Comparison of Various Extenders for Freeze-Preservation of Semen from Selective Captive Wild Ungulates

J. G. Howard, DVM; V. G. Pursel, PhD; D. E. Wildt, PhD; M. Bush, DVM

SUMMARY

Semen collected by electroejaculation from selected captive wild ungulates was used to evaluate the cryoprotective ability of 4 semen diluents. Three species of ungulates were used: blesbok *(Damaliscus dorcas phillipsi),* dorcas gazelle *(Gazella dorcas),* and onager *(Equus hemionus onager).* Fresh semen was immediately evaluated for the percentage of motility and the progressive motility, and an aliquot was fixed for evaluation of the acrosomal integrity of spermatozoa. The remaining volume of ejaculate was divided into equal aliquots and either frozen raw on dry ice or extended with each of the 4 diluents. Samples were concurrently equilibrated at 5 C, frozen on dry ice, and stored in liquid nitrogen. Aliquots were thawed for evaluation of motility traits and spermatozoal acrosomes. Each diluent provided cryoprotection in each species. However, spermatozoa varied in ability to survive cryopreservation, and postthaw viability was influenced by the semen diluent used. Although ineffective in the onager, the study of morphologic forms of spermatozoal acrosomes proved useful for assessing the cryoprotective property of semen diluents in the blesbok and dorcas gazelle.

NATURAL BREEDING of nondomestic species is desirable but not always possible because of: (1) behavior incompatibility, (2) limited gene pools, which can result in inbreeding, and (3) inherent problems associated with geographically separated individuals, including the risk and expense of animal shipment. In such cases, artificial breeding techniques used in domestic animal production can have application in enhancing captive propagation of certain nondomestic species. Successful procedures for collecting semen from zoological specimens by electroejaculation have been developed and are currently in use.[1-12] Investigations into the handling and freeze-preservation of spermatozoa from nondomesticated species have been sparse, and further data on methods for obtaining optimal recovery and minimal morphologic damage of spermatozoa after cryopreservation are needed.

The primary objective of the present study was to compare various diluents for freeze-preservation of semen from the following captive wild ungulates, blesbok *(Damaliscus dorcas phillipsi),* dorcas gazelle *(Gazella dorcas),* and onager *(Equus hemionus onager).* A secondary objective was to determine whether the morphologic forms of spermatozoal acrosomes, a useful criterion for assessing sperm viability in domestic species,[13-15] might have similar application in these species.

Materials and Methods

An adult male of each of the 3 species was housed in a zoological setting in sensory contact with females.

Electroejaculation—Each animal was anesthetized for electroejaculation by administering the following agents IM: blesbok, etorphine[a] (0.01 mg/kg of body weight) and xylazine[b] (0.14 mg/kg); dorcas gazelle, xylazine (0.23 mg/kg) and ketamine hydrochloride[c] (11.54 mg/kg); onager, etorphine (0.017 mg/kg). When etorphine was used and the electroejaculation procedure was completed, diprenorphine[d] was administered IV as the antagonist. Electroejaculation was initiated when a surgical plane of anesthesia was evident. A Teflon rectal probe containing 3 raised, longitudinal, stainless steel electrodes and a tapered tip was lubricated, inserted into the rectum, and positioned so that the electrodes were positioned ventrally. Probe diameter, probe length, and electrode length for each species were, respectively: blesbok, 2.7 cm, 38.5 cm, and 10 cm; dorcas gazelle, 1.6 cm, 20 cm, and 6.5 cm; and onager, 4.5 cm, 55 cm, and 15 cm.

The ejaculator was capable of monitoring voltage and amperage and used an AC current of 120 V, with a transformer producing a maximum of 60 V and 1 A.[e] A plastic collection vial was placed over the prepuce and gentle retraction was applied to the prepuce to expose the penis. The electrical stimuli were administered in sets of 10 serial stimulations applied at the same voltage and amperage. The stimuli were given in a 3-sec-on and 3-sec-

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The authors thank Dr. Don Janssen, Dr. Mike Loomis, Ms. Leah L. Schulman, Mr. Jon Camp, and Ms. Pat Schmidt for assistance in data collection and Ms. Jessie Cohen for photographs.

[a]M99 (1 mg/ml), D-M Pharmaceuticals Inc, Rockville, Md.
[b]Rompun (100 mg/ml), Haver-Lockhart, Shawnee, Kan.
[c]Ketaset (100 mg/ml), Bristol Laboratories, Syracuse, NY.
[d]M50-50 (2 mg/ml), D-M Pharmaceuticals Inc, Rockville, Md.
[e]PT Electronics, Sugarland, Tex.

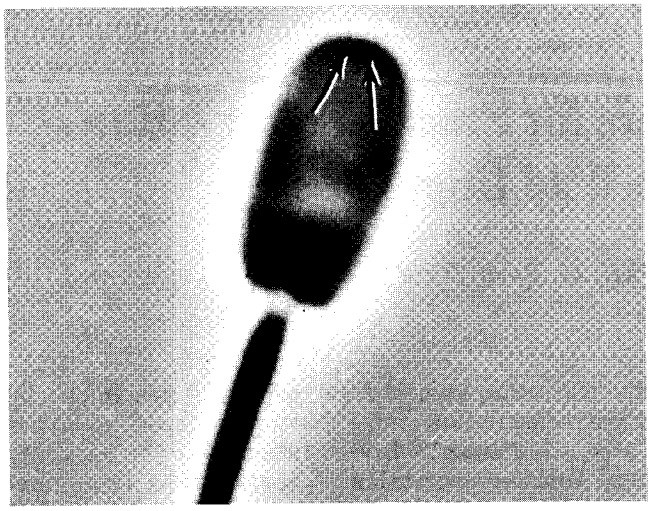

Fig 1—Phase contrast micrograph of dorcas gazelle spermatozoa with normal apical ridge (arrows).

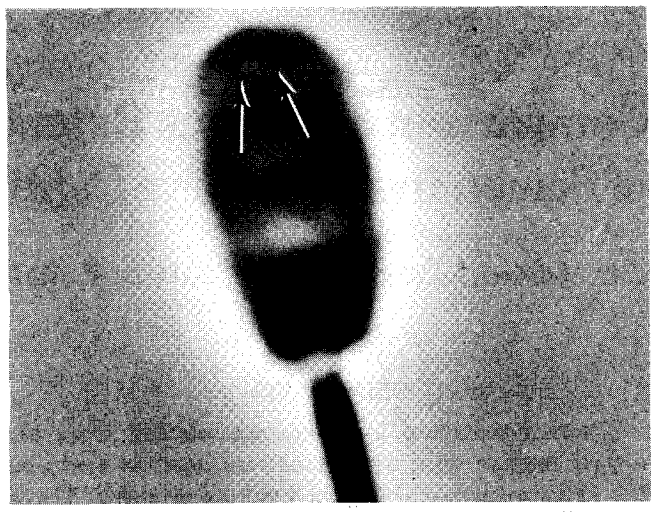

Fig 2—Phase contrast micrograph of dorcas gazelle spermatozoa with damaged apical ridge (arrows).

off pattern, with a continuous rise in voltage from 0 V to the desired peak, then returning to 0. Initial voltage was selected on the basis of the animal's response (hindlimb extension) during stimulation.[16] After the first 10 stimulations at initial voltage, the next set of 10 stimulations was increased 1 V. After 3 sets (a total of 30 stimulations), the procedure was discontinued and the ejaculate was evaluated. If additional ejaculate was required, a subsequent series of 30 stimulations (3 sets of 10 stimuli each) was administered. During the 2nd series, the first 10 stimulations were initiated at the intermediate voltage of the 1st series and increased in increments of 1 V for each additional 10 stimuli.

Semen Processing—Semen was evaluated immediately for ejaculate volume, percentage of motility, and progressive motility, and was processed for determination of sperm concentration, using the hemacytometer method. Progressive motility was an evaluation of the degree of forward movement of the spermatozoa, using a scale of 0 (lowest rating) to 5 (highest rating).[16] An aliquot of the unfrozen semen was fixed in 1% glutaraldehyde for evaluation of the morphologic features of spermatozoal acrosomes by phase contrast microscopy, as described by Pursel and Johnson.[17] The remaining ejaculate volume was divided into 5 aliquots and either maintained undiluted or diluted in a 1:1 semen-to-diluent ratio. The undiluted sample, processed in the same manner as the diluted aliquots, was used to demonstrate the most harmful effects of cryopreservation on undiluted semen. Four diluents (1–4) were tested. Diluent 1[f] consisted of 20% egg yolk, 11% lactose, and 4% glycerol in distilled water. Diluent 2[g] consisted of 20% egg yolk, 1.6% glucose, 1.6% fructose, 4% glycerol, 1.2% tes-n-tris (hydroxymethyl) methyl 2 aminoethane sulfonic acid, 0.2% tris (hydroxymethyl) aminomethane, and a 0.5% surfactant mixture of sodium and triethanolamine lauryl sulfate, in distilled water.[18] The remaining diluents were commercially prepared concentrates for bovine semen. Diluent 3[h] consisted of 20% egg yolk, 6% glycerol, antibiotics, and distilled water. Diluent 4[i] consisted of 3.6% egg yolk, 6% glycerol, antibiotics, and distilled water.

The undiluted and diluted samples were concurrently equilibrated at 5 C for 20 minutes, the temperature of the sample declining at the rate of approximately 1-degree C/minute. At the end of this 1st equilibration period, a 2nd dilution was made in an amount equal to the first. After equilibrating at 5 C for an additional 10 minutes, the samples were frozen by a technique originally described for bull semen.[19] Single drops of the semen were pipetted into 3 × 4-mm indentations in a block of solid CO_2 (dry ice). After 3 minutes, the frozen pellets were deposited in liquid nitrogen and transferred into vials[j] for storage.

Evaluation of Thawed Spermatozoa—Within 2 months of freezing, sperm aliquots were evaluated. Three pellets from each aliquot were thawed rapidly in 0.5 ml of 0.9% NaCl solution warmed to 37 C. The thawed pellets were examined microscopically for assessment of the percentage of motility and the progressive motility and then were fixed in 1% glutaraldehyde for evaluation of the acrosomal integrity. For each aliquot, 100 acrosomes were categorized by phase contrast microscopy into 4 morphologic classes: normal apical ridge (Fig 1), the acrosomes possessing a smooth crescentic apical ridge; damaged apical ridge (Fig 2), the acrosomes possessing irregular-shaped apical ridge; missing apical ridge (Fig 3), the apical ridge absent but the acrosomal cap firmly adhered to the nucleus; and loose acrosomal cap (Fig 4 and 5), the acrosomal cap loosened and vesiculated.

Correlation Coefficients—To determine the relationship of postthaw sperm motility and progressive motility to acrosomal integrity, correlation coefficients (r values) were calculated on a preprogrammed computer, using results obtained from individual and grouped species data.

Results

Semen was successfully collected by electroejaculation in the 3 species studied. Voltage, amperage, and number of sets of 10 stimulations used in each species, respectively, were: blesbok, 5 to 6 V, 50 to 60 mA, 2 sets; dorcas gazelle, 3 to 6 V, 25 to 60 mA, 8 sets; and onager, 2 to 4 V, 30 to 80 mA, 4 sets. Ejaculation usually commenced prior to full erection in the blesbok and onager, but the dorcas gazelle failed to obtain a full erection during the procedure. Ejaculate volume and sperm concentration per

[f]PDV-62, Life Forces Inc, College Station, Tex.

[g]BF5F, US Department of Agriculture, Agricultural Research Center, Beltsville, Md.

[h]Triladyl, MINITUB GmbH Abfull- und Labortechnik, West Germany.

[i]Plus-Ex, Edwards Agri-Supply Inc, Baraboo, Wis.

[j]Nalgene, Sybron Corp, Rochester, NY.

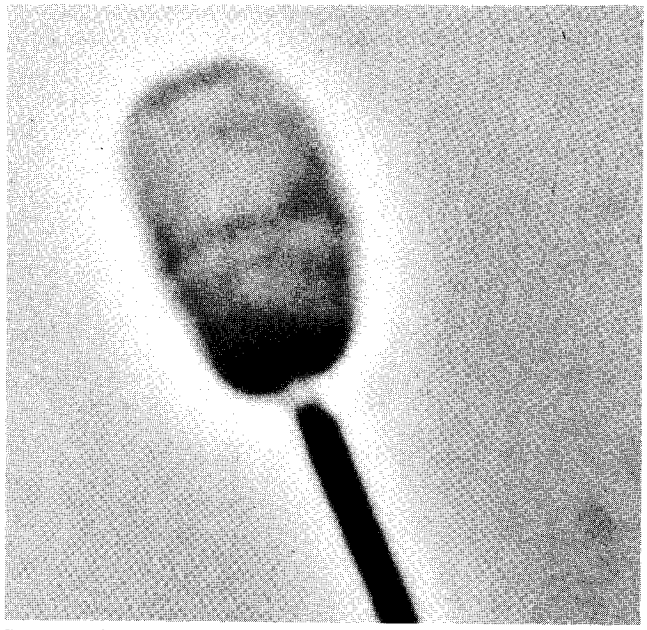

Fig 3—Phase contrast micrograph of blesbok spermatozoa with missing apical ridge.

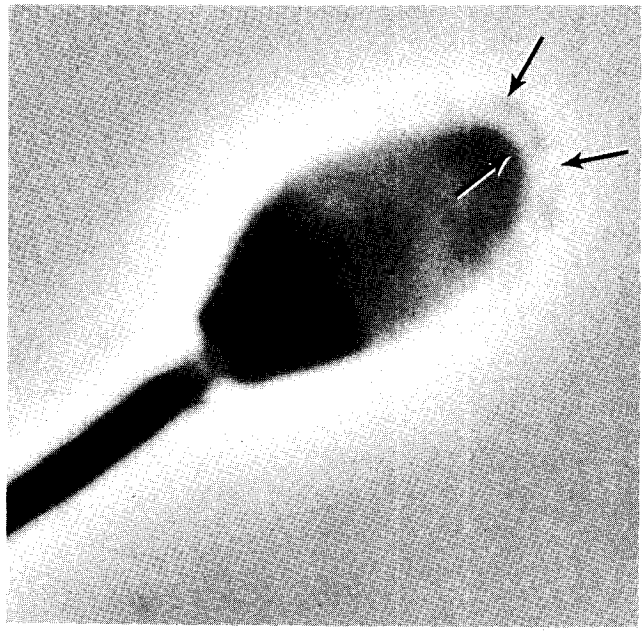

Fig 4—Phase contrast micrograph of blesbok spermatozoa with loose acrosomal cap (arrows).

milliliter for each animal were, respectively: blesbok, 7 ml, $1,548 \times 10^6$; dorcas gazelle, 2 ml, 317×10^6; and onager, 10 ml, 604×10^6. Dimensions of the spermatozoan head length (6.65 μm) and width (3.8 μm) were the same for each species.

Blesbok—Prefreeze sperm motility and progressive motility for the blesbok were 70% and 4, respectively, with most (92%) of the spermatozoa possessing a normal apical ridge (Table 1). Freezing raw undiluted semen resulted in 0% postthaw motility, 0 progressive motility, and considerable aberrance of acrosomal morphologic features. Most damage (42%) consisted of loose acrosomal caps. All diluents had satisfactory cryoprotective ability, with postthaw motility and progressive motility ranging from 40% to 50% and 3.5 to 4.5, respectively. Although the percentage of sperm with a normal apical ridge was less in the diluted frozen semen than in unfrozen raw semen, each diluent reduced acrosomal damage, when compared with the frozen raw aliquot. The ability of each diluent to reduce the percentage of spermatozoa with loose acrosomal caps was particularly evident. A general evaluation of data including postthaw sperm motility, progressive motility, and acrosomal integrity indicated that the 4 diluents were similar in cryoprotective ability. However, semen frozen in diluent 3 had the highest percentage of normal apical ridges and lowest percentage of loose acrosomal caps of the 4 diluents tested.

Dorcas Gazelle—Prefreeze sperm motility and progressive motility for the dorcas gazelle were 90% and 5, respectively, with 79% of the spermatozoa possessing a normal apical ridge (Table 1). Freezing raw undiluted semen resulted in 0% postthaw motility and 0 progressive motility, and most (89%) of the acrosomal damage was loose acrosomal caps. Considerable variation in postthaw results was evident among the spermatozoa frozen in the 4 diluents. Diluent 3 produced the greatest percentage of postthaw motility (40%) and the lowest percentage of loose acrosomal caps (16%), although a considerable percentage of cells had damaged (32%) or missing (17%) apical ridges. The remaining diluents did afford some cryoprotection, when compared with the frozen raw aliquot; however, postthaw motility ranged from 10%–30%, and large proportions (31%–56%) of spermatozoa frozen in diluents 1, 2, and 4 had loose acrosomal caps. The total percentage of spermatozoa with normal apical ridges did not appear different among the aliquots in

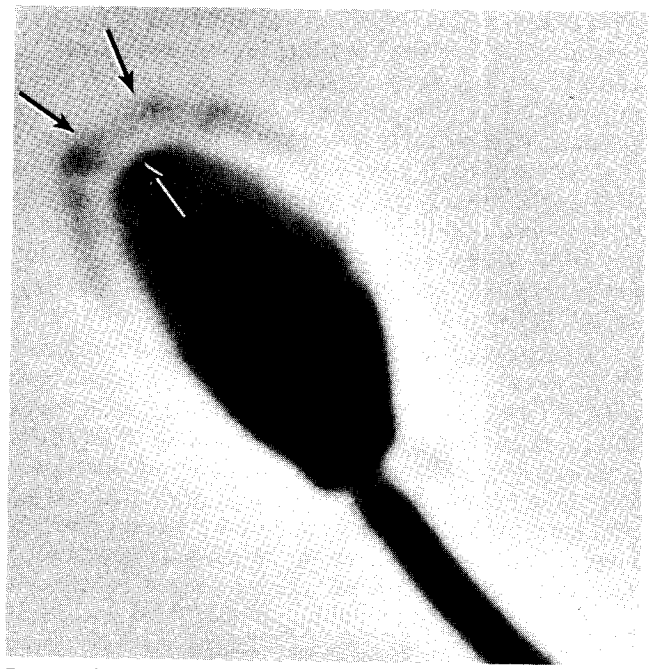

Fig 5—Phase contrast micrograph of onager spermatozoa with loose acrosomal cap (arrows).

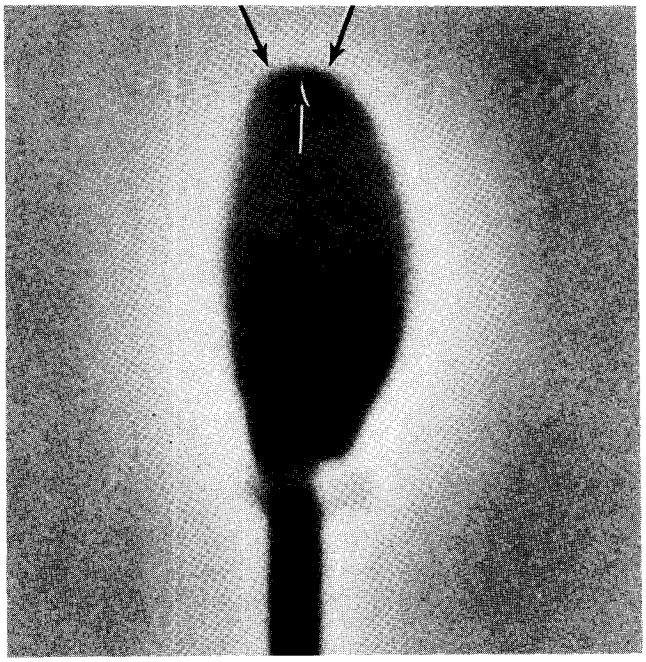

Fig 6—Phase contrast micrograph of onager spermatozoa with normal acrosomal cap (arrows).

diluents 2, 3, and 4. Overall data assessment indicated that, of the 4 diluents tested, diluent 3 afforded the most suitable cryoprotection of dorcas gazelle semen.

Onager—Prefreeze sperm motility and progressive motility for the onager were 90% and 5, respectively (Table 1). Apical ridges were not morphologically distinguishable on the spermatozoa of this species (Fig 6). Spermatozoa with loose acrosomal caps were readily detectable and, therefore, were used as the sole criterion for assessing acrosomal deterioration. Freezing raw undiluted semen resulted in 0% postthaw motility, 0 progressive motility, and 84% loose acrosomal caps. The percentage of postthaw motility (60%) and progressive motility (4) were similar for diluents 1 and 2, and the values were greater than values for the 2 commercial bovine diluents (40% motility, 2 progressive motility). The proportion of loose acrosomal caps did not vary markedly among tested diluents (0%–14%). Based on an overall evaluation of the semen characteristics examined, diluents 1 and 2 provided improved cryoprotection of onager spermatozoa when compared with results obtained with diluents 3 and 4.

Relationship of Sperm Motility and Progressive Motility to Acrosomal Integrity—Within and across species lines, a definite relationship existed between the proportion of thawed spermatozoa with the loose acrosomal cap characteristic and the percentage of sperm motility and progressive motility rating (Table 2). Relatively high negative *r* values for each species indicated that, as the number of sperm with loosened caps increased, there was a decreased percentage of motile sperm, as well as a decline in progressive motility. The correlation coefficients for these traits were particularly significant in the blesbok and onager.

TABLE 1—Motility, Progressive Motility, and Acrosomal Integrity of Blesbok and Dorcas Gazelle Spermatozoa Before and After Freezing

	Motility %	Progressive motility*	Acrosomal integrity (%)†			
			NAR	DAR	MAR	LAC
		PREFREEZE				
Blesbok						
Unfrozen raw	70	4	92	4	3	1
		POSTTHAW				
Frozen raw	0	0	20	10	28	42
Diluent 1	50	4.5	49	17	27	7
2	50	4	55	18	19	8
3	50	4	63	3	33	1
4	40	3.5	54	24	16	6
		PREFREEZE				
Dorcas Gazelle						
Unfrozen raw	90	5	79	17	1	3
		POSTTHAW				
Frozen raw	0	0	10	1	0	89
Diluent 1	30	3.5	24	15	5	56
2	10	3	36	23	10	31
3	40	3.5	35	32	17	16
4	20	3	31	17	10	42
		PREFREEZE				
Onager						
Unfrozen raw	90	5	100	...	0	...
		POSTTHAW				
Frozen raw	0	0	16	...	84	...
Diluent 1	60	4	97	...	3	...
2	60	4	100	...	0	...
3	40	2	94	...	6	...
4	40	2	86	...	14	...

*Based on degree of forward motility of spermatozoa, using a scale of 0 (lowest rating) to 5 (highest rating). †Based on evaluation of 100 spermatozoal acrosomes. NAR = normal apical ridge, DAR = damaged apical ridge, MAR = missing apical ridge, LAC = loose acrosomal cap.

TABLE 2—Correlation Coefficients Between the Proportion of Postthaw Spermatazoa with Loose Acrosomal Caps and Postthaw Percentage of Motility and Progressive Motility

	Blesbok	Dorcas gazelle	Onager	All species
r, LAC, and percentage of motility*	−0.97	−0.68	−0.95	−0.80
r, LAC and progressive motility	−0.96	−0.64	−0.86	−0.76

*Calculations based on aliquots of semen frozen either raw or in diluents 1–4. *r* = correlation coefficient, LAC = loose acrosomal caps.

Discussion

For commercial reasons, artificial breeding techniques, including the use of frozen semen, have centered in domestic farm industries. To date, the routine application of such procedures to assist propagation of captive wild species has been limited. In this study, we demonstrated the feasibility of collecting semen from selected wild ungulates, using the technique of electroejaculation. More important, the data suggest that spermatozoa in the ejaculates of these ungulates vary in ability to survive cryopreservation and that this survival can be influenced markedly by the semen diluent used. All 4 diluents tested provided sperm cryoprotection, as determined from the subjective and morphologic criteria. However, no diluent was determined superior across species lines. This suggests that comparative semen diluent testing similar to that used in the present study may be a prerequisite to the intensive cryobanking of spermatozoa from any particular zoological species.

Postthaw motility and progressive motility have been frequently used traits for assessing ejaculate quality in domestic species. However, acceptable motilities have been observed in thawed porcine semen in which morphologic evaluations have revealed high percentages of damaged or disintegrat-

ing spermatozoa.[20-21] Because the acrosome and its enzymes play an essential role in fertilization, it would appear beneficial to determine the fertilizing potential of frozen-thawed semen on the basis of a morphologic analysis of the spermatozoa, as well as postthaw motility and progressive motility. Acrosomal integrity has proved to be a valuable criterion for determining cryoprotective properties of porcine semen diluents and is highly correlated with fertilization capacity.[18,20,22] During freezing of the spermatozoon, the acrosome can deteriorate progressively so that the apical ridge can become damaged or completely lost or the acrosomal cap can become loosened. Recent investigations have determined that loose acrosomal caps most adversely affect fertilizing capacity of porcine semen.[23]

Although the composition of the 4 diluents varied, all were similar in ability to protect blesbok semen during freezing. However, in the other ruminant species, the dorcas gazelle, postthaw percentage of motility and the proportion of spermatozoa with loose acrosomal caps varied dramatically among aliquots frozen in each of the 4 diluents. Spermatozoa of the onager survived freezing particularly well in diluents 1 and 2. This observation is interesting, since the onager is an equid and its domestic counterpart *(Equus caballus)* traditionally does not experience routine success in the area of artificial insemination with previously frozen spermatozoa.[24] Demick et al demonstrated that stallion spermatozoa were adversely affected by diluents containing 7% or greater concentrations of glycerol.[25] In our study, the poorer postthaw results for onager spermatozoa diluted with diluents 3 and 4 may have been the result of the greater glycerol concentration (6%), in comparison with the 4% glycerol in the other diluents.

Acrosomal integrity may be a valuable additional criterion for future studies involving cryoprotective properties of semen diluents or for improving assessments of fertility potential of semen from selected wild ungulates. However, as evidenced by the results in the onager, apical ridge status may not be a dependable trait in all species. The morphologic features of the apical ridge were not readily detectable by use of phase contrast microscopy in the onager. Results in the blesbok and dorcas gazelle resembled the results in previous studies involving swine and sheep[15,26] in that the integrity of the apical ridge could be assessed by phase contrast microscopy. In contrast, the apical ridge of domestic bovine spermatozoa was not commonly discerned by phase contrast microscopy and such evaluation required interference contrast microscopy.[14] The morphologic features of spermatozoal acrosomes in the onager were similar to those reported in the domestic horse.[27] In the horse, the acrosome was small and the apical ridge was not readily detectable by phase contrast or interference contrast microscopy. The structural integrity of the acrosomal cap could be observed in all 3 ungulate species, and this particular feature eventually may prove to be a reliable indicator of male fertility potential. This trait may be particularly valuable because it was closely correlated with both sperm motility and progressive motility in each species studied.

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