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Pretreatment with cholesterol-loaded cyclodextrins prevents loss of motility associated proteins during cryopreservation of addra gazelle (*Nanger dama ruficollis*) spermatozoa

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ABSTRACT

Sperm cryopreservation is challenging, often resulting in irreversible damage to spermatozoa, as indicated by decreased motility, viability, and/or acrosomal integrity. Developing cryopreservation protocols for gametes of endangered species compounds the complexity of technique optimization; samples are difficult to obtain and numbers are limited. Cryopreservation of sperm collected from the critically endangered addra gazelle (*Nanger dama ruficollis*), a member of the Bovidae family, resulted in significant loss of motility, which was prevented by pretreatment with cholesterol-loaded cyclodextrin (CLC). This study investigated the proteome of sperm (fresh and cryopreserved), processed in the absence and presence of 0.5 mg/ml CLC in the addra gazelle. The proteome of *Bos taurus*, the closest domestic relative, was used as a reference. Mass spectrometry analysis of the addra gazelle sperm proteome revealed 287 proteins. The concentrations of 85 proteins differed between fresh and frozen/thawed samples; nearly all were decreased. Most were associated with metabolic processes, specifically glycolysis, which may explain the decrease in post-thaw motility observed in this species. CLC pretreatment partially prevented the loss of various proteins involved in metabolism including CAPZB (gene = CAPZB), HS90A (gene = HSP90AA1), and PGAM2 (gene = PGAM2). To our knowledge, this is the first study to evaluate the proteome of any wild bovids' sperm, and the first to compare protein levels in sperm pretreated with CLC.

1. Introduction

The addra gazelle (*Nanger dama ruficollis*) is a subspecies of one of the most critically endangered antelope species, the dama gazelle (*Nanger dama*) [45]. Once wide spread in the Sahelo-Saharan region of Northern Africa, poaching, habitat destruction, competition with livestock, and prolonged drought has caused numbers to plummet below an estimated 1000 individuals world-wide, most of which are managed in captivity [28]. The majority of large vertebrates sympatric to the addra gazelle are threatened or extinct; a symptom of an ever-growing problem, which is the accelerated extinction of species globally [16]. There are over 25,000 species listed as threatened by extinction by the International Union for Conservation of Nature (IUCN)'s Red List of Threatened Species, and as only 5% of the world's described species have been evaluated, actual numbers are likely much greater [28]. Extinctions are occurring at an estimated 100–1000x historical rates and the challenges faced by species are not diminishing [14,16]. While preservation of habitat, development of policies to protect listed species, and efforts to mitigate climate change are useful in alleviating some stressors *in situ*, in many cases, species survival depends heavily upon the development of captive breeding programs and assisted reproductive technologies (ARTs), such as artificial insemination and gamete cryopreservation, to propagate offspring and maximize diversity [42].

Sperm cryopreservation is a useful tool in genetic management and conservation [23,42], allowing for the preservation of valuable genetic materials and facilitating shipment and use of preserved gametes in ARTs. Spermatozoa are highly specialized cells with unique morphology, physiology, and function and therefore, there are many challenges associated with cryopreservation. Sperm are believed to be transcriptionally silent, relying on post-translational modification of proteins for functional or physiological changes [7,34]. Though mechanisms regulating these changes are not fully understood [34], many proteins are altered during freezing/thawing [9,53,57]. Proteins

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involved in maintaining membrane structure [13], the acrosome reaction, capacitation [7,47], apoptosis [31], and sperm metabolism [26,53] are modified during cryopreservation, which can be deleterious to sperm motility, acrosomal integrity, viability, and fertilizing capacity [23]. These changes may be attributed to post-translational modifications, protein degradation, or physical loss of proteins due to membrane damage [9,53,57]. It is important to expand our understanding of the impact of cryopreservation on sperm protein levels as such information could facilitate efforts to minimize cryodamage and to optimize preservation protocols [1].

There are significant differences in cryosurvival rates among species, creating a need for techniques tailored to each [23,25]. Often extenders are specifically optimized for a particular species. Modification of the sperm plasma membrane through the incorporation of cholesterol prior to cryopreservation, via incubation with cholesterol-loaded cyclodextrins (CLC), has proven to be useful in improving post-thaw motility and viability in a number of species including the bull [43], stallion [11], camel [10], elephant [32], and the addra gazelle [54]. It is hypothesized that increasing the cholesterol: phospholipid ratio of the membrane increases flexibility during freeze-thawing [12,43]. There is no information on the effects CLC might exert on sperm proteins.

The objectives of this study were to: 1) characterize the proteome of addra gazelle spermatozoa, 2) compare protein levels in fresh versus frozen-thawed spermatozoa to elucidate the mechanisms resulting in the significant decrease in post-thaw motility, and 3) evaluate the impact of CLC pretreatment on the proteome of addra gazelle sperm in order to investigate how incorporation of cholesterol benefits sperm survival during the cryopreservation process.

2. Materials and methods

2.1. Animals

Semen was collected via electroejaculation from four adult male addra gazelles (1 ejaculate/male; n = 4), 6–10 years old, managed at Smithsonian Conservation Biology Institute (SCBI) in Front Royal, Virginia, USA. The gazelles were housed individually in stalls (3.0 m × 3.7 m) with adjoining outdoor paddocks (3.7 m × 24.4 m). All animals had visual access, but no direct contact, with females and males of the same species. The males were fed approximately 0.9 kg of Zeigler ADF-25 (Zeigler Bros, Inc. Gardners, PA) pellets daily and provided ad libitum access to water and hay. All procedures were approved by the Animal Care and Use Committee of SCBI.

2.2. Semen collection and sample processing

Cholesterol-loaded cyclodextrin (CLC) was prepared and semen collection procedures were conducted as previously described [54]. In brief, anesthesia was induced using a combination of etorphine (ZooPharm, Windsor, CO; ~0.04–0.05 mg kg^{-1} body weight) and xylazine HCl (X-Ject E, Henry Schein Animal Health, Dublin, OH; ~0.1–0.5 mg kg^{-1} body weight) administered intramuscularly (i.m.), and maintained with intravenous (i.v) propofol (Propoflo, Abbot Animal Health, Abbot Park, IL; ~0.1–0.4 mg kg^{-1} body weight per min) or ketamine (Ketathesia, Henry Schein Animal Health; ~0.2–0.5 mg kg^{-1} body weight). Some animals were given a dose of azaperone (ZooPharm; ~0.1 mg kg^{-1} body weight, i.m.) following semen collection. Anesthesia was antagonized using naltrexone (ZooPharm; ~4–5 mg kg^{-1} body weight, split between i.m. and i.v.) and atipamezole (ZooPharm; ~0.01–0.05 mg kg^{-1} body weight, i.m.) [54]. A rectal probe, 2.6 cm in diameter, with three longitudinal electrodes, was inserted into the rectum, and a total of approximately 80 stimuli, 2–5 V were provided over the course of 2–3 series [24,54] with the use of a sine-wave electrostimulator (P.T. Electronics, Boring, OR). Rest periods of at least 5 min followed each series and collections were completed simultaneously with veterinary procedures to minimize the number of anesthetic procedures conducted on an individual. Ejaculates were collected into pre-warmed (37 °C) collection vials, that were switched out frequently to prevent urine contamination of the entire sample if urination occurred. Samples were evaluated for motility and progressive status (scale of 0–5; 5 being rapid straight-forward movement). Only samples displaying > 60% motility were included in the study.

Samples (n = 4) were each divided into 2 aliquots designated 'fresh' and 'frozen' (cryopreserved). Both aliquots were diluted to 120 × 10^6 sperm/ml with Ham's F10 supplemented with Hepes (Irvine Scientific, Santa Ana, CA), divided again and then incubated (15 min; 37 °C) with either 0 (control) or 0.5 mg/ml CLC (found to be most beneficial to addra gazelle sperm post-thaw survival) [54]. In total, there were four treatment groups: fresh control, frozen control, fresh CLC, and frozen CLC. Post-incubation, samples were centrifuged (300 × *g*; 8 min) and supernatant discarded. Samples designated 'fresh' were washed with Ham's F10 and divided into aliquots of 100 × 10^6 sperm/tube, centrifuged (300 × *g*; 8 min), supernatant was removed, and the samples were plunged into liquid nitrogen (LN2). Samples were stored at −80 °C, until protein digestion.

Samples designated 'frozen' were resuspended in TEST-Yolk Buffer Refrigeration Medium (Irvine Scientific) containing 6% (v/v) glycerol, loaded into 0.25 ml straws, and cooled in a water bath for approximately 4 h to 5 °C. Straws were then placed 4 cm over LN2 for 10 min, then plunged. All straws were stored for a minimum of 3 months. Straws were thawed in a water bath at 37 °C for 30 s, then processed as described above for the fresh samples: dilution to 100 × 10^6 sperm per tube, flash frozen, and stored at −80 °C.

2.3. Protein digestion

Protein concentration was determined using a nanodrop (Thermo Fisher Scientific, Waltham, MA) and samples (n = 16; 4/treatment group) diluted to 1 mg/ml with 0.1 M tetraethylammonium bicarbonate (TEAB); final volumes ranged from 50 to 100 µl. Each sample was diluted 1:1 with 16 M urea (50–100 µl) and incubated at room temperature (RT) for 5 min. Then 1 µl of 50 mM dithiothreitol (DTT) was added for every 12 µl of the urea mixture (4.2–16.6 µl) and samples were incubated for 30 min at 65 °C. Samples were allowed to cool, then 55 mM indole-3-acetic acid (IAA) was added at 2X the amount of DTT (8.4–33.2 µl) and samples incubated in the dark for 1 h at RT before being diluted 1:1 with TEAB. Trypsin/LysC mix (Promega, Madison, WI) was added and samples were incubated at 37 °C for 2–4 h. Samples were diluted with 0.1 M TEAB to bring the concentration of urea below 1 M to activate trypsin. Digestion continued at 37 °C overnight. Digests were acidified with 1 µl acetic acid. Tryptic peptide was subjected to LCMS/MS analysis.

2.4. LCMS/MS analysis

Each tryptic digest (1 µg) was loaded and desalted in an Agilent Zorbax 300 SB-C18 trapping column (0.3 × 5 mm) at 15 µl/min for 10 min. Peptides were then eluted into a New Objective PicoChip column (0.075 × 105 mm) packed with Reprosil-pur material (3 µm) and chromatographically separated using a binary solvent system consisting of A: 0.1% formic acid and 5% acetonitrile and B: 0.1% formic acid and 95% acetonitrile at a flow rate of 300 nl/min. A gradient was run from 0% B to 35% B over 90 min, followed by a 5-min gradient to 80% B, the gradient was held at 80% B for 10 min, and then equilibrated at 0% B for 10 min before the next sample was injected. The Orbitrap XL mass spectrometer was set to complete a full mass scan of m/z 400–1800 with resolution at 60,000 (m/z 400) in the orbitrap followed by data dependent MS/MS analysis of up to 10 most intense ions with CID in the linear ion trap at unit mass resolution. Peaks eluting from the LC column that have ions above 5000 arbitrary