Semen collection, evaluation, and cryopreservation in the domestic feline
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Abstract
Semen collection, cryopreservation, and subsequent use in the domestic cat has its own unique challenges, but is important for the improvement of advanced reproductive techniques. The number of publications on this topic has increased dramatically over the past ten years. The domestic cat serves as an important model for several human diseases, as well as a model for the preservation of the rare and endangered feline species. Techniques for the preservation of spermatozoa in the domestic cat are vital to preserve valuable genetics in pure breed catteries and valuable research models. Semen collection is most often performed by use of an artificial vagina, or by electroejaculation under general anesthesia. The intent of this review is to describe a detailed protocol for the successful collection, cryopreservation, and use of feline semen.

Keywords: Feline, cat, cryopreservation, sperm collection, electroejaculation

Introduction
Interest in domestic cat semen collection, evaluation, and cryopreservation has increased in the past ten years. Because the domestic cat can serve as a model for reproduction of exotic and endangered feline species, and as an animal model for human disease, interest in assisted reproduction has increased. Semen collection and evaluation in the cat present a unique set of challenges due to the small volume and comparatively low sperm numbers present in each ejaculate. In the domestic cat, semen collection, evaluation, and cryopreservation are used extensively in catteries and feline research colonies to preserve important or valuable genetic materials.

Semen collection
The two common methods of semen collection in the tom cat are an artificial vagina (AV) and electroejaculation. The AV is most commonly constructed using an Eppendorf tube (Eppendorf North America, Hauppauge, NY) and a rubber pipette bulb. The male is allowed to mount a queen, and the AV is held in place to facilitate and collect the ejaculate. If the male has been adequately trained to the AV, or a queen in estrus is not available, the male may be allowed to mount a gloved arm. The advantages of using an AV are that collection can be performed readily in the unanesthetized tom and a complete ejaculate is obtained. Disadvantages are the requirement for training and the frequent necessity of a teaser queen. Often, two to three weeks of conditioning and training are required before a tom is consistently producing ejaculates, and training may not be successful in all toms. Collection using an AV is an excellent method for situations where a single male or group of toms are collected on a regular basis, such as in a cattery or research colony, but is impractical for a single evaluation in a clinical setting using an untrained tom.

Electroejaculation is the most common method of obtaining an ejaculate from a tom which is not trained to an AV. The procedure requires general anesthesia. This author’s preferred anesthesia protocol includes dexmedetomidine (30-40 µg/kg) and ketamine (3-5 mg/kg) injected intramuscularly, followed by intubation and supplemental oxygen. Inhalant anesthesia (isofluorane) can be added if necessary, but the short procedure time generally does not require it. On occasion, electroejaculation using inhalation anesthetics may result in urination and contamination of semen samples. Zambelli, et al. compared the quality of ejaculates collected by electroejaculation using medetomidine alone or ketamine alone. These researchers found that the use of an α2 agonist (medetomidine) produced higher numbers of spermatozoa in the ejaculate than using ketamine alone, and did not increase the incidence of retroejaculation. However, these researchers did not evaluate the use of these medications in combination. To prevent the perception of discomfort during the procedure, it is recommended that an anesthetic, such as ketamine, be added to balance the sedative and analgesic effects of dexmedetomidine (personal communication, Johnson 2010). Each tom should be monitored appropriately while under general anesthesia to minimize anesthetic complications.

Procedure for electroejaculation:
- Electroejaculation is performed using a rectal probe one cm in diameter and 12-13 cm long (Figure 1). Appropriate electroejaculators are available commercially (P-T Electronics, Boring, OR; Figure 2).
- Lubricate the rectal probe with non-spermicidal lubricant and insert the probe gently into the rectum approximately 5-7 cm. The electrodes should be oriented ventrally.
• If feces in the rectum prevent the placement of the probe, a lubricated gloved finger may be used to evacuate the rectum but is not always necessary.
• Manually extend the penis and clean with gauze moistened with saline (no alcohol or soap). Dry the penis with clean or sterile dry gauze.
• Place a sterile vial (Eppendorf) over the penis. Alternatively, one could use a sterile 5 mL sample collection vial (Nalgene, Thermo Fisher Scientific, Rochester, NY).
• Turn on the ejaculator. Make sure the rheostat dial is set to zero prior to activating the power switch.
• Rotate the rheostat to provide a series of electrical stimuli by turning the dial to the desired voltage for 2-3 seconds, then abruptly back to zero for 2-3 seconds. The stimuli should be administered in the following order:
  o Set one: 10 times with 2 volts, 10 times with 3 volts, 10 times with 4 volts, rest 3-5 min.
  o Set two: 10 times with 3 volts, 10 times with 4 volts, 10 times with 5 volts, rest 3-5 min
  o Set 3: 10 times with 4 volts, 10 times with 5 volts, 10 times with 5 volts (or 6 if needed, depending on previous response).
• The sample obtained between each electroejaculation set should be evaluated for the presence of sperm. This is usually readily evident, as an ejaculate containing spermatozoa will be cloudy. A new, sterile tube should be used between each set after collection to prevent contamination or loss of the sample.

Figure 1: A rectal probe designed for use in the domestic feline measures 1 cm in diameter and 12-13 cm long.

Figure 2. Several models of electroejaculators are available commercially. This one is produced by P-T Electronics (Boring, OR) and features a manual rheostat that offers complete control of the stimuli provided.
The tom’s response to the stimuli should be monitored and the probe location adjusted accordingly. During the stimulation, both hind limbs typically would extend symmetrically. If they are not extending, or if one extends more than the other, confirm that the probe is in contact with the rectal wall, and that the electrodes are on ventral midline.

As an alternative to an ejaculate, collection of epididymal sperm by flushing post-castration or postmortem followed by cryopreservation has been described. Research with this technique in the domestic cat has provided a model for the preservation of genetic material from endangered feline species.

Ejaculated sperm for morphologic evaluation may be collected by aspiration or lavage of the queen’s vaginal vault following mating. Collection by this method may also be useful to rule out azoospermia. Because retroejaculation is common in the tom, cystocentesis and analysis of the urine after ejaculation may yield enough sperm cells for a limited analysis but both sperm motility and morphology are likely to be compromised.

**Semen evaluation**

Following collection, the volume of the ejaculate is recorded, and the sample is immediately extended 1:1 using a slow, drop-wise addition with mixing of a suitable medium (Ham’s F-10 with 25 mM Hepes, 1mM pyruvate and glutamine, penicillin/streptomycin/neomycin, and 5% fetal bovine serum). Motility (total and progressive) should be recorded by estimation under low power microscopy on a warmed microscope slide, or using a computer assisted analysis (CASA) calibrated for feline spermatozoa. Concentration is determined using a hemacytometer at a 1:100 dilution. The Nucleocounter® (Chemometec, Allerød, Denmark) has been used clinically to determine feline sperm concentration but requires at least 10 µL of sample. The low volume of the feline ejaculate often precludes routine use of this instrument. Volume of the ejaculate and sperm concentrations will vary between cats and collection method. The reported ranges for samples obtained using electroejaculation are 0.001-0.7 mL and 0.05-153 million sperm per ejaculate.

Evaluation of sperm morphology is an integral part of the semen evaluation. Teratospermia in the domestic cat has been defined as less than 40% morphologically normal spermatozoa. There is a very high degree of teratospermia in many of the exotic feline species studied, complicating genetic preservation. Teratospermia is also observed in small populations of cats where inbreeding has occurred. A single generation of inbreeding (offspring bred to parent) produced male offspring with less than 15% morphologically normal sperm compared to 55% morphologically normal sperm in control animals, indicating that loss of genetic diversity leads to increased teratospermia in as little as one generation. The degree of teratospermia and the nature of the defects present affect the post-thaw survival of cryopreserved sperm, and the freezing method may need to be adjusted to compensate for these defects. For example, rapid cooling of semen collected from teratospermic cats resulted in larger number of damaged acrosomes compared to semen collected from normal controls. The number of damaged acrosomes is decreased when using a slower cooling rate.

In other species, the number of morphologically normal sperm in an ejaculate and motility of the sperm are often closely correlated. In the cat, many teratospermic ejaculates demonstrate adequate motility (greater than 70%), in spite of low number of morphologically normal spermatozoa. Teratospermic cats appear to compensate for the lower number of morphologically normal spermatozoa by increasing spermatogenesis and frequency of copulatory activity. These strategies may improve breeding success by allowing an adequate number of normal sperm to be deposited into the tract. Other measured parameters of the ejaculate may include pH, osmolality, membrane integrity, sperm chromatin structure, bacterial culture, or seminal plasma chemistry.

**Cryopreservation**

Successful cryopreservation of feline semen was first reported in 1978 by Platz, et al. with a conception rate of 11% following intravaginal insemination. Subsequent research has compared various cooling rates, extenders, cryoprotectants, and techniques to achieve the optimal post-thaw motility for several feline species. For the domestic cat, TEST yolk buffer comprised of TES buffer, Tris lactose, and 20% egg yolk is an effective extender. Glycerol at a final concentration of 4-5% is a commonly used as a cryoprotectant. Feline sperm appear to be sensitive to glycerol, and higher concentrations result in lower post-thaw sperm motility.

Appropriate cooling rates optimize post-thaw sperm quality. Using an ultra-rapid (14 ºC/min) or a rapid (4 ºC/min) cooling rate from room temperature to either 0 or 5 ºC caused a significant decrease in the number of intact acrosomes, thereby affecting the post-thaw semen quality in teratospermic cats. This effect was diminished when a slower cooling rate of 0.5 ºC/min was used. A comparison of five freezing rates indicated that a rate of 3.85 ºC/min from 5 ºC to -40 ºC resulted in the greatest post-thaw motility and lowest number of damaged acrosomes.

The authors use a protocol designed and tested by Pukazhenthi, et al. at the Smithsonian National Zoological Park. Following collection, the ejaculate is immediately extended 1:1 in Ham’s F-10 with Hepes. An
aliquot of the extended sample is used to obtain an initial motility and concentration using a hemacytometer. The remainder of the ejaculate is centrifuged at 300 x g for 8 minutes. The supernatant is removed. The resulting sperm pellet is re-suspended in a quantity of TEST medium (Refrigeration Medium; Irvine Scientific, Santa Ana, CA) sufficient to achieve a concentration of 100-120 x 10^6 motile sperm/mL (no fewer than 50 x 10^6 motile sperm/mL). Feline spermatozoa appear to require a lower concentration of glycerol.12 A final concentration of 4% glycerol is used during the final cryopreservation step. Initially, TEST-Freezing Medium (12% glycerol) is diluted to an 8% solution by combining two parts of TEST-Freezing Medium with one part TEST-Refrigeration Medium (0% glycerol). An equal volume of this 8% glycerol medium is added to the sperm solution in a three step process: add one-fourth of the total volume to the ejaculate, wait five minutes, add another one-fourth of the total volume, wait five minutes, then add the remaining volume. This results in a final glycerol concentration of 4%, and a final concentration of approximately 50-60 million motile spermatozoa/mL. The ejaculate is loaded into 0.25 mL straws and sealed. If loading less than 0.25 mL in a straw, a small amount of TEST medium with 4% glycerol can be loaded first, followed by an air bubble, then the sperm suspension. Straws are placed in a sealed plastic bag and submerged in a room temperature water bath. A plastic bottle (a squirt bottle or 0.5 L drinking bottle) can function well as a water bath. The water bath containing the straws is placed in a refrigerator or in a cooler with ice packs for two to three hours, until the water temperature reaches 5 ºC. Once reaching 5 ºC, the extended semen in the loaded straws is ready to freeze.

The procedure can be modified if working in a laboratory with a “cold room.” The ejaculate is initially processed as above, but freezing medium containing glycerol is not added until after the cooling step. The processed ejaculate (in Refrigeration Medium) is placed in a room temperature water bath (300-350 mL) and placed in the cold room until the water bath reaches 5 ºC. An equal volume of 8% TEST-Freezing Medium is prepared and cooled with the ejaculate. After reaching 5 ºC, the freezing medium is added in a stepwise fashion (as described above) and the extended semen is loaded into cooled straws (5 ºC) immediately prior to freezing.

Semen is frozen in a two step liquid nitrogen vapor method (Figure 3). Initially, straws are placed on a rack 7.5 cm above the liquid nitrogen (-30 ºC) for one minute. The straws are then lowered to a second rack 2.5 cm above the liquid nitrogen (-130 ºC) and held there for an additional one minute before plunging into the liquid nitrogen and transferred to canes for long term storage. To thaw, straws are removed from liquid nitrogen storage, held in the air for ten seconds, and transferred to a 37 ºC water bath for 30 seconds. Immediately following thawing, the straws are thoroughly dried, opened, and the semen placed in a vial. The thawed semen is diluted in a drop wise fashion with an equal volume of warmed Ham’s F-10 medium with Hepes buffer and 5% fetal calf serum. Following dilution, the sample is centrifuged at 300 x g for 8 minutes. The supernatant containing the egg yolk, glycerol, and diluent is immediately removed, and the pellet re-suspended in 100-200µL of the Ham’s F-10 medium prior to insemination.

Figure 3: Diagram depicting the measurements for the two step freezing process. Two stainless steel test-tube racks of appropriate size; 1 placed on side, 1 placed flat, can be secured together with plastic ties. Pour adequate liquid nitrogen into styrofoam box and place the steel test-tube rack apparatus inside. Replace lid and allow to equilibrate prior to placing straws onto top level.

Use of cryopreserved sperm

Prior to insemination, estrus queens should be given an ovulation induction agent. A single dose of 100 IU human chorionic gonadotropin (hCG) or 25 µg gonadotropin-releasing hormone (GnRH; Cystorelin®, Merial, Duluth, GA) IM successfully induces ovulation approximately 25-27 hours after administration.15,16 Queens are usually inseminated between 28-30 hours after ovulation induction.16,17 When using frozen-thawed semen, intrauterine insemination is preferred to intravaginal insemination.16,17 The birth of kittens following intravaginal...
insemination of frozen-thawed semen has been reported, but success rates are low. More recent studies failed to produce pregnancies following intravaginal insemination with frozen-thawed spermatozoa. Intrauterine insemination is easily and quickly accomplished by laparotomy. A benefit to this procedure is it allows visualization of the uterus and ovaries. When inseminating at 30 hours after ovulation induction, corpora lutea can be observed, confirming ovulation has occurred. A pregnancy rate of 57% (8/14 queens) following surgical insemination with 50 x 10^6 frozen-thawed spermatozoa has been reported. Additionally, a 27% pregnancy rate (3/11 queens) following insemination with 5 x 10^7 frozen-thawed epididymal spermatozoa was achieved. A third study resulted in a pregnancy rate of 75% (6/8 queens) in 2009 using approximately 40 x 10^6 motile spermatozoa. Laparoscopic intrauterine insemination has been reported as an alternative to a laparotomy. However, this study indicated that pre-ovulatory anesthesia may affect ovulation. Fewer corpora lutea, decreased embryo recovery, and reduced pregnancy rates were reported in cats that underwent laparoscopic intrauterine insemination under general anesthesia prior to ovulation compared to queens undergoing the same procedure performed after ovulation suggesting that it may be beneficial to perform surgical intrauterine inseminations after ovulation.

Nonsurgical, transcervical insemination has been reported. Seventeen attempts resulted in 12 successful cervical catheterizations, four unsuccessful attempts, and one vaginal fornix penetration. Several methods and variations of this technique have since been described. Success rates with transcervical insemination are dependent on operator experience.

Summary
Collection of semen from the domestic cat can be accomplished with the necessary equipment. Evaluation of the ejaculate is similar to evaluation in other species. The cat often has a higher incidence of teratospermia due to the reduced genetic pool available in many catteries, research colonies, and exotic species. In natural breeding situations, cats appear to compensate for teratospermia by increased numbers of matings per estrus. Spermatozoal defects adversely affect the quality of the semen following cryopreservation. By adjusting the cooling rate and concentration of glycerol, many teratospermic cats have been successfully cryopreserved. The use of cryopreserved sperm can result in acceptable pregnancy rates if semen is placed directly into the uterus.

References
