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A Study on Encapsulation of Buffalo Bull (*Bubalus bubalis*) Semen

V.N. Gite[1], M.S. Patil[1], A.M. Narkhede[1], D.S. Raghuwanshi[1], K.P. Kharkar[2], S.A. Ingle[3], A.P. Gawande[1]

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ABSTRACT

Background: Since coordination of oestrus with artificial insemination remains a major challenge in Buffalo, the protection of spermatozoa from the *in utero* environment, combined with controlled sperm release achieved by encapsulation, is expected to extend the fertilization period post-insemination.

Methods: A total of 36 semen ejaculates were collected from 06 buffalo bulls by the artificial vagina method. At a concentration of 1.5 per cent Sodium Alginate, 1 per cent Calcium Chloride and 0.01 percent Poly–L–Lysine, encapsulated semen capsules of a spherical shape, having an average volume of 4.55 µl containing 3.34×10^6 spermatozoa/capsule were prepared. Average semen motility, viability and plasma membrane integrity of spermatozoa, pre and post freeze, were studied.

Result: Encapsulation of buffalo bull semen resulted in significantly lower motility, higher viability and plasma membrane integrity after dilution (Pre freeze) and post-thaw (Post Freeze) than in the conventional method of freezing buffalo bull semen.

Key words: Buffalo, Encapsulation, Semen.

INTRODUCTION

Artificial insemination (AI) remains the principal field-level biotechnological intervention for genetic improvement and enhancement of production potential in domestic buffalo (*Bubalus bubalis*). The most influential factors affecting the conception rate following artificial insemination in buffalo are the quality of frozen-thawed semen (Saacke, 1984).

Endogenous antioxidant systems present in seminal plasma, like superoxide dismutase, catalase, glutathione peroxidase and some reductases, inactivate reactive oxygen species (ROS) and thus protect spermatozoa (Aitken and Clarkson, 1987). However, conventional semen processing techniques involving dilution cryopreservation, thawing and subsequent AI procedures result in substantial loss of seminal plasma components, including antioxidant enzymes and cytoplasmic constituents (Blesbois, 2007). This damage to membranes depletes sperm viability and impairs capacitation, ultimately resulting in a false acrosomal reaction and reduction in fertilization capacity of spermatozoa. More than 50% of the sperm viability is reduced during the cryopreservation process (Watson, 1992).

Microencapsulation, defined as the complete enclosure of solid particles, liquid droplets, gases, or living cells within inert polymeric materials (Chang, 1972), has emerged as a potential strategy to improve controlled delivery systems. In the context of spermatozoa, encapsulation techniques were developed to maintain elevated sperm concentrations within the bovine uterus during ovulation, thereby enhancing fertility and prolificacy (Nebel *et al*., 1993). Encapsulation-based approaches have also been evaluated in rams, where administration of encapsulated ovalbumin–LHRH protein resulted in partial suppression of reproductive traits, indicating the practical use of controlled-release systems in reproductive modulation (Yılmaz *et al*., 2018).

Alginic acid is a chemically inert mixture of two sugars, -D-mannuronic and L-guluronic acid. Polymerization occurs in the presence of calcium ions, forming stable gel matrices. These alginate microcapsules can subsequently be liquefied through competitive chelation of calcium using trisodium citrate. The resulting microbeads permit bidirectional diffusion across the polymerized membrane. Furthermore, alginate-poly-L-lysine microcapsules exhibit selective permeability to cellular metabolites and provide

[1]Department of Animal Reproduction, Gynaecology and Obstetrics, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur-440 002, Maharashtra, India.
[2]Department of Animal Genetics and Breeding, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur-440 002, Maharashtra, India.
[3]Department of Veterinary Biotechnology, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur-440 002, Maharashtra, India.

Corresponding Author: M.S. Patil, Department of Animal Reproduction, Gynaecology and Obstetrics, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur-440 002, Maharashtra, India.
Email: vgonvc@gmail.com

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immuno-isolation of encapsulated cells from host cellular and humoral immune responses.

Therefore, the present study was undertaken to evaluate the macroscopic and microscopic characteristics of spermatozoa following semen encapsulation during the peak summer season of April and May at Nagpur Veterinary College, Nagpur, Maharashtra.

MATERIALS AND METHODS

The present study was carried out at the Regional Frozen Semen Laboratory, Telangkhedi, Nagpur and the Department of Animal Reproduction, Gynaecology and Obstetrics, Nagpur Veterinary College, Nagpur, Maharashtra State, in the months of April and May 2022. Nagpur is located in a subtropical region with extreme climatic conditions. The average maximum and minimum temperatures in summer are 48°C and 17°C, with an average of 1405 mm of rainfall. The experimental animal consisted of 6 adults, healthy Murrha buffalo bulls having an average body weight of 650 kgs. All standard feeding and management practices were being followed at the farm.

The breeding buffalo bulls were regularly washed and groomed a day before and on the day of semen collection. Semen was collected twice a week from all 6 breeding buffalo bulls over a period of 3 weeks in the months of April to May. Thus, a total of 36 ejaculates were collected (06 from each buffalo bull) by the artificial vagina method.

Immediately after collection, the ejaculates were transferred to the laboratory and stored at 37°C in a water bath for further evaluation of seminal attributes which included Volume (ml) - determined in a graduated glass collection tube, Sperm Concentration - by using Neubaur's hemocytometer, Sperm Motility (per cent) - A 10 µl drop of semen was placed on pre-warmed glass slide and covered with cover slip. The percentage of progressively motile sperm was observed by subjective microscopic examination, using a light microscope at 400X magnification. Viability (Per cent) - The percentage of sperm viability was estimated by using Eosin Y staining, Plasma Membrane Integrity (Per cent) - by using the hypo-osmotic swelling test (Jeyendran *et al*., 1984).

Cryopreservation of semen

The collected semen was divided into two equal parts. Part one was utilized for conventional cryopreservation (Group I), whereas the second part was used for preparation of encapsulated semen capsules (Group II) and subsequent cryopreservation.

Semen encapsulation

A neat semen sample was mixed (1:2) with 1.5% (w/v) sodium alginate solution dissolved in physiological saline to reach a final concentration of 1.5% sodium alginate. The sperm suspension was passed through a 24-gauge needle attached to a 2 mL syringe into a 100 ml glass beaker containing 1.5% (w/v) calcium chloride dissolved in physiological saline. The distance between the tip of the needle and the surface of the calcium chloride solution was maintained at 8.0 cm to ensure the round shape of the capsules. The sperm suspension immediately upon contact with the calcium chloride solution resulted in a solidification of the entire droplets to form a microgel. The microgels were swayed gently and allowed to react with calcium ions for 30 seconds. The microgels were then collected by filtration using a muslin cloth, rinsed three times with physiological saline. Then, the microgels were transferred into 0.1% (w/v) poly-L-lysine in physiological saline for 5 min to make a semipermeable membrane on the surface of the microgels. These poly-L-lysine membrane bound microgels were filtered with the muslin cloth and then rinsed three times with physiological saline. The membrane bound capsules were filtered and used for cryopreservation.

The volume of each microcapsule was obtained by measuring the number of capsules from the total volume of sperm suspension used for encapsulation. In the present study, in a sodium alginate mixed semen sample of 0.5 ml, an average total of 110 encapsules were prepared, each of 4.55 µl capacity, containing 3.34×10^6 sperm/capsule.

The encapsules were suspended in a tris egg yolk citrate based dilutor. These encapsulated semen sample was then stored at 4°C in a cold handling cabinet and equilibrated for the next 5, 30 and 60 minutes duration, followed by manual filling of these capsules (each straw comprises of 6 capsules) in 0.50 ml capacity French Medium Straw for further cryopreservation by conventional method.

All the procedures described above for the encapsulation of buffalo bull semen were performed at room temperature in a sterile environment.

Conventional cryopreservation of semen

The remaining semen sample, having a sperm concentration of 20 million sperm per milliliter, was cryopreserved as per the standard method of semen cryopreservation.

The obtained data was analyzed by applying Factorial Randomized Block Design and Student "t" test as per Kaps and Lamberon (2009).

RESULTS AND DISCUSSION

Encapsulation of buffalo bull semen

An average volume of 3.03 ml of semen was collected from the buffalo bulls by the artificial vagina method, having an average concentration of 1222.34×10^6 ml in the present study.

Encapsulation procedure resulted in a polymerization of alginate with calcium, resulting in the formation of calcium alginate, which is presented in the form of capsules, each containing 4.55 µl neat semen in its core, having an average semen concentration of 3.34×10^6 capsule. Alginate-based biomaterials have been shown to possess favourable biocompatibility and structural stability in animal tissue systems, supporting their suitability for controlled biological applications (Qin and Guan, 2017).

After equilibrating the encapsules in Tris - Egg Yolk - Citrate dilutor for 5, 30 and 60 minutes duration and subsequent conventional vapour freezing of semen capsules in 0.50 ml French Medium Straws, thawing at

37°C for 60 seconds and dissolving the capsules in normal saline solution instead of sodium citrate solution for 2 minutes, the post freeze semen sample showed arrested motility for first 10 - 15 minutes followed by resumption of progressive motility in the semen sample.

Alginate concentration above 1.0 per cent is required to maintain the spherical shape of the semen capsule, as stated by Shah *et al.* (2010) and is in agreement with the findings of the present study. In the present experiment, the spermatozoa encapsulated with physiological seminal plasma were suspended rather than diluted in the semen dilutor, thus ensuring molecular nutrient and metabolite exchanges but providing pH buffering to avoid ''dilution shock'' facilitating a virtual dilution after microencapsulation, which is in complete agreement with the earlier findings of Torre *et al.* (2000) and Johnson *et al.* (2000).

Pre- and post-freeze characteristics of buffalo bull semen

Semen motility

Average semen motility (+SE) in Group I (Conventional) and Group II (Encapsulated) cryopreserved buffalo bull semen is shown in Table 1.

After dilution in tris-egg yolk-citrate dilutor, the mean post-dilution motility of buffalo bull semen in Group II (Encapsulation) (49.44±2.12%) was numerically lower than that observed in Group I (Conventional) (53.29±2.51%), however, no significant difference was seen between the groups statistically (p>0.05). Earlier findings of Nebel *et al.* (1985), who observed 46.7 and 48.3 percent motility for encapsulated swine semen against the 51.7 per cent motility for the control group, are in accordance with the present findings.

Torre *et al.* (2000) also found that the motility of encapsulated spermatozoa was lower than that of the free sperm, which is in agreement with the present findings. This may be because alginate matrix residues could interfere with sperm kinetic activity, thereby reducing sperm motility.

After dilution and equilibration for 5, 30 and 60 minutes, no significant difference in motility was observed in Group I (Conventional) and Group II (Encapsulation) at any equilibration intervals (P>0.05). Our findings are consistent with those of Olar *et al.* (1989), who suggested that the maintenance of motility and viability in encapsulated sperm at different concentrations may be due to the porosity of the capsule membrane being adequate to facilitate the exchange of nutrients and waste products at the surface-to-volume ratio presented by capsule sizes.

However, significantly higher post-thaw motility was observed in Group I (Conventional) (38.75±2.75 per cent) than in Group II (Encapsulated) (19.46±1.35 per cent) (P<0.01), suggesting that encapsulation may not provide sufficient protection against cryoinjury during freezing and thawing. These findings are similar to those of Vishwanath *et al.* (1997), who observed a comparatively lower motility (11.7±6.2%) in bovine encapsulated semen than in control semen samples (36.7±7.5%) after both were incubated at 37°C for 96 hours.

Table 1: Average semen motility (± SE) in Group I (Control) and Group II (Encapsulation) cryopreserved buffalo bull semen.

Group	Semen motility %								
	Initial	After dilution equilibration period			Average	Post thaw			Average
		5 Min	30 Min	60 Min		5 Min	30 Min	60 Min	
Group I (Conventional)	66.11±2.24	51.94±2.66	53.47±2.61	54.44±2.53	53.29±2.51^{Aa}	29.86±2.93^{F}	41.39±2.86^{GI}	45.00±2.80^{HI}	38.75±2.75^{bIIi}
Group II (Encapsulation)		50.97±2.39	47.64±2.36	49.72±2.08	49.44±2.12^{A}	9.06 ±1.27^{B}	20.64±1.49^{CE}	28.69±1.83^{DE}	19.46±1.35^{II}

Significant at p<0.01.

Following the conventional method of cryopreservation, El-Sheshtawy *et al.* (2008) and Gupta and Saxena (2000) showed similar (32.50±1.53 and 39.58±8.06 per cent) post-thaw motility in cryopreserved buffalo bull semen when compared with the control semen samples of the present study. A similar decline in post-thaw motility has been reported in crossbred bull semen using computer-assisted sperm analysis, emphasising the detrimental impact of cryopreservation on the fertilizing capacity of the sperm (Patel and Dhami, 2013)

In the present study, after an equilibration period of 5, 30 and 60 minutes, the post-thaw motility of Group I (Conventional) is significantly higher (P<0.01) than that of Group II (Encapsulation), with significantly higher (P<0.01) post-thaw motility observed after an equilibration period of 30 and 60 minutes compared to 5 minutes.

These observations are in agreement with the findings of Shah *et al.* (2011), who studied canine semen encapsulation and reported that a decrease in sperm characteristics was observed in frozen-thawed semen due to cryopreservation, indicating that sufficient equilibration may mitigate, but not completely prevent, changes caused by cryodamage.

Viability of semen

Average sperm viability (+SE) as observed in Group I (Conventional) and Group II (Encapsulation) cryopreserved buffalo bull semen is shown in Table 2.

It was observed that in Group I (Conventional) and Group II (Encapsulation) cryopreserved buffalo bull semen, had significantly (P<0.01) higher (34.10±1.46 and 57.57± 2.00 per cent) viable spermatozoa after dilution than after post-thaw (24.28±1.14 and 32.10± 1.61 per cent), respectively (Table 2).

Comparatively, higher initial viability of spermatozoa was recorded by Shah *et al.* (2011) for canine sperm (89.4±3.6 and 88.8±3.0 per cent) in unencapsulated and microencapsulated sperm. The difference was attributed to variation in species.

Average viability percentage as recorded in the present study is in accordance with the earlier findings of Torre *et al.* (1998), who noted 60 per cent of viability for a swine semen sample encapsulated in hydroxyl propylmethyl cellulose and calcium chloride and of Herrler *et al.* (2006) for human spermatozoa encapsulated in alginic acid - calcium chloride.

Shah *et al.* (2010) reported comparatively higher viability in polycation microencapsulated sperm (85.0±3.4% and 86.9±1.0%) and in unencapsulated (conventional) sperm (89.0±1.8%) from beagle dogs (*Canis familiaris*), which differs from the present observations. The variation may be attributed to inter-species differences and the use of alternative encapsulating polymers compared to the alginate-based system employed in the present study.

Viability of microencapsulated sperm with glycerol exposure for 5, 30 and 60 min as recorded by Shah *et al.* (2011) is comparatively higher (86.1±1.7, 85.6±3.7 and 86.5±3.2 per cent) and hence not in agreement with the findings of the present study.

Table 2: Average sperm viability (± SE) in Group I (Conventional) and Group II (Encapsulation) cryopreserved buffalo bull semen.

Semen vability %									
Group	Initial	After dilution equilibration period			Average	Post Thaw			Average
		5 Min	30 Min	60 Min		5 Min	30 Min	60 Min	
Group I (Conventional)	76.05±2.22	32.00±2.12	35.25±1.89	35.04±1.90	34.10±1.46^{BI}	20.30±1.25^{G}	25.28±1.53^{HJ}	27.27±1.89^{IJ}	24.28±1.14^{IIii}
Group II (Encapsulation)		56.00±2.54	54.39±2.95	62.31±2.16	57.57±2.00^{Aa}	25.86±2.53^{BE}	33.58±2.13^{CEF}	36.87±13.13^{DF}	32.10±1.61^{bi}

Significant at p<0.01.

Table 3: Average plasma membrane intact spermatozoa (± SE) in Group I (Conventional) and Group II (Encapsulation) cryopreserved buffalo bull semen.

Group	Plasma membrane intact %								
	Initial	After dilution equilibration period			Average	Post thaw			Average
		5 Min	30 Min	60 Min		5 Min	30 Min	60 Min	
Group I (Conventional)	69.74±2.15	26.39±1.52	29.00±1.51	28.81±1.71	28.07±1.12$^{b\ I}$	17.72±1.29	21.64±1.14	24.47±1.96	21.28±1.08$^{II\ 2}$
Group II (Encapsulation)		54.71±2.24	53.59±2.73	60.23±1.49	56.18±1.62^{Aa}	26.78±2.42^{i}	31.02±2.22^{i}	33.95±1.91^{ii}	30.58±1.60^{B1}

Significant at p<0.01.

After cryopreservation and thawing of bull semen, significantly (P<0.01) higher (32.10± 1.61 per cent) viable spermatozoa are observed in Group II (Encapsulation) than in Group I (Conventional) (24.28±1.14 per cent).

Using conventional cryopreservation methods in buffalo bull semen, Ansari *et al.* (2010) reported comparatively higher sperm viability.

Following equilibration periods of 5, 30 and 60 minutes, the post-thaw viability observed in both Group I (Conventional) and Group II (Encapsulation) was consistent with the findings of Shah *et al.* (2011). They reported that microencapsulated sperm exposed to glycerol for 30 and 60 minutes exhibited higher post-thaw viability as compared to unencapsulated sperm, however, a 5-minute glycerol exposure resulted in comparatively lower viability in both encapsulated and unencapsulated samples.

Plasma membrane integrity of buffalo bull semen by HOST

Average plasma membrane intact spermatozoa (+SE) in Group I (Conventional) and Group II (Encapsulation) cryopreserved buffalo bull semen are shown in Table 3.

Immediately after semen collection, the mean plasma membrane integrity observed in the present study (69.74±2.15%) was comparable to the values reported by Khan and Ijaz (2007), who recorded 58.8±7.36% intact spermatozoa in undiluted buffalo semen and by El-Sheshtawy *et al.* (2008), who reported 71.10±1.57% plasma membrane integrity in buffalo bull spermatozoa. After dilution of the buffalo bull semen, the plasma membrane intact spermatozoa observed in Group I (Conventional) were 28.07±1.12 and in Group II (Encapsulation) were 56.18±1.62 per cent. The values observed in the encapsulated group were comparable to those reported by El-Sheshtawy *et al.* (2008), who recorded 57.60±1.69% plasma membrane integrity in conventionally diluted buffalo bull semen.

An average of 21.28±1.08 and 30.58±1.60 per cent plasma membrane intact spermatozoa, respectively, were observed in the present study in post-thaw buffalo bull semen samples from Group I (Conventional) and Group II (Encapsulation).

The findings of this study are lower than those reported by Wankhade (2008), who observed 58.75±1.07 per cent plasma membrane intact spermatozoa in the post-thawed semen sample of an encapsulated electro ejaculated black buck (*Antelope cervicapra*) semen, whereas it was 64.38±1.11 per cent in the conventional sample. The differences recorded may be because of variation in species.

Comparatively higher percentage of plasma membrane intact spermatozoa were reported by Khan and Ijaz (2007), El-Sheshtawy *et al.* (2008), Ansari *et al.* (2010) and Akhter *et al.* (2010) for buffalo bull semen cryopreserved by the conventional method.

CONCLUSION

Encapsulation of buffalo bull semen resulted in significantly lower post-thaw motility but higher viability and plasma membrane integrity as compared to the conventional

method of freezing buffalo bull semen. After dilution of neat and encapsulated buffalo bull semen in tris-egg yolk-citrate dilutor, an equilibration period of 30 and 60 minutes before freezing showed significantly higher post-thaw motility, viability and plasma membrane integrity compared to a 5 minute equilibration period.

Conflict of interest

On behalf of all the authors, I confirm that there are no conflicts of interest associated with this manuscript.

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