Sperm Motility, Plasma Membrane Integrity, and Binding Capacity to Homologous Zona Pellucida of Cryopreserved Epididymal Spermatozoa in the Domestic Cat

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Abstract. We examined motility, plasma membrane integrity, and binding capacity to homologous zona pellucidae (ZP) of frozen/thawed epididymal cat sperm as a model species for endangered felines. Epididymal spermatozoa from 20 domestic cats were frozen with freezing egg-yolk extender containing 3.0% glycerol in 0.25-ml straws. Post-thaw motility and plasma membrane integrity of the frozen/thawed spermatozoa were 31.8 ± 2.4% and 32.2 ± 4.2%, respectively. The frozen/thawed spermatozoa were co-cultured with frozen/thawed immature homologous oocytes with intact ZP for 3 h to examine their ability to bind to the ZP. Sixteen of the 20 frozen/thawed sperm samples demonstrated the ability to bind to ZP. These results indicated that the freezing system for epididymal sperm used in the present study gives appropriate information for banking the genetic resources of wild felid species.

Key words: Cat, Epididymal sperm, Freezing, Zona pellucida binding

Excluding the domestic cat, all of the felidae family are considered threatened or endangered, including Iriomote wild cats (Felis iriomotensis) and Tsushima leopard cats (Felis bengalensis euptilura), which are both mammals native to Japan [1]. Effective conservation and maintenance of an adequate diversity of animals can be achieved by preserving gametes and/or embryos as genetic resources in order to prevent the extinction of species [2]. Cryopreservation of epididymal spermatozoa offers a potential tool for rescuing genetic material from males that die unexpectedly, thus preserving gametes that could be used to maintain genetic variety within depleted...
populations and preventing the loss of diversity for future generations.

As a first step, we examined sperm motility, plasma membrane integrity, and binding capacity to homologous zona pellucidae (ZP) of frozen/thawed epididymal spermatozoa in the domestic cat to develop a method for cryopreservation of feline spermatozoa with the ultimate aim of applying the method to preservation of the genetic resources of wild felines.

Materials and Methods

Preparation and freezing of spermatozoa

Epididymides were collected from 20 domestic cats (hybrid) during routine neutering procedures between September and December of 1999 (Table 1). They were then placed in phosphate-buffered saline contained in a Dewar vessel at 20–24°C and transported to the laboratory within 3 h of removal. Two sections from the caudal portion of the epididymides were placed into 1,000 µl of extender-I [3, 4] containing 23.0% (v/v) egg-yolk, 8.0% (w/v) lactose monohydrate, and antibiotics (1,000 IU/ml penicillin G potassium, 1.0 mg/ml streptomycin sulfate; Sigma, St. Louis, MO, USA) in a petri dish (35 × 10 mm; Falcon 351008; Becton Dickinson and Company, Franklin Lakes, NJ, USA). The caudal portions were dissected with scalpel blades to release spermatozoa. Tissue debris was removed from the sperm suspension using forceps. A portion of the sperm suspension (10 µl) was transferred to an examination chamber (Fujihira Industry Co., Ltd. Tokyo, Japan), placed on a micro-warm plate (Kitasato Supply, Shizuoka, Japan) kept at 37°C, and the motility was evaluated under a phase contrast microscope at a magnification of 100 ×. The semen was transferred to a refrigerator at 5°C, and kept there for 180 min. The cooled semen was then diluted with 500 µl of extender-II, which consisted of extender-I with 9.0% (v/v) glycerol. The final concentration of glycerol was 3.0% (v/v). The semen was loaded into 0.25-ml plastic straws (Fujihira Industry Co., Ltd.), and exposed to liquid nitrogen vapor for 15

Table 1. Post-thaw sperm motility, plasma membrane integrity, and ability to bind to homologous zona pellucidae of epididymal spermatozoa in the domestic cat

<table>
<thead>
<tr>
<th>Male #</th>
<th>Age (year)</th>
<th>BW (kg)</th>
<th>Motility (%) at collection</th>
<th>Motility (%) at 5°C</th>
<th>Motility (%) post-thaw</th>
<th>SPMI* (%) post-thaw</th>
<th>ZP-binding test**</th>
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<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>4.3</td>
<td>80</td>
<td>70</td>
<td>35</td>
<td>22.0</td>
<td>P***</td>
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<tr>
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<td>4.5</td>
<td>80</td>
<td>70</td>
<td>35</td>
<td>28.0</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
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<td>4.4</td>
<td>85</td>
<td>70</td>
<td>35</td>
<td>68.5</td>
<td>P</td>
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<tr>
<td>4</td>
<td>1.5</td>
<td>6.5</td>
<td>90</td>
<td>75</td>
<td>45</td>
<td>45.0</td>
<td>P</td>
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<tr>
<td>5</td>
<td>6.0</td>
<td>6.5</td>
<td>70</td>
<td>55</td>
<td>10</td>
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<td>65</td>
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<tr>
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<td>3.0</td>
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Total 1.4 ± 0.3 4.2 ± 0.3 80.8 ± 2.0 63.0 ± 2.3 31.8 ± 2.4 32.2 ± 4.2 P: 16/20 N: 4/20

*SPMI: Sperm plasma membrane integrity (%) assessed by LIVE/DEAD Sperm Viability Kit (Molecular Probes, USA).
**Frozen/thawed spermatozoa were co-cultured with homologous zona pellucidae for 3 h under 5% CO₂ in humidified air at 37°C.
***Positive for the sperm binding test.
****Negative for the sperm binding test.
CRYOPRESERVATION OF EPIDIDYMAL CAT SPERM

Afterward, the straws were plunged into liquid nitrogen and stored for at least one week. The straws were thawed in a warm water bath kept at 37 C for 20 sec. The thawed semen in the straws were diluted with 1.0 ml of tissue culture medium 199 (TCM-199, Earle’s salts), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) at 37 C, and then incubated at 37 C under a humidified atmosphere of 5% CO2 in air.

Sperm motility and plasma membrane integrity
The rate of motile spermatozoa was assessed microscopically after thawing and dilution with extender-II at 5 C. The sperm plasma membrane integrity was also determined using a commercially available test kit (Live/Dead Sperm Viability Kit, Molecular Probes, Inc., Eugene, OR, USA), which differentiates between cells with intact sperm plasma membranes and those with damaged membranes according to fluorescent staining patterns observed using a fluorescence microscope (Olympus, BX-50, Tokyo, Japan). The nucleus of spermatozoon with an intact plasma membrane exhibited green fluorescence, whereas a spermatozoon with a damaged plasma membrane emitted orange-red fluorescence [3, 4]. At least 300 sperm cells were counted in duplicates for each sample. The data for sperm motility and plasma membrane integrity were expressed as means ± SEM.

Homologous zona pellucida binding assay of frozen/thawed spermatozoa
Ovaries collected from 7 domestic cats (hybrid; more than 6 months old) during routine neutering procedures were placed in phosphate-buffered saline contained in a Dewar vessel at 20–24 C and transported to the laboratory within 3 h of removal. The oocytes were recovered by slicing the ovaries in a petri dish (90 × 15 mm; CSPD 90–15; Kanto Chemicals Co., Inc., Tokyo, Japan) with TCM-199 containing 0.4% (w/v) bovine serum albumin (BSA; Sigma). Recovered oocytes were cryopreserved with 1.5 M glycerol in 0.25-ml straws (Fujihira Industry) according to the method described by Kashiwazaki et al. [5] and thawed just before the sperm binding test. After thawing, oocytes with cumulus cells were denuded by pipetting gently using a narrow-bore Pasteur pipette. Post-thaw oocytes with intact ZP were used for the sperm binding test in the present study. Five to 10 oocytes were introduced into a 100 µl droplet of TCM199 + 10% FCS. The final sperm concentration was 1 × 106 sperm cells/ml. Co-culture was performed for 3 h under 5% CO2 in humidified air at 37 C. After co-culture, the oocytes were vigorously pipetted to dislodge loosely attached types using a narrow-bore Pasteur pipette, and observed using a phase contrast microscope (Olympus, CK40). An oocyte to which the ZP sperm was tightly bound was judged to be a sperm-binding oocyte. We determined that a sperm sample was positive when there was more than one sperm-binding oocyte after co-culture with the sperm sample.

Results
Although the epididymal sperm motility at collection from the twenty male cats was 80.8 ± 2.0%, the post-thaw sperm motility (including faint sperm motility) was 31.8 ± 2.4%. The post-thaw sperm plasma membrane integrity was 32.2 ± 4.2%, and 16 of the 20 sperm samples were positive for the ZP binding test (Table 1).

Discussion
The present study showed that epididymal spermatozoa can be frozen in egg-yolk extender with 3.0% glycerol in 0.25-ml straws, and that the frozen/thawed spermatozoa have the ability to attach to homologous ZP in the domestic cat. Cryopreservation of spermatozoa in mammals is a valuable tool for preserving genetic material. Improvement of sperm freezing protocols and related technologies would increase the efficacy of gene banking from endangered felines, and the domestic cat can be used as a model for the wild felines. The first pregnancies in the domestic cat using a cryopreservation technique were obtained using semen frozen in pellets [6]. Although freezing pelleted semen is often the standard method for the cat [7], freezing semen in straws has been found to produce equivalent results [8]. It has been reported that a pregnancy rate of only about 10% was obtained in the domestic cat after the use of frozen/thawed semen with vaginal insemination [6]. The fertilizing ability of frozen/thawed
epididymal spermatozoa has been proven [9]. Cryopreservation of epididymal spermatozoa is a potentially valuable procedure for banking genetic resources from individuals of endangered species that die accidentally. Tsutsui et al. [10] reported a conception rate of 27.3% (3/11) for eleven female cats that received unilateral intruterine AI of \(5 \times 10^7\) sperm. This report was the first case of conception obtained with frozen/thawed epididymal sperm in the domestic cat. In the report, the mean motility and viability of epididymal sperm at time of recovery from 10 males were 67.0% and 82.5%, respectively, and the post-thaw motility was 24.0%. The rate of post-thaw motility was similar to the results of the present study, as well as another study [11]. However, the plasma membrane integrity of frozen/thawed epididymal sperm in the present study seems to be lower than other reports [10, 11], and maintenance of integrity differs depending upon the cat (0% to 68.5%). Therefore, a certain method to maintain the integrity should be developed. Recently, Axnér et al. [12] reported that the addition of Equex STM paste into the freezing extender protects the acrosomes of epididymal cat spermatozoa during the freezing/thawing process.

The procedure used in the present study for epididymal cat sperm cryopreservation may be applicable for banking the genetic resources of wild felid species. However, development and improvements in reproductive technologies such as AI, in vitro fertilization (IVF), and intracytoplasmic sperm injection will be required to generate offspring from the frozen/thawed spermatozoa of wild felid species. Although reproductive technologies have only rarely been studied in wild felid species, Pope et al. [13] reported that a litter of two kittens of the Indian desert cat (IDC, Felis silvestris ornata) was born after IVF following the interspecies transfer of IDC embryos into domestic cat recipients. Further studies are required to develop reproductive technologies in wild felid species to support conservation and maintenance of genetic diversity for these important species.

Sperm binding to homologous ZP is a prerequisite for fertilization, and tests that evaluate this function have been described for several species (the cat [14–16], the dog [17], the pig [18], and the human [19]). Spermatozoa attachment to the ZP, a cellular glycoprotein coat surrounding oocytes, is a crucial step in mammalian fertilization. It is generally accepted that in the majority of mammalian species the fertilizing spermatozoon has an intact acrosome that initiates binding to the ZP with the surface of the plasma membrane overlying the acrosome [20]. In the present study, semen sample #5, which did not show any plasma membrane integrity, could not bind to the zona pellucidae. When conducting ZP binding assay for evaluation of spermatozoa, a sufficient number of oocytes with intact ZP must be obtained at time of testing. In the present study, oocytes were used that had an intact ZP after being cryopreserved in liquid nitrogen. Frozen/thawed samples were used because we wished to evaluate the ability of sperm to bind to the ZP [21–24]. In addition, Strom Holst et al. [25] reported that deep freezing of ZP sources appears to be a better storage method than storing oocytes in salt solution, as is done in many studies.

In conclusion, the freezing system for epididymal spermatozoa used in the present study is a workable protocol in the domestic cat that gives appropriate information for banking the genetic resources of wild felid species.

References

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