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Comparison of soy lecithin, coconut water, and coconut milk as substitutes for egg-yolk in semen cryodiluent for black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*)

Jessye Wojtusik*, Monica A. Stoops, Terri L. Roth

Cincinnati Zoo and Botanical Garden, Center for Conservation and Research of Endangered Wildlife, 3400 Vine St., Cincinnati, OH, 45220, USA

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ABSTRACT

Semen cryopreservation for the black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*) relies on extenders containing egg-yolk (EY). Use of such media is not ideal as inter-batch composition varies and there is risk of pathogenic contamination. The goal of this study was to test animal protein-free extenders. Semen collected via electroejaculation from 10 rhinoceros (6 black, 4 Indian) was diluted with extender containing EY, 1% or 2% soy lecithin (1%SL; 2%SL), coconut water (CW), or coconut milk (CM), cryopreserved and evaluated for sperm motility, viability, morphology, progression, and acrosomal integrity at 0, 1, 3, 6 and 24 h post-thaw. Mean ± SD fresh ejaculate motility was 84.5 ± 7.6%, progression: 3.6 ± 0.6 (scale 0–5), viability: 83.4 ± 7.1%, intact acrosomes: 71.3 ± 6.9%, and morphologically normal: 78.8 ± 13.6%. Motility and progression decreased in all groups post-thaw, were greatest in EY, and decreased over time ($P \leq 0.05$). Motility and progression did not differ ($P > 0.05$) between 1%SL and 2%SL, but were lower ($P \leq 0.05$) in CM and CW, and acrosomal integrity was higher ($P \leq 0.05$) in EY, 1%SL and 2%SL than in CM and CW. Post-thaw viability was greatest in EY and 2%SL followed by 1%SL, then CM and CW ($P \leq 0.05$). Morphology did not differ among treatments ($P > 0.05$). Morphology, acrosomal integrity, and viability were maintained over time ($P > 0.05$). Although some rhinoceros sperm survived cryopreservation in SL treatments, reduced post-thaw motility rendered all treatments inadequate substitutes for EY-based extenders.

1. Introduction

The black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*) are considered critically endangered and vulnerable, respectively, by the IUCN [1,2]. Successful captive breeding programs may soon play an important role in promoting species survival, as poaching and habitat loss, among other threats, severely hinder population growth *in situ*. Semen cryopreservation in tandem with other assisted reproductive technologies (ARTs) can facilitate genetic management [3,4] and though semen collection and cryopreservation protocols have been developed for black, white (*Ceratotherium simum*), Indian, and Sumatran (*Dicerorhinus sumatrensis*) rhinoceros, they rely on extenders that contain an animal protein, egg-yolk (EY) [5–10].

The use of animal protein, such as EY or skim milk, in semen extenders is common practice [11]. EY protects spermatozoa from damage during cryopreservation, as is often evidenced by increased viability, motility and fertilizing capacity post-thaw when compared to extenders that do not contain EY [11,12]. Membrane composition, particularly cholesterol and phospholipid content, and the interaction of various cryoprotectant agents and extenders with the membrane components, greatly influence cryosurvival [12–16]. It is generally accepted that the protective effect provided by EY is attributable to low-density lipoproteins (LDLs) and phospholipids [11,17], though direct mechanism is unclear. There is speculation that EY may stabilize the membrane, replace phospholipids lost during the cryopreservation process [15], or prevent the efflux of cholesterol and phospholipids [11].

The efficacy of animal protein based media has come into question prompting the search for an equally effective vegan or chemically defined alternative [11,12,18–21]. EY composition can vary among batches, potentially influenced by the diet and health status of the hen [22], whereas plant composition can be better controlled. More importantly, there is risk of bacterial or viral

* Corresponding author.
E-mail address: jessye.wojtusik@cincinnatizoo.org (J. Wojtusik).

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contamination in EY, which may be a source of endotoxins potentially impacting fertilizing capacity of the spermatozoa [23] and the transport of samples containing animal protein could result in the spread of disease [24].

Soy lecithin (SL) plays a role equivalent to EY, protecting the sperm membrane during cryopreservation [12] by possibly mitigating the efflux of cholesterol or phospholipids. Phosphatidylcholine, a key component of SL, performed equally well to EY as an additive to extenders for stallions [25] and it is hypothesized that any extenders with choline phospholipids will improve spermatozoa cryosurvival [11]. SL is an effective alternative to EY in semen cryopreservation for many species including humans [26], bulls [18], stallions [27], goats [28], rams [29], and domestic cats [21] and dogs [30]. SL concentrations appear optimal at approximately 1% of extender volume [21,26,28–30], resulting in high sperm quality post-thaw; higher concentrations appear to have a toxic effect [28,29]. No bacterial contamination was found in samples that used SL as a diluent [23]. Furthermore, fertility rates of cows were equivalent when inseminated with semen frozen with SL as compared to an EY based extender [23]. Bull sperm frozen using commercially available SL extender displayed higher motility and equal measures of viability, acrosomal integrity, and acrosome reaction induction as compared to samples frozen using EY based diluents [18]. However, contrasting reports indicate the use of SL extenders resulted in decreased motility in bulls [31] and mitochondrial damage in rams [19].

Coconut (*Cocos nucifera*) water (CW), the liquid endosperm of green coconuts, and coconut milk (CM), the extract of fruit pulp from mature coconuts, contain many fatty acids (including poly unsaturated fatty acids (PUFAs)), amino acids, antioxidants, vitamins, minerals, and sugars, contributing to their usefulness as semen extenders and culture media [32–34]. PUFAs are thought to contribute to fluidity and flexibility of membranes and, as precursors to prostaglandins and leukotrienes, may influence motility [13,16]. Incorporating PUFA, docosahexaenoic acid (DHA) to extenders, increased membrane integrity and sperm motility in boars [35]. Furthermore, CW is an effective diluent for cryopreservation of ram [36] and goat [37] semen. CM prolonged bull sperm viability at room temperature when used as an additive to a citrate buffer medium [38], and did not impact fertilizing capacity [39]. When used in addition to EY, CM did not impact fertility rates following AI in goats [40], protected against acrosome damage in boars [41], and promoted bull sperm viability over time in chilled samples [42].

The goal of this study was to evaluate soy lecithin, coconut water, and coconut milk as animal protein-free alternatives to egg-yolk in traditional rhinoceros semen extenders. Sperm quality measures including motility, progressive status, viability, acrosomal integrity, and morphology, were used to assess the efficacy of the substitutes following cryopreservation of black rhinoceros and Indian rhinoceros spermatozoa.

2. Materials and methods

2.1. Animals and semen collection

All procedures were reviewed and approved by the Cincinnati Zoo and Botanical Garden's Animal Care and Use Committee (protocol #14-120). Semen was collected via electroejaculation [8,10] from 10 adult male rhinoceros (6 black, 4 Indian; 1 ejaculate/male) maintained at institutions across the United States (Table 1). A surgical plane of anesthesia was induced in each male, though specific drug combinations varied (Table 1). Rectal probes designed specifically for each rhinoceros species [8] (Innovative Zoological Solutions, Cincinnati, OH 45205, USA) with 3 longitudinal electrodes and an electroejaculator (Innovative Zoological Solutions; P-T Electronics, Boring, OR 97009, USA) were used to provide stimuli over the course of 2–3 series (Table 2). Each series was followed by a resting period of at least 5 min, during which motility and progressive status of each sample were assessed. Semen was collected into whirlpak bags (Nasco, Fort Atkinson, WI 53538, USA) that were frequently changed between stimulations and stored in an insulated container until prepared for cryopreservation. Prior to cryopreservation, samples were assessed for motility and progressive status, and sperm concentration was determined using a hemocytometer (American Optical, Buffalo, NY 14215) [43].

2.2. Semen cryopreservation

Unless otherwise indicated, all chemicals were obtained from Sigma Aldrich (St. Louis, MO 63146). Semen samples (exhibiting $\geq$ 60% motility) were prepared for cryopreservation as described in O'Brien and Roth [6]. Briefly, 250 μL of semen per treatment group were diluted 1:1 with semen extender and contained in a water bath at room temperature (RT). Treatment groups differed by extender type and included a control which employed an equine extender (EQ) containing EY (20% v/v), lactose (5.5% v/v), disodium EDTA (0.25% w/v), glucose (1.5% w/v), Equex STM (0.25% v/v; Nova Chemical, Moon Township, PA 15108, USA), 25 iu penicillin G mL^{-1}, and 25 iu streptomycin mL^{-1} [6]. Soy lecithin (1%SL and 2%SL), coconut milk (20% v/v; CM), and coconut water (20% v/v; CW) were used in lieu of EY for the respective treatment groups. The source of SL (Swanson health products, Fargo ND) and concentrations used were chosen based on the beneficial effects reported by similar studies in other species [21,26,28–30]. The CW (Harmless Harvest, San Francisco, CA 94111, USA) and CM (Native Forest, Edwards and Sons, Carpinteria, CA 93014, USA) were organic and did not contain any preservatives or additives. All extenders were brought up to a pH of 7.0 and stored at −80 °C until use. Samples were cooled to 4 °C then diluted further 1:1 with chilled extender containing 10% glycerol in a stepwise manner (25, 25, 50% v/v every 20 min) to a final concentration of 5% glycerol and final volume of 1 mL per treatment. Samples equilibrated for 1 h at 4 °C, and were loaded into cooled 0.5 mL straws (2 straws/treatment/male), placed into cooled canes and lowered into a charged dry shipper (depth: 42 cm, capacity: 3.6 L; Chart MVE Biomedical, Ball Ground, GA 30107) for 10 min [44] before being plunged into liquid nitrogen and stored until post-thaw assessment.

Straws were thawed in random order and treatment group was not revealed to the assessor until after all assessments were complete. All assessments were conducted by the same individual for consistency. Two straws of each treatment group were processed and evaluated separately, and therefore each assessment was carried out in duplicate. Values from duplicate straws were averaged for each individual prior to statistical analysis. Straws were thawed (10 s at RT in air; 20 s in 37 °C water bath) and maintained, protected from light, at RT, then evaluated for sperm motility, viability, morphology, progressive status, and acrosomal integrity at 0, 1, 3, 6 and 24 h post-thaw.

2.3. Motility and progressive status assessment

The percentage of motile sperm was assessed on pre-warmed slides under 200× magnification using phase contrast optics (Axiostar plus; Carl Zeiss Microscopy, Thornwood, NY 10594). Progressive status, straight-forward movement, was ranked on a scale of 0–5 (5 = all sperm exhibiting rapid, forward progression).

2.4. Acrosomal integrity assessment

To assess acrosomal integrity, a 5 μL aliquot of each sample was