Pretreatment of Addra gazelle (*Nanger dama ruficollis*) spermatozoa with cholesterol-loaded cyclodextrins improves cryosurvival

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Abstract

Preserving genetic diversity of the critically endangered Addra gazelle (*Nanger dama ruficollis*) could be enhanced through the use of frozen-thawed sperm and artificial insemination. Our aim was to characterize Addra ejaculate traits and to assess the effects of cholesterol-loaded cyclodextrin (CLC) on sperm cryosurvival. Fresh ejaculates were treated with CLC (0, 0.5, 1.5, 3.0, and 6.0 mg/ml) prior to cryopreservation. All males produced spermic ejaculates with >75% sperm motility. The mean $\pm$ SEM seminal volume, sperm concentration, percent motility, forward progression, and percent morphologically normal spermatozoa were 3.2 ± 0.3 ml, $1.2 \pm 0.3 \times 10^9$, $75.8 \pm 2.7\%$, 3.2 ± 0.3 (0-5 scale; 5 = most progressive), and $57.1 \pm 3.8\%$, respectively. More than 92% contained an intact acrosome. There was no effect of time or *in vitro* incubation on progression or acrosomal integrity on thawed samples ($P > 0.05$). Spermatozoa pre-treated with 0.5 mg/ml CLC retained higher ($P < 0.05$) motility post-thaw than aliquots treated with 0, 3.0, or 6.0 mg/ml of CLC. Spermatozoa pre-treated with 0.5, 1.5, or 3.0 mg/ml CLC exhibited greater viability than counterparts ($P < 0.05$). Sperm kinetics including beat cross frequency (BCF), average path velocity (VAP), curvilinear velocity (VCL), and straight line velocity (VSL) did not differ among samples ($P > 0.05$). Linearity (LIN) and straightness (STR) were different among samples after thawing. Results demonstrate treatment with CLC (0.5 mg/ml) protects Addra spermatozoa from cryo-damage. Reported advances will facilitate establishment of a frozen repository and support the genetic management of this critically endangered north African desert antelope.

Keywords: Cryopreservation; Cholesterol; Antelope; Acrosome Reaction; Endangered Species

Introduction

The Addra gazelle (*Nanger dama ruficollis*), an antelope and member of the Bovidae family, is listed as critically endangered by the International Union for the Conservation of Nature (IUCN) and under Appendix I by the Convention of the International Trade in Endangered Species of Wild Flora and Fauna [6, 33]. Addra gazelle are one of three subspecies constituting the Dama gazelle species (*Nanger dama*), the other two being the Mohor gazelle (*N. d. mhor*) and the Dama gazelle (*N. d. dama*). Indigenous to the Sahelo-Saharan region of northern Africa, Dama gazelle once ranged across the grasslands, wooded savanna, and sub-desert steppes of countries including Libya, Morocco, Mauritania, Nigeria, and Tunisia [25, 33]. Now assumed to be extinct in northern Africa; small populations are limited to fragmented areas within Chad, Niger, and Mali [33]. Adverse impacts to the wild population include habitat degradation and loss through overgrazing by domestic species, agricultural expansion, unregulated hunting and extended drought [25, 40, 56]. It is estimated that fewer than 2,000 Dama gazelle remain worldwide and >75% are managed in *ex situ* collections [56, 58]. The number of Addra gazelle in the wild remains unclear but approximately 168 individuals are managed in North American zoos as part of the Association of Zoos and Aquariums' Species Survival Plan [46]. Extant populations (*in situ* and *ex situ*) are extremely segregated increasing the possibility of inbreeding and potentially exerting negative impacts on reproduction [24, 53].

Segregation of *ex situ* populations creates a challenge for managers striving to maintain optimum genetic diversity. Shipping animals for breeding purposes is logistically difficult, costly, and mating pairs, though genetically compatible, may not be behaviorally compatible, possibly leading to injury or failure to mate [50]. To maintain genetic diversity and limit the effects of inbreeding, preserving gametes and developing reproductive technologies to facilitate successful offspring production and reduce risks and costs associated with shipping animals, are

necessary [50, 57]. However, there is minimal information on the reproductive characteristics of the Addra gazelle. In the wild, Dama gazelle females calve during seasons when food resources are not limited [56, 70]. Mohor gazelle males in captivity are considered reproductively competent by 2 years of age [1] but no clear reproductive seasonality has been observed [28]. Furthermore, the ejaculate traits have been characterized in the Mohor gazelle [1], Cuvier's gazelle (*Gazella cuvieri*), and Dorcas gazelle (*Gazella dorcas neglecta*) [21]. but not the Addra gazelle. Garde et al. (2003) also reported that in the Mohor gazelle, sperm motility was inversely related to age and inbreeding coefficient [1, 21]. Therefore, it is essential to gain further insight into the basic reproductive biology of the Addra gazelle including general ejaculate characteristics and sensitivity to cryopreservation, and to develop strategies to mitigate cryo-damage. The development of sperm cryopreservation in conjunction with artificial insemination (AI) can facilitate offspring production [30, 50, 64]. Species-specific differences can create a challenge in optimizing such tools [31, 65, 66]. Sperm morphology, membrane structure and permeability, and cell surface area:volume ratio differ among species, and each characteristic may influence spermatozoa tolerance to cold and osmotic changes encountered during freezing and thawing [8].

Spermatozoa encounter multiple stressors during the cryopreservation process, some of which can lead to irreversible damage to the phospholipid bilayer plasma membrane resulting in loss of cell function and/or cell death [26, 61]. Addition of cryoprotective agents (CPAs) creates a hypertonic environment in the extracellular milieu resulting in changes in cellular volume as water moves out of the cell, while the CPA moves in [20, 26, 27]. Cooling sperm at a supra-optimal rate can result in sub-optimal dehydration and formation of intracellular ice crystals, damaging organelles and altering cellular function [15, 27, 43]. Despite the use of

95 cryoprotectants, there is sometimes extensive cryo-damage observed in sperm after freezing and
96 thawing, as indicated by decreased motility, viability, and membrane (plasma and acrosomal)
97 integrity [48, 59].

98 The ability to modify the sperm membrane prior to cryopreservation has been shown to
99 increase post-thaw survival in the domestic bull [2, 38, 51, 52], stallion [37, 41, 60], ram [36],
100 goat [17], boar [18, 62]) and non-domestic (camel [9] and elephant [34]) mammals, as well as in
101 fish [39]. It has been demonstrated that cholesterol content and the cholesterol:phospholipid ratio
102 are among several factors influencing cold shock sensitivity of cells. The spermatozoa of species
103 with an inherently higher cholesterol:phospholipid ratio, such as the human (0.99) and rabbit
104 (*Oryctolagus cuniculus*; 0.88), appear to withstand cryopreservation better than those with lower
105 ratios, including the bull (0.45), rat (*Rattus norvegicus*; 0.58), ram (*Ovis aries*; 0.24), and boar
106 (*Sus scrofa domesticus*; 0.23) [11, 13, 31, 44, 63]. In bulls, artificially increasing cholesterol
107 content of spermatozoa by 2-3 times the normal amount, thereby changing the
108 cholesterol:phospholipid ratio from 0.45 to 0.90, is shown to increase post-thaw motility and
109 viability [51, 52]. However, there is an adverse influence of increasing the cholesterol content 4-
110 5 times, which for bull sperm, has led to premature cell death, likely by disrupting normal cell
111 function [51, 52]. Therefore, although altering the lipid composition by incorporating lipids into
112 the plasma membrane can increase spermatozoon cryosurvival, there appears to be a threshold
113 beyond which such alterations are detrimental [27].

114 Cyclodextrins are cyclic oligosaccharides with a hydrophilic exterior and hydrophobic
115 interior that have a high affinity for sterols [5]. Capacitation is induced in spermatozoa by using
116 cyclodextrins to remove cholesterol from the plasma membrane in the mouse [3], goat [32], and
117 human [10]. Cyclodextrins can also be used to incorporate cholesterol into the membrane when

preloaded with cholesterol, thereby preventing membrane destabilization during cryopreservation. Pre-treatment with cholesterol-loaded cyclodextrins (CLC) has been shown to increase sperm osmotic tolerance limits [62] and improve post-thaw motility and viability of spermatozoa in the bull [51, 52] stallion [7] ram [36], donkey [42], boar [18], elephant [34], and dromedary camel [9].

Earlier attempts to cryopreserve Addra gazelle spermatozoa have demonstrated susceptibility to cryo-damage as demonstrated by a decrease in total motility post-thaw (Pukazhenth, unpublished data). Our goals were to further our knowledge regarding the biology of this rare species and to gain perspective into the damage caused to sperm during cryopreservation. In addition, we hoped to improve cryopreservation techniques in order to mitigate such damage. The aims of this study were to 1) characterize the ejaculate traits in the Addra gazelle, 2) assess the effects of CLC on Addra gazelle sperm cryosurvival including motility, longevity, and acrosomal integrity, and to 3) compare subjective and objective approaches to evaluating sperm motility parameters.

Materials and Methods

Animals

All procedures were reviewed and approved by the Animal Care and Use Committees of the Smithsonian Conservation Biology Institute (SCBI) and White Oak Conservation (WOC). Semen was collected from nine adult (2-4 years) Addra gazelles (*Nanger dama ruficollis*), six of which were managed at SCBI near Front Royal, VA and the remaining at WOC near Yulee, FL. The individuals at SCBI were housed separately in stalls (3.1 m W x 3.7 m L) with access to outdoor runs (3.7 m W x 24.4 m L) and with visual and olfactory contact with other males and a

herd of females. Water and hay were provided ad libitum as well as approximately 0.9 kg of Zeigler ADF-25 (Zeigler Bros, Inc., Gardners, PA) pellets were provided daily. Males managed at WOC were housed as a bachelor herd and received 0.5 kg of Mazuri ADF 16 pellets (PMI Nutrition International, LLC, St. Louis, MO) and 0.5 flakes of alfalfa hay/animal/day with ad libitum access to water. The herd had access to a corral and barn area and approximately 1 acre of pasture but no contact with females.

Preparation of Cholesterol-loaded Cyclodextrin

Cholesterol-loaded cyclodextrin (CLC) was prepared as previously described [34, 51]. Briefly, 1 g of methyl- β -cyclodextrin was dissolved in 2 ml of methanol and 200 mg cholesterol dissolved into 1 ml of chloroform. A 450 μ l aliquot of the cholesterol solution was added to the cyclodextrin, mixed, poured into a glass Petri dish, and allowed to evaporate for 24-36 h at room temperature (21°C). CLC crystals were scraped from the dish and stored protected from light at room temperature until use.

Semen Collection, Cryopreservation, and Evaluation

For ejaculate characterization, 22 ejaculates were collected from nine males (ages 2-4 years; 1-3 samples/male). A surgical plane of anesthesia was induced in each gazelle using a combination of etorphine (ZooPharm, Windsor, CO; $\sim 0.04 - 0.05 \text{ mg kg}^{-1}$ body weight) and xylazine HCl (X-Ject E, Henry Schein Animal Health, Dublin OH; $\sim 0.1 - 0.5 \text{ mg kg}^{-1}$ body weight) administered i.m. using an air-propelled dart into the hind leg musculature. The desired plane of anesthesia was maintained using intravenous propofol (Propoflo, Abbot Animal Health, Abbot Park, IL; $\sim 0.1 - 0.4 \text{ mg kg}^{-1}$ body weight per min) in small boluses given to effect by a

164 butterfly catheter secured in a forelimb vein, or ketamine (Ketathesia, Henry Schein Animal
165 Health, Dublin, OH; 0.2 - 0.5 mg kg⁻¹ body weight) i.v. All animals underwent endotracheal
166 intubation and positive pressure ventilation was administered to supplement spontaneous
167 respirations. Upon completion of semen collections, some animals were given a single dose of
168 azaperone (ZooPharm, Windsor, CO; ~ 0.1 mg kg⁻¹ body weight) i.m. and anesthesia was
169 antagonized using naltrexone (ZooPharm, Windsor, CO; ~4 - 5 mg kg⁻¹ body weight) split
170 equally between i.v. and i.m. routes, and atipamezole (ZooPharm, Windsor, CO; ~ 0.01 – 0.05
171 mg kg⁻¹ body weight) given by i.m. injection. Anesthesia at WOC used a combination of
172 Carfentanil (ZooPharm; ~20 µg kg⁻¹) citrate, Medetomidine HCl (ZooPharm; ~35 µg/kg⁻¹, and
173 Ketamine (ZooPharm; ~0.9 mg kg⁻¹).

174 A rectal probe (2.6 cm diameter) with 3 longitudinal electrodes and an electrostimulator
175 (P.T. Electronics, Boring, OR) were used to provide a total of 80-100 stimuli (2 – 5 volts) over a
176 30 min interval [28, 29]. Semen was collected in pre-warmed (37°C) sterile collection vials, and
177 an aliquot (3 µl) immediately assessed for sperm total motility, forward progressive status (scale
178 of 0-5; 5 being rapid straight-forward movement), and concentration using a hemocytometer [1].
179 An aliquot (15 µl) of fresh ejaculate was fixed in 500 µl of 4% paraformaldehyde and stored at
180 4°C for assessment of acrosomal integrity (see below). An additional aliquot (20 µl) of fresh
181 ejaculate was fixed in 200 µl of 0.3% glutaraldehyde in phosphate buffered saline (340 mOsm;
182 pH 7.4) and stored at 4°C for morphological assessment (see below).

183 For sperm cryopreservation studies, four ejaculates (four males; single ejaculate/male)
184 were diluted (120 x 10⁶ sperm/ml) with Ham's F10 supplemented with Hepes (Irvine Scientific,
185 Santa Ana, CA), aliquoted into 5 (1.5 ml) Eppendorf centrifuge tubes and incubated (37°C; 15
186 min) with 0, 0.5, 1.5, 3.0 and 6.0 mg/ml CLC, respectively. These concentrations were chosen

based on the beneficial effects reported by similar studies completed in other species [9, 34]. Aliquots were centrifuged (300 x g; 8 min) and the resulting sperm pellets resuspended (250 x 10⁶ sperm/ml) in TEST-Yolk Buffer Refrigeration Medium (TYB; Irvine Scientific, Santa Ana, CA) containing 6% (v/v) glycerol. Sperm suspensions were loaded in plastic straws (0.25 ml; 125 x 10⁶ sperm/ml), cooled to 5°C in a water bath for approximately 4 h, and placed 4 cm over liquid nitrogen (LN₂) for 10 min before being plunged into LN₂. All straws were stored for at least 2 months before analysis [28].

Computer Assisted Semen Analysis

Due to acquisition of the Computer Assisted Sperm Analyzer (CASA; Hamilton Thorne, Beverly, MA) instrument several months following semen collection and cryopreservation, fresh samples were assessed subjectively while all post-thaw samples were analyzed objectively using CASA, in addition to subjective analysis. Samples were thawed at 37°C (30 s) and stored protected from light at 21°C during evaluation. Aliquots were removed from thawed samples for motility (37°C), viability, acrosomal integrity, morphological assessments and CASA at 0 (immediately post-thaw), 1, 2, and 3 h. For CASA, the aliquots were diluted 1:1 with TYB (Irvine Scientific) then 2 µl was loaded on a 4-chamber slide (Cytonix Corporation, Beltsville, MD) for motility analysis; samples were not diluted for subjective analysis.

Computer assisted semen analysis is capable of evaluating metrics that describe sperm motility and progressive status, allowing for standardization of objective sperm quality measures among studies and laboratories [4, 14, 16]. Standard parameters measured by CASA include percent total motility (% motility), percent total progression (% progressive), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/sec), straight line velocity (VSL;

$\mu\text{m}/\text{sec}$), linearity (LIN; VSL/VCL), straightness (STR; VSL/VAP), and beat cross frequency (BCF; Hz). VAP, VCL, VSL are measurements of velocity, LIN and STR are ratios of velocity measurements and used to determine progressiveness, and BCF measures cell oscillation; all parameters provide general information regarding structure and function of the spermatozoa [1, 14, 16, 28]. CASA was set up with a frame capture speed of 60 Hz and a frame count of 30. Additional parameters were as follows: elongation (ratio of head width to head length) 1-96%; head size, 8-49 μm^2 ; head brightness minimum, 106; and tail brightness minimum, 104. Progressive STR was defined as 70%. Progressive VAP was defined as 50 $\mu\text{m}/\text{s}$, slow: 30 $\mu\text{m}/\text{s}$, and static: 4 $\mu\text{m}/\text{s}$. Slow VSL was defined as 15 $\mu\text{m}/\text{s}$ and static VSL as 1 $\mu\text{m}/\text{s}$ (settings modified from those used for bull spermatozoa) [16].

Acrosomal Integrity and Morphological Assessment

Protocols for acrosomal integrity and morphological assessment were as previously described [34]. Briefly, fixed samples were centrifuged (2,000 g; 8 min), then washed twice with 0.5 ml of 0.1 M ammonium acetate (pH 9.0). The pellets were resuspended in 50 μl of the ammonium acetate, and 20 μl was smeared on slides then allowed to air dry at room temperature. The slides were then immersed in Coomassie stain (0.22% (w/v) Coomassie blue G-250, 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) water) for 2 min at room temperature, rinsed with water, allowed to air-dry at room temperature and mounted permanently using Permount (Fisher Scientific, Pittsburgh, PA). Spermatozoa (200/sample) were examined with brightfield microscopy under 1000x magnification [34]. Spermatozoa exhibiting uniform staining over the acrosomal region were designated as intact, those with patchy staining as damaged, and those with no staining as non-intact (Fig 1).

For morphological assessment, spermatozoa fixed in 0.3% glutaraldehyde were examined under 1000x magnification using phase contrast optics (200 spermatozoa/sample; Fig 2). Sperm displaying normal morphology were considered 'normal'. Those displaying abnormalities, including microcephaly, bicephaly, misshaped and detached heads, proximal and distal cytoplasmic droplets, and/or a bent or coiled flagellum were considered 'abnormal'.

Viability Assessment

The post-thaw integrity of the plasma membrane, indicative of viability, was assessed using a dual SYBR14 and propidium iodide (PI) fluorescence staining protocol (Sperm Viability Kit; Molecular Probes, Eugene, OR) [22, 49]. Spermatozoa were stained using 8 µl SYBR14 (1:50 dilution; 0.03%) and 2 µl PI (2%) added to 100 µl of the sperm suspension. Samples were incubated for 15 min at 37°C in the dark then examined under 1000x magnification using epifluorescence. The percentage of sperm staining green (SYBR 14; intact membranes) versus red (PI, non-intact membranes) was determined by counting 200 cells/sample.

Statistical Analysis

Ejaculate traits were analyzed using descriptive statistics. The influence of CLC concentration on each CASA parameter, acrosomal integrity, viability, and morphology were analyzed using analysis of variance (ANOVA) followed by a post-hoc Tukey's range test for pair-wise analysis. Percentage values were log-transformed prior to analysis. Statistical significance was defined as $P < 0.05$ and data presented as mean $\pm$ SEM. Statistical analysis was performed using SPSS for Windows (Version 23; IBM Corp. Armonk, NY).

Results

Characteristics of Addra gazelle semen collected via electroejaculation are summarized in Table 1 and morphological and acrosomal assessments represented in Fig. 1 and 2. Sperm total motility as determined by CASA, as well as subjectively, declined ($P < 0.05$) immediately after thawing compared with fresh samples (Fig. 3). *In vitro* incubation at 21°C resulted in a further ($P < 0.05$) decline in motility by ~10% in all samples over the 3 h assessment period (Fig. 3). Motility values differed (26.3 – 42.4%, dependent upon treatment group) when determined subjectively as compared to CASA assessment; motility decline was similar for both assessment types (Table 2). Dilution of thawed sperm suspensions to facilitate CASA assessments resulted in a significant decline in sperm motility. Freezing and thawing had no ($P > 0.05$) influence on sperm acrosomal integrity or progressive status immediately after thawing (Table 2) or after three hours of *in vitro* incubation (data not shown). Total percent progressive motility was not impacted by *in vitro* incubation ($P > 0.05$; Table 2).

Immediately after thawing, samples pre-treated with 0.5 mg/ml CLC retained higher ($P > 0.05$) total motility than samples cryopreserved in the absence of CLC. There was no ($P > 0.05$) difference in total motility between samples cryopreserved in the presence of 0.5 mg/ml and 1.5 mg/ml CLC (Table 2). Samples pre-treated with 0.5, 1.5, or 3.0 mg/ml CLC exhibited greater viability than samples cryopreserved in the absence of CLC (0 mg/ml) or 6.0 mg/ml. There was no ($P > 0.05$) difference in sperm viability among samples cryopreserved in the presence of 0.5, 1.5, or 3.0 mg/ml CLC (Table 2).

BCF, VAP, VCL, and VSL did not differ ($P > 0.05$) among treatment groups (Table 3) or over time (data not shown). LIN and STR were not ($P > 0.05$) impacted by time (data not shown). However, LIN differed among samples treated with 3.0 and 6.0 mg/ml as compared to 0

and 0.5 mg/ml ($P < 0.05$; Table 3); while those treated with 1.5 mg/ml did not differ from any treatment ($P > 0.05$). Samples treated with 3.0 and 6.0 mg/ml CLC displayed decreased STR as compared to all other samples ($P < 0.05$; Table 3); those treated with 0, 0.5, and 1.5 did not differ from each other immediately post-thaw ($P > 0.05$).

Discussion

It is estimated that there are less than 500 Addra gazelle remaining in the wild and population size is experiencing a downward trend due to habitat degradation and unregulated hunting [33]. Surviving individuals are highly segregated, limiting mating opportunities, and thus, hindering offspring production. Due to small population size as well as high levels of inbreeding, genetic diversity within the species is dwindling which will negatively impact the Addra's ability to adapt to environmental changes, furthering their susceptibility to extinction. Populations managed in captivity act as a genetic reservoir against extinction and genetic diversity can be maximized through careful management. However, these populations are also segregated and costs and risks associated with moving animals makes pairing genetically compatible individuals difficult and expensive [47, 50]. It is imperative to develop assisted reproductive technologies (ARTs) such as artificial insemination in conjunction with semen cryopreservation, as alternatives to support natural mating for offspring production and species survival [47, 50]. In order to optimize such technologies it is crucial to have a firm understanding of Addra gazelle reproductive biology, as species-specific differences in spermatozoa tolerance to cold and osmotic changes associated with cryopreservation can impact cryosurvival [8]. There is minimal information available regarding the reproductive biology of the Addra gazelle. Our goal was to further our fundamental understanding of seminal traits; in addition we were keen to

examine the impact of cholesterol-loaded cyclodextrins supplementation on Addra gazelle sperm cryosurvival. We made a number of important discoveries: firstly, Addra gazelle spermatozoa suffer damage to mechanisms responsible for motility during cryopreservation as evidenced by a significant decrease in percent motile sperm post-thaw. Secondly, pre-treatment of Addra spermatozoa with 0.5 and 1.5 mg/ml of cholesterol-loaded cyclodextrin (CLC) protects these mechanisms as indicated by greater % motility in these treatment groups. Thirdly, higher concentrations of CLC (3 and 6 mg/ml) further damage spermatozoa, observed as additional decreases in % motility in those treatment groups. Finally, subjective and objective (use of CASA) analysis of sperm motility kinematics demonstrate similar outcome patterns, though values differ.

Addra gazelle ejaculate characteristics were similar to those reported in the Mohor gazelle (*Nanger dama mhorr*), a closely related subspecies, Cuvier's gazelle (*Gazella cuvieri*), and Dorcas gazelle (*Gazella dorcas neglecta*) [1, 21]. Both ejaculate volume and sperm concentration/ml of ejaculate were similar between Addra and Mohor gazelle (range, 1.1 – 1.2 x 10⁹/ml) [1]. Total motility and acrosomal integrity of the Addra gazelle spermatozoa prior to cryopreservation (~75% and ~90%, respectively) were similar to values reported in the Mohor gazelle, Cuvier's gazelle, and the Dorcas gazelle (~80% motility and ~90% acrosomal integrity) [21]. However, percentage of morphologically normal sperm was lower for the Addra gazelle (~57.1%) compared to the Mohor, Cuvier's or Dorcas gazelle (~80 to 90%) [21]. The reason for differences among these closely related species remains unclear, but may be related to inbreeding. The North American Addra gazelle population descended from less than 15 founding individuals [56, 58] and the current inbreeding coefficient is 0.1, suggesting a high incidence of inbreeding and limited genetic variation within the population [46]. Higher levels of

morphological and acrosomal abnormalities have previously been reported in other inbred ungulates [53, 54, 55]. However, despite these differences, gazelles in this taxon appear to share similar sperm traits.

Cryopreservation resulted in a significant decline in post-thaw motility as determined by both subjective and objective analysis. Subjective analysis of motility during post-thaw evaluations exhibited a pattern similar to that of the CASA data, but was higher by ~40%. However, in order to analyze samples using CASA, aliquots had to be diluted 1:1 with Test yolk buffer refrigeration medium which resulted in a sharp decline in total motility. The observed decline in motility may be attributed to osmotic stress experienced by spermatozoa upon dilution, albeit with an isotonic diluent [27]. Species vary in their osmotic stress tolerance, as indicated by functional responses to osmotic changes. Bull [26] and boar [23] spermatozoa appear to be highly sensitive to osmotic changes; both species display an irreversible loss in motility. Other species, including mice [67] and humans [19], appear to be more tolerant. Our findings have major implications on the use of frozen-thawed sperm in ARTs. Specifically, it may not be prudent to attempt removal of cryoprotectants from frozen-thawed sperm prior to insemination; doing so could further the osmotic damage to spermatozoa. However, allowing spermatozoa to remain in the cryoprotectant may also have a deleterious effect. Hence, additional studies are warranted to develop processing approaches to mitigate osmotic shock induced loss in sperm motility.

In general, the addition of CLC prior to cryopreservation improves cryosurvival in many species, though the extent of the benefit differs among species and depends upon concentration [35]. Despite reports describing a beneficial effect of CLC use in the boar [18] other studies in the same species suggest the opposite, that further removal of cholesterol from the membrane

supports cryosurvival [68, 69]. Addra gazelle sperm, similar to Asian elephant sperm [34] experienced a negative effect when exposed to higher CLC concentrations (> 3.0 mg/ml). Species that have a higher cholesterol:phospholipid ratio and are less sensitive to cryodamage (i.e., rabbit) do not appear to benefit from CLC treatment [35]. Although the precise mechanism by which CLC exerts a benefit is not fully understood, providing opportunities for incorporating more cholesterol into the membrane may offer stability and therefore lower the temperature of the lipid phase transition and prevent associated damage [12, 51]. Species similar to the Addra gazelle, such as the domestic bull, have a low cholesterol:phospholipid ratio, increasing susceptibility to cryodamage [11, 52]. Results from this study demonstrate that Addra gazelle spermatozoa are sensitive to cryopreservation possibly due to a low cholesterol:phospholipid ratio, and therefore increasing cholesterol content of the membrane likely stabilized it, enhancing their tolerance to cryopreservation. Further studies measuring cholesterol content throughout the cryopreservation process are necessary in order to validate or disprove this hypothesis.

Addra gazelle spermatozoa displayed increased total motility post-thaw when pre-incubated with lower CLC concentrations (0.5 and 1.5 mg/ml) compared to no or higher doses. This is consistent with reports in the bull [2, 38, 51], Asian elephant [34] and camel [9], all of which reported an increase in post-thaw sperm motility after pre-treatment with a 1.5 mg CLC concentration compared to no supplementation perhaps indicating conservation of CLC dosage effect across species [35]. This is not a universal observation as findings have been mixed in the horse where some investigators [37, 60], but not others [41] have reported improved sperm motility with the 1.5 mg concentration. Differences in results among various studies may arise from collection techniques or male-male sensitivity to cryopreservation [35].

In the Addra gazelle, VAP, VCL, and VSL (~ 44, 83, and 37 $\mu\text{m}/\text{sec}$) were all lower than values reported in both fresh (~141, 150, and 130 $\mu\text{m}/\text{sec}$, respectively) [16] and thawed bull samples (~111, 193, and 90 $\mu\text{m}/\text{sec}$, respectively) [45], but similar to those reported in fresh Mohor gazelle samples (19-130, 76-170, and 8-26 $\mu\text{m}/\text{sec}$, respectively and dependent upon subpopulation) [1]. Despite belonging to the Bovidae family, our findings demonstrate a species-specific difference between *Bos taurus* and *Nanger dama*. None of the velocity measures (VAP, VCL, VSL) were impacted by CLC treatment and did not differ over time, however, Abaigar et al. [1] report that characteristics of Mohor gazelle sperm stored at 17°C, did not differ until 48-72 hours of storage; perhaps explaining why we did not observe changes within the first few hours post-thaw. Addra gazelle sperm BCF (~30 Hz) was approximately 2 to 5 fold higher than in fresh bull (~15 Hz) and Mohor (~0.02-6 Hz) samples, but equivalent to frozen bull sperm (~30 Hz) [1, 16, 45], suggesting cryopreservation results in an increased flagellar beat. However, BCF did not change in response to CLC treatment or time. Straightness (STR) values (~80) were not different when compared to fresh [16] and frozen-thawed bull samples [45] (~80-90%; STR was not reported for the Mohor), suggesting straightness is not impacted by freezing and is similar among related species. Linearity (LIN; ~45) was similar to cryopreserved bull sperm (~45) [45] but different from the fresh counterparts (Mohor: ~8-20, bull: ~88) [1, 16] indicating an effect of cryopreservation on LIN. However, LIN was lower than fresh bull samples but higher than reported in the Mohor [1]; there may be additional confounding factors influencing this measure. Both LIN and STR were similar in samples pretreated with 0, 0.5, and 1.5 mg/ml CLC, but were significantly decreased in those treated with 3.0 and 6.0 mg/ml CLC. Total motility is also significantly decreased by higher CLC treatments indicating an effect on membrane integrity that disrupts normal cell function, impacting sperm motility and progression [52].

This study is the first to evaluate semen characteristics and the impact of cryopreservation on Addra gazelle spermatozoa. Addra spermatozoa display high motility values post-collection, but suffer significant losses during freezing and thawing. These losses can be partially mitigated by loading the plasma membrane with cholesterol using 0.5 to 1.5 mg CLCs, which significantly increases cryosurvival likely by protecting the mechanisms associated with motility. Higher concentrations of CLC have a detrimental effect, possibly increasing the rigidity of the membrane to the point that it decreases permeability, such that the cell can no longer react to osmolarity changes. These results provide information critical for establishing frozen repositories of Addra gazelle sperm and developing ARTs for improved genetic management of extant *ex situ* populations. Our findings will allow us to optimize sperm cryopreservation techniques, facilitating ARTs such as AI, and improve reproductive rates as well as genetic diversity. Each of these components will aid in the creation of self-sustaining populations and preventing extinction of a species.

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Figure Legends:

Figure 1. Coomassie blue staining of Addra gazelle spermatozoa collected via electroejaculation to assess acrosomal integrity (1,000x). Spermatozoa with intact acrosomes (IA) exhibit a uniform blue staining overlying the acrosomal region. Spermatozoa with non-intact acrosomes (NIA) display a clear or patchy staining pattern.

Figure 2. Common morphological forms of spermatozoa in ejaculates obtained from Addra gazelle via electroejaculation (1,000x): A) normal ($57.1 \pm 4.0\%$); B) abnormal acrosome ($3.7 \pm 0.8\%$); C) retained proximal cytoplasmic droplet ($4.9 \pm 0.9\%$); D) retained distal cytoplasmic droplet ($16.4 \pm 3.0\%$); E) bent midpiece with a retained cytoplasmic droplet ($5.3 \pm 1.1\%$); and F) bent tail with a retained cytoplasmic droplet ($2.0 \pm 0.4\%$).

Figure 3. Impact of time on electroejaculated Addra gazelle sperm motility (%) post-thaw. Dotted line indicates average % motility value in fresh samples. All values (except fresh data which was subjectively evaluated) were generated by CASA and represent mean $\pm$ SEM. Different letters represent statistically significant differences ($P < 0.05$; $n = 4$ ejaculates; 1 ejaculate/male).

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Figure 1

Figure 2

Figure 3

Figure 3.

Table 1: Ejaculate characteristics of Addra gazelle immediately following collection via electroejaculation (fresh) and post-thaw (n = 22 ejaculates from 9 males; 1-3 ejaculates/male).

Trait	Fresh Mean ± SEM	Post-thaw Mean ± SEM
Seminal volume (ml)	3.2 ± 0.3	---
Sperm motility (%) [#]	75.8 ± 2.7	range: 40.0 ± 5.8 to 73.3 ± 3.3
Sperm forward progression status (FPS) ^{*#}	3.2 ± 0.3	3.5 ± 0.2
Total sperm concentration (x10 ⁹)	1.2 ± 0.2	---
Intact acrosomes (%)	92.5 ± 3.9	84.4 ± 6.6
Morphologically normal sperm (%)	57.1 ± 4.0	67.3 ± 6.4

^{*}FPS scale = 0 – 5 with 5 being the most rapid, straightforward progression.

[#]Sperm motility and forward progression were assessed subjectively.

Values represent mean ± SEM.

Table 2: Changes in post-thaw electroejaculated Addra gazelle sperm total motility, progressive motility, and viability following cryopreservation in the absence and presence of CLC (n = 4 ejaculates; 1 ejaculate/male).

CLC (mg/ml)	Subjective % Motility	CASA % Motility	CASA % Progressive	% Viability
0	56.7 ± 8.8 ^A	16.4 ± 2.3 ^A	4.3 ± 1.6	18.8 ± 0.9 ^A
0.5	73.3 ± 3.3 ^B	30.9 ± 3.4 ^B	6.5 ± 1.3	40.0 ± 1.3 ^B
1.5	60.0 ± 5.8 ^A	21.9 ± 6.3 ^{AB}	5.1 ± 0.8	39.9 ± 3.9 ^B
3	41.7 ± 14.2 ^C	15.4 ± 4.3 ^A	9.4 ± 6.2	41.3 ± 1.5 ^B
6	40.0 ± 5.8 ^C	1.1 ± 0.4 ^C	0.3 ± 0.1	24.9 ± 1.3 ^A

Values represent mean ± SEM. Different letters represent statistically significant differences ($P < 0.05$) within each measure.

Table 3: Computer-assisted post-thaw semen assessment of Addra gazelle spermatozoa collected via electroejaculation and cryopreserved in the absence or presence of CLC (n = 4 ejaculates; 1 ejaculate/male).

CLC (mg/ml)	BCF	LIN	STR	VAP	VCL	VSL
0	30.6 ± 2.6	46.9 ± 1.0 ^A	83.6 ± 0.9 ^A	44.3 ± 3.9	83.0 ± 8.6	37.2 ± 3.7
0.5	33.3 ± 1.0	50.6 ± 2.0 ^A	86.7 ± 1.6 ^A	42.4 ± 1.8	77.6 ± 5.0	36.6 ± 1.5
1.5	26.4 ± 1.0	43.8 ± 1.8 ^{AB}	79.7 ± 2.7 ^A	45.7 ± 1.5	86.7 ± 3.3	36.1 ± 0.6
3	22.4 ± 1.8	36.9 ± 0.8 ^B	70.4 ± 0.8 ^B	54.0 ± 1.8	107.3 ± 5.4	37.0 ± 0.8
6	28.1 ± 6.4	38.5 ± 3.6 ^B	61.4 ± 3.0 ^C	55.6 ± 5.3	96.6 ± 6.9	33.4 ± 3.5

Values represent mean ± SEM.
BCF, beat cell frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; and VSL, straight line velocity.
Different letters represent statistically significant differences (*P* < 0.05) within column.