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A simple, field-friendly technique for cryopreserving semen from Asian elephants (Elephas maximus)

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ABSTRACT

The specific objectives of the present study were to investigate the effects of manual seeding, differing freeze and thaw rates as well as storage for 24 h at 4 °C prior to cryopreservation on post-thaw sperm quality in Asian elephants. Extended semen was cooled in an equitainer to 4 °C, frozen in liquid nitrogen vapour at various rates with and without manual seeding or in a dry shipper and thawed at 37, 50 and 75 °C. There was a significant effect of freeze rate on post-thaw motility ($P < 0.0001$) and acrosomal integrity ($P < 0.005$). The faster freeze rates in the dry shipper and at 1 cm or 2 cm above liquid nitrogen consistently provided better cryopreservation than slower freezing rates. Thaw temperature had no effect on post-thaw semen quality but there was an interaction between freeze and thaw rates with higher thaw rates resulting in superior post-thaw semen quality in straws frozen at fast rates. Storage of samples prior to freezing had a detrimental effect on post-thaw semen quality. In summary, our results indicate cooling extended semen in an equitainer and cryopreserving it by placing straws directly in a dry shipper is a simple technique for effectively cryopreserving Asian elephant semen in the field or zoo.

1. Introduction

Asian elephants are considered endangered according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species and there are estimated to be only 50,000 to 70,000 individuals left worldwide with 15,000 representing the captive population (Choudhury et al., 2008). The population of Asian elephants in captivity is currently not self-sustaining (Choudhury et al., 2008; Wiese, 2000). There are many factors that reduce the breeding success of captive Asian elephants including single sex housing, ovarian acyclicity and reproductive tract pathologies in aging, non-reproductive females. (Brown et al., 2004a, 2004b; Hildebrandt et al., 2000a; Taylor and Poole, 1998). Assisted reproduction techniques (ART) such as artificial insemination (AI) using cryopreserved sperm, have the potential to become a valuable tool in the management of zoo elephant populations.

Current AI techniques in Asian elephants usually utilize chilled semen stored at 4 °C (Brown, 2000) and has produced viable calves. However, the quality of semen collected from bull elephants is inconsistent and, with a shelf life of less than 48 h for chilled semen, high quality ejaculates are not always available at the predicted time of ovulation (Graham et al., 2004; Hermes et al., 2013; Hildebrandt et al., 2012; Kiso et al., 2011). Cryopreserved semen can be collected at any time and frozen indefinitely from both wild

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and captive elephants, overcoming the limitations of using chilled semen.

Unfortunately, Asian elephant semen has been difficult to cryopreserve. To date there has been only one report of a successful conception following AI with cryopreserved semen in Asian elephants and the pregnancy ended prematurely (Thongtip et al., 2009). Although a post-thaw motility of > 50% has been reported using directional freezing, a method that controls the rate of ice crystal propagation thereby minimizing damage to the cells, the directional freezing equipment is bulky and expensive and not easily afforded by many zoos (Saragusty et al., 2009b). Manual seeding is an alternative way to control ice crystal formation with no special equipment needed and has resulted in improved post-thaw quality of cryopreserved spermatozoa in a number of species including turkeys (Zavos and Graham, 1983), humans (Critser et al., 1987), rabbits (Chen and Foote, 1994) and mice (Songsasen and Leibo, 1997) but has not been attempted with Asian elephant semen. In addition, the effect the freezing rate has on post-thaw sperm motility has also not been investigated in the Asian elephant despite the fact that it has been shown to profoundly affect sperm survival during cryopreservation in bovine (Robbins et al., 1976), equine (Cochran et al., 1984) and in Scimitar-horned oryx semen (Roth et al., 1999).

The specific objectives of the present study were to investigate the effects of manual seeding, differing freeze rates and thaw rates as well as storage for 24 h at 4 °C prior to cryopreservation on post-thaw sperm quality in Asian elephants. Ultimately, the goal was to develop a method for cryopreservation of Asian elephant semen that could be employed successfully and cost-effectively in a zoo or field situation.

2. Materials and methods

Unless otherwise stated, all materials and stains were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

2.1. Animals

Semen samples were collected from three Asian elephant bulls housed in North American zoos accredited by the American or Canadian Association of Zoos and Aquariums. The bulls had proven fertility. Bulls 1, 2 and 3 were 12, 25 and 17 years of age, respectively. All three bulls were managed in protected contact systems and had controlled access to females. All research protocols were approved by the Animal Care Committee at the University of Guelph and by the equivalent Institution-specific Animal Care and Use Committees.

2.2. Semen collection and initial processing

A total of 29 semen collection attempts were made and 14 ejaculates (N = 7, 6 and 1 from Bull 1, Bull 2 and Bull 3, respectively) that met the criteria of a minimum of 60% initial motility were used (Table 1). Samples were collected via rectal stimulation by massaging the pelvic portion of the rectum, dorsal to urethra and ampullae (Schmitt and Hildebrandt, 1998). The fullness of the ampullae before each collection was assessed by a transrectal 3D ultrasonography (GE Voluson-I, Providian Medical Equipment, Ohio, US) to ensure an adequate semen reservoir was present and to avoid urine contamination from overstimulation (Hildebrandt et al., 2000b; Kiso et al., 2011; Schmitt and Hildebrandt, 1998). Semen was immediately extended 1:1 (v:v) in a modified Berliner Cryomedium (BC) extender (16% egg yolk, 69.8 mM TES, 47.9 mM Tris, 5.6 mM fructose, 0.1526 M lactose, 1 ml Gentamycin, 1 ml Orvus Equex Paste (OEP) and 25 IU alpha-tocopherol in 100 ml − Saragusty et al., 2009b) with an osmolarity of 285 mOsm. The initial sperm concentration was determined using a hemocytometer after immobilizing 10 µl of raw semen in 990 µl of 10% buffered formalin (Fisher Scientific) (Graham et al., 2004). The acrosomal status was also determined as described below. The extended sample was further extended to a concentration of 200 × 10^6 sperm/ml and placed in the payload tray of an equine equitainer (EST-XL-H (Hard Case) containing a frozen ice brick − Plastilite Corporation, Washington, PA) for cooling to 4 °C. Preliminary tests indicated that extended semen in a 50 ml tube cooled at an approximate rate of 0.23 °C/min, thus taking 2.5–3 h to cool to 4 °C.

2.3. Semen cooling and pre-freeze processing

Each ejaculate was split into two aliquots with one undergoing cryopreservation treatments immediately after cooling to 4 °C (0 h) and the other undergoing cryopreservation treatments after storage at 4 °C for at least 24 h (range: 24–28 h). The following procedures were performed on both the 0 h and 24 h aliquots. Before any further processing of the semen samples, 0.5 ml of extended

Table 1
Initial and pre-freeze motility and acrosomal integrity of semen collected from 3 Asian elephant bulls (N = 14 ejaculates).

Time	Motility (%)	Acrosomal Integrity (%)
Fresh Ejaculate	80.9 ± 1.8 [a]	87.9 ± 2.5 [a]
0 h storage at 4 °C	77.6 ± 2.5 [b]	84.9 ± 3.1 [b]
24 h storage at 4 °C	72.8 ± 3.2 [c]	81.5 ± 3.7 [b]

Different superscripts represent significant differences within measures ($P < 0.05$) at each time point.

semen was removed for the assessment of pre-freeze motility and acrosomal status prior to the addition of glycerolated extender. Immediately prior to cryopreservation, the extended semen aliquot was further diluted 1:1 (v:v) with glycerolated BC extender solution (containing 14% glycerol for a final concentration of 7% glycerol). The volume of glycerolated extender was divided into four parts and added slowly to the extended semen solution every 15 min, allowing the solution to equilibrate over an hour (Thongtip et al., 2004) for a final concentration of 100×10^6 sperm/ml. Extended samples were then loaded into 0.5 ml straws and sealed with sealing powder (IMV Technologies; Maple Grove, MN, US).

2.4. Semen cryopreservation treatments

The following procedures were performed on both the 0 h and 24 h aliquots. Straws (N = 6 per cryopreservation treatment) were frozen in liquid nitrogen vapour using five different methods described below. Straws were placed on styrofoam racks at 1, 2, or 5 cm above 10 cm of liquid nitrogen in a closed styrofoam box (30d x 90 w x 35 h cm) for 80 s before plunging them directly into liquid nitrogen. Straws frozen at 5 cm above LN2 vapour were frozen with or without seeding. Straws were seeded by briefly touching the straw with a metal hanger cooled in liquid nitrogen 10 s after the straws were placed 5 cm over liquid nitrogen vapour. The straws remained in the vapour for an additional 70 s before being plunged into liquid nitrogen. The fifth cryopreservation treatment was performed by placing the straws in a cooled (4 °C) dry shipper canister and plunging it straight to the bottom of a charged, but empty dry shipper (~ −150 °C; model SC 4/3 v; MVE, EastGen, Guelph, Canada) and holding them there for 10 min (method modified from Roth et al., 1999). The seeding protocol was a modification of one used for seeding embryos during cryopreservation (Leibo, 1990). Straws were stored in liquid nitrogen for at least 48 h prior to thawing.

2.5. Semen thaw treatments

Straws (1 per treatment) were thawed in a water bath (StableTemp Digital Utility Water Bath; Cole-Parmer, Montreal, Canada) for 30 s at 37 °C, 10 s at 50 °C, 15 s at 50 °C or 6 s at 75 °C. Immediately after thawing, straws were emptied into vials and samples were diluted with 1 ml of unglycerolated BC extender for a final concentration of 33×10^6 sperm/ml. Vials were then incubated for 1 h in a 37 °C water bath to assess the stability of the cryopreserved sperm at physiological temperatures. Motility and acrosomal status were assessed immediately after thawing and after 1 h incubation at 37 °C as described below.

2.6. Freezing and thawing rates

Trials (N = 3 per treatment) were done to assess the thermal changes in the straws that were associated with the different freezing and thawing treatments. Freezing rates and thawing rates were assessed by placing a thermocouple (Digi-Sense Dual JTEK; Cole-Parmer, IL, US) probe inside a straw filled with extended semen (Roth et al., 1999) and frozen as described above. The temperature within the straw was recorded every 2 s from the time of contact with liquid nitrogen vapour until the temperature reached −80 °C. Immediately after freezing, straws were thawed at each of the four thawing temperatures as described above with the temperature being recorded every 2 s until the designated thawing duration was complete.

2.7. Semen evaluation

Initial motility was roughly assessed by observing a 10 µl pre-warmed (37 °C in for 5 min) aliquot under light microscopy (model DM 750; Leica Microsystems) using the 40× objective and estimating the percentage of motile sperm. Motility at all other time points was determined in aliquots that were cooled to 25 °C to slow the sperm motility and allow accurate counting of the number of motile cells. Spermatozoa were considered non-motile if there was no flagellar movement. Sperm were considered to be motile if there was flagellar movement, regardless of the velocity or linearity of forward progression and total motility was defined as the percentage of sperm with flagellar movement (Graham et al., 2004). Acrosomal status was evaluated using Coomassie Blue Stain (Larson and Miller, 1999). Briefly, 250 µl aliquots were centrifuged (500 x g for 5 min) to remove extender and seminal plasma, re-suspended in 250 µl Tyrodes medium (0.1369 M NaCl, 9.99 mM Fructose, 10.1 mM Hepes, 4.0 mM KCl, 0.98 mM $MgCl_2$), and diluted 1:100 in 10% buffered formalin preservative to fix the sperm and stored at 4 °C until staining. For staining, fixed sperm were centrifuged at 500 x g for 5 min and the pellet re-suspended in 100 µl of 100 mM ammonium acetate. Ten microliters of sperm suspension was smeared on a glass slide and left to air dry. Once the slide was dry, it was placed in Coomassie Blue stain solution (0.22% w/v Coomassie Blue G-250 in 50% methanol, 10% acetic acid, 40% water) for 2 min, washed with distilled water and air-dried. Coverslips were sealed to the slide using Cytoseal™ 60 (Thermo Fisher Scientific, MI, US) and examined under light microscopy (model DM 750; Leica Microsystems) using the 40X objective. Acrosome-intact sperm were identified by a dark stained cap on the sperm head, whereas acrosome-reacted sperm had very faint or no dark staining. At least 100 spermatozoa were assessed per slide to estimate the percentage of sperm with intact acrosomes.

2.8. Statistical analyses

All analyses were done using the mixed procedure (SAS version 9.4; SAS Institute). All models accounted for bull, ejaculate within bull and sample within each bull/ejaculate with sample, ejaculate and bull as random effects and freezing rate, thaw temperature and time after thawing plus all interactions as fixed effects. Post-hoc differences among means were assessed using the LSD test. The

proportions of motile sperm, both pre and post freezing, and the proportions of sperm with intact acrosomes both pre and post freezing were all transformed (arc-sine square-root) prior to analysis as is commonly done for proportional data. Post-thaw proportions of motile sperm and sperm with intact acrosomes frozen at 0 or 24 h were analyzed separately, each as a split plot design with 2 repeated measurements taken on each subsample. The freezing rate applied to each sample from each ejaculate within each bull was considered the main plot factor, and the thaw temperature applied to each subsample within each sample was the subplot factor. Repeated measurements taken on each subsample were handled as given by Wang and Goonewardene (2004). The pre-freezing proportion of motile sperm/acrosome formation was included as a covariate in the analysis of post-thaw motility/acrosome formation. All treatments were combined and comparisons between straws frozen after 0 and 24 h of storage were performed using a Paired *T*-Test. Values in the figures are presented as the mean ± s.e.m. fraction of pre-freeze values for clarity unless otherwise indicated and for all analyses, a $P < 0.05$ was considered significant.

3. Results

3.1. Initial ejaculate characteristics

Approximately half (48.5%) of the ejaculates met the minimum standard for use in the study. Ejaculates (N = 14) had an initial average (± s.e.m.) concentration, motility and acrosomal integrity of 650.5 ± 103.5 × 10^6 sperm/ml, 80.9 ± 1.8% and 87.9 ± 2.5%, respectively.

3.2. Pre-Freeze Cooling and Storage

There was significant decrease (< 10%; $P < 0.05$) in motility and acrosomal integrity after cooling to 4 °C (0 h storage) and a further decrease in motility, but not acrosomal integrity, after 24 h storage at 4 °C (Table 1). Pre-freeze motility and acrosomal integrity were significant ($P < 0.05$) predictors of post-thaw motility and acrosomal integrity.

3.3. Freezing rates

Freezing curves are shown in Fig. 1. No difference was observed between straws dropped in the dry shipper or held in vapour 1 or 2 cm above liquid nitrogen. However, the rate of freezing (slope) decreased when the distance between straws and liquid nitrogen vapour was increased. Moreover, straws held 5 cm above liquid nitrogen had an extended time interval at which they remained at around −10 °C, presumably the point of spontaneous ice nucleation, and seeding appeared to shorten the time interval.

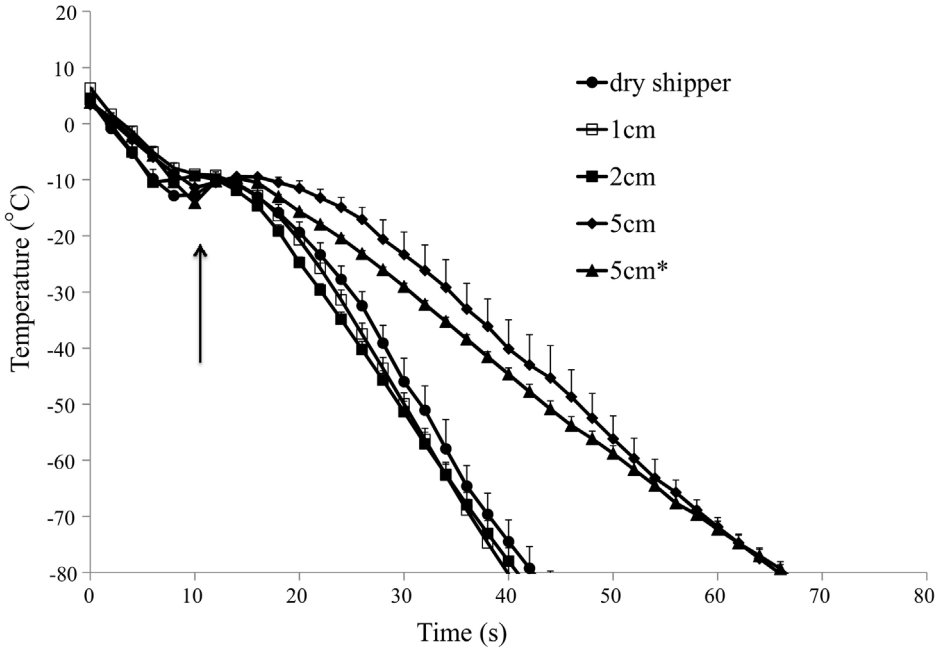

Fig. 1. Freezing rates of 0.5 ml straws frozen over liquid nitrogen vapour 1 (open square), 2 (closed square), 5 (open circle) or 5 (closed circle) cm with seeding (5*cm) or in a dry shipper (open triangle). Values are the mean of three trials ± s.e.m. The arrow represents the increase in temperature associated with ice nucleation.

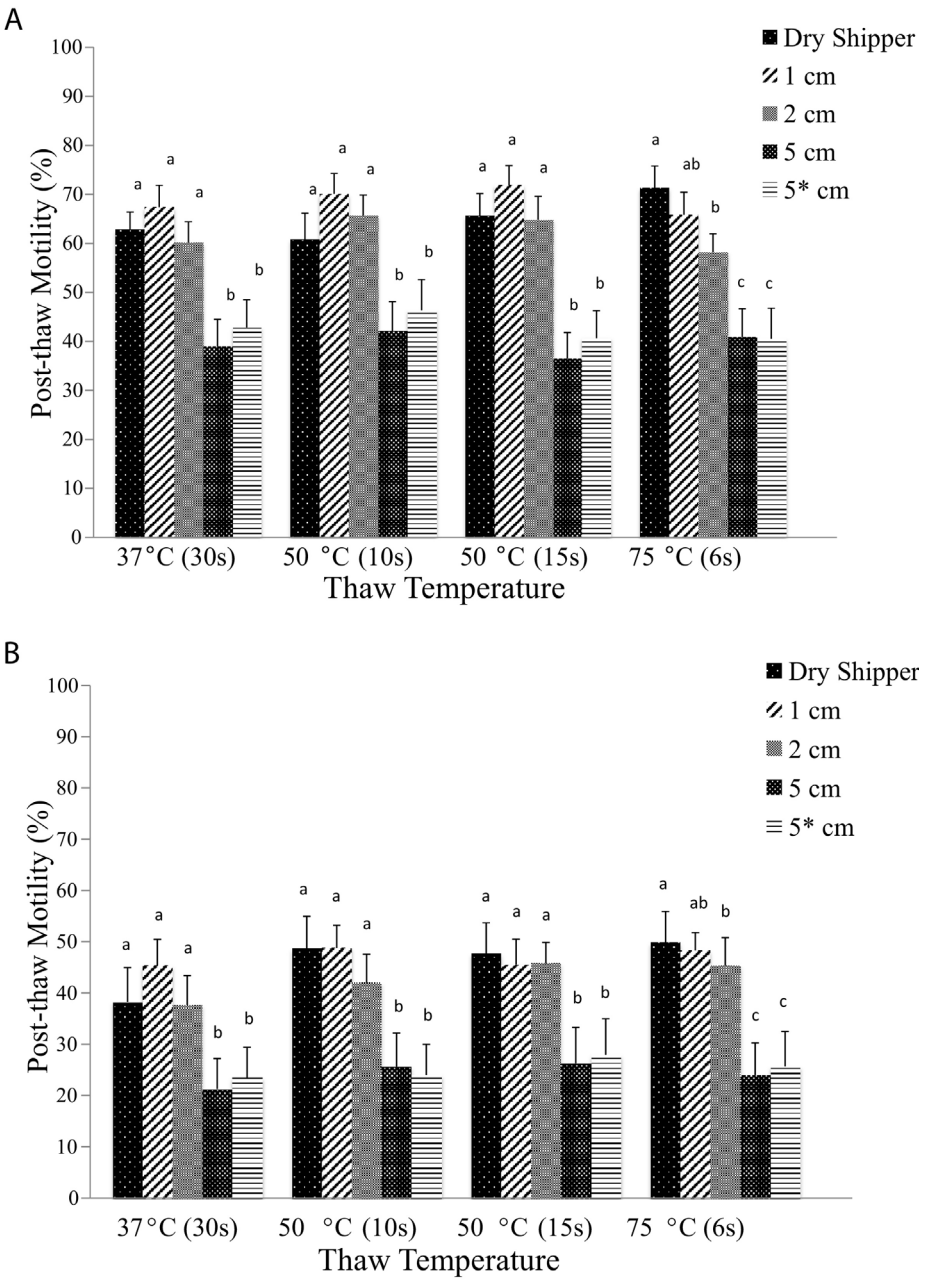

Fig. 2. (a) Mean ± s.e.m. post-thaw motility after cryopreservation at 5 different freeze rates followed by thawing at 4 different temperatures (* indicates manual seeding was performed) for straws stored for 0 h at 4 °C prior to cryopreservation. Within each thaw temperature, different letters represent significant differences ($P < 0.05$) between freeze rates. All post-thaw data is presented as the percentage of fresh ejaculate values. (b) Mean ± s.e.m. post-thaw motility after cryopreservation at 5 different freeze rates followed by thawing at 4 different temperatures (* indicates manual seeding was performed) for straws stored for 24 h at 4 °C prior to cryopreservation. Within each thaw temperature, different letters represent significant differences ($P < 0.05$) between freeze rates. All post-thaw data is presented as the percentage of fresh ejaculate values.

3.4. Effect of freeze rate

There was a significant effect of freeze rate on post-thaw motility ($P < 0.0001$) and acrosomal integrity ($P < 0.005$) regardless of the duration of storage at 4 °C prior to cryopreservation (Figs. 2 and 3a,b respectively). The faster freeze rates obtained in the dry shipper and at 1 cm or 2 cm above liquid nitrogen consistently provided better cryopreservation than at 5 cm above liquid nitrogen (Figs. 2 and 3a,b). Manual seeding had no significant effect ($P > 0.05$) on post-thaw motility or acrosomal integrity (Figs. 2 and 3a,b respectively).

There was no significant decrease ($P > 0.05$) in motility after 1 h post-thaw incubation at 37 °C in straws frozen after 0 h storage

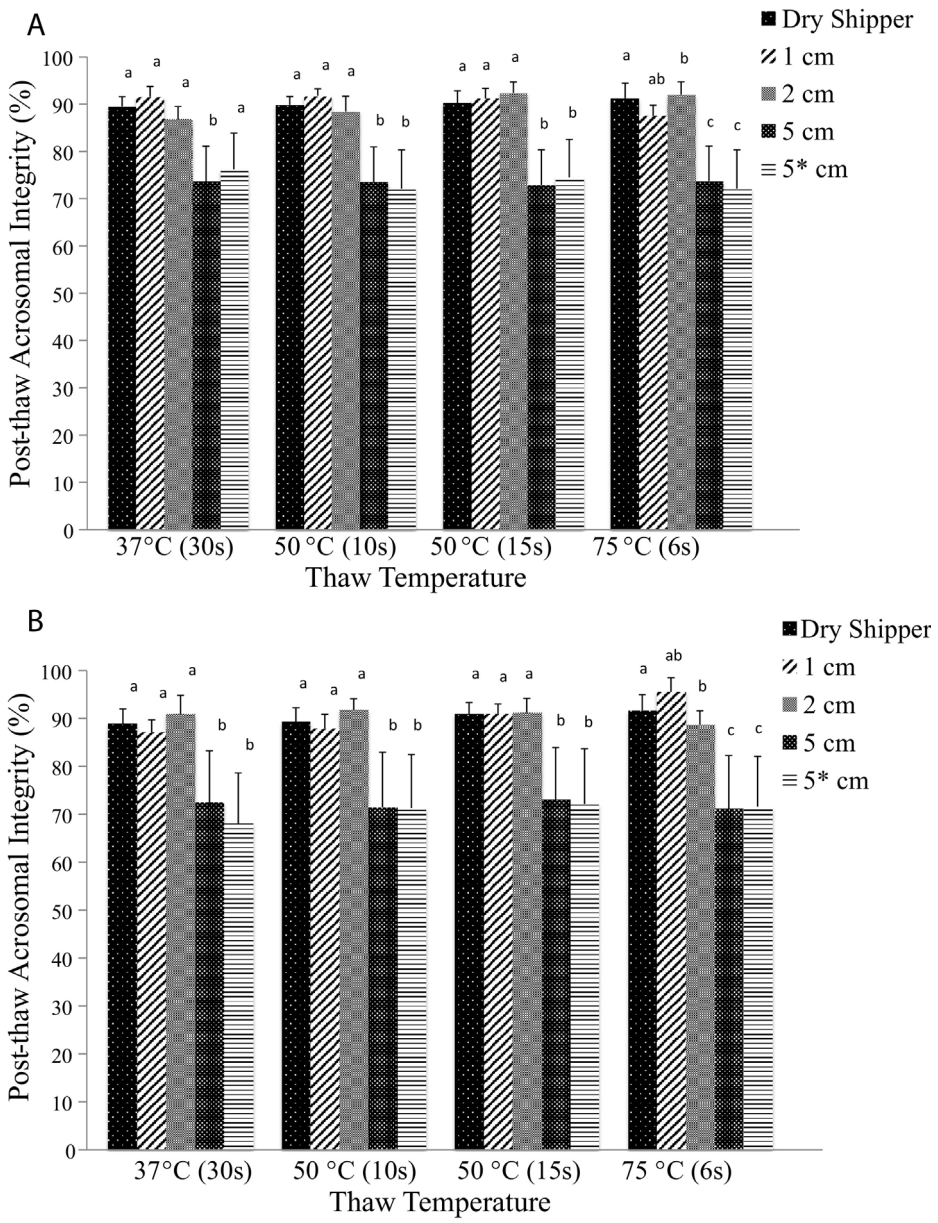

Fig. 3. (a) Mean ± s.e.m. post-thaw acrosomal integrity after cryopreservation at 5 different freeze rates and thawing at 4 different temperatures (* indicates manual seeding was performed) for straws stored for 0 h at 4 °C. Within each thaw temperature, different letters represent significant differences ($P < 0.05$) between freeze rates. All post-thaw data is presented as the percentage of fresh ejaculate values. (b) Mean ± s.e.m. post-thaw acrosomal integrity after cryopreservation at 5 different freeze rates and thawing at 4 different temperatures (* indicates manual seeding was performed) for straws stored for 24 h at 4 °C prior to cryopreservation. Within each thaw temperature, different letters represent significant differences ($P < 0.05$) between freeze rates. All post-thaw data is presented as the percentage of fresh ejaculate values.

at 4 °C (Fig. 4). There was a significant decrease (< 2%; $P < 0.05$) in motility after 1 h post-thaw incubation at 37 °C in straws frozen after 24 h storage at 4 °C. There was a significant decrease (< 5%; $P < 0.05$) in sperm with intact acrosomes after 1hr post-thaw incubation at 37 °C regardless of how long the straws were stored at 4 °C (Fig. 4).

3.5. Effect of thawing temperature

Straws that were thawed at 75 °C for 6 s reached −5 °C, while straws thawed at 50 °C for 10 s reached 3 °C and for 15 s reached 27 °C, and straws thawed at 37 °C for 30 s reached 32 °C. Immediately following the thaw treatment all straws were held at 37 °C in a water bath. Thawing temperature had no effect on post-thaw motility of straws frozen after 0 h storage at 4 °C. However there was an interaction ($P < 0.05$) of freeze rate and thaw temperature, with a linear relationship between post-thaw motility and thaw

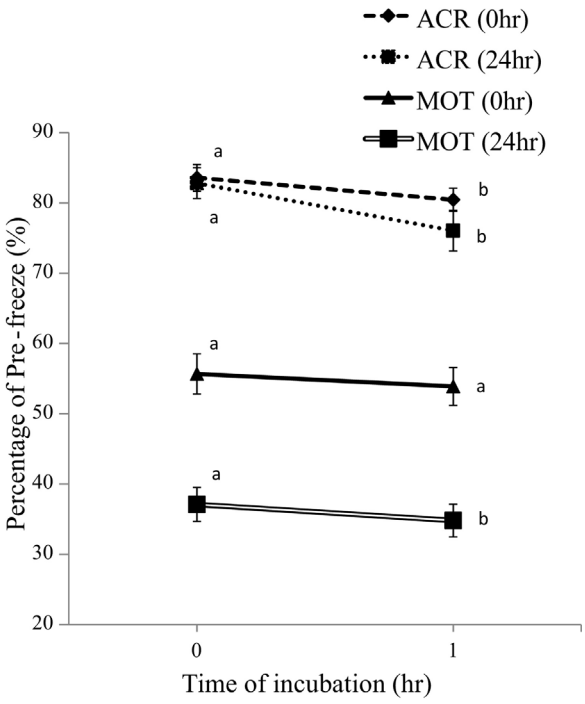

Fig. 4. The effect of 1 h post-thaw incubation at 37 °C on motility (MOT) and acrosomal integrity (ACR) in samples cryopreserved in the dry shipper and thawed at 75 °C. Samples were stored at 4 °C for either 0 (0 h) or 24 (24 h) prior to cryopreservation. Different letters represent significant differences ($P < 0.05$) within parameters between immediately after thawing and after 1 h post-thaw incubation.

temperature in straws frozen in the dry shipper. This also held true for straws frozen after 24 h storage (Fig. 5). There was no effect ($P > 0.05$) of thawing temperature on acrosomal integrity.

3.6. Effect of storage for 24 h prior to cryopreservation

Storage at 4 °C for 24 h prior to freezing had a significant detrimental effect on post-thaw motility and acrosomal integrity ($P < 0.05$) compared to samples frozen immediately upon reaching 4 °C. Samples that had been stored for 24 h prior to freezing had an average of 64% of the post-thaw motility and 95% of the acrosomal integrity of samples frozen immediately after cooling to 4 °C

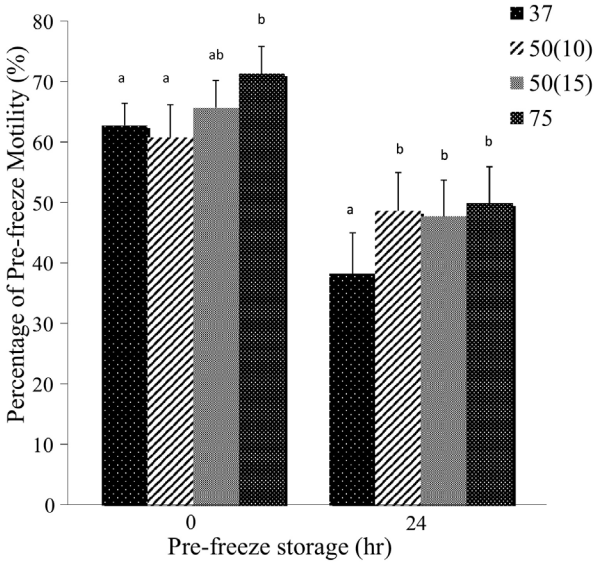

Fig. 5. Effect of thaw temperature on post-thaw motility in straws frozen in a dry shipper after 0 and 24 h of storage at 4 °C. Different letters represent significant differences ($P < 0.05$) within storage times.

(Fig. 4). These declines were observed consistently across freezing and thawing treatments.

4. Discussion

The present study is the first to report the effect of manual seeding, differing freeze and thaw rates and extended storage on post-thaw quality of Asian elephant semen. We compared the effect of different freeze rates by placing straws at 1, 2 and 5 cm above liquid nitrogen and in a dry shipper. Freeze rate had a significant effect on post-thaw semen quality. Similar to findings with bulls and buffalos (Mohammed et al., 1998) and boars (Torreta et al., 1996), better post-thaw sperm quality was achieved in Asian elephants when faster freeze rates were employed, either by using the dry shipper or by placing the straws 1 or 2 cm above liquid nitrogen instead of 5 cm and resulted in post-thaw motility and acrosomal integrity similar to the range previously reported for Asian elephants (Kiso et al., 2012; O'Brien et al., 2013; Sa-Ardrit et al., 2006; Saragusty et al., 2009b; Thongtip et al., 2004). Dry shippers are designed to transport cryopreserved material at temperatures below −150 °C without the dangers of handling liquid nitrogen (Batista et al., 2012; Bielanski, 2005). However, they can also be used in the cryopreservation process and are field friendly eliminating the need to handle liquid nitrogen during cryopreservation (Roth et al., 1999). Successful cryopreservation using a dry shipper has previously been reported in other wildlife species including oryx and rhinoceros semen (Roth et al., 1999; Stoops et al., 2010).

During the freezing process, the physical and biological injury to cells at subzero temperatures, especially those caused by the water phase changes in the extra and intracellular environments can be detrimental to the sperm cells (ie. cryoinjuries − Gao and Critser, 2000). The directional freezing technique is a method to control ice nucleation and has been used with Asian elephant semen to obtain a post-thaw motility of > 50% (Saragusty et al., 2009b). However, the equipment is large and cumbersome and expensive for many zoos. Manual seeding requires no special equipment and has not previously been tested with Asian elephant semen. However, our data indicated that it did not enhance Asian elephant sperm motility or acrsomal integrity post-thaw, suggesting that the elephant sperm may be more similar to that of boars in response to this technique since boar semen does not improve when seeding is employed in samples stored in straws (Fiser et al., 1991).

The rate of warming can exert effects on cell survival. For example, thawing at low temperatures potentially can cause small intracellular crystals to form larger lethal crystals during warming (Mazur, 1984; Gao and Critser, 2000). In contrast, if the samples are thawed quickly, the small intracellular ice crystals don't recrystallize into large ice crystals and lethal damage is avoided (Mazur, 1984). In some species, thawing cryopreserved sperm at high temperatures (e.g. 50 °C for 10 s) has produced superior post-thaw quality compared to thawing at 37 °C (Bovine (Chandler et al., 1983; Dhami et al., 1992), porcine (Fiser and Fairfull, 1990), lupine (Chen et al., 1993), ovine (Soderquist et al., 1997), canine (Pena and Linde-Forsberg, 2000) and equine (Cochran et al., 1984)). In our study, thaw temperature had no effect on post-thaw motility or acrosomal integrity of straws frozen immediately after cooling, however there was an interaction of freeze rate and thaw temperature, with a linear relationship between post-thaw motility and thaw temperature in straws frozen in the dry shipper. This suggests that the faster freeze rate in the dry shipper resulted in the formation of small intracellular ice crystals which did not recrystallize when subjected to faster thaw rates. A similar interaction between freeze rate and thaw rate has been observed in bovids (Robbins et al., 1976; Rodriguez et al., 1975), rams (Fiser et al., 1986) and boars (Fiser et al., 1993). Mazur (1984) suggested that the effect of thaw temperature would not be as pronounced in cells that were frozen more slowly because there would be little intracellular ice formation and, therefore, little risk of lethal recrystallization. This fits well with our observation that thaw temperature had no effect on post-thaw semen quality when samples were frozen at slower rates. The data from our investigations on post-thaw semen quality suggest that Asian elephant sperm cells are more sensitive to the exposure of high solute concentrations during slow freezing than the formation of small intracellular ice crystals during fast freezing. This is supported by a study by Saragusty et al. (2009a) that indicated Asian elephant semen has a lower osmolarity compared to other species and is sensitive to high concentrations of solutes (high osmolarity) in extenders and freezes better in an extender that is of similar osmolarity to the semen.

The fact that we observed only small, if any, decreases in motility and acrosomal integrity 1 h post-thaw regardless of treatment is similar to results obtained by others. Kiso et al. (2012), O'Brien et al. (2013) and Saragusty et al. (2009b) found no large decrease in post-thaw semen quality after incubation of at least an hour at 37 °C. Taken together, results from all of these studies suggest that the sub-population of Asian elephant sperm cells that survive the cryopreservation process is relatively stable under physiological conditions.

The pre-freeze motility and acrosomal integrity was a significant ($P < 0.05$) predictor for post-thaw motility and acrosomal integrity in the Asian elephant in our study. Imrat et al. (2013) demonstrated that semen volume, pH, sperm concentrations and percentages of motile, viable and morphologically abnormal sperm all affected the post-thaw quality of Asian elephant spermatozoa. Studies done on Asian elephants collected via rectal stimulation suggest the majority of ejaculates are unfit for cryopreservation and that ejaculates differ within a bull and between bulls (Imrat et al., 2012; Kiso et al., 2011; Saragusty et al., 2009b). Similar differences have been found in other species as well, such as in stallions and bulls (Loomis and Graham, 2008) and in boars (Yeste et al., 2013). In our study we observed a wide range of motility and acrosomal integrity both within and among the three bulls collected. Both Imrat et al. (2012) and Saragusty et al. (2009b) have suggested that specific protocols for Asian elephant semen cryopreservation may have to be adjusted to suit specific bull cryopreservation. However, in our study, high quality ejaculates from all bulls resulted in similar post-thaw semen quality at the faster freeze rates. Therefore, optimising the collection protocol to obtain high quality ejaculates from all bulls and improving the quality of poor ejaculates prior to cryopreservation may be warranted in lieu of modifying cryopreservation protocols for individual bulls.

Cooling semen to 4 °C reduces cellular metabolic activity, which can increase the life span of the spermatozoa (see review: Barbas

and Mascarenhas, 2009). However, if cooled too quickly sperm can experience 'cold shock' which can result in physical and metabolic damage prior to cryopreservation (see reviews: Gao and Critser, 2000; Holt, 2000; Leibo and Songsasen, 2002; Medeiros et al., 2002; Watson, 2000). Saragusty et al. (2009b) reported that rapid chilling of Asian elephant semen by diluting semen in cold (4 °C) extender was very detrimental to semen quality. However, Saragusty et al. (2009b) and others (Graham et al., 2004; Kiso et al., 2012; O'Brien et al., 2013) have reported only small decreases in sperm quality after cooling the semen from room temperature to 4 °C at a relatively slow rate of < 0.2 °C/min in an equine equitainer. Therefore, we employed a similar procedure for sperm cooling and transport in our study. Since the equitainer eliminates the need for refrigeration equipment it is very advantageous under certain field conditions.

In many species, the storage of semen for 24 h or more is necessary for transportation from the site of collection to a location where it can be subjected to sex-sorting procedures, cryopreservation or used for artificial insemination (Kiso et al., 2011; O'Brien et al., 2013; Purdy et al., 2010). In our study, motility and acrosomal integrity decreased slightly after cooling to 4 °C and motility further decreased after 24 h storage at 4 °C. This decrease in Asian elephant semen quality with cooling and storage has been reported previously (Graham et al., 2004; Kiso et al., 2011; O'Brien et al., 2013). Storage at 4 °C for 24 h prior to freezing had a significantly detrimental effect on the proportion of sperm cells that survived cryopreservation. The only other study that investigated the effect of storage at above zero temperatures prior to cryopreservation on post-thaw Asian elephant semen quality reported no decrease in total post-thaw motility with 24 h storage at 8 °C but there was a decrease in progressive motility and other motility parameters (O'Brien et al., 2013). However, they had a much greater decrease in pre-freeze motility with storage than we observed in our study. This may be because different extenders were used or possibly because the semen was stored at a higher temperature than in our study (8 °C vs. 4 °C, respectively). In both these studies, the extender in which the semen was stored was unglycerolated and it is possible that adding glycerol to extenders used for sperm storage could help maintain sperm quality over time. The addition of glycerol to the extender has been shown to be advantageous in preserving the function of sperm stored at 4 °C in bulls (Anzar et al., 2011; Foote, 1969) but not in rams (Purdy, 2006; Purdy et al., 2010), boars (Guthrie and Welch, 2005) or in white and Sumatran rhinoceros (O'Brien and Roth, 2000; Williams et al., 1995). Although the effects of glycerol exposure on the extended storage of elephant semen at 4 °C have not been studied, Kiso et al. (2012) reported that the addition of glycerol to semen at 22 °C had a detrimental effect on semen quality as it was cooled to 4 °C over 2–3 h.

5. Conclusions

To our knowledge this was the first study to assess different freeze rates, including the use of a dry shipper, different thaw temperatures and seeding on post-thaw quality of cryopreserved Asian elephant semen. Our results indicate that faster freeze rates and higher thaw temperatures can significantly improve post-thaw semen quality in Asian elephants. Our overall goal was to develop a method of Asian elephant semen cryopreservation that can be employed successfully and cost-effectively in a zoo or field situation. Our results indicate that the use of an equitainer to cool semen, a dry shipper to cryopreserve semen and thawing at temperatures of 50 °C or 75 °C is a field-friendly protocol that results in post-thaw semen quality as high as any previously reported in Asian elephants. This is a breakthrough for the cryopreservation of Asian elephant semen in a non-laboratory setting, employing simple and inexpensive techniques and equipment that can be used in zoos and in the field alike.

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