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ASSESSMENT OF VIRAL PRESENCE IN SEMEN AND REPRODUCTIVE FUNCTION OF FROZEN-THAWED SPERMATOZOA FROM PALLAS' CATS (*OTOCOLOBUS MANUL*) INFECTED WITH FELINE HERPESVIRUS

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Abstract: Although herpesviruses are known to contaminate the semen of several mammalian species, the occurrence of feline herpesvirus type 1 (FHV-1) in semen of infected cats has not been reported. Our objectives in this study were to investigate the presence of FHV-1 DNA in seminal fluid and frozen-thawed spermatozoa from FHV-1 infected Pallas' cats (*Otocolobus manul*) and assess the functionality of their frozen-thawed spermatozoa in vitro. Over a 3-yr period, semen ($n = 33$ ejaculates) was collected periodically via electroejaculation from four Pallas' cats chronically infected with FHV-1. Spermic ejaculates were frozen by pelleting on dry ice and stored in liquid nitrogen. After thawing, sperm motility and acrosome status were assessed over time during in vitro culture. For vitro fertilization (IVF), viable domestic cat (*Felis silvestris catus*) oocytes were inseminated with frozen-thawed Pallas' cat spermatozoa and evaluated for embryo cleavage. For FHV-1 polymerase chain reaction (PCR) analysis, DNA was extracted from seminal fluid, frozen-thawed spermatozoa, inseminated oocytes, heterologous IVF embryos, and conjunctival biopsies and analyzed for presence of a 322–base pair region of the FHV-1 thymidine kinase gene. Immediately post-thaw, sperm motility and percentage of intact acrosomes were decreased ($P < 0.05$) compared to fresh samples, and declined further ($P < 0.05$) during culture. However, all frozen-thawed IVF samples were capable of fertilizing domestic cat oocytes (overall, 46.1 ± 6.0% cleavage). PCR analysis did not identify FHV-1 DNA in any reproductive sample despite the repeated detection of FHV-1 DNA in conjunctival biopsies. These results suggest that semen collected from Pallas' cats infected with FHV-1 does not contain cell-associated or non–cell-associated virus and that frozen-thawed spermatozoa exhibit adequate function for potential genetic rescue with minimal risk of FHV-1 transmission.

Key words: Assisted reproduction, cryopreservation, herpesvirus, *Otocolobus manul*, Pallas' cat, semen.

INTRODUCTION

Feline herpesvirus type 1 (FHV-1) infection in cats can produce both acute clinical disease, characterized by upper respiratory tract and ocular symptoms (i.e., sneezing, nasal discharge, conjunctivitis, keratitis, and corneal ulceration), and latent carrier states with periodic reactivation and viral shedding.[16,34] In felids, the virus is shed primarily in ocular and nasal secretions.[15] In several other mammalian species, including humans,[14,51] bovids,[49] swine,[18] and equids,[10,30] herpesviruses also have been demonstrated in the semen of infected individuals. Based on these findings and the experimental observation that vaginal exposure of female domestic cats (*Felis silvestris catus*) to FHV-1 can cause genital infections,[4] FHV-1 has been suggested as a potential pathogen in cat semen.[29,41] However, there has been no published empirical evidence demonstrating the presence or absence of FHV-1 in the semen of domestic cats or any nondomestic felid species.

Clinical disease caused by FHV-1 infection occurs infrequently in vaccinated captive populations of most nondomestic felids, but three species, the cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), and Pallas' cat (*Otocolobus manul*), have experienced disease outbreaks in U.S. zoos.[5,27,28] Although FHV-1 infection rarely causes mortality in adult cats, neonates are more vulnerable, especially to secondary bacterial infections.[16,34] FHV-1 also can cause high morbidity in susceptible populations and become a significant barrier to movement of cats between zoologic institutions, impairing the genetic management of these small, threatened populations. For chronically-infected male cats, the application of assisted reproductive technology, such as artificial insemination (AI) or in vitro fertilization (IVF) and embryo transfer

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(ET), may offer one means to rescue their genetic potential,[21,53] provided that cat semen does not contain infectious virus.

The Pallas' cat is a small-sized felid species endemic to Central Asia that is facing threats to its survival in the wild.[22,32] Between 1995 and 2000, 24 Pallas' cats were imported from Russia and Mongolia to establish a founder population of this species in North American zoologic parks.[9] At two zoos, several of these founders and their offspring developed clinical signs consistent with FHV-1 infection, possibly related to vaccination with modified live FHV-1 vaccines. As an alternative to euthanasia, six FHV-1–infected cats (four males and two females) were donated to North Carolina State University to establish the core population of a Pallas' cat research colony. One goal of this colony is to intensively study the immunology and reproductive physiology of Pallas' cats and, if possible, to rescue the genetic contribution of FHV-1–infected founders through natural breeding and cross-fostering of offspring or by application of assisted reproduction. In the present study, our objectives were to 1) investigate potential FHV-1 infectivity of seminal fluid and frozen-thawed spermatozoa from Pallas' cats chronically infected with FHV-1, and 2) assess in vitro motility, acrosome status, and functionality of frozen-thawed Pallas' cat spermatozoa for future genetic rescue.

MATERIALS AND METHODS

Animals

Four adult male Pallas' cats (ages, 2–8 yr), chronically infected with FHV-1, served as semen donors for this study. Two of these males (Studbook [SB] Nos. 291 and 297) were wild-born in Russia and two (SB Nos. 390 and 391) were the captive-born offspring of one of the wild-born males (SB No. 297).[9] Infection with FHV-1 had been diagnosed originally in these Pallas' cats based on clinical signs and virus isolation,[28] and all cats showed variable recurrence of upper respiratory and ocular disease symptoms consistent with FHV-1 infection during the study period. Males were housed as singletons in adjacent wire mesh pens in an indoor laboratory animal facility and maintained under an artificial lighting schedule timed to mimic natural photoperiods. Ambient temperature (68–72°C) and humidity (20–70%) were maintained within recommended ranges for laboratory animal facilities. During the breeding season (January–April) each year, one of the wild-born males was housed with a FHV-1–infected female in the same enclosure. Cats were provided with a daily diet consisting of raw horsemeat, supplemented with vitamins and minerals (Milliken Feline Diet, Scarborough, Ontario M1V3F1, Canada). Once a week, each cat also was fed one whole mouse from a gnotobiotic colony.

Female domestic cats served as the source of in vivo–matured oocytes for heterologous IVF procedures. All laboratory cats were obtained from an antibody-defined breeding colony (Liberty Research, Inc., Waverly, New York 14892, USA) and received vaccination only with killed viral products (Felovax-IV, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA). Females were group-housed in large communal animal rooms and provided with a commercial cat diet (Iams Chicken & Rice, Iams Company, Dayton, Ohio 45414, USA). All animals were maintained in compliance with U.S. federal guidelines, and research procedures were reviewed and approved by the respective Institutional Animal Care and Use Committees at North Carolina State University and the Cincinnati Zoo & Botanical Garden.

Semen collection, processing, and cryopreservation

Over a 3-yr (2000–2003) time span, semen was collected periodically from male Pallas' cats with a minimum of 1 mo between procedures. All collections were conducted from December to May to bracket the normal breeding season of this species.[7] For semen collection, males were anesthetized using a combination of ketamine hydrochloride (Ketaset, Fort Dodge; 2 mg/kg body weight, i.m.), medetomidine (Dormitor, Pfizer Animal Health, Exton, Pennsylvania 19341, USA; 0.04 mg/kg, i.m.) and butorphanol (Torbugesic, Fort Dodge; 0.4 mg/kg, i.m.), supplemented as needed with isoflurane (0.5–1%; Aerrane, Baxter Healthcare Corporation, Deerfield, Illinois 60015, USA) delivered by face mask. Following sample collection, medetomidine was reversed with atipamazole (Antisedan, Pfizer; 0.2 mg/kg, i.m.). For a companion study of viral pathogenesis, bilateral conjunctival biopsies were collected from each male concurrent with the electroejaculation procedures. Biopsy samples were frozen in cryovials containing sterile phosphate-buffered saline and subsequently analyzed for viral DNA presence with polymerase chain reaction (PCR) analysis.

Semen was collected using a standardized electroejaculation protocol with slight modification.[42] Recovered raw semen was evaluated (×100 magnification) initially for the presence or absence of spermatozoa. For aspermic or oligospermic ejaculates, raw seminal fluid was centrifuged (1,700 *g*,

10 min) and cell-free supernatants transferred into labeled cryovials and frozen for later herpes PCR analysis. For spermic ejaculates, samples were assessed (×100 magnification) for progressive sperm motility (0–100%) and rate of forward progression (on a scale of 0–5, with 0 being nonmotile and 5 being rapid linear movement) to calculate a sperm motility index (SMI = [% sperm motility + (20 × rate of progressive movement)]/2).[20] Spermic samples were diluted (1:1) in warm (37°C) Ham's F10 culture medium (Sigma Chemical Company, St. Louis, Missouri 63178, USA), containing $NaHCO_3$ and 5% fetal bovine serum (v/v). Aliquots (3–5 μl) of diluted semen were used to determine sperm concentration[20] and for acrosome staining with a rose bengal/fast green stain.[35] The remaining semen was centrifuged (600 *g*, 10 min) to concentrate spermatozoa and remove seminal fluid and culture medium.

After centrifugation, washed sperm samples were resuspended slowly in cryoprotectant (TEST Egg Yolk; Irvine Scientific Inc., Santa Ana, California 92705, USA), supplemented with 4% glycerol (v/v), to a final concentration of 5–40 × 10^6 motile sperm/ml. Microcentrifuge tubes containing extended sperm samples were inserted into foam floats and placed into a glass beaker (100 ml) filled with room-temperature (22°C) water. Samples were slowly cooled to 4°C over 2 hr in a refrigerator and then frozen by pelleting (~30 μl/pellet) on dry ice before plunging into liquid nitrogen (LN_2).[20,42] Sperm pellets were transferred into labeled cryovials and placed in a LN_2 dewar for transport and subsequent storage in the Cincinnati Zoo's biologic resource bank.

Heterologous in vitro fertilization

For oocyte recovery, female domestic cats in the Cincinnati Zoo's research colony were treated with exogenous gonadotropins and subjected to laparoscopic oocyte aspiration, as previously described with slight modification.[45] Anestrual domestic cats (n = 9) were injected (i.m.) with equine chorionic gonadotropin (150 IU; Sigma) followed 85 hr later with an injection (i.m.) of human chorionic gonadotropin (hCG; 100 IU; Sigma). At 25–28 hr after hCG, females were anesthetized and evaluated laparoscopically for aspiration of ovarian follicles. Mature follicles (≥2 mm diameter) were aspirated percutaneously into sterile collection tubes containing pre-equilibrated (38°C; 5% CO_2 in air) Ham's F10 culture medium, supplemented with 5% fetal bovine serum (v/v) and sodium heparin (40 U/ml). Recovered oocytes were assessed microscopically (×10–40) for morphologic characteristics, and high-quality oocytes, exhibiting a dark, uniform cytoplasm, distinct corona radiata, and expansive cumulus cell mass, were selected for insemination.[17] Oocytes were washed repeatedly through 100-μl microdrops of pre-equilibrated Ham's F10 medium and transferred into petri dishes containing insemination drops (10–17 oocytes/drop; 95-μl volume) under washed mineral oil.

For IVF, frozen sperm samples (n = 17) were thawed (1 pellet/100 μl Ham's F10 with Hepes buffer; 37°C), diluted (1:3) in additional Ham's F10, centrifuged (600 *g*, 10 min), and then resuspended in equilibrated Ham's F10 (with $NaHCO_3$) to a concentration of 10 × 10^6 motile sperm/ml. Recovered oocytes in microdrops were inseminated with frozen-thawed spermatozoa (5 μl or 5 × 10^4 motile sperm/drop; 10–17 oocytes/sample), cocultured (38°C; 5% CO_2 in air) for 12–18 hr, and then rinsed repeatedly to remove loosely-bound spermatozoa and cumulus cells. Oocytes were transferred into pre-equilibrated culture drops for an additional 30–36 hr of culture and evaluated for embryo cleavage and developmental stage (i.e., 1-cell, 2-cell, 3–4-cell, 5–8-cell, 9–16-cell) at 30 and 48 hr post-insemination. After 48 hr of culture, all oocytes and embryos were stripped repeatedly using a fine-bore glass pipette to remove any remaining cumulus cells and washed through three microdrops of medium. For each IVF sperm sample, oocytes and embryos were pooled and frozen (−80°C) in a labeled cryovial (containing 1 ml of sterile culture medium) for later PCR analysis. Because of concerns that fluorescent staining would interfere with PCR analysis, routine staining of embryos and oocytes with Hoescht 33342 could not be used for further evaluation of fertilization and embryo development.

Residual IVF sperm samples were used for motility, acrosome, and viral assessments. Sperm motility was evaluated at 0, 1, 3, and 6 hr post-thaw in aliquots (3 μl) pipetted from microdrops (25 μl/drop; 2 × 10^6 motile sperm/ml, 0 h) under oil (38°C; 5% CO_2 in air) and an SMI calculated for each time point. Spermic aliquots from microdrops also were stained (0 and 6 hr post-thaw) using a rose bengal/fast green stain and assessed for acrosome status. Remaining aliquots of frozen-thawed spermatozoa (>5 × 10^5 sperm/sample; suspended in 9–500 μl of Ham's F10 medium) were transferred into labeled cryovials and frozen (−80°C) for later PCR analysis.

FHV-1 PCR Analysis

For FHV-1 PCR, DNA was extracted from samples of cell-free seminal fluid, frozen-thawed sper-

matozoa, inseminated domestic cat oocytes, hybrid embryos, and conjunctival biopsies, and analyzed for the presence of a 322–base pair fragment of the FHV-1 thymidine kinase gene.[52] This protocol reliably detects ≥240 copies of FHV-1 DNA. The assay was performed as previously described except for omission of the 10-min hot-start. DNA was extracted from sample vials using a commercially available kit (DNeasy Tissue Kit, Qiagen Inc., Valencia, California 91355, USA). The DNA concentration of each extracted conjunctival biopsy sample was estimated using a biophotometer, and approximately 250 ng of DNA was used in each PCR assay. The DNA concentration of all other samples was too low to quantify, and thus 25 or 35 μl of extracted DNA was used in each assay.

Positive and negative controls were included in all PCR assays. Negative controls were not subjected to DNA extraction; however, no batch of samples produced only positive results, suggesting that contamination at the DNA extraction step did not occur in this study. For negative controls, DNAse-RNAse-free water was substituted for sample DNA in the PCR mixture. For positive controls, DNA extracted from FHV-1 strain 727 (passage 8) cultured in Crandell-Reese feline kidney cells was substituted for sample DNA in the PCR mixture. This strain is a plaque-purified field isolate of FHV-1 previously verified as FHV-1 by immunofluorescence assay with FHV-1-specific antiserum.[31]

Following thermocycling, 10 μl of reaction mixture underwent electrophoresis on ethidium bromide–stained 1.5% agarose gels, and PCR products were identified by visual examination and then digitally photographed (Kodak Electrophoresis documentation and analysis system [EDAS] 290, Eastman Kodak Company, Rochester, New York 14650, USA). To assess the potential for PCR interference or inhibition by semen, two representative samples of seminal fluid (that did not contain detectable FHV-1 DNA) were 'spiked' with the FHV-1 DNA used as the positive control and processed as described. Both samples tested in this manner were positive.

Statistical analysis

Values are presented as means ± SEM. Variation in sperm motility (SMI) and acrosome status (percentage intact) over time for freshly-collected and frozen-thawed spermatozoa were assessed using a repeated measures analysis of variance (ANOVA) and differences evaluated using a Tukey-Kramer means comparison (Statview; SAS Institute, Cary, North Carolina 27513, USA). Differences in embryo cleavage percentage among males were assessed using a two-way ANOVA and Tukey-Kramer means comparison. All percentage data were subjected to arcsin transformation prior to ANOVA. Correlation of cleavage percentage with post-thaw sperm motility and acrosomal data also was assessed (Statview). For all analyses, a P value of <0.05 was considered significant.

RESULTS

Electroejaculation of male Pallas' cats resulted in recovery of seminal fluid (>10 μl) during most (80%, 33/41) collection attempts, with the majority (52%, 17/33) of these ejaculates containing adequate numbers of motile spermatozoa ($>0.5 \times 10^6$) for cryopreservation. A total of 17 ejaculates were processed for cryopreservation. The remaining aspermic and oligospermic samples ($n = 16$) were centrifuged for seminal fluid recovery and freezing (Table 1). After transport to the Cincinnati Zoo, one or two frozen sperm pellets representing each ejaculate were thawed to assess post-thaw sperm motility and function. For pre-freeze ejaculates, SMI averaged 71.6 ± 1.4, and 94.8 ± 1.0 % of acrosomes were intact (Fig. 1). Immediately post-thaw (0 hr), mean SMI (52.8 ± 2.7) was decreased ($P < 0.05$) relative to pre-freeze values and fewer ($P < 0.05$) acrosomes (39.0 ± 3.4%) were classified as intact. Values for both parameters continued to decline ($P < 0.05$) over 6 hr of culture (SMI: 20.7 ± 1.4; acrosomes intact: 32.1 ± 3.5%; Fig. 1). There was a significant interaction between the individual sperm donor and SMI, but pairwise means comparison did not reveal significant differences between individuals. Individual sperm donor also affected ($P < 0.05$) acrosome status, with spermatozoa from the one captive-born male (SB No. 391) having fewer intact acrosomes over time than samples from the two wild-born males.

Laparoscopic follicular aspiration of female domestic cats allowed recovery of 201 high-quality oocytes (22.3 ± 5.5 oocytes/female) for IVF procedures. The only frozen sperm sample available from one of the captive-born males (SB No. 390) had inadequate motility (10% progressive motility, SMI < 25) and total sperm numbers ($\sim 7 \times 10^4$) immediately after thawing to provide enough motile spermatozoa for IVF, and was not used. However, the entire sample was refrozen for subsequent FHV-1 PCR analysis. All other frozen-thawed sperm samples ($n = 16$) were used for IVF and proved capable of fertilizing domestic cat oocytes, based on cleavage to the 2–16 cell stage within 48 hr of insemination (Fig. 2). Following IVF and culture, embryo cleavage percentage for each sperm sample ranged from 13 to 80%, with a mean value

Table 1. PCR analysis of FHV-1 DNA in reproductive tissue samples obtained from male Pallas' cats chronically infected with feline herpesvirus.[a]

Cat SB No.	Sample date	Reproductive tissue samples			FHV-1 PCR results	
		Seminal fluid (μl)	Spermatozoa ($\times 10^4$)	No. embryos and oocytes	Reproductive samples	Conjunctival biopsies[b]
291	14 Dec 2000	29			negative	positive
	19 Feb 2001		30	11	negative	negative
	16 Apr 2001		49	15	negative	negative
	14 Jan 2002	12			negative	negative
	11 Feb 2002		31	12	negative	negative
	11 Mar 2002		26	11	negative	negative
	16 Apr 2002		8	11	negative	negative
	20 May 2002	180			negative	negative
	18 Mar 2003		114	15	negative	negative
	18 Apr 2003	160			negative	negative
297	19 Feb 2001		7	10	negative	negative
	16 Apr 2001		25	10	negative	negative
	11 Feb 2002		64	14	negative	negative
	11 Mar 2002		16	7	negative	negative
	16 Apr 2002		16	10	negative	negative
	20 May 2002	90			negative	negative
	17 Feb 2003		32	10	negative	negative
	18 Mar 2003		14	16	negative	positive
390	14 Dec 2000	40			negative	positive
	19 Feb 2001	50			negative	negative
	11 Mar 2002		7		negative	negative
	20 May 2002	70			negative	negative
	18 Mar 2003	32			negative	positive
391	14 Dec 2000	14			negative	positive
	19 Feb 2001	51			negative	positive
	16 Apr 2001		23	14	negative	negative
	14 Jan 2002	46			negative	negative
	11 Feb 2002		11	13	negative	negative
	11 Mar 2002		7	17	negative	negative
	20 May 2002	60			negative	negative
	17 Feb 2003	43			negative	positive
	18 Mar 2003	85			negative	positive
	18 Apr 2003	70			negative	negative

[a] PCR, polymerase chain reaction; FHV-1, feline herpesvirus type 1; SB, Studbook.
[b] Bilateral conjunctival tissue samples; for only those procedures that resulted in semen recovery.

of 46.1 ± 6.0%. For the three males (SB Nos. 291, 297, and 391) that provided IVF sperm samples (n = 3–7 ejaculates/male), cleavage percentages averaged 48.0 ± 9.6%, 52.6 ± 9.8% and 27.3 ± 8.3%, respectively, and did not differ ($P > 0.05$) among individuals (Fig. 3a). Most heterologous IVF embryos (72/92, 78%) exhibited initial cleavage by 30 hr post-insemination, with one or two subsequent divisions over the following 18 hr (Fig. 3b). Cleavage percentage was positively correlated ($P < 0.05$; $r = 0.54$) with acrosome status at 6 hr post-thaw, but was not correlated ($P > 0.05$; r = 0.11–0.43) with acrosome status immediately post-thaw (0 hr) or with SMI at any time point after thawing.

PCR analysis was conducted on cell-free seminal fluid samples (n = 16 ejaculates), frozen-thawed sperm samples (n = 17 ejaculates), noncleaving domestic cat oocytes (n = 107 oocytes; separated by IVF sperm sample into 16 vials), hybrid IVF embryos (n = 89 embryos; separated by IVF sample and combined with noncleaving oocytes into 16 vials), and bilateral conjunctival biopsies (n = 41). Three embryos and one oocyte were lost during processing prior to PCR. Reproductive samples used for PCR analysis averaged 64.5 ± 11.8 μl of

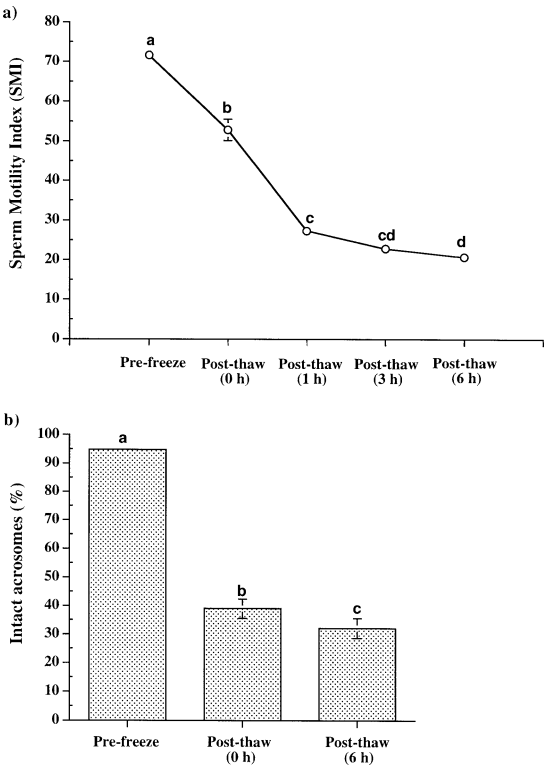

Figure 1. (**a**) Sperm motility index (SMI*) and (**b**) percentage of intact acrosomes for Pallas' cat sperm samples before and after cryopreservation and thawing. Values with different superscripts are different ($P < 0.05$). *SMI = (% sperm motility + [20 × rate of progressive movement])/2.

raw seminal fluid, 28.2 ± 6.0 × 10^4 frozen-thawed spermatozoa per ejaculate, and 12.3 ± 0.7 oocytes/embryos per IVF sperm sample. PCR analysis did not identify FHV-1 DNA in any of these reproductive samples (Table 1). However, FHV-1 DNA was identified in all four males from 13 of the 41 bilateral conjunctival biopsies collected at the time of electroejaculation. Semen was recovered concurrently with positive FHV-1 results from conjunctival biopsies on 8 occasions, including at least one semen sample obtained from each male coincident with FHV-1 detection in conjunctiva (Table 1).

DISCUSSION

In several mammalian species, herpesviruses have been identified using PCR or virus isolation as contaminants of ejaculated semen.[14,18,49,51] Although felids are highly susceptible to FHV-1 infection and display both acute and chronic disease syndromes, to the authors' knowledge, the presence or absence of herpesvirus in the semen of any cat species has not been reported. The results of this study indicate that for at least one felid species, the Pallas' cat, detectable amounts of FHV-1 DNA are not found associated with either cellular or noncellular components of semen collected from males infected with FHV-1, despite concurrent viral detection in multiple conjunctival biopsies.

The absence of FHV-1 DNA in semen from infected Pallas' cats is not totally unexpected, given previous observations of FHV-1 etiology in domestic cats. Herpesviruses have a propensity for infecting mucosal surfaces in all affected species, possibly related to slightly lower body temperatures found at these peripheral locations. In males of some species, herpesviruses colonize the preputial and penile mucosa, causing balanoposthitis and permitting semen to become infected through direct contact during ejaculation and coitus.[11,18,30,49] Herpesvirus infection in females of some species also may cause vulvovaginitis, endometritis, and/or abortion.[6,11,39,47] Although alphaherpesviruses such as FHV-1 share a strong concordance across species for in vitro culture traits and molecular genetic phylogeny,[16,54] most tend to be species-adapted with strong tropism for specific mucosal surfaces. In felids, it appears that FHV-1 does not have a strong affinity for reproductive tissues[16] nor for inducing abortion, even during widespread clinical outbreaks.[19]

Our inability to identify FHV-1 in Pallas' cat semen may indicate the complete absence of viral DNA in these samples, but also might be attributable to several other explanations. The PCR assay used for viral detection in this study can reliably detect ≥240 copies of FHV-1 DNA,[52] providing a more sensitive test than either viral isolation or immunofluorescent antibody assay.[8,40,46] However, it is possible that even greater PCR sensitivity might provide different conclusions about the presence or absence of FHV-1 in Pallas' cat semen. For example, a PCR assay used in bovids can detect as few as 5 copies of herpesvirus DNA in 50 μl of semen.[48] Although vaginitis has been induced in domestic cats following experimental intravaginal inoculation with high viral loads (~3,000 infectious viral particles),[4] the minimum number of viral particles necessary to induce genital infection in felids is unknown. In bovids, herpes infection after AI typically requires more than 200 infectious viral particles.[49] Accordingly, the clinical relevance of viral levels in cat semen lower than our current detectable limit has not been established.

Alternatively, FHV-1 may be shed in Pallas' cat semen but on an inconsistent basis or only associated with certain seminal components, making de-

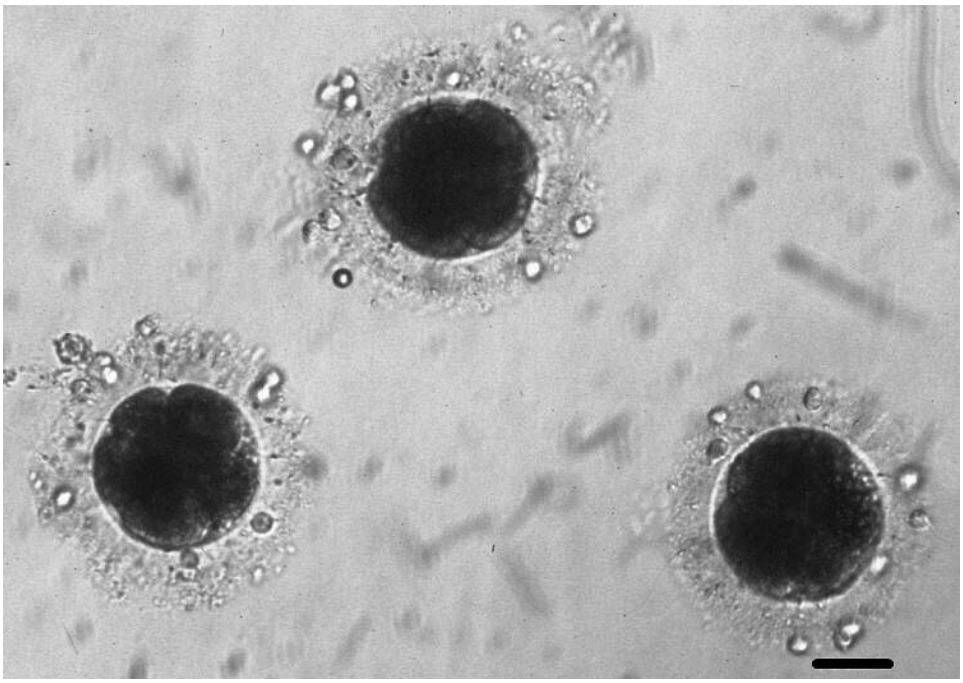

Figure 2. Pallas' cat–domestic cat hybrid IVF embryos (3–4-cell and 5–8-cell stage) at 48 hr post-insemination. ×100, scale bar = 50 μm.

tection difficult with infrequent collection attempts or variable sample recovery. The sporadic detection of FHV-1 in conjunctival biopsy samples of chronically-infected Pallas' cats suggests that Pallas' cats are similar to domestic cats in exhibiting intermittent viral reactivation and shedding.[16,34] Although positive PCR results were obtained on 13 occasions from Pallas' cat conjunctival biopsies, only one ejaculate containing appreciable numbers of spermatozoa was recovered concurrently. If FHV-1 is associated primarily with Pallas' cat sperm cells during periods of active viral replication, it is possible that the lack of sperm recovery during these periods may have biased our results. In bovids and humans, however, herpesviruses are found predominantly in the seminal fluid and are not strongly associated with spermatozoa only.[33,48]

To our knowledge, feline immunodeficiency virus (FIV) is the only other virus that has been investigated for its affinity for seminal shedding in cats. Unlike our findings with FHV-1, FIV has been shown by PCR and virus isolation to occur associated with seminal plasma and spermatozoa of both experimentally-infected and naturally-infected domestic cats.[24,26] In addition, transmission of FIV in domestic cats following laparoscopic intrauterine insemination with freshly-collected spermatozoa has been demonstrated.[25] However, insemination with frozen-thawed spermatozoa did not result in FIV transmission, suggesting that the cryopreservation process itself or prolonged cold storage (>4 mo) could affect viral infectivity.[25] In the present study, all frozen sperm samples were maintained in liquid nitrogen tanks for a minimum of 6 mo before thawing. Studies in other species, however, have shown that herpesviruses can readily survive cryopreservation and remain infective within liquid nitrogen tanks.[3] Assuming that the target region of viral DNA detected by this PCR protocol was not altered during cryopreservation, PCR should have been able to detect FHV-1 DNA regardless of viral viability at the time of PCR analysis.

Our findings are promising for the prospect of using frozen-thawed Pallas' cat spermatozoa for attempted genetic rescue. Results indicate that Pallas' cat spermatozoa subjected to cryopreservation exhibit adequate motility, acrosome status, and functionality after thawing for routine use in IVF procedures. Although post-thaw sperm motility decreased rapidly in most samples and acrosomal integrity was compromised, sperm viability and function were sufficient in all insemination drops to fertilize domestic cat oocytes. In addition, high-quality sperm samples were obtained for cryopreservation from three of the four study animals, with no differences observed in fertilization success

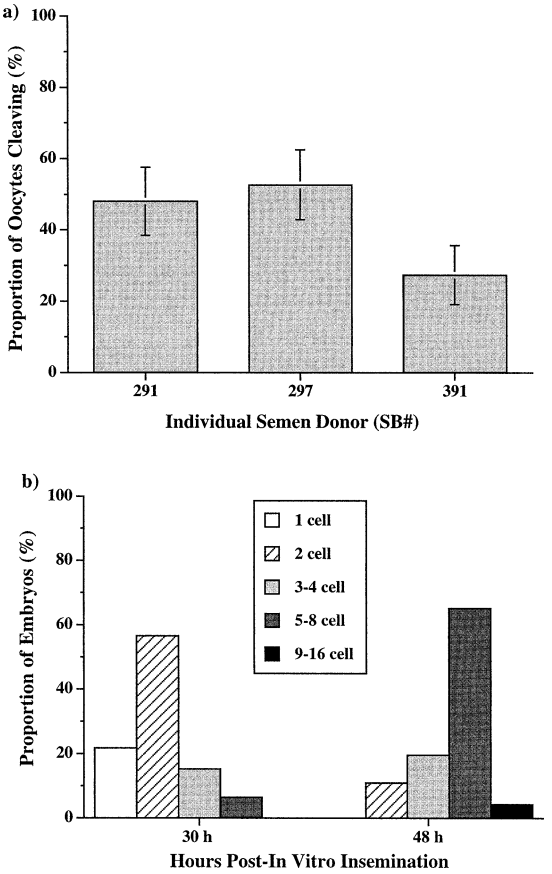

Figure 3. Cleavage and developmental kinetics of Pallas' cat–domestic cat hybrid IVF embryos. **a.** Proportion of domestic cat oocytes cleaving at 48 hr post-insemination with frozen-thawed Pallas' cat spermatozoa from individual sperm donors. **b.** Developmental stages of hybrid embryos at 30 and 48 hr post-insemination.

among frozen-thawed IVF samples from these males. Few studies have examined post-thaw sperm function in cat species using homologous or heterologous IVF, but fertilization percentages in this study (~46%) were comparable to those reported (50–70%) for homologous IVF using frozen-thawed spermatozoa in domestic cats[43] and tigers[12] and heterologous IVF with nonfrozen spermatozoa in nine nondomestic cat species, including Pallas' cats (Swanson, unpubl. data).[44]

Our IVF findings support these earlier results showing that spermatozoa from Pallas' cats can readily penetrate and fertilize viable oocytes from domestic cats. In the current study, it was not possible to confirm the presence of blastomeric nuclei in cleaving embryos with fluorescent staining. However, based on gross embryo morphology and the kinetics of embryo cleavage, Pallas' cat–domestic cat hybrid embryos showed similar developmental traits to that of IVF- and in vivo–generated domestic cat embryos.[17,37] Furthermore, in our earlier Pallas' cat studies, fluorescent staining of hybrid embryos determined that number of stained nuclei corresponded to visual assessments of embryo cleavage through the morula stage (Swanson, unpubl. data). Lastly, parthenogenic cleavage of in vivo–matured domestic cat oocytes typically is very low (0–6%) during in vitro culture.[7,23]

Heterologous IVF allowed assessment of the functionality of frozen-thawed Pallas'cat spermatozoa without requiring the sacrifice of valuable Pallas' cat oocytes. Heterologous insemination of viable oocytes from a closely-related species offers several advantages over other methods of fertility assessment by allowing evaluation of all sequential aspects of sperm function from capacitation to fertilization to embryo cleavage.[36,38] In this study, heterologous IVF also facilitated PCR analysis of hybrid embryos and noncleaving oocytes to determine whether herpesvirus was transmitted to the embryos from the IVF sperm sample. In bovids, IVF-derived embryos exposed to herpesvirus during culture may retain viral particles on their zonae pellucidae.[2,50] Because none of the Pallas' cat semen samples tested positive for FHV-1, PCR analysis could not address this potential for viral transmission to embryos but served to reconfirm the absence of FHV-1 in the sperm samples used for IVF.

These findings may have important implications for the genetic rescue of Pallas' cats and other felids infected with herpesviruses. In North American zoos, Pallas' cats are managed within a Species Survival Plan (SSP) under the auspices of the American Zoo and Aquarium Association.[1] The SSP monitors demographic and genetic parameters, issues breeding recommendations, and seeks to optimize genetic diversity in the captive population. Of the original 24 founders imported from Asia to establish this population, the genetic contribution of three individuals (i.e., two wild-born males and one wild-born female) or one-eighth of the SSP's existing genetic heterozygosity is represented by the sperm samples collected for this study. Our results suggest that frozen-thawed spermatozoa from these founders (or their offspring) exhibit adequate function for use in assisted reproductive procedures with Pallas' cat females.

Given the limited availability of frozen-thawed spermatozoa from these males, the adverse impact of cryopreservation on sperm viability, and any lingering concerns of FHV-1 presence in frozen samples, IVF and ET may present the most viable strategy for genetic rescue. Our earlier attempts with AI

in Pallas' cats proved unsuccessful for producing offspring, possibly because of insemination with low numbers of compromised frozen-thawed spermatozoa.[7] More recently, IVF of in vivo–matured Pallas' cat oocytes with frozen-thawed spermatozoa from a FHV-1–infected male resulted in 11 of 15 oocytes (73%) cleaving and most (9/11, 82%) embryos developing to the late morula–blastocyst stage during culture (Swanson, unpubl. data). For added security, IVF embryos also can be treated enzymatically using trypsin to remove any residual herpesvirus adhering to the zona pellucidae prior to transfer, as reported for bovine IVF embryos.[2,13]

In conclusion, we have shown that semen collected from FHV-1–infected male Pallas' cats does not contain detectable amounts of FHV-1 DNA. Furthermore, frozen-thawed Pallas' cat spermatozoa are functional after thawing and are capable of fertilizing viable cat oocytes. Collectively, these results suggest that frozen-thawed spermatozoa from infected males may be used in female Pallas' cats with minimal risk of transmitting FHV-1 to noninfected individuals, and the rescue of founder genes from these Pallas' cats may be possible through the application of assisted reproductive technology. These findings also may have positive implications for reproductive management of other felid species, such as domestic cats, clouded leopards, and cheetahs, which are susceptible to herpesvirus infections.

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