BIOLOGY OF REPRODUCTION 60, 493–498 (1999)

Scimitar-Horned Oryx (*Oryx dammah*) Spermatozoa Are Functionally Competent in a Heterologous Bovine In Vitro Fertilization System after Cryopreservation on Dry Ice, in a Dry Shipper, or over Liquid Nitrogen Vapor[1]

Terri L. Roth,[2,3] Lena May Bush,[4] David E. Wildt,[4] and Rachael B. Weiss[4]

Center for Research of Endangered Wildlife,[3] Cincinnati Zoo and Botanical Garden, Cincinnati, Ohio 45220
Conservation & Research Center,[4] National Zoological Park, Smithsonian Institution, Front Royal, Virginia 22630

ABSTRACT

A heterologous bovine in vitro fertilization (IVF) system was used to study the functional competence of scimitar-horned oryx spermatozoa after cryopreservation. Four sperm-freezing methods were compared after dilution of ejaculates from six oryx with an equine semen extender: 1) dry ice, 2) dry shipper one-step, 3) dry shipper two-step, and 4) liquid nitrogen vapor. Post-thaw sperm motility, longevity, and acrosomal status were assessed and zona pellucida penetration, fertilization, and embryo cleavage were evaluated after coincubation of thawed oryx spermatozoa with in vitro-matured domestic cow oocytes. Sperm motility index (SMI) decreased ($p < 0.05$) over a 6-h period, but a high percentage (≥ 65%) of spermatozoa contained intact acrosomes in all treatments. Despite differences in sperm motility among methods, oocyte penetration, fertilization, and embryo cleavage did not differ ($p \geq 0.05$). However, cleavage success was < 50% across all treatments. There were positive correlations ($p < 0.05$; $r = 0.81–0.97$) between sample SMI at 3 and 6 h and fertilization, penetration, and cleavage, but no correlations ($p \geq 0.05$) between SMI at 0 or 1 h and IVF success. This study demonstrates that compatible heterologous gamete interaction allows thorough assessment of post-thaw sperm function in an endangered antelope. Scimitar-horned oryx spermatozoa appear relatively tolerant of varied cryopreservation methods, and preserved samples exhibit adequate post-thaw function to warrant use for assisted reproduction.

INTRODUCTION

The concept of using genome resource banking to facilitate the management and conservation of endangered species is being promoted extensively [1–3]. In fact, germ plasm cryopreservation is becoming increasingly popular among zoos for helping to preserve genetic variability. The production of offspring from preserved sperm samples now has been realized in several wildlife species [4–8]. One recent example was the production of offspring in an endangered African antelope, the scimitar-horned oryx (*Oryx dammah*), after artificial insemination (AI) with cryopreserved spermatozoa [9]. These results are encouraging, but with pregnancy success usually less than 50% for inseminated females, efficiency still could be improved. One factor possibly affecting success rate is the quality of frozen-thawed sperm samples used for AI.

Sperm cryopreservation in endangered species is challenging. Typically, there is little (if any) available information about processing semen or about the effects of diluents, cryoprotectants, and freeze-thaw methods on sperm cells of these rare species. Additionally, species within a common taxon can express unique characteristics requiring fundamental studies to determine basic sperm-handling and cryopreservation requirements [10–12]. Furthermore, samples frequently are of poor quality, either because of factors inherent to the species (usually a lack of genetic diversity) [13] or because they are "rescued" from animals postmortem. Logistical complications also can hinder sperm cryopreservation success. For instance, if samples are collected under field conditions it often is difficult to obtain the necessary supplies, maintain appropriate temperatures, and avoid sample contamination. After samples are collected and cryopreserved, there is the challenge of thoroughly evaluating post-thaw sperm function so that the potential of producing embryos is known.

Often, post-thaw sperm quality is evaluated by a single subjective assessment of motility within minutes of thawing. Although providing useful information, this approach is known to be an insufficient predictor of fertility in many species [14]. With a number of fluorescent stains now readily available for assessing sperm plasma membranes, acrosomes, and mitochondria [15, 16], it seems logical to conduct a more comprehensive sample evaluation when one is comparing sperm processing and cryopreservation procedures. However, in vitro fertilization (IVF) still offers the best method for assessing overall post-thaw sperm function in vitro. Recently it was determined that freshly collected oryx spermatozoa readily interact with and fertilize in vitro-matured (IVM) domestic cow oocytes [17]. Therefore, a heterologous IVF system is available to fully assess the functionality of cryopreserved spermatozoa in this antelope species.

The general goals of this study were to compare scimitar-horned oryx sperm quality and function after cryopreservation on dry ice, in a dry shipper, and over liquid nitrogen vapor and to confirm that spermatozoa cryopreserved by each method were functionally competent. These three test methods were chosen because each is simple and theoretically could be done under field conditions. Specifically, post-thaw sperm motility, longevity, acrosomal status, and functionality in a heterologous IVF system were compared among cryopreservation methods.

MATERIALS AND METHODS

Semen Collection

Six male scimitar-horned oryx (ages 3–14 yr) served as semen donors. Animals were maintained on diets consisting of alfalfa hay and pelleted feed, with unlimited access to a mineral block and water. Anesthesia and semen collection by electroejaculation were conducted according to Roth et al. [17] during the months of January and March, since

Accepted September 29, 1998.
Received July 14, 1998.
[1]This research was supported, in part, by the Morris Animal Foundation.
[2]Correspondence: Terri L. Roth, Center for Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220. FAX: 513 569 8213; e-mail: terri.roth@cincyzoo.org

semen quality is high year-round [18]. In brief, food and water were withheld for 24 h before a surgical plane of anesthesia was induced using a combination of etorphine HCl (0.022–0.024 mg/kg, M99; Lemmon Co., Sellersville, PA), xylazine (0.25–0.27 mg/kg, Rompun; Mobay Corp., Shawnee, KS), and ketamine HCl (0.62–0.69 mg/kg, Ketaset; Fort Dodge Laboratories Inc., Fort Dodge, IA) delivered i.m. via a projectile dart (Cap-Chur Dart; Palmer Chemical and Equipment Co., Douglasville, GA). Supplemental ketamine HCl i.v. (100–200 mg) was administered as needed during the 15- to 30-min procedure.

Electroejaculation was conducted according to previously described methods [17]. Each male received one to four series of stimulations delivered using a rectal probe (3.9 cm in diameter). A series typically consisted of 10 stimuli at each of 2, 3, and 4 volts or 3, 4, and 5 volts (maximum voltage and total number of stimuli per animal were 6 and 100, respectively). Semen samples were evaluated immediately for sperm motility (0–100%), and good-quality samples (those containing $\geq$ 70% motile spermatozoa and not contaminated with urine) were transferred immediately into a sterile 15-ml tube suspended in a 37°C water bath. Sperm concentration was determined using a hemacytometer, and a 5-μl aliquot was fixed in PBS containing 6% glutaraldehyde for later morphological analysis.

Anesthesia was reversed using yohimbine HCl (0.15–0.17 mg/kg i.v. and 0.15–0.17 mg/kg i.m., Antagonil; Wildlife Laboratories, Fort Collins, CO) and diprenorphine HCl (0.022–0.048 mg/kg i.v., M50–50; Lemmon Co.).

Sperm Processing

Ejaculates from each oryx (n = 6) were diluted 1:1 with EQ extender (20% egg yolk, 5.5% lactose, 1.5% glucose, 0.25% sodium triethanolamine lauryl sulfate) prewarmed to 37°C. Warm water baths containing the extended semen samples were placed in a cold room (~4°C) and allowed to cool. At 1.5 h, concurrently cooled EQ containing 10% glycerol was added incrementally to the extended semen (25%, 25%, and 50% of volume) with 20-min equilibration intervals between each addition. The final sample consisted of 1 part semen to 3 parts EQ in 5% glycerol. After an additional 1-h equilibration, 0.5-ml straws were filled with semen and sealed using a heat sealer.

Sperm Cryopreservation

At least three straws per ejaculate were subjected to each cryopreservation method: dry ice (DI), dry shipper one-step (DS1), dry shipper two-step (DS2), and liquid nitrogen vapor (LNV). For the DI method, straws were placed directly on a block of dry ice in a covered polystyrene cooler for 10 min and then plunged into liquid nitrogen. A fully charged but empty dry shipper (model BDS-5; MVE, New Prague, MN) was used for DS1 and DS2 methods. Straws were placed in the canister (at 4°C), which was lowered into the core of the dry shipper. For DS1, the canister was lowered rapidly (within 1–2 sec) to the bottom of the dry shipper in one step. For the DS2 method, the canister was lowered until the top rim of the canister was level with the top rim of the dry shipper; the canister was then held for 1 min before being lowered rapidly (1 sec) to the bottom of the shipper. After 10 min in the dry shipper, straws were plunged into liquid nitrogen. For the LNV method, straws were placed horizontally on a plastic rack that was lowered slowly over liquid nitrogen vapor. The cooling rate was monitored using a thermocouple (Cole-Parmer Instrument Co., Vernon Hills, IL), and straws typically were submerged in liquid nitrogen within 4 min.

Cooling rates were monitored using a thermocouple probe secured inside an unsealed straw of semen that was cryopreserved by each method alongside the sealed straws of semen. Temperature readings on the thermocouple were recorded approximately every 10 sec for up to 5 min.

Semen Thawing

After 2 yr of liquid nitrogen storage, straws (n = 1 per method per male) were removed and held in air 5 sec before they were plunged into a 37°C water bath and shaken vigorously for 25 sec. Thawed semen was emptied into an Eppendorf tube, and a 5-μl aliquot was used to prepare a slide for acrosome evaluations. For IVF and all other assessments, approximately 400 μl of semen was diluted with 1 ml IVF-Talp (IVF-Talp: Tyrode's albumin lactate pyruvate [TALP] without glucose [19] supplemented with 4 mg/ml BSA [Sigma Chemical Co., St. Louis, MO]), 100 IU/ml penicillin and streptomycin, 0.1 mM pyruvate, and 2 μg/ml heparin (Sigma); the sample was then centrifuged (600 × *g*; 10 min). The resulting sperm pellet was layered under 0.5 ml IVF-TALP in an Eppendorf tube in an incubator (38°C; 5% CO_2), and spermatozoa were allowed to swim up for 1 h. The swim-up supernatants were recovered (~300 μl), sperm concentration was determined, motility was evaluated, and an aliquot (5 μl) was fixed in 6% glutaraldehyde in PBS for morphological evaluation.

Heterologous IVF

IVM domestic cow oocytes were prepared by Bomed Incorporated (Madison, WI). Vials of maturing oocytes were shipped overnight to our laboratory in Cincinnati in a heated (~39°C) transportable incubator (Minitube of America, Verona, WI). Upon arrival, vials were placed in a 38°C incubator until all semen samples were thawed. Oocytes were removed from the maturation medium (22–24 h after IVM was initiated), rinsed through three dishes containing 3 ml IVF-TALP (5-min incubation at 38°C in each dish), and transferred randomly into 90-μl IVF drops under oil (~10–15 oocytes per drop).

Each IVF drop was inseminated with 1×10^5 motile spermatozoa (delivered in a 10-μl drop). Two IVF drops were inseminated with each sample (20–30 oocytes per sample). At the time of insemination, percentage motile spermatozoa and forward progressive motility (on a 0–5 scale; 0 = no movement, 5 = rapid forward progression) [20] for each sample were recorded (0 h), and a sperm motility index (SMI) was calculated (SMI = [percentage motile spermatozoa + (forward progressive motility × 20)] ÷ 2). Additionally, a 5-μl aliquot was used to prepare a slide for acrosomal assessment. Motility evaluations and acrosome slide preparation were repeated at 1, 3, and 6 h postinsemination (hpi) with the remaining swim-up sperm samples contained within an open Eppendorf tube in a humidified incubator (38°C; 5% CO_2 and air). For each shipment of oocytes, one IVF drop containing ~5 oocytes was not inseminated. These oocytes served as parthenogenetic controls.

At 18 hpi, IVM/IVF oocytes were removed from IVF drops, pipetted through a small-bore pipette to remove cumulus cells, and transferred into fresh 100-μl culture drops of IVF-TALP. Oocytes were evaluated 24 h later (42 hpi) for cleavage, and all uncleaved oocytes were fixed and stained with Hoechst 33342 to determine maturation status

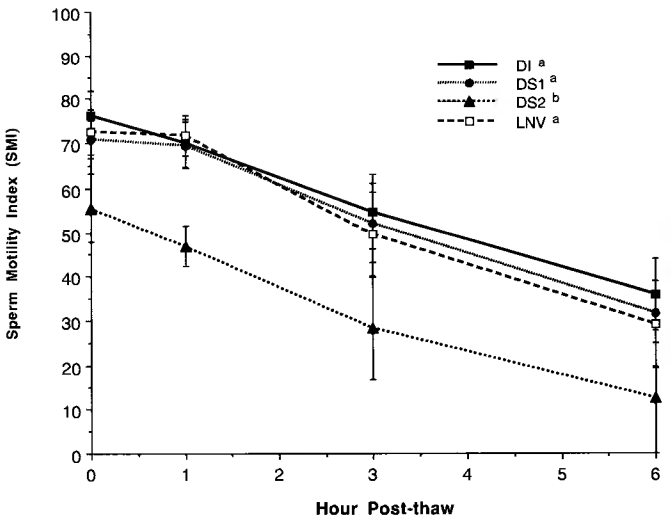

FIG. 1. SMI over time for thawed, swim-up processed scimitar-horned oryx spermatozoa cryopreserved on dry ice (DI), in a dry shipper (DS1, DS2), or over liquid nitrogen vapor (LNV). Treatments with different superscripts differ ($p < 0.05$).

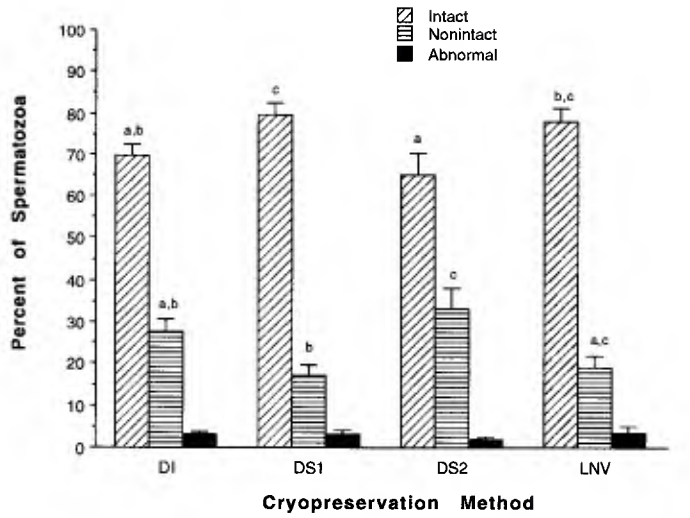

FIG. 2. Percentages of scimitar-horned oryx spermatozoa with intact, nonintact, and structurally abnormal acrosomes after cryopreservation on dry ice (DI), in a dry shipper (DS1, DS2), or over liquid nitrogen vapor (LNV). Different superscripts within sperm categories denote differences between treatments ($p < 0.05$).

and/or fertilization by the presence of $\geq$ 2 pronuclei or decondensed sperm heads. Numbers of spermatozoa penetrating the zonae also were determined. Developing embryos were evaluated 24 and 48 h later (66 and 90 hpi) for cleavage stage; and at 90 hpi, all embryos were fixed and stained with Hoechst 33342 to determine the number of nuclei.

Sperm Acrosome Evaluations

To assess sperm acrosomal status, slides were stained with fluorescein isothiocyanate-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA; Sigma) according to Roth et al. [17]. In brief, 10- to 20-μl aliquots of FITC-PNA (0.1 mg/ml PBS) were gently spread across slides that were incubated for 15 min in a dark, humidified container at 4°C. After incubation, slides were rinsed by dipping in cold PBS and air dried in the dark. Just before evaluation, a drop of mounting medium (4.5 ml glycerol, 0.5 ml PBS, and 5 mg *p*-phenylenediamine [Sigma]) was placed on the slide and covered firmly with a coverslip. Using fluorescent microscopy, acrosomal status of 100 spermatozoa per slide was evaluated (×400). Sperm cells were considered acrosome intact if the acrosome stained bright green, the acrosomal membrane was intact, and the acrosomal cap was located over the head region of the spermatozoa [17]. Sperm without intact acrosomes were those with no or only faint, patchy green staining of the head region, a single band of green stain at the equatorial segment, acrosomal caps lifted halfway or more off the head, or ballooning acrosomes. A third category included those spermatozoa with structurally abnormal heads and/or acrosomes.

Statistics

Data for sperm post-thaw motility and acrosomal status and for oocyte penetration, fertilization, and cleavage were assessed by ANOVA, and means were compared using a least significant difference test [21]. All percentage data were converted using arcsine transformation prior to ANOVA. For sperm motility and acrosomal status evaluations, independent variables were treatment and time. Across treatments, correlation coefficients were calculated between sperm characteristics (acrosomal status and motility at 0, 1, 3, and 6 h) and oocyte penetration, fertilization, and cleavage. Polynomial regression analysis was used to assess the cooling rate data and to compare the slopes of the curves. Data are presented as means ± SEM.

RESULTS

Sperm Motility and Acrosomal Status

Ejaculates contained 7.9–92.7 × 10^8 total sperm. Mean SMI for the six fresh ejaculates was 75.6 ± 2.4. Most spermatozoa in each ejaculate contained intact acrosomes (> 75%) and were morphologically normal (range, 58–94.5%). Final sperm concentration in straws ranged from 41 to 321 × 10^6/ml.

Post-thaw SMI for unprocessed samples ranged from 44.4 ± 4.1 for the DS2 treatment to 55.4 ± 5.0 for the DI method. Even after swim-up processing, the SMI for the DS2 method was lower ($p < 0.05$) than for the other three methods (Fig. 1). Across all treatments, SMI decreased ($p < 0.05$) over time.

There were no differences ($p > 0.05$) in the mean percentages of acrosome-intact spermatozoa at 0 and 6 h post-thaw for any treatments, so data for the time points were combined (Fig. 2). Most spermatozoa (> 65% on average) retained intact acrosomes regardless of the cryopreservation method (Fig. 2). However, fewer ($p < 0.05$) spermatozoa had intact acrosomes in the DS2 than in the DS1 or LNV treatments.

Heterologous IVF

A total of approximately 100 oocytes per treatment were evaluated. Percentage of oocytes with penetrated zonae did not differ ($p \geq 0.05$) among methods, ranging from 47.2 ± 5.9 (DS2) to 87.4 ± 9.4 (DI) (Fig. 3). Similarly, mean number of penetrating spermatozoa did not differ ($p \geq 0.05$), ranging from 1.6 ± 0.4 (DS2) to 4.4 ± 0.6 (LNV). Hybrid embryos were produced using samples cryopreserved by all four methods. Incidence of fertilization and polyspermy (range, 37.8 ± 14.8% [DS2] to 55.6 ± 4.8% [DS1] and 4.1 ± 2% [DS1] to 11.1 ± 3.7% [DS2], respec-

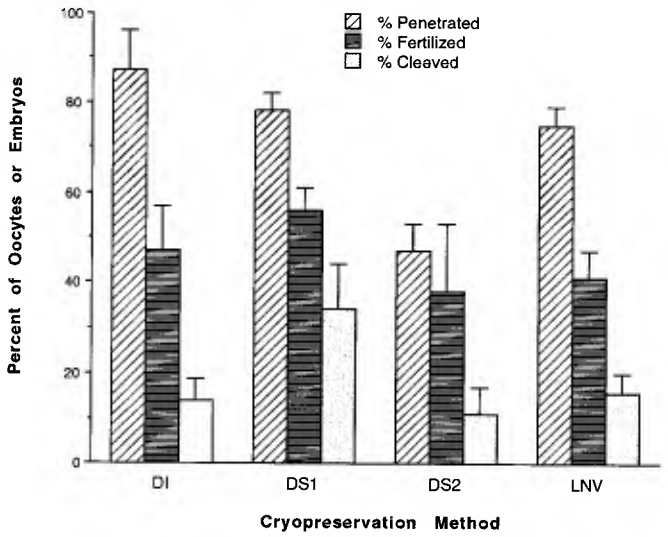

FIG. 3. Percentages of penetrated, fertilized, and cleaved hybrid embryos after insemination of IVM domestic cow oocytes with scimitar-horned oryx spermatozoa cryopreserved on dry ice (DI), in a dry shipper (DS1, DS2), or over liquid nitrogen vapor (LNV).

tively) did not differ ($p \geq 0.05$) among treatments. Cleavage success varied among sperm samples (range, 0–63%) but was similar ($p \geq 0.05$) across treatments (Fig. 3), with < 40% of inseminated oocytes cleaving in any treatment. However, ≥ 50% of cleaved embryos developed at least to the 5- to 8-cell stage. None of the parthenogenetic controls (n = 22) cleaved.

There were no correlations ($p > 0.05$) between cryopreservation method and post-thaw sperm quality traits examined. Across all treatments, there were positive correlations ($p < 0.05$; $r = 0.81$–0.97) between sample SMI at 3 and 6 h and penetration, fertilization, and cleavage success. In contrast, there were no significant correlations ($p > 0.05$) between IVF success and SMI at 0 or 1 h.

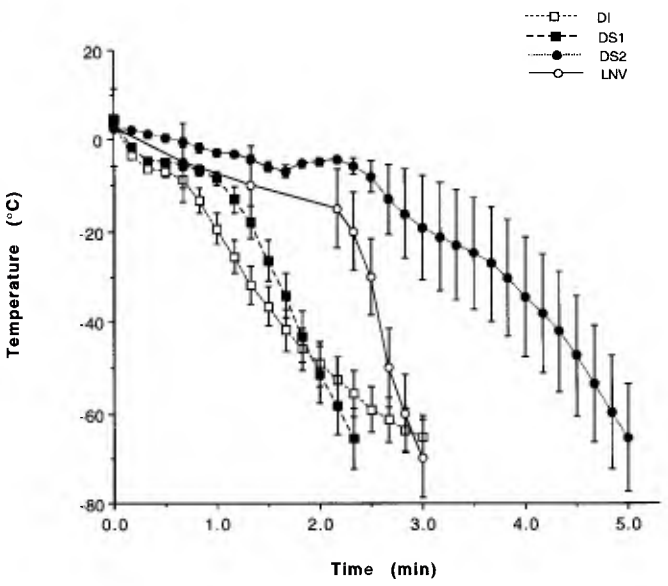

FIG. 4. Cooling rates associated with the four sperm cryopreservation methods tested: 1) dry ice (DI), 2) dry shipper one-step (DS1), 3) dry shipper two-step (DS2), and 4) liquid nitrogen vapor (LNV). No two methods produced similar cooling curve slopes ($p < 0.05$; n = 6 replicates).

Cooling Rates

On the basis of polynomial regression analysis, all cooling rate profiles followed quadratic functions with $r = 0.993$ (DI), 0.994 (DS1), 0.993 (DS2), and 0.951 (LNV). However, no two curves had similar slopes ($p < 0.05$; Fig. 4). The most apparent difference in cooling rate was associated with the DS2 method. Compared to what occurred with the DS1 and DI methods, temperatures in straws cryopreserved by the DS2 method decreased slowly, taking twice the amount of time to reach −60°C.

DISCUSSION

It is generally known that semen cryopreservation reduces sperm quality compared to that in fresh ejaculates [16]. However, a reduction in sample quality does not necessarily translate into reduced fertility. Because optimal laboratory cryopreservation protocols used in domesticated animals often are impractical when one is working with endangered species, especially in the field, it is important to identify alternative methods for storing spermatozoa under more challenging environments. Sperm preserved by these alternative methods may exhibit suboptimal post-thaw traits but still demonstrate high fertilizing ability. Results in the present study suggested that oryx spermatozoa tolerate a range of cooling rates associated with diverse cryopreservation methods. Even though post-thaw sperm motility varied, zona pellucida penetration, fertilization, and embryo cleavage were similar across methods.

Because the acrosome is particularly sensitive to damage during cryopreservation [22, 23], it was surprising that the percentage of acrosome-intact spermatozoa after thawing was high for all methods. The percentage of spermatozoa with intact acrosomes in freshly collected oryx ejaculates typically ranges from 70% to 90% [17], a finding consistent with this study. Interestingly, post-thaw samples contained 65–80% acrosome-intact spermatozoa, suggesting that only 10% of spermatozoa sustained acrosomal damage during the freeze-thaw process. A much higher incidence (40%) of post-thaw acrosomal damage was reported for the spermatozoa of other African hoof stock, including the greater kudu (*Tragelaphus strepsiceros*) and wildebeest (*Connochaetes taurinus*), in a study in which EQ extender also was used [10]. However, in that study, spermatozoa were cryopreserved by pelleting on dry ice, which may have accounted for some of the damage. Yet even after extensive studies in domestic species like the bull, ram, and boar, the freeze-thaw process still typically results in ≥ 20% of spermatozoa experiencing acrosomal damage [16, 22, 24]. Different methods of evaluating acrosomal status are unlikely to account for the higher proportions of acrosome-intact spermatozoa reported here, since results among methods are highly correlated [15]. The FITC-PNA stain is easy to use and reliable for evaluating oryx sperm acrosomes [17], so it was our method of choice.

A significant challenge to studying sperm quality in endangered species is identifying a method for fully assessing sperm function. Typically, it is not possible to conduct large-scale fertility trials or homologous IVF studies because the animals are not available. Even in domestic species, such trials can be expensive and time-consuming. Therefore, alternative sperm-oocyte interaction assays like the hemi-zona, salt-stored egg, and hamster egg penetration assays have been developed to evaluate sperm function in vitro [25–28]. Some of these approaches have proven useful in studies of wildlife species [29–31]. However, assays uti-

lizing nonviable, preserved oocytes or zonae may be inadequate and unreliable [17, 32, 33]. Furthermore, sperm penetration success in these assays does not always correlate with in vivo fertility or IVF success [17, 34, 35]. A heterologous IVF system developed previously for assessing oryx sperm function in vitro [17] offers a useful alternative.

The most significant finding in this study was the ability of oryx spermatozoa to fertilize domestic cow oocytes and produce hybrid embryos in vitro after cryopreservation by several different methods. These data provided strong evidence that oryx spermatozoa are fully functional after thawing and that various cryopreservation methods can be effective for sperm banking. Sperm cryopreserved by all methods maintained motility for > 6 h post-thaw. Despite reduced motility in the DS2 treatment, embryo production did not differ from that of spermatozoa cryopreserved by the other three methods. These results suggest that sample fertilizing ability is not necessarily compromised when post-thaw sperm motility is reduced by 20%.

Heterologous IVF success varied considerably among oryx bulls regardless of cryopreservation method. This was not surprising, since no fertility trials or ejaculate quality criteria were used to select study animals. Furthermore, there can be inherent variation among males in ability of spermatozoa to survive cryopreservation [36, 37]. It was noteworthy that spermatozoa from the animal producing the poorest sample were capable of producing embryos. In domestic livestock species, semen donors for assisted reproduction are chosen, in part, on the basis of semen quality and fertility ratings. In contrast, sperm banking in endangered species is dictated by genetics and driven by efforts to maintain optimal genetic diversity among populations [3, 38]. Therefore, animals targeted for genome resource banking are not necessarily producing the highest-quality ejaculates, and it is important that cryopreservation protocols maintain sperm function even of lower-quality samples.

On the basis of sperm functional competency post-thaw, all cryopreservation methods tested appeared acceptable for freezing oryx spermatozoa. This information is important because it provides alternatives that are better suited for field conditions. For example, dry ice can be difficult to acquire in some countries and dissipates rapidly in hot, arid climates. A tank of liquid nitrogen and the LNV method could provide a viable alternative. The dry shipper method obviates the need for dry ice or liquid nitrogen. Considering the simplicity of the method and post-thaw sample performance, DS1 might be the cryopreservation protocol of choice, even in the laboratory.

Overall, embryo cleavage success after insemination with frozen oryx spermatozoa was relatively low compared to the typical $\geq$ 70% cleavage achieved in homologous bovine IVM/IVF systems [39, 40]. Furthermore, cleavage can average as high as 70% in this heterologous system with the use of freshly collected oryx spermatozoa [17]. Lower fertilization and cleavage success with cryopreserved samples might suggest a reduction in sperm functionality compared to that of freshly collected samples. However, a high percentage of zonae pellucidae were penetrated by spermatozoa. Therefore, it is possible that the spermatozoa are fully functional but the IVF system simply is inadequate for supporting optimal fertilization and embryo cleavage. The goals of this study did not include refining post-thaw sperm processing and/or the IVM/IVF system to optimize IVF success. Instead, a standardized method was used throughout the study to determine whether spermatozoa could fertilize oocytes post-thaw and to compare results across treatments. Despite low cleavage success, $> 50\%$ of cleaving embryos developed to the $\geq$ 5–8 cell stage. These developmental data are similar to those reported for embryos produced using freshly collected spermatozoa [17]. Additional studies are under way to determine whether the functional competence of frozen-thawed oryx spermatozoa is inferior to that of the domestic bull and/or freshly collected oryx spermatozoa [17].

The lack of a significant correlation between sperm quality assessments and cryopreservation method or IVF success was likely observed because sample quality, overall, was relatively high. The positive correlation between IVF success and sperm motility at 3 and 6 h was not surprising. A similar relationship between sperm longevity and IVF success has been reported for another endangered species, the cheetah [41]. It was interesting that there were no correlations with IVF success and sperm motility at 0 or 1 h, but this also was probably due to relatively high motility assessments for all samples. These data suggested that a single post-thaw evaluation of sperm motility before AI or IVF may not be an accurate method for estimating a sample's potential fertility. Instead, sample longevity may be a more valuable predictor. Although caution should be exercised when one is extrapolating in vitro results to predict in vivo fertility, these data indicated that cryopreserved oryx spermatozoa are functionally competent and should be adequate for use in AI. In any case, because of the gradual decrease in SMI over time, as well as the correlation between post-thaw sperm longevity and embryo cleavage success, it might be important that females are inseminated within the 6-h interval preceding ovulation to maximize fertility.

Heterologous IVF systems substantially enhance our ability to study gamete function in endangered species. It seems likely that spermatozoa from diverse bovids will be capable of interacting with and fertilizing domestic cow oocytes. Therefore, this assay could have wide-reaching applications with regard to sperm function and cryopreservation studies among nondomestic hoof stock species. In this study, heterologous IVF was used to determine that spermatozoa from an endangered antelope are functionally competent in vitro after cryopreservation on dry ice, in a dry shipper, or over liquid nitrogen vapor. These results support the suggestion that these protocols can be used for sperm banking and that the quality of frozen-thawed spermatozoa is sufficient for assisted reproduction in the scimitar-horned oryx.

ACKNOWLEDGMENTS

The authors thank Linwood Williamson for his exceptional care of the animals and his assistance with semen collection. The authors also thank Drs. Mitch Bush, Steve Monfort, Suzan Murray, Barbara Wolfe, and Lisa Ware for veterinary support and assistance with semen collection.

REFERENCES

1. Wildt DE. Genetic resource banking for conserving wildlife species: justification, examples and becoming organized on a global basis. Anim Reprod Sci 1992; 28:247–257.
2. Wildt DE, Rall WF, Critser JK, Monfort SL, Seal US. Genome resource banks: living collections for biodiversity conservation. Bioscience 1997; 47:689–698.
3. Ballou JD. Potential contribution of cryopreserved germplasm to the preservation of genetic diversity and conservation of endangered species in captivity. Cryobiology 1995; 28:19–25.
4. Densmore MA, Bowen MJ, Magyar SJ, Amoss MS Jr, Robinson RM,

Harms PG, Kraemer DC. Artificial insemination with frozen, thawed semen and pregnancy diagnosis in Addax (*Addax nasomaculatus*). Zoo Biol 1987; 6:21–29.
5. Howard JG, Bush M, Morton C, Morton F, Wildt DE. Comparative semen cryopreservation in ferrets (*Mustela putorius furo*) and pregnancies after laparoscopic intrauterine insemination with frozen-thawed spermatozoa. J Reprod Fertil 1990; 92:109–118.
6. Monfort SL, Asher GW, Wildt DE, Wood TC, Schiewe MC, Williamson LR, Bush M, Rall WF. Successful intrauterine insemination of Eld's deer (*Cervus eldi thamin*) with frozen-thawed spermatozoa. J Reprod Fertil 1993; 99:459–465.
7. Swanson WF, Howard JG, Roth TL, Brown JL, Alvarado T, Burton M, Starnes D, Wildt DE. Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen-thawed spermatozoa in ocelots (*Felis pardalis*). J Reprod Fertil 1996; 106:87–94.
8. Howard JG, Roth TL, Swanson WF, Buff JL, Bush M, Grisham J, Marker-Kraus L, Kraus D, Wildt DE. Successful intercontinental genome resource banking and artificial insemination with cryopreserved sperm in cheetahs. J Androl 1997; (suppl 1):55 (abstract 123).
9. Morrow CJ, Wolfe BA, Roth TL, Wildt DE, Bush M, Blumer ES, Atkinson MW, Monfort SL. Endocrine response in scimitar-horned oryx (*Oryx dammah*) treated with progesterone and prostaglandin $F_{2\alpha}$ to synchronize ovulation for artificial insemination. Biol Reprod 1997; 56(suppl 1):131 (abstract 194).
10. Schiewe MC. Species variations in semen cryopreservation of nondomestic ungulates. Proc Wild Cattle Symp 1991; 56–64.
11. Howard JG, Pursel VG, Wildt DE, Bush M. Comparison of various extenders for freeze-preservation of semen from selective captive wild ungulates. Am Vet Med Assoc 1981; 179:1157–1161.
12. Roth TL, Swanson WF, Collins D, Burton M, Garell DM, Wildt DE. Snow leopard (*Panthera uncia*) spermatozoa are sensitive to alkaline pH, but motility in vitro is not influenced by protein or energy supplements. J Androl 1996; 17:558–566.
13. Wildt DE, Phillips LG, Simmons LG, Chakraborty PK, Brown JL, Teare A, Bush M. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard and puma. Biol Reprod 1988; 38: 245–255.
14. Amann RP. Can the fertility potential of a seminal sample be predicted accurately? J Androl 1989; 10:89–98.
15. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997; 56:991–998.
16. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Effect of cryopreservation on bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod 1998; 58:786–793.
17. Roth TL, Weiss RB, Buff JL, Bush LM, Wildt DE, Bush M. Heterologous in vitro fertilization and sperm capacitation in an endangered African antelope, the scimitar-horned oryx (*Oryx dammah*). Biol Reprod 1998; 58:475–482.
18. Roth, TL, Williamson LR, Bush M, Wildt DE, Monfort SL. Seasonal reproductive characteristics and steroid metabolite excretion in male scimitar-horned oryx (*Oryx dammah*). Biol Reprod 1997; 56(suppl 1): 198 (abstract 463).
19. Parrish JJ, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.
20. Howard JG, Bush M, Wildt DE. Semen collection, analysis, and cryopreservation in nondomestic mammals. In: Morrow DA (ed.), Current Therapy in Theriogenology. Philadelphia, PA: WB Saunders Co.; 1986: 1047–1053.
21. Steel RGD, Torrie JH. Principles and Procedures of Statistics. New York: McGraw-Hill Book Company; 1980.
22. Salamon S, Maxwell WMC. Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. Anim Reprod Sci 1995; 38:1–36.
23. Watson PF. The preservation of semen in mammals. In: Finn CA (ed.), Oxford Reviews of Reproductive Biology, Vol 1. Oxford, Clarendon Press; 1979: 283–350.
24. Maxwell WMC, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling or cryopreservation. Mol Reprod Dev 1997; 46:408–418.
25. Yanagimachi R, Lopata A, Odom CB, Bronson RA, Mahi CA, Nicolson GL. Retention of biologic characteristics of zona pellucida in highly concentrated salt solution: the use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa. Fertil Steril 1979; 31: 562–574.
26. Yanagimachi R. Zona-free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. Gamete Res 1984; 10:187–232.
27. Burkman LJ, Kruger TF, Coddington CC, Rosenwaks Z, Franken DR, Hodgen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. Fertil Steril 1988; 49:688–697.
28. Howard JG, Bush M, Wildt DE. Teratospermia in domestic cats compromises penetration of zona-free hamster ova and cat zonae pellucidae. J Androl 1991; 12:36–45.
29. Andrews JC, Howard JG, Bavister BD, Wildt DE. Sperm capacitation in the domestic cat (*Felis catus*) and leopard cat (*Felis bengalensis*) as studied with a salt-stored zona pellucida penetration assay. Mol Reprod Dev 1992; 31:200–207.
30. Howard JG, Wildt DE. Ejaculate-hormonal traits in the leopard cat (*Felis bengalensis*) and sperm function as measured by in vitro penetration of zona-free hamster ova and zona-intact domestic cat oocytes. Mol Reprod Dev 1990; 26:163–174.
31. Boatman DE, Bavister BD. Stimulation of rhesus monkey sperm capacitation by cyclic nucleotide mediators. J Reprod Fertil 1984; 71: 357–366.
32. Franken DR, Coddington CC, Burkman LJ, Oosthuizen WT, Oehninger SC, Kruger TE, Hodgen GD. Defining the valid hemizona assay: accounting for binding variability within zonae pellucidae and within semen samples from fertile males. Fertil Steril 1991; 56:1156–1161.
33. Lynham JA, Harrison RAP. Use of stored pig eggs to assess boar sperm fertilizing functions in vitro. Biol Reprod 1998; 58:539–550.
34. Codde JM, Berger T. In vivo fertility of rams in relation to sperm-zona pellucida binding and sperm-zona pellucida penetration of ovine oocytes. Theriogenology 1995; 44:901–906.
35. Swanson WF, McRae MA, Davidson JL, Levens GP, Campbell MK. Comparative sperm function and gamete interaction among small felid species from divergent evolutionary lineages. Biol Reprod 1998; 58(suppl 1):176 (abstract 334).
36. Schroeder-Jenkins M, Rothman SA. Causes of donor rejection in a sperm banking program. Fertil Steril 1989; 51:903–906.
37. McLaughlin EA, Ford WCL, Hull MGR. Motility characteristics and membrane integrity of cryopreserved human spermatozoa. J Reprod Fertil 1992; 95:527–534.
38. Johnston LA, Lacy RC. Genome resource banking for species conservation: selection of sperm donors. Cryobiology 1995; 32:68–77.
39. Keskintepe L, Brackett BG. In vitro developmental competence of in vitro-matured bovine oocytes fertilized and cultured in completely defined media. Biol Reprod 1996; 55:333–339.
40. Reed WA, Tae-kwang Suh, Bunch TD, White KL. Culture of in vitro fertilized bovine embryos with bovine oviductal epithelial cells, buffalo rat liver (BRL) cells, or BRL-cell-conditioned medium. Theriogenology 1996; 45:439–449.
41. Donoghue AM, Howard JG, Byers AP, Goodrowe KL, Bush M, Blumer E, Lukas J, Stover J, Snodgrass K, Wildt DE. Correlation of sperm viability with gamete interaction and fertilization in vitro in the cheetah (*Acinonyx jubatus*). Biol Reprod 1992; 46:1047–1056.