ICAR-NRCE Technical Bulletin

CRYOPRESERVATION OF HORSE AND DONKEY SEMEN

S K Ravi, R A Legha, T R Talluri, R K Dedar
R C Sharma, Yash Pal, B N Tripathi, R K Singh

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ICAR-National Research Centre on Equines
(Indian Council of Agricultural Research)

Equine Production Campus
Post Box No. 80 Bikaner 334001 (Rajasthan) India

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Publication Committee : S K Ravi
T R Talluri
R A Legha
Yash Pal

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T R Talluri

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PREFACE

Horses and donkeys being important animals for draught and transport play significant role in the rural economy and constitute main source of income for sizeable underprivileged section of society in many parts of the country. The horse breeds in India are well adapted to different agro-climatic regions and possess certain unique characteristics. However, owing to indiscriminate breeding and lack of sound breeding policies, the breed's characteristics are being diluted. Assisted reproductive technologies like semen cryopreservation and artificial insemination (AI) in equines are the answer to address such issues and conserve the equine germplasm. AI technique has been widely used in the domesticated animals worldwide for rapid genetic improvement and propagation of superior germplasm. Cryopreservation of semen has aided new dimension in AI technique in terms of easy transport of semen and semen storage for years by cryopreservation, and control on spread of venereal disease transmission.

ICAR-NRCE has standardized the technique for collection and cryopreservation of semen from horses and donkeys. The frozen semen is being used successfully for AI in mares and jennies. Mules have been produced at the centre and in the field at farmer's doorstep. The centre has also been providing frozen semen doses to the field veterinarians for AI to propagate purebreds. Semen from purebred horses and elite stallions were cryopreserved at owner's doorstep from many places in Rajasthan, Gujarat and Punjab, which were brought to this centre for breed conservation and propagation.

This technical bulletin will be useful for academicians and to the researchers engaged in equine semen cryopreservation and the related techniques. This can be used as teaching and training material to learn the process and techniques of semen collection and cryopreservation from horses and donkeys. This will serve as a reference material for future research work to study components of semen diluents, cryoprotectants and the freezing technique to improve post-thaw sperm motility. The technique of semen cryopreservation can be applied to enhance equine production for the benefit of equine owners and for sustainment of the species.

It is a matter of pleasure to present the technical bulletin on 'Cryopreservation of horse and donkey semen'. I congratulate the team of authors for bringing out this technical bulletin.

Tripathi

(B.N. Tripathi)
Director, ICAR-NRCE

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Introduction

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 with frozen semen (Loomis, 2001) 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). Though, 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). There are several advantages of semen cryopreservation like transport of frozen semen is much less expensive than to transport a horse or a mare for breeding, semen doses can be stored theoretically for indefinite period without loss of fertility, it can be used worldwide whenever required and less wastage of an ejaculate from quality stallions/jacks since the entire ejaculate can be subdivided into many semen doses for use in AI. Moreover, cryopreservation technique allows stallion's semen to be used for AI while he is busy in competition, ill or injured and cryopreserved semen can be used even after death of donor. Other benefits of this technique include avoiding spread of venereal diseases, less risk of disease transmitted through semen and making breeding possible in mare/jenny that do not stand for stallion. Use of frozen semen has great importance in producing superior mule due to ease for AI in jennies. This technique provides great aid in germplasm conservation and its multiplication at faster rate. Therefore, preliminary research work on freezing of the semen was initiated at ICAR-National Research Centre on Equines (ICAR-NRCE) at Bikaner sub campus during the year 1997 in exotic jacks. Roy *et al* (2003) reported higher sperm concentration in summer than in autumn and

winter in exotic donkeys as wells as non-significant effect of season on live sperm percentage. Effect of season on characteristics of jacks semen were studied (Gupta *et al*., 2003) and physical, biochemical characteristics like total motility, progressive motility, p^H, colour, consistency, total spermatozoa count were performed (Gupta *et al*., 2008) as prerequisite to semen freezing. Similarly, comparative study of physical, morphological and biochemical characteristics of donkey and horse stallions were studied by pal *et al*. (2009). Pal *et al*. (2007) cryopreserved the semen of true to breed Marwari stallions at farmers' door. Overall post thaw motility of sperm for thawing protocol 37°C for 30 seconds in the breeding and non breeding season was reported 25.11 ±0.82 and 39.46 ±0.84 per cent, respectively (Kumar, 2008). The semen of Marwari stallion and Poitou jack was characterized (Pal *et al*. 2009) as well as cryopreserved using vapor freezing technique in Marwari stallion (Pal *et al*. 2011) and Poitou jack (Legha and Pal, 2012a; 2012b). Semen from Marwari horses were cryopreserved at owner's doorstep from places including Udaipur, Ladnu, Dundlodh (Jhunjhunu) in Rajasthan and Gumjal in Punjab with 35-50 % post thaw sperm motility. Similarly, semen from Kathiawari horses (Ravi *et al*., 2013) in field at Junagarh (Gujarat), Zanskari horses (Talluri *et al*., 2012) and indigenous donkeys (Pal *et al*. 2013; Legha *et al*. 2013) at ICAR-NRCE were characterized and cryopreserved. Arangasamy and Tandon (2008) assessed frozen semen of Marwari stallions and Poitou jacks for acrososmal integrity using Peanut Agglutinin (PNA) coupled with fluoroscienisothiocynate (FITC). Hypo-osmotic swelling test (HOST) was performed for quality assessment of frozen Poitou jack semen (Arangasamy *et al*., 2009; Talluri *et al*., 2012), Marwari and Zanskari horses (Talluri *et al*., 2012). Stallions were categorized as poor, moderate and good freezers which had post-thaw motility <20, 20–40 and >40%, respectively (Pal *et al*., 2012).

AI with frozen semen is being practiced at ICAR-NRCE since last one decade in farm mares and donkeys which was extended further to mares visiting to centre as well as in field mares. Jhamb *et al*. (2006) reported pregnancy rate as 61 and 36%, using fresh semen vs. frozen semen, respectively, in Thoroughbred horses. The conception rate of 72.73% (8/11) and 66.67% (10/15) were reported in Marwari mares (Arangasamy *et al*., 2008) and exotic donkeys (Arangasamy *et al*., 2009), respectively, using fixed time insemination scheme with frozen semen. Encouraging results obtained from farm and the field with positive attitude of the farmers towards AI with frozen semen for future use. Though, risk of disease transmission is less with use of frozen semen, semen could act as a vehicle for the wide distribution of pathogens, therefore, it acquires importance to verify the microbial

contamination of semen in order to find out the risk involved in transmission of pathogens to the mare in AI. Microbial quality of fresh and frozen semen samples were studied for presence of aerobic pathogenic and non-pathogenic bacteria, and fungi (moulds and yeasts) in Indian horses and exotic donkeys (Vaid *et al*., 2012).

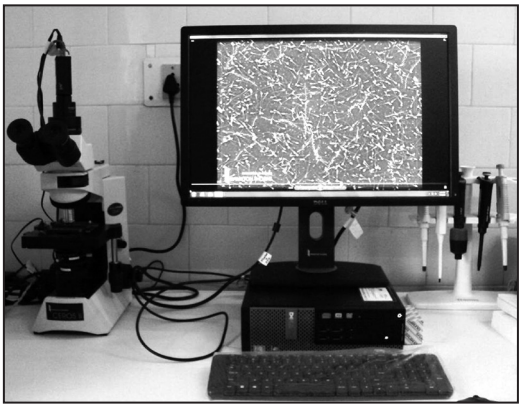
Cryopreservation of semen is a technique where sperm cells being preserved are exposed to sub zero temperature such as -196°C. This technique is based on principle to minimize biological activities of sperm cells to its least and thus production of negligible amount of waste products; by gradual cooling to sub zero temperature. Media which is used to dilute sperm cells helps to supplement energy, nutrient requirement and also resist change in its p^H. To save sperm cells from adverse effect of sub zero 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 sub zero 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 (Leopold Sybil, www.livinglegends.org.au).

List of essential requirements for semen freezing

Equipments

1. Electronic weighing machine
2. Dummy for stallion to mount
3. Artificial vagina (AV) for semen collection
4. Microscope for semen evaluation/ Computer assisted semen analyzer
5. Refrigerated centrifuge machine
6. Vacuum pump with sucking glass nozzle or pipette pump for semen plasma separation

Electronic weighing machine



Computer assisted semen analyzer

Artificial vagina

Liquid nitrogen transport container

7. Automatic straws filling and sealing machine or kit for manual filling and sealing, including straws holding clamp, disposable cups, cup and comb holding stand
8. Cooling cabinet/refrigerator
9. Automatic controlled rate freezer machine with UPS battery or alternative thermocol box with wired net/freezing racks to hold straws

Frozen semen storage containers

10. Magnetic stirrer
11. Liquid nitrogen transport and storage containers
12. Water bath/Thawing kit
13. Hot air oven
14. Automatic autoclave machine for sterilization of heat labile objects.

Chemicals

Chemicals (analytical grade): for preparation of media, staining etc

1. Lactose
2. Glucose
3. Sodium citrate dehydrate
4. Di-sodium EDTA
5. Sod-bicarbonate
6. Streptomycin (antibiotic)
7. Benzyl penicillin (antibiotic)

8. Glycerol/Dimethyl formamide/ Dimethyl sulfoxide as cryoprotectant
9. Poly vinyl alcohol powder for sealing of straws
10. Eosin-nigrosin stain for sperm live : dead between and sperm morphology visualization

Glassware and plasticware

1. Measuring cylinders (at least one each of 100, 250 and 500 ml capacity)
2. Conical flask (at least two of 250 and one of 500 ml capacity)
3. Glass slides and cover slips
4. Glass pipette (1 ml and 10 ml)
5. Straws of 0.5 ml capacity (in thousands of same or different colors)
6. Plastic tubes of 50 ml capacity (50 in number)
7. Funnels (two)

Miscellaneous items

1. Disposable plastic hand gloves, Disposable plastic liner for AV
2. Liquid paraffin, Lab wash detergent
3. Helmets, Shoes/gumboot
4. Aprons, lab slippers, rubber gloves, hand wash
5. Bucket, Mug, tub and dustbin
6. Water heater, Thermometer, Hemocytometer chamber and pipette
7. Micro pipette, pipette pump, Scissors, Marker pen
8. Fresh eggs
9. Tissue paper, Gauze, Cotton, Alumunium foil
10. Syringes (20 ml), Pipette tips (200 µl), Microcentrifuge tubes (2.5 ml)
11. Cotton bandages for tail bandage and rope for animal restraining
12. Test tube stands

Cleaning and sterilization of equipments and accessory items

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. Articles are kept inverted in a clean tray allowing water to dry. AV should also be washed thoroughly with detergent solution using brush and there is no need to separate inner rubber liner from the hard rubber cylinder. Application of corrosive substances on rubber articles should be avoided. AV should then be washed with water. The laboratory should be cleaned preferably with vacuum cleaner. All used items should be washed immediately with water otherwise allowing semen or media to dry and thereafter it requires more effort to clean.

Openings of glassware and metallic ware after cleaning should be closed for their sterilization. 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

Hot air oven for hot sterilization and drying

Autoclave for sterilization

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. Buffer solutions should be cooled down to room temperature and should be stored in refrigerator. Straws, gauze etc may be sterilized using ultraviolet radiation in closed chamber. Heat labile and moisture sensitive objects may be sterilized using ethylene oxide gas sterilization in closed chamber. This uses feroxide which is mixture of ethylene oxide and (12%) and freon (88%).

Preparation of diluents for semen freezing

Primary and secondary extenders

Processing of equine semen for cryopreservation involves use of two extenders: a primary for initial dilution, sperm washing and secondary cryoprotective extender for final dilution. Both the primary and secondary extenders contain energy source (sugar); protein source (egg yolk or milk); electrolytes (buffers) and antibiotics. Extenders help to sustain as well as extend the viability of sperm in fresh and cryopreserved semen. Secondary extender contains additional cryoprotectant mixed into it.

Primary extender : Primary extender is to maintain sperm motility, also act to protect sperm while centrifugation. Primary extender is aspirated off after centrifugation called sperm washing, prior to addition of a secondary extender. This is required to mitigate deleterious effect of seminal plasma on long term sperm viability.

Composition of primary extender (Citrate- EDTA) : 100 ml volume

(i)	Glucose	0.15 g.
(ii)	Sodium citrate dehydrate	2.60 g.
(iii)	Di-sodium EDTA	0.37 g.
(iv)	Sod-bicarbonate	0.12 g.
(v)	Streptomycin	0.10 g.
(vi)	Benzyl penicillin	0.10 g.

Preparation : All above are dissolved in conical flask by addition of double distilled water and making final volume up to 100 ml. This can be stored at 4°C for use within 6 hrs.

Secondary extender : Secondary extenders are those that provide dilution to ejaculate and also protect spermatozoa from cold shock. Cryoprotectant is thus an integral part of secondary extender. This is constituted by mixture of solution A and B :

Composition of Solution A (EDTA- glucose) : 100ml volume

(i)	Glucose	6.00 g.
(ii)	Sodium citrate dehydrate	0.37 g.
(iii)	Di-sodium EDTA	0.37 g.
(iv)	Sod-bicarbonate	0.12 g.
(v)	Streptomycin	0.10 g.
(vi)	Benzyl penicillin	0.10 g.

Preparation: Above is dissolved in conical flask by addition of double distilled water and making final volume up to 100 ml. This can be stored at 4°C for use within 6 hrs.

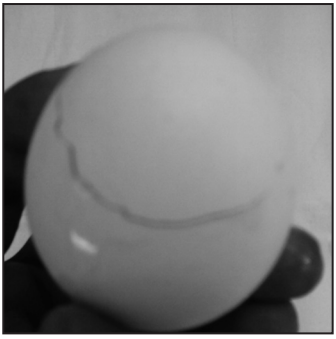
Composition of Solution B (Lactose solution) : 100 ml volume

(i)	Lactose	11.00 g.
(ii)	Streptomycin	0.08 g.
(vi)	Benzyl penicillin	0.08 g.

Preparation : Above is dissolved in conical flask by addition of double distilled water and making final volume up to 100 ml. This can be kept at 4°C if not using and discarded if not used within 6 hrs.

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



Breaking egg from narrow end

Collection of egg yolk

Egg yolk added with solution A and B

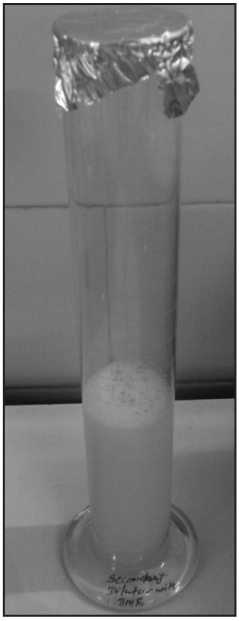
caution not to disturb yolk. Albumin is discarded and yolk is placed cautiously over sterilized piece of blotting paper to avoid rupture of yolk membrane. Syringe (20 ml) adaptor without needle is pierced through the yolk membrane and yolk is aspirated as much as possible by pulling plunger. Syringe 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 secondary extender: Lactose-Glucose-EDTA-egg yolk

Solution A and B is taken as 25 and 50 ml, respectively in an appropriate measuring cylinder (500 ml). Add 20 ml of egg yolk, cover with aluminium foil, mix well by shaking measuring cylinder up and down holding both the ends. Now, mixture of solution is taken into 50 ml sterilized plastic tubes and centrifuged at 3000 rpm in refrigerated centrifuge (temp 10-12°C) for 30 minutes. Tubes after centrifugation are taken out and the supernatant is filtered through sterilized cotton gauze leaving the remaining egg yolk. Suitable cryoprotectant such as Glycerol, Di-methyl formamide (DMF) or Di-methyl sulfoxide (DMSO) are added @ 2-5% to total volume of supernatant. This can be kept at 4°C if not using immediately within 6 hrs.

Refrigerated centrifuge machine

Primary extender ready for use



Secondary extender ready for use

Cryoprotectants

Cryoprotectants are classified as either penetrating or non-penetrating based on their action. Penetrating cryoprotectant can act both intra and extracellular level as being able to penetrate sperm plasma membrane whereas non-penetrating acts only extracellularly.

Cryoprotectants used for semen cryopreservation

An optimal cryoprotectant should have a low molecular weight and high water solubility; rapidly enters the cell, decreases temperature dependence and toxicity to cell. Glycerol has been the most frequently used cryoprotectant; however, at high concentrations, glycerol can be toxic to sperm and depress sperm fertility. Glycerol is typically used at concentrations of 2.5 to 6%. More recently, other cryoprotectant have been explored, including Ethylene glycol (EG), DMF, Methyl formamide (MF), Glutamine and DMSO. These alternative cryoprotectants have a lower molecular weight than glycerol which may enable them to penetrate the sperm plasma membrane more readily and decrease osmotic toxicity. Currently, DMF appears to be the best option to be used in combination with or to replace glycerol, particularly for stallions classified as poor freezers. Not only does DMF improve the quality of semen post-thaw, but also DMF has low or no apparent osmotic toxicity.

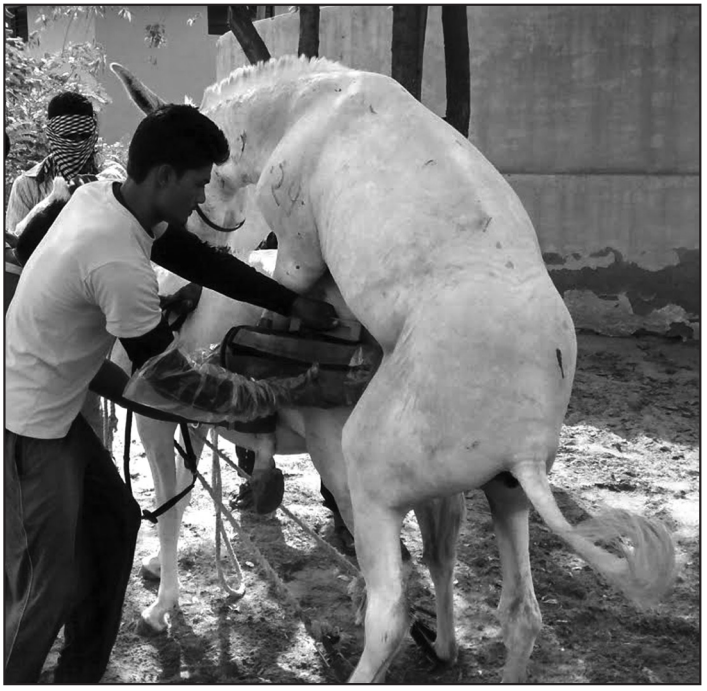
Semen collection

Semen collection using AV is an ideal method in equines. A dummy or estrous female is used for this purpose. In case dummy is not available and using an estrous 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

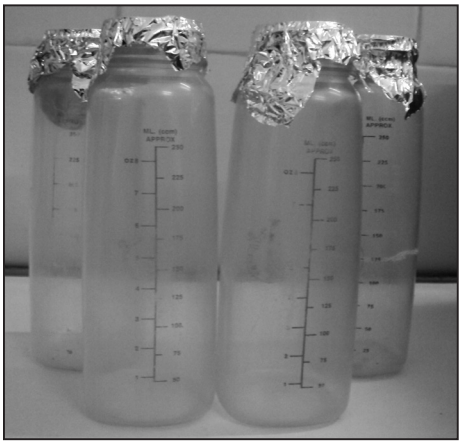
Preparation of Artificial vagina for semen collection

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.

Artificial vagina ready for semen collection



Semen collection from an Indigenous Jack



Plastic bottles of 250 ml used for semen collection

Ejaculate soon after semen collection

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.

Semen evaluation

Evaluating the semen has diagnostic value in determination of cause, severity and the degree of pathological conditions of testes as well as accessory organs in addition to check semen suitability for freezing. Good quality semen has predictive value for fertility of male. Semen should be evaluated as soon as possible after collection, kept maintained at 37°C until examination protected from changes in temperature, exposure to light, and any chemicals, lubricants, water

that can adversely affect sperm motility and fertility. Common tests for assessment of semen quality are:

Appearance and consistency: This is done by visual observation. A good sample should appear milky white in color, evenly turbid and without clots. Abnormal ejaculates may be yellow (urine contamination, white blood cells) or pink (hemorrhage). 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.

Volume: 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. 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 vasiculitis. Teasing of jack/stallion result in higher ejaculate volume.

Osmolarity: This is done with help of an osmometer. Normal osmolarity of semen range between 290 to 310 mOsm. Hypo tonicity/ low osmolarity cause swelling and deformation of the sperm cell (especially tail) and may result in rupture of the plasma membrane leading to its adverse effect on spermatozoa function.

Seminal fluid pH: This is noted by putting a drop of semen on pH strip or with help of pH 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. A higher pH indicates soap or urine contamination, inflammation, or problems with the accessory sex gland fluid. Second ejaculate in the succession has usually higher pH.

Sperm motility: Motility is assessed either visually or using a computerized motility analysis system from gel-free semen on a pre-warmed glass slide. In visual method, undiluted raw semen is placed on a warmed slide (~35°C) and viewed using a light microscope. Mass motility is observed as oscillatory or progressive sperm, i.e. those that are alive but are moving on the spot and those that are actively moving forward, respectively. Progressive motility is observed as 10

sperm cells/high power field (40X) in order to accurately estimate the number of cells that are progressively moving across the field. This method is highly subjective and very dependent on the experience of the examiner.

The percentage of motile spermatozoa and in particular those showing progressive movement is a good indication of the number of viable spermatozoa. The correlation between motility and morphologically normal spermatozoa is reported to be 0.63. Mass and progressive motility is observed higher during summer compared to autumn and winter. Sperm from a normal fertile stallion should have mass motility more than 80% and progressive motility more than 60%.

Sperm longevity: This is done by periodic observation of sperm motility in raw or extended semen under incubation. Raw semen samples are incubated at 37°C and an extended sample (1:1) is kept at 5°C. Samples are then assessed for progressive motility at 15 minutes intervals for the first hour followed at every hour until motility is <10%. The purpose of longevity test is to give an idea of the expected survival time for the spermatozoa within the female tract. Semen samples showing motility <10% within a three hour period, generally have poor fertility.

Sperm concentration: The sperm concentration is one of the most important parameter. It will provide not only an indication as to whether the sample is worth using but it will also determine the number of mares that can be covered from a single ejaculate. The values for spermatozoa concentration in semen sample vary widely; commonly from 100 to 350 x 10^6 per ml. The sperm concentration of semen, preserved in diluting fluid (1:100) is estimated by hemocytometer.

Preparation of diluting fluid for sperm count

0.05 gm eosin Y (water soluble) and 1 gm sodium chloride are dissolved 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.

$$\text{Sperm concentration (per ml)} = \frac{N \times D \times 4000 \times 1000}{n}$$

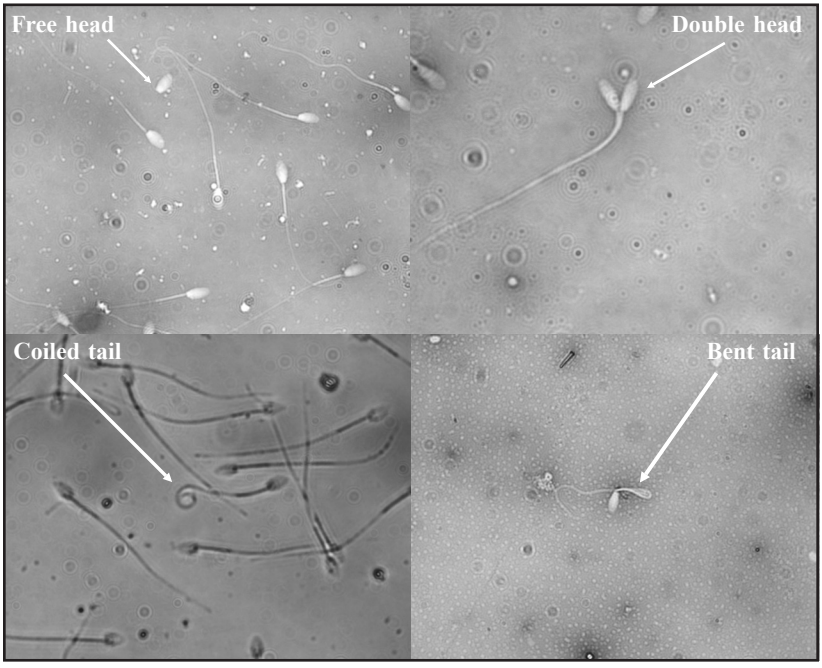
Where, N=No. of Sperms counted
D=Dilution rate (here 100)
n=No. of tertiary squares counted

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.

Sperm morphology: Spermatozoa morphology is usually assessed by microscopic examination either of an unstained semen sample fixed in buffered formal saline or of a stained semen sample with eosin-nigrosin stain. In sperm evaluation by staining, a drop of well-mixed semen is mixed with a drop of stain on a glass slide and a second slide is used to make a smear. The slide is allowed to dry and can be evaluated immediately or stored and evaluated later. Sperm abnormalities are optimally detected using immersion oil and 100X magnification. On average 200 spermatozoa are usually examined, allowing a reasonably accurate estimate of the percentage normal and abnormal sperms to be made.

Sperm cells are examined for abnormalities such as abnormal head, detached head, abnormal acrosome, proximal or distal cytoplasmic droplet, abnormal mid piece, bent or coiled tail, premature sperm cells etc. Heads separated from tails are undesirable. Some heads are abnormally shaped and are assumed as infertile. Sperm abnormalities

are classified primary (failure of spermatogenesis process), secondary (failure of sperm maturation) or tertiary (sperm damage during or after ejaculation). A high proportion of primary abnormalities suggest a testis defect, whereas a high proportion of secondary abnormalities suggest an epididymal defect. Changes in seminal plasma may affect sperm morphology and so render them more susceptible to damage from manipulation after ejaculation. Spermatogenesis and/or the passage of sperm through the epididymis may be affected by local increases in temperature, a generalized rise in body temperature, the action of some drugs and over-conditioning. Morphologic evaluation of sperm can be used as a predictor of fertility in stallions. Samples with high percentage of abnormalities can be compensated to a certain extent if the total volume and sperm concentration are high, as abnormal spermatozoa have no apparent detrimental effect on viable spermatozoa in the semen sample.



Microscopic view of abnormal sperms (100X)

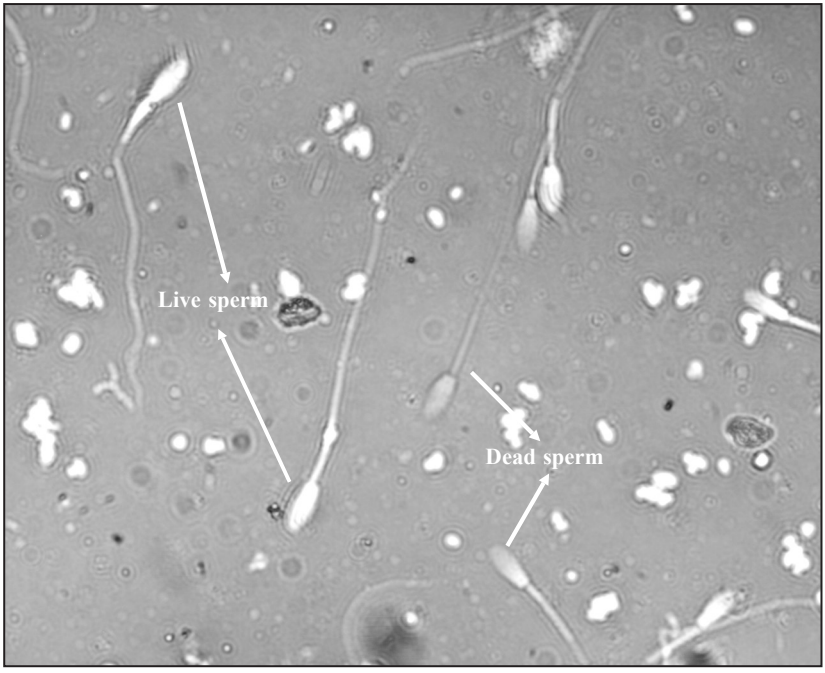
Live and dead sperm ratio: This is calculated using eosin-nigrosin stained semen smear under microscope. To prepare smear, one drop of semen is placed on clean, grease free, pre warmed glass slide and mixed with one drop of eosin-nigrosin stain using blunt fine glass rod. After a minute, a thin smear 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 NaH_2PO4 or 0.1 M Na_2HPO4 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.

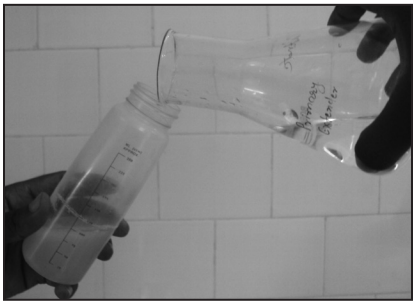


Microscopic view of live and dead sperm (100X)

Semen processing for cryopreservation

Primary dilution and removal of semen plasma

Gel free semen is mixed with an equal volume of primary extender within 10 minute of collection, filled in 50 ml tubes and centrifuged in refrigerated centrifuge to get soft sperm pellet at the base. Addition of primary extender prior to centrifugation makes spermatozoa more resistant to cold stress and damage while centrifugation. Centrifugation is done at 2000 rpm for 3 minutes. After centrifugation, the supernatant is removed carefully and discarded. A small portion (a minimum of 5%) of the seminal plasma must be left with the sperm to preserve viability. The pellet is then re-suspended using sufficient extender to achieve a final concentration of 150-200x10^6 progressively motile sperm per ml.



Semen dilution with primary extender

Sperm soft pellet at the base after seminal plasma removal

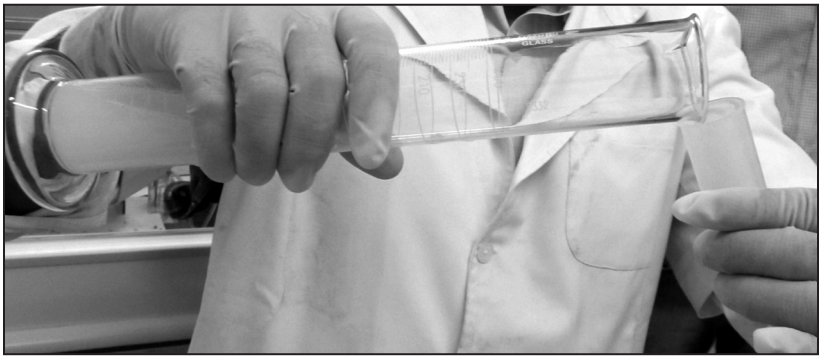
Sperm soft pellet at the base after seminal plasma removal

Secondary dilution and the dilution rate

The aim of final secondary dilution is to store semen samples with an appropriate concentration and for immediate insemination without any further treatment. Adequate dilution is required to ensure optimum sperm survival whereas excessive dilution results in low concentration that required large volume to be inseminated for acceptable fertilization rates. It is agreed that 400-500 million progressive sperm per insemination is required in order to have optimal fertilization. Considering the damage (approximately 50%) that occurred to sperm while freezing and thawing as well as volume of frozen semen for AI (4-5 ml), a final sperm concentration of 150-200 millions/ml extender is recommended. After final dilution, semen samples are kept in cooling cabinet before being packaged into straws.

Calculation of dilution rate :

$$\text{Dilution rate : } = \frac{\text{No of progressive motile sperm per ejaculate (ml)}}{\text{Required sperm concentration per ml}}$$



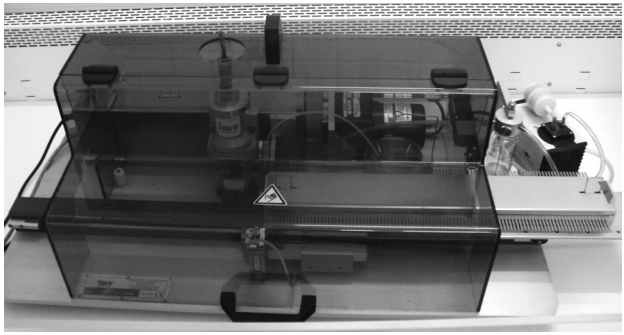
Semen dilution with secondary extender

Cooling and equilibration of diluted semen

A cooling rate of -0.05 to -0.1°C/min is desired between 20 and 5°C. Ideal storage temperature is 4-6°C. Diluted semen is kept in cooling cabinet or refrigerator temperature (4-5°C) for 2 hours. This is required so that sperm cells get adjusted with the dilutor and loss of sperms does not occur excessively during the freezing process. The pre-freeze motility is recorded after 1 hr of equilibration before freezing.

Packaging of diluted semen

The diluted semen after equilibration is filled and sealed into straws with automatic filling-sealing machine or using manual method. Poly-vinyl chloride (PVC) straws of 0.5 ml capacity is used commonly for packaging but other options are also available, including 0.25 ml and



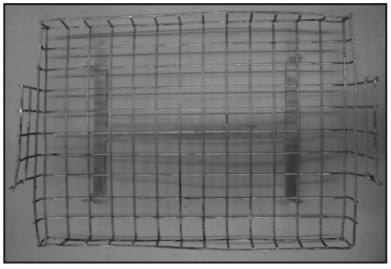
Automatic straws filling and sealing machine

5 ml straws, 0.1-0.2 ml pellets and 3.6 ml vials. Name of the jack/ stallion, institute/company name, date of freezing etc can be printed over straws before being used. Manual method includes filling diluted semen into straws using kit assembly and sealed with poly vinyl alcohol powder.

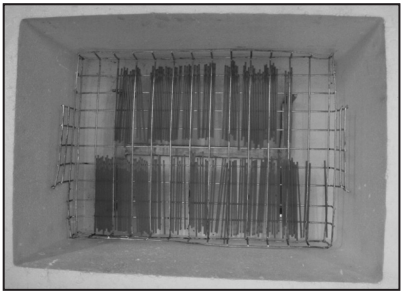
Methods of semen freezing

Semen is frozen into straws either by traditional vapor freeze technique or controlled rate freezer machine. Initial processing in both the methods is same. The semen samples having progressive sperm motility more than 60% in fresh is processed further for cryopreservation. The rate at which extended semen is cooled is critical. If the cooling rate is too fast or too slow, sperm viability is decreased. The straws loaded in controlled rate freezer machine were cooled at the rates of 0.3°C per minute from 18°C to 5°C; 10°C per

Straws (0.5ml) used for semen freezing



Freezing racks to hold straws



Vapor freezing technique of semen freezing

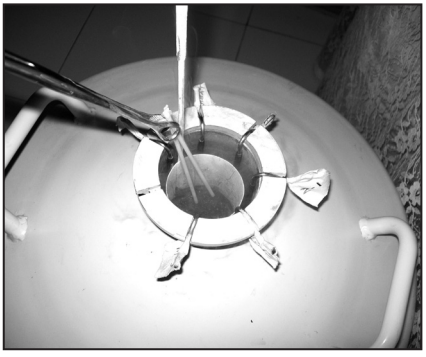
Automatic controlled rate freezing machine

minute from +5°C to –15°C and 19°C per minute from –15°C to –100°C. After reaching –100°C, the straws are taken out and finally plunged into liquid nitrogen.

Vapor freezing technique is used due to unavailability of bio-freezer being a costly instrument. The results obtained are comparable and satisfactory to automatic controlled rate freezer. In this technique, straws with diluted semen are laid horizontally onto a freezing rack and lowered into a styrofoam box that contains at least one inch of liquid nitrogen level. The freezing rack is designed to support the straws 3 cm above the liquid nitrogen level. After being held in that position for 12 minutes, the straws were then plunged into liquid nitrogen and stored at -196°C. During vapor freezing, the cooling rate at the bottom of the straw is generally much faster than at the top and to avoid variation in temperature the position of straws are turned around.

Thawing procedure

To use frozen semen for AI, straws are taken out from storage container, dipped in waterbath maintained at 37°C for 30 second to 1 minute or thawing kit adjusted to 37°C. In equines, large volume is needed for insemination so 8-10 frozen straws (0.5 ml) are taken out for thawing to make 4-5ml of volume. Straw are wiped with tissue paper to remove water and cut to load into syringe for insemination. A small drop from thawed semen is taken for evaluation of post thaw motility before use in AI. Semen samples having post thaw motility ≥35% is considered suitable for use in AI.



Straw removal from storage container

Thawing kit

Factors affecting semen freezability

Equine semen is less tolerant to freezing and thawing than the bull semen, in particular due to higher levels of docosapentaenoic acid (DPA) relative to DHA (docosahexaenoic acid). Further, some jack/ stallions' semen freezes better than others. So, there is species and individual variations. Semen from some stallions freezes fairly better than others with certain freezing media. At different times of the years and with different ejaculates, tolerance to freezing and thawing may show some variations in an individual stallion. Season is known to affect reproductive efficiency in stallion due to low libido; low sperm concentration and hence low total sperm count evident during the non breeding season. There is practice of semen collection and freezing during non-breeding season to avoid the clash with natural covering season. The decrease in total sperm count can be compensated by appropriate adjustment while semen dilution. The quality of frozen/ thawed semen also varies laboratory wise and depending on the technician that is involved in the production and handling of the frozen semen as well as the method adopted for freezing. Selection of ejaculates is usually based on post-thaw motility, where less than 35% motile sperm after thawing is considered unacceptable.

Approaches to reduce microbial load in frozen semen

Examination of new animals entering to herd for diseases and isolation from other animals at the centre is general precaution. All stallions should be serologically screened negative for the presence of Equine Arteritis Virus (EAV) antibodies and cultures negative for causative agent of Contagious Equine Metritis (CEM) within 30 days prior to available for semen collection. All stallions should be tested negative for Equine Infectious Anemia (EIA) within 6 months prior to entering collection facility. Other diseases of concern that need tested negative are Vesicular Stomatitis, Dourine and Glanders. Fresh or frozen semen to be used for AI should be cultured prior to the start of the season and periodically during the season to confirm that they are not harboring and shedding potential pathogens. Prior to semen collection on kuchcha floor, water should be sprinkled to reduce dust, stallion prepuce should be cleaned with normal water, tail of estrus mare should tie aside and perineum is cleaned. Proper cleaning with lab wash detergent followed with water and then, sterilization of glassware and autoclave of plasticware, rubber article should be done. Eggs to be used for media should be cleaned and wiped with 70 percent alcohol before breaking. Antibiotic is added in freezing

media to reduce growth of microbes. Standard laboratory hygiene and practices should be adopted. Thawed semen should be free of potential pathogens and significant environmental contaminants.

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Semen freezing laboratory setup at ICAR - NRCE, EPC, Bikaner

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