Technical bulletin

Chicken Semen Cryopreservation

Dr. Shanmugam M
Dr. R.K. Mahapatra

ICAR-Directorate of Poultry Research
Rajendranagar, Hyderabad-500030, India

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ICAR-Directorate of Poultry Research
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PREFACE

Poultry farming in India has got transformed from backyard rearing to commercial industry over the past few decades. There are nineteen recognized poultry breeds that are spread around the country. Chicken lines having specific characteristics were developed and maintained by veterinary universities and research organizations. These diverse chicken breeds/lines are threatened or decreasing in numbers due to various reasons such as repeated occurrence of avian influenza, cross breeding and selective maintenance of lines dictated by the commercial returns. The different native breeds are well adapted to the local environmental conditions and have distinguishing specific features. The population of native breeds is small and scattered. Conservation of the diverse chicken genetic resources is required for any future utilization.

Chicken genetic resources may be conserved as live birds or through *ex-situ* conservation programme. Semen is the only gamete in chicken that can be cryopreserved and used. Alternatively, blastodermal or primordial germ cells can be cryopreserved and reimplanted in recipient embryos. However, the recovery rate is very low and costly for implementing in large scale. Thus, semen cryopreservation is the feasible *ex-situ* method currently available. There are challenges associated with chicken semen cryopreservation. The semen cryopreservation protocol has to be standardized for each breed or line of chicken, since the results obtained in a breed/line may not be replicated in other breed/line. We have standardized semen cryopreservation protocols for selected breeds and lines maintained at ICAR-DPR. In this manuscript the procedure for chicken semen cryopreservation and evaluation has been detailed.

We express our sincere gratitude to Dr. R. N. Chatterjee, Director, ICAR-DPR for his guidance and moral support. We sincerely thank all scientific colleagues and technical staff for their wholehearted support.

Shanmugam M

R.K. Mahapatra

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INTRODUCTION

India is having a wealth of chicken genetic resources with nineteen registered native breeds. In addition, there are many chicken parent lines developed and maintained by different research institutes/universities. At ICAR-Directorate of Poultry Research, different parent lines are being maintained and selected for specific traits for many generations. These lines are crossed to develop different varieties for the benefit of poultry farmers of the country. Under conservation programme few native chicken breeds are maintained. The nineteen chicken lines/breeds maintained at this Directorate are valuable resources that are to be safeguarded against any adverse events which may lead to the loss of these birds. Cryopreservation of avian gametes is one of the different strategies for conserving chicken resources. Cryopreservation of female gametes is not feasible with the present knowledge due to the non-availability of technologies to preserve the whole egg. Cryopreservation of chicken semen is a relatively easy and cheaper method compared to other methods such as cryopreservation of testis, ovary, primordial germ cells and blastodermal cells. Furthermore, the other preservation methods involve higher-end technology, cost, and the lower rate of success during regeneration of the birds. In short, the chicken lines maintained at this directorate need to be conserved ex-situ with the feasible technology available i.e., semen cryopreservation with the best-suited protocol.

Chicken semen cryopreservation is a challenging area where the success in the semen cryopreservation protocol has been shown to vary with the line/breed of chicken and also with individual males. A single cryopreservation protocol standardized for a line/breed cannot be applied for all the chicken lines and get similar results. This contrasts with that of cattle where semen cryopreservation is a success with optimized and standardized protocols are available. At field level cryopreserved semen is routinely used for insemination and production of calf. The unique reproductive biology of chicken further poses several challenges. The requirement in chicken is that several chicks have to be produced over a period of time of at least 8-10 consecutive days after a single intravaginal insemination for commercial application. For this relatively high number of sperm should remain viable in the hen's sperm storage tubules for fertilizing the released ova between any two inseminations. Thus, there is a need for the development of separate semen cryopreservation protocol or validation of existing protocols for individual breed/line maintained at the Directorate. This publication presents the cryopreservation protocols evaluated for a few selected lines/breeds maintained at the Directorate.

HISTORY

Artificial insemination (AI) involves the collection, evaluation, dilution, preservation and placement of semen into the female reproductive tract usually per vagina. Artificial insemination research was first performed in dogs by Italian physiologist Spallanzani in 1780. The Russian AI pioneer, Ivanoff, has first reported work on AI in birds (Ivanoff, 1922). An abdominal massage technique for semen collection in chickens was reported by Burrows and Quinn (1937) that is extensively used in poultry today. In India, artificial insemination of poultry was implemented in the early 1970s at Poultry Research Division, Indian Veterinary Research Institute that subsequently became Central Avian Research Institute, Izatnagar.

The freezing of fowl spermatozoa was first reported by Shaffner et al. (1941), where fructose was used as a cryoprotective agent, however, there was low post-thaw motility. In a subsequent report, Shaffner (1942) obtained fertile eggs with development arrested embryos after insemination with frozen-thawed semen. Live chicks from frozen-thawed semen were first obtained by Polge (1951). This result was possible after the accidental discovery of the cryoprotective action of glycerol. It was also observed that glycerol used at concentration 10-15% protected the sperm during freezing but the fertilizing ability was very less after insemination. The fertilizing power of glycerol cryopreserved sperm was improved after dialysis and the reduction of the glycerol concentration. The removal of glycerol by washing and centrifugation further adds to the stress of the sperm. To avoid the contraceptive effect of glycerol, other cryoprotectants such as dimethyl sulphoxide, dimethylacetamide, ethylene glycol, propylene glycol, dimethylformamide, methylformamide, sucrose and polyvinyl pyrrolidone for cryopreserving fowl semen were explored and reported. Semen can be cryopreserved as pellets or in plastic straws. Due to issues in storage and traceability in pellet semen cryopreservation, preserving in plastic straws is widely practiced.

In India, there is only one published report on chicken semen freezing by Omprakash et al. (2000). At ICAR-Directorate of Poultry Research work on cryopreserving semen from different lines maintained at the farm was undertaken. The information generated from the research work is compiled and reported in this publication.

Advantages

- Semen from genetically superior males is not wasted and stored in the frozen state for a long duration even after their death.
- Frozen donor semen can be easily transported to distant places.

- The cost of rearing males can be reduced.
- Ex-situ conservation of important or endangered lines/breeds.
- Transgenic or any genetically manipulated sperm can be stored for future use.

Disadvantages

- Trained personnel with suitable knowledge are required.
- Cryopreservation protocol with the type of cryoprotectant, diluent and thawing conditions has to be standardized for each breed/line.
- Fertility results from cryopreserved semen vary with the line/breed of hens inseminated.Fertility results from cryopreserved semen vary with the line/breed of hens inseminated.
- Maintenance of cryopreserved semen repository on a smaller scale is not cost-effective.

HANDLING OF CRYOGENIC CONTAINERS

Maintenance of cryogenic containers

The cryogenic containers or cryogens are purpose-built double-walled vessels which are insulated and vacuum jacketed. The inner wall is made of austenitic stainless steel or other suitable steel containing nickel whereas the outer wall is made of stainless steel or carbon steel. These materials are resistant to corrosion. Different types of insulation such as vacuum, materials like polyisocyanurate and polyurethane rigid foam, or cellular glass may be used as thermal insulators in a cryogenic container. The containers should be used to store only the specific material. Rough handling of the containers should be avoided. The container should always be kept in an upright position, preferably on a mat. Similarly, when transported the containers should be supported with soft paddings. The containers should not be exposed to direct sunlight, and frequent cooling and warming should be avoided. Any frosting or appearance of moisture on the outer wall of the container indicates damage and defect in insulation which has to be promptly attended to. The evaporation rate of liquid nitrogen from containers varies with the type of container and the storage environment. Considering these two factors the periodicity of refilling should be scheduled.

Assessment of liquid nitrogen level

The liquid nitrogen evaporation from the container varies with the size of the container, environmental temperature and frequency and duration of opening and closing of the container. Therefore, the containers should be periodically checked for the liquid nitrogen level and refilling done accordingly. The minimum level of liquid nitrogen is that level that completely submerges the straws placed in the container.

The level of liquid nitrogen is measured using a measuring scale. The scale should be gently lowered into the container till the bottom is touched. The scale should be kept in the position for few seconds, slowly removed and shaken in the air. The moisture in the air condenses on the scale up to the liquid nitrogen level. The volume of liquid nitrogen can then be estimated using a calibration chart.

Care in handling liquid nitrogen

- `Use only longhand metal forceps for removing objects from liquid nitrogen. Avoid using commonly available plastic materials since they may become very brittle at cryogenic temperature and shatter causing damage to the handler.
- Avoid direct contact of any body parts with liquid nitrogen since it

may result in frostbite injury. Wear protective clothing, cover face with face shield and comfortably fitting cryo gloves.

- Handle liquid nitrogen in a well-ventilated area to avoid suffocation since nitrogen gas is colorless, odorless and tasteless, therefore not detected by the human senses.
- Liquid nitrogen containers should not be overfilled. Use proper transfer equipment while transferring and avoid splashing and spilling.
- Liquid nitrogen containers should not be tightly sealed. If done so it will lead to build-up of pressure as the liquid boils and may lead to explosion after a short time.

SEMEN COLLECTION

Handling of birds

The roosters should be handled gently and quickly. Catching birds housed in individual cages are easier than those housed in floor pens. Birds struggling during handling and rough manipulation get excited and that may result in inhibition of ejaculatory reflex or chance of defecation. Feathers surrounding the vent area should be clipped off to obtain unsoiled semen. Birds should be trained for semen collection during the beginning of the breeding programme. This will help in establishing a better erectile and ejaculatory responses to the massaging procedure.

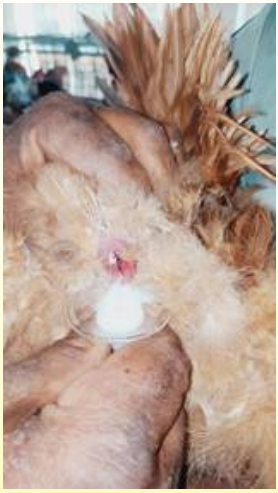
Use of clean equipment

The materials used for semen collection and processing should be clean and dry. All the materials (glassware and rubber ware) after use should be first rinsed in tap water and washed in a mild soap solution. Final rinsing in distilled water will prevent salt deposits on the materials after drying. The washed materials should be stored in a dust-free environment. A glass cup of 4-5 cm diameter with stem for holding is used in the laboratory. A 1ml graduated syringe is used for inseminating a known volume of semen into the oviduct of a hen. After each use, the syringes are washed, dried and checked for smooth piston movement.

Collection

Semen can be collected by 'one man' or 'two man' methods. In the one-man method, a single person handles the bird and collects semen himself. In

Materials used for semen collection and insemination



Semen collection from rooster

the two-man method, one person holds the bird while another strokes the back and collects semen into a funnel. Upon application of pressure by the collector, the tissue in the cloaca which forms part of the papillae gets swollen through which semen is ejaculated. The swollen papilla is gently squeezed for collecting the semen. Hard squeezing may rupture the vascular tissue and traces of blood appears in the semen.

Semen evaluation and processing laboratory setup

Soon after collection of semen a proportion of sperm quality starts deteriorating due to high sperm concentration. Therefore, immediately after collection, the semen has to be evaluated and processed. The semen should be hygienically transported on ice to the laboratory. The laboratory should preferably be located nearby the farm. The workspace in the laboratory should be hygienic and dust-free. There should be a separate room for cleaning and sterilization of equipment. The evaluation room should be spacious, fitted with good work tables, well-lit and air-conditioned. The laboratory should be well equipped with a refrigerator, cold handling box and microscope. For processing a large volume of samples additional equipment such as filling and sealing machines, straw printing machine and programmable freezer may be required.

SEMEN EVALUATION

The semen samples used for freezing and insemination should have the best fertilizing ability. After semen collection, each batch should be routinely examined before processing to achieve the objective. During evaluation, a fair assessment of the fertilizing capacity is made and furthermore, an idea is obtained on the dilution rate before processing.

Methods of semen evaluation

Macroscopic evaluation

Soon after semen collection, a gross evaluation of the ejaculate is done with the naked eye for the following parameters.

- Colour: Normal poultry semen should be pearly white in colour and any other colour indicates contamination. Greenish and white deposits in the semen are indicative of faecal contamination. Contamination with blood is identified by the presence of brownish-red pigments.
- Volume: The volume is measured using a graduated 1 ml syringe. Semen volume has to be measured for calculating the rate of dilution before processing. The volume of semen produced by broiler breeders will be 0.1-0.9 ml and layers will be 0.08-0.5 ml. Indigenous breeds like Aseel and Kadaknath produce 0.1-0.4 ml of semen. The volume is influenced by the frequency of collection.
- Appearance: The appearance has to be scored soon after collection. The appearance is influenced by the sperm concentration. General classification of appearance is watery or clear semen, watery with white streaks, medium, thick white and viscous or chalky white.
- Presence of contaminating material: The collected semen should be free from any contaminating materials. The general contaminating materials are faeces, feather particles, dust and feed particles

Microscopic evaluation

In the laboratory, semen samples are evaluated microscopically using a light or phase-contrast microscope.

- Individual motility: Freshly collected semen is evaluated under a microscope for mass motility that is generally ranked on a subjective scale of 1 to 5. Diluted semen is evaluated for motility of individual sperm and expressed in percentage (0 to 100). Samples with poor motility are not likely to possess good fertilizing capacity as it may be difficult for the sperm to reach the site of fertilization. Alternatively, the semen samples may be evaluated by computer assisted sperm analysis (CASA) where additional information on

motility like curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN) and straightness (STR) of the sperm are obtained that gives additional objective information on the quality of the sample.

- Sperm concentration: The number of spermatozoa per unit volume of semen is sperm concentration. A simple method of sperm concentration determination is by using haemocytometer. For processing large numbers of samples indirect methods of determining the sperm concentration like packed cell volume (PCV), spectrophotometry and turbidity are available. For these indirect methods conversion factor or a standard curve is needed. For spectrophotometric evaluation, a standard curve is prepared with the sperm concentration and optical density.

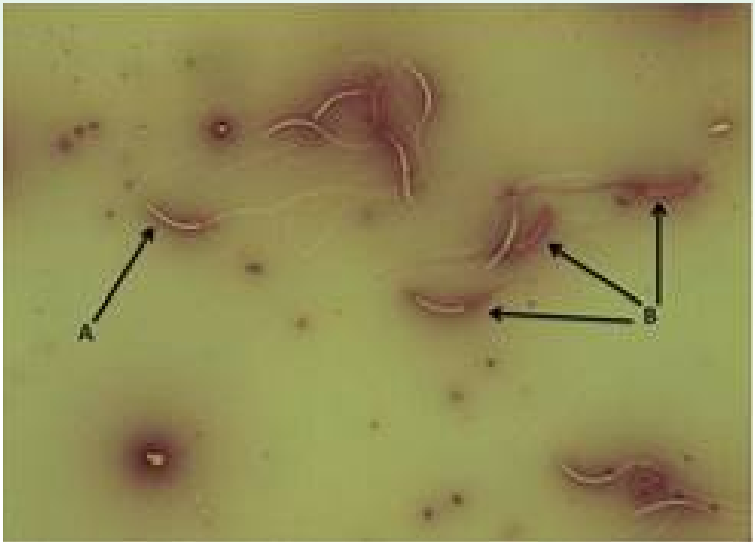
  Procedure: A pooled semen sample is serially diluted in test tubes and the optical density is measured at 540 nm. The sperm concentration of these serially diluted samples is determined using haemocytometer and used for standard curve preparation. The unknown samples are diluted and optical density measured. Using the optical density values the sperm concentration is calculated from the standard curve already prepared. A separate standard curve should be prepared for each breed/line of birds.

  The application of CASA for sperm concentration determination is more advanced and sophisticated for continuous evaluation of samples, which can be processed and concentration values obtained instantaneously. The broiler breeders' sperm concentration will range from 3-8 billion sperm/ml and that of layer from 3.5-6 billion sperm/ml. Aseel and Kadaknath breed have sperm concentration of 5-8 billion sperm/ml.

- Live and dead sperm: The percent of live and abnormal sperm is estimated by differential staining using Eosin-Nigrosin stain (Campbell et al. 1953). Semen smear prepared by mixing one drop of semen with two drops of Eosin-Nigrosin stain is air-dried and observed under high power (1000x). All pink-stained and partially stained sperm are considered dead and unstained sperm are considered as live. The percentage of live sperm is determined by counting at least 200 sperm. The same slides can be used for estimating the percentage of abnormal sperm that has different morphological abnormalities.

- Abnormal sperm: Abnormal sperm are those having any morphological deviation from the normal sperm structure. Abnormal sperm may occur in an ejaculate due to inheritance, adverse

environmental conditions and improper handling of semen. Abnormalities may be primarily arising due to impaired spermiogenesis and secondary due to damage during maturation, collection and preparation of semen smear.



A-Live Sperm B-Dead Sperm

CRYOPRESERVATION PROCEDURE

Evaluation of semen

The collected semen is immediately evaluated for volume, colour, appearance, presence of contaminating materials and motility. Samples having good quality are only to be processed for freezing. The semen evaluation is done on an aliquot of the sample. Samples fulfilling the parameter requirements are used for further processing. The samples are to be stored in ice-cool temperature till dilution.

Diluent preparation

In chicken, the post-thaw semen quality and fertility are affected by the semen diluent used for cryopreservation. The diluent suitable for a chicken line/breed has to be evaluated before fixing a cryopreservation protocol for the particular line/breed. The semen diluents evaluated and used in our laboratory are Lake and Ravie (LR) diluent, Sasaki diluent, Red Fowl Extender (RFE) and Beltsville Poultry Semen Extender (BPSE). The diluent to be used for freezing semen should be kept at a cool temperature throughout the procedure.

Dilution of semen

The rate of dilution of the sample is based on the sperm concentration of the sample. The volume of semen to be prepared is determined. To the raw semen equal volume of the cool diluent containing double the cryoprotectant concentration is added to the beaker so that the final cryoprotectant concentration is obtained.

Equilibration of semen

Equilibration is the time period when semen with cryoprotectant diluent is stored at a particular temperature. This is done so that the cryoprotectant brings its beneficial action on the sperm before freezing. This period varies with the cryopreservation protocol and has to be standardized. In our laboratory, an equilibration of semen samples to be frozen are done at 5°C for 30 min.

Marking/printing of straws

Marking or printing of 0.5 ml French straws should be done before filling straws. The number of doses to be made from the processed semen is determined. Information about the breed, cryoprotectant and diluent used and date of freezing are legibly marked using a cryo marker. For a higher

number of doses, printing is done using automatic straw printing machine. Before filling straws, it should be ensured that the print dries.

Filling of straws

The manual filling method is preferred where a lesser number of straws are to be filled. In this method, a custom-made plastic syringe tip that snugly holds the straw is used. The open end of the straw is dipped into the diluted semen and the plunger of the syringe is pulled so that the semen is filled in the straw by suction. During filling it is to be ensured that a small air space is created at the open end. This air space is required for allowing the expansion of semen during freezing.

Alternatively, for filling many numbers of straws manually a filling comb, straw clip and a vacuum pump are used. Using a straw clip, the straws are clipped together and precooled to 5°C. The factory seal side of the straws is clipped to the filling comb which in turn is attached to a vacuum pump through a rubber tube. In a bubbler, the diluted semen is taken and filling is done by operating the vacuum pump and slowly dipping the open end of the straw. Due to the negative pressure, the semen is drawn into the straws. A uniform air space is created in the filled straws by pressing the open end of straws on to the teeth of the bubbler.

Sealing and cleaning of straws

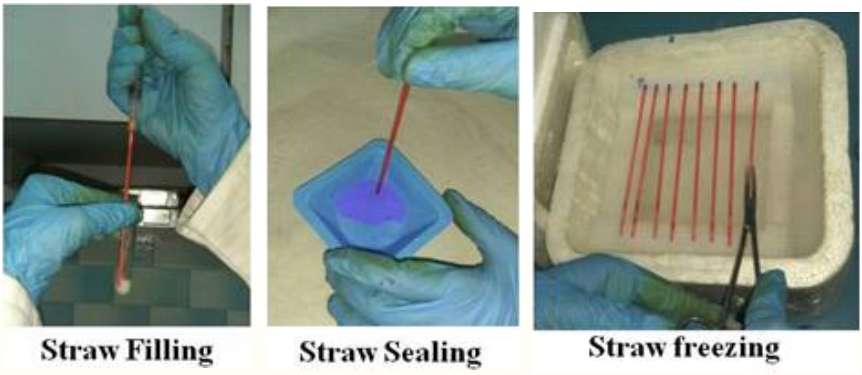
The open ends of the filled straws are sealed using polyvinyl alcohol powder as a sealant. The powder is uniformly spread to a thickness of 4-5 mm in a sterile Petri dish or plastic boat. The open ends of filled straws are dipped into the powder 2-3 times. The straw is satisfactorily filled when the powder penetrates 4-5 mm into the straw. This is the laboratory seal which will appear as a single band in contrast to the factory seal that appears as two distinct bands. Immediately after sealing the filled straws are immersed in water at 5°C. The water binds the polyvinyl alcohol powder and the seal becomes firmer.

Automatic filling and sealing machines are available from commercial companies. These machines operate in a shorter time with the least handling of semen. In such machines sealing is done by compressing the end of the straw and sealing powder is not required.

The sealed straws are held in bundles in the hand and tapped on a moist tissue paper to ensure good plugging. On a precooled paper towels the straws are rolled and dried of any water adhering on the straws. If drying is not done the moisture on the straws will lead to ice formation and reduction in the freezability.

Racking freezing and storing of straws

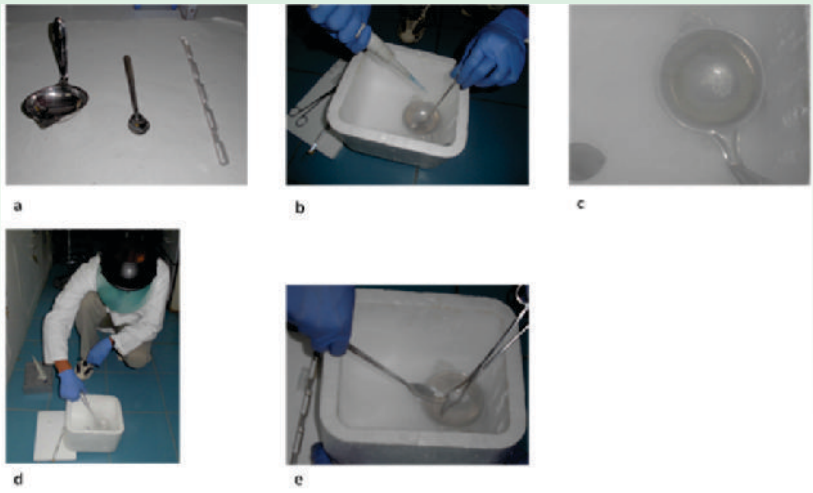
A custom-made styrofoam rack is used for racking the straws in our laboratory. The styrofoam is 4-4.5 cm in height and floats on liquid nitrogen. In a thermocol box, liquid nitrogen is filled up to halfway mark and the styrofoam rack is placed floating on the liquid nitrogen. The straws are placed one by one on the cooled rack carefully. The freezing of straws is carried out by this procedure. The straws will be exposed to liquid nitrogen vapours at a height of 4-4.5 cm. The straws will reach a temperature of approximately -135°C in about 10-15 minutes. The straws are held in the position for 30 minutes and then plunged into the liquid nitrogen. The goblets are immersed into the liquid nitrogen and cooled. In this cooled goblet, the frozen straws are placed and transferred to a canister of frozen semen container for storage at -196°C. This method of freezing is simple and efficient without the requirement of any costly equipments. The freezing styrofoam racks hold the straws at a constant height where the straws are frozen evenly over the length of the straw.



Steps in semen cryopreservation by straw method

Pellet method of semen cryopreservation

The semen and cryodiluent mixture was equilibrated for 5 min at 5°C. Semen pellets were formed by dropping the DMF mixed semen directly into liquid nitrogen drop by drop from 1ml pipette and stored in plastic cryovials in liquid nitrogen



Equipment and steps in pellet method of semen cryopreservation

a. Stainless steel sieve, spoon and cane for holding cryovials

b. Placing semen cryomixture drop by drop above the sieve

c. semen pellets inside the sieve

d. Equilibration of cryovials in liquid nitrogen

e. Filling cryovials with semen pellets

FROZEN SEMEN EVALUATION

The frozen semen is evaluated for different parameters after thawing the samples that were stored in straws or as pellets.

Thawing of straws

The semen straws were thawed either in ice-cold water maintained at 5°C for 100 sec or in a water bath maintained at 37°C for 30 sec depending on the protocol. The content of the straw is collected in a glass funnel after cutting the powder-sealed end.

Thawing

Semen Pooling

Artificial Insemination

Thawing of pellets

The semen pellets stored in plastic cryovials should be transferred to a glass beaker and for thawing the beaker is kept on a hotplate constantly maintained at 60°C for few seconds so that the pellets melt and turns into liquid.

Post-thaw motility

A small drop of semen is placed on a Makler chamber, covered and examined under a microscope. The percentage of progressive motile sperm is subjectively assessed and recorded.

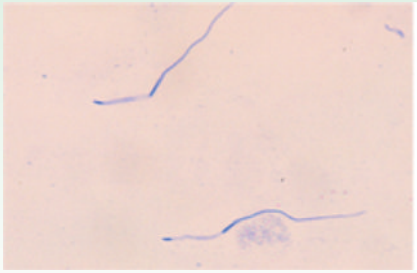
Live and dead sperm

The percent live and abnormal sperm is estimated by differential staining using Eosin-Nigrosin stain.

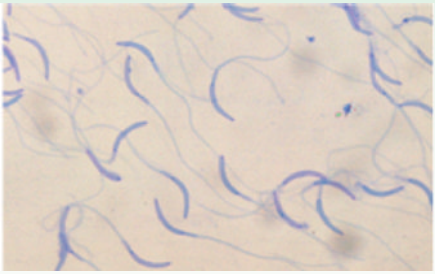
Acrosomal integrity

The acrosomal integrity in sperm is assessed as described by Pope et al. (1991). Diluted semen (10 µl) is mixed with 10 µl of stain solution (1% (wt/vol) rose Bengal, 1% (wt/vol) fast green FCF and 40% ethanol in citric acid (0.1 M) disodium phosphate (0.2 M) buffer (McIlvaine's, pH 7.2-7.3) and kept for 70 sec. A smear from the mixture is made on a glass slide, dried and examined under high magnification (1000x). The acrosomal caps are stained blue in

acrosome-intact sperm and no staining is observed in the acrosome region of the acrosome reacted sperm. A minimum of 200 sperm is counted in each smear sample for calculating the percent acrosome intact sperm.



Acrosome intact sperm



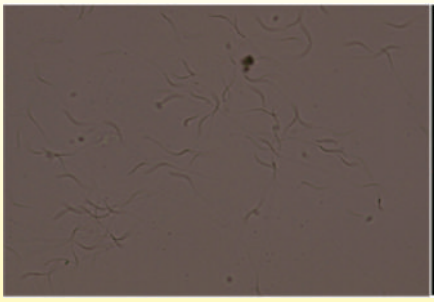
Acrosome reacted sperm

MTT assay

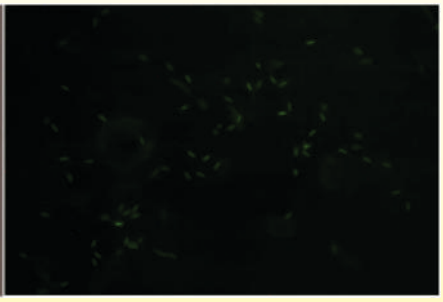
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction test is carried out in duplicate samples as described by Hazary et al. (2001). In a glass tube, 900 µl NaCl–TES, 100 µl of 100 mM glucose, 30 µl semen sample and 50 µl of 4 mM MTT dye is added, mixed and incubated in a shaking water bath at 37°C for one hour. After incubation, the tubes are removed and 200 µl of 10 % SDS in 0.01 M HCl solution is added to each tube, mixed well and allowed to stand for one hour. The optical density of each sample is measured against blank at 570 nm in a colorimeter/spectrophotometer and the dye reduction activity of sperm is calculated using extinction co-efficient.

Rhodamine 123 assay

Rhodamine 123 (R123) staining assay is done to study sperm mitochondrial activity (Yoon et al. 2015). To 100 µl of the semen sample, 15 µl of R123 (0.01 mg/ml) is added and incubated at 37°C for 15 min. This is followed by the addition of 1 ml of PBS. From this mixture one drop of sample is placed on a glass slide and covered with glass slip. These slides are observed under 60x magnification in a fluorescent microscope. Sperm cells displaying green



Light microscopy



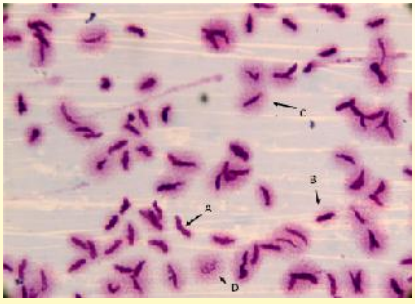
Fluorescent microscopy

fluorescence at the mid-piece region are considered viable sperm with functional mitochondria. For each sample, a minimum of 200 sperm is to be examined and classified as sperm with or without functional mitochondria.

Sperm chromatin dispersion (SCD) test

The SCD test for chicken sperm is carried out as described by Shanmugam et al. (2015). An equal volume of diluted semen sample and 1% low-melting agarose is mixed at 37°C. From this mixture, an aliquot of 30 µl is pipetted onto a glass slide that was precoated with 0.65% normal melting agarose. Cover the drop with a glass slip and keep it on an ice pack for 4 min. The glass slip is carefully removed and immediately the slide is to be kept immersed horizontally in a tray containing acid denaturation solution (0.08 N HCl) for 3 min at 22°C in dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. The denaturation process is stopped and proteins are removed by transferring the slide to a tray with neutralizing and lysing solution (0.4 M Tris, 0.8 M DTT, 1% SDS, 50 mM EDTA, pH 7.5) for 5 min at room temperature. The slide is then washed in Tris–borate–EDTA buffer (0.09 M Tris–borate and 0.002 M EDTA, pH 7.5) for 2 min and dehydrated sequentially in 70%, 90% and 100% ethanol (2 min each), air-dried and stored in dark until staining.

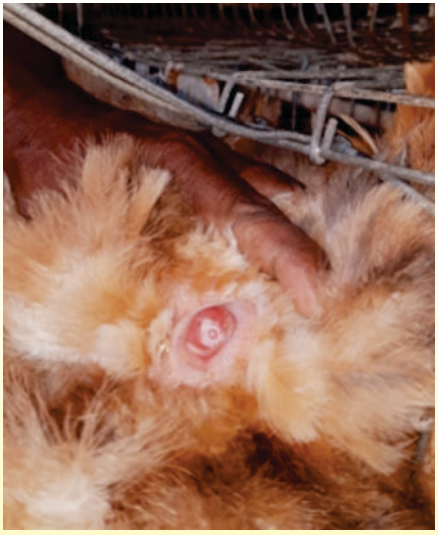
For bright-field microscopy, the slides are placed horizontally and covered with Wright's stain for 3-5 minutes taking care that the stain does not dry on the slide. Then stain should be diluted with an equal quantity of buffer (3.8 g Na_2HPO_4 and 5.47 g KH_2PO_4 and volume made up to one liter using distilled water). The diluted stain is allowed to act for 10 minutes with continuous air blowing, flood off with tap water and air dry the slide. The stained slides are evaluated under oil immersion. About 500 sperm on each slide is counted for halo size and dispersion pattern. The nuclei with large to medium size halo are considered sperm with non-fragmented DNA while nuclei with small size halo, without halo or without a halo and degraded are to be considered as sperm with fragmented DNA.



A-No halo; B-Small halo; C-Medium halo; D-Large halo

ARTIFICIAL INSEMINATION IN HEN

The thawed semen is pooled in a glass cup. The hen is held by its leg with one hand and with another hand gentle pressure is applied to the left side of the abdomen around the vent. This causes the cloaca to evert and the oviduct to protrude. For insemination, a syringe or plastic straw is inserted 1-2 inches into the oviduct and the appropriate amount of semen is deposited. As the semen is deposited by the inseminator, pressure around the vent is slowly released, which assists the hen in retaining sperm in the vagina or the oviduct. In chicken, a volume of 0.05-0.1 mL of pooled raw semen is deposited.



Artificial insemination in a hen

Cryopreservation protocols using 0.5ml French straw for breeds/lines maintained at DPR

Breed/Line	Cryoprotectant-Diluent	Thawing	Insemination in	Average Fertility (%)
Ghagus	8% EG-SD	37°C for 30 sec	Ghagus	18
	8% EG-LR	37°C for 30 sec		48
	8% EG-RFE	37°C for 30 sec		38
	6% DMF-SD	5°C for 100 sec in ice water		25
	6% DMF-LR	5°C for 100 sec in ice water		30
	6% DMF-BPSE	5°C for 100 sec in ice water		19
Kadaknath	4% DMSO-SD	5°C for 100 sec in ice water	Kadaknath	14
PD-1	4% DMSO-SD	5°C for 100 sec in ice water	PD-1	16
PD-6	4% DMSO-SD	5°C for 100 sec in ice water	PD-3 (for getting Gramapriya chicks)	78
	12% MA-SD	5°C for 100 sec in ice water	PD-3 (for getting Gramapriya chicks)	4-10
	4% DMSO-SD	5°C for 100 sec in ice water	PD-6	6.6
PB-2	8% EG-BPSE	5°C for 100 sec in ice water	PB-2	30
	6% DMF-BPSE	37°C for 30 sec		47
IWK	4% DMSO-SD	5°C for 100 sec in ice water	IWK	7.54
IWH	4% DMSO-SD	5°C for 100 sec in ice water	IWH	5-16

EG- Ethylene Glycol
DMF- Dimethylformamide
DMSO-Dimethyl sulphoxide
MA-Methylacetamide
SD- Sasaki diluent
LR-Lake and Ravie diluent
RFE-Red fowl extender
BPSE-Beltsville poultry semen extender

FACTORS AFFECTING SEMEN CRYOPRESERVATION OUTCOME

The freezability and post-thaw fertility from cryopreserved chicken semen are influenced by factors such as cryoprotectant, diluent, thawing temperature and additives employed during the cryopreservation procedure.

Cryoprotectant

The cryoprotectant used for semen cryopreservation is an important factor determining the result of post-thaw semen parameters and fertility. Cryoprotectants can be broadly classified as permeable and non-permeable. The permeable cryoprotectants cross the cell membrane and bring about their effect. Among the permeable cryoprotectants, glycerol is the most widely used compound in cryopreserving semen in other species, particularly cattle. In chicken, glycerol was found to be the least toxic and effective semen cryoprotectant, however, when the concentration is above 1% during insemination it results in contraception and no fertile eggs are obtained. Therefore, it has to be removed from the cryopreserved semen before insemination. The removal step before insemination involves centrifugation that further adds stress and possible damage to the sperm. Furthermore, under field conditions the necessary equipment and setup may not be available. Alternatively, other cryoprotectants such as dimethylsulfoxide, ethylene glycol, dimethylacetamide (DMA), dimethylformamide and methylacetamide were used for cryopreserving poultry semen and insemination without removal. These cryoprotectants were evaluated and used for cryopreserving semen of breeds/lines maintained at the institute.

Non-permeable cryoprotectants do not cross the cell membrane and are high molecular weight compounds. Polyvinylpyrrolidone acts as non-permeable cryoprotectant and is a component in Lake and Ravie diluent. Disaccharides such as trehalose and sucrose were the reported non-permeating sugars in chicken semen cryopreservation. The non-permeating disaccharides increases tonicity of the extender and stability of cell membrane. Combining permeable and non-permeable cryoprotectants may provide added protection to sperm in withstanding damage during freeze-thaw procedure. Sucrose was used at 100 mM concentration along with 6% DMA and evaluated during pellet method of semen cryopreservation. This combination of cryoprotectants had no positive effect on post-thaw fertility in PD1 line. Dextran is a complex branched glucan polysaccharide and different molecular weights (10 and 20 kDa) of dextran were evaluated in control broiler semen cryopreservation. It was observed that dextran 10 and 20 kDa were toxic for sperm when used at 5 and 10% concentrations. There was very low or no post-thaw motility and live sperm. Dextran (10 and 20 kDa) at 8.2% was combined with 9%DMA and evaluated. The combinations produced very low or no fertility. Dextran (10 and 20 kDa) at 5 and 10% concentrations were also evaluated in Nicobari chicken semen cryopreservation. Though post-thaw motility ranged between 10-15% and acrosome intact sperm above 80% no fertile eggs were obtained.

Diluent

The diluent employed during semen cryopreservation should essentially help in maintaining the structure and functional capacity of the sperm during and after cryopreservation. The available chicken semen diluents composition widely varies. Apart from energy and buffering components in the diluent substances such as polyvinylpyrrolidone and glutamic acid are included that supports the sperm during cryopreservation procedure. The disaccharide trehalose reported to support sperm function is also used as a component in Sasaki diluent. We observed that the NaCl/TES diluent with minimal components that may support sperm during liquid storage may not be of use in cryopreserving chicken semen. Therefore, the suitable diluent for semen cryopreservation has to be evaluated and then used.

Thawing temperature

In most reports of chicken semen cryopreservation, a thawing temperature of 5°C was used. In very few reports a thawing temperature of 37°C was used. We found that the thawing temperature influences fertility outcome. During Ghagus chicken semen cryopreservation using ethylene glycol, fertility was obtained when straws were thawed at 37°C for 30 secand used. The cryopreserved semen thawed at 5°C for 100 sec did not produce any fertile eggs.

Additives

During cryopreservation sperm undergoes stress and there is structural as well as functional damages. The cryopreservation process results in reduced sperm survivability and fertilizing ability because of the effect of the freeze-thawing stages on sperm membrane integrity and other functional parameters. High levels of reactive oxygen species (ROS) are formed during the freeze-thawing process. The chicken sperm have high level of membrane polyunsaturated fatty acids such as arachidonic and docosatetraenoic acids that predisposes to deleterious effects of lipid peroxidation (LPO) due excessive production of ROS. The cryopreservation procedure may compromise the antioxidant system of semen. This further increases the intensity of LPO and the consequent damaging effects. Hence, antioxidant protection of sperm is an important step in sustaining membrane integrity, motility and fertilizing ability. Therefore, additives are included in the cryopreservation mixture to improve the post-thaw semen quality and fertilizing ability. Below are few additives evaluated at the indicated final concentrations during semen cryopreservation of different breeds/lines.

1. Trolox - a water-soluble vitamin E analogue 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (0.2 mM, 0.4 mM and 0.8 mM)
2. Butylated hydroxytoluene (BHT) - a lipophilic phenolic antioxidant and synthetic analogue of vitamin E (0.25 mM, 0.5 mM and 1 mM)
3. Zinc as zinc oxide (6.25 and 12.5 µM) and zinc sulphate (100 and 200 µM)

4. Tempol (4-hydroxy2, 2, 6, 6-tetramethylpiperidine-1-oxyl) (1 and 5 mM)
5. Betaine (0.2 and 0.4 mM)

Vitamin E is a chain breaking antioxidant and the water soluble analogue Trolox is easier to dissolve in the diluents and use. BHT reduces the auto-oxidation reaction by converting peroxy radicals to hydroperoxides and acts as a membrane lipid perturbant. BHT considerably reduces the permeability changes of sperm plasma membrane when they are cold-shocked. Zinc has important role in testicular development and steroidogenesis. Zinc acts as a second messenger in sperm and has role in motility and fertility. It is an important component of metalloproteins. Tempol is a low molecular weight cyclic nitroxide compound that mimics superoxide dismutase (SOD) enzyme activity. Betaine is a trimethyl derivative of glycine widely distributed in animal's body with high concentration in testes.

The experimental results indicated beneficial effect for only Trolox at 0.2 mM concentration where the fertility from post-thaw semen was significantly higher compared to control. BHT had deleterious effect on in vitro post-thaw semen parameters, but no effect on fertility. Other additives did not improve the post-thaw fertility in comparison to control with no additive.

PRACTICAL ASPECTS OF CHICKEN SEMEN CRYOPRESERVATION

In chicken semen cryopreservation it was reported and we have observed as well that the protocol has to be standardized for each breed or line due to variation in results depending on the breed/line. In the process of standardization different combinations of diluent, cryoprotectant and thawing temperature has to be evaluated. Preliminary evaluation has to be carried out in the laboratory with few major parameters such as progressive sperm motility, live sperm and abnormal sperm. The evaluation of sperm intact acrosome is one another essential parameter to be carried out. It was observed that many cryoprotectants gave comparatively better post-thaw motility but there were no acrosome intact sperm resulting in infertile eggs after insemination. Furthermore, few cryoprotectants induced spontaneous sperm acrosome reaction soon after addition before freezing. The molecular mechanisms involved in this spontaneous reaction is not known and protocol with such cryoprotectants need not be taken further. Thus, acrosome integrity in post-thaw sperm may be used as an important screening test before proceeding further. Having a higher post-thaw acrosome intact sperm is not a guarantee for fertility. Some protocols gave higher post-thaw *in vitro* sperm parameters including acrosome intact sperm, however, there were no fertile eggs. The in vitro evaluation of cryopreserved semen should be followed by pilot artificial insemination trials in few birds to observe fertility. The unique reproductive physiology in birds where sperm are stored in hens for few days influences the function of the post-thaw sperm. It was also observed that the breed or line of hen inseminated influenced the fertility.

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APPENDIX

Sasaki diluent

D (+)-glucose	:0.2 g
D (+)-trehalose dehydrate	:3.8 g
L-glutamic acid monosodium salt	:1.2 g
Potassium acetate	:0.3 g
Magnesium acetate tetrahydrate	:0.08 g
Potassium citrate monohydrate	:0.05 g
BES	:0.4 g
Bis-Tris	:0.4 g

Distilled water to make up to 100 ml and final pH 6.8

Lake and Ravie diluent

Sodium glutamate	:1.92 g
Glucose	:0.8 g
Magnesium acetate $4H_2O$	:0.08 g
Potassium acetate	:0.5 g
Polyvinylpyrrolidone [relative molecular mass (Mr) = 10 000]	:0.3 g
Osmolality	:343 mOsm/kg

Distilled water to make up to 100 ml and final pH 7.08

Red fowl extender

Fructose	:1.15 g
Sodium glutamate	:2.1 g
Polyvinylpyrrolidone [relative molecular mass (Mr) = 10 000]	:0.6 g
Glycine	:0.2 g
Potassium acetate	:0.5 g
Osmolality	:380 mOsm/kg

Distilled water to make up to 100 ml and final pH 7

Beltsville poultry semen extender

Fructose	:0.3 g
Potassium citrate	:0.0384 g
Sodium glutamate	:0.5202 g
Magnesium chloride	:0.0204 g
di-Potassium hydrogen phosphate	:0.762 g
TES	:0.317 g

Potassium di-hydrogen phosphate	:0.039 g
Sodium acetate	:0.258 g
Osmolality	:330 mOsm/kg

Distilled water to make up to 100 ml and final pH 7.3

0.01M Hcl

Hcl (35.6% purity)	:0.8688 ml
Distilled water	:999.1312 ml

5% Eosin stain

Eosine	:5 g3%
Sodium citrate buffer	:0.8 g

10% Nigrosine stain

Nigrosine	:10 g
3% Sodium citrate buffer	:0.8 g

4mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stain

MTT	:165.732 mg
Normal saline	:100 ml

NaCl-TES

NaCl	:0.877 g
TES	:0.4585 g

Distilled water to make up to 100 ml and final pH 7.4

This stock solution will have 150 mM NaCl and 20 mM TES

McIlvaine's Buffer

Na_2HPO_4	:2.8392 g in 100 ml distilled water
Citric acid	:2.1014 g in 100 ml distilled water

The above preparations will give 0.2 M Na_2HPO_4 and 0.1 M Citric acid. Combining different volumes of these two solutions will give McIlvaine buffer having different pH values. To get McIlvaine buffer with pH 7.2-7.3 combine 17.39 ml of 0.2 M Na_2HPO_4 and 2.61 ml of 0.1 M Citric acid.

ICAR-Directorate of Poultry Research
Rajendranagar, Hyderabad-500030, India
www.pdonpoultry.org