# YAK FROZEN SEMEN PRODUCTION AND CRYOPRESERVATION IN BHUTAN: A TECHNOLOGICAL BREAKTHROUGH

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ABSTRACT: This is a short research communication to inform on yak semen collection and processing, successfully conducted under Bhutanese condition. Although the technology existed in yak rearing countries elsewhere, this is the first time it was successfully done in Bhutan. Two rounds of semen collection were attempted: one during non-breeding season on induced heat using exogenous hormones, and another on natural heat during the yak's breeding season. The first attempt conducted during non-breeding season did not succeed mainly due to poor response of female yaks to heat inducing hormones and failure of yak bulls to mount female not manifesting heat. The second semen collection trial was timed during yak breeding season. Yak bulls were trained to mount in-heat females restrained in a chute. Several trials were conducted prior to the actual successful semen collection. There was a major technological breakthrough in yak semen processing in Bhutan wherein over 220 doses of yak frozen semen could be processed and cryopreserved. The semen was of acceptable standard with >40% post thaw motility and had concentration of 27 million spermatozoa/dose when assessed after 24 hours. The scientific procedures and knowledge established including prior training of donor bulls to mount, timing of semen collection during breeding season and techniques to chill semen during transportation appropriate for Bhutanese farming environment is now understood. The procedures and techniques can further be improved and applied for quality semen production in Bhutan.

Keywords: cryopreservation; technological breakthrough; yak frozen semen

## 1. INTRODUCTION

The Yak (*Poephagus grunniens or Bos grunniens*) is an integral part of pastoral system in Bhutan and has considerable national importance as the animals play a key role in maintaining rangeland ecosystem and preserving highland culture and tradition. Yak herding in high altitudes thus have unique relationships that have evolved overtime between people and nature. Bhutan 2020 states 'we will take pride in such occupation and wisdom accumulated over centuries concerning the sustainability of human activities in fragile often inhospitable environment' (Bhutan 2020; Planning Commission 1999).

Some 1100 farm families in 11 out of 20 Dzongkhags (districts) in Bhutan rear yaks (Wangda 2016; NSB 2022). Yak is a unique bovine species of economical importance surviving in snow bound areas under hypoxic and extreme cold conditions above 3000 meter above sea level (masl), where major agriculture is not rewarding due to non-availability of arable lands. Gerald et al (2003) remarked that the yak must be regarded as one of the world's most remarkable domestic animals as it thrives in conditions of extreme harshness and deprivation while providing a livelihood for highland people. However, decreasing yak population and yak farming households due to deterioration of rangeland pasture, and out-migration of educated youth seeking

alternative economic opportunities is reported (Derville & Bonnemaire 2010).

Yaks are managed under free range system with seasonal migrations based on changes in weather conditions and are reared at 5000 masl during breeding season from June to October as grass grows continually in response to favorable weather (Ura 1993). Yaks are mated at the rangelands through natural services of the bulls selected among growing young stock from own herd or purchased from other herds. Holding period of breeding bulls within a herd is about eight years and such practice might result into inbreeding (Dorji et al 2013). These factors accompanied by geographical isolation of yak herds increases the risk of inbreeding that can adversely affect the genetic potential of the animals. Lu Hongji (1987) supports the view that there are circumstantial evidences of inbreeding happening in yak population due to insufficient interchange of breeding bulls across the national boundaries. Gerald et al (2003) also mentioned that traditional breeding practices may encourage inbreeding, with its effect especially on reduced reproductive capacity, growth rate, body size and milk production and it should be considered in relation to prevailing yak herding system.

To inject fresh blood for genetic improvement of Bhutanese yaks and to minimize inbreeding, limited doses of frozen semen was imported from China in mid 1990s through Food & Agriculture Organization of United Nations. Artificial Insemination (AI) in yaks was tried on a small scale in Bumthang, Merak Sakten gewogs (sub district) in Tashigang and Dagala gewog, Thimphu. The success rate at Dagala is reported to be 44 percent (Tshering et al. 2000). Though this technology is popular in yak rearing countries elsewhere, it could not be applied in appreciable scale in Bhutan due to lack of yak frozen semen.

To address the issue of lack of yak frozen semen in Bhutan, National Dairy Research and Development Centre (NDRDC) Yusipang conducted two trails on semen collection, transport from highland areas to semen processing station at Yusipang for processing within stipulated timeframe to establish scientific procedure for the production of yak semen; collection, processing and cryopreservation, and document techniques on cryopreservation of yak frozen semen for future references.

## 2. MATERIALS AND METHODS

### 2.1 First trial

Initial trial was conducted at the Integrated Yak Farm (IYF), Chanaphu, Haa in April, 2022. During the trial period four potential young yak semen donor bulls between two to four years of age were selected. As the trial period was before the breeding season, four female yaks (to be used as dummies for semen collection trials) were estrus synchronized to induce heat. Potential donor bulls (>3 years of age) were tethered and taken near to mount the females that are inside the chute. Standard procedure was followed to prepare Bovine Artificial Vagina (AV) to collect semen. The trial was carried out twice daily (early morning & evening) for a week. But semen collection was futile because female yaks were not in natural heat and untrained bulls just smelled the female and refused to mount.

#### 2.1.1 Testing semen preservation methods prior to trial 2

Yak herds remain in summer pasture at high altitude during the breeding season from June to September. Though it is ideal to process semen immediately after collection to avoid deterioration of sperm quality, it is impossible to do so at high altitude summer pasture due to lack of electricity, equipment and other facilities. Hence, techniques have to be devised for long distance transportation (about a day journey) from summer yak herding areas to NDRDC,

Yusipang. Hence, prior to the second trial, several trials on different method of semen preservation were conducted at the National Semen Processing Laboratory, NDRDC Yusipang for a period of six weeks (June-July, 2022). Preservation of semen was tried from nine semen donor bulls (cattle) during the routine semen collection as per the collection schedule. Each time 2ml of freshly collected semen were transferred in separate semen collection vials, extended with BioXcell (IMV, France) which is animal protein free semen extender and an excellent media for frozen semen cryopreservation (at -196°C) or preservation of fresh bovine semen (4-5°C) and processing. Different methods tried for semen preservation were:

i. The fresh semen ejaculates were diluted (1:3) with BioXcell and kept in dry bath at 37$^{o}$C.
ii. The fresh ejaculates were diluted (1:3) with BioXcell and kept at room temperature of 18-24 $^{o}$ C.
iii. The fresh ejaculates were diluted (1:3) with BioXcell and stored at sub zero temperature (<0$^{o}$C) by plunging in liquid nitrogen.
iv. The fresh ejaculates were diluted (1:3) with BioXcell and stored at 4-5$^{o}$C inside Cool box cushioned with ice packs.

### 2.1.2 Familiarization of yak attendants to train breeding bulls

To eliminate the bull's hostility, training of yak bulls is an important aspect of semen collection (Zang 2000). Hence, a month long training (August – September, 2021) was imparted to two yak attendants of IYF, Haa at NDRDC, Yusipang to develop knowledge and skills on proper care, handling, general management of semen donor bulls, technique of semen collection and other activities related to bovine semen collection. The knowledge gained was applied by the attendants to train young yak bulls before conducting the semen collection trial 2.

## 2.2 Second trial

Yak being a seasonal breeder, estrous/heat is manifested in summer (June to September) when the animals have migrated to remote high altitude summer pasture at about 5000 masl. Hence, yak semen collection was tried at this altitude in Haa Dzongkhag in August 2022.

### 2.2.1 Training of yak bulls

Prior to the second trial, based on lesson learnt from the first trial, the yak attendants were attached at the summer yak grazing areas from June to August, 2022 to care, handle and train donor bulls to mount for semen collection.

Zhang (2000) explained that through proper feeding and care, yak bull should be accustomed to herdsman or attendants approaching the animals, so that it allows to even touch the animals. The herdsman or attendant scratches the bull from the front to the rear of the body, and from the back to the abdomen. When the bull is more accustomed to being handled, scrotum and sheath be handled. Since Yaks dislikes being touched on the head, herdsman avoids this area.

### 2.2.2 Improvising procedure for semen collection

The female yaks in heat were restrained in a chute. Potential yak bulls with docile temperament were tethered and introduced to mount female yak in estrus. When the bulls mounted, the sheath was held to guide the penis into artificial vagina (internal temperature, 39-42$^{o}$C).

Yak bulls were handled in a gentle manner and their surroundings kept quiet and familiar. The team tried semen collection from more than five potential bulls for a week in August 2022. The team finally managed to collect semen from one yak bull on 18 August 2022 after trial and error for weeks.

### 2.2.3 Semen collection method applied

Yak semen was collected using bovine Artificial Vagina (AV) methods (Figure 1). Prior to collection all the parts of AV were cleaned, sterilized and assembled into artificial vagina. The rubber cone of AV was attached with graduated semen collection tube. Water at 39– 42 $^0$C (degree centigrade) was filled in through the water valve / outlet to ½ to ¾$^{th}$ full to maintain required temperature for bull semen collection.

**Figure 1.** Yak Semen collection at Yak Summer Pasture, on 18$^{th}$ August 2022

### 2.2.4 Yak semen chilling and transportation

Fresh yak semen collected was diluted with Bioxcell (Semen extender) and vial with semen was cushioned with ice pack for its gradual chilling to 4-5$^0$ C. The semen being chilled was transported in cool box by pre-arranged herdsman/porter to the nearest road head at Shana in Paro in shortest possible time (10 hours walk) and to NDRDC Yusipang, Thimphu (another two and half hour drive from the road head), to reach the consignment within 24 hours for best results.

### 2.2.5 Semen evaluation and processing

With planned and coordinated move, semen sample reached NDRDC Yusipang within 24 hours of collection and dispatch. It was evaluated rapidly but carefully so that samples can be processed to maintain quality and fertility. The major criteria considered for evaluation includes ejaculate volume, sperm motility, sperm concentration, proportion of abnormal sperm and proportion of live sperm cells. For physical test: semen volume, color and consistency were macroscopically examined. Mass Activity was determined with phase contrast microscope under low power objective (10x) and to estimate initial motility or percentage of motile cell, 20x objective at a magnification of 200 times (10x eye piece and 20x objective of same microscope).

Sperm concentration is assessed using spectrophotometer. Eosin-Negrosin staining was done for live-dead count. When semen samples received, met all the criteria the semen was processed overnight (late 18 August to early morning of 19 August 2022) at the National Semen Processing Laboratory, NDRDC Yusipang following Standard Operating Procedure.

## 3. RESULTS AND DISCUSSION

### 3.1 Experience and outcome from first trial

The first trial was unsuccessful but it gave a good learning experience. It was difficult to restrain and collect semen from un-trained/untamed yak bulls in the wild. The researchers had following leanings from that experience:

- The young bulls smelled and licked the rear and vulva of the dummies (estrous synchronized yaks) and showed Flehmen

response to detect non volatile organic compounds (certain scents, pheromones & hormones) excreted from genital regions or urine to detect reproductive state of the female

- The yak donor bulls did not show any other signs of mounting the dummies though grooming and massaging the rear of the bulls was tried to stimulate sexual arousal.
- The failure of the female yaks to come to heat even after estrus synchronization is the possible reason as to why the donor bulls did not mount the female dummies.
- The period from March – April was not the right time because during this time the donor bulls were having poor body condition score (BCS) due to unavailability of adequate pasture. This could have resulted in poor libido in the young bulls.
- Adequate and continuous training of young semen donor bulls with adequate BCS with availability of female yaks in natural estrus/heat is needed to facilitate mounting and semen donation.

Hence, lessons from this trial were applied for improvement of procedure/ techniques for yak semen collection during the second trial.

### 3.2 Laboratory trial on semen preservation

Liquid semen collection, chilling and then thawing it to inseminate within 24-48 hours is scientifically possible with viable spermatozoa in domestic animals- bovine, porcine and canine (FAO undated, Ferguson 2022). This scientific fact was validated through four different techniques tried for semen preservation: storage at 37$^{o}$C in dry bath; storage at room temperature; storage at sub zero temperature and storage at refrigeration temperature / chilled at 4-5$^{o}$C. The result indicated that in first and second techniques, semen quality was preserved only up to 4–6 hours post ejaculation but sperm motility decreased after 6 hours and was below the standard <40% sperm motility with passing time (Table 1). Further, storage at sub zero temperature (method three-storage of collected semen, diluted with semen extender and directly plunged in Liquid Nitrogen) drastically reduced semen motility within few hours, probably due to sudden cold shock and this technique was also found to be ineffective. However, storage of bovine semen diluted/extended with BioXcell and chilled semen at 4 to 5 $^{o}$C (technique four) was found most effective method since the sperm cells maintained required viability for more than 24 hours. The predominant effect of initial storage of diluted semen at 4-5 $^{o}$C could be due to lowering of metabolic rate of spermatozoa, which contributed to its extended survival. Research on yak semen characteristics in China also indicated that survival time of spermatozoa and the survival index are 54 hours and 18.72 hours respectively, at <15$^{o}$C (Zhang 2000). Thus,

**Table 1:** Semen quality tested at different time interval

| Methods used | Time intervals (hours) of microscopic examination | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 16 | 18 | 20 | 22 | 24 | >24 |
| 1. Semen storage in dry bath (37$^{o}$C) | VG | VG | G | G | P | P | P | P | P | P | P | P |
| 2. Semen storage at room temperature (18-24 $^{o}$C) | VG | VG | VG | G | P | P | P | P | P | P | P | P |
| 3. Semen storage at subzero temperature (< 0$^{o}$C) | P | P | P | P | P | P | P | P | P | P | P | P |
| 4. Semen storage at refrigeration temperature / chilled (4-5 $^{o}$C) | VG | VG | VG | VG | VG | VG | VG | VG | VG | VG | VG | VG |

*Note: VG= Very good (>40% motility), G=Good (30-<40%) Motility, P=Poor (<30% motility)*

application of method four was thought to be useful while transporting semen from yak summer pasture to NDRDC Yusipang for further processing.

### 3.3 Outcome of the second trial

#### 3.3.1 Training of yak bulls

Based on the lessons learnt from the previous trial, to have trained bulls and yak cows in heat, the second trial was conducted during the yak breeding season. Yak attendants familiarized on care, handling and training of yak bulls to mount were sent to yak summer pastures to train yak breeding bulls. Yak bulls were trained continuously for two months to mount which eased semen collection. Unlike other species/breed of cattle such as Jersey and Nublang that are much docile and easy to handle, training to tame bulls of semi-wild species such as Mithun and Yak are crucial without which managing them for semen collection will be very difficult (Tshering & Gurung, Pers. Comm 2018).

#### 3.3.2 Semen evaluation and processing

Semen samples received within the stipulated time from the highland was evaluated rapidly but carefully, and found to be fit for processing. The yak semen had similar morphology of bovine semen. Color of Yak semen from Bhutan was found to be milky to creamy with a volume of 4.5 ml in two ejaculations. Sperm concentration was 1350x10$^6$/ml, Mass Activity and Initial Motility was 2++ and 85% respectively. Further, the live cell count was 83.3 % which indicates semen quality is good. The detail characteristics of the yak semen sample transported and evaluated are shown in Tables 2, 3 and Figure 2.

**Table 2:** Characteristics of yak semen

| Particulars | Details | Remarks |
|---|---|---|
| Mass Activity (MA) | 2 (++) | The MA & IM were good for semen cryo preservation with sperm concentration of about 1350x10$^6$/ml. % abnormal cell count was very low. Abnormal sperm cells observed were dag defect, bent tails and distal cytoplasmic droplets |
| Initial Motility (IM) | 85% | |
| Live Spermatozoa % | 83.3% | |
| Abnormal Spermatozoa % | 6.7 % | |
| Concentration | 1350 M/ml | |

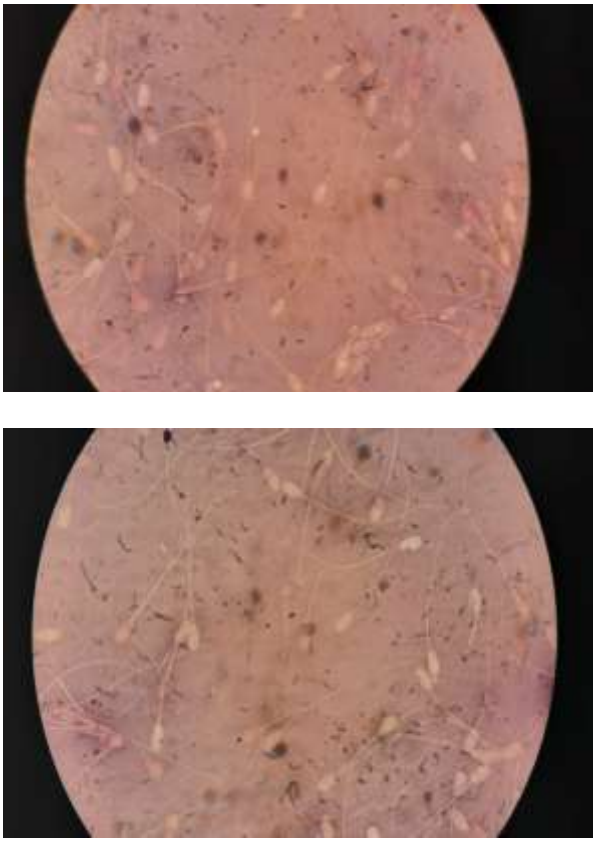

**Figure 2:** Yak semen quality when evaluated at NDRDC Yusipang

Similarly, characteristics of ejaculates of adult yak bulls from Datong Yak Farm, Quangai, China was reported to be milky-white in color, average volume ranged from 2-5 ml per collection and spermatozoa concentration ranged from 750-1600×10$^6$/ml, with 70-85% progressively motile (Zhang 2000). Hence, semen collected from Bhutanese yak is of good quality as it falls within the range described.

**Table 3.** Morphological examination of yak semen

| Particulars | Nos. | Percentage (%) |
|---|---|---|
| Live spermatozoa | 250 | 83.3 |
| Dead Spermatozoa | 50 | 16.7 |
| Total cells counted | 300 | |
| Major Spermatozoa abnormalities | | |
| Proximal Protoplasmic droplet | 0 | 0.0 |
| Pyriform Head | 0 | 0.0 |
| Dag Defect | 3 | 2.0 |
| Deformities of Mid Piece | 1 | 0.7 |
| Under development-Double form | 0 | 0.0 |
| Craters/ Diadem | 0 | 0.0 |
| Knobbed Acrosomes | 0 | 0.0 |
| Total Major abnormalities (%) | 4 | 2.7 |
| Minor spermatozoa abnormalities | | |
| Distal Protoplasmic droplets | 3 | 2.0 |
| Head normal without tail | 1 | 0.7 |
| Bent/Coiled tail | 2 | 1.3 |
| Tapered /narrow/ small/ giant head | 0 | 0.0 |
| Abaxial tail | 0 | 0.0 |
| Abnormal Acrosome | 0 | 0.0 |
| Others | 0 | 0.0 |
| Total Minor abnormalities (%) | 6 | 4.0 |
| Total Major + Minor abnormalities (%) | 10 | 6.7 |

### 3.3.3 Semen processing and cryopreservation

Following Standard Operating Procedure (SOP) 220 doses of yak semen processed was cryopreserved, immersing in liquid nitrogen at -196$^0$C after filling and sealing it in French mini straw (0.25 ml per dose) with blue color straw allotted to distinguish Yak frozen semen. Bovine semen cryopreservation world-wide follows standardized methods for extension, cooling, freezing, and thawing (Rodriguez-Martinez et al 2013) and semen processing SOP applied in Bhutan is consistent with international standard.

### 3.3.4 Post thaw motility check

As per standard protocol for Bovine frozen semen production (NDRDC 2017), post thaw motility (PTM) test was done after 24 hours yak semen cryopreservation. The cryopreserved semen is found to be of acceptable standard of >40% PTM and concentration of 27 million spermatozoa per dose, which therefore is above the standard requirement.

## 4. CONCLUSION & RECOMMENDATION

Based on field trials, it can be concluded that semen collection from yak bulls should be best attempted during the breeding season from June to September when female yaks are in natural heat. Induction of heat in female yaks with hormonal drugs during non breeding season often leads to poor manifestation of heat resulting in failure of bulls to attempt mounting/semen donation. Besides, collection of semen from untrained bulls is difficult owing to free range yak management system. Hence to avoid bull hostility, yak breeding bulls' needs to be cared, handled properly and trained to mount for fruitful semen collection venture. Further, most feasible techniques for liquid semen preservation are to extend semen with semen extender, cushion the semen vial with icepack and place it in a box soon after collection to enable gradual cooling to a temperature of about 4-5$^0$C. This can ensure viability of spermatozoa for more than 24 hours, assuring survival during long distance transportation and semen has to be processed within 24 hours of collection for desired results. Consistent efforts to refine the scientific procedures in collection of yak semen, validation of semen preservation techniques that ensure sperm viability essential during long distance transportation; and timely processing of semen, within the

stipulated time of <24 hours after collection has enabled cryopreservation of yak frozen semen. This is the first successful event in the history of Yak breeding in Bhutan and this no doubt is a major technological breakthrough for advancement of science. This scientific procedure for the cryopreservation of yak frozen semen, with semen transported in chilled form, appropriate for Bhutanese farming environment, is now understood. This established procedure or knowledge can be further researched and validated in future with improvement where necessary. The foundation laid on production and cryopreservation of yak frozen semen in Bhutan is expected to go a long way in accelerating yak breed improvement in the country.

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