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Species-Specific Sperm-Egg Interaction Affects the Utility of a Heterologous Bovine In Vitro Fertilization System for Evaluating Antelope Sperm

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ABSTRACT

The purpose of this study was to evaluate cryopreserved fringe-eared (FE) oryx (*Oryx gazella callotis*) sperm function using a heterologous in vitro fertilization (IVF) system previously developed to study scimitar-horned (SH) oryx (*Oryx dammah*) spermatozoa. Semen was collected by electroejaculation from FE oryx (n = 2) and SH oryx (n = 2), evaluated immediately postcollection, and cryopreserved. Thawed spermatozoa were evaluated for motility, forward progression, and acrosomal status immediately post-thaw, after Percoll-separation, and 1, 2, 3, and 8 h after culture in IVF medium. In vitro-matured cow oocytes (n = 924) were inseminated with either domestic bull, FE, or SH oryx spermatozoa and after an 8-h coincubation period, half the oocytes were fixed and examined for sperm penetration, polyspermy, and male pronuclear formation. The remaining oocytes were placed into in vitro culture and evaluated for cleavage after 48 h. Overall, there were no between-species differences in sperm motility and acrosome integrity. However, an effect of time ($P < 0.05$) and a species-by-time interaction ($P < 0.05$) were detected for both parameters. Penetration, male pronuclear formation, and embryo cleavage were high (>90%, >85%, and >70%, respectively) for oocytes inseminated with domestic bull and SH oryx spermatozoa and did not differ ($P > 0.05$) between species. In contrast, very few oocytes (2.8%, 4 of 141) inseminated with FE oryx sperm were penetrated. Cleavage was rare (8.0%, 16 of 200) in oocytes inseminated with FE oryx spermatozoa and did not differ ($P > 0.05$) from that in parthenogenetic controls (4.2%, 3 of 72). Furthermore, FE oryx spermatozoa were incapable of penetrating zona-free cow oocytes. These results indicate that species-specific differences in gamete interaction may exist even between very closely related nondomestic bovids.

fertilization, IVF/ART

INTRODUCTION

Genome resource banking is becoming increasingly popular among zoos for preserving genetic variability [1, 2]. These genome banks containing frozen gametes and embryos have existed for decades, serving mainly as genetic and research resources [3]. However, the ability of thawed gametes or embryos from most nondomestic Bovidae to establish successful pregnancies is largely unknown. In contrast to the several breeding cooperatives in the United States that provide nonreturn rate data on domestic bull sperm fertility, zoos have very little information on the potential fertility of cryopreserved sperm from most endangered species. Regardless, pregnancies have been established in exotic species when cryopreserved spermatozoa were used in tandem with other reproductive technologies such as artificial insemination (AI) [4–7] or in vitro fertilization (IVF) and embryo transfer [8, 9]. However, the efficiency of these procedures in nondomestic animals has been considerably less than that routinely achieved in domestic species.

One challenge of developing reproductive technologies for nondomestic species is in determining appropriate protocols. Often, procedures proven highly effective in laboratory or domestic species are adopted for use in related nondomestic species. For example, hoofstock semen typically is cryopreserved using standard bovine and/or equine methods [10]. However, these protocols may not be optimal and could produce sperm samples compromised in their ability to fertilize oocytes [11]. To ensure that reduced sperm function is not a factor affecting pregnancy success following assisted reproduction in endangered species, samples should be tested rigorously post-thaw before being used.

Typically, post-thaw sperm quality is evaluated by cell survivability, motility, the ability to undergo capacitation, and the acrosome reaction [12]. However, the best indicator of post-thaw sperm quality is fertilizing capacity [13]. Heterologous IVF is an attractive method for evaluating the fertilizing capacity of frozen-thawed sperm in rare species because it does not require the use of valuable homologous gametes. Furthermore, compared to AI, heterologous IVF requires fewer sperm cells. Therefore, sperm function can be thoroughly tested while sparing valuable male gametes. However, the extent to which heterologous IVF can be used among related species remains largely a mystery.

Cross-species fertilization of oocytes from domestic farm or laboratory species has proven successful using cryopreserved sperm from endangered felids [14, 15] and nondomestic Bovidae [16–18]. Recently, cryopreservation and post-thaw sperm function analyses were performed in an endangered African antelope, the scimitar-horned (SH) oryx (*Oryx dammah*) [17, 18]. Heterologous inseminations of zona-intact domestic cow oocytes with SH oryx spermatozoa resulted in the production of oryx-cow hybrid embryos [17, 18]. The success of heterologous IVF with the SH oryx suggests that this system may be useful for testing sperm function in a range of bovid species. Moreover, the recent birth of several SH oryx calves produced by AI using pretested sperm [19] suggests that IVF performance of post-thaw sperm may provide useful selection criteria when choosing antelope sires and/or ejaculates for AI.

Another antelope species that may benefit from the use of assisted reproductive technologies is the fringe-eared (FE) oryx (*Oryx gazella callotis*). Although this animal thrives in the wild, the North American captive population of FE oryx is highly inbred, originating from five or fewer

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founders [20]. In order to reduce inbreeding depression in this captive population, conservation efforts designed to introduce new genetic material through AI are being explored. The goal of the current project was to apply the same semen collection and cryopreservation techniques proven effective in the SH oryx to the FE oryx and confirm that post-thaw sperm are equally functional. However, preliminary data with freshly collected semen indicated that FE oryx spermatozoa do not fertilize cow oocytes. Therefore, additional in-depth studies were designed to gain further insight into the functional differences between FE and SH oryx sperm.

Specific objectives were to 1) characterize post-thaw FE oryx sperm motility and acrosome integrity over time, 2) evaluate sperm penetration, polyspermy, and male pronuclear formation in zona-intact and zona-free cow oocytes after coincubation with oryx sperm, 3) determine the cleavage rate of FE oryx-cow hybrid embryos, and 4) compare results to those from parallel studies with domestic bull and SH oryx spermatozoa.

MATERIALS AND METHODS

Materials

Estradiol, heparin, Hepes, sodium pyruvate, antibiotics, mineral oil, and tissue culture medium-199 (TCM-199) were purchased from Sigma-Aldrich (St. Louis, MO). Folltropin was purchased from Agtech, Inc. (Manhattan, KS). Fetal calf serum (FCS) was obtained from Hyclone Laboratories, Inc (Logan, UT). Minimum essential medium (MEM) without glutamine was obtained from Life Technologies (Grand Island, NY), and unsupplemented embryo culture medium (simplex optimization medium, KSOM) [21] was purchased from Cell and Molecular Technologies Inc. (Phillipsburg, NJ). Modified Tyrode albumin lactate pyruvate (TALP) solutions (Hepes-TALP, SPERM-TALP, and IVF-TALP) were prepared as described by Parrish et al. [22]. However, IVF-TALP was modified to contain essentially fatty acid-free BSA (6 mg/ml) instead of fraction V. Frozen domestic bull semen (Jersey bull, n = 2 ejaculates) was acquired from Sire Technology Int. (Springfield, OH). Unless otherwise stated, all other chemicals and supplies were from either Sigma-Aldrich or Fisher Scientific (Orlando, FL). Investigations described herein were approved by the Center for Research of Endangered Wildlife's Animal Care and Use Committee.

Semen Collection and Cryopreservation

Two male FE oryx (Ogac 1 [4 yr old] and Ogac 2 [8 yr old]) and two male SH oryx (Oda 7 [11 yr old] and Oda 9 [8 yr old]) served as semen donors. Animals were fed diets of alfalfa or grass hay ad libitum and pelleted feed three times/day with continuous access to water and mineral blocks. Anesthesia and semen collection by electroejaculation for the SH oryx were as previously described [17, 18]. The FE oryx were immobilized on three separate occasions using a combination of 2.6 mg carfentanil (Wildlife Laboratories Inc., Fort Collins, CO) and 15 mg xylazine (Phoenix Scientific, St. Joseph, MO) by means of a projectile dart. Surgical anesthesia was achieved 4–6 min after darting, and animals were supplemented with 100 mg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) administered intravenously. An endotracheal tube was passed following recumbence as a precaution against regurgitation or apnea. Electroejaculation was performed as previously described [17, 18] with a series of stimuli, ranging from 2 to 6 V. An i.v. injection of 20 mg yohimbine (Wildlife Laboratories Inc.) and 250 mg naltrexone (Wildlife Laboratories Inc.) reversed anesthesia, and animal recovery was closely monitored for 24 h. Semen samples were evaluated immediately upon collection for volume and motility. Sperm concentrations were determined using a hemacytometer, and a 5-μl aliquot was fixed in PBS containing 6% (v/v) glutaraldehyde for sperm morphological analysis.

Sperm processing and cryopreservation were conducted as previously described [18]. In brief, ejaculates from each oryx were diluted 1:1 with EQ extender prewarmed to 37°C. Tubes containing the diluted semen were placed into a waterbath to which ice was occasionally added allowing samples to cool slowly for 1.5 h. When samples reached ≤8°C, similarly cooled EQ containing 10% (v/v) glycerol was added incrementally to the extended semen, with 20-min equilibration intervals between each addition (25%, 25%, and 50% of volume). After an additional 1 h incubation, 0.5-ml straws were filled with extended semen, sealed, and frozen in a liquid nitrogen dry shipper as previously described [18].

Oocyte Collection and In Vitro Maturation

Collection and in vitro maturation of domestic cow oocytes were as previously described [23, 24]. Abattoir ovaries were obtained from cows and washed several times with warm sterile saline (0.9% [w/v] NaCl) containing 100 U/ml penicillin-G and 100 μg/ml streptomycin (30–35°C). Cumulus oocyte complexes (COCs) were retrieved by slicing follicles (2–6-mm diameter) with a scalpel blade and then washing the ovaries vigorously in 200 ml of collection medium (TCM-199 with Hanks salts, 10 mM Hepes, 2% [v/v] FCS, 40 U/L heparin, 250 IU/ml penicillin-G, and 250 μg/ml streptomycin). Only COCs having one or more layers of cumulus cells and evenly granulated ooplasm were selected. Groups of 10 COCs were washed several times and placed into 50-μl drops of maturation medium (TCM-199 with Earle salts, 10% [v/v] FCS, 50 μg/ml gentamicin, 0.2 mM sodium pyruvate, 2 μg/ml estradiol, and 20 μg/ml FSH [Folltropin]) covered with mineral oil. COCs were allowed to mature for 22–24 h at 39°C in an atmosphere of 5% CO_2 in humidified air.

Sperm Preparation, IVF, and Embryo Culture

Sperm preparation and IVF of oocytes were conducted according to previously described methods [22–24] with some modifications. Straws of semen were thawed and washed by centrifugation through a Percoll gradient (45%/90% [v/v]) at 2000 × *g* for 20 min. The sperm pellet was collected from the bottom of the tube, diluted with 8 ml of sperm-TALP, and centrifuged at 1000 × *g* for 5 min. The supernatant was removed and the pellet resuspended in 100 μl of IVF-TALP. Sperm concentration was determined using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel).

After maturation, COCs were washed twice in Hepes-TALP, and groups of 30 randomly selected oocytes were transferred into four-well plates containing 600 μl IVF-TALP per well. Each well received 25 μl of the sperm suspension (for a final concentration of 1×10^6 motile sperm/ml) and 25 μl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl). Oocytes and sperm were allowed to coincubate for 8 h at 39°C in an atmosphere of 5% CO_2 in humidified air. After coincubation, presumptive zygotes were placed in microcentrifuge tubes containing 250 μl Hepes-TALP, vortexed for 5 min, and washed twice in Hepes-TALP. Half the presumptive zygotes (30/drop) inseminated with each sperm sample were placed into 50-μl drops of embryo culture medium (KSOM) supplemented with 2 mg/ml fatty acid-free BSA, 2.5 μg/ml gentamycin, 1× essential and nonessential amino acids, cultured at 39°C in 5% CO_2 in humidified air, and were evaluated for cleavage 48 h after insemination. The remaining presumptive zygotes were pipetted through a small-bore pipette to remove any remaining sperm and cumulus cells, mounted, and placed into fixative (25% [v/v] acetic acid in ethanol, room temperature) for 48–72 h. Presumptive zygotes were then stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined under a phase-contrast microscope at 200× and 400× magnification. Oocytes were considered penetrated when one or more sperm heads and/or male pronuclei and corresponding sperm tails were observed in the ooplasm.

Sperm Morphology, Motility, and Acrosomal Evaluations

Sperm morphology was evaluated in samples after Percoll gradient washing by placing 15 μl of sperm into 5 μl of fixative (8% [v/v] glutaraldehyde in PBS). After Percoll separation and washing, 50 μl of the sperm suspensions were added to 75-μl drops of IVF media containing 5.2 μl of PHE under oil, and maintained at 39°C in 5% CO_2. These drops provided a reservoir of sperm for evaluations over time. Sperm motility and acrosome integrity were evaluated as previously described [18]. The percentage of motile spermatozoa and forward progression (scale of 0–5, 0 = no forward movement and 5 = rapid forward movement) were evaluated immediately post-thaw, after Percoll gradient separation, and 1, 2, 3, and 8 h after insemination. At each time point, a sperm motility index (SMI = [percentage motile sperm + (forward progressive motility × 20)] ÷ 2) was determined, and a 10-μl aliquot of sperm was smeared on a slide and allowed to air dry. These slides were evaluated for sperm acrosome integrity by staining them with 20 μl of fluorescein isothiocyanate-conjugated *Arachis hypogaea* (peanut) agglutinin (0.1 mg/ml in PBS) for 30 min in a dark humidified chamber at 4°C. Slides were rinsed in PBS and air dried before evaluation. A drop of mounting medium (4.5 ml glycerol, 0.5 ml PBS, and 5 mg *p*-phenylenediamine [Sigma]) was placed

TABLE 1. Ejaculate and sperm characteristics from two fringe-eared oryx (Ogac 1 and Ogac 2) immediately after semen collection by electroejaculation (n = 3 ejaculates/animal); values are means ± SD.

Ejaculate characteristics	Animal	
	Ogac 1	Ogac 2
Total ejaculate volume (ml)	3.59 ± 2.05	1.73 ± 1.07
Sperm count/ml ($\times 10^8$)	2.16 ± 0.09	3.05 ± 2.95
Sperm motility (%)	86.7 ± 2.80	81.7 ± 2.80
Sperm progressive motility (scale 0–5)	4.00 ± 0.50	4.30 ± 0.30
Acrosome intact (%)	38.7 ± 12.50	87.7 ± 4.90
Viable sperm (%)	77.0 ± 10.40	80.3 ± 5.80
Morphologically normal (%)	74.7 ± 6.00	66.2 ± 9.40

under firmly mounted coverslips, and the acrosomal status of 100 spermatozoa/slide was evaluated (×400) using fluorescent microscopy (450–490 nm). Descriptions of oryx acrosomal status have been reported previously [18].

Zona-Free IVF

Domestic cow oocytes were collected and matured as described above. After maturation, oocytes were washed twice in Hepes-TALP and then vortexed for 5 min in Hepes-TALP containing hyaluronidase (300 μg/ml; Sigma) to remove cumulus cells. After vortexing, oocytes were washed and incubated in 0.1% pronase (w/v) in Hepes-TALP for 5–10 min to remove the zona pellucida. Zona digestion was observed continuously using an inverted microscope. When zonae pellucidae were no longer visible, oocytes were immediately washed twice in Hepes-TALP and placed into IVF medium. Oocytes were allowed to recover for 30 min prior to insemination. Zona-free oocytes were randomly assigned to two groups. One group was inseminated with FE oryx spermatozoa, whereas the second group was inseminated with domestic bull spermatozoa. Presumptive zygotes were fixed 8 h after insemination and evaluated for penetration as described above.

Statistics

Cleavage rate, penetration, polyspermy, mean number of sperm/oocyte, and pronuclei formation were analyzed by least-squares ANOVA using Statview (SAS Institute Inc., Cary, NC). All experiments were replicated three times and percentage data were subjected to arcsine transformation before analysis. Values are expressed as means ± SEM and are considered significant at $P \leq 0.05$. Differences between means were evaluated by Fisher protected least significant difference. For cleavage analysis, the microdrop was considered the experimental unit with cleavage being recorded separately for each drop. Individual means (n = 2/species; except domestic bull) were pooled for motility and acrosome analysis as an overall species estimation. For evaluation of motility and acrosome status over time, a two-way repeated-measures ANOVA was used. Analyses included the main effects of species and time and the species-by-time interaction.

RESULTS

Sperm Characteristics

Fresh ejaculate characteristics of the two FE oryx (Ogac 1 and Ogac 2) used in this study are shown in Table 1. There were no differences in morphology among thawed semen samples from the two SH oryx, FE oryx, and domestic bull after Percoll separation. At the time of insemination, >80% of sperm from all three species were morphologically normal.

Overall, sperm motility (calculated as SMI) over time did not differ ($P > 0.05$) among species (Fig. 1). After Percoll separation, SMI increased ($P < 0.05$) for all species compared to initial post-thaw values and then decreased ($P < 0.01$) for all species during in vitro culture. Furthermore, there was a species-by-time interaction ($P < 0.01$), indicating that the pattern of change in motility over time differed by species, with the domestic bull having a faster decline in SMI over time than either oryx species. Regardless, even after 8 h of culture, the SMI for the FE oryx and SH oryx were high (>45%).

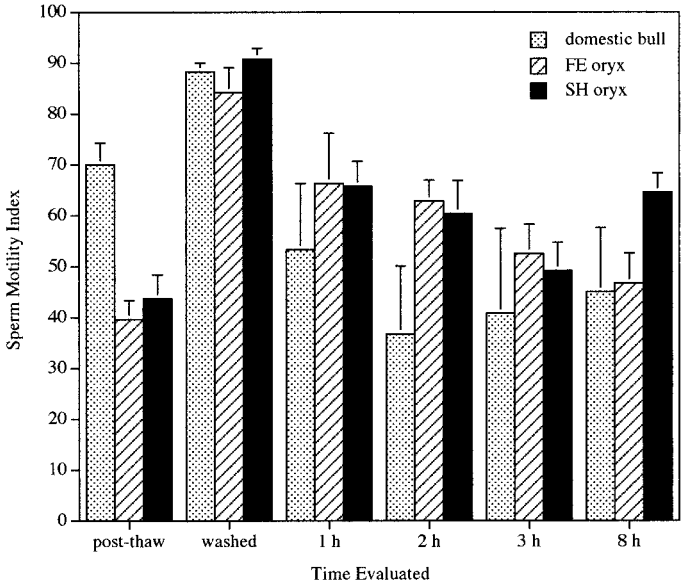

FIG. 1. Sperm motility index (SMI) post-thaw, after Percoll separation, and over time for domestic bull, FE oryx, and SH oryx spermatozoa maintained under IVF conditions. Columns are means ± SEM of three to four replicates/animal (domestic bull n = 1; FE oryx n = 2; SH oryx n = 2). Species had no effect ($P > 0.05$) on SMI; however, an effect ($P < 0.05$) of time and a species-by-time interaction ($P < 0.05$) were detected.

Similar to SMI results, the percentage of sperm with intact acrosomes did not differ ($P > 0.05$) across species. However, there was an effect of time on acrosome integrity ($P < 0.05$) and a species-by-time interaction ($P = 0.05$), indicating that the pattern of change in acrosome status over time differed by species (Fig. 2). After Percoll separation, the acrosome status of sperm from FE oryx remained rel-

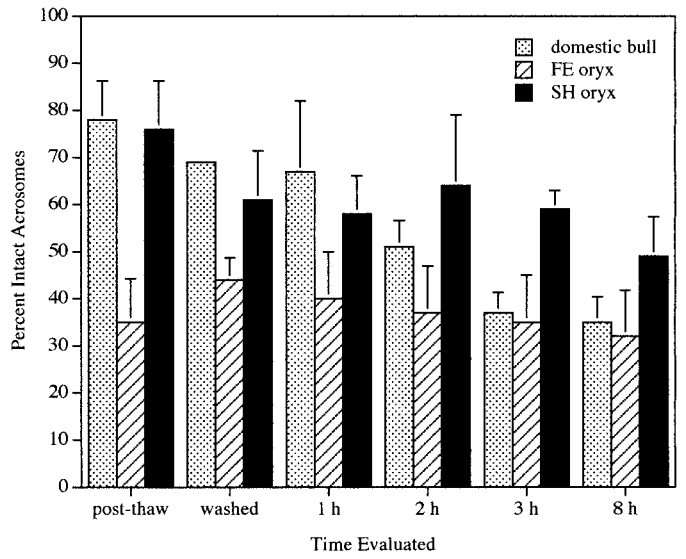

FIG. 2. Percentage of intact acrosomes post-thaw, after Percoll separation, and over time for domestic bull, FE oryx, and SH oryx spermatozoa maintained under IVF conditions. Columns are means ± SEM of three to four replicates/animal (domestic bull n = 1; FE oryx n = 2; SH oryx n = 2). Species had no effect ($P > 0.05$) on the percentage of intact acrosomes; however, an effect ($P < 0.05$) of time and a species-by-time interaction ($P < 0.05$) were detected.

TABLE 2. In vitro fertilization results following insemination of zona-intact and zona-free domestic cow oocytes with bull, FE oryx, and SH oryx spermatozoa.

Species	Oocytes				Mean no. spermatozoa in penetrated oocyte
	Examined (no.)	Penetrated (%)	Polyspermic (%)[a]	With MPN* (%)*	
Zona-intact					
Bull	83	94.0 ± 1.0[b]	28.0 ± 2.3[b]	88.0 ± 0.7[b]	1.40 ± 0.04[b]
FE oryx	141	3.5 ± 1.7[c]	0.0 ± 0.0[c]	8.3 ± 8.3[c]	0.50 ± 0.22[c]
SH oryx	147	88.3 ± 3.5[b]	13.8 ± 3.6[d]	92.7 ± 4.2[b]	1.24 ± 0.88[b]
Zona-free					
Bull	17	100	100	100	9.1
FE Oryx	72	0	0	0	0.0

* Male pronucleus.
[a] Percentage of the number of oocytes penetrated.
[b–d] Within a column, values with different superscripts are significantly different ($P < 0.05$). For zona-intact IVF, percentage values are means ± SEM of six replicates.

atively constant (35–45% intact) during the 8-h culture period in IVF medium. In contrast, the percentage of intact acrosomes decreased by 28% in the SH oryx, and by 50% in the domestic bull during the 8-h culture period.

Fertilization

For each of the four functional criteria employed to evaluate sperm-egg interaction, the performance of FE oryx spermatozoa was inferior ($P < 0.05$) to that achieved with SH oryx or domestic bull spermatozoa (Table 2). For all parameters evaluated, no differences ($P > 0.05$) were detected between individual ejaculates. The percentage of cow oocytes penetrated by sperm from SH oryx was high (>80%) and similar ($P > 0.05$) to that for domestic bull spermatozoa. In contrast, ≤6% of oocytes inseminated with FE oryx spermatozoa were penetrated. The majority (>95%; 137 of 141) of cow oocytes not penetrated by FE oryx sperm were found to be in metaphase II of meiosis, indicating these eggs were mature when coincubated with sperm. Across all species, polyspermy was observed in <30% of penetrated oocytes with percent polyspermy lower ($P < 0.05$) in the SH oryx compared to the bull, and absent in the FE oryx. Male pronuclear formation was similar ($P > 0.05$; ≥88%) in oocytes fertilized with either SH oryx sperm or domestic bull sperm. Of the four oocytes penetrated by FE oryx sperm, only a single oocyte contained a male pronucleus. Mean numbers of SH oryx and bull sperm per oocyte did not differ ($P > 0.05$). FE oryx spermatozoa (>50) were found tightly associated with the zona pellucida of cow oocytes. This tight association was not disturbed even when oocytes were vortexed to remove the cumulus cells. Furthermore, the number of FE oryx spermatozoa adhered to the zona pellucida of cow oocytes appeared greater than that of bull spermatozoa (<10) adhered to homologous oocytes.

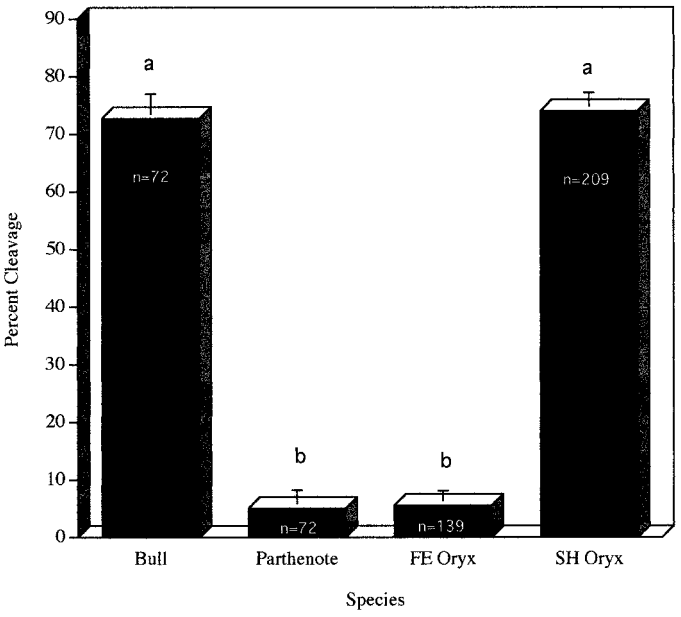

FIG. 3. Cleavage (44 h postinsemination) of domestic cow oocytes inseminated with either domestic bull sperm, FE oryx sperm, or SH oryx sperm. Included is the parthenogenetic control. Columns are means ± SEM of three to four replicates/animal (domestic bull n = 1; FE oryx n = 2; SH oryx n = 2). In each column, n = total number of oocytes within species. Different superscripts indicate significant differences ($P < 0.05$).

Zona-Free Penetration Assay

Removal of the zona pellucida by pronase digestion did not facilitate penetration of zona-free cow oocytes by FE oryx spermatozoa (Table 2). In fact, none of the zona-free oocytes were penetrated. Although numerous sperm (>50) were found tightly associated with the vitelline membrane, fusion of gamete membranes was never confirmed. In contrast, all zona-free cow oocytes inseminated with domestic bull sperm were penetrated and polyspermic with an average of 9.1 sperm per fertilized oocyte.

Cleavage

Cleavage clearly was affected by species ($P < 0.01$; Fig. 3). The percentage of oocytes cleaving after insemination with SH oryx spermatozoa was similar ($P < 0.05$) to that for oocytes inseminated with domestic bull spermatozoa (≥71%). In contrast, very few oocytes (<6%) inseminated with FE oryx spermatozoa cleaved, and this cleavage rate was not different ($P > 0.05$) from that (≤5%) for the parthenogenetic control oocytes. No differences ($P > 0.05$) in cleavage were detected between ejaculates within individuals.

DISCUSSION

In this study, species-specific differences were noted in the ability of oryx spermatozoa to fertilize cow oocytes. SH oryx spermatozoa readily fertilized cow oocytes, whereas no hybrid embryos resulted after oocytes were inseminated with FE oryx spermatozoa. A closer evaluation of these oocytes indicated penetration failure as the primary

cause for a lack of fertilization by FE oryx spermatozoa. These results were unexpected because previous studies have demonstrated that cow oocytes can be fertilized by SH oryx [17, 18], yak, bison, banteng, and gaur [16] spermatozoa, suggesting little cross-species specificity with regard to gamete interaction among Bovidae.

When IVF fails, it is typical to suspect sperm quality, especially when frozen-thawed samples are involved. However, in this study, deficiencies in FE oryx sperm quality were not apparent. A standard TALP medium established for domestic bovids [22] supported post-thaw sperm motility, morphology, viability, and acrosome integrity for both oryx species during the 8-h postinsemination evaluation period, suggesting that similar sperm requirements may exist among Bovidae. After Percoll gradient centrifugation and washing, a high percentage of FE oryx spermatozoa were motile and appeared morphologically normal. In fact, the recovered sample appeared to be of higher quality than the fresh sample initially collected. Thus, poor post-thaw sperm quality was not considered the primary factor responsible for fertilization failure when cow oocytes were inseminated with FE oryx spermatozoa. Instead it appears that species specificity, with regard to sperm-egg interaction, effectively prohibits heterologous fertilization between the domestic cow and FE oryx.

Among mammals, fertilization is comprised of several ordered steps. It begins in the oviduct with the binding of spermatozoa to the egg's extracellular coat and ends with the fusion of egg and sperm plasma membranes [25, 26]. Throughout this process, several important events must take place to ensure successful fertilization. During ovulation, oocytes must undergo meiotic maturation to prepare them for interacting appropriately with the sperm, and within the oviduct, sperm must undergo capacitation, a process that enables them to bind to eggs [26, 27]. The binding between homologous sperm and oocytes involves recognition between sperm receptors on the oocyte's zona pellucida and egg-binding proteins on the spermatozoa. After binding, capacitated sperm undergo the acrosome reaction and subsequently penetrate through the zona pellucida. Once inside the perivitelline space, sperm plasma membrane proteins will recognize and bind the complementary molecules on the oocyte's oolema, leading to membrane fusion and culminating in fertilization. Although the goal of this study was not to delineate precisely why fertilization failed between the cow and FE oryx, results do provide some insight into the species-specific block to fertilization.

Domestic cow oocytes recovered from slaughterhouse ovaries must undergo in vitro maturation before they are capable of being fertilized. An evaluation of domestic cow oocytes inseminated with FE and SH oryx spermatozoa indicated that >95% of all inseminated oocytes were mature. Furthermore, oocytes inseminated with bull or SH oryx spermatozoa were successfully fertilized. Therefore, oocyte quality or immaturity were not factors contributing to fertilization failure between FE oryx sperm and cow oocytes.

Another essential prebinding event, sperm capacitation, sometimes can be difficult to induce in an in vitro system. However, sufficient evidence exists to rule out capacitation failure as the cause of fertilization failure. First, the concentration of heparin in the IVF medium [22] should have been suitable for inducing capacitation as it has been for other nondomestic Bovidae [16, 17]. Moreover, samples that have undergone the freeze-thaw process typically contain a large proportion of capacitated spermatozoa due to cryodamage of sperm structures [28]. In addition, preliminary investigations of the capacitation status in FE oryx spermatozoa revealed that after a short incubation period in IVF medium the majority of sperm exhibited a capacitated staining pattern (data not shown). Perhaps the strongest evidence that capacitation occurred was the large number (~50) of spermatozoa tightly bound to the oocyte's vitelline membrane in the zona-free experiment. Only capacitated, acrosome-reacted spermatozoa are capable of binding to the vitelline membrane [29, 30].

The importance of zona proteins in initial sperm attachment and penetration through the zona pellucida is well documented [26, 31, 32]. One possible explanation for failed penetration of zona-intact oocytes is that those ligands and/or receptors present on the surface of the gametes failed to recognize each other. The zona pellucida has classically been recognized as the primary block to cross-species fertilization [25, 33], although the large numbers of FE oryx sperm adhered to the oocyte's zona pellucida argue against a lack of recognition as a primary cause. The association between the sperm and oocytes persisted even after vortexing (5 min) to remove the cumulus cells. In addition, FE oryx spermatozoa were observed within the zona pellucida, supporting the notion that binding and the acrosome reaction may have functioned normally. However, the presence of sperm in the perivitelline space of zona-intact cow oocytes was never confirmed. Therefore, it is possible that zona pellucida penetration was initiated but rarely successfully completed. The number of FE oryx spermatozoa bound to the zona pellucida of cow oocytes was greater than that of domestic bull spermatozoa bound to the zona pellucida of homologous cow oocytes. This phenomenon is likely due to the failure of the spermatozoa to fuse with and activate the oocytes; thus, cortical exocytosis and the subsequent zona reaction would not take place [34]. In the absence of the zona reaction, FE oryx spermatozoa would then continue to bind to the zona, accounting for the excessive numbers of sperm bound.

We hypothesized that once the zona pellucida was removed, zona-free cow oocytes would be fertilized by FE oryx spermatozoa, thereby confirming that the zona pellucida was the major block to fertilization. Surprisingly, membrane fusion and fertilization failed to occur even though the vitelline membranes of the zona-free oocytes were covered with tightly associated FE oryx spermatozoa that could not be removed by forceful pippeting. Hence, gametic incompatibility appears to exist at the vitelline membrane and possibly also the zona pellucida. This vitelline membrane block to cross-fertilization has also been observed in several other species including the rat, mouse, and rabbit [35, 36].

Whereas the vitelline membrane appears to be a major barrier preventing cross-fertilization, this block may only exist for some intergeneric (or more distant) crosses. A similar barrier does not seem to operate in intrageneric (closely related) crosses, as matings involving *O. dammah* × *Oryx leucoryx* and *Oryx gazella beisa* × *Oryx gazella gazella* can produce viable offspring [37]. Interestingly, intrageneric hybridizations have not been reported for the FE oryx. The sex chromosomes of *Oryx* and *Bos* differ widely in morphology [38], suggesting that genetic differences within the Bovidae family may be one reason for gametic incompatibility. However, why two evolutionarily related animals within the same genus, *Oryx*, exhibit vastly different fertilization specificities has yet to be determined.

The present study indicates that cryopreservation protocols established in the SH oryx [17, 18] can be success-

fully applied to the closely related FE oryx. However, full functional capacity of post-thaw sperm could not be evaluated because of apparent gamete incompatibilities between the FE oryx and domestic cow. Although blocks to cross-species fertilization are common, these results were surprising because the SH oryx, a close relative of the FE oryx, produces sperm that readily fertilize cow oocytes [17, 18]. While disappointing from an applied perspective, these findings are important because they suggest taxonomic relatedness is not always useful in predicting gamete function among the Bovidae family. Thus, these species of antelope may constitute interesting models for the study of interspecific isolating mechanisms, the genetics of sperm function, and molecular aspects of sperm-egg recognition.

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REFERENCES

1. Wildt DE, Rall WF, Critser JK, Monfort SL, Seal US. Genome resource banks: living collections for biodiversity conservation. Bioscience 1997; 47:689–698.
2. Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999; 4:143–150.
3. Soule' M. Conservation: tactics for a constant crisis. Science 1991; 253:744–750.
4. Howard JG, Donoghue AM, Goodrowe KL, Blumer E, Snodgrass K, Starnes D, Tucker M, Bush M, Wildt DE. Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (*Acinonyx jubatus*). J Zoo Wildl Med 1992; 23:288–300.
5. 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.
6. Loskutoff NM, Bartels P, Meintjes M, Godke RA, Schiewe MC. Assisted reproductive technology in nondomestic ungulates: a model approach to preserving and managing genetic diversity. Theriogenology 1995; 43:3–12.
7. Swanson WF, Howard JG, Roth TL, Brown JL, Alvarado T, Burton M, Starnes D, Wildt DE. Responsiveness of ovaries to exogenous gonadotropins and laparoscopic artificial insemination with frozen-thawed spermatozoa in ocelots (*Felis pardalis*). J Reprod Fertil 1996; 106:87–94.
8. Pope CE, Dresser BL, Chin NW, Liu JH, Loskutoff NM, Behnke EJ, Brown C, McRae MA, Sinoway CE, Campbell MK, Cameron KN, Owens OM, Johnson CA, Evans RR, Cedars MI. Birth of a western lowland gorilla (*Gorilla gorilla gorilla*) following in vitro fertilization and embryo transfer. Am J Primatol 1997; 41:247–260.
9. Swanson WF, McRae MA, Bond JB, Melniczek JR, Haskins ME. Homozygous and heterozygous mucopolysaccharidosis kittens produced by transfer of frozen-thawed IVF embryos. Biol Reprod 2000; 62:318.
10. 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.
11. Schiewe MC. Species variation in semen cryopreservation of nondomestic ungulates. In: Proc Wild Cattle Symposium; 1991; Omaha, NE; 56–64.
12. Holt WV. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology 2000; 53:47–58.
13. Brahmkshtri BP, Edwin MJ, John MC, Nainar AM, Krishnan AR. Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim Reprod Sci 1999; 54:159–168.
14. 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.
15. Swanson WF, Wildt DE. Strategies and progress in reproductive research involving small cat species. Int Zoo Yearb 1997; 35:152–159.
16. McHugh JA, Rutledge JJ. Heterologous fertilization to characterize spermatozoa of the genus *Bos.* Theriogenology 1998; 50:185–193.
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, Bush LM, Wildt DE, Weiss RB. 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. Biol Reprod 1999; 60: 493–498.
19. 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 F2 to synchronize ovulation for artificial insemination. Biol Reprod 1997; 56(suppl 1):131.
20. International Species Information System (ISIS). June Abstract Report; 1998: 243–244.
21. Lawitts JA, Biggers JD. Culture of preimplantation embryos. Methods Enzymol 1993; 225:153–164.
22. Parrish JJ, Susko-Parrish JL, Critser ES, Eyestone WH, First NL. Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology 1986; 25:591–600.
23. Edwards JL, Hansen PJ. Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes. Biol Reprod 1996; 55:340–346.
24. Paula-Lopes FF, de Moraes AAS, Edwards JL, Justice JE, Hansen PJ. Regulation of preimplantation development of bovine embryos by interleukin-1β. Biol Reprod 1998; 59:1406–1412.
25. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 189–318.
26. Wassarman PM. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. Cell 1999; 96:175–183.
27. Harrison RAP. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev 1996; 8:581–594.
28. Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000; 62:3–22.
29. Yanagimachi R. Penetration of guinea-pig spermatozoa into hamster eggs in vitro. J Reprod Fertil 1972; 28:477–480.
30. Hanada A, Chang MC. Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to sperm capacitation. J Reprod Fertil 1976; 46:239–241.
31. Evans JP. Getting sperm and egg together: things conserved and things diverged. Biol Reprod 2000; 63:355–360.
32. Rankin T, Dean J. The zona pellucida: using molecular genetics to study the mammalian egg coat. Rev Reprod 2000; 5:114–121.
33. Hanada A, Chang MC. Penetration of zona-free eggs by spermatozoa of different species. Biol Reprod 1972; 6:300–309.
34. Roldan ERS, Yanagimachi R. Cross-fertilization between syrian and chinese hamsters. J Exp Zool 1989; 250:321–328.
35. Hanada A, Chang MC. Penetration of the zona-free or intact eggs by foreign spermatozoa and the fertilization of deer mouse eggs *in vitro.* J Exp Zool 1978; 203:277–286.
36. Quinn P. Failure of human spermatozoa to penetrate zona free mouse and rat ova *in vitro.* J Exp Zool 1979; 210:497–506.
37. Gray AP. Mammalian Hybrids—A Check-List with Bibliography, 2nd ed. Farnham Royal: Commonwealth Agriculture Bureaux; 1972.
38. Kumamoto AT, Charter SJ, Kingswood SC, Ryder OA, Gallagher DS Jr. Centric fusion differences among *Oryx dammah, O. gazella,* and *O. leucoryx* (Artiodactyla, Bovidae). Cytogenet Cell Genet 1999; 86: 74–80.