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Standardisation of Microencapsulation Protocol for Cryopreservation of Malabari Goat Semen

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ABSTRACT

The study was conducted to characterise fresh semen and to standardise a microencapsulation protocol for cryopreservation of Malabari buck spermatozoa. Fresh semen evaluation revealed the ejaculate volume (1.10 ± 0.24 ml), progressive motility (87.50 ± 2.14 %), concentration of spermatozoa (3829.16 ± 172.01 million/mL), viability (92.16 ± 1.62 %), morphological abnormalities (1.91 ± 0.23 %), functional membrane integrity (64.00 ± 1.93 %) and acrosome integrity (93.16 ± 1.01 %) within normal range. Pooled good quality ejaculates from bucks with good semen freezability were extended in Tris egg yolk based extender; microcapsules were prepared after mixing with sodium alginate at different concentrations (2.5% and 3%), at different ratios of alginate and extended semen (1:0.6 and 1:1) and extruding through different sizes of hypodermic needle (22 G, 23 G and 24 G) to obtain microcapsules of different size and shape. The size, shape and wall integrity of microcapsules were evaluated in all combinations of production parameters. Alginate concentration of 3 per cent, when mixed with extended semen at the ratio 1:1 and extruded through a 24 G needle into a five per cent barium chloride solution at a fixed distance of 3 cm from the needle tip yielded globular, 1.7 mm sized capsules with 65 per cent wall integrity.

Keywords: Semen evaluation, Microencapsulation, Alginate, Microcapsule, Malabari, Goat.

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INTRODUCTION

The decline of pure goat breeds from their native tracts is becoming a global concern, leading to the disappearance of pure germplasm lines. The low male-to-female ratio due to the indiscriminate slaughter and early castration of males (Khandoker *et al.,* 2011) raises serious apprehension about the existence of goat breeds. Although artificial insemination (AI) with cryopreserved semen is an alternative to compensate for a low male-to-female ratio in this species,

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due to the challenges associated with cryopreservation and lower success rate after frozen semen AI, it is not widely accepted as in cattle. Further, the protracted oestrus period of 36 – 48 h in goat makes it difficult to achieve the precision of AI timing, which is very much essential for the success of AI using frozen semen.

Sperm encapsulation technology is already in vogue among cattle and some other species to overcome the ill-effects of cryopreservation and to minimise the risk of mistiming AI. Microencapsulation involves encapsulating sperm cells within a semipermeable membrane, enabling the exchange of nutrients and metabolites. Packaging sperm in hydrogel matrices is recognised for its potential to facilitate controlled sperm release around oestrus, thereby potentially increasing the success rate of AI (Nebel *et al.,* 1985). Cell encapsulation offers a favourable environment that shields sperm from the adverse effects of the freezing process, thereby enhancing post-thaw longevity, motility and viability during storage. It also significantly reduces acrosomal and sperm plasma membrane damage, improving sperm preservation (Shah *et al.,* 2011). The present study was conducted to standardise a protocol for microencapsulation of Malabari goat semen for its cryostorage.

MATERIALS AND METHODS

Selection of animals and semen collection: The study was carried out with good quality semen collected from three healthy adult, Malabari bucks aged two to three years and weighing 42 to 46 kg, having good semen freezability. The bucks with more than 35 per cent post-thaw progressive motility of spermatozoa were considered as of good semen freezability (Bhai et al., 2023). These bucks were maintained under uniform feeding, housing and management conditions at the Artificial Insemination Centre, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy. Six ejaculates were collected from the bucks, twice weekly at an interval of three days by a single ejaculate regime, using Danish type artificial vagina (12×3.8 cm) fitted with a warm sterile graduated glass collection vial.

Fresh semen evaluation: The ejaculates were assessed for progressive motility, sperm viability, morphological abnormalities, functional membrane integrity and acrosome integrity of spermatozoa. Progressive motility (%) of spermatozoa was assessed after mixing two drops of 2.96 percent sodium citrate solution to a small drop (10 µL) of the fresh semen taken on a warm, clean, grease-free glass slide, covered with a clean coverslip and observed under high power of bright field microscope (400×). Viability and morphological abnormalities (%) of spermatozoa were assessed by the eosin-nigrosin staining technique (Campbell *et al.,* 1956). Functional membrane integrity (%) of spermatozoa was assessed by means of hypo osmotic sperm swelling test (Jeyendran *et al.,* 1984). Per cent intact acrosome (PIA) was assessed by Giemsa staining technique (Tamuli and Watson, 1994).

Microencapsulation of semen: After preliminary evaluation, ejaculates with sperm concentration of more than 2500×10^6 /mL and progressive motility of more than 70 per cent, collected from the three selected bucks were pooled and used for microencapsulation.

Microencapsulation procedure was done as a modification of the method adopted by Nivethitha *et al.* (2024). The pooled semen was diluted in Tris-egg yolk-based extender at a dilution rate of 1:2. Microcapsules were prepared after mixing the extended semen with sodium alginate at different concentrations (2.5% and 3%), at different ratios of alginate and extended semen (1:0.6 and 1:1) and extruding through different sizes of hypodermic needle (22 G, 23 G and 24 G) to obtain microcapsules of different size and shape. Sodium alginate and extended semen mixture in a 5 ml glass syringe was extruded through the hypodermic needle from a height of 3.0 cm from the needle tip perpendicularly into a five per cent barium chloride solution. The microcapsules formed were allowed to react with the barium chloride solution for 15 min, followed by aspiration of the remaining solution and washing with physiological saline. The microcapsules were then treated with 0.1 per cent poly-L-lysine solution for 5 min to facilitate formation of the outermost membrane of the capsule, followed by washing with physiological saline. Finally, the microcapsules were treated with 2.96 percent sodium citrate solution for the liquefaction of the gel core.

Evaluation of microcapsules: The size, shape and wall integrity of capsules were evaluated in different combinations of production parameters. A bright field microscope with a micrometer scale was used to determine the capsule size. Capsule size was measured in mm as the maximum diameter of the capsule observed under the 4× objective of the microscope. Capsule shapes were recorded as irregular, tear drop, oval or globular shaped. Capsule wall integrity was assessed by vortexing 10 microcapsules in 1 mL of five per cent barium chloride solution in a microcentrifuge tube at 1000 rpm and percent of intact capsules that survived vortexing was counted. Considering all the criteria to evaluate microcapsules, the best procedure that resulted in globular capsules of less than 2 mm size with maximum wall integrity was identified.

RESULTS AND DISCUSSION

Fresh semen evaluation: The results of fresh Malabari buck semen evaluation are summarised in Table 1. The mean volume of semen collected was 1.10 ± 0.24 mL, which was within the normal range and in agreement with the results of Urmila (2022), Bhai *et al.* (2023) and Nivethitha (2024), who also reported similar volumes. Semen ejaculate volume is influenced by breed, age, season and method of semen collection (Purdy, 2006). Dorado *et al.* (2010) reported that ejaculate volume within breed varied with individual animals. Sultana *et al.* (2013) demonstrated heterogeneity in ejaculate volume of Black Bengal bucks of identical age, management, nutritional status and health condition, due to differences in genetic potential.

Table 1. Fresh semen characteristics of Malabari bucks (n=6)

Semen characteristics	Mean ± SE	Minimum	Maximum
Volume (mL)	1.10 ± 0.24	0.4	2
Sperm concentration (millions/mL)	3829.16 ± 172.01	3384	4387
Progressive motility (%)	87.50 ± 2.14	80	95
Viability (%)	92.16 ± 1.62	86	97
Morphological abnormalities (%)	1.91 ± 0.23	1	2.5
Functional membrane integrity (%)	64.00 ± 1.93	58	70
Per cent intact acrosome (%)	93.16 ± 1.01	89	96

The mean sperm concentration recorded in the present study was 3829.16 ± 172.01 million/mL. The result matches with reports of Bhai (2012), Urmila (2022) and Bhai *et al.* (2023) in Malabari bucks. Lower values of 3149.91 ± 60.07 and 3267.00 ± 196 million/mL were reported in Malabari bucks by Pawshe (2016) and Nivethitha (2024), respectively. Breed, age, nutrition, season, technique of semen collection, degree of sexual excitement and scrotal circumference influenced the concentration of spermatozoa (Belkadi *et al.,* 2017).

The mean sperm progressive motility recorded in the study was 87.50 ± 2.14 per cent Similar values of 86.00 ± 0.58 (Pawshe, 2016), 85.50 ± 1.40 (Bhai *et al.,* 2023) and 85.50 ± 1.40 per cent (Nivethitha, 2024) were reported earlier in Malabari bucks. Higher (John, 2016) and lower (Rajan, 2010 and Urmila, 2022) values were reported earlier in the same breed. Variations in the progressive motility of spermatozoa may be attributed to differences in breed, climatic condition, season, nutrition, body condition score, scrotal circumference and method of semen collection and handling (Agossou and Koluman, 2018).

The mean per cent of viable sperm in fresh buck semen was 92.16 ± 1.62 in the present study. Similar percentages of 92.22 ± 0.52, 92.00 ± 0.37 and 90.88 ± 0.36 were reported in the same breed by John (2016), Krishnan (2017) and Bhai *et al.* (2023), respectively. Higher (Shiny, 2011) and lower (Behera, 2012 and Nivethitha, 2024) values were reported earlier in Malabari bucks. Sperm viability can be affected by breed, age, nutrition and season (Agossou and Koluman, 2018); heat stress, temperature fluctuations and distance from the point of semen collection to the laboratory (Hahn *et al.,* 2019).

The mean morphological sperm abnormality per cent recorded in the present study were 1.91 ± 0.23. Comparable results of 1.90 ± 0.12 (John, 2016), 1.89 ± 0.28 (Krishnan, 2017) and 1.78 ± 0.24 (Urmila, 2022) per cent were reported earlier in the same breed. Higher (Shiny, 2011 and Pawshe, 2016) as well as lower (Bhai, 2012 and Bhai *et al.,* 2023) abnormality per cent were also reported in Malabari buck semen. Increased sperm abnormalities were induced by genetic conditions, age, nutrition, heat stress and laboratory errors during semen handling.

Osmotic stress is commonly employed as a criterion to evaluate the functional membrane integrity of spermatozoa. Spermatozoa with intact plasma membranes exhibit tail coiling when exposed to a hypo-osmotic solution. In the present study, 64.00 ± 1.93 per cent of Malabari buck spermatozoa had functionally intact plasma membrane which was comparable with reported plasma membrane integrity per cent by Bhai (2012), Urmila (2022) and Nivethitha (2024) in the same breed. Higher sperm plasma membrane integrity of 83.25 ± 0.62, 81.61 ± 1.18 and 82.44 ± 0.24 per cent in Malabari bucks were reported by John (2016), Krishnan (2017) and Bhai et al. (2023), respectively whereas lower value of 54.53 ± 1.99 per cent was also reported (Behera, 2012). Being an effective semi permeable barrier for molecules, an intact plasma membrane maintains and modulates the intracellular composition of spermatozoa also (Azeredo *et al.,* 2001).

Giemsa stain has low molecular weight and is able to pass through the cell membrane that protects the acrosome and stains acrosomal region of spermatozoa. Only acrosome-intact spermatozoa can penetrate the zona pellucida and fuse with the oocyte plasma membrane. The PIA of semen in the present study was 93.16 ± 1.01, which was within the normal range. Analogous PIA was reported in Malabari bucks by Rajan (2010), Bhai (2012), Krishnan (2017) and by Bhai et al. (2023). Agossou and Koluman

(2018) reported that sperm acrosome integrity can be affected by breed, age, nutrition, heat stress and season.

The results of fresh semen evaluation of Malabari goats showed that all seminal characteristics evaluated were within the normal reported values for Malabari goats, which is a direct indicator of good fertility in these animals. The seminal parameters are affected by many factors such as body weight, body condition score, breed, age, managemental conditions, nutrition, scrotal circumference and testicular parameters, climatic and seasonal conditions, diseases, the method of semen collection and degree of sexual stimulation. Semen samples with a sperm concentration of more than 2500×10^6 /mL and more than 70 per cent progressive motility were used for preservation and artificial insemination (Bhai *et al.,* 2023 and Nivethitha, 2024).

Microencapsulation of semen: The results of microencapsulation are presented in Table 2. Extended semen (1:2) when mixed with three per cent sodium alginate (1:1) to get net alginate concentration of 1.5 per cent and extruded through a 24 G needle yielded globular capsules of 1.7 mm size and 65 percent capsule wall integrity (Fig.1).

Table 2. Sperm capsule parameters recorded at different concentrations and ratios of alginate with extended semen and different extrusion needle bore sizes

Alginate conc.	Ratio of alginate to semen (Net alginate conc.)	Bore size of needle	Capsule parameters		
			Size (mm)	Shape	Wall integrity (%)
2.5%	1:0.6 (1.5%)	22 G	4	Oval	50
		23 G	3	Irregular	40
		24 G	2	Globular	60
	1:1 (1.2%)	22 G	5	Irregular	30
		23 G	3	Oval	40
		24 G	2.5	Tear drop	45
3.0%	1:0.6 (1.8%)	22 G	4	Plate like	35
		23 G	3.5	Irregular	30
		24 G	3	Oval	45
	1:1 (1.5%)	22 G	5	Irregular	45
		23 G	2.5	Tear drop	50
		24 G	1.7	Globular	65

In an earlier study by Nivethitha et al. (2024), the procedure of preparation of microcapsules was standardised for the chilled preservation of microencapsulated Malabari buck spermatozoa, where the capsule size obtained was 1.90 ± 0.03 mm. Since the microencapsulated semen needed to be packaged in French straws for cryopreservation, where the inner diameter of the straws is only 2.0 mm and 2.5 mm for French mini and medium straws respectively, the microcapsules produced should have a lesser diameter compared to the previous study and accordingly the procedure was modified.

Sodium alginate concentration of 1.5 per cent was used in the present study, which was similar to that employed by Nivethitha et al. (2024) for Malabari buck sperm encapsulation. A similar concentration of sodium alginate was used in encapsulating bovine (Nebel *et al.,* 1985) and canine (Shah *et al.,* 2011; Lakde *et al.,* 2018) spermatozoa. When the alginate concentration was below one per cent, non-spherical capsules formed, due to an insufficient number of carboxyl groups necessary for the gelling process (Chan *et al.,* 2011).

The concentrated semen was mixed with an equal volume of 15 per cent alginic acid in the case of boar spermatozoa (Huang *et al.,* 2005). A sperm suspension diluted with EYT was combined in a 1:2 ratio with a 1.5 per cent (w/v) sodium alginate solution dissolved in physiological saline, resulting in a final concentration of 1.0 per cent sodium alginate in canine sperm (Shah *et al.,* 2011).

Sodium alginate has the property of preserving the morphology as well as functional characteristics of encapsulated cells (Torre *et al.,* 2002). The alginate structure allowed the exchange of nutrients and metabolites (Paredes-Juarez *et al.,* 2014). Alginate capsules preserve sperm cells in a non-capacitated state, extending their fertile period after AI and making ovulation timing less critical. The spermatozoal release can be controlled by factors such as moisture, pH, temperature, physical force or a combination of these. The release mechanism may involve processes like leaching, erosion, rupture, enzymatic deg-

radation, or similar actions, depending on the structure of the wall (Nebel *et al.,* 1993). The capsules gradually release sperm through enzymatic degradation, allowing a prolonged presence of fertile sperm cells in the uterus, which is expected to improve fertilisation rates (Weber *et al.,* 2006).

It could be noted that the reduction of the bore size of the needle from 22G to 24G resulted in the capsular size reduction from 5.0 to 1.7 mm. The shape of the capsules improved from irregular through tear-shaped to globular on reducing the bore size of the needle. A notable increase in capsule wall integrity from 45 to 65 per cent was also recorded with a reduction in the bore size of the needle. The size and shape of microcapsules were

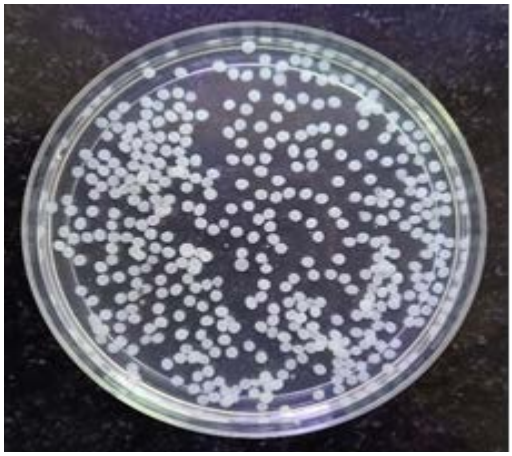

Fig. 1: Globular microcapsules of Malabari goat semen

influenced by the needle gauge and its distance from the hardener solution (Nebel *et al.,* 1985). Lakde et al. (2018) used a 24 G needle for microencapsulation of canine spermatozoa. Nivethitha et al. (2024) used a 23 G needle for the microencapsulation of Malabari buck spermatozoa, which resulted in globular capsules with a mean size of 1.90 ± 0.03 mm.

Since the sperm concentration in a buck is much higher than in the bull, more spermatozoa will be encapsulated in a single capsule of buck semen, which can result in reduced stability of microcapsules. Capsule fragility was affected by the poly-L-lysine concentration as well as the concentration of spermatozoa encapsulated (Nebel *et al.,* 1993). Nivethitha (2024) obtained capsule wall integrity of 79 per cent during microencapsulation of Malabari buck spermatozoa. In this study, reduced wall integrity of 65 per cent was obtained which may be attributed to the reduced size of the capsule with incorporation of higher concentration of spermatozoa.

The globular shape of capsules is important in encapsulation because it can incorporate the maximum volume of semen. Barium, at equivalent ion concentrations, produced a stronger alginate gel compared to calcium, reducing the chemical stress on sperm cells during encapsulation and preventing premature capacitation (Huang *et al.,* 2012).

Normal cryopreserved semen has a limited lifespan in the female reproductive tract due to the capacitation of spermatozoa and lack of formation of sperm reservoirs. The microencapsulated semen has the advantage of reduced membrane damage, DNA damage, acrosome damage and capacitation during cryopreservation, which may also mimic sperm reservoir formation by controlled release of spermatozoa. Also, it protects the spermatozoal loss from phagocytosis by leukocytes and backflow of semen (de Lamirande *et al.,* 1997). A standardised protocol for buck semen encapsulation and cryopreservation would provide a reliable, repeatable process that ensures high post-thaw sperm quality, viability and fertility. By making the process more affordable and user-friendly, such a protocol could enhance the widespread use of AI, improve genetic diversity, preserve valuable goat breeds, and ultimately increase productivity and profitability in the goat farming sector.

CONCLUSIONS

Extended Malabari buck semen, when mixed with 1.5 per cent sodium alginate at 1:1 ratio, extruded through a 24 G needle from a distance of 3 cm to five per cent barium chloride hardening solution and cross-linking with poly-L-Lysine produced stable, globular capsules of 1.7 mm size, which are suitable for packaging in French semen straws for cryostorage.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in the conduct of this experiment.

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