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Standardisation of microencapsulation protocol for chilled preservation of Malabari buck spermatozoa#

K.K. Nivethitha[1], B. Bibin Becha[1*], C. Jayakumar[1], K. Lekshmi Bhai[1] and Suresh N. Nair[2]

[1]Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur – 680 651, [2]Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur – 680 651, Kerala Veterinary and Animal Sciences University, Kerala, India

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Abstract

The present study was conducted to standardise the protocol for microencapsulation of buck spermatozoa for its chilled preservation. Semen ejaculates collected from Malabari bucks of age two to three years, weighing 42 to 46 Kg and maintained under uniform managemental conditions were utilised. Sodium alginate of concentrations 0.5, 0.75 and 1.5 per cent with varying gauge sized hypodermic needles of 18G, 20G and 23G were used. The distance between the tip of the needle and the upper meniscus of $BaCl_2$ solution was adjusted to varied distance like ≥ 7 cm, 3.5 to 5 cm, ≤ 2cm. This resulted in capsule sizes of 1 mm, 2 mm, 3 mm, 4 mm, 4.5 mm, 6 mm and 7 mm. Different shape of capsules like globular, plate like, irregular, tear-drop and oval were obtained and the capsule stability varied from 35 to 75 per cent. Capsule parameters were assessed at 0 h, 24 h, 48 h, 72 h and 96 h of chilled preservation in EYC extender at ratio of (1:2). The results of the present study cited that 1.5 per cent sodium alginate concentration, with 23G sized hypodermic needle and distance of 3.5 cm were ideal to acquire uniform spherical shaped stable capsule of size 1.90 ± 0.03 and stability of 75 per cent.

Keywords: *Microencapsulation, chilled preservation, buck spermatozoa, Malabari*

According to 2019 livestock census goat population in the state of Kerala is about 13.59 lakhs, which was increased by 9.08 per cent compared to previous census. Kerala has about 0.91 per cent of goat population of the country mainly represented by native Malabari breed. Malabari breed is known for its quality meat with less fat and remarkable prolificacy (Animal Husbandry Department, 2021). The doe is a seasonal breeder with a 21-day cycle. Oestrus typically lasts for 36–48 h, with ovulation occurring near the end of oestrus. Due to the prolonged oestrus period the farmers are advised to bring the animals 24-30 h after detection of heat and if the farmers are unable to identify the exact time of heat, double insemination is advised. Standard AI recommendation is to breed the oestrus does two times at 24 h intervals. Sperm loss and phagocytic removal of sperms occur at the time of artificial insemination due to which the conception rate is compromised. In order to overcome these problems microencapsulation of spermatozoa was introduced. Sodium alginate is a naturally occurring anionic polysaccharide derived from brown seaweed which is biocompatible, non-immunogenic and non-toxic (Sachan *et al*., 2009). Encapsulating sperm in alginate may shield spermatozoa and

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**Corresponding author: bibinbecha@gmail.com, Ph. 9447971872*

prolong their release, while simultaneously avoiding sperm backflow due to its bio-adhesive properties. The present study was conducted to standardise the protocol for alginate microencapsulation of buck spermatozoa for its chilled preservation.

Materials and methods

Semen was collected from three healthy adult Malabari bucks of 4-6 years of age maintained under uniform managemental conditions at Artificial Insemination Centre, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy. Semen was collected by artificial vagina method by single ejaculate regime and pooled to eliminate the buck effect after preliminary evaluation. Semen samples with 70 per cent progressive motility and a concentration of 2500×10^6 spermatozoa/ mL were utilised for the study.

Procedure for microencapsulation of semen was standardised with trial-and-error method, sodium alginate of concentrations 0.5, 0.75 and 1.5 per cent with varying gauge sized needle like 18G (Inner diameter: 0.838 mm), 20G (Inner diameter 0.603 mm) and 23G (Inner diameter: 0.337 mm) were used. The distance between the tip of the needle and $BaCl_2$ solution was adjusted by varying the distance like ≥ 7, 3.5 to 5, ≤ 2 cm. The semen alginate mixture was delivered into a 90 × 14 mm petri dish with 20 mL of 25 Mm (w/v) $BaCl_2$ dissolved in physiological saline. These microgels were further allowed to react for 30 seconds.

The microgels were obtained by filtering using a muslin cloth, followed by washing three times with physiological saline solution. Microgels were then submerged for five minutes in 0.1 per cent (w/v) poly-L-lysine in physiological saline to create a semipermeable membrane. The muslin cloth was used to filter these poly-L-lysine membrane bound microgels, which were then rinsed three times with physiological saline. By estimating the number of capsules from the total amount of sperm suspension used for encapsulation, the volume of each microcapsule was determined. Finally, these capsules were transferred to Tris-Egg yolk extender in the ratio of (1:2) and stored in 5 mL glass vials under chilled preservation at 5°C.

Microcapsule properties like its size, wall integrity, spermatozoa concentration per capsule and wall thickness were studied. Bright field microscope fixed with scale bar in the stage was used to determine the capsule size. Capsule size was measured in mm as the highest diameter of the capsule ranged up to 2 mm was observed under the microscope at 100× objective. Capsule wall integrity was assessed by vortexing 100 capsules in 30 mL normal saline in a 250 mL glass beaker placed in orbital shaker incubator at 120 rpm for five minutes. The percentage of intact capsules that survived vortexing for five minutes was assessed, which was repeated at 24, 48, 72 and 96 h of chilled preservation. Sperm concentration per capsule was assessed by taking five capsules in microcentrifuge tube and agitated with 0.5 mL of 2.96 per cent sodium citrate to ensure complete dissolution of barium alginate capsule gel. From the solution 0.5 mL of semen sample was withdrawn in a WBC pipette and five per cent of sodium citrate was withdrawn up to 101 mark. Concentration of spermatozoa per capsule was estimated using hemocytometer method. For measuring capsule wall thickness, capsules were fixed in 70 per cent alcohol for 24 h, followed by orienting in a metallic mould filled with melted paraffin wax. The mould with infiltrated sample and paraffin surrounding the sample were left at room temperature until solidification of paraffin. Capsules embedded in paraffin were mounted and sectioned. Sectioned samples were placed in glass slide and viewed under microscope (100×) and measured for capsule wall thickness (µm).

The data was tabulated and analysed statistically using SPSS software version 24 for capsule parameters like capsule size, wall thickness, wall integrity and concentration of spermatozoa/capsule between different days of preservation by repeated measures ANOVA.

Results and discussion

When alginate concentration was increased from 0.5 to 1.5 per cent the size of the capsule was reduced from 4 mm to 2 mm and resulted in globular shaped capsule. Concentration of sodium alginate of ≤ 0.5 per cent resulted in the formation of a tear drop shaped capsule (Plate 1). The stability of the capsule showed an increase, when alginate concentration was raised from 0.5 per cent to 1.5 per cent. With 0.5 per cent alginate, the stability varied from 35 to 45 per cent and with 0.75 per cent, the stability was 40 to 55 per cent. With 1.5 per cent, sodium alginate the stability per cent varied from 40 to 75 per cent (Table 1). Gel microcapsules with a concentration of 0.75 per cent alginate had a fragile tear drop-shaped capsule, whereas those with a concentration of 1.0 per cent alginate had a rigid spherical structure for canine spermatozoa (Shah *et al*., 2010). When the concentration of alginate was less than one per cent, non-spherical capsules were formed, most likely due to a lack of carboxyl groups during gelling process.

When the bore size of the needle was reduced from 18G to 23G, there was reduction in capsule size from 6 to 2 mm. Capsules produced using a needle with smaller gauge size exhibited greater stability than those produced by using a larger gauge sized needle (Table 1). When the bore size of the needle was increased from 23G to 18G, the capsule formed was flat in shape (Plate 2). Varying needle sizes used for encapsulation of canine spermatozoa were 21G and 24G, respectively by Shah *et al*. (2010) and Lakde *et al*. (2018), whereas 30G needle was used for ram spermatozoa by Thiangthientham *et*

Table 1. Capsule parameters obtained with different alginate concentrations, distance between needle tip to $BaCl_2$ meniscus and bore size of needle used

Alginate concentration	Dist. between needle tip and $BaCl_2$ meniscus	Bore size of needle	Capsule parameters		
			Size	Shape	Fragility (%)
0.5%	≥7cm	18 G	7 mm	Irregular	35
		20 G	6 mm	Irregular	40
		23 G	4 mm	Irregular	44
	3.1 to 6.9 cm	18 G	6 mm	Plate like	35
		20 G	5 mm	Tear drop	40
		23 G	4 mm	Tear drop	45
	≤ 3cm	18 G	5 mm	Tear drop	43
		20 G	5 mm	Plate like	38
		23 G	1 mm	Tear drop	45
0.75%	≥7cm	18 G	6 mm	Irregular	47
		20 G	5 mm	Irregular	49
		23 G	3 mm	Oval	48
	3.1 to 6.9 cm	18 G	6 mm	Irregular	45
		20 G	4 mm	Irregular	47
		23 G	2 mm	Globular	50
	≤ 3cm	18 G	6 mm	Plate like	42
		20 G	4 mm	Oval	49
		23 G	3 mm	Globular	55
1.5%	≥7cm	18 G	6 mm	Oval	44
		20 G	6 mm	Irregular	42
		23 G	3 mm	Slightly globular	40
	3.1 to 6.9 cm	18 G	5 mm	Oval	58
		20 G	4.5 mm	Oval	60
		23 G	2 mm	Globular	75
	≤ 3cm	18 G	6 mm	Globular	68
		20 G	4 mm	Globular	72
		23 G	4 mm	Irregular	65

al. (2020) and 19 or 22G needle for bovine spermatozoa (Nebel *et al*., 1993). The size and shape of the resulting capsules were determined by the bore size of needle and distance from the hardener solution.

Poly-L-lysine at 0.1 per cent concentration was used for encapsulation of canine sperm (Shah *et al*., 2010; Lakde *et al*., 2018). Different poly-L-lysine concentrations of 0.025, 0.05, 0.075 and 0.1 per cent were used for interfacial polymerisation by Nebel *et al*. (1996). The microcapsule wall thickness gradually increased with increasing concentrations of 0.025, 0.05, and 0.075 per cent poly-L-lysine, with no further increase noticed from 0.075 to 0.1 per cent poly-L-lysine (Nebel *et al*., 1996).

The reduction in the distance between the needle tip and $BaCl_2$ meniscus from ≥ 7 cm to 3.0 cm, led to decrease in capsule size from 6 mm to 2mm. However, when the distance was further reduced to ≤ 3 cm, capsule formation occurred with increased size. When the distance between the tip of the needle and $BaCl_2$ was increased to ≥ 7 cm, the capsule shape was irregular or oval (Plate 3 and 4). With lower distance between the needle tip and $BaCl_2$ solution, the capsule was more stable compared to increased distance (Table 1). Distance between the tip of the needle and the $BaCl_2$/ $CaCl_2$ meniscus was kept as 8.5 cm distance for canine sperm encapsulation by Shah *et al*. (2010) whereas, distance of 4.5 cm for canine sperm (Lakde *et al*., 2018), 8.5 cm for ram spermatozoa (Thiangthientham *et al*., 2020), 3.54 cm (Nebel *et al*., 1993) and 7.5 cm for bovine spermatozoa (Nebel *et al*., 1996) were adopted.

Capsule size

The capsule size at 0 h and 24 h of storage was 1.90 ± 0.03 and 2.06 ± 0.05 mm, respectively and the size during 48 h, 72 h and 96 h of preservation was 3.22 ± 0.06, 3.56 ± 0.04 and 3.78 ± 0.06 mm, respectively. The size of the capsule showed a non-significant increase within 24 hours, but it significantly increased after 96 hours of storage. The data was presented in Table 2 and Plate 5.

In this study the average size of the capsule obtained was 1.90 ± 0.03mm. There was no significant difference in capsule size during 24 h of preservation, but the size significantly ($p<0.05$) increased on daily basis up

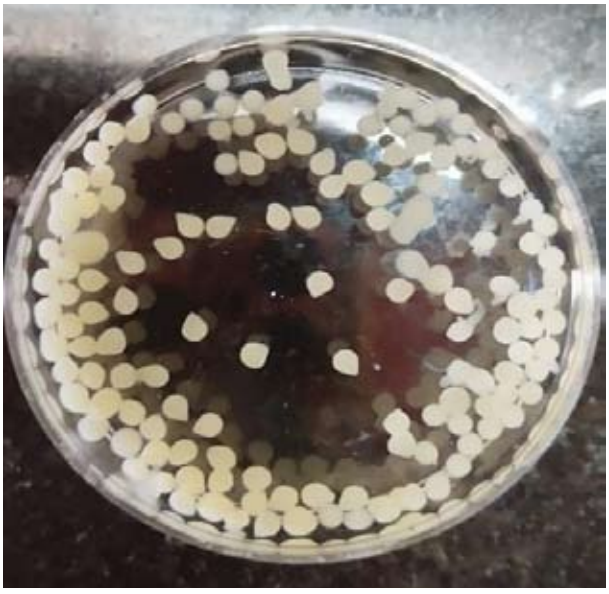

Plate 1. Tear drop shaped capsules

Plate 2. Plate shaped capsules

Plate 3. Irregular shaped capsules

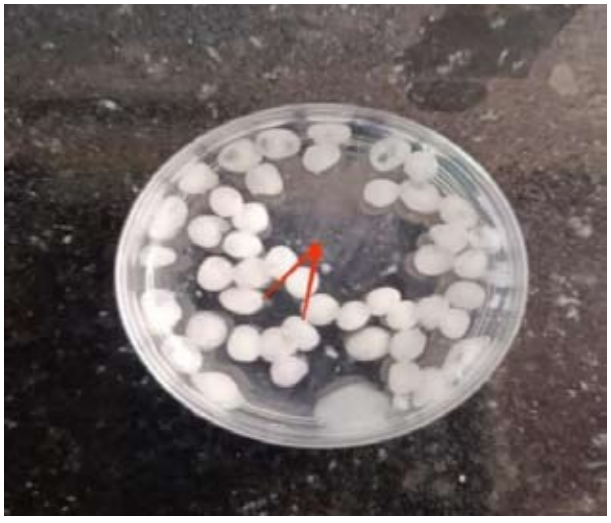

Plate 4. Oval shaped capsule

to 96 h of chilled preservation. This was in accordance with the observations of Shah *et al*. (2010) who observed swelling of polycation microcapsules during storage period, resulted in increased diameter, indicating absorption of the extender utilized for preservation of the alginate capsule and influx of the extender *via* the semipermeable polycation membrane. Large capsules were discovered to have a central necrotic core after implantation, because of nutritional shortages produced by the long diffusion path. The shape and size of an encapsulating substance constitute important considerations that could affect the survival of implanted cells as well as the recipient's immunological response.

Capsule wall thickness

At 0 h of storage, the capsule wall thickness was 5.40±0.05 µm and during 24 and 48 h of storage, the wall thickness was 5.03 ± 0.04 and 4.35 ± 0.08 µm, respectively. The capsule wall thickness was 4.04 ± 0.04 and 3.02 ± 0.13 µm at 72 h and 96 h of preservation, respectively. Significant reduction ($p<0.05$) in capsule wall thickness was observed throughout the storage period from 24 h interval, which was in accordance to the findings of Shah *et al*. (2010). The results are presented in Table 2 and Plate 6. The capsule wall thickness at 0 h of preservation in the present study was similar to the findings by Nebel *et al*. (1993) which was 5.32 ± 0.54 µm using 0.1 per cent poly-L-lysine. Nebel *et al.* (1996) measured capsule wall thickness using scanning electron microscopy. Polycation microcapsule membrane thickness reduced during the storage period. Max *et al.* (1994) observed that the membrane thickness was inversely proportional to capsule volume expansion during membrane production.

Capsule wall integrity

The capsule wall integrity was 79.00 ± 1.01,

Table 2. Microcapsule characteristics (Mean ± SE) on different days of chilled preservation of Malabari buck semen

Microcapsule characteristics	Different time of preservation				
	0 h	24 h	48 h	72 h	96 h
Capsule size (mm)	$1.90^{a} \pm 0.03$	$2.06^{a} \pm 0.05$	$3.22^{b} \pm 0.06$	$3.56^{c} \pm 0.04$	$3.78^{d} \pm 0.06$
Capsule wall thickness (µm)	$5.40^{a} \pm 0.05$	$5.03^{b} \pm 0.04$	$4.35^{c} \pm 0.08$	$4.04^{d} \pm 0.04$	$3.02^{e} \pm 0.13$
Capsule wall integrity (%)	$79.00^{a} \pm 1.01$	$73.00^{b} \pm 0.70$	$61.00^{c} \pm 1.65$	$44.00^{d} \pm 1.17$	$26.4^{e} \pm 1.50$
Concentration of spermatozoa per capsule (million)	3.74 ± 0.06	3.26 ± 0.28	3.34 ± 0.30	3.22 ± 0.40	3.68 ± 0.15

Values with different superscripts within row (lower case) differ significantly ($p<0.05$)

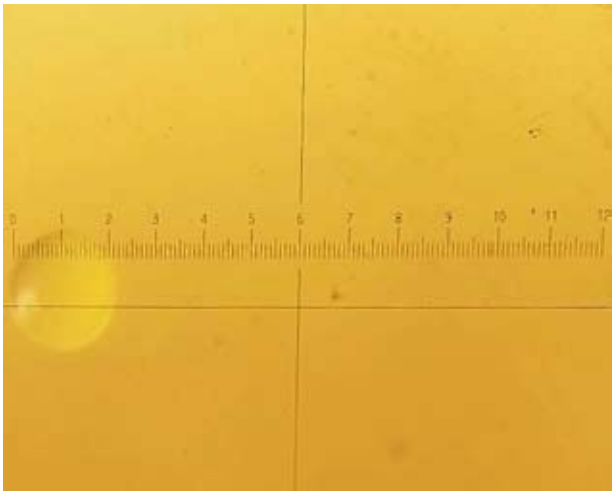

Plate 5. Capsule size (2mm) under light microscopy

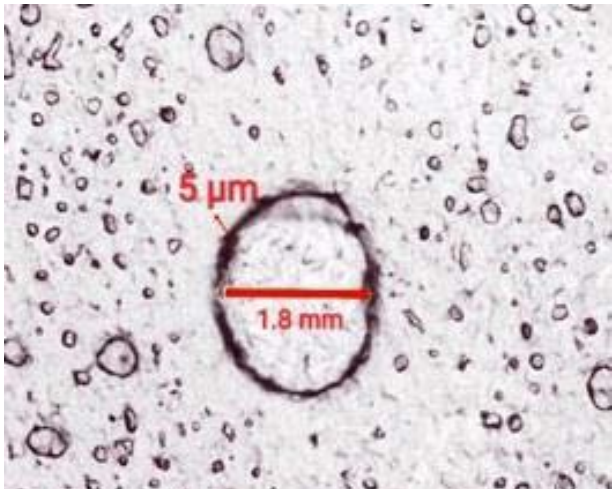

Plate 6. Capsule wall thickness

73.00 ± 0.70 and 61.00 ± 1.65 per cent respectively, during 0 h, 24 h and 48 h of preservation period and 44.00 ± 1.17 and 26.4 ± 1.50 per cent integrity was maintained at 72 h and 96 h of preservation, respectively. Significant daily reduction ($p<0.05$) in capsule wall integrity was observed during the preservation period.

Capsule wall integrity was 79.00 ± 1.01 per cent in the present study which could be correlated with 75.0 ± 10.6 per cent reported by Nebel *et al*. (1993). Significant reduction in the capsule wall integrity was observed during entire storage period. This might be due to increased sperm concentration in buck semen. Similar result was observed by Nebel *et al*. (1993), who stated that fragility of capsules increased over a 24 h period at 37°C as the concentration of encapsulated sperm increased from 45 to 180 × 10^6 sperm/mL; more tears in the capsule membrane occurred due to incomplete cross-linking during membrane formation, and was proportionate with higher sperm numbers. In the present study, the concentration of encapsulated sperm was 234 million sperms/mL, which might had increased the fragility of capsules leading to its breakage when stored beyond 24 hours.

Membranes produced with 0.05 mg mL poly-L-lysine resulted in greater capsule fragility than with less than or equal to 0.15 mg/mL when subjected to vortexing, indicating that maximum cross-linking between poly-L-lysine and alginate occurred at higher concentration (Nebel *et al*., 1993).

Concentration of spermatozoa per capsule

Average sperm concentration per capsule was 3.74 ± 0.06, 3.26 ± 0.08, 3.34 ± 0.30, 3.22 ± 0.40 and 3.68 ± 0.15 million/mL during the preservation period of 0 h, 24 h, 48 h, 72 h and 96 h, respectively. There was no significant difference in the concentration of spermatozoa per capsule during the entire storage period. The results are demonstrated in Table 2.

Average sperm concentration per capsule in the present study was 3.74 ± 0.06 million. Lower sperm concentration of 1.25 × 10^5 sperm per capsule which had diameter of 0.75 mm was noticed by Munkittrick *et al*. (1992). Capsules with a core containing 7.50 µl of sperm-rich canine semen had an average sperm concentration of 1.85 × 10^6 sperm per capsule (Lakde *et al*., 2018). Higher concentration of spermatozoa such as 5 × 10^6 sperm/capsule was observed by Thiangthientham *et al*. (2020). In the present study, nearly about 0.6 × 10^6 sperm/mL were lost during the capsule preparation.

Conclusion

It was concluded that microencapsulation of buck spermatozoa could be obtained with 1.5 per cent sodium alginate concentration, 23 G needle and when the distance between the tip of the needle and $BaCl_2$ solution was maintained at 3.5 cm. This extrusion procedure produced uniform, spherical shaped and stable microcapsules of

1.9 ± 0.03 mm size with good capsule preservability under chilled preservation conditions at 5° C for four days.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Animal Husbandry Department. 2021. Report on Twentieth Quinquennial Livestock Census – 2019. Directorate of Animal Husbandry, Govt. of Kerala, Thiruvananthapuram, 203p.

Lakde, C., Patil, M., Sahatpure, S., Gawande, A. and Umesh, K. 2018. Standard protocol for encapsulation of canine semen. *Int. J. Environ. Sci.* **7**: 1296-1300.

Max, X., Vacek, I. and Sun, A. 1994. Generation of alginate-poly-l-lysine-alginate (APA) biomicrocapsules: the relationship between the membrane strength and the reaction conditions. *Artif. Cells Blood Substit. Immobil. Biotechnol*. **22**(1): 43-69.

Munkittrick, T.W., Nebel, R.L. and Saacke, R.G. 1992. Accessory sperm numbers for cattle inseminated with protamine sulfate microcapsules. *J. Dairy Sci.* **75**: 25–31.

Nebel, R.L., Vishwanath, R., Mc Millan, W.H. and Saacke, R.G. 1993. Microencapsulation of bovine spermatozoa for use in artificial insemination: a review. *Reprod. Fertil. Dev*. **5**: 701-712.

Nebel, R.L., Vishwanath, R., McMillan, W.H. and Pitt, C.J. 1996. Microencapsulation of bovine spermatozoa: effect of capsule membrane thickness on spermatozoal viability and fertility. *Anim. Reprod. Sci.* **44**: 79-90.

Sachan, N.K., Pushkar, S., Jha, A. and Bhattcharya, A., 2009. Sodium alginate: the wonder polymer for controlled drug delivery. *J. Pharm. Res.* **2**(8): 1191-1199.

Shah, S., Nagano, M., Yamashita, Y. and Hishinuma, M. 2010. Microencapsulation of canine sperm and its preservation at 4˚C. *Theriogenology*. **73**: 560–567

Thiangthientham, P., Suwimonteerabutr, J., Tharasanit, T. and Techakumphu, M. 2020. The optimal divalent cations and storage temperatures for the encapsulation of ram spermatozoa. *Thai J. Vet. Med.* **50**: 89–96. ■