Zygote

cambridge.org/zyg

Optimization of sperm freezability in Bactrian camel using various dilution rates and equilibration times

Vahid Vahedi[1], Mohsen Mostafaei[2], Hossein Vaseghi Dodaran[3] and Nemat Hedayat Evrigh[4]

[1]Department of Animal Science, Moghan College of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran; [2]Research Centre for Agriculture and Natural Resources, Ardabil 56491-11169, Iran; [3]Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran and [4]Department of Animal Science, Faculty of Agricultural Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

Research Article

Cite this article: Vahedi V *et al.* (2019) Optimization of sperm freezability in Bactrian camel using various dilution rates and equilibration times. *Zygote* **27**: 362–366. doi: 10.1017/S0967199419000261

Received: 29 July 2018
Revised: 16 December 2018
Accepted: 18 April 2019
First published online: 30 September 2019

Keywords:
Bactrian camel; Dilution rate; Equilibration time; Semen cryopreservation

Address for correspondence:
Vahid Vahedi. Department of Animal Science, Moghan College of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran. Tel: +98 45 32715417. Mobile: +98 914 4322355. Fax: +98 45 32715417. E-mail: vahediv@uma.ac.ir
Nemat Hedayat Evrigh. Department of Animal Science, Faculty of Agricultural Sciences, University of Mohaghegh Ardabili, Ardabil , Iran Fax: +98 45 33512204. E-mail: nhedayat@uma.ac.ir

Summary

The effect of different dilution rates and equilibration times on the cryopreservation of Bactrian camel spermatozoa was evaluated in the current study. Semen samples from four healthy adult males were collected, processed and pooled. They were then subjected to a completely randomized 4×2 factorial design including four dilution rates (DR; 1:1, 1:2, 1:4 or 1:8; v:v with SHOTOR diluent) and two equilibration times (ET; 1 or 2 h at 5°C). After freezing and thawing, sperm kinematic parameters as well as viability, plasma membrane integrity, abnormality and seminal malondialdehyde level were assessed. According to the results, four-fold diluted samples recorded significantly higher values ($P < 0.05$) for sperm total (39.58 vs 31.83 and 33.33,%) and progressive motility (19.50 vs 14.00 and 14.25,%), viability (55.37 vs 43.50 and 48.75,%) and plasma membrane integrity (46.75 vs 37.25 and 37.37,%) than those of both less (1:1) and high (1:8) concentrated samples, respectively. By contrast, the percentage of abnormal spermatozoa and the concentration of seminal malondialdehyde were comparable among all treated groups. Moreover, ET revealed that 1 h equilibration had significantly higher sperm motility (37.04 vs 33.33%), linearity (42.29 vs 32.26%), beat cross-frequency (13.15 vs 8.70 Hz), plasma membrane integrity (42.25 vs 39.75%) and viability (51.37 vs 48.12%) compared with 2 h of ET ($P < 0.05$). Taken together, a four-fold dilution along with 1 h equilibration can be an optimal procedure to cryopreserve Bactrian camel sperm.

Introduction

Semen cryopreservation is an efficient method to extend genetic materials among a population especially those on the brink of extinction. Furthermore, the success of breeding programmes namely artificial insemination (AI) relies mostly on the quality of fresh or cryopreserved semen as in the case with camelid species. It is speculated that Bactrian camel (*Camelus bactrianus*) was domesticated on the eastern border of the Caspian Sea in 2500 BC (Fowler, 1997). However, its population is on the edge of extinction in Iran, with less than 156 camels (Niasari-Naslaji *et al.*, 2009). Although the initial success report on AI in Bactrian camel dates back to early 1960s (Elliot, 1961), limited numbers of studies have reported pregnancies from AI either by fresh or frozen–thawed semen (Zhao *et al.*, 1994; Bravo *et al.*, 2000). Some biochemical and biophysical traits of camelid semen have been characterized although its manipulation and cryopreservation is still a tricky process because of poor quality and highly viscous ejaculates (Bravo *et al.*, 2000). This situation, in turn, ends in ineffective cryopreservation, therefore hindering the extensive application of AI in this family.

Previous reports have suggested that the diluent, cryoprotectant type and cryoprotectant exposure time, as well as freezing and thawing rates, are important criteria to develop suitable and species-specific cryopreservation protocols (Morton *et al.*, 2010; Ahmad *et al.*, 2015). As for freezing extender, introducing SHOTOR diluent in Bactrian camel (Niasari-Naslaji *et al.*, 2007) and Tris–citrate–fructose buffer in Dromedary camel (Malo *et al.*, 2017) resulted in satisfactory sperm quality after freezing–thawing.

The freezability of Bactrian camel spermatozoa in different dilution rates (DR) has not been assessed.

In other words, being an important determinant of cryopreservation outcome, Camelid sperm DR has been mostly studied in liquid storage with contradictory results. For instance, motility and acrosome integrity of ejaculated alpaca sperm with different DRs (1:1, 1:2 or 1:4; v:v) stored at 5 or 15°C for different storage time (0, 24, 48 or 72 h) showed the highest value of four-fold diluted samples in all temperatures and storage times (Morton *et al.*,

© Cambridge University Press 2019.

2009). Conversely, 1:3 and 1:10 DRs were utilized for Dromedary and Bactrian sperm storage, respectively (Deen *et al.*, 2004; Niasari-Naslaji *et al.*, 2006).

The equilibration time (ET) is another contributing factor defined as a pre-freezing period during which glycerol quickly penetrates into the spermatozoon to establish equilibrium between its intracellular and extracellular compartments (Shah *et al.*, 2016). Although long exposure to glycerol seems to be detrimental for spermatozoa, an optimum period is required for the plasma membrane to adopt to lower temperatures. Water outflow through cells resulted in lower damage caused by ice crystals during freezing–thawing (Fleisch *et al.*, 2017). A broad range of equilibration periods from no equilibration at all to 3 h has been reported in different species (Leite *et al.*, 2010; Kaka *et al.* 2015; Pradiee *et al.*, 2016). A greater range (0–4 h) with no direct comparison and with contradictory results has also been reported in camelid semen cryopreservation (Niasari-Naslaji *et al.*, 2007; Abd El-Salaam 2013; Malo *et al.*, 2017).

Generally, studies on the Bactrian camel spermatozoa cryopreservation are scant in the literature and, indeed, a broad range of 25–70 × 10^6 sperm cell/straw was applied with no direct comparison (Niasari-Naslaji *et al.*, 2007). Additional research is warranted to develop a feasible strategy to cryopreserve Bactrian camel spermatozoa. Therefore, this study was conducted to determine an optimum DR and ET for its semen freezing ability.

Materials and methods

Ethics

The local Ethic Committee of Mohaghegh Ardbili University (Ardabil, Iran) approved issues concerning the experimental setups and evaluation techniques.

Chemicals

All chemicals were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Animals

Four healthy adult male camels, aged 6–12 years old, were housed individually at Bactrian Camel Research Center, Jahadabad, Meshkinshahr, Ardabil Province, Iran. Animals were exposed to natural day length with ambient temperature and fed a diet containing 7.5 kg alfalfa hay and 2.5 kg mixed concentrate including 68% barley, 12% cotton seed meal, 17% wheat bran, 2% molasses, and 1% mixed vitamins and minerals.

Semen collection, processing and cryopreservation

Semen sampling was carried out weekly during the breeding season via a modified bovine artificial vagina. Fresh semen samples were immediately transported to the laboratory and kept in a water bath at 37°C. Samples of good quality (>60% total motility, >80% viability) were pooled and subjected to a two-step freezing procedure. Different DRs achieved after extending pooled sample with various amount of pre-warmed SHOTOR extender without cryoprotectant (2× 1:1, 1:2, 1:4 and 1:8 ratios) and cooled to 4°C in 2 h. After inclusion of an appropriate amount of cryoprotectant (glycerol, a final concentration of 5%) samples were subjected to two ETs (1 or 2 h). They were then loaded in 0.25 ml straws (IMV, L'Aigle, France), sealed with polyvinyl alcohol powder (IMV, L'Aigle, France) and placed horizontally 4 cm above liquid nitrogen in a cryobox for 8 min. Finally, straws were plunged into liquid nitrogen and stored until evaluation. The thawing procedure was performed in a water bath at 37°C for 30 s. In total, eight straws were considered for each treatment and this experiment was repeated eight times. SHOTOR diluent composed of 214.6 mM Tris, 64.2 mM citric acid, 66.6 mM glucose, and 49.9 mM fructose with an osmolarity of 330 mOsm/kg (Niasari-Naslaji *et al.*, 2007).

Gross assessment

Computer-assisted semen analysis parameters

For analyzing motility parameters, sperm samples were incubated after thawing in a water bath at 37°C for 5 min. A 5 µl aliquot of semen was placed directly on a pre-warmed microscope slide (76.2 × 24.5 mm; Pearl, China), covered by a coverslip (24 mm × 24 mm; Menzel-Glaser, Germany) and sperm motility characteristics were determined using a phase-contrast microscope (Nikon, Japan) equipped with computer-assisted semen analysis (CASA) software (Hoshmand Fanavaran, Version 6, Amirkabir Medical Engineering Co., Tehran, Iran). The assessed parameters included total motility (TM, %), progressive motility (PM, %), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, %), straightness (STR, %), linearity (%), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz). CASA parameters for the camel species were adapted from Malo *et al.* (2019). The system parameters for CASA were a frame rate of 30/s, Particles of size 13–101 µm, progressive STR (%) 70, progressive VAP (µm/s) 40, slow VAP (µm/s) 20, slow VSL (µm/s) 30, static VAP (µm/s) 4, and static VSL (µm/s) 1. For each evaluation, 10 fields were assessed to include at least 200 spermatozoa.

Viability

Sperm viability was assessed using the eosin–nigrosin staining method (eosin Y 0.67 g, nigrosin 10 g, NaCl 0.9 g dissolved in 100 ml double-distilled water). Briefly,10 µl of diluted semen was mixed with 20 µl of stain on a pre-warmed slide and smeared. Each slide was prepared in duplicate and the percentages of unstained heads of spermatozoa (live) and stained/partial stained heads of spermatozoa (dead) were determined by counting 200 spermatozoa under a phase-contrast microscope at ×400 magnification (Seifi-Jamadi *et al.*, 2016).

Lipid peroxidation

Lipid peroxidation is simply the oxidative degradation of mainly plasma membrane lipids from which free radicals grab electrons and subsequently results in cell damage. Malondialdehyde is the most prevalent byproduct of lipid peroxidation, so that its measurement by thiobarbituric acid assay is widely acknowledged as the technique to assess oxidative damage (Esterbauer and Cheeseman, 1990). Here, 1 ml of diluted semen (1:2 in 2.9% sodium citrate solution) was first mixed with 2 ml of trichloroacetic acid (TCA; 612 mM), 1 ml of ethylenediaminetetraacetic acid (EDTA; 12.66 mM) and 1ml of butylated hydroxytoluene (BHT; 90.76 mM in ethyl alcohol 100%). After centrifugation (900 *g*, 18°C for 10 min), 1 ml of supernatant was incubated with 1 ml of thiobarbituric acid (TBA; 3.6 mM in acetic acid solution; pH: 4) in a boiling water bath for 10 min. After cooling, the absorbance at 532 nm was read using a spectrophotometer.

https://doi.org/10.1017/S0967199419000261 Published online by Cambridge University Press

Hypo-osmotic swilling test

The hypo-osmotic swelling (HOS) test was used to determine spermatozoa with intact plasma membranes. Briefly, 10 µl of diluted semen was mixed with 100 µl of a 100 mOsm hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate per litre of bi-distilled water) in a 0.5-ml test tube. After 45 min incubation at room temperature, the mixtures were homogenized. Then, a drop of fixed semen was placed on a warm slide and spread with a coverslip. The percentage of spermatozoa with a curled or swollen flagellum was estimated by counting 200 sperm per slide using a phase-contrast microscope at ×400 magnification (Niasari-Naslaji *et al.*, 2007).

Sperm morphology

The Schafer and Holzmann (2000) method was applied in Hancock's solution to evaluate sperm morphology. Briefly, sodium saline solution (9.01 g NaCl/500 ml double-distilled water) and buffer solutions (A, 21.682 g Na_2HPO_4/500 ml double-distilled water; B, 22.254 g KH_2PO_4/500 ml double-distilled water). Then 200 ml buffer A and 80 ml buffer B was mixed to prepare 280 ml of buffer solution C. The final Hancock solution was composed of 62.5 ml formalin (37%) + 150 ml sodium saline solution + 150 ml buffer solution C, and 500 ml double-distilled water. A 5-µl aliquot of diluted semen was mixed with 500 µl Hancock's solution. A sample of this mixture was deposited on a slide, covered with a cover slide and sperm abnormality percentage was estimated by counting those with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails and protoplasmic droplets in 200 spermatozoa per slide using a phase-contrast microscope at ×400 magnification

Statistical analysis

Data were tested for normality using the univariate procedure and the Shapiro–Wilk test. Arc sin transformation (Arc sin) was performed when necessary. Statistical analysis of measurement data was performed using PROC GLM of SAS version 9 (SAS Institute Inc., Cary, NC, USA). The statistical model was as follows: $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ijk} is the parameter evaluated, µ is the mean of the population, T_i is the mean of treatments and e_{ijk} is residual effect. Tukey's test was applied to compare lsmeans and a cut-off of 5% was used to indicate statistical significance. Results were presented as the Lsmean ± standard error (SE).

Results

Objective parameters

Post-thaw sperm kinematic parameters in two ETs and at four DRs are presented in Table 1. Overall, it can be seen that, despite some fluctuations, a four-fold dilution along with 1 h of ET recorded more satisfying results compared with other treatments ($P < 0.05$).

Moving to a more detailed analysis, sperm total motility percentage was significantly higher in samples with four-fold dilution (39.58%) than in both the lowest (31.83%) and highest (33.33%) diluted samples (Table 1). Similarly, a 1:4 DR could significantly enhance sperm PM percentage compared with other DRs (19.50 vs 14.00, 16:00 and 14.25 for 1:4, 1:1, 1:2 and 1:8 DR, respectively).

For ET, increasing the ET from 1 to 2 h adversely affected sperm total motility after freezing–thawing. A 1 h ET prior to cryopreservation resulted in an ~4% increase in sperm total motility

Table 1. Effect of different dilution rates and equilibration times on post-thaw quality of Bactrian camel spermatozoa assessed by CASA

Factors	Levels	CASA parameters (LSmean ± SE)								
		TM	PM	STR	LIN	VSL	VCL	VAP	ALH	BCF
Dilution rate (DR) v:v	1:1	31.83^{b} ± 1.93	14.00^{b} ± 1.17	82.72 ± 1.19	37.27 ± 3.51	13.76 ± 1.43	40.40 ± 5.21	16.56 ± 1.68	1.02 ± 0.12	9.94 ± 2.45
	1:2	36.00^{ab} ± 1.65	16.00^{b} ± 1.01	80.33 ± 1.32	38.10 ± 3.18	14.00 ± 1.69	40.21 ± 6.02	17.34 ± 2.06	1.09 ± 0.13	9.00 ± 2.39
	1:4	39.58^{a} ± 2.11	19.50^{a} ± 1.15	80.78 ± 1.51	34.06 ± 3.61	13.65 ± 2.20	41.96 ± 6.12	16.78 ± 2.64	1.11 ± 0.15	10.57 ± 2.33
	1:8	33.33^{b} ± 1.23	14.25^{b} ± 0.62	83.50 ± 1.11	39.66 ± 3.86	12.08 ± 1.26	33.22 ± 4.13	14.41 ± 1.43	0.94 ± 0.10	8.22 ± 2.53
Equilibration time (ET) (h)	1	37.04^{A} ± 1.39	16.50 ± 0.76	82.93 ± 1.09	42.29^{A} ± 2.40	13.95 ± 1.12	33.80^{B} ± 3.04	15.16 ± 1.28	1.22^{A} ± 0.10	13.15^{A} ± 1.5
	2	33.33^{B} ± 1.22	15.37 ± 0.88	80.74 ± 0.06	32.26^{B} ± 2.11	12.76 ± 1.21	40.10^{A} ± 3.93	17.39 ± 1.43	0.86^{B} ± 0.05	8.70^{B} ± 1.5
P-value	DR	0.01	<0.01	0.23	0.68	0.85	0.63	0.75	0.77	0.88
	ET	0.03	0.28	0.08	<0.01	0.50	0.04	0.27	<0.01	<0.01
	DR ×ET	0.49	0.84	0.31	0.81	0.52	0.61	0.63	0.90	0.83

Lsmeans with different superscripts (A–B, a–b) in a column are significantly ($P < 0.05$) different. The assessed parameters included motility (TM, %), progressive motility (PM, %), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, %), straightness (STR, %), and linearity (%), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz).

https://doi.org/10.1017/S0967199419000261 Published online by Cambridge University Press

Table 2. Effect of various dilution rates and equilibration times on some parameters of Bactrian camel spermatozoa after freezing–thawing

Factors	Levels	Parameters (LSmean ± SE)			
		Viability (%)	Abnormality (%)	Membrane integrity (%)	Malondialdehyde (ng/ml)
Dilution rate (DR), v:v	1:1	43.5^{c} ± 1.18	21.37 ± 1.18	37.25^{c} ± 1.34	5.68 ± 0.60
	1:2	51.75^{ab} ± 1.56	22.25 ± 1.56	42.62^{b} ± 1.37	4.63 ± 0.48
	1:4	55.37^{a} ± 1.99	21.50 ± 1.99	46.75^{a} ± 0.95	5.10 ± 0.31
	1:8	48.75^{b} ± 1.25	18.75 ± 1.25	37.37^{c} ± 1.17	5.82 ± 0.61
Equilibration time (ET) (h)	1	51.37^{A} ± 1.35	20.62 ± 1.25	42.25^{A} ± 1.06	4.96 ± 0.35
	2	48.12^{B} ± 1.51	21.31 ± 1.31	39.75^{B} ± 1.47	5.66 ± 0.38
P-value	DR	<0.01	0.58	<0.01	0.35
	ET	0.03	0.71	0.03	0.18
	DR × ET	0.92	0.64	0.12	0.46

Lsmeans with different superscripts (A–B, a–c) in a column are significantly ($P < 0.05$) different.

after thawing, compared with 2 h of ET ($P < 0.05$). Furthermore, linearity (42.29 vs 32.26%), ALH (1.22 vs 0.86%) and BCF (13.15 vs 8.70%) percentages were significantly higher in 1 h of ET than in 2 h. Conversely, a significant enhancement in VCL (~15%) was recorded in the samples belonging to 2 h of ET (Table 1).

Subjective parameters

Samples in the 1:2 and 1:4 dilution groups had significantly higher live spermatozoa than the 1:1 group (Table 2; 51.75 and 55.37 vs 43.50%, respectively). Similarly, the highest significant value ($P < 0.01$) of membrane integrity was noted only at a 1:4 ratio compared with other ratios (46.75 vs 37.25, 42.62 and 37.37% for 1:4, 1:1, 1:2 and 1:8 DRs, respectively).

For ETs, samples that underwent 2 h of ET compared with 1 h of ET, experienced a significant decline in sperm viability (51.37 vs 48.12%) and plasma membrane integrity percentages (42.25 vs 39.75%) (Table 2; $P < 0.05$). Although not statistically significant ($P = 0.12$), peroxidation increased following a longer ET (4.96 vs 5.66 for 1 and 2 h of ET, respectively).

Discussion

This study examined the effect of varying DRs and ETs on the post-thaw quality of Bactrian camel spermatozoa. A four-fold DR resulted in more satisfying results in terms of sperm motion parameters, viability and plasma membrane integrity. However, a lower dilution of spermatozoa before freezing led to poor post-thaw quality parameters. Although it has been proposed that increasing semen concentration, especially for animals with lower freezability of spermatozoa, can compensate quality loss during cryopreservation (Alvarez *et al.*, 2012), this, in turn, resulted in increased free radical production, modification of sperm metabolism, change in medium due to the catabolism products, and release of toxic products from damaged spermatozoa, all of which can contribute to the destabilization of membranes and other structures in intact spermatozoa (Chatterjee and Gagnon, 2001; Perez-Crespo *et al.*, 2008).

A considerable reduction in sperm motility, viability and membrane integrity was noted following cryopreservation at the higher DR (1:8). Negative effects of higher DR with sperm motility have been well documented for fowl (Austin and Natarajan, 1991), boar (Bamba and Cran, 1988), and bull (Garner *et al.*, 2001) spermatozoa. This situation is believed to be caused by the interaction of seminal plasma with the diluent (Blesbois and De Reviers, 1992). In fact, the proportion of cryoprotectant per sperm cell might regulate its function during cryopreservation (Daskin *et al.*, 2011). An optimum ratio may help sperm to survive ice crystal formation during freezing and thawing. Conversely, a higher DR might cause more glycerol to penetrate sperm cells, as a higher glycerol ratio per sperm cell is available, and consequently sperm quality would be diminished. Higher DRs may also reduce sperm post-thaw quality through dilution of several proteins or antioxidants present in seminal plasma that protect sperm against freezing–thawing (Viveiros and Leal, 2016).

Regarding ET, 1 h compared with 2 h ET resulted in significantly higher sperm motility, LIN, ALH and BCF percentages in the present study. Given that cryoprotectant exposure time plays a pivotal role in cryopreservation success, it is important to determine the most appropriate time for each species. Although no comparison was made, Niasari-Naslaji *et al.* (2007) applied an ET of 1 h for Bactrian camel sperm cryopreservation. In Dromedary camel, studies on the length of ET are contradictory. Considering a broad range of ET (10 min to 2 h), for instance, Malo *et al.* (2017) reported no significant difference in post-thaw motility parameters. In another study, however, samples with no ET compared with 2 h or 4 h ET had significantly higher post-thaw motility and acrosome integrity (Abd El-Salaam, 2013). In rooster, extremely short ET (no more than 1 min) resulted in lower viability of post-thaw sperm (Santiago-Moreno *et al.*, 2011). Similarly, higher acrosome and plasma membrane damage, as well as motility loss, were noted in bull sperm given no ET (Leite *et al.*, 2010). In the current study, samples subjected to 2 h of ET had lower plasma membrane integrity and viability compared with 1 h of ET. This finding might be the result of prolonged exposure to glycerol. The toxicity of cryoprotectant on sperm vital reactions such as glycolysis and fructose diphosphates has been well documented (Fahy *et al.*, 1990). In addition, the process of cryopreservation imposes various stresses not only on physical features of sperm, but also on sensitive organelles such as mitochondria that are required to produce ATP (Long, 2006). It is also worth mentioning that mitochondrial ATP is required for the maintenance of sperm motility and membrane integrity (Davila *et al.*, 2016). Consequently, spermatozoa experience arduous conditions in terms of cryoprotectant

https://doi.org/10.1017/S0967199419000261 Published online by Cambridge University Press

toxicity and cryoinjuries during cryopreservation. Although the present study fails to disclose the plausible scenario behind sperm quality loss and higher cryoprotectant exposure time, all or at least some of the above-mentioned mechanisms might be involved.

In conclusion, the highest improvement in kinematic parameters, viability and membrane integrity of post-thawed sperm was obtained with a 1 h ET and 1:4 DR using the SHOTOR diluent. Therefore, this procedure may become an appropriate approach for cryopreservation of Bactrian camel sperm.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflicts of interest. None.

Ethical standards. The local Ethic Committee of Mohaghegh Ardbili University (Ardabil, Iran) approved issues concerning the experimental setups and evaluation techniques.

References

Abd El-Salaam A (2013) Freezability of camel spermatozoa as affected by cryoprotective agents and equilibration periods added with pentoxifylline. *Egypt J Basic Appl Physiol* **12**, 17–48.

Ahmad M, Nasrullah R and Ahmad N (2015) Effect of cooling rate and equilibration time on pre-freeze and post-thaw survival of buck sperm. *Cryobiology* **70**, 233–8.

Alvarez M, Tamayo-Canul J, Anel E, Boixo JC, Mata-Campuzano M, Martinez-Pastor F, Anel L and de Paz P (2012) Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. *Theriogenology* **77**, 1111–8.

Austin A and Natarajan N (1991) Effect of buffers and dilution rates on motility percent of chicken spermatozoa. *Ind J Poult Sci* **26**, 34–8.

Blesbois E and De Reviers M (1992) Effect of different fractions of seminal plasma on the fertilizing ability of fowl spermatozoa stored *in vitro*. *J Reprod Infertil* **95**, 263–8.

Bravo PW, Skidmore JA and Zhao XX (2000) Reproductive aspects and storage of semen in Camelidae. *Anim Reprod Sci* **62**, 173–93.

Chatterjee S and Gagnon C (2001) Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol Reprod Dev* **59**, 451–8.

Daskin A, Kulaksiz R, Ergun A and Halil E (2011) The effect of different dilution rates of Angora buck semen frozen with Bioxcell® extender on the post-thaw quality. *J Fac Vet Med Univ Erciyes* **8**, 23–6.

Davila MP, Munoz PM, Bolanos JM, Stout TA, Gadella BM, Tapia JA, da Silva CB, Ferrusola CO and Pena FJ (2016) Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. *Reproduction (Camb.)* **152**, 683–94.

Deen A, Vyas S, Jain M and Sahani M (2004) Refrigeratory preservation of camel semen. *J Camel Pract Res* **11**, 137–9.

Elliot F (1961) Artificial insemination of a Bactrian camel (*Camelus bactrianus*). *Int Zoo Year Book* **3**, 94–5.

Esterbauer H and Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* **186**, 407–21.

Fahy GM, Lilley TH, Linsdell H, Douglas MS and Meryman HT (1990) Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. *Cryobiology* **27**, 247–68.

Fleisch A, Malama E, Witschi U, Leiding C, Siuda M, Janett F and Bollwein H (2017) Effects of an extension of the equilibration period up to 96 h on the characteristics of cryopreserved bull semen. *Theriogenology* **89**, 255–62.

Fowler M (1997) Evolutionary history and differences between camelids and ruminants. *J Camel Pract Res* **4**, 99–105.

Garner DL, Thomas CA, Gravance CG, Marshall CE, DeJarnette JM and Allen CH (2001) Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology* **56**, 31–40.

Kaka A, Wahid H, Rosnina Y, Yimer N, Khumran A, Sarsaifi K, Behan AA, Kaka U and Ebrahimi M (2015) α-Linolenic acid supplementation in Bioxcell® extender can improve the quality of post-cooling and frozen–thawed bovine sperm. *Anim Reprod Sci* **153**, 1–7.

Leite TG, Do Vale Filho VR, De Arruda RP, De Andrade AF, Emerick LL, Zaffalon FG, Martins JA and De Andrade VJ (2010) Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved bull semen evaluated by CASA and flow cytometry. *Anim Reprod Sci* **120**, 31–8.

Long JA (2006) Avian semen cryopreservation: what are the biological challenges? *Poult Sci* **85**, 232–6.

Malo C, Crichton EG and Skidmore JA (2017) Optimization of the cryopreservation of Dromedary camel semen: cryoprotectants and their concentration and equilibration times. *Cryobiology* **74**, 141–7.

Malo C, Elwing B, Soederstroem L, Lundeheim N, Morrell JM and Skidmore JA (2019) Effect of different freezing rates and thawing temperatures on cryosurvival of Dromedary camel spermatozoa. *Theriogenology* **125**, 43–8.

Morton K, Gibb Z, Bertoldo M and Maxwell WC (2009) Effect of diluent, dilution rate and storage temperature on longevity and functional integrity of liquid stored alpaca (*Vicugna pacos*) semen. *J Camelid Sci* **2**, 15–25.

Morton KM, Evans G and Maxwell WM (2010) Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen–thawed epididymal alpaca (*Vicugna pacos*) sperm. *Theriogenology* **74**, 311–6.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gerami A, Gharahdaghi AA, Abarghani A and Ghanbari A (2007) Semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using SHOTOR diluent: effects of cooling rates and glycerol concentrations. *Theriogenology* **68**, 618–25.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gharahdaghi AA, Abarghani A, Ghanbari A and Gerami A (2006) Effectiveness of a Tris-based extender (SHOTOR diluent) for the preservation of Bactrian camel (*Camelus bactrianus*) semen. *Cryobiology* **53**, 12–21.

Niasari-Naslaji A, Nikjou D, Skidmore JA, Moghiseh A, Mostafaey M, Razavi K and Moosavi-Movahedi AA (2009) Interspecies embryo transfer in camelids: the birth of the first Bactrian camel calves (*Camelus bactrianus*) from Dromedary camels (*Camelus dromedarius*). *Reprod Fertil Dev* **21**, 333–7.

Perez-Crespo M, Moreira P, Pintado B and Gutierrez-Adan A (2008) Factors from damaged sperm affect its DNA integrity and its ability to promote embryo implantation in mice. *J Androl* **29**, 47–54.

Pradiee J, O'Brien E, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, Marcos-Beltran JL, Vega RS, Guillamon FG, Martinez-Nevado E, Guerra R and Santiago-Moreno J (2016) Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. *Anim Reprod Sci* **171**, 121–8.

Santiago-Moreno J, Castaño C, Toledano-Díaz A, Coloma MA, López-Sebastián A, Prieto MT and Campo JL (2011) Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: optimization of freezing rate and equilibration time. *Poult Sci* **90**, 2047–53.

Schafer S and Holzmann A (2000) The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa. *Anim Reprod Sci* **59**, 201–11.

Seifi-Jamadi A, Kohram H, Shahneh AZ, Ansari M and Macías-García B (2016) Quercetin ameliorate motility in frozen–thawed turkmen stallions sperm. *J Equine Vet Sci* **45**, 73–7.

Shah SA, Andrabi SM and Qureshi IZ (2016) Effect of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. *Andrology* **4**, 972–6.

Viveiros AT and Leal MC (2016) Sperm dilution ratio affects post-thaw motility rate and velocity of *Prochilodus lineatus* (Characiformes) sperm. *Zygote* **24**, 662–7.

Zhao XX, Huang YM and Chen BX (1994) Artificial insemination and pregnancy diagnosis in the Bactrian camel (*Camelus bactrianus*). *J Arid Environ* **26**, 61–5.

https://doi.org/10.1017/S0967199419000261 Published online by Cambridge University Press