Improving Storage Stability and Kinetics of Pasundan Bull Sperm Encapsulation Using Alginate Solid Beads

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ABSTRAK

Manan MA, Khan A, Samsudewa D, Pamungkas FA, Abbas F, Khan BN, Haidari K, Zulfiqar H, Darussalam I, Widaningsih W. 2024. Peningkatan stabilitas penyimpanan dan kinematik enkapsulasi *solid bead* alginat pada sperma sapi Pasundan. JITV 29(2):103-113. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3427.

Penelitian ini bertujuan untuk mengembangkan proses enkapsulasi sperma sapi pasundan dan menyelidiki apakah enkapsulasi *solid bead* alginat dapat meningkatkan kemampuan bertahan hidup sperma dalam penyimpanan suhu dingin. Proses enkapsulasi dilakukan dengan meneteskan sperma-alginat 0,5% dan 0,25% kedalam larutan yang mengandung 1,5% kalsium klorida ($CaCl_2$), lalu dibiarkan mengendap selama satu menit sehingga sperma berasa dalam enkapsulasi *solid bead* alginat. Enkapsulasi *solid bead* alginat dan yang tidak dienkapsulasi dalam pengencer kuning telur tris dengan alginat 0,25% dan 0,5% diamati 0, 24, 48, 72, dan 96 jam penyimpanan pada 5°C. Parameter yang diamati selain viabilitas dan integritas membran spermatozoa, juga mencakup parameter motilitas total, motilitas progresif, dan kinematika spermatozoa yang diukur menggunakan sistem *computer-assisted sperm analysis* (CASA). Data yang diperoleh dianalisis secara statistik menggunakan *analysis of variance* dan *Duncan Multi Range Test*. Hasil penelitian menunjukkan bahwa meskipun proses awal enkapsulasi menghasilkan penurunan motilitas total, motilitas progresif, dan nilai kinematika, enkapsulasi *solid bead* alginat lebih stabil dibandingkan sperma yang tidak dienkapsulasi hingga 4 hari penyimpanan pada suhu 5 °C. Pergerakan spermatozoa pada enkapsulasi *solid bead* alginat dibatasi karena viskositas media alginat tanpa mengganggu viabilitas dan integritas membran spermatozoa. Dapat disimpulkan bahwa enkapsulasi *solid bead* alginat dalam semen sapi pasundan dapat meningkatkan stabilisasi spermatozoa selama proses penyimpanan.

Kata Kunci: Alginat, Kinematika, Sperma Sapi Pasundan, Preservasin, Alginate Solid Bead Enkapsulasi

ABSTRACT

Manan MA, Khan A, Samsudewa D, Pamungkas FA, Abbas F, Khan BN, Haidari K, Zulfiqar H, Darussalam I, Widaningsih W. 2024. Improving storage stability and kinetics of Pasundan bull sperm encapsulation using alginate solid beads. JITV 29(2):103-113. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3427.

The study aims to develop a pasundan bull sperm encapsulation process and investigate whether alginate solid bead encapsulation improves sperm lifetime and survivability in cold storage. In order to make sperm encapsulation, 0.5% and 0.25% sperm-alginate droplets were added to a solution containing 1.5% dissolved calcium chloride (CaCl2) in physiological saline, and droplets were allowed to settle for one minute, resulting in sperm embedded in solid beads of alginate matrix. Solid beads and unencapsulated sperms in a diluent of tris egg yolk with 0.25% and 0.5% alginate were assessed following 0 h, 24 h, 48 h, 72 h, and 96 h of refrigerated storage at 5°C. The observed parameters, in addition to sperm viability and membrane integrity, also include total motility, progressive motility, and sperm kinematics, which were measured using computer-assisted sperm analysis (CASA) systems. To determine if differences amongst data were statistically significant, analysis of variance was used, and the Duncan Multi Range Test was performed. The result showed that although the initial process of encapsulation resulted in a decrease in total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was found to be more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The movement of spermatozoa is restricted to the viscosity of the alginate medium without disturbing the membrane's viability and integrity. It can be concluded that alginate solid bead encapsulation in pasundan bull semen can improve sperm stabilization during refrigerator storage.

Key Words: Alginate, Kinematics, Pasundan Bull Sperm, Preservation, Solid Bead Encapsulation

INTRODUCTION

The Pasundan cattle, endemic to Indonesia, primarily occupy the Southern coast of West Java and the boundary along the Northern Priangan region, retaining its importance as an emblematic breed among the Sundanese people (Arifin et al. 2019). According to 2015 research by the West Java Central Statistics Agency, there was a significant reduction in the Pasundan cattle population, dropping from 50,000 to 40,000 heads in 2013 (Dwitresnadi et al. 2015). Improvements in the breeding process and the genetic quality of livestock are necessary to maintain the population. As part of these efforts, Artificial Insemination (AI) employing frozen semen has been identified as a vital technology to raise the population and genetic traits of Pasundan cattle (Widyastuti et al. 2022). Agriculture Regulation No. 10/Permentan/PK210/2016 mandates that 60% of AI procedures must employ indigenous breeds (Baharun et al. 2023).

Artificial Insemination uses treated semen, both liquid and frozen. This procedure involves the dilution of semen using centrifugation in an appropriate extender, followed by further preservation and freezing for cryogenic storage (Galarza et al., 2023). However, during these storage processes, sperm incur issues arising from temperature-induced biomolecular changes, physiological imbalances, and oxidative damage. These factors may reduce sperm viability, motility, and functional fertilization capability (Sharafi et al., 2022).

Improving sperm resistance against stress and enhancing sperm lifetime during storage would have tremendous practical advantages for the breeding business. In contrast, it improves the period available for transit and usage for artificial insemination. One feasible method would be to preserve sperm cells inside microcapsules to shield them from environmental stress (Pruß et al., 2022). Within the discipline of zootechnics, the first concept for encapsulating male gametes is dedicated to facilitating a regulated discharge of spermatozoa. Over the past 30 years, researchers have actively investigated the encapsulation process and increased its application to several animal species (Perteghella et al. 2015a). Increased fertility and prolificacy can be ensured by sperm encapsulation, which can keep a high concentration of sperm in the uterus during estrus (Gordon 2017).

Alginate encapsulation of sperm is vital in reproductive technology and animal reproduction. The gelation process frequently uses calcium chloride (Feyzmanesh et al., 2022). A significant advantage of alginate encapsulation is its efficacy in enhancing sperm viability during preservation or cryopreservation. Sperm encapsulation has been a focal point in bovine and pig breeding efforts, mainly aimed at enabling more prolonged sperm release within the female reproductive system after a single insemination (Roca et al. 2016; Alm-Kristiansen et al. 2018). Solid bead encapsulation, a novel technique for sperm encapsulation, was introduced to create a regulated semen delivery system, mainly for stallion semen (Pruß et al., 2022). This experiment involved dropping semen that contained calcium chloride into an alginate solution. A semi-permeable calcium alginate gel membrane is formed when calcium ions diffuse out of the droplets and react with the alginate. This one-step method yields capsules containing spermatozoa that have stability and vitality preserved (Pruß et al. 2022).

On the other hand, most research has evaluated semen properties using traditional techniques, which provide little and frequently inconsistent information regarding sperm motility across laboratories (Alipour et al. 2017). Objective computer-assisted sperm analysis (CASA) systems are often used to analyze sperm motility, including sperm kinematics. These methods enable the precise and repeatable evaluation of sperm motility characteristics (Knox 2015). Using a CASA system, sperm kinematics are assessed by taking pictures of individual sperm cells. After that, the system tracks the cells' trajectories using algorithms to describe their movement (Van de Hoek et al., 2022).

This work aimed to design a procedure for alginate solid bead encapsulation optimized for pasundan bull sperm. Additionally, the research intended to determine whether encapsulation leads to enhanced stability during cold storage and maximizes sperm survival in contrast to unencapsulated by assessing sperm motility and kinematics using CASA.

MATERIALS AND METHODS

Animals

The study was conducted at the Center for Artificial Insemination Breeding and Development of Beef Cattle in Ciamis, West Java, Indonesia, and authorized by Ethical Clearance and Foreign Research Permit, Directorate of Management for Research and Innovation Permit and Scientific Authorities, BRIN No. 011/KE.02/SK/01/2023. The research involved three Pasundan bulls aged 3-6 years, with body weights ranging from 380 to 430 kg. The bulls were fed a diet of Pennisetum purpureum grass up to 10% of body weight and commercial concentrate up to 1% of body weight, retaining 16% crude protein, fed twice a day, and water provided ad libitum.

Semen collection and evaluation

The semen was collected using an artificial vagina once a week from each pasundan bull, from 07.30 AM to 10.00 AM, following the Operational Standard

Procedure of the Center for Artificial Insemination Breeding and Development of Beef Cattle. After collection, the semen samples were immediately subjected to microscopic and macroscopic evaluation.

Unencapsulated sperm

Semen was collected using an artificial vagina, and ejaculated semen was immediately evaluated following collection and diluted with Tris Egg Yolk (TEY) extender in alginate with concentrations of 0.25% and 0.5% (Kumar et al. 2019). The diluted semen was packaged in a corning tube (15 ml). The samples were then placed in a beaker glass with a water jacket cooled from 37 °C to 5 °C and stored at 5 °C. Microscopic evaluation of unencapsulated sperm was determined after diluting with sodium alginate and Tris Egg Yolk (TEY) extender and after storage at 5 °C for four days.

Sperm alginate encapsulation in solid bead structures

Microencapsulation procedures developed for canine and stallion sperm (Shah et al. 2011; Pruß et al. 2022) were used with minor modifications. Briefly, the sperm diluted with the TEY diluent was supplemented with 0.25% and 0.5% alginate. The sperm was forced through a 21-gauge needle attached to a 5 mL syringe into a beaker glass containing the 1.5% calcium chloride ($CaCl_2$) dissolved in physiologic saline. The distance between the tip of the needle and the surface of the calcium chloride solution was maintained at 8.5 cm to ensure the shape of the microcapsule. The sperm suspension immediately upon contact with the calcium chloride solution solidified the entire droplet to form a high-viscosity microgel. The microgels were swayed gently and allowed to react for 30 sec. A schematic presentation of the procedures resulting in a calcium-alginate solid bead is presented in Figure 1.

The microgels were then collected and rinsed two times with physiologic saline. After that, sperm/alginate solid bead capsules were transferred to TEY diluent and packaged in a Corning tube (15 ml). The samples were then placed in a beaker glass with a water jacket cooled from 37°C to 5°C and stored at 5°C. Microscopic evaluation of sperm was determined after encapsulation and storage at 5°C for four days.

To assess sperm evaluation, shell structures/spheres were pierced, using a 100 µL plastic micropipette tip to aspirate sperm residing in the inner core. Sperm inside alginate solid bead capsules were recovered by repeated gentle pipetting of the concentrated bead-containing solution, disintegrating the beads. Recovered sperm were diluted in TEY diluent, and the sperm was ready for evaluation.

Microscopic evaluation of sperm

Sperm concentration was evaluated using a photometer (SDM 6, Minitub, Germany). Semen with ≥70% progressive motility of sperm, a concentration of sperm $\geq 500 \times 10^6$ ml^{-1}, and morphologically abnormal sperm ≤20% were used for the experiment. Computer-Assisted Sperm Analysis (CASA) using the Hamilton Thorne IVOS II ver. 12.1 was utilized to analyze sperm motility. An 8 µl semen sample was diluted with 4 ml of 0.9% NaCl. The combination was mounted on a slide, covered with a coverslip, and viewed using a microscope with a 10x10 objective magnification linked to a computer containing CASA. Five fields of view were tested, and the motility was evaluated on a scale from 0%

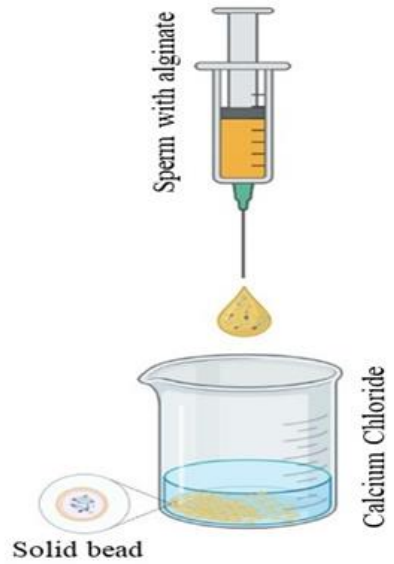

Figure 1. Formation of alginate solid bead encapsulation

Table 1. Sperm kinematic descriptors and their corresponding definition (Barbas et al. 2018)

Kinematic Descriptor	Measurement Unit	Descriptor Definition
Curvilinear velocity (VCL)	(μm/s)	The average path velocity of the sperm head along its true
Straight-line velocity (VSL)	(μm/s)	The average path velocity of the sperm head along its true trajectory per unit time
Average path velocity (VAP)	(μm/s)	The average velocity of the sperm head along its average trajectory per unit time
Linearity index (LIN)	(%)	The ratio between VSL and VCL (x 100)
Straightness index (STR)	(%)	The ratio between VSL and VAP (x 100)
Wobble coefficient index (WOB)	(%)	The ratio between VAP and VCL (x 100)
Amplitude of lateral head displacement (ALH)	(μm)	The average value of the extreme side-to-side movement of the sperm head in each beat cycle
Beat cross-frequency (BCF)	(Hz)	The frequency with which the actual sperm trajectory crosses the average path trajectory

to 100%. The CASA system measured different motility characteristics, including total motility (TM), progressive motility (PM), fast motility (FM), slow motility (SM), local motility (LM), and immotile (IM). The kinematic variables assessed were the curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), wobbles (WOB), the amplitude of lateral displacement (ALH). They beat cross frequency (BCF), shown in Table 1**.**

Sperm viability was assessed using eosin-nigrosin staining, a technique adapted from Barth and Oko (Chenoweth, 2022) with modification. The integrity of the plasma membrane was asses using a hypoosmotic swelling solution (HOS test) consisting of 0.735 g Na citrate, 1.351 g fructose, and 100 ml distilled water. (Baldaniya et al. 2021). The solution was incubated at 37°C for 30 minutes, and then 10 μL of the mixture was placed on a microscope slide and examined at 400× magnification.

Statistical analysis

Data were analyzed using Statistical Analysis Software (SAS Institute, Cary, NC, USA). ANOVA (analysis of variance) was used to determine if there were differences among statistically significant data, and the Duncan Multi-Range Test was performed. Data are presented as mean values ± standard deviations. Differences are considered statistically significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

The total motility and kinematic values of sperm are rapid, slow, circle, local, and immotile between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. These are presented in Figure 2, Tables 2 and 3. The results showed that the total motility value of sperm, both encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. The encapsulation process showed lower total motility values than the unencapsulated sperm, especially for up to 48 hours refrigerated at 5°C ($P<0.05$). However, encapsulated sperm did not experience a significant decrease in total motility values compared to unencapsulated during refrigerator storage; this can be seen from the total motility values found to be numerically superior in encapsulation 0.25% alginate groups after 96 hours refrigerated at 5°C without statistical differences.

The slow motility value of encapsulated sperm at 0 hours at room temperature is lower than that of unencapsulated sperm. However, after 96 hours of refrigerated storage, the decrease in the slow motility value of sperm is less than that of the unencapsulated sperm. The immotile value influences the total motility value. The immotile values after 96 hours at refrigerated storage were found to be numerically superior in the encapsulated 0.25% alginate group, followed by unencapsulated 0.25% alginate, encapsulated 0.5% alginate, and unencapsulated 0.5% alginate groups without statistical difference, respectively.

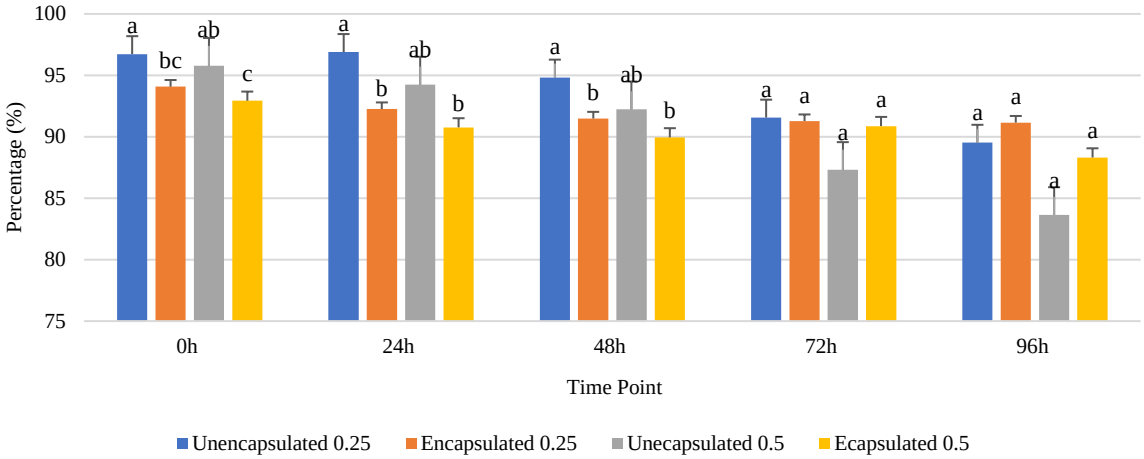

Figure 2. Total motility of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, 96h refrigerated at 5 °C. Statistical significance difference (P≤ 0.05) was shown with the different superscripts

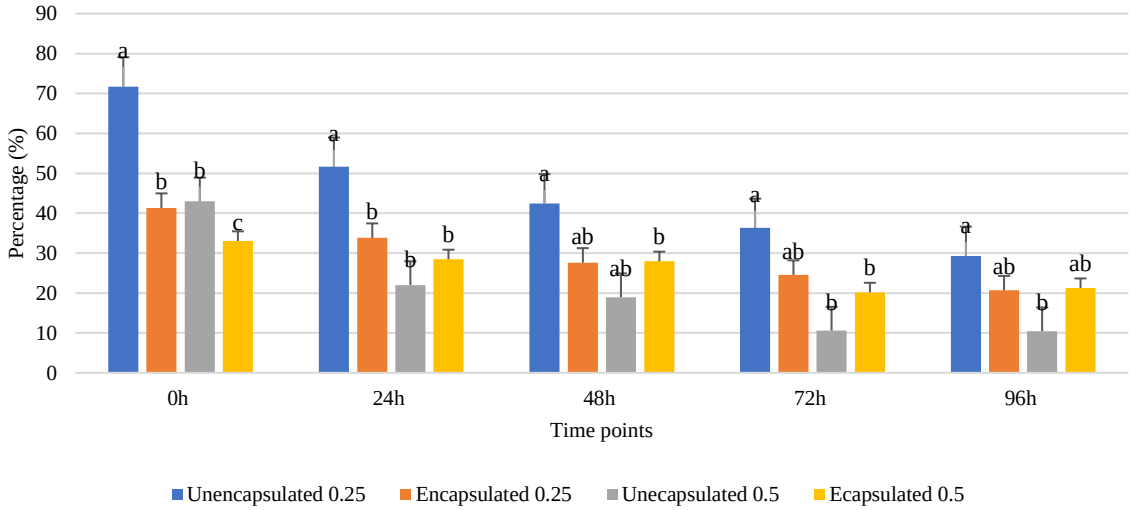

Figure 3. Progressive motility of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, 96h refrigerated at 5 °C. Statistical significance difference (P≤0.05) showed with the different superscripts

The results of the analysis of the kinematic value of sperm at the time of 0 h storage showed that the values of VCL, VSL, VAP, and ALH in the unencapsulated 0.25 group were higher, followed by the encapsulation 0.25, unencapsulated 0.50. They encapsulated 0.50 alginate groups with a statistical difference, respectively. However, after 96 h of refrigerated storage, VCL, VSL, VAP, and ALH values showed no significant difference and found that the unencapsulated group 0.5 had the lowest value. I f we look deeper, it turns out thatduring the storage process, the unencapsulated group experienced a more drastic decrease in VCL, VSL, VAP, and ALH values than the encapsulated group.

LIN, WOB, and BCF values at 0 h storage indicate that the encapsulated group is higher than the unencapsulated group. However, after 96 h of refrigerated storage, the values of LIN, WOB, and BCF showed no significant difference. Similarly, the STR values from 0h to 96h storage showed no difference statistically.

Alginate solid bead encapsulation is a viable strategy to reduce harmful effects following sperm preservation. During the preservation process, many spermatozoa die or are seriously damaged. Refrigerated semen storage causes structural and functional changes in sperm as indicative of a natural aging process influenced by storage temperature, time of day, and species (Wiebke et al., 2022). When freshly ejaculated sperm is rapidly cooled from body temperature to temperatures below 5°C, a cold shock occurs, resulting in a loss of sperm viability. Cold shock during the period of adaptation and storage of spermatozoa at low temperatures leads to changes in the composition of the spermatozoa membrane (Wiebke et al. 2022); this

Table 2. Evaluation of sperm motility between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at the different time points of 0h at room temperature and four days refrigerated at 5°C

Parameters	Groups	Time Points				
		0h	24h	48h	72h	96h
Fast Motility	Unencapsulated 0.25	2.64±0.38^{a}	1.03±0.30^{a}	1.02±0.36^{a}	0.71±0.26^{a}	0.57±0.21^{a}
	Encapsulated 0.25	1.62±0.32^{a}	0.90±0.26^{a}	0.55±0.21^{b}	0.30±0.09^{b}	0.08±0.04^{b}
	Unencapsulated 0.5	0.20±0.04^{b}	0.14±0.01^{a}	0.24±0.12^{b}	0.15±0.03^{b}	0.12±0.06^{b}
	Encapsulated 0.5	0.45±0.12^{ab}	0.70±0.10^{a}	0.54±0.28^{b}	0.17±0.00^{b}	0.16±0.05^{b}
Slow motility	Unencapsulated 0.25	69.10±0.95^{a}	50.60±3.79^{a}	41.42±6.33^{a}	35.59±6.99^{a}	28.74±6.68^{a}
	Encapsulated 0.25	37.31±1.65^{bc}	32.58±4.21^{b}	26.68±5.88^{ab}	24.07±3.75^{ab}	20.52±3.84^{ab}
	Unencapsulated 0.5	42.76±1.47^{b}	21.88±2.89^{b}	18.72±4.76^{b}	10.46±2.00^{b}	10.32±3.88^{b}
	Encapsulated 0.5	32.49±3.12^{c}	27.53±4.48^{b}	27.08±6.98^{ab}	19.82±3.36^{b}	20.96±5.79^{ab}
Circular Motility	Unencapsulated 0.25	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
	Encapsulated 0.25	2.40±0.65^{a}	0.38±0.14^{a}	0.34±0.14^{a}	0.18±0.09^{a}	0.05±0.02^{a}
	Unencapsulated 0.5	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
	Encapsulated 0.5	0.12±0.08^{b}	0.33±0.21^{a}	0.23±0.09^{ab}	0.17±0.06^{a}	0.14±0.08^{a}
Local Motility	Unencapsulated 0.25	25.00±0.66^{b}	45.28±4.28^{c}	52.38±6.47^{b}	55.26±5.79^{b}	60.22±5.15^{a}
	Encapsulated 0.25	52.75±1.11^{c}	56.45±2.32^{b}	60.88±4.67^{ab}	66.73±3.52^{a}	70.50±4.28^{a}
	Unencapsulated 0.5	52.82±1.82^{b}	72.23±3.20^{a}	73.27±4.52^{a}	76.7±1.45^{a}	73.20±2.35^{a}
	Encapsulated 0.5	59.88±2.37^{a}	62.31±2.99^{b}	61.99±5.81^{ab}	70.72±2.44^{a}	67.06±5.48^{a}
Immotile	Unencapsulated 0.25	3.27±0.47^{c}	3.10±0.32^{b}	5.18±0.99^{b}	8.44±2.07^{a}	10.48±2.38^{a}
	Encapsulated 0.25	5.92±0.41^{ab}	9.75±2.27^{a}	11.51±2.01^{a}	8.72±0.86^{a}	8.85±1.05^{a}
	Unencapsulated 0.5	4.22±0.51^{bc}	5.75±0.37^{ab}	7.76±1.21^{ab}	12.70±2.32^{a}	16.36±4.20^{a}
	Encapsulated 0.5	7.06±1.12^{a}	9.24±1.91^{a}	10.05±1.80^{a}	9.13±1.08^{a}	11.69±0.90^{a}

Statistical significance difference ($P \leq 0.05$) is shown with the different superscripts along the column.

Table 3. Kinematic study of unencapsulated and encapsulated sperms of pasundan bulls

Time	Groups	Kinematic parameters							
		VCL (µm/s)	VSL% (µm/s)	VAP (µm/s)	STR (%)	LIN (%)	WOB (%)	ALH (µm)	BCF (Hz)
0h	Unencapsulated 0.25	54.67±1.20^{a}	31.25±0.65^{a}	29.44±2.78^{a}	0.95±0.00^{a}	0.57±0.01^{c}	0.61±0.01^{c}	1.45±0.03^{a}	0.61±0.01^{c}
	Encapsulated 0.25	38.05±1.21^{b}	26.91±2.11^{a}	28.01±2.10^{a}	0.96±0.00^{a}	0.71±0.03^{a}	0.74±0.03^{a}	0.98±0.02^{b}	0.74±0.04^{a}
	Unencapsulated 0.50	32.78±0.93^{b}	20.63±0.80^{b}	17.99±2.89^{b}	0.95±0.00^{a}	0.63±0.01^{bc}	0.67±0.01^{bc}	0.98±0.03^{b}	0.67±0.01^{bc}
	Encapsulated 0.50	25.04±4.46^{c}	16.92±5.45^{b}	17.81±2.83^{b}	0.95±0.00^{a}	0.68±0.01^{ab}	0.72±0.01^{ab}	0.75±0.09^{c}	0.72±0.01^{ab}
24 h	Unencapsulated 0.25	42.56±3.17^{a}	22.69±1.98^{a}	20.03±4.68^{a}	0.93±0.00^{b}	0.53±0.01^{b}	0.57±0.00^{b}	1.24±0.04^{a}	0.57±0.01^{b}
	Encapsulated 0.25	28.96±6.04^{b}	17.61±3.69^{ab}	18.61±3.86^{a}	0.95±0.00^{a}	0.61±0.02^{a}	0.65±0.02^{a}	0.90±0.15^{b}	0.65±0.04^{a}
	Unencapsulated 0.50	23.57±1.58^{b}	13.67±1.16^{a}	15.45±2.23^{a}	0.93±0.00^{b}	0.58±0.01^{a}	0.63±0.01^{a}	0.81±0.03^{b}	0.63±0.02^{a}
	Encapsulated 0.50	26.13±4.81^{b}	16.14±2.84^{ab}	17.05±2.99^{a}	0.95±0.00^{a}	0.62±0.01^{a}	0.66±0.01^{a}	0.79±0.12^{b}	0.66±0.03^{a}
48 h	Unencapsulated 0.25	39.02±4.83^{a}	20.33±2.72^{a}	16.47±4.40^{a}	0.93±0.01^{a}	0.52±0.01^{b}	0.56±0.01^{b}	1.15±0.10^{a}	0.56±0.01^{b}
	Encapsulated 0.25	24.93±6.06^{a}	16.92±4.57^{a}	16.63±4.47^{a}	0.94±0.01^{a}	0.61±0.04^{a}	0.66±0.04^{a}	0.75±0.15^{a}	0.66±0.04^{a}
	Unencapsulated 0.50	20.59±3.64^{a}	12.03±2.05^{a}	10.98±2.94^{a}	0.92±0.01^{a}	0.59±0.00^{a}	0.64±0.01^{a}	0.71±0.08^{a}	0.64±0.01^{a}
	Encapsulated 0.50	22.05±7.16^{a}	13.75±4.53^{a}	14.47±4.69^{a}	0.95±0.01^{a}	0.62±0.01^{a}	0.66±0.01^{a}	0.71±0.20^{a}	0.66±0.02^{a}
72 h	Unencapsulated 0.25	33.81±5.75^{a}	17.70±3.05^{a}	16.29±3.50^{a}	0.92±0.01^{a}	0.53±0.01^{b}	0.57±0.01^{c}	1.06±0.14^{a}	0.57±0.01^{b}
	Encapsulated 0.25	24.35±3.55^{ab}	14.73±2.62^{a}	15.67±2.69^{a}	0.94±0.01^{a}	0.60±0.04^{ab}	0.64±0.04^{ab}	0.74±0.08^{b}	0.64±0.07^{ab}
	Unencapsulated 0.50	17.41±2.41^{b}	9.95±1.21^{a}	10.63±1.21^{a}	0.92±0.00^{a}	0.58±0.02^{ab}	0.63±0.01^{ab}	0.62±0.07^{b}	0.63±0.02^{ab}
	Encapsulated 0.50	20.21±2.98^{b}	12.54±2.33^{a}	13.32±2.31^{a}	0.94±0.00^{a}	0.61±0.02^{a}	0.66±0.02^{a}	0.65±0.07^{b}	0.66±0.04^{a}
96 h	Unencapsulated 0.25	29.07±5.79^{a}	15.30±6.67^{a}	13.24±3.61^{a}	0.91±0.01^{a}	0.52±0.01^{a}	0.57±0.01^{a}	0.91±0.13^{a}	0.62±0.01^{a}
	Encapsulated 0.25	20.02±2.42^{ab}	10.33±6.67^{a}	12.59±2.25^{a}	0.93±0.01^{a}	0.58±0.04^{a}	0.62±0.03^{a}	0.74±0.08^{ab}	0.62±0.06^{a}
	Unencapsulated 0.50	14.46±4.19^{b}	9.91±3.74^{a}	11.69±2.44^{a}	0.90±0.01^{a}	0.58±0.02^{a}	0.64±0.02^{a}	0.54±0.12^{b}	0.64±0.04^{a}
	Encapsulated 0.50	21.54±3.90^{ab}	15.85±6.68^{a}	14.04±2.98^{a}	0.94±0.02^{a}	0.60±0.04^{a}	0.64±0.04^{a}	0.71±0.07^{ab}	0.64±0.07^{a}

VCL= curvilinear velocity; VSL= straight-line velocity; VAP= average pathway velocity; STR= straightness; LIN= linearity; WOB= wobbles; ALH= amplitude of the lateral head displacement; BCF= beat cross frequency. The data is shown in the form of Mean±S.E at the ($P<0.05$)

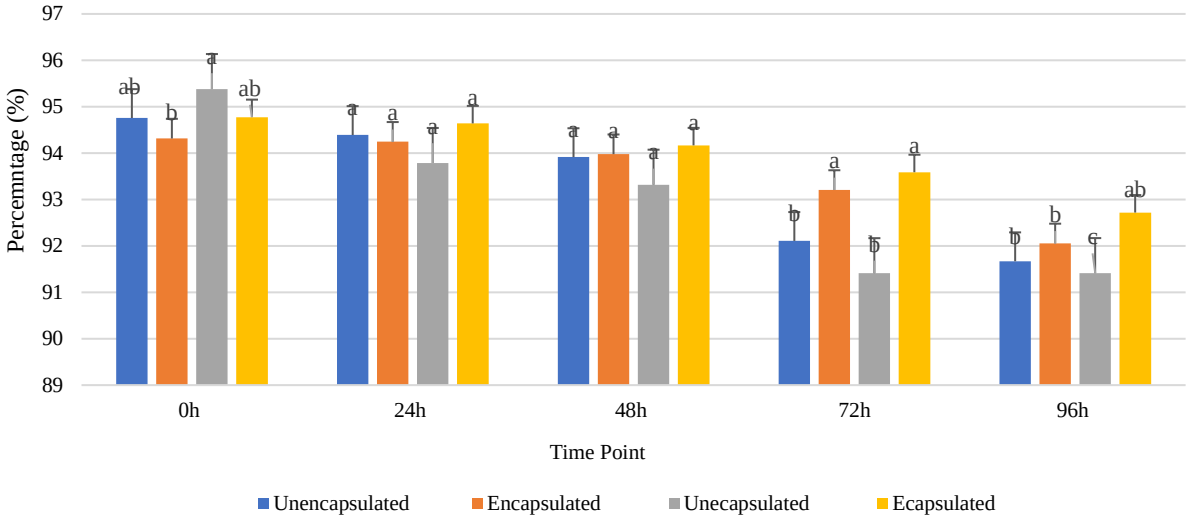

Figure 4. Plasma membrane integrity of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, and 96h refrigerated at 5 °C. Statistical significance difference (P≤0.05) showed with different superscripts.

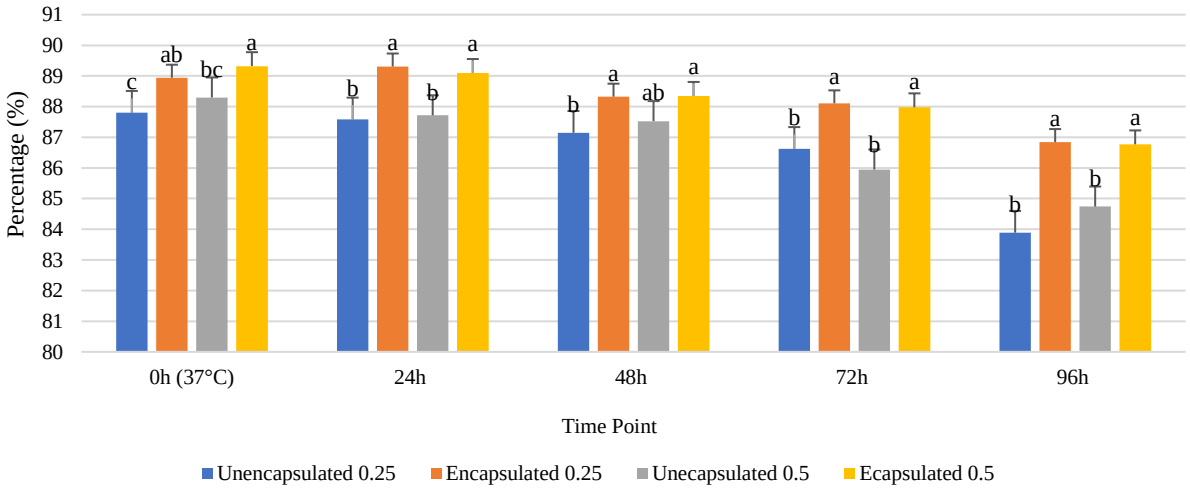

Figure 5. Sperm viability between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, and 96h refrigerated at 5°C. Statistical significance difference (P≤0.05) showed with the different superscripts

indicates that cold shock changes the composition of the membrane lipid bilayer and the fluidity of the plasma membrane (Gunde-Cimerman et al. 2014).

The cold sensitivity of spermatozoa changes with time and temperature (Paschoal et al., 2020). Other essential factors include seminal plasma (Höfner et al. 2020), aging during incubation and diluents used (Rahman et al. 2023), and the cholesterol content (Batissaco et al. 2020). The inner sperm membrane is more sensitive to damage from cold shock. The consequence of cold-induced membrane, dilution, and cooling during sperm preservation increases the permeability of the plasma membrane, resulting in a change in phase transition and the entry of free calcium ions from the external environment into the cell, inducing capacitation processes and making cell membranes more fusogenic and unstable (Bernabò et al. 2018).

Although the initial encapsulation process decreased total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was found to be more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The primary purpose of sperm encapsulation is to increase the stability of sperm during cold storage (Gosálvez et al. 2021). In addition, encapsulation has explicitly been tried to facilitate prolonged sperm release in the female reproductive tract after insemination (Perteghella et al. 2015b; Perteghella et al. 2017; Pruß et al. 2022). A decrease in total motility, progressive motility, and kinematics value in the initial

process of encapsulated sperm may be caused by alginate increasing the medium viscosity; this was evident from the decrease in sperm velocity upon incubation in sperm alginate solid bead encapsulation.

In the results, it was observed that sperm motility and kinematics value decreased in alginate solid bead encapsulated sperm as compared to unencapsulated sperm, which was in line with the study by Pruß et al. (2022). One of the factors that seems to be causing sperm motility and kinematics to decline is the presence of alginate particles on the surface of the sperm. Alginate is widely used as a gelling agent, and it was anticipated that it would increase the viscosity of extenders, leading to lesser values for sperm velocities (Kumar et al. 2019). Sperm motility is actively regulated by the fibrous sheaths that make up the cytoskeletal structure of sperm flagella (Lehti & Sironen 2017). Retaining the alginate residue on the sperm may hinder its ability to move its tail actively and decrease its motility (Pruß et al. 2022). Sperm motility was found to be reduced by Torre et al. (2000) when encapsulated in porcine semen with 0.5% alginate; this was attributed to the presence of residual alginate particles in the sperm. Furthermore, Ebel et al. (2023) report that the concentration of crosslinking agents, type of alginate, total amount of alginate, method used to encapsulate cells, and procedure moderating hydrogel disintegration can affect how successfully alginate hydrogel is formed and can independently affect sperm motility.

Figure 3 shows the progressive motility values of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. The results showed that the progressive motility value of sperm, both encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. The encapsulation process showed lower progressive motility values than the unencapsulated sperm at 0 hours at room temperature. However, after storage in a refrigerator at 5°C for up to 96 hours, the progressive motility was highest in the unencapsulated 0.25% alginate group was followed by the encapsulated 0.25% alginate, encapsulated 0.5% alginate, and unencapsulated 0.5% alginate groups, respectively.

The plasma membrane integrity value of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points is presented in Figure 4. Similar to the total and progressive motility values, the plasma membrane integrity value, encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. While the plasma membrane integrity value was highest in the unencapsulated 0.5% alginate group, a statistical difference was found between the unencapsulated 0.5% alginate and the encapsulated 0.25 and 0.5% alginate groups ($P<0.05$). No significant difference was found between the groups at 24 and 48 hours. However, at 72 and 96 hours, the encapsulated groups had significantly more intact sperm than the unencapsulated groups; this can be seen from the plasma membrane integrity values that were numerically superior in the encapsulation 0.5% alginate group than the unencapsulated groups.

Figure 5 presents the viability value of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. As with the previous parameter, the viability value, both encapsulated and unencapsulated, decreased for up to 96 hours during storage. The viability values were numerically superior in the encapsulated groups than the unencapsulated groups at all point times.

Although sperm motility is hampered by the viscosity of the alginate in the encapsulation process, the results of the present study indicate that alginate encapsulation maximized the viability and membrane damage during the storage process for up to 96 hours. Sperm viability was observed to be more significant in the alginate-encapsulated groups than in the unencapsulated groups based on the results of the present experiment. Alginate is known to be a biodegradable polymer with high biocompatibility. It also has a remarkable ability to form a three-dimensional matrix around cells, like the extracellular matrix. It is possible to maintain the appropriate cell viability in various in vitro and in vivo settings using this porous matrix (Pahlevanzadeh et al., 2020). Alginate has a unique structure that facilitates the transport of nutrients and signaling molecules. It was established that poly (propylene fumarate)-co-alginate blocks the entrance of ROS (Tram et al. 2020).

In comparison to previous studies on human and canine sperm encased in solid beads, the present study shows that using alginate encapsulation improves sperm vitality during long-term preservation (Gosálvez et al. 2021). Additionally, Kumar et al. (2019) used alginate to enhance the buffalo semen extender and then used a programmed biological freezer to cryopreserve the samples. Their results showed that supplementing with alginate increased sperm vitality while maintaining membrane integrity.

CONCLUSION

Overall, this study demonstrates the feasibility of pasundan bull sperm in alginate solid bead encapsulation structures to improve sperm stabilization. The sperm integrity and viability remain unaffected until 4 days of storage at 5°C. Although the initial encapsulation process decreased total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The movement of spermatozoa is restricted to the viscosity of the alginate medium without disturbing the membrane's viability and integrity.

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