Optimal and Tolerant Conditions for Alginate and Calcium Chloride for the Semen Encapsulation of Pasundan Bull

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ABSTRAK

Khan A, Manan MA, Samsudewa D, Pamungkas FA, Zulfiqar H, Irfan S, Haidari K, Wulandari V, Hadi DN. 2024. Kondisi alginat dan kalsium klorida yang optimal dan toleran untuk enkapsulasi semen sapi Pasundan. JITV 29(2):56-66. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3430.

Penelitian ini bertujuan untuk menguji toleransi spermatozoa sapi pasundan dengan konsentrasi alginat dan kalsium klorida ($CaCl_2$) yang berbeda untuk mengidentifikasi kondisi optimal enkapsulasi spermatozoa. Sampel semen sapi pasundan dikoleksi setiap minggu dengan vagina buatan. Semen yang dikoleksi dibagi menjadi 9 volume yang sama. Sampel pertama diencerkan dengan tris kuning telur (kontrol), 4 sampel diencerkan dengan tris kuning telur dengan konsentrasi alginat yang berbeda (0,25; 0,5; 0,75, dan 1%), dan 4 sampel lainnya $CaCl_2$ yang berbeda (2,5; 5; 10, dan 20 mM). Evaluasi semen ditentukan setelah inkubasi 5 menit pada suhu ruang dan setelah penyimpanan selama 24 jam pada suhu 5 °C. Hasil penelitian menunjukkan setelah penyimpanan 24 jam, nilai motilitas total dan progresif pada kelompok $CaCl_2$ 2,5 mM dan alginat 0,25% tidak menunjukkan perbedaan dengan kelompok kontrol, perbedaan yang signifikan ($P<0,05$) diperlihatkan oleh kelompok $CaCl_2$ dan alginat lainnya. Nilai motilitas progresif pada kelompok dengan konsentrasi alginat lebih besar dari 0,25% menurun secara signifikan ($P<0,05$). Tidak ada perbedaan antara kelompok alginat dan $CaCl_2$ dalam nilai viabilitas dan integritas membran plasma spermatozoa. Kesimpulan dari penelitian ini adalah spermatozoa sapi pasundan dalam pengencer tris kuning telur ditambah dengan 2,5 mM kalsium klorida atau 0,25% alginat lebih toleran terhadap paparan yang lebih lama dan proses enkapsulasi spermatozoa.

Kata Kunci: Alginat, Kalsium Klorida, Toleransi Sperma, Enkapsulasi, Semen Sapi Pasundan

ABSTRACT

Khan A, Manan MA, Samsudewa D, Pamungkas FA, Zulfiqar H, Irfan S, Haidari K, Wulandari V, Hadi DN. 2024. Optimal and tolerant conditions for alginate and calcium chloride for the semen encapsulation of Pasundan bull. JITV 29(2):56-66. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3430.

The study aimed to determine the tolerance of Pasundan bull's sperm with different alginate and calcium chloride (CaCl2) concentrations to identify optimal conditions for sperm encapsulation. Semen samples were collected weekly with artificial vaginas from pasundan bulls. Pooled semen was divided into 9 equal volumes. The first sample was diluted with tris egg yolk extender (control), 4 samples were diluted with tris egg yolk extender supplemented with different concentrations of alginate (0.25, 0.5, 0.75, and 1%), and 4 other samples with CaCl2 (2.5, 5, 10, and 20 mM). Evaluation of semen was determined after 5 min incubation at room temperature and after overnight storage at 5 °C. Results showed that after 24h of refrigerated storage, the values for total motility and progressive motility in the groups with 2.5 mM CaCl2 and 0.25% alginate showed no difference compared to the control group. In contrast, a significant difference ($P<0.05$) was found between the other groups with CaCl2 and alginate. The progressive motility value in the group with alginate concentration greater than 0.25% decreased significantly ($P<0.05$). There was no difference between the groups (both alginate and CaCl2) in the spermatozoa viability and plasma membrane integrity variable. In conclusion, sperm with 2.5 mM calcium chloride and 0.25 % alginate was more tolerant of appropriate prolonged exposure and the sperm encapsulation process.

Key Words: Alginate, Calcium Chloride, Sperm Tolerance, Encapsulation, Pasundan Bull Semen

INTRODUCTION

Pasundan cattle are local Indonesian livestock recognized as a genetic resource based on the Decree of the Minister of Agriculture of the Republic of Indonesia Number 1051/Kpts/SR.120/10/2014 dated 13 October 2014. The production of frozen pasundan bull semen and its distribution through artificial insemination (AI) technology was used to preserve and maintain this local Indonesian genetic heritage (Sutarno & Setyawan 2015).

The success of the application of AI technology is determined by the high percentage of pregnancies achieved, which are influenced by the reproductive health of females, the quality of the inseminated semen, and the practical and timely application of AI techniques (Abdel Aziz et al. 2023). The low probability of a pregnancy resulting from incorrect timing of AI can be due to either a decrease in sperm viability while waiting for the ovum to be ready for fertilization or loss of the sperm due to contraction and phagocytosis processes in the female reproductive tract (Anchordoquy et al. 2022).

Sperm encapsulation technology can potentially solve the problems and challenges mentioned above. From a male perspective, it increases the viability and stability of the sperm membrane over a more extended period. The sperm are gradually released from the microcapsule to remain available for a more extended period, waiting to be released from the encapsulation membrane when the egg is ready for ovulation (Kang et al. 2014). From a female perspective, on the other hand, sperm reflux is reduced during AI, and the process of phagocytosis in the female reproductive tract is prevented, allowing the technology to remain available for longer and potentially solving problems related to the duration of artificial insemination (Sánchez-Sánchez et al. 2022).

Biomaterials, both natural and synthetic polymers, have been used as matrices in the sperm encapsulation process. Alginate is an example of an organic anionic polysaccharide derived from brown seaweed and is known for its general properties of biocompatibility, non-immunogenicity, and non-toxicity (Ahmad et al. 2021). Numerous studies have shown that alginate has a rich reservoir of antioxidants (Król et al. 2017), antibacterial (Karbassi et al. 2014), antiviral (Ahmadi et al. 2015), and fungicidal (Tøndervik et al. 2014; Hernández-Figueroa et al. 2024) properties. Calcium chloride ($CaCl_2$) is a versatile chemical known for its use in de-icing highways in the winter, as it can lower the freezing point of water so that ice cannot form, making it the best choice for encapsulating sperm at low temperatures (Nutile & Solan 2019). Some studies use a combination of alginate and $CaCl_2$ in sperm encapsulation, including in goat (Silva et al. 2015), dairy cattle (Kusumaningrum et al. 2015), sheep (Thiangthientham et al. 2020), mice (Veisi et al. 2022), and horses (Pruß et al. 2022).

However, some of these studies still use very different concentrations of alginate and $CaCl_2$, although too high concentrations would impair sperm viability or motility. In contrast, an insufficient concentration would not provide sufficient protection against oxidative stress during storage (Pruß et al. 2022). Therefore, in order to maintain sperm in animal breeding over a long period, it is necessary to perform tolerance tests to facilitate the creation of a stable environment that balances structural integrity in the encapsulation matrix while preserving sperm function (Kunkitti et al. 2016). The research aims to evaluate the optimal and tolerant conditions of pasundan bull semen at different concentrations of alginate and $CaCl_2$ for the sperm encapsulation approach.

MATERIALS AND METHODS

Animals

The study is conducted at the Center for Artificial Insemination Breeding and Development of Beef Cattle, Ciamis, West Java, Indonesia. A total of 3 Pasundan bulls aged 3-6 years with body weights range of 380-430 kg were used in this study. The bulls were fed similar diets consisting of 10% Pennisetum purpureum grass and 1% commercial concentrate with 16% crude protein. Fresh drinking water is given ad libitum, and feed is administered twice daily. Approval (Protocol No. 011/KE.02/SK/01/2023) was obtained from the Ethical Clearance and Foreign Research Permit, Directorate of Management for Research and Innovation Permit, and the Scientific Authorities, National Research and Innovation Agency before the start of the experiments. Animal care and experimental protocols followed the Manual for the Care and Use of Laboratory Animals.

Diluents preparation

The primary diluent used for this study is Tris egg yolk. Tris buffer consisted of 3.03 g Tris (hydroxymethyl) aminomethane, 1.78 g citric acid, and 1.25 g fructose in 100 ml aquabidest, as Darussalam et al. (2020) used. Tris egg yolk consisted of 85% Tris buffer, 15% egg yolk, 1000 IU/mL penicillin, and 1 mg/mL streptomycin, which were thoroughly mixed and used as a diluent as done by Kusumaningrum et al. (2015) for the preservation of dairy bull semen.

Semen collection

By the Standard Procedure of the Center for Artificial Insemination, Breeding and Development of Beef Cattle, semen samples were collected from each pasundan bull once a week for one month using an artificial vagina in the morning between 8:00 to 10:30 a.m. Before pooling, the Computer Assisted Sperm Analyzed (CASA) instrument Androvision® (Minitube-Germany) was used to measure the motility of the sperm at 100X magnification using a heating plate. In this study, semen samples with less than 75% motility values were not used. Nine equal volumes of pooled semen from each bull were obtained. One sample was diluted with tris egg yolk extender (control); four samples were diluted with tris egg yolk extender supplemented with various concentrations of sodium alginate or sodium salt from brown algae (Sigma, A2033 CAS Number. 9005-38-3),

0.25, 0.5, 0.75, and 1%. The remaining four samples were diluted with tris egg yolk extender supplemented with different concentrations of Calcium chloride or $CaCl_2$ (Sigma SA C4901), 2.5, 5, 10, and 20 mM. The final concentration of sperm was around 100×10^6 sperm ml^{-1}. After that, the diluted semen was placed into a 15 ml corning tube. Then, the samples were placed in a glass beaker with a water jacket. Sperm microscopic analysis was assessed during a 5-minute incubation period at room temperature and following an overnight storage period at 5°C.

Evaluation of the tolerance of sperm to alginate and calcium chloride

Sperm motility was assessed using the CASA instrument Androvision® (Minitube-Germany). An 8 µl semen sample was mixed with 4 ml of 0.9% NaCl; the sample was dropped on a slide with a coverslip, observed using a microscope with objective magnification of 10x10 connected to a computer and installed with CASA, observing five fields of view, and scoring from 0% to 100 %. The motility value variables of CASA are total motility (TM), progressive motility (PM), fast motility (FM), slow motility (SM), local motility (LM), immotile (IM), the curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), wobbles (WOB), the amplitude of lateral displacement (ALH), and beat cross frequency (BCF), as shown in Table 1.

Sperm viability was determined using eosin-nigrosin staining (each 100 mL contains 1.1 g eosin Y, 0.5 g Na citrate, 6.67 g nigrosin, and distilled water to 100 mL) according to Singh et al. (2022) with modifications. A semen sample of 2 µL and 16 µL eosin-nigrosin dye were homogenized. A test was made on a glass object, dried on a hot plate, and viewed with a microscope at 10×40 magnification. The preparation is tested in at least five fields of view or >200 sperm. Alive (viable) sperm are marked with an unstained (transparent) head, and dead sperm are marked with a purple head.

Plasma membrane integrity was assayed with hypoosmotic swelling solution (HOS test) (each 100 ml has 0.736 g Na citrate, 1.352 g fructose, and 100 ml distilled water) according to Hufana-Duran et al. (2015). Three hundred (300) µl of the HOS test solution was placed in microtubes, and 3 µl of the semen sample was dropped and incubated in a water bath at 37 °C for 30 min. Then, 15 µL of the mixture was removed and dropped onto a microscope slide, covered with a cover glass, and observed with a microscope at 400× magnification. Observations were made in at least two hundred cells. Sperm with intact plasma membrane show a reaction, namely a circular on the end of the tail.

Statistical analysis

The data collected from the different groups were analyzed using statistics according to various criteria. A One-way ANOVA was explicitly used to calculate each parameter's mean values and group differences, supported by the SPSS software when there was only one independent variable (such as the control) and several dependent variables. The results of the ANOVA test indicated significant differences between at least two

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

groups if the p-value was below a predetermined significance level (P≤0.05). Post-hoc tests, such as the Duncan test, were used to detect group differences in significant cases (Chen et al. 2019).

RESULTS AND DISCUSSION

The results of the analysis of total motility, progressive motility, and kinematics of sperm (fast, slow, circle, local, and immotile) after incubation in a tris egg yolk diluent containing different concentrations of alginate and calcium chloride are shown in Figure 1 and Table 2-5. In the $CaCl_2$ groups, there were no differences in the total and progressive motility characteristics that were assessed at 0 hours. However, after 24h of cold storage, the total motility value was nsignificant in the 2.5 mM $CaCl_2$ compared with a control group. In contrast, a significant difference was found between the other groups (P<0.05). At the same time, the values of progressive motility in the 2.5 and 5 mM $CaCl_2$ groups were almost identical to those of the control group.

As for fast and slow motility in the $CaCl_2$ groups, after 24h of cold storage, the numerically highest rate was found in the 2.5 mM $CaCl_2$ group, and a significant statistical difference was found compared with the 20 mM $CaCl_2$ groups (P<0.05). The local and immotile values were higher in the 20 mM group than in the other groups (P<0.05). In addition, there were no differences between groups in all kinematic sperm characteristics assessed at 0 hours in the $CaCl_2$ groups. For kinematic sperm such as VSL, VAP, LIN, and ALH, values found a significant difference between the other groups (P<0.05) after 24 hours of cold storage. However, the values in the 2.5 mM $CaCl_2$ groups were almost identical to the control.

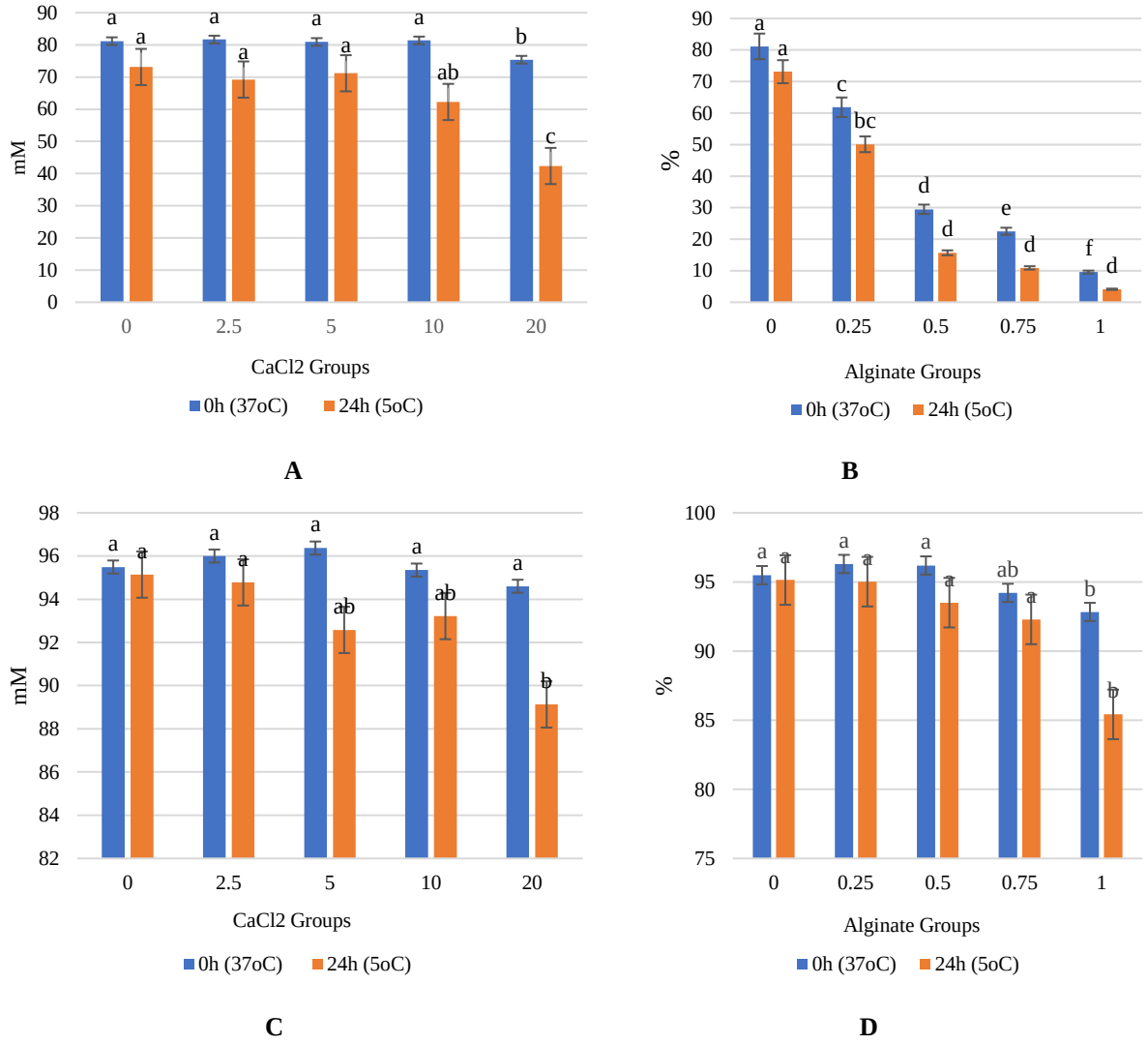

Figure 1. Evaluated progressive motility (A and B) and total motility (C and D) of sperm after incubation in a tris egg yolk diluent containing different amounts of alginate and calcium chloride, and the data were examined at 0hours (37ºC) and 24hours (5ºC). Statistically significant differences (P≤0.05) between the analysis times are labeled with different letters

Table 2. Assessment of sperm motility after incubation in a tris egg yolk diluent containing different amounts of calcium chloride and the data were examined at 0 hours (37ºC) and 24 hours (5ºC).

Calcium Chloride Group (mM)	Sperms (%)									
	Fast Motility		Slow Motility		Circle Motility		Local Motility		Immotile	
	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours
0	11.33±2.48^{ab}	10.47±1.99^{a}	67.53±4.05^{a}	56.67±3.92^{a}	1.14±0.94^{a}	0.26±0.07^{a}	14.33±0.92^{b}	27.72±5.08^{b}	4.50±0.74^{b}	4.85±0.86^{c}
2.5	12.07±1.95^{ab}	11.18±1.61^{a}	69.14±1.46^{a}	57.58±3.17^{a}	0.46±0.19^{b}	0.45±0.15^{a}	14.32±1.37^{b}	25.56±4.01^{c}	3.99±0.60^{b}	5.21±0.74^{c}
5	15.09±1.82^{a}	10.44±2.23^{a}	66.59±1.58^{a}	50.76±6.99^{ab}	0.65±0.16^{b}	0.25±0.07^{a}	15.45±2.88^{b}	31.12±7.43^{a} b	3.62±0.29^{b}	7.42±1.90^{ab}
10	13.61±1.65^{ab}	9.77±2.10^{a}	65.47±2.71^{a}	52.15±3.97^{a}	0.46±0.16^{b}	0.31±0.19^{a}	13.95±1.72^{b}	30.96±4.99^{b} c	4.65±0.69^{b}	6.78±0.92^{bc}
20	10.05±1.86^{b}	5.11±1.79^{b}	64.87±1.81^{a}	38.18±8.77^{b}	0.46±0.26^{b}	0.04±0.01^{b}	20.05±2.76^{a}	45.78±7.73^{a}	5.39±0.99^{a}	10.87±2.68^{a}

Statistical results are given as Mean ± SE; the One-Way ANOVA analysis revealed significant differences (P≤0.05) between the alphabetically separated groups

Table 3. Assessment of sperm motility after incubation in a tris egg yolk diluent containing different amounts of alginate and the data were examined at 0 hours (37ºC) and 24 hours (5ºC)

Alginate Group (%)	Sperms (%)									
	Fast Motility		Slow Motility		Circle Motility		Local Motility		Immotile	
	0h	24h	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours
0	11.33±2.48^{a}	10.47±1.9^{a}	67.53±4.05^{a}	56.67±3.92^{a}	1.14±0.94^{a}	0.26±0.07^{a}	14.33±0.92^{a}	27.72±5.08^{a}	4.50±0.74^{a}	4.85±0.86^{a}
0.25	1.69±0.49^{b}	0.12±0.02^{b}	60.17±2.07^{a}	48.93±2.45^{a}	0.00±0.00^{b}	0.00±0.00^{b}	36.01±3.74^{b}	44.91±3.12^{b}	3.69±0.68^{a}	4.97±1.04^{a}
0.5	0.22±0.73^{b}	0.12±0.02^{b}	29.26±0.46^{b}	15.53±1.90^{b}	0.00±0.00^{b}	0.00±0.00^{b}	66.70±0.66^{b}	77.83±1.47^{c}	3.81±0.40^{a}	6.50±0.78^{a}
0.75	0.24±0.03^{b}	0.12±0.05^{b}	22.26±1.45^{c}	10.76±1.73^{b}	0.00±0.00^{b}	0.00±0.00^{b}	71.70±1.59^{c}	81.41±1.67^{c}	5.78±0.76^{a}	7.70±0.98^{a}
1	0.05±0.02^{b}	0.07±0.03^{b}	9.52±1.67^{d}	4.03±0.48^{b}	0.00±0.00^{b}	0.00±0.00^{b}	83.26±1.29^{c}	81.32±1.86^{c}	7.16±0.77^{a}	14.57±1.58^{b}

Statistical results are shown as Mean ± SE; the One-Way ANOVA analysis revealed significant differences (P≤0.05) between the alphabetically distinct groups

Calcium chloride is an essential component in extenders because it prevents the formation of ice crystals from water and thus protects the sperm from potential damage (Nutile and Solan 2019). The biocompatibility results of $CaCl_2$ at a concentration of 2.5 mM and 5 mM against sperm of pasundan bulls show no significant difference in terms of the tested factors after exposure at 0 hours at room temperature and after 24 hours of cooling at 5 °C. According to Ashizawa et al. (2013), $CaCl_2$ does an excellent job restoring sperm motility. The results show that sperm has a good tolerance to these $CaCl_2$ concentrations at room temperature, but prolonged exposure to high concentrations (20Mm $CaCl_2$) significantly decreases these parameters. Faustini (2011) reported that CaCl2 concentrations of 5-25 mM were used for sperm tolerance in other species. The loss of sperm motility of pasundan semen from 81.39% to 62.25% after 24 hours of cold storage was attributed to calcium chloride and previously reported in numerous studies for other species (Fernandez-Novo et al. 2021).

The increase in the internal calcium ion concentration stimulates the cyclic adenosine monophosphate (cAMP) signaling pathway, activating sperm motility patterns in different species. Calcium chloride is a prerequisite for regulating sperm motility in different species (Bondarenko et al. 2017). Ionic calcium concentration not only plays a role in triggering sperm motility but is also related to the flagellar beating pattern (Bondarenko et al. 2017; Dzyuba et al. 2017). Consequently, increased extracellular calcium concentration promotes asymmetrical movement and curvilinear velocity (VCL) of sperm (Sandoval-Vargas et al. 2021). However, sperm activated with the calcium-free solution tended to reduce VCL and VAP values. Pérez et al. (2016) also found a moderate reduction in VCL and VAP values and the VSL and BCF of sperm activated with a calcium-free solution.

In the alginate groups, the total motility values in the 0.25 and 0.5 % alginate groups were numerically almost the same in the control, with no statistical difference. However, after 24h of cold storage, the total motility value in the 0.25% alginate group was numerically almost the same. It did not differ from the control group, while a statistical difference was found in the 1% alginate group ($P<0.05$). On the other hand, the value of progressive motility in the alginate groups was found to decrease with increasing alginate concentration. The control group had the highest value of progressive motility, which gradually decreased, followed by the 0.25, 0.5, 0.75, and 1% alginate groups. However, after the 0.25% alginate group, the rate of progressive sperm motility decreased dramatically, and as Fig. 1 shows, there was a significant difference ($P<0.05$) between the other groups. In addition, in the alginate groups, after 24 hours of cold storage, the highest rate of fast and slow motility was found in the control group, and a statistical difference was found between the other groups ($P<0.05$). Fast and slow motility was highest in the control group, followed by the 0.25, 0.5, 0.75, and 1 % alginate groups. VCL, VSL, VAP, and ALH values decreased with increasing alginate concentration in the alginate groups. The control group had the highest value, which gradually decreased, followed by the groups with 0.25, 0.5, 0.75, and 1% alginate.

Alginate is commonly employed as a gelling agent. Therefore, it was expected to increase the viscosity of extenders and cause the sperm velocities in samples containing the alginate-fortified extender to be lower (Kumar et al. 2019). Fertilization requires the female reproductive tract to maintain sperm motility for the whole course of the reproductive cycle. As demonstrated by the outcomes of the in vitro incubation test, the current study's findings suggest that supplementing semen extenders with alginate can result in sperm motility being sustained for extended durations of time. Additionally, a spermatozoon needs a functioning and undamaged plasma membrane for progressive motility to be sustained for the cell to have the ability to fertilize. The current study suggests that adding alginate to egg yolk extender reduced membrane damage during preservation. This discovery supports the outcomes of an earlier investigation wherein the incorporation of alginate showed efficacy in preserving the plasma membrane integrity of salmonid fish sperm following brief preservation at 4°C (Merino et al. 2017). Alginate is expected to increase the viscosity of the extender as it is widely used for gelling agents. Due to the high viscosity, sperm velocity decreases, resulting in sperm energy conservation. The result showed that alginate is also used as a food and helps in the controlled release of nutrients; in this way, it increases sperm lifespan and helps in storage (Gheorghita Puscaselu et al. 2020). An increase in alginate content causes a decrease in progressive motility and an increase in local sperm motility. Possibly due to the higher viscosity of the medium, higher alginate levels were associated with decreased progressive motility and increased local sperm. As in Feyzmanesh et al. (2022), sperm motility decreases due to alginate, but the other parameters, like viability and membrane integrity, remain the same as the control. Pruß et al. (2022) also reported similar findings while working on the alginate encapsulation of stallion sperm to increase its storage stability. These concentrations show that sperm have an excellent tolerance to alginate hydrogel. Besides that, Veisi et al. (2022) conclude that alginate hydrogel enhances the proliferation of spermatogonial stem cells.

The results of viability and membrane integrity analysis after incubation in a tris egg yolk diluent with different concentrations of alginate and calcium chloride are shown in Figure 2. In the $CaCl_2$ groups, the viability

Table 4. Kinematic characteristic of Pasundan bull sperm in extender with different concentrations of $CaCl_2$ during storage

Kinematic	Hour	Calcium chloride group (mM)				
		0	2.5	5	10	20
VCL (µm/s)	0	76.54±1.89^{a}	72.05±3.20^{a}	73.21±4.05^{a}	76.89±4.12^{a}	70.83±4.10^{a}
	24	55.70±8.88^{a}	62.26±4.48^{a}	54.37±9.62^{a}	50.22±7.02^{a}	37.79±11.11^{a}
VSL (µm/s)	0	42.55±3.39^{a}	41.74±8.71^{a}	41.08±1.73^{a}	46.24±4.72^{a}	40.19±1.98^{a}
	24	35.41±4.14^{ab}	39.96±1.92^{a}	29.84±5.51^{ab}	30.03±3.16^{ab}	19.56±6.24^{b}
VAP (µm/s)	0	46.46±3.23^{a}	42.64±2.00^{a}	43.85±1.81^{a}	48.89±4.89^{a}	42.63±2.01^{a}
	24	35.43±3.83^{ab}	36.78±2.38^{a}	31.73±5.71^{ab}	32.05±3.29^{ab}	21.06±6.52^{b}
LIN (%)	0	0.57±0.02^{a}	0.55±0.01^{a}	0.56±0.01^{a}	0.59±0.03^{a}	0.56±0.01^{a}
	24	0.53±0.01^{ab}	0.55±0.01^{a}	0.54±0.01^{ab}	0.54±0.01^{ab}	0.50±0.01^{b}
STR (%)	0	0.94±0.00^{a}	0.94±0.00^{a}	0.93±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}
	24	0.93±0.00^{a}	0.93±0.00^{a}	0.93±0.00^{a}	0.93±0.00^{a}	0.91±0.01^{a}
WOB (%)	0	0.60±0.02^{a}	0.60±0.01^{a}	0.60±0.01^{a}	0.64±0.02^{a}	0.59±0.01^{a}
	24	0.57±0.01^{a}	0.58±0.00^{a}	0.58±0.01^{a}	0.57±0.01^{a}	0.54±0.01^{a}
ALH (µm)	0	1.94±0.02^{a}	1.95±0.06^{a}	1.95±0.09^{a}	1.96±0.09^{a}	1.82±0.09^{a}
	24	1.66±0.12^{a}	1.66±0.10^{a}	1.42±0.20^{ab}	1.49±0.13^{ab}	1.06±0.24^{b}
BCF (Hz)	0	1.16±0.05^{a}	1.34±0.14^{a}	1.27±0.09^{a}	1.20±0.07^{a}	1.20±0.12^{a}
	24	1.58±0.17^{a}	1.54±0.08^{a}	1.41±0.14^{a}	1.55±0.11^{a}	1.40±0.04^{a}

VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, LIN= Linearity index, STR= Straightness index, WOB= Wobble coefficient index, ALH= Amplitude of lateral head displacement, BCF= Beat cross-frequency. The One-Way ANOVA analysis showed significant differences (P<0.05) between the alphabetically separate groups; the statistical data are shown as Mean±S.E

Table 5. Kinematic performance of Pasundan bull sperm diluted in extender with varied concentrations of alginate during different storage durations

Kinematic	Observation time (hour)	Alginate group (%)				
		0	2.5	5	0.75	1
VCL (µm/s)	0	76.54±1.81^{a}	46.66±2.00^{b}	29.03±0.66^{c}	25.60±1.33^{c}	17.71±2.04^{d}
	24	55.70±8.88^{a}	40.64±1.81^{b}	21.87±0.95^{c}	18.44±1.20^{c}	12.52±0.75^{c}
VSL (µm/s)	0	42.55±3.39^{a}	27.18±1.35^{b}	16.45±0.76^{c}	16.37±0.69^{c}	11.97±1.37^{c}
	24	35.41±4.14^{a}	23.28±1.03^{b}	12.97±0.42^{c}	11.16±0.68^{c}	8.57±1.06^{c}
VAP (µm/s)	0	46.46±3.23^{a}	28.01±1.11^{b}	18.68±0.43^{c}	16.88±0.67^{cd}	11.98±1.36^{d}
	24	35.43±3.83^{a}	23.55±1.07^{b}	13.85±0.52^{c}	12.05±0.69^{c}	8.47±0.58^{c}
LIN (%)	0	0.57±0.02^{bc}	0.56±0.00^{c}	0.60±0.00^{abc}	0.61±0.01^{b}	0.62±0.00^{a}
	24	0.53±0.01^{b}	0.54±0.00^{b}	0.58±0.00^{a}	0.60±0.01^{a}	0.61±0.02^{a}
STR (%)	0	0.94±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}	0.93±0.00^{a}
	24	0.93±0.00^{a}	0.93±0.00^{a}	0.92±0.00^{ab}	0.92±0.00^{ab}	0.91±0.00^{b}
WOB (%)	0	0.60±0.02^{bc}	0.60±0.00^{c}	0.64±0.00^{ab}	0.66±0.01a	0.67±0.00^{a}
	24	0.57±0.01^{c}	0.57±0.00^{c}	0.63±0.00^{b}	0.65±0.01^{ab}	0.67±0.01^{a}
ALH (µm)	0	1.94±0.02^{a}	1.29±0.04^{b}	0.90±0.02^{c}	0.87±0.02^{c}	0.62±0.04^{d}
	24	1.66±0.12^{a}	1.21±0.03^{b}	0.77±0.04^{c}	0.66±0.02^{cd}	0.49±0.02^{d}
BCF (Hz)	0	1.16±0.05^{b}	1.59±0.11^{a}	1.63±0.06^{a}	1.57±0.04^{a}	1.54±0.14^{a}
	24	1.58±0.17^{a}	1.62±0.18^{a}	1.40±0.17^{a}	1.25±0.19^{a}	1.16±0.11^{a}

VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, LIN= Linearity index, STR= Straightness index, WOB= Wobble coefficient index, ALH= Amplitude of lateral head displacement, BCF= Beat cross-frequency. The One-Way ANOVA analysis showed significant differences (P<0.05) between the alphabetically separate groups; the statistical data are shown as Mean±S.E

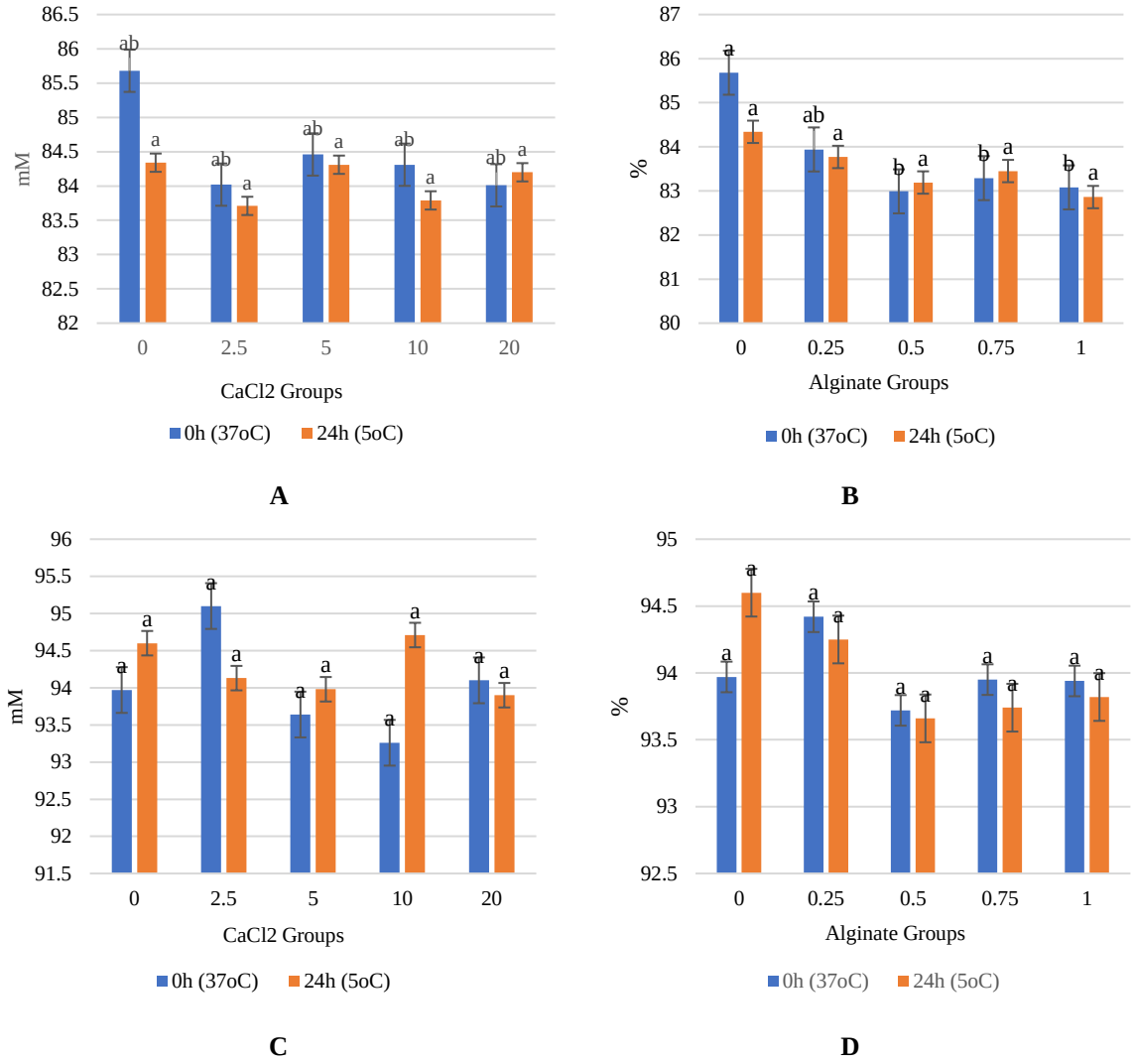

Figure 2. Evaluation of viability (A and B) and membrane integrity (C and D) of sperm after incubation in a tris egg yolk diluent containing different concentrations of alginate and calcium chloride and the data were examined at 0 hours (37ºC) and 24 hours (5ºC). Statistically significant differences (P≤0.05) between the analysis times are indicated with different letters

values of the 2.5 mM $CaCl_2$ groups were numerically the same as the control group, with a statistical difference between the other groups (P<0.05) when tested at 0 hours. However, after 24 hours of cold storage, there was no difference between the groups regarding viability parameters. Viability assessment provides information about the overall health of sperm under different experimental or clinical conditions (Eckel et al. 2017). The interaction of different elements, such as the presence of chemicals like calcium chloride and alginate, in combination with physical properties like temperature and nutrition availability, controls cell survival and metabolic activity (Yuan et al. 2022).

However, in the alginate groups, there was no difference in viability parameters after incubation in a tris egg yolk diluent containing different alginate groups, which were examined at 0 and 24 hours. In addition, the results of many studies indicate that a slight increase in the viscosity of alginate leads to an improvement in the semen quality in sheep (Yániz et al. 2005), rabbits (Rosato & Iaffaldano 2011) and boars (Gil et al. 2014). The results of the present study indicate that supplementations of semen extenders with alginate may destabilize the plasma membrane components when temperatures decrease during preservation (Swami et al. 2017). The results of the present study suggest that supplementation of alginate in the extender minimized the membrane damage during preservation. This finding confirms the results of a previous study in which adding alginate to boar semen improved the integrity of the plasma membrane after thawing (Hu et al. 2014).

In addition, the statistical analysis results show no difference between the groups in terms of plasma membrane integrity parameters after incubation in a tris

egg yolk diluent containing different alginate and calcium chloride concentrations at 0 and 24 hours. Since the plasma membrane serves as a barrier that regulates the flux of ions and molecules, maintaining its integrity is essential for cell activity and overall survival (Dias & Nylandsted 2021); this means that, within the range studied, neither the different concentrations of alginate nor calcium chloride had a significant effect on the plasma membrane integrity. The stability of the plasma membrane integrity under these different experimental conditions demonstrates that the different concentrations of calcium chloride and alginate did not affect the structural integrity of the cell's plasma membrane.

CONCLUSION

In this study, we demonstrate the sperm tolerance of Pasundan bulls to calcium chloride and alginate concentrations to select conditions for sperm encapsulation. Sperm with 2.5 mM calcium chloride and 0.25 % alginate were more tolerant to corresponding prolonged cold storage and can be used as a concentration for sperm encapsulation.

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