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Collection and preservation of pygmy hippopotamus (*Choeropsis liberiensis*) semen

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Abstract

Knowledge about the reproduction of the endangered pygmy hippopotamus is almost non-existent. This study takes the first step toward changing this by devising a protocol for the collection, evaluation, and short-term preservation of semen of this endangered species. Semen was collected successfully from seven bulls by electroejaculation, using a specially designed rectal probe. Mean ± SEM values of native sperm parameters from combined best fractions were: motility—80.0 ± 4.1%, concentration—2421 ± 1530 × 10^6 cells/mL, total collected cell number—759 ± 261 × 10^6 cells, intact acrosome—87.8 ± 1.2%, intact morphology—52.7 ± 4.3%, and, for some, hypoosmotic swelling test—79.3 ± 4.4% and seminal plasma osmolarity—297.5 ± 3.3 mOsm. Seven different extenders were tested for sperm storage under chilling conditions: Berliner Cryomedium (BC), Biladyl®, modification of Kenney modified Tyrode's medium (KMT), MES medium, Androhep®, boar M III™ extender and Human Sperm Refrigeration Medium. While differences between males were apparent, the BC was consistently superior to all other extenders in sperm motility and facilitated storage for 7 d with up to 30% motility and some motility even after 3 weeks. With this knowledge in hand, the obvious two directions for future research are to conduct artificial insemination and to develop a technique for sperm cryopreservation.

Keywords: Conservation; Electroejaculation; Spermatozoa; Endangered species; *Hexaprotodon liberiensis*

1. Introduction

The endangered pygmy hippopotamus (*Choeropsis liberiensis*), an endemic species to waterways and forests of West Africa (Sierra Leone, Guinea, Ivory Coast, and Liberia), resembles the common hippopotamus but is much smaller in size. Unlike its larger relative, the pygmy hippopotamus lives a solitary life and joins only for mating. While practically nothing is known about their reproduction in the wild, the captive population reproduces well. Years ago they were already considered one of the few endangered species with self-sustaining populations in captivity [1], as indicated by the fact that the last animal to be captured in the wild was captured in 1974 [2]. Despite the availability of a captive population and the fact that the wild population is in constant decline, numbering today is probably at less than 3000 individuals [3], these animals were hardly studied and, to date, nothing was done to unravel their reproductive biology. Running a search in online literature databases for "pygmy hippopotamus" OR "*Choeropsis liberiensis*" yielded only 27 publications, just two of which are reproduction related—one on placentation and one on caesarian section. In the absence of

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relevant information on the male pygmy hippopotamus, any activity in the field of assisted reproductive technologies (ART) is practically impossible. It was decided to take the first step in order to change this situation—develop a procedure for semen collection and evaluation, establish for the first time fertility parameters, and find a suitable way to preserve chilled semen, thus laying the grounds for further assisted reproduction technologies.

2. Materials and methods

2.1. Materials

Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from either:

- Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany.
- Carl Roth GmbH+ Co., Karlsruhe, Germany.
- Merck KGaA, Darmstadt, Germany.

2.2. Animals

Semen was collected from seven pygmy hippopotamus males housed at various zoological institutions across Europe. Ages of the males ranged between 9 and 40 yr. Semen collection procedures were conducted as part of a general reproductive and health assessment.

2.3. Anesthesia

All semen collection procedures were conducted under general anesthesia. Animals were injected in the neck muscles by remote darting with a combination of medetomidine (Zalopine®, Orion Farmos Corporation, Espoo, Finland) and ketamine (WDT eG, Garbsen, Germany); the dosages of 0.08 mg/kg and 1.2 mg/kg, respectively, were based on estimated body weights. Animals were then intubated using a long-blade laryngoscope and maintained with isoflurane (Isobo®, Nbl. Der Essex Pharma Gmbh, Munich, Germany) and air or air mixed with oxygen throughout the procedure. Anesthesia was partially antagonized by intramuscular injection of atipamezole hydrochloride (Antisedan®, Pfizer GmbH, Karlsruhe, Germany) at 5 times the dosage of medetomidine. All animals were up and standing 10–15 min after application of the antidote.

2.4. Semen collection and evaluation

Semen from all males was collected by electroejaculation under general anesthesia (Seager model 14, Dalzell USA Medical Systems, The Plains, VA). A specially designed probe, 130 mm long with a diameter of 45 mm and three longitudinal slightly raised electrodes, was introduced into the male's rectum. On each occasion, a total of 10–15 stimulations, divided into sets of 3–4 stimulations, were applied with increasing voltage (range 2–15 Volts) and amperage (maximum 350 mA). After collection, the total volume was noted, subjective motility of the native semen was evaluated by dark field microscope (Olympus CH 40, Olympus, Hamburg, Germany) equipped with a heating stage (37 °C) and sperm concentration was estimated using an improved Neubauer haemocytometer. Smears were prepared for later evaluation of sperm morphology using the Kovács-Foote staining technique as was previously described [4] and aliquots of native semen were fixated in Hancock's fixative for evaluation of acrosome integrity [5]. Sperm morphology evaluation included search for abnormalities of the acrosome (missing, dissolving, swollen, deformed, asymmetrical, small, or with acrosomal granules), the head (deformed, corded, bulb-, spear- or spade-shaped, round, conical, narrow, small, or large), the neck (broken, tail-less, para- or retro-axial bend, plasma droplet), the mid-piece (fibrilar, deformed, double tail, narrow, broken, plasma droplet), the principal piece and end-tail (looped, coiled, bent, stunted, plasma droplet) and other (multiple heads, more than two tails). On three occasions the hypoosmotic swelling test (150 mOsm/kg fructose/trisodium-citrate solution) was also used to evaluate membrane integrity as was previously described [6]. When sufficient seminal plasma was available its osmolarity was evaluated. These measurements were performed on samples obtained from the supernatant after centrifugation of native sperm at 1000 × g for 20 min immediately after collection. Osmolarity of the samples was measured in triplicate with automatic osmometer (Roebling, Berlin, Germany). Samples were evaluated on the day of collection or kept at −20 °C pending evaluation.

2.5. Chilled storage of semen

After collection and evaluation, aliquots of only the combined best fractions containing approximately 100 × 10^6 cells were transferred into 7 pre-warmed Eppendorf tubes and suspended in seven different isothermic extenders: 1) Berliner Cryomedium (BC) basic solution—a TEST, fructose, lactose, egg yolk extender with osmotic strength of 330 mOsm/kg [7]. This extender has been used successfully for a wide variety of species [7–10], 2) Biladyl®—a Tris, citric acid, fructose, egg yolk extender that has been used for various artiodac-