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Original Article

The optimal divalent cations and storage temperatures for the encapsulation of ram spermatozoa

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Abstract

The aim of this study was to investigate the effects of encapsulation with different divalent cations and temperatures on the quality of ram spermatozoa during cold storage. Experiment 1: diluted semen was allocated randomly into three groups: control, encapsulation with calcium (Ca^{2+}) alginate and encapsulation with barium (Ba^{2+}) alginate. The samples were stored at two different temperatures (4 °C or 16 °C) for 24 h and subsequently examined for spermatozoa quality. Experiment 2: determination of sperm functionality by means of sperm binding was performed. Spermatozoa with and without Ba^{2+} alginate were cooled for 24 h and then used for sperm binding assay. Experiment 1: the progressive motility of spermatozoa in Ba^{2+} alginate stored at 16 °C was better than that stored at 4 °C ($P = 0.042$). Encapsulation with Ba^{2+} alginate and storage at 16 °C significantly improved progressive motility when compared to Ca^{2+} alginate ($P < 0.001$). The motility characteristics of curve velocity, straight linear velocity and amplitude lateral head displacement of Ba^{2+} alginate at 16 °C were higher than Ca^{2+} alginate ($P = 0.045$, $P = 0.005$ and $P = 0.013$, respectively). A low storage temperature (4 °C and 16 °C) did not markedly decrease the viability or acrosome integrity of spermatozoa, irrespective the type of crosslinking. Although the spermatozoa released from Ba^{2+} alginate were motile and could bind to the zona pellucida, the numbers of bound spermatozoa were significantly lower than in the control group ($P < 0.001$, Experiment 2). In conclusion, Ba^{2+} alginate is preferable to Ca^{2+} alginate for the encapsulation of ram spermatozoa with cold storage at 16 °C.

Keywords: cold storage, encapsulation, ram, spermatozoa, temperature

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Introduction

In farm animals, artificial insemination (AI) is used worldwide for effective breeding. This technique promotes breeding strategies aimed especially at decreasing inbreeding and rapid genetic improvement (Evans, 1988). However, pregnancy rates of ewes following AI have been reported to be lower than those obtained from natural breeding (Deligiannis *et al.*, 2005). Several reports have indicated that the time of insemination plays an important role in high pregnancy outcome (Wulster-Radcliffe *et al.*, 2004; Faigl *et al.*, 2012), in which the ovulation times can be variable among individuals and times of the year. Oestrous synchronisation and fixed-time AI have, therefore, been successfully used in the sheep industry (Donovan *et al.*, 2001; De *et al.*, 2015). The success of these techniques depends on spermatozoa viability and longevity following AI. However, fixed-time AI is labour-intensive with a high cost of animal management (Vigo *et al.*, 2009). Alternatively, encapsulation with the slow release of viable spermatozoa can be used principally to increase the *in utero* longevity of spermatozoa and thus address improper insemination time with cost effectiveness. Encapsulation of spermatozoa has been successfully reported in several species, such as bovines (Nebel and Saacke, 1994; Nebel *et al.*, 1996) and boars (Spinaci *et al.*, 2013a). This encapsulation technology can be combined with cold storage, or cryopreservation, in order to increase spermatozoa quality following encapsulation. For example, encapsulated spermatozoa can be preserved at 5 °C (Huang *et al.*, 2005), 15 °C (Spinaci *et al.*, 2013b), 18 °C (Torre *et al.*, 2002; Faustini *et al.*, 2004) and in liquid nitrogen (Weber *et al.*, 2006). In addition, the fertility of encapsulated bovine spermatozoa has been reported to be similar to that of non-encapsulated spermatozoa (49.4% and 48.6%, respectively; Nebel *et al.*, 1996). Therefore, this technology may be used for ram spermatozoa in order to maintain its viability in the female reproductive tract and to mitigate inappropriate insemination time.

Alginate is a brown algae composed of copolymer of guluronic acid (G-block) and mannuronic acid (M-block; Davis *et al.*, 2003). This alginate has been intensively used to preserve the quality of bioactive compounds (Shishir *et al.*, 2018) because its structure permits the exchange of nutrients and metabolites (Paredes Juárez *et al.*, 2014). Moreover, it can maintain the morphology and functional characteristic of encapsulated cells (Torre *et al.*, 2002). The process of encapsulation involves crosslinking between block chains (G- or M-block) and divalent cations to create the junction zone: the so-called egg-box model. Among divalent cations, Ca^{2+} and Ba^{2+} have frequently been used for encapsulation. In spermatozoa, crosslinking of alginate with $CaCl_2$ has been reported for bovines (Nebel *et al.*, 1985), swine (Huang *et al.*, 2005) and canines (Shah *et al.*, 2010), while barium alginate has also been used in bovines (Perteghella *et al.*, 2017) and swine (Torre *et al.*, 2002). However, information on the encapsulation of ram spermatozoa has been limited, and no recent data has been reported. The aim of this study was therefore to investigate the effects of encapsulation with different divalent cations and temperatures on spermatozoa quality during cold storage.

Materials and Methods

Chemicals: All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA). For fluorescent staining, ethidium homodimer-1 (EthD-1), fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and SYBR-14 were purchased from Invitrogen (Thermo Fisher Scientific, USA).

Experimental animals: This study was conducted with the permission of Chulalongkorn University of Animal Care and Use (approval number 1931056). Semen samples were selected from three crossbred Dorper rams, aged 4–5 years. All animals were fed with concentrates, mineral salts and water *ad libitum*. Bovine ovaries were collected from a local slaughterhouse and used for heterogenous zona pellucida (ZP) binding assay.

Semen collection: First-ejaculated semen was collected every 2 weeks from three fertility-proven rams using an artificial vagina. The samples were evaluated for sperm motility, viability and sperm concentration. The sperm concentration was determined using a haemocytometer. Semen samples with > 70% viability and motility were included in this experiment. The concentrated semen was diluted with an egg yolk Tris-citric extender (Tris 36.3 g/L, citric acid 18.2 g/L, trehalose 5.68 g/L, 20% (v/v) egg yolk, streptomycin 1 g/L, penicillin 1×10^6 IU/L and distilled water to 1 L) to achieve a sperm concentration of 1×10^9 spermatozoa/mL and transported to the laboratory within 2 h in a thermal protective container.

Sperm evaluation: Sperm motility

A warm slide (37 °C) was loaded with a 8-µL sample of diluted semen with phosphate buffered saline (PBS; 1:90). Sperm movement was evaluated using Sperm Class Analyzer (SCA; Microptic, Spain), fitted on a microscope (Olympus, Spain) under 100× magnification. At least 1000 spermatozoa were determined in five different fields for each sample. The following kinematic parameters were evaluated in the study: 1) curve velocity (VCL; µm/s), the average velocity measure between point-to-point track followed by the cell, 2) straight linear velocity (VSL; µm/s), the time average velocity of the sperm head along the straight line between the first and last sperm positions, 3) average path velocity (VAP; µm/s), the average velocity of a sperm head along its average path, and 4) amplitude lateral head displacement (ALH; µm), the average distance of the sperm head from the average sperm-swimming path.

4.2 Sperm viability: The semen samples were mixed with 1 µM SYBR-14 and 4.65 µM EthD-1 and incubated at 37 °C for 15 min. A total of 200 spermatozoa were evaluated dead and alive under a fluorescent microscope (Olympus, Spain; 400× magnification).

Acrosome integrity: The semen samples were mixed with 4.65 µM EthD-1 and incubated at 37 °C for 15

mins and the mixed samples smeared onto a glass slide and air-dried. The spermatozoa were permeabilised by 95% ethanol and air-dried. The slides were stained with 100 µg/mL FITC-PNA at 4 °C for 30 mins. Subsequently, the slides were rinsed with cooled PBS. The acrosomal region in an intact acrosome exhibited even staining with apple-green fluorescence. A total of 200 spermatozoa were evaluated under a fluorescent microscope (1000× magnification).

Sperm encapsulation: For encapsulation of spermatozoa with Ca^{2+} alginate, the diluted semen (1×10^9 spermatozoa/mL) was mixed with 2% (w/v) sodium alginate in Tris-citric buffer solution at a ratio of 1:1 to reach a final concentration of 1% (w/v) sodium alginate. The solution containing the spermatozoa was slowly dropped through a blunt 30-gauge needle connected to a 1 mL syringe into a 100 mL beaker containing 30 mL of 125 mM $CaCl_2$ dissolved in egg yolk Tris-citric medium. The needle tip was 8.5 cm above the surface of the $CaCl_2$ solution. Encapsulated spermatozoa were incubated in the $CaCl_2$ solution for 10 min to achieve full Ca^{2+} alginate crosslinking. The procedure and concentrations of sodium alginate and $CaCl_2$ solution of spermatozoa for Ca^{2+} alginate encapsulation were modified from Huang *et al.* (2005) and Shah *et al.* (2010). The process for encapsulation of spermatozoa using Ba^{2+} alginate was similar to the aforementioned with minor modifications: modified concentrations of sodium alginate solution and $BaCl_2$ were 1% (w/v) and 25 mM, respectively (Perteghella *et al.*, 2017). The alginate solution (1% w/v) was mixed with diluted semen in a ratio of 1:1. The concentration of the sodium alginate was 0.5% (w/v). In all cases, the encapsulated spermatozoa were incubated in an egg yolk Tris-citric medium and stored at either 4 °C or 16 °C until evaluation. Prior to evaluating the spermatozoa, five encapsulated spermatozoa were dissolved in 100 µL of 0.3% (v/v) sodium citrate and 0.5% (v/v) ethylenediamine tetraacetic acid (EDTA) saline solution (pH 7.4) at 37 °C for 15 min. The final capsule concentration after dissolving the alginate was 5×10^8 spermatozoa/mL (100 capsules/mL). The concentration of spermatozoa in each capsule was approximately 5×10^6.

Sperm binding assay: This experiment used heterologous oocytes for sperm binding assay. Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 h. The ovaries were sliced with a surgical blade in order to release the cumulus oocyte complexes. Subsequently, the cumulus cells were enzymatically removed from the oocytes by incubating with hyaluronidase (50 U/mL) for 5 min. Cumulus-denuded oocytes (DOs) were preserved at 4 °C in a salt storage solution (0.5 M $(NH_4)_2SO_4$), 0.75 M $CaCl_2$, 0.2 mM $ZnCl_2$, 40 mM HEPES, pH 7.4, and 0.01% PVA) for 2 days (Herrick and Swanson, 2003).

Control spermatozoa were incubated at 37 °C for 15 mins, while five encapsulated spermatozoa were dissolved in 100 µl of 0.3% (v/v) sodium citrate and 0.5% (v/v) EDTA before incubation. Spermatozoa were washed with fertilising medium (9.6 mg/mL Tyrode's salt, 1 mM $NaHCO_3$, 20 µM penicillamine, 1 µM epinephrine, 0.2 mM pyruvate, 10 µg/mL heparin, 0.4% BSA, gentamicin and penicillin, pH 7.6) by centrifugation at 1000 rpm for 5 mins. One million resuspended spermatozoa per mL were incubated with salt-stored oocytes, which were randomly allocated into two groups for fertilisation with non-encapsulated spermatozoa (control) and encapsulated spermatozoa. Salt-stored oocytes and spermatozoa were co-incubated at 38.5 °C for 24 h in fertilisation medium with culture condition of 5% CO_2 humid air.

Assessment of sperm binding: The oocytes were fixed in 4% (w/v) paraformaldehyde overnight. After washing twice, the oocytes were stained with 50 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) for 15 mins. The number of tightly bound spermatozoa onto the ZP was counted under a fluorescence microscope.

Experimental design: Experiment 1: The experiment was replicated three times with first-ejaculated diluted semen samples from three rams. Semen samples were pooled and allocated randomly into three groups. Pooled semen was diluted with an egg yolk Tris-citric extender and then allocated randomly into three groups: control (no encapsulation), encapsulation with calcium (Ca^{2+}) alginate and encapsulation with barium (Ba^{2+}) alginate. The samples were stored at different temperatures (4 °C or 16 °C) for 24 h and subsequently examined for sperm progressive motility, viability and acrosome integrity.

Experiment 2: The oocytes were allocated randomly into two groups and fertilised with either control spermatozoa (non-encapsulated) or Ba^{2+} alginate-encapsulated sperm. First-ejaculated diluted semen samples from three rams were pooled and allocated randomly for control spermatozoa and Ba^{2+} alginate-encapsulated spermatozoa. The spermatozoa samples were stored at 16 °C for 24 h, and the spermatozoa and oocytes were co-incubated for 24 h. Subsequently, the number of spermatozoa tightly bound to the ZP was determined under a fluorescence microscope. Three replicates were performed in this experiment.

Statistical analyses: Data is presented as mean ± standard error (SE) of the mean. For Experiment 1, the quality of spermatozoa (progressive motility, VCL, VSL, VAP, ALH, viability and acrosome integrity) in each group (control, Ca^{2+} and Ba^{2+} alginate-encapsulated spermatozoa) were compared using one-way Analysis of Variance (ANOVA). The effects of different temperatures used for cold storage were compared using independent t-tests. For Experiment 2, an independent t-test was used to compare the numbers of ZP tightly bound spermatozoa. The SPSS statistical software version 22 was used to analyse for normality and equality of variances. A P-value of less than 0.05 was considered statistically significant.

Results

Experiment 1: The sperm motility in term of progressive motility, VCL, VSL, VAP and ALH in cold storage is shown in Table 1. Ca^{2+} alginate and Ba^{2+} alginate stored at a cold temperature of 4 °C

significantly reduced progressive motility of the spermatozoa compared with the control (P = 0.003 and P = 0.007, respectively). The VCLs of Ca^{2+} and Ba^{2+} alginates were significantly reduced when compared to the control group at 4 °C ($P < 0.001$ and P = 0.007, respectively). The VCL of Ba^{2+} alginate was significantly higher than that of Ca^{2+} alginate (P = 0.002). The VSL, VAP and ALH did not differ among groups. With cold storage at 16 °C, motility characteristics in term of progressive motility, VCL, VAP and ALH of the control did not significantly differ from those of Ba^{2+} alginate ($P > 0.05$). Encapsulation with Ba^{2+} alginate and storage at 16 °C significantly improved the progressive motility of spermatozoa compared to Ca^{2+} alginate ($P < 0.001$). The VCL of control and Ba^{2+} alginate were significantly higher than that of Ca^{2+} alginate (P = 0.009 and P = 0.045, respectively). The VSL of control was significantly higher than those of Ba^{2+} and Ca^{2+} alginate ($P < 0.001$ and $P < 0.001$, respectively) while that of of Ba^{2+} alginate was significantly higher than that of Ca^{2+} alginate ($P < 0.005$). The VAP of Ca^{2+} alginate was significantly different from that of the control (P = 0.007) but did not differ from that of Ba^{2+} alginate (P = 0.074). The ALHs of the control and Ba^{2+} alginate were significantly higher than that of Ca^{2+} alginate (P = 0.002 and P = 0.013, respectively). For cold storage at 4 °C, control and Ca^{2+} alginate were significantly decreased in terms of ALH (P = 0.025) and VSL (P = 0.047) when compared with those at 16 °C, respectively. The viability and acrosome integrity are shown in Table 2. In all cases, the encapsulation, irrespective of the storage temperatures, did not affect the viability or acrosome integrity ($P > 0.05$).

Experiment 2: As indicated in Experiment 1 cold storage at 16 °C for 24 h of Ba^{2+} alginate was better than Ca^{2+} alginate in terms of progressive motility, VCL, VSL and ALH. This condition was therefore used in this study. The numbers and binding capability of encapsulated and control spermatozoa after incubation with salt-stored oocytes are presented in Table 3. The spermatozoa released from Ba^{2+} alginate were motile and could bind to the ZP (Figure 1). However, the numbers of bound spermatozoa originating from encapsulated spermatozoa were significantly lower than the control group ($P < 0.001$).

Table 1 Effect of divalent cations and temperatures on sperm motion parameters after 24 h storage

Groups	Progressive motility (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	ALH (µm)
4 °C					
Control	21.17 ± 2.14^{aA}	113.20 ± 6.84^{aA}	26.55 ± 7.18^{aA}	41.44 ± 10.16^{aA}	2.6 ± 0.56^{aB}
Ca^{2+} alginate	5.55 ± 0.48^{bA}	28.17 ± 0.66^{cA}	15.04 ± 5.85^{aA}	36.95 ± 2.49^{aA}	1.27 ± 0.13^{aA}
Ba^{2+} alginate	8.19 ± 2.24^{bB}	75.98 ± 5.8^{bA}	19.08 ± 1.76^{aA}	35.60 ± 1.76^{aA}	2.0 ± 0.28^{aA}
16 °C					
Control	57.58 ± 3.06^{aA}	136.38 ± 5.66^{aA}	68.96 ± 3.03^{aA}	84.50 ± 3.58^{aA}	3.00 ± 0.06^{aA}
Ca^{2+} alginate	8.5 ± 4.91^{bA}	58.54 ± 19.50^{bA}	5.66 ± 1.12^{cA}	34.37 ± 12.11^{bA}	1.17 ± 0.23^{bA}
Ba^{2+} alginate	51.12 ± 0.52^{aA}	112.28 ± 2.8^{aA}	30.20 ± 8.30^{bA}	62.88 ± 1.16^{abA}	2.48 ± 0.28^{aA}

[a-b] Within a column, values with different superscripts differ significantly ($P < 0.05$)
[A-B] Within a column in the same group, values with different superscripts differ significantly ($P < 0.05$)
Values are presented as Mean ± SE

Table 2 Effect of divalent cation and temperature on sperm viability and acrosome integrity after 24 h storage

Treatments	Viability (%)		Acrosome integrity (%)	
	4 °C	16 °C	4 °C	16 °C
Control	43.33 ± 3.16^{aA}	59.11 ± 2.22^{aA}	35.16 ± 2.92^{aA}	56.33 ± 4.66^{aA}
Ca^{2+} alginate	42.42 ± 0.72^{aA}	39.40 ± 8.44^{aA}	25.36 ± 2.74^{aA}	36.26 ± 4.79^{bA}
Ba^{2+} alginate	46.90 ± 5.15^{aA}	53.53 ± 1.27^{aA}	26.00 ± 3.51^{aA}	55.16 ± 2.77^{abA}

[a-b] Within a column, values with different superscripts differ significantly ($P < 0.05$)
[A-B] Within a row, values with different superscripts differ significantly ($P < 0.05$)
Values are presented as Mean ± SE

Table 3 The number of Ba^{2+} alginate-encapsulated spermatozoa and the control group after incubation with oocytes

Group	Number of salt-stored oocytes	Number of sperm binding
Control	45	$251.58 \pm 11.25(113–300)^{a}$
Encapsulated spermatozoa	52	$110.36 \pm 5.37(21–200)^{b}$

[a-b] Within a column, values with different superscripts differ significantly ($P < 0.05$)
( ) ranges of sperm tightly bound to zona pellucida

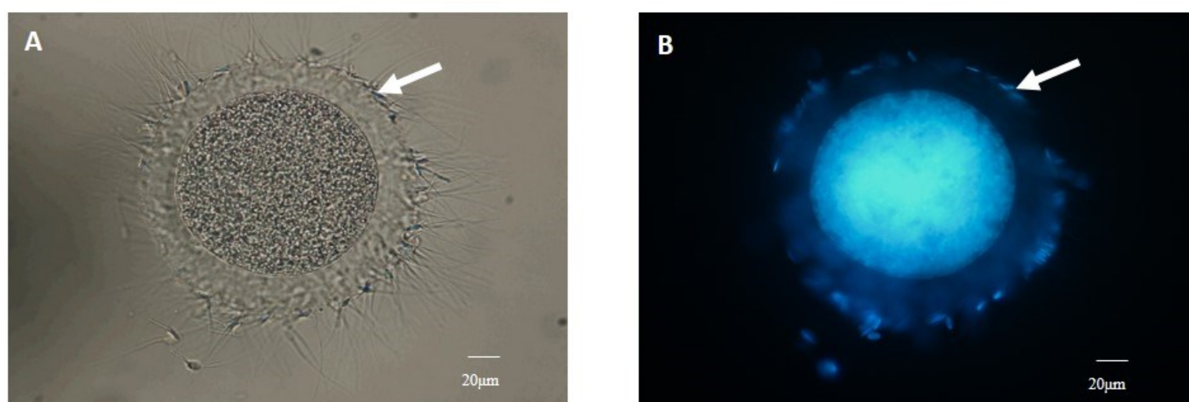

Figure 1 Encapsulated spermatozoa bound to the zona pellucida after 24 h incubation (A; oocyte and spermatozoa under a phase-contrast microscope, B; DAPI-stained oocyte and spermatozoa under fluorescence microscope; white arrow: spermatozoa)

Discussion

This study demonstrated that encapsulation could be used for short-term preservation of ram spermatozoa. It also demonstrated that the storage temperature and type of divalent cation used affected sperm quality during storage of encapsulated spermatozoa. This encapsulation was effective for the preservation of sperm functions, especially progressive motility and binding capacity. This encapsulation technology has been demonstrated to improve conception rate following AI (Perteghella *et al.*, 2017). According to the findings that the encapsulated spermatozoa are released slowly after insemination, the longevity of spermatozoa will therefore be ameliorated *in utero*. However, it was found in this study that the motility characteristics (progressive motility, VCL, VSL and ALH) of encapsulated spermatozoa were dependent on the type of alginate crosslinking (Table 1) and also the temperature during cold storage. The percentage of progressive motility was a more sensitive indicator of the adverse effects of storage than the characteristics of sperm motility (VCL, VSL, VAP and ALH; Arman *et al.*, 2006). However, a previous study in humans reported that VSL and VAP correlated with fertilisation, while ALH was associated with the ability of sperm to penetrate the cervical mucus and fuse with the oocyte (Hirano *et al.*, 2001). Both Ca^{2+} and Ba^{2+} could be used for crosslinking the alginate to form Ca^{2+} and Ba^{2+} alginates, respectively. These two types of crosslinkings have been used successfully for the encapsulation of several cell types, including spermatozoa (Lakde *et al.*; Vigo *et al.*, 2009; Perteghella *et al.*, 2017). In rams, the quality of spermatozoa encapsulated with Ca^{2+} crosslinking was significantly inferior to that obtained from Ba^{2+} crosslinking. Calcium has been reported to have positive and negative effects on the quality of spermatozoa, depending on Ca^{2+} concentration and species (Simpson and White, 1987). A low concentration of calcium in the medium causes hyperactivation of spermatozoa (Yanagimachi and Usui, 1974), while too high a concentration of calcium negatively affects sperm motility (Rosado *et al.*, 1970; Brokaw *et al.*, 1974; Morton *et al.*, 1974; Davis, 1978) and also decreases adenylyl cyclase activity in spermatozoa (PGoh and White, 1988).

Alginate chains are composed principally of guluronic acid (G) and mannuronic acid (M) and form G–G or G–M blocks. For gel formation, Ca^{2+} ions bind to G–G or G–M blocks. In contrast, Ba^{2+} ions bind to G–G and M–M blocks (Paredes Juárez *et al.*, 2014). The binding affinity of Ca^{2+} to alginate has been reported to be lower than that of Ba^{2+} (Smidsrod and Haug, 1972; Zimmermann *et al.*, 2007) and results in gel swelling, increasing pore size and sphere destabilisation (Mørch *et al.*, 2006). Due to the high binding affinity of Ba^{2+} to alginate, the concentration of Ba^{2+} used in this study was therefore lower than that of Ca^{2+}. The lower concentration of Ba^{2+} than Ca^{2+} also appeared to mitigate the adverse effects of encapsulation on spermatozoa.

In this study, the sperm quality of encapsulated spermatozoa at 16 °C was seen to be better than at 4 °C, as shown in Table 1. Cold shock affected sperm quality adversely, which may be a reason for the lower sperm quality in the semen stored at 4 °C. Cold storage of encapsulated spermatozoa causes irreversible thermal shock that negatively affects spermatozoa quality. Thermal shock has been reported to decrease sperm adenosine triphosphate, which is necessary for sperm motility (Appell *et al.*, 1977). Moreover, improper thermal change can also disrupt the organisation of spermatozoa membranes (Lee *et al.*, 2006; Hermansson and Axner, 2007). However, preservation of the spermatozoa at too high a temperature of cold storage can promote bacterial growth (Appell and Evans, 1977) and metabolism of the spermatozoa (Mohamed *et al.*, 2012). After 12 h semen storage, there was no bacterial growth at 4 °C while there was growth at 20 °C (1×10^3 colonies/mL) and dramatic growth at 37° C (1×10^5 colonies/mL). Gram-negative bacteria are the primary organisms that can inhibit the motility of spermatozoa (Appell and Evans, 1977). To reduce the number of bacteria in the samples, this experiment examined spermatozoa quality after 24 h storage at a low temperature.

To examine the fertilising ability of encapsulated spermatozoa stored at 16 °C, the spermatozoa were tested for binding capability to the ZP of the oocytes. Fertilisation in mammals consists of three critical events: 1) movement of spermatozoa through cumulus cells; 2) sperm attachment and movement through the specific receptors of the ZP; and 3) fusion of spermatozoa and plasma membrane of the oocytes

(Hafez and Hafez, 2013). In this study, bovine oocytes were used to examine heterologous binding assays, as the oocytes were readily available and compared and predicted the fertility of ram spermatozoa (García-Álvarez *et al.*, 2009). It was found in this study that ram spermatozoa can bind to the ZP of bovine oocytes. Previous studies have reported that salt-stored oocytes do not show different sperm binding capability compared to fresh oocytes (Liu *et al.*, 1988). The ZP changes during the process of maturation, which increases sperm binding to the matured oocyte (Oehninger *et al.*, 1991). However, previous studies have used immature salt-stored oocytes (Franken *et al.*, 1991) and immature oocytes (Roth *et al.*, 1998) to determine sperm binding capacity. It was clearly seen in this study that the encapsulated spermatozoa stored for 24 h retained their functions, especially for hyperactivation induced by heparin, which was supplemented in the fertilisation medium. Heparin induces plasma membrane changes during capacitation (Ferrari *et al.*, 2000; Parrish, 2014) by increasing intracellular calcium, pH and cAMP (Parrish *et al.*, 1985). Movement of flagella are necessary for hyperactivation of spermatozoa (Hafez and Hafez, 2013). Following hyperactivation and ZP binding, the spermatozoa undergo an acrosome reaction and release acrosomal enzymes to digest the ZP (Georgadaki *et al.*, 2016). Although we did not examine the acrosomal status of the penetrated spermatozoa, the tightly bound spermatozoa within the ZP indicated convincingly that the acrosomal activation of spermatozoa encapsulated with Ba^{2+} crosslinking had been initiated.

In summary, barium alginate is preferable for the encapsulation of ram spermatozoa, as it maintains the quality of the encapsulated spermatozoa. Encapsulation with calcium alginate and cold storage at 4 °C was detrimental to sperm quality. Encapsulation of ram spermatozoa with barium alginate and cold storage at 16 °C for 24 h can also be used. However, longevity and fertilisation test by means of AI should be further performed.

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