Theriogenology 119 (2018) 91–95

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Characterization and cryopreservation of Amur leopard cats (*Prionailurus bengalensis euptilurus*) semen collected by urethral catheterization

Dong-Hyuk Jeong [a, 1], Jeong-Ho Kim [b, 1], Ki-Jeong Na [c, *]

[a] Species Restoration Technology Institute of Korea National Park Service, Gurye, 57616, Republic of Korea
[b] Cheongju Zoo, Cheongju, 28311, Republic of Korea
[c] College of Veterinary Medicine, Chungbuk National University, Cheongju, 28644, Republic of Korea

ARTICLE INFO

Article history:
Received 6 February 2018
Received in revised form
7 June 2018
Accepted 8 June 2018

Keywords:
Amur leopard cat
Computer-assisted semen analysis
Cryopreservation
Motility
Semen
Urethral catheterization

ABSTRACT

The Amur leopard cat (*Prionailurus bengalensis euptilurus*) is a globally endangered species, and there is thus an urgent need to increase its population. The objectives of this study were to: (1) confirm the utility of urethral catheterization (UC) for semen collection from Amur leopard cats; (2) investigate proper dilution media for fresh semen; and (3) investigate the effectiveness of sperm cryopreservation, including examining the effect of glycerol concentration. Six adult males in captivity (mean weight 5.03 ± 0.44 kg, aged 2–6 years) were included. All study procedures were performed during the breeding season (February to April) over two consecutive years. Semen samples (n = 28) were collected four or five times from each animal (four times for two animals and five times for four animals) by UC under general anaesthesia, and their characteristics (including sperm motility) were evaluated. First, the sperm motility in semen diluted in Ham's F-10 or phosphate buffer saline (PBS) was compared. Next, semen diluted with TEST-yolk buffer containing 2%, 4%, or 6% glycerol was frozen in a liquid nitrogen tank, and sperm motility and acrosome integrity were evaluated after thawing. No difference in motility was observed between sperm diluted in Ham's F-10 and PBS. The percentages of sperm motility and kinetic values in semen frozen in 2% or 4% glycerol were higher than those in semen stored in 6% glycerol. In conclusion, the UC method for semen collection is recommendable for Amur leopard cats and should be useful for artificial insemination. Although sperm motility decreased after thawing, samples thus preserved may be usable for advanced reproductive techniques, such as intracytoplasmic sperm injection or in-vitro fertilization.

1. Introduction

The Amur leopard cat (*Prionailurus bengalensis euptilurus*) is an endangered species that inhabits only the Korean Peninsula, Northeast China, and East Siberia. Protection of Amur leopard cats and other endangered species could benefit from effective captive breeding and genetic management, including the application of assisted reproductive technologies (ARTs), such as genome resource banking, in-vitro fertilization, embryo transfer, and artificial insemination (AI) [1–3]. Maintaining adequate genetic variation could be facilitated by storing frozen gametes and embryos in liquid nitrogen tanks, permitting easier transport of genetic resources and long-term preservation [1,4]. However, success depends on establishing effective protocols for collecting and cryopreserving these fragile biological samples and employing assisted reproduction to routinely generate viable offspring.

Semen from cheetahs (*Acinonyx jubatus*) [5], ocelots (*Felis pardalis*) [6], and fishing cats (*Prionailurus viverrinus*) [7] can be stored effectively after collection by electro-ejaculation and used for intrauterine AI in Amur leopard cats [8]. However, little is known regarding the natural history of Amur leopard cats or their reproductive physiology, or ART applications with this species. Moreover, semen was mostly collected by electro-ejaculation in previous studies, and microscopic visual inspection was used to assess the

* Corresponding author. College of Veterinary Medicine, Chungbuk National University, Cheongju, 28644, Republic of Korea.
E-mail addresses: africabear@knps.or.kr (D.-H. Jeong), In-africa@korea.kr (J.-H. Kim), sigol@cbnu.ac.kr (K.-J. Na).
[1] The first two authors (Drs. Jeong and Kim) contributed equally to this research.

https://doi.org/10.1016/j.theriogenology.2018.06.004
0093-691X/

semen quality, including sperm motility. Although electroejaculation is commonly used for semen collection with various animal species, it involves high equipment costs and sperm death when a high current is applied to the bladder [9]. Thus, urethral catheterization (UC) after administering an alpha-2 agonist (such as medetomidine) has been investigated in domestic cats [10], lions (*Panthera leo*) [11], and jungle cats (*Felis chaus*) [12], and more concentrated, high-quality semen was collected. Microscopic assessment of semen quality is subjective, although this can be improved using the computer-assisted semen analysis (CASA) program [13–15].

Securing high-quality semen during captive breeding is important, and the quality of sperm depends on the collection, dilution, and storage methods. Therefore, selecting appropriate dilution and storage media is essential for stable semen preservation. In various feline species, Ham's F-10 and phosphate-buffered saline (PBS) have mainly been used for semen dilution, and TEST-Yolk buffer (TYB) with glycerol is commonly used for cryopreservation, although optimal dilution and freezing medium or glycerol concentrations have not been investigated in Amur leopard cats. Thus, we evaluated the potential for semen collection and cryopreservation with Amur leopard cats to establish a species-specific reproductive database and develop an efficient strategy for genome-resource banking and ART. Our objectives were to: (1) confirm the applicability and usefulness of UC for semen collection from Amur leopard cats; (2) evaluate the sperm characteristics; (3) investigate proper dilution media for fresh semen; and (4) investigate the effectiveness of sperm cryopreservation, including examining the effect of the glycerol concentration.

2. Materials and methods

2.1. Animals

The study protocol was approved by the Animal Care and Use Committee (CBNUA-1127-1702) of Chungbuk National University. Six clinically healthy adult males (mean weight 5.03 ± 0.44 kg, aged 2–6 years) housed at the Cheongju Zoo were included. All study procedures were performed during the breeding season (February to April) over two consecutive years. Each animal was housed individually in a small room (~4 m × 2 m × 2.5 m) with a cement floor, and spent 8 h per day in a pen with an earthen floor (~4 m × 9 m × 2.5 m). A wooden cat tower was present in all enclosures to induce natural behaviour. The animals were fed 150 g of chicken daily, 50 g of commercial carnivore feed (Exotic Feline Diet Canned®, ZuPreem, Mission, TX, USA) daily, and a whole mouse once a week.

2.2. Anaesthesia

All animals were captured using a hoop net for anaesthesia after a 24 h fast and immobilized with ketamine (4 mg/kg IM; Ketamine 50®, Yuhan Co., Ltd., Seoul, Korea) and medetomidine (0.05 mg/kg IM; Domitor®, Orion Pharma Animal Health, Espoo, Finland). Subsequently, the animals were weighed and monitored (pulse, peripheral-oxygen saturation, and respiration) using a Datex-ohmada S/5 patient monitor (GE Healthcare, Buckinghamshire, UK). Finally, atipamezole (0.2 mg/kg im; Antisedan®, Orion Pharma Animal Health, Espoo, Finland) was administered to counteract the medetomidine and expedite recovery from anaesthesia. During the procedures, meloxicam (0.1 mg/kg im; Metacam®, Boehringer Ingelheim Co., Ltd., Ingelheim am Rhein, Germany) and cefazolin (5 mg/kg im; Cefazolin®, Yuhan Co., Ltd., Seoul, Korea) were administered to reduce pain and to minimize the risk of later wound infection. Total anaesthesia times (AT, time from recumbency to raising of the head) were recorded.

2.3. Blood sampling and serum testosterone measurement

After inducing general anaesthesia, blood was collected to confirm the relationship between semen quality and serum testosterone concentration (TC) from the cephalic or jugular vein (12 times for six animals during the study; 2.5 mL in each sampling). Serum was separated by centrifugation (600 × *g*, 10 min) and stored at −40 °C until hormone measurement. The TC was measured using a Cobas 8000 automatic electrochemiluminescence measurement device (Roche Diagnostics International Ltd, Rotkreuz, Switzerland).

2.4. Semen collection

Twenty-eight semen samples from six Amur leopard cats (four times from two animals and five times from four animals = 28) were collected using a urinary Tomcat catheter under general anaesthesia, and the samples were processed individually. Before semen collection, the pubic region was cleaned, and the penis was washed with sterile physiological saline. Catheterization (~9 cm) was initiated after 20 min of medetomidine treatment to induce the pharmacological effect. A urinary Tomcat catheter (1.0 mm × 110 mm; Portex, St. Paul, MN, USA) was lubricated with non-spermicidal sterile lubricant (Priority Care, First Priority, Elgin, IL, USA) before insertion into the urethral opening. At about 3 s after insertion, it was removed and placed in a pre-warmed Eppendorf tube. The time for semen collection (ST, time from inserting to removing the catheter) was recorded.

2.5. Semen evaluation

Collected semen was diluted with culture media. Then, the volume, sperm motility, sperm concentration, abnormal sperm morphology, and acrosome integrity were immediately investigated. To determine sperm concentration and motility including kinetics, the samples were dispensed onto a Makler chamber (Sefi Medical, Haifa, Israel) on a pre-heated warm plate (Thermo Plate, Tokai, Tokyo, Japan), and analysed via CASA (FSA sperm analyzer, Medical Supply, Wonju, Korea) in 10 randomized microscopic fields. The variables included the percentage of total motile spermatozoa (TM), progressive fast spermatozoa (PM; spermatozoa moving at >60 μm/s and a straightness of >80%), average path velocity (VAP; velocity over a calculated smoothed path), curvilinear velocity (VCL; velocity over the actual sperm track, including all deviations of sperm head movement), straight line velocity (VSL; velocity over the straight line distance between the beginning and the end of the sperm track), linearity (LIN; departure of sperm track from a straight line [VSL/VCL] × 100), beat-cross frequency (BCF; frequency with which the sperm crosses the smoothed path), and amplitude of lateral displacement (ALH; average time of absolute values for the instantaneous turning angle of the sperm head along its curvilinear trajectory). The percentages of spermatozoa with damaged acrosomes and abnormal morphology were determined using the Spermac® (Minitübe GmbH, Tiefenbach, Germany) staining technique and by assessing at least 100 sperm cells/slide at 1000 × magnification [16].

2.6. Experimental design

2.6.1. Experiment 1: Sperm motility over time during cool storage in different culture media

The collected semen samples (n = 18) were diluted in Ham's F-10 medium (liquid 9056, Irvine Scientific, Santa Ana, CA, USA;