

## The Qualities of Cryopreserved Epididymal Sperm Collected from Feline Epididymides Stored at Low Temperature

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**ABSTRACT.** We observed the influences of low-temperature storage of the feline epididymis on the epididymal semen qualities before and after cryopreservation to identify the optimal duration for low-temperature storage of the epididymis. After excision, the feline epididymis was stored at 4°C for 0–72 hr and then subjected to epididymal sperm collection. When sperm from the refrigerated cauda epididymis were frozen and thawed, there was no significant difference in sperm motility between the 0- and 24-hr low-temperature storage groups, but sperm motility was significantly decreased in the 48-hr storage group. The above findings suggested that low-temperature storage of the epididymis until 24 hr is useful for frozen sperm collected from the feline cauda epididymis.

**KEY WORDS:** cryopreservation, epididymal sperm, feline, refrigeration.

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The total number of animals worldwide has decreased in many wildcat species, and various assisted reproductive technologies (ART) are necessary to maintain and increase their numbers. Many studies on ART for endangered feline species are underway using domestic cats as a model [1–5, 10–15].

In a previous study, we collected feline sperm from the cauda epididymis immediately after excision and performed intrauterine insemination of frozen semen ( $5 \times 10^6$ ) [14]. The mean sperm motility following freeze-thawing was  $24.0 \pm 4.0\%$  (SE), and the conception rate was only 27.3% (3/11). Two female cats delivered five and one kittens, respectively, and another one aborted on 30 days of gestation. This was our initial success rate achieved using epididymal frozen semen in cats. Our previous study is also the only one to report successful conception cases achieved using frozen feline epididymal semen thus far.

The duration and temperature of storage before collection of sperm after excision of the epididymis have marked influences on semen qualities in various animal species [6–9]. In cats, Hay and Goodrowe [5] reported that semen qualities did not differ between sperm collected from the cauda epididymis immediately after excision and after 24-hr storage at 5°C. Furthermore, Chatdarong *et al.* [3] compared the quality of caudal epididymal semen collected from the epididymis after storage for 2 and 4 days at 4°C and reported that the sperm motility after storage for 2 days is significantly higher than that after storage for 4 days. Filliers *et al.* [4] observed that the qualities of semen from the feline epididymis stored in sterile physiological saline for 24 hr at 5°C were good. However, Chatdarong *et al.* [3] and Filliers *et al.* [4] did not

observe the semen quality immediately after excision. Tittarelli *et al.* [13] also stored the feline epididymis in sterile physiological saline or egg yolk-Tris for 24, 48 and 72 hr and observed the semen qualities. Sperm motility was apparently decreased at all storage time points, and egg yolk-Tris was suggested to be superior as a storage medium compared with physiological saline. However, the mean sperm motility after 24-hr storage was  $27.7 \pm 3.9\%$ , which was far lower than that ( $71.0 \pm 2.0\%$ ) reported by Hay and Goodrowe [5]. Additionally, they did not present data concerning epididymal semen qualities immediately after the excision [13]. Thus, precise comparison of semen qualities with regard to storage may be difficult. Moreover, egg yolk-Tris, one of the storage media they used, is problematic because it is not readily available when a male of an endangered feline species dies unexpectedly.

To efficiently use gametes in such a situation, sperm cryopreservation under improved conditions is important. However, in our report [14] on artificial insemination using frozen feline semen from the cauda epididymis, sperm were collected immediately after castration.

In this study, we investigated the influences of storage time of the feline epididymis on epididymal semen qualities before and after cryopreservation to identify the optimal duration for low-temperature storage of the epididymis applicable to artificial insemination. For the storage medium, sterile physiological saline was used because it is readily available.

**Animals:** Fifty-four male mixed cats of known age brought to an animal hospital for castration were used. The male cats were 0.6 to 7.5 years of age, with a mean of  $2.2 \pm 0.2$  years, and weighed 2.0–6.8 kg, with a mean of  $4.4 \pm 0.1$  kg.

The study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Life Science

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**Preservation of the epididymis:** The testes and epididymides were placed in sterile physiological saline immediately after excision and stored at 4°C for various durations.

**Sperm collection and semen quality test:** Epididymal sperm were collected as previously reported [14].

The collected sperm suspensions were centrifuged at  $1,200 \times g$  for 5 min, the supernatants were removed, 200  $\mu$ l of egg yolk Tris-fructose citrate (EYT-FC) was added to the semen and the sperm motility, sperm viability, sperm abnormality, immature sperm and number of sperm were evaluated as previously reported [11].

**Cryopreservation of sperm:** EYT-FC was used as the extender of frozen semen. After the semen quality test, semen was subjected to the 1st dilution at 20°C to adjust the sperm count to  $1 \times 10^8$ /ml and was then kept in a water bath at 4°C for 1 hr for a 1st cooling. After the 1st cooling, using a secondary extender supplemented by dripping at 4°C, taking approximately 10 min while stirring a stirrer, with 14% glycerol and 2.0% Orvus Es Paste (OEP, Nova Chemical Sales Inc., Situate, MA, U.S.A.). The semen was diluted with an equal volume of the extender, giving a final sperm concentration of  $0.5 \times 10^8$ /ml and the final glycerol and OEP concentrations of 7 and 1%, respectively. After the 2nd dilution, the semen was put into 250  $\mu$ l straws, and equilibrated with glycerol for 1 hr. For freezing, liquid nitrogen (LN<sub>2</sub>) was poured into a styrene foam box [30.5  $\times$  23  $\times$  16.5 (height) cm] to a level of 5 cm, and the straws were maintained horizontally at a height of 7 cm from the surface for 10 min for sensitization in LN<sub>2</sub> vapor. The straws were the plunged into the LN<sub>2</sub>.

**Semen quality test after thawing:** Frozen straws were stored for more than 1 week and thawed by immersing them in 37°C water for 30 sec. The semen quality test was performed immediately after thawing to determine the sperm motility, sperm viability and incidence of abnormal sperm. Sperm that had proximal droplets were judged as immature.

**Experimental design:** In Experiment 1, the epididymis was stored for 0, 24, 48 and 72 hr (n=6, 15, 8 and 10, respectively) after excision. The epididymal sperm were released from the caput and cauda epididymis, and the semen qualities were investigated.

In Experiment 2, sperm were released from the cauda epididymis 0, 24, and 48 hr (n=5 each) after excision, based on

the findings of Experiment 1 and then frozen and the semen qualities were investigated after thawing.

**Statistical analysis:** The significance of semen quality was analyzed among the groups by two-way ANOVA and Tukey-Kramer test. The lateral differences in the number of sperm were analyzed by Student's *t*-test for after analyzing the homogeneity of variance. A *P* value of less than 0.05 was regarded as significant.

**Experiment 1:** There were no bilateral differences in the testicular or epididymal weights and number of sperm in the cats used in Exp.1, which is in agreement with the results of our previous report [14]. The mean number of sperm collected from the caput epididymis was significantly lower than that collected from the cauda epididymis ( $54.5 \pm 5.3 \times 10^6$  vs.  $95.3 \pm 5.3 \times 10^6$ ).

The qualities of the epididymal sperm collected after low-temperature storage of the epididymis for various durations are shown in Table 1.

The mean motility of sperm collected from the caput epididymis stored for 0, 24, 48 and 72 hr decreased with the prolongation of storage and was significantly higher in the 0-hr storage group compared with the 48- and 72-hr storage groups ( $P < 0.01$ ) and in the 24-hr storage group compared with the 48- and 72-hr storage groups ( $P < 0.01$ ). Cauda epididymal sperm motility decreased with the storage time and was significantly higher in the 0-hr storage group compared with the 48- and 72-hr storage groups ( $P < 0.01$ ), in the 48-hr storage group compared with the 72-hr storage group ( $P < 0.01$ ) and in the 24-hr storage group compared with the 72-hr storage group ( $P < 0.01$ ). The motility of sperm from the caudal region was significantly higher than that of the caput region in the 0-, 24- and 48-hr storage groups ( $P < 0.01$ ).

Caput and cauda epididymal sperm viability decreased with time but was not significantly different among the experimental groups or between the caput and cauda epididymides. Sperm abnormality increased with time but was not significantly different among the experimental groups or between the caput and cauda regions. No significant difference was noted in the frequency of immature sperm among the 0- and 72-hr storage groups.

**Experiment 2:** There were no lateral differences in the testicular or epididymal weights and number of sperm, as in Experiment 1.

The qualities of the sperm collected from the feline cauda

Table 1. The qualities of epididymal sperm collected after low-temperature storage of the epididymis for various durations (mean  $\pm$  SE)

Storage time (hr)	Sperm motility (%)		Sperm viability (%)		Sperm abnormality (%)		Immature sperm (%)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda	Caput	Cauda
0	45.0 $\pm$ 5.7 <sup>a,1)</sup>	66.4 $\pm$ 3.2 <sup>a,2)</sup>	74.0 $\pm$ 4.9	84.6 $\pm$ 2.4	3.8 $\pm$ 0.7	7.0 $\pm$ 2.5	0.9 $\pm$ 0.3	0.5 $\pm$ 0.2
24	27.3 $\pm$ 3.1 <sup>a,1)</sup>	51.7 $\pm$ 3.0 <sup>a,b,2)</sup>	80.7 $\pm$ 3.3	87.5 $\pm$ 0.6	12.8 $\pm$ 1.7	13.9 $\pm$ 1.5	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1
48	10.9 $\pm$ 2.3 <sup>b,1)</sup>	37.5 $\pm$ 3.4 <sup>b,2)</sup>	71.5 $\pm$ 5.8	77.1 $\pm$ 2.9	16.7 $\pm$ 2.9	15.0 $\pm$ 2.4	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1
72	2.0 $\pm$ 1.4 <sup>c,1)</sup>	9.8 $\pm$ 2.2 <sup>c,1)</sup>	71.4 $\pm$ 4.4	73.5 $\pm$ 2.1	24.2 $\pm$ 2.9	34.4 $\pm$ 4.2	1.1 $\pm$ 0.4	0.7 $\pm$ 0.3

a-c: Different superscripts indicate significant differences among storage times ( $P < 0.01$ ).

1-2: Different superscripts indicate significant differences between the caput and caudal regions ( $P < 0.01$ ).

Table 2. The qualities of sperm collected from the feline cauda epididymis stored at a low temperature and those after thawing (mean  $\pm$  SE)

Storage time (hr)	Sperm motility (%)		Sperm viability (%)		Sperm abnormality (%)	
	Initial	Post-thaw	Initial	Post-thaw	Initial	Post-thaw
0	80.0 $\pm$ 4.6 <sup>a)</sup>	38.0 $\pm$ 2.2 <sup>a)</sup>	86.8 $\pm$ 3.1	54.8 $\pm$ 5.3 <sup>a)</sup>	3.7 $\pm$ 2.1	7.1 $\pm$ 1.6 <sup>a)</sup>
24	66.0 $\pm$ 8.4 <sup>a,b)</sup>	36.0 $\pm$ 5.7 <sup>a)</sup>	78.5 $\pm$ 7.1	44.3 $\pm$ 3.8 <sup>a,b)</sup>	7.6 $\pm$ 1.6	16.2 $\pm$ 2.6 <sup>a,b)</sup>
48	50.0 $\pm$ 12.7 <sup>b)</sup>	14.0 $\pm$ 5.1 <sup>b)</sup>	66.0 $\pm$ 11.9	29.3 $\pm$ 5.0 <sup>b)</sup>	11.1 $\pm$ 7.5	39.0 $\pm$ 8.5 <sup>b)</sup>

a, b): Different superscripts indicate significant difference among storage times ( $P < 0.05$ ).

epididymis stored at a low temperature and those after thawing are shown in Table 2.

The mean sperm motilities immediately after collection from the epididymides stored for 0, 24 and 48 hr showed that the motility of the sperm from the epididymides stored for 0 hr was significantly higher than that from the epididymides stored for 48 hr ( $P < 0.01$ ). No significant difference was noted in sperm viability, the incidence of abnormal sperm and the frequency of immature sperm among the storage time groups.

Sperm motility following freeze-thawing was similar for the 0- and 24-hr storage groups but was significantly decreased in the 48-hr storage group compared with the 0-hr storage group ( $P < 0.05$ ). Sperm viability was significantly higher in the 0-hr storage group compared with the 48-hr storage group ( $P < 0.05$ ). The incidence of abnormal sperm was significantly lower in the 0-hr storage group compared with the 48-hr group ( $P < 0.05$ ). No such difference was noted in the frequency of immature sperm among the storage time groups.

The motility of the sperm collected from the feline cauda epididymis immediately stored in sterile physiological saline at 4°C decreased with time. This finding was consistent with the quality of sperm collected from the feline cauda epididymis and stored in PBS at 5°C for 24 hr reported by Hay and Goodrowe [5]. However, in our study, the sperm motility before freezing was different between sperm collected from the cauda epididymis and stored at a low temperature for 0 or 24 hr, but not after freeze-thawing. The post-thaw motility of the sperm collected from the epididymis and stored at a low temperature for 24 hr was apparently lower than those of the sperm collected without storage in the study reported by Hay and Goodrowe [5], which is inconsistent with our results. The incidence of abnormal sperm was higher in the 48-hr storage group compared with the 0-hr storage group. This was also inconsistent with the findings reported by Hay and Goodrowe [5], who reported that the incidence of abnormal sperm collected after 24-hr storage was higher. These differences may be due to variations in the storage medium, evaluation method and freezing method, particularly the extender composition. Regarding the storage medium for the feline epididymis, Tittarelli *et al.* [13] investigated 2 extenders, sterile saline and egg yolk-Tris, and found that the motility of caudal epididymal sperm was higher when collected after low-temperature storage in egg yolk-Tris for 24–72 hr. However,

comparison of their data with our findings is not possible because they did not designate a 0-hr storage group as a control. They described that egg yolk-Tris protected the feline epididymis from the influences of the low temperature, but the mechanisms were not clarified. We used sterile physiological saline because it is readily available. Tittarelli *et al.* [13] suggest that consideration of the influences of the osmotic pressure and pH of the storage medium on epididymal sperm is necessary. Therefore, further investigation of the storage medium for the feline epididymis is required.

Generally, many epididymal sperm possess cytoplasm droplets. The frequency of immature sperm reported in feline caudal epididymal sperm varies markedly among researchers [1,10,12,15]. Furthermore, Axné *et al.* [2] reported that the ratio of immature sperm decreases from the caput to the cauda. They reported values that were lower than those in other reports, but similar to the values for ejaculated sperm in our previous study [11]. Considering the possible influences of the mincing method and EYT-FC on the loss of cytoplasm droplets, we obtained the epididymis immediately after excision, but the frequency of immature sperm was as low as that in the minced epididymis. Further investigation is necessary to clarify whether these findings are specific to cats maintained in Japan.

The number of motile sperm collected from the cauda epididymis was significantly greater than that collected from the caput epididymis. Since sperm motility before freezing is important for cryopreservation of semen for artificial insemination, sperm of the caput epididymis may not be applicable for clinical use.

In conclusion, for use of frozen sperm collected from the feline epididymis and stored at a low temperature for artificial insemination, storage of the epididymis for 24 hr may be useful. The fertilization competence of the frozen-thawed semen should be examined by artificial insemination.

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