. Cells that are dead will stain red, whereas live cells are white ("red is dead"). The ease with which this stain is utilized for determining the morphologic characteristics of spermatozoa makes it a widely used stain in reproductive practice.

"Sperm viability" is an ambiguous but commonly used term. Most measures of sperm viability refer to the integrity of the sperm plasma membrane. Techniques used to measure sperm viability rely on penetration of special stains such as eosin or propidium iodide. Differences in sperm viability values determined by eosin-nigrosin staining versus PI staining may reflect differences in penetration rates or a subjective interpretation of degree of staining in eosin-nigrosin staining. Sperm viability measurements are usually correlated with estimates of sperm motility. However, there appear to be populations of sperm that are non-motile yet contain an intact plasma membrane (i.e., "viable").

Materials

Hancock's Stain (Hancock 1951)

1.67 g eosin Y. 10 g nigrosin.

Dilute to 100 mL DW.

Blom's Stain (Blom 1950)

5 g eosin B. 10 g nigrosin.

Dilute to 500 mL DW.

To prepare both stains, add the nigrosin to the water while stirring and heating until all is dissolved. Thereafter, add the eosin to the nigrosin solution. Boiling should be avoided throughout the procedure. However, it is difficult to dissolve stain at this concentration and is quite hypotonic.

Modified Eosin-Nigrosin Formula

1. 3.3 g eosin Y. 2. 20.0 g nigrosin. 3. 1.5 g sodium citrate.

Dilute to 300 mL DW.

Preparation of the Stain

(a) Dissolve the ingredients by stirring and heating as described above.

(b) Adjust the pH to 6.8–7.0 if necessary.

(c) Allow the stain to stand a few days and then filter it.

(d) Since Hancock's and Blom's stains are slightly hypotonic, a small amount of sodium citrate is added to increase the osmolarity. This prevents formation of bent tail, which is a common problem with the above two stains.

(e) Stock solution of eosin-nigrosin can be stored for several years if refrigerated. At RT these stains may be kept without harm up to 6 months. However, many times bacteria and fungi may multiply in the stains at RT

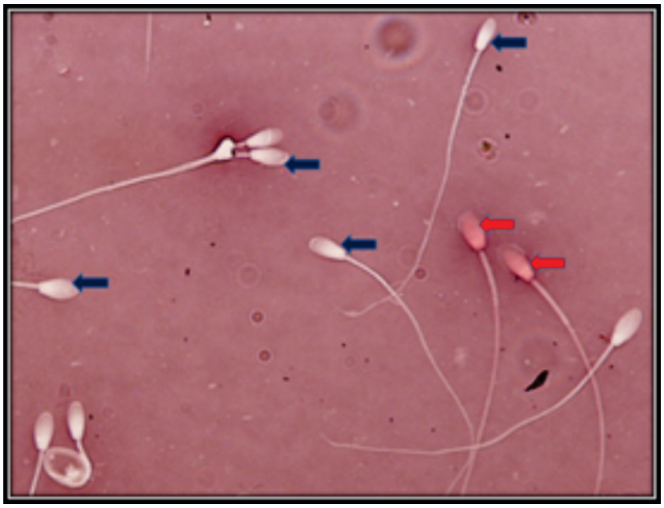


Fig. Picture showing live (Blue arrows) and dead (Red arrows) spermatozoa stained with eosin-nigrosin.

To prepare smear, one drop of semen is placed on clean, grease free, pre warmed glass slide and mixed with one drop of eosin-nigrosin stain using blunt fine glass rod. After a minute, a thin smear from the mixture is prepared on glass slide and air dried. A total of 200 sperm are counted from each smear for live and dead sperm percentage. The stain penetrates through plasma membrane of dead sperm and thus appears pink in color whereas unstained sperm are considered live. Partially stained sperm are considered as dead. The results are variable but it should be a minimum of 50% live and usually more than 60% for use in AI. An indication of the percentage of live: dead spermatozoa within a sample can be obtained initially by a motility assessment. Preparation of eosin nigrosin stain for sperm live-dead count and morphology 1. 3 gm of Sodium citrate (dihydrate) is dissolved in 100ml distilled water to make 3% solution. 2. 1 gm Eosin B and 5 gm Nigrosin are taken to dissolve in 100ml 3% Sodium citrate (dihydrate). 3. pH of the stain is adjusted to 7.0 by adding a few drops of 0.1 M NaH2 PO4 or 0.1 M Na2 HPO4 and filtered. This mixture has remained stable for more than one year without refrigeration. However, it should be refrigerated to prevent growth of bacteria, and warmed to room temperature before it is used for staining sperm.

Observation

Live spermatozoa remain unstained, while dead spermatozoa take pink or red stain against blue black dark background. Count partially stained sperm as dead ones.

Calculation

$$\text{Percent live sperm} = \frac{\text{Number of live sperm} \times 100}{\text{Total number of live and dead sperm}}$$

Acrosome Integrity

Equine sperm that have been freshly ejaculated are unable to fertilize an oocyte without undergoing a series of cellular modifications known as capacitation. Changes occurring in the spermatozoa during capacitation include loss of cholesterol from the plasma membrane, hyperactivated motility, and increases in intracellular calcium, pH, and cAMP. The phenomenon of capacitation opens receptor sites on the spermatozoon, which allows binding to the zona pellucida of the oocyte and the acrosome reaction to occur. The normal acrosome reaction will only occur in fully capacitated sperm, and will be triggered by the sperm binding to the zona pellucida.

The acrosome is an enzymatic cap that is located on the rostral end of the sperm's head. The acrosomal enzymes are released during the acrosome reaction, which digest a hole through the zona pellucida, allowing the sperm to pass through the zona pellucida and fertilize the oocyte. The acrosome reaction is an exocytotic event in which the plasma membrane fuses with the outer acrosomal membrane leading to small, separate, membrane-bound vesicles, thereby releasing the enzymatic contents of the acrosome. The acrosome reaction of mammalian spermatozoa is an essential contributor to fertilization because only acrosome-reacted spermatozoa can penetrate the zona pellucida and fuse with an oocyte. Sperm that have undergone an acrosomal reaction are no longer able to participate in fertilization hence visualization of sperm acrosome integrity may be beneficial in the evaluation of semen quality, since male infertility could be caused by a lack of sperm with intact acrosomes at ejaculation. Once acrosome-reacted, the life span of the sperm is very short and if fertilization does not occur the sperm will die.

Giemsa word comes from the German bacteriologist and chemist Gustav Giemsa. The stain is widely used in sperm morphology evaluation and cytogenetics and for the histopathological diagnosis of malaria and similar other parasites. Giemsa stain is specific for binding with the phosphate groups of DNA where high amount of adenine-thymine bonding is present.

Materials

Giemsa powder/solution, methanol, glycerol,
sodium citrate dihydrate 2.9%, formalin 40%, potassium dihydrogen phosphate (anhydrous), disodium hydrogen phosphate, sodium chloride, sodium bicarbonate

Giemsa Stain Solution (Stock)

1 g	Giemsa stain
98 mL	Methanol
32 mL	Glycerol

Stain Preparation

(a) Take Giemsa stain powder in a glass mortar.
(b) Add small quantity of absolute methanol and ground well using pestle.
(c) Repeat the process after adding small quantities of methanol until dissolved completely.
(d) Filter the solution using Whatman filter paper.
(e) Add glycerol and store at 37 °C for 1 week.
(f) Mix the stain mixture for few minutes every day for 1 week.

Sorenson's 0.1 M Phosphate Buffer (pH 7.0)

17 mL solution A.
33 mL solution B.
Adjust pH to 7.0.

Solution A:

0.1 M Potassium Dihydrogen Phosphate Solution
13.609 g potassium dihydrogen phosphate (anhydrous).
Dilute to 1000 mL DW.

Solution B:

0.1 M Disodium Hydrogen Orthophosphate Solution
14.198 g disodium hydrogen phosphate.
Dilute to 1000 mL DW.

Hancock's Fixative

10 g sodium chloride.
0.5 g sodium bicarbonate.
125 mL formalin.
Dilute to 1000 mL DW.

Giemsa Working Solution

3.0 mL stock Giemsa solution
2.0 mL Sorenson's 0.1 M phosphate buffer
45.0 mL double glass DW

Procedure

(a) Prepare a smear of neat (thin) or diluted (if thick) semen sample on a glass slide and air-dry.
(b) Dilute thick semen with 2.9% sodium citrate dihydrate solution.
(c) Keep slide in a coupling jar with Hancock's fixative for 15 min.
(d) Wash fixed smear in slow running water for 10 min.
(e) Rinse with DW and air-dry it.
(f) Stain slide in Giemsa working solution in a coupling jar for 3–4 h.
(g) Remove slide from the stain solution and rinse quickly in DW.
(h) Air-dry slide and mount in DPX mountant.
(i) Examine the slide under oil immersion objective (100 x) of the microscope.
(j) Count at least 200 spermatozoa and categorize as acrosome intact or acrosome reacted

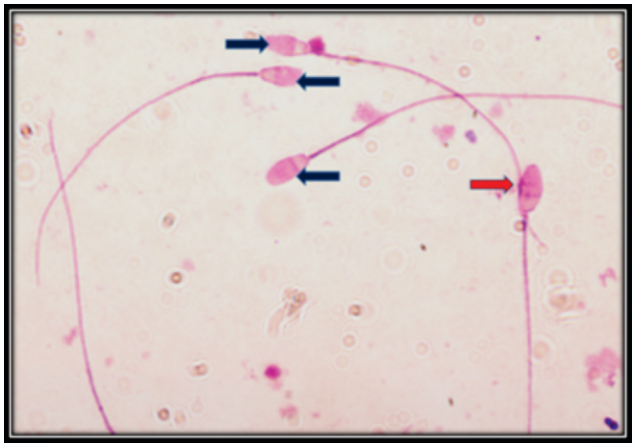


Fig. Picture showing stallion spermatozoa with intact acrosome (Blue arrwos) and reacted acrosome (Red arrows).

Plasma membrane integrity (Hypo-osmotic swelling test)

There is a high correlation between the Hypo-osmotic swelling (HOS) test and progressive motility. However, the relationship between the HOS test and fertility is not well established in the horse. One study reported that stallions with a “low swelling score” (<40%) had doubtful fertility. It has been suggested that the HOS test may be especially valuable in the evaluation of membrane integrity of frozen-thawed equine spermatozoa. The goal of the hypo-osmotic swelling (HOS) test is to evaluate the functional integrity of the plasma membrane of spermatozoa. The test is simple to perform, accurate, and consistent, with good reliability and repeatability. It has been validated for spermatozoa of stallions and several other species.

The basis of the test is the exposure of spermatozoa to a hypo-osmotic solution, such as a 50–100 mOsm lactose or sucrose solution. Fluid from the hypo-osmotic solution will be transported across the plasma membrane. Spermatozoa with intact plasma membranes will exhibit characteristic swelling and/or coiling of the tail as water enters the intact sperm cell. Sperm with plasma membrane damage do not swell or exhibit coiling of the tail since fluid is not confined within the sperm cell.

The HOS test can be used to evaluate sperm plasma membrane integrity in lieu of more expensive diagnostic tests, such as supravital stains (SYBR-14 and propidium iodide) which require fluorescent microscopy or a flow cytometer.

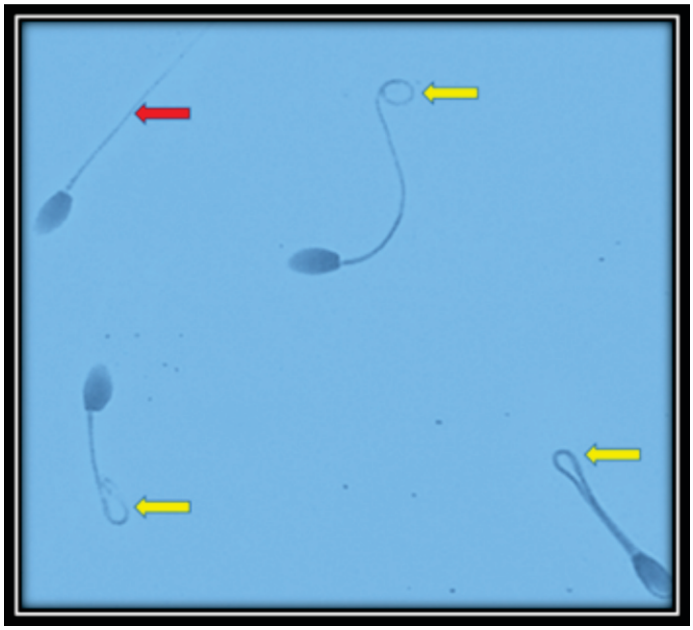


Fig. Spermatozoa with intact plasma membrane (yellow arrows) and damaged plasma membrane (Red arrow)

Technique

- A 100 µl volume of raw semen is added to 1.0 ml of pre-warmed hypo-osmotic solution in a glass test tube or micro centrifuge tube.
- The contents are gently mixed.
- The sample is incubated at 37°C (99°F) for 30–60 minutes.
- A small drop of sample (approximately 30 µl) is placed onto a glass slide and a coverslip applied.
- Sperm morphology is evaluated in the wet mount sample at 400× or 1,000× using either bright field, differential interference contrast (DIC), or phase-contrast optics
- The percentage of sperm with swollen or coiled tails (HOS+) is determined to acquire the percentage of plasma membrane-intact spermatozoa.

ASSESSMENT OF SPERM FUNCTIONAL PARAMETERS WITH FLUORESCENT STAINS

Sperm Plasma Membrane Integrity and Viability: Propidium Iodide/SYBR-14

Viability is usually highly correlated with spermatozoal motility. However, in some cases a significant percentage of sperm may be viable (membrane intact), but not motile. Unfortunately, sperm motility and/or viability alone do not necessarily correlate with fertility in a stallion. Spermatozoa are considered as viable when they have an intact plasma membrane. SYBR-14 is a fluorescent stain that can penetrate all cells, membrane damaged or intact, and bind to DNA. SYBR-14 is used in the evaluation of semen to identify spermatozoa from other non-cellular debris in the sample. Propidium iodide (PI) is a fluorescent stain that cannot penetrate an intact plasma membrane and therefore can only enter spermatozoa and bind to DNA of spermatozoa with a damaged plasma membrane (i.e., a non-viable sperm). A dual stain of PI and SYBR-14 is commonly used to evaluate the viability of stallion spermatozoa. The two stains are added to an aliquot of spermatozoa and when excited by a laser, non-viable cells will fluoresce red from the PI and live, membrane-intact cells will fluoresce green with SYBR-14. Fluorescent stains allow for greater contrast and interpretation between viable and non-viable cells as compared with dried smears with an eosin-nigrosin (Hancock) stain.

Stock Solutions

Stock solutions of SYBR-14 can be prepared and stored in small aliquots until used. To prepare the stock solution add 900 µl of dimethyl sulfoxide (DMSO) to the 100 µl of SYBR-14 that comes in the viability kit. An aliquot of 30 µl can then be placed into micro centrifuge tubes and stored at –20°C (–4°F) until use. To make a working solution to stain cells, add 270 µl DMSO to the thawed 30 µl aliquot of SYBR-14 stock solution. This results in a final concentration of 10 µM SYBR-14 and will remain stable at 5°C (40°F) for 3–4 weeks.

Stock solutions of PI do not have to be made if using the Molecular Probes sperm viability kit; however, the solution can be divided into smaller aliquots and stored at –20°C (–4°F) until use. Once thawed, the stain is stable at 5°C (40°F) for 4–8 weeks.

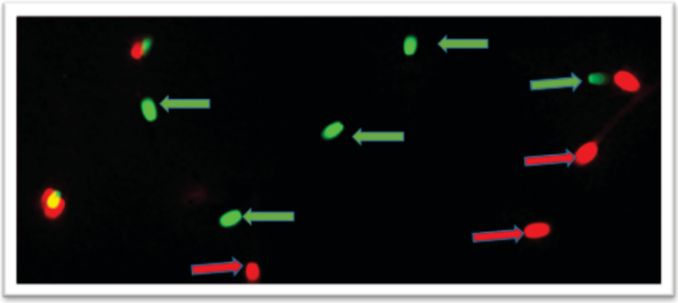


Fig. Picture showing Live and Dead Spermatozoa stained with SYBR-14 and PI (Green Arrow= Live; Red arrows= Dead spermatozoa)

Technique

- Semen must first be diluted to a concentration of 25–50 × 106 sperm/ml in either a commercial skim milk/ glucose semen extender or TALP diluent
- In a glass test tube add 20 µl SYBR-14 (working solution), 10 µl PI, 200 µl TALP, and 100 µl diluted sperm. The samples should be incubated in a container protected from light exposure for 10–15 minutes at 20°C (68°F) before being read on a fluorescent microscope.
- Two separate slides should be analyzed with a minimum of 200 cells counted per slide. Cells that fluoresce red are considered non-viable, while cells that fluoresce green are considered viable. These cells can also be analyzed via flow cytometry. Flow cytometry allows a large number of cells (>50,000) to be automatically analyzed.

Assessment of Sperm Acrosomal Status: PE-PNA

Equine sperm that have been freshly ejaculated are unable to fertilize an oocyte without undergoing a series of cellular modifications known as capacitation. Changes occurring in the spermatozoa during capacitation include loss of cholesterol from the plasma membrane, hyperactivated motility, and increases in intracellular calcium, pH, and cyclic adenosine monophosphate. The phenomenon of capacitation opens receptor sites on the spermatozoon, which allows binding to the zona pellucida of the oocyte and the acrosome reaction to occur. The normal acrosome reaction will only occur in fully capacitated sperm, and will be triggered by the sperm binding to the zona pellucida.

The acrosome is an enzymatic cap that is located on the rostral end of the sperm's head. The acrosomal enzymes are released during the acrosome reaction, which digest a hole through the zona pellucida, allowing the sperm to pass through the zona pellucida, bind to the oocyte plasma membrane, and ultimately fertilize the oocyte. The acrosome reaction is an exocytotic event in which the plasma membrane fuses with the outer acrosomal membrane leading to small, separate, membrane-bound vesicles, thereby releasing the enzymatic contents of the acrosome. Once acrosome-reacted, the lifespan of the sperm is very short and if fertilization does not occur the sperm will die.

The process of cooling and freezing equine semen can damage either the plasma or acrosomal membrane and can render a sperm infertile by causing a loss of intracellular molecules or premature release of the acrosomal contents, respectively. Conventional dried smear staining techniques cannot be used to detect the acrosomal status of stallion sperm, because of its small size relative to the sperm's head and the lack of an acrosomal ridge that is present on the sperm of many other species. However, fluorescent microscopy can be used to accurately detect the integrity of the acrosome. Certain lectins, plant proteins, bind only to the enzymatic contents of the acrosome. These proteins are large and cannot pass through an intact membrane. These lectins can be tagged with fluorescent probes and if membrane damage has occurred, they will contact and bind to any acrosomal enzymes adhering to the sperm head, causing the acrosomal area to fluoresce. Sperm that have intact acrosomes, however, will not allow any lectin

binding and will not fluoresce.

Two lectins are commonly used for these analyses; the common pea plant (Pisum sativum) agglutinin (PSA) and the peanut (Arachis hypogea) agglutinin (PNA). These lectins each bind to different sugar groups that are attached to proteins, and while both bind to proteins of the acrosome, PNA binds to fewer non-acrosomal proteins scattered over the entire sperm surface, making damaged acrosomes stand out much brighter than the rest of the sperm.

Lectins must be conjugated to fluorescent molecules in order to view them under a fluorescent microscope. Many different probes can be used, but fluorescein isothiocyanate (FITC), a molecule that excites at 488 nm and provides a green fluorescent emission at 518 nm, is most commonly used, as it fluoresces brighter than other probes. However, lectins conjugated to phycoerythrin (PE), which also excites at 488 nm but provides a yellow-orange fluorescence at 578 nm, can be used too. A triple staining procedure is used for the detection of acrosomal damage by fluorescent microscopy. Cells are stained with a combination of propidium iodide (PI), SYBR-14, and PE-PNA. The PI/SBYR-14 combination is necessary to differentiate between live and dead cells, while PE-PNA is used to determine if acrosomal damage has occurred.

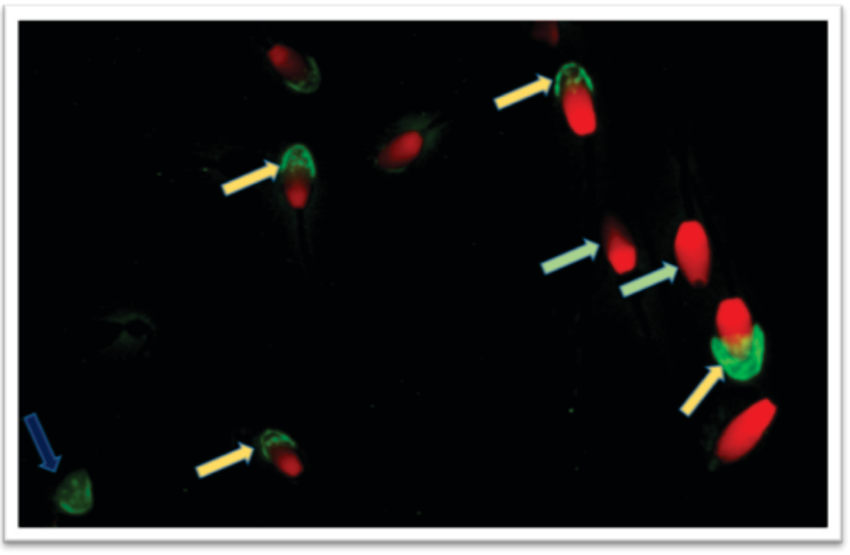


Fig. Picture showing acrosome reacted and acrosome non-reacted Spermatozoa stained with FITC-PNA and PI (Yellow Arrow= Dead acrosome reacted; Green Arrow= Dead non-reacted; Blue arrows= Live reacted spermatozoa)

Stock Solutions

- Stock solutions of SYBR-14 and PI should be
- Stock solutions of PE-PNA do not have to be made. Store the PE-PNA solution at 5°C (40°F), and do not freeze.
- Prepare a stock solution of sperm TALP media

Technique

- Semen must be diluted to a concentration of 25–50 × 10^6 sperm/ml in the TALP diluent.
- In a glass test tube add 20 µl SYBR-14 (working solution), 10 µl PI, 5 µl PE-PNA, 200 µl TALP, and 100 µl diluted sperm.

The samples should be incubated for 10–15 minutes at 20°C (68°F) before being read on a fluorescent microscope.

- Two separate slides should be analyzed with a minimum of 200 cells counted per slide.

There will be three separate populations of sperm analysed 1): viable, acrosome-intact sperm (fluoresces green), viable, acrosomal- damaged sperm (fluoresces green with orange acrosome cap), and non-viable sperm (fluoresces red). The acrosomal status of the non-viable sperm is hard to discern because of the similar fluorescence of PI and PE-PNA.

Interpretation

Equine sperm must possess an intact acrosome when it reaches the oocyte in order for fertilization to occur. If the acrosome is lost or damaged during processing, cryopreservation, or in transit through the female reproductive tract, the sperm cannot bind to the zona pellucida and fertilization will not occur. Therefore it is necessary to have intact acrosomal membranes at the time of insemination.

Measuring acrosomal integrity as a single attribute has been poorly correlated with a stallion's potential fertility. However, it can be expected that samples containing a high percentage of acrosome-damaged sperm (>10% of the population) will most likely exhibit reduced fertility. One potential way to increase the fertility of these frozen samples would be to inseminate an increased number of sperm into the mare compared with the normal insemination dose. This increase in sperm numbers will increase the number of viable, acrosome-intact sperm present in the oviduct during the time of fertilization. Changes in the processing and cryopreservation of sperm from a stallion that experience an increase in acrosomal damage after thawing may also improve the integrity of the acrosome and fertility. Sperm from individual stallions respond differently to cryopreservation and changes in the freezing extender, cryoprotectant, centrifugal force, and freeze rate may be necessary to preserve the acrosome.

Assessment of Sperm Mitochondrial Function: JC-1 and Rhodamine 123

The evaluation of sperm motility has been an important standard method of assessing sperm quality. The mitochondria of sperm provide the energy source for motility and any changes in mitochondrial function may be reflective of changes in sperm motility. Visual or computer-assisted sperm analysis (CASA) assessment of spermatozoal motility is not always correlated with the estimated fertility of a stallion. The energy source for motility is produced in the mitochondria of the middle piece of a spermatozoon. Changes in mitochondrial membrane potential may result in changes in sperm motility and be more indicative of spermatozoal function.

Rhodamine 123 (R123) is the most widely used mitochondrial probe. R123 excites with visible light (488 nm) and emits a green fluorescence at 515–575 nm. This probe has been validated for stallion sperm; however, this stain cannot differentiate between high and low mitochondrial membrane potential, only the presence of mitochondria.

The lipophilic probe JC-1 (5,5′,6,6′–tetrachloro-1,1′,3,3′– tetraethylbenzimid-azolyl carbocyanine iodide) has been reported as distinguishing sperm cells with high or low mitochondrial membrane potential. JC-1 is excited by visible light (488 nm) and accumulates in the mitochondria of the sperm. With an increase in transmembrane electrical potential, JC-1 forms aggregates that will cause the mitochondria to fluoresce orange (590 nm). However, if the sperm has low membrane potential, JC-1 will form monomers in the mitochondria which will fluoresce green (530 nm). It is hypothesized that sperm with high mitochondrial membrane potential are more functionally correct and are able to produce the necessary adenosine triphosphate (ATP) energy to provide sperm motility.

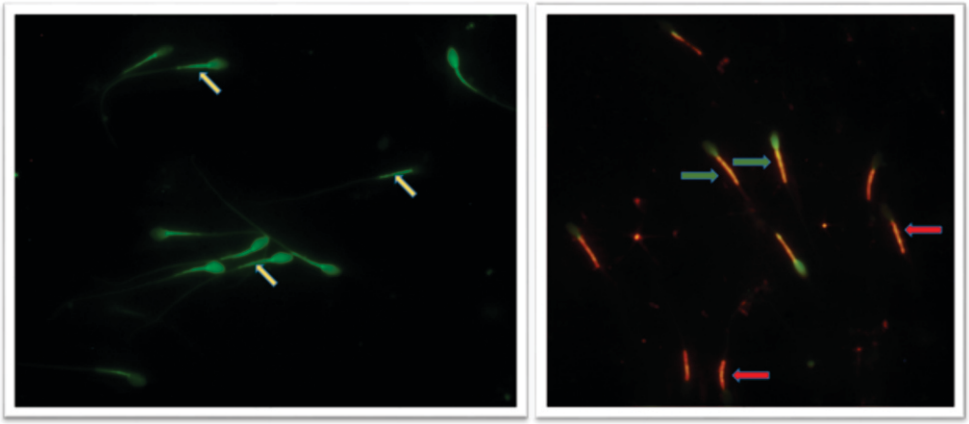


Fig. Picture showing spermatozoa having high and low mitochondrial potential stained with JC-1 (Yellow and Green Arrows= Live sperm with high mitochondrial potential; Red Arrows= Live spermatozoa with low mitochondrial potential)

Stock Solutions

- Stock solutions of JC-1 are made by dissolving 5 mg of JC-1 powder into 1.094 ml of DMSO.
- To make a working solution, place 11 µl of the stock solution into a microcentrifuge tube and add 189 µl of DMSO.
- Sperm TALP (Tyrode's albumin–lactate–pyruvate) medium consists of 37 mM NaCl, 10 mM KCl, 1 mM KH 2PO4, 36 mM NaHCO3, 2.4 mM MgSO4, 10 mM HEPES, 2 mM CaCl2, 84 mM fructose, 6 mM glucose, 182 µM Na-pyruvate, 0.37 ml/l Na-lactate, and 3 g/l bovine serum albumen (BSA), with the pH adjusted to 7.2.

Technique

- Semen must first be diluted to a concentration of 25–50 × 10^6 sperm/ml in a commercial skim milk/glucose diluent or TALP diluent.
- In a glass test tube add 20 µl JC-1 (working solution) and 500 µl of diluted sperm. Incubate sperm in a 37°C (99°F) water bath or incubator for 20 minutes.
- After incubation add 250 µl of JC-1-stained sperm to 250 µl TALP in a separate glass test tube and mix gently.
- The sperm can now be read using a fluorescent microscope. Two separate slides should be analyzed with a minimum of 200 cells counted per slide.

With the use of JC-1, two distinct sperm populations can be observed. The percentage of cells fluorescing orange indicates the amount of sperm in the sample that contains functioning mitochondria. A higher percentage of functioning mitochondria in the sample provides information that the sperm are able to produce the energy needed for motility. An increase in the percentage of cells staining green may indicate a functional loss in motility and fertility may be compromised. Changes in the procedures used to cryopreserve these sperm cells may help to increase the amount of cells with high mitochondrial membrane potential after thawing

Table 2. Fluorescent probes used for the evaluation of sperm attributes

Attributes	Probes
Plasma membrane integrity (or) Viability	SYBR14, PI, CFDA, YO-PRO-1, Hoechst 33258, Hoechst 33342, 7-AAD, Ethidium Homodimer, Ethidium bromide, TO-PRO-3
Acrosome reaction	Lysotracker Green, Lectins (PSA, PNA, ConA, UEA and WGA) tagged with FITC, PE, Alexa Flour 488, Alexa Flour 647 or TRITC
DNA integrity	Chromomycin A3, Acridine Orange (SCSA)
Capacitation changes	CTC, Fluo-3, Fluo-4, Indo-1, Mercyanine540, eFluor 514 Calcium sensor, Rhod5, Fura2, BCECF AM, SNARF-1
Mitochondrial status	JC-1, JC-9, JC-10, Rhodamine 123, DiOC6, TMRE, TMRM, Mitotracker green, MitoTracker deep red, MitoTracker red, MitoTracker Red CMXRos, MitoTracker Orange CMH2TMRos, RedoxSensor Red CC-1, Nonyl Acridine Orange, Mito-ID Red, MitoFluor Green, MitoFluor Red 589
Apoptosis	Annexin V & PI assay, YO-PRO-1, SYTO16, Carboxyfluorescein-tagged caspase inhibitor probe
Lipid peroxidation & ROS	BODIPY, MitoSOX, Dihydroethidium, H2DCFDA, DAF FM and DAF-FM diacetate, Monobromobimane, 5-iodoacetamido?uoresceine (5-IAF).

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