Breeding soundness examination in a clouded leopard

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Abstract

A 3-year male clouded leopard was presented to the University of Tennessee College of Veterinary Medicine Theriogenology services for a breeding soundness examination. On physical examination, the left testis felt abnormally small. On ultrasonography, no abnormalities were observed; testis and prostate were of normal size. Semen was collected with a pulsator electroejaculator and a ram electroejaculator probe (commonly used to collect semen in small ruminants). The purpose of using this electroejaculator and probe was to determine if they could be used to collect semen from a clouded leopard. Using manual settings with this device we collected ~ 0.2 - 0.3 ml of semen. Motility and morphology of sperm were acceptable. We concluded that the Pulsator IV electroejaculator and ram probe can be used to collect semen as part of breeding soundness evaluation in a clouded leopard.

Keywords: Nondomestic feline, electroejaculator, manual settings, semen evaluation

Background

Clouded leopards (*Neofelis* genus) are medium-sized nondomestic felines, and the genus consists of 2 species.[1] One species, *Neofelis nebulosa*, is present in Southeast Asia, and the other, *Neofelis diardi*, is in the islands of Borneo and Sumatra.[2] Under human care, the average lifespan of clouded leopards is 13 to 15 years, and they reach sexual maturity between 20 and 30 months.[2-4] Reproduction is highest between 2 and 4 years in males and between 1 and 5 years in females. Reproduction usually starts to decline after 6 years of age.[4] Clouded leopards have long estrous cycles, varying from 15 to 40 days and estrus typically lasts for 3 - 6 days.[3] Additionally, > 40% of females display spontaneous ovulation.[3,6] However, unlike domestic cats, they rarely exhibit obvious signs of sexual receptivity under human care.[6] A seasonal influence has also been observed in clouded leopards. In males, motile sperm number peaked in June and July.[7] In western zoos, short periods of anestrus may occur in late summer and early fall.[3] Pregnancy lasts for ~ 90 days.[3]

Clouded leopards are considered vulnerable in the wild, and their numbers are continuing to decrease.[1,3] Habitat destruction and illegal hunting are big contributors to their declining numbers.[4,5] Therefore, breeding is important to maintain their survival and genetic diversity. However, breeding clouded leopards under human care is challenging, even in exhibits that mimic their natural environment.[3] In addition to an inconsistent ovulation pattern in females, males exhibit high levels of aggression that can potentially lead to serious injury and female's death.[2,8] Successful breeding had occurred with pairs that have been introduced before puberty.[3,7]Additionally, male clouded leopards have a high percentage of abnormal sperm. Teratospermia and pleiomorphism are common findings in clouded leopards, and this is likely related to low genetic diversity in the breeding population under human care.[2-3,6-8] Clouded leopard sperm have abnormal acrosomes, coiled tail, and midpiece defects[2,7,9] Motility ranged from 55 to 80% and normal morphology ranged from 20 to 35%.[2,5,9]

Electroejaculation under general anesthesia is a method adopted to collect semen. This procedure is relatively safe, with anesthesia usually being the biggest concern.[10] Ketamine hydrochloride has been used to anesthetize these animals.[2,5,11,12] With ketamine, one must carefully monitor for increased heart rate, increased blood pressure, apneustic breathing, and bronchodilation, as these are common side effects.[13] A disadvantage of electroejaculation in cats has been urine contamination. This has occurred with high voltage or when the electrode is positioned cranially.[11] Historically, electroejaculation probes used in wild cats have varied. In general, the probe has had 3 longitudinal strips of copper that are 0.4 cm apart and positioned ventrally, and the diameter varied among species.[11] Stimulations and voltage have also varied. A probe (2.5 cm in diameter and 7 cm in length) with electrodes (4.6 mm diameter and 56.8 mm length) was used in clouded leopards, and it was inserted into the rectum ~ to 10 - 12 cm. Stimulation consisted of 2 - 3 series that were divided into 2 - 3 sets of 10 stimuli each, using 1- 5 volts with 4 - 5 seconds of rest between each stimulus.[2] Furthermore, a 1.6 cm diameter probe was used and stimuli were divided into 3 series of 2 - 6 volts.[12] A 1.6 cm in diameter and 23 cm in length probe also was used and stimuli were divided into 3 series of 5 - 8 volts.[7]

Currently, a nondomestic feline semen collection procedure has been developed by the Smithsonian Conservation Biology Institute that is used in clouded leopards. Personal communication (Dr. Adrienne Crosier from the Smithsonian Conservation Biology Institute) provided us with the 2018 updated procedures used by the institute to conduct felid semen collection, evaluation, and freezing. This procedure uses either a 1.6 or 1.9 cm diameter probe, depending on the size of the animal. The probe and the rectum should be well lubricated with a nonspermicidal gel, and the probe should be gently inserted into the rectum. Electrodes should be positioned over the accessory sex glands, with the central electrode positioned ventrally. This procedure consists of 3 series and with each series consisting of 30 stimulations. For the first series, 10 stimulations are given at 2 volts, 10 at 3 volts, and 10 at 4 volts. For the second and third series, 20 stimulations are given at 3 volts and 10 at 4 volts. Two seconds rest should be given between each stimulus. However, each electroejaculation attempt should be modified to that specific patient, since it can vary among species, individuals, and the individual's response on various days.[10]

Collected semen should be evaluated for volume, pH, appearance, and consistency and sperm motility, morphology, and concentration.[2,5,10] None of these factors alone sufficiently predicts fertility, so they should be assessed together to determine a more accurate prediction of male fertility.[10] Fresh semen may be used for artificial insemination or can potentially be cryopreserved for later use. However, motility is lower in postthaw semen with possible lower fertility rates.[5]

We performed a breeding soundness examination on a clouded leopard imported from Thailand to determine his fertility. Electroejaculation was performed with a pulsator electroejaculator (Pulsator IV, Lane Manufacturing Inc., Denver, CO) and ram probe (12 cm long, 2 cm diameter, 2 electrodes [Figure 1]), traditionally used in small ruminants. This electroejaculator and probe,

Figure 1. Pulsator IV electroejaculator and ram probe

commonly used by large animal theriogenologists, were used to determine if a more widely available electroejaculator and probe could be used to collect a clouded leopard. Semen volume, motility, and morphology were analyzed after collection.

Case presentation

A 3-year 33 kg male clouded leopard (*Neofelis nebulosa*) at the Nashville Zoo (recently brought from Thailand) was presented to the University of Tennessee College of Veterinary Medicine Theriogenology services for a breeding soundness examination on September 15, 2020. He was fasted for 8 - 16 hours and was moderately active prior to anesthesia. He was anesthetized intramuscularly with a total of 280 mg of ketamine hydrochloride (Zetamine 100 mg/ml, VetOne, Boise, ID) and 1,680 µg of dexmedetomidine hydrochloride (Dexdomitor 0.5 mg/ml, Zoeitis inc, Kalamazoo, MI). He was initially given 100 mg of ketamine (3 mg/kg) and 690 µg of dexmedetomidine (20 µg/kg). However, he was not sedated enough and was given another 100 mg of ketamine and 690 µg of dexmedetomidine. Since he was not fully sedated another 50 mg of ketamine and 300 µg of dexmedetomidine was given. Subsequently, another 30 mg of ketamine was given. Once he was fully sedated, he was maintained on 3% of isoflurane via facemask. Animal was placed in right lateral recumbency and appeared healthy on physical examination. He was initially thought to be a cryptorchid. The zoo medicine team also collected urine samples (using a retrograde urinary catheter coated with nonspermicidal sterile lube) for pheochromocytomas, the most common neoplasia in clouded leopards.[14] Pheochromocytomas were identified in 45% of clouded leopards that were necropsied.[15] However, pheochromocytomas only consist of 2% of mortality in clouded leopards.[14] Urethral catherization was performed prior to electroejaculation to avoid semen contamination.

Penis was exteriorized and cleaned with saline and gauze. Scrotum was palpated and testes were measured with calipers. Transcutaneous ultrasonography of testes was performed using a 3.5 MHz convex transducer. Prostate was examined via transrectal ultrasonography using a 5 MHz rectal transducer. Approximately, 60 ml of nonspermicidal sterile lube was applied to the probe. More nonspermicidal sterile lube was applied into the rectum prior to electroejaculation. Manual settings on the ejaculator were used to avoid applying too high voltages. Voltages applied were manually manipulated (quarter to half-way up to the maximum volts on each step) that have been previously established by the manufacturer. With this approach, voltages were able to stay similar to voltages used in a standardized nondomestic feline procedure. Each step was held for 5 seconds, and steps were repeated 3 - 5 times, based on twitching response by the animal. A 2 second rest at 0 volts was given in between steps. Frequency (hertz) remained at 15.02 +/- 0.02.

Semen was analyzed for motility and morphology. For motility analysis, a drop of semen was placed on a prewarmed microscope slide, and a cover-slip was placed on top. Motility was subjectively evaluated under a phase contrast microscope at 10, 20, and 40 x objectives. For morphology analysis, a drop of Eosin-Nigrosin stain was placed on a prewarmed microscope slide, and then a drop of semen was added to the slide. This slide was mixed with another slide to make a thin layer to avoid sperm overlapping. Morphology was evaluated

by counting 100 individual sperm under oil objective (1,000 x). Semen was not sufficient for extending to have further examinations.

Outcome

Penis appeared normal and healthy (Figure 2), and had spines indicating sexual maturity. Both testes were palpable. However, the right testis was bigger compared to the left (2.3 cm in height, 4.1 cm in length, and 2.6 cm in width.) On ultrasonography, right testis measured 4.2 cm in length and calculated[3] scrotal volume was 14.5 cm. A consistent measurement of left testis with calipers was not possible (unable to maintain a consistent grip). However, it was measured (1.5 cm in height and 3 cm in length) via ultrasonogram. Testes and prostate appeared normal.

Figure 2. Extended penis

Electroejaculation was accomplished (~ 0.2 - 0.3 ml of ejaculate) using the Pulsator IV electroejaculator probe (2.5 cm diameter and 12 cm in length with 2 electrodes). A phase contrast microscope was used for semen analysis. Forty percent of sperm had normal motility (400 x objective) and 66% had normal morphology (eosin-nigrosin stain under 1000 x oil immersion objective). Primary abnormalities were acrosome defects (e.g. knobbed acrosomes), narrow heads, tightly coiled tails, and bent midpieces (Figure 3). Semen appeared viable for reproduction.

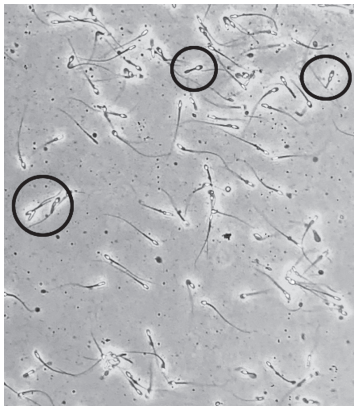

Figure 3. Abnormal sperm

Discussion

Objective was to determine if this genetically valuable clouded leopard would be fertile producing viable sperm. A breeding soundness examination was conducted that included semen collection and evaluation. An electroejaculator probe, commonly utilized in small ruminants (widely available), was used to collect semen.

Clouded leopards are categorized as vulnerable and their numbers are on the decline.[1,3] Therefore, zoos have important roles in clouded leopard conservation. However, breeding clouded leopards presents several challenges. Inconsistent ovulation pattern, high incidence of male aggression, and high percentage of abnormal sperm were observed.[2,8] Therefore, sperm collection and evaluation are important.

Clouded leopards have a high percentage of abnormal sperm (acrosomal defects, midpiece defects, and coiled tail defects).[2-3,5-9] Therefore, sperm morphology and motility are usually lower. Morphology of normal sperm has ranged from 20 to 35%, and motility ranged from 55 to 80%.[2,5,9] These defects are possibly due to a low genetic diversity in the clouded leopard population under human care.[2,6,7] In this case, normal morphology was 66% and motility was 40%. Higher percent of normal morphology in this clouded leopard could be due to its genetically diverse (Thailand) nature, supporting the theory that the lower genetic variation in the breeding population under human care could be causing low normal morphology. More studies are needed to confirm this observation. Abnormal sperm in this clouded leopard's ejaculate include acrosome defects, narrow heads, tightly coiled tail defects, and bent midpieces. Similar to this case, 40% of clouded leopard sperm had abnormal acrosomes (increased to 70% with Coomassie blue).[5] We did not use Coomassie blue, so it is possible that this clouded leopard had a higher percent of abnormal acrosomes that was not obvious under the phase contrast microscope.

A variety of techniques (probe size and stimulation procedures) have been used.[2,7,10-12] A nondomestic felid semen collection, evaluation, and freezing procedure was developed by the Smithsonian Conservation Biology Institute that used a 1.6 or a 1.9 cm diameter probe. However, we used a 2.5 cm diameter and 12 cm length probe with 2 electrodes, a probe, commonly used in small ruminants, and therefore, more widely available to veterinarians than a probe specifically used for wild felids. With this probe, we collected ~ 0.2 - 0.3 ml of ejaculate, demonstrating that this commonly available probe can be used to collect semen in clouded leopards. Semen volume, motility, and morphology were analyzed in this case. However, since the objective of this case was primarily to determine if this male could be fertile producing viable sperm, the semen was not cryopreserved.

Although this clouded leopard had lower percent of normal and motile sperm, percent normal sperm was higher compared to earlier observations. Additionally, it had an abnormally small testis and apparently the other testis was able to compensate to produce adequate sperm. Furthermore, his penis and prostate were normal based on physical examination and ultrasonographic observations. In conclusion, this cloud-

ed leopard was considered fertile and capable of producing viable sperm.

Learning points

- Pulsator IV electroejaculator with the ram probe (12 cm long and 2 cm diameter with 2 electrodes) can be used to collect semen in male clouded leopards.
- Pulsator IV electroejaculator and ram probe are easily available, and they can be used as an alternate method to collect semen in clouded leopards for data collection, semen evaluation, and potential artificial insemination.

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