FULL PAPER *Theriogenology*

Unilateral Intrauterine Insemination with Cryopreserved Caudal Epididymal Sperm Recovered from Refrigerated Canine Epididymides

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ABSTRACT. Canine epididymides were excised and immediately stored at 4°C for 48 hr, and the qualities of caudal epididymal sperm after recovery and cryopreservation were evaluated. To confirm the fertility of the cryopreserved caudal epididymal sperm, artificial intrauterine insemination was performed. The sperm motility (61.0%) immediately after recovery from caudal epididymis stored at 4°C for 48 hr was significantly lower than those of sperm stored for 0 and 24 hr (88.6 and 80.7%, respectively), but there was no significant difference after freeze-thawing (0-, 24-, and 48-hr storage groups: 27.9, 24.3, and 28.3%, respectively). The incidence of abnormal sperm immediately after recovery was significantly higher in the 24-hr and 48-hr storage groups (19.3 and 27.7%, respectively) than in the 0-hr storage group (5.6%), and a significant difference was also observed after freeze-thawing. The incidence of immature sperm with cytoplasmic droplets was significantly higher in the 48-hr storage group (18.4%) than in the 0-hr storage group (4.7%), but there was no difference after freeze-thawing. By unilateral intrauterine insemination (2×10^8 sperm), 4 of 5 bitches (80%) conceived. The above findings demonstrated that sperm motility was good even enough the incidence of abnormal sperm was high in canine epididymal sperm that were recovered from the epididymis stored at 4°C for 48 hr and cryopreseved, and that artificial intrauterine insemination resulted in a high conception rate.

KEY WORDS: canine, cryopreservation, epididymal sperm, intrauterine insemination, refrigeration.

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Many wild animals, including those in the canine family such as the gray wolf (*Canis palus*) and American red wolf (*Canis rufus*), are on the verge of extinction [4]. At the deaths of these animals by unexpected accidents, recovery and cryopreservation of sperm from the epididymis are important for assisted reproductive techniques [1, 16].

Since it may take time to recover and cryopreserve sperm from the epididymis after the death of an animals, development of a technique to store the epididymides until recovery of sperm is necessary, and such techniques have been investigated in many animal species [2, 7, 9, 10, 13–15, 18, 21, 26–28]. For example, in dogs, when Yu and Leibo [27] stored canine epididymides at 4°C for 8 days after excision, sperm motility was significantly decreased after 5 hr, but sperm membrane integrity was maintained even after storage for 48 hr, and the zona pellucida-binding ability was maintained even after 192 hr. In sheep, it has been reported that sperm from recovery of the epididymis stored at 5°C showed better sperm quality after 24 and 48 hr than sperm recovered from epididymides stored at room temperature for the same period [13]. In mice, it has been reported that sperm motility decreased to between 10 and 15% within 24 hr when the epididymis was stored at room temperature after the animal died, but epididymis stored at 4°C maintained 30% of the sperm motility even after 10 days, and could be successfully used for *in vitro* fertilization [14, 21]. These reports clarified that storage at a low temperature after excision of the epididymides is capable of maintaining good qualities in epididymal sperm.

Regarding cryopreservation of canine caudal epididymal sperm, several studies, including two we have previously conducted, have been reported [7, 10, 11, 15, 28]. Marks *et al.* [15] recovered sperm from the cauda of the epididymis and the seminal duct of a 9-year-old boxer that developed epileptic seizures. Thereafter, the sperm were used for artificial insemination after cryopreservation, and 1 puppy was obtained. In our studies, when caudal epididymal sperm was recovered, cryopreserved, and inseminated into the uterus, the conception rate was low (1/16, 6.3%) [10], but use of prostate fluid (PF) for transmigration of sperm resulted in a high conception rate (8/10, 80%) [11]. In studies reported by Hewitt *et al.* [7] and Yu *et al.* [28], *in vitro* sperm motility, sperm membrane integrity, and the ability of sperm to bind to the zona pellucida were investigated after cryopreservation of epididymal sperm, but no insemination tests were performed. In these studies of cryopreservation of canine caudal epididymal sperm [7, 10, 11, 28], the time between excision of the epididymis and recovery of the sperm was not investigated.

Thus, in this study, canine epididymides were excised and immediately stored at 4°C for 48 hr, and the qualities of caudal epididymal sperm after recovery into PF and cryopreservation and the fertility of the sperm were evaluated.

MATERIALS AND METHODS

Animals: The animals were 12 males bred at our colony that were 0.9 to 2.6 (mean ± SE: 1.7 ± 0.1) years old. The weights of the 12 dogs ranged from 8.5 to 15.2 (mean: 10.9 ± 0.6) kg. For artificial insemination, 5 female dogs aged

1.5 to 4.3 (mean: 3.0 ± 0.5) years old were used. All dogs were beagles. The experimental dogs were kept in 160 × 75 × 65 cm cages, each containing 2 dogs. Commercial dog food (Hill's Canine Maintenance, Hill's-Colgate Ltd., Tokyo, Japan) was given once daily and drinking water was given three times daily. Pregnant dogs were given food twice a day (morning and evening) starting at 35 days of gestation.

This study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Animal Science University.

Excision and preservation of epididymis: The testes and epididymides were excised under general anesthesia according to the method we previously reported [10, 11]. The excised testes and epididymides were ligated at the ductus deferens and pampiniform plexus and immediately placed in sterile physiological saline (0.9% NaCl, pH: 5.4 ± 0.2, osmotic pressure: 277 mOsm/*l*) at 20°C to avoid drying.

In 9 of the 12 dogs, the caudal epididymal sperm were recovered from either the left or right epididymides after being stored in a refrigerator at 4°C for 24 or 48 hr (24-hr and 48-hr storage groups, respectively), whereas epididymal sperm were immediately recovered from the contralateral epididymis (0-hr storage group). In the remaining 3 dogs, the caudal epididymal sperm were recovered from the bilateral epididymis after being stored at 4°C for 48 hr. The duration of epididymal storage was within 48 hr, assuming that the maximal interval between removal of an epididymis and its arrival at a laboratory at which it can be treated might be 48 hr after a male animal dies.

Sperm collection and semen quality test: After the epididymides were removed, the excised testes and epididymides were weighed. Sperm was recovered from the caudal epididymides at room temperature (22 to 23°C) by the mincing method, as previously reported [11, 12]. As the solution for the recovery of the sperm, PF (pH: 6.9 ± 0.1, osmotic pressure: 311 ± 7.0 mOsm/*l*) was used. PF was previously collected from 4 other beagle dogs and centrifuged at 600 × g for 5 min, and the supernatant was stored at –40°C.

For comparison with the quality of caudal epididymal sperm, ejaculated sperm was collected once from 12 dogs by digital manipulation 7 to 251 days (mean 63.5 days) before excision of the epididymis and frozen according to the method previously reported (ejaculated group) [23].

Sperm recovered from caudal epididymides and ejaculated sperm were examined by semen quality test through a microscopic examination [25]. The sperm concentration was determined by hematocytometer counts, sperm motility was examined as the percentage of actively motile sperm using a semen quality examination plate and a warm-plate, and the percentages of viable sperm, morphologically abnormal sperm, and immature sperm were assessed by eosin-nigrosin staining. Sperm demonstrating cytoplasmic droplets on their midpiece were judged as immature. Acrosome integrity was investigated using the triple-stain technique reported by Talbot and Chacon [22].

Cryopreservation of sperm: Caudal epididymal sperm and ejaculated sperm were frozen according to the method previously reported [10, 23]. The semen was centrifuged (600 × g) for 5 min to remove PF or seminal plasma. As an extender for canine frozen semen, egg yolk Tris-fructose citrate (EYT-FC, pH: 6.5 ± 0.1, osmotic pressure: 301 ± 5.1 mOsm/*l*) solution [24] was used. Primary and secondary dilutions with EYT-FC were performed at 20°C. The final sperm concentration was adjusted to 1×10^8/m*l*. Using a second extender supplemented with 1.5% Orvus ES Paste (OEP, Nova Chemical Sales, Inc., Scituate, MA, U.S.A.) and 14% glycerol, the semen was diluted by the drip method and stirried for approximately 10 min. The final concentrations of OEP and glycerol were 0.75 and 7%, respectively. The semen was subjected to a first refrigeration at 4°C for 1 hr using a programmable cooling system (UH-JF, Chino Ltd., Tokyo, Japan). Sperm were then frozen in 0.5-m*l* straws in a conventional freezer using liquid nitrogen (Simple-type quick LNG freezer FA-1652, Fujihira Industry Co., Ltd., Tokyo, Japan).

Semen quality test after thawing: Semen straws were thawed in warm water at 37°C for 45 sec no earlier than 1 week after freezing. After the general semen quality test described above, the semen was stored at 20°C and the time-course sperm motility and sperm viability of semen preparations from the four groups were examined after 1, 2, 4, and 6 hr. The presence or absence of acrosomes in viable and dead sperm was observed by the triple-staining technique [22] immediately after thawing, and after 6 hr.

Intrauterine insemination: Artificial insemination was performed during the optimal mating period 3 to 5 days after ovulation, as estimated from the peripheral blood progesterone level, which was determined by the EIA method [17] as reported previously [6]. The bitches were laparotomized under general anesthesia, and semen was introduced into a uterine horn with many corpora lutea, as reported previously [23]. Atropine sulfate (0.05 mg/kg s.c.; Tanabe Seiyaku Co., Ltd., Osaka, Japan) and acepromazine maleate (0.025 mg/kg s.c.; TechAmerica Group, Inc., Elwood, KS, U.S.A.) were administered for pretreatment, and anesthesia was induced by ketamine hydrochloride (KETALAR®50, 7 mg/kg i.v.; Sankyo Co., Ltd., Tokyo, Japan) and maintained by isoflurane (ISOFLU®, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Five bitches received insemination of 2×10^8 sperm. Following centrifugation of the thawed semen for 5 min at 600 × g, a sperm volume of 150 to 200 μl was inseminated.

Determination of the number of ovulations and newborns: The number of corpora lutea in both ovaries was counted by observing the fat-thin areas at the mesosalpinx of the ovarian bursa at the time of intrauterine artificial insemination, and this was regarded as the number of ovulations on each side. Newborns were counted at delivery.

Diagnosis of pregnancy: Pregnancy was determined by the detection of fetal sac, 20 to 30 days after artificial insemination, with an ultrasonographic diagnostic system (ECHOVISON SSD-500EV, 7.5 MHz, Aloka Co., Ltd., Tokyo, Japan). Pregnant animals were examined by ultra-

sonography every 5 days until delivery to confirm normal maintenance of pregnancy.

Statistical analysis: The sperm parameters; percentages of motile, viable, morphologically abnormal, and immature sperm; and percentages with an intact acrosome were compared between pre-freezing and post-thawing groups, and the findings were analyzed by paired Student's *t*-test. Sperm motility, sperm viability, and acrosome integrity over time were analyzed by repeated-measures ANOVA. Sperm parameters among the 0-hr, 24-hr, and 48-hr groups were analyzed by one-way ANOVA and Duncan's multiple-range test. A probability value of less than 5% was considered significant.

RESULTS

Total number of caudal epididymal sperm: The total numbers of epididymal/ejaculated sperm in the dogs are shown in Table 1. The numbers of left and right caudal epididymal sperm were 1.8 to 9.0 $\times 10^8$ and 1.6 to 9.4 $\times 10^8$, respectively, with means of 4.4 $\pm$ 0.6 (SE) $\times 10^8$ and 5.2 $\pm$ 0.7 $\times 10^8$, respectively. There were no difference between the left and right epididymides in individual animals. The number of ejaculated sperm ranged from 2.7 to 11.1 $\times 10^8$, with a mean of 6.2 $\pm$ 0.7 $\times 10^8$. The ratio of ejaculated sperm to that of total number of epididymal sperm (ejaculated sperm/left+right epididymal sperm) ranged from 21.7% to 171.9%, with a mean of 76.8 $\pm$ 12.3%.

Semen qualities after recovery: The semen qualities of caudal epididymal sperm after the epididymis was stored at 4°C for 0, 24, or 48 hr are shown in Fig. 1. The mean sperm motilities were 88.6 and 92.1% in the 0-hr storage and ejaculated groups, respectively; there was no significant differences between the two groups. The mean sperm motilities were 80.7 and 61.0% in the 24-hr and 48-hr storage groups, respectively. The percentage in the 24-hr group was lower than that for the 0-hr storage group ($p<0.05$) and the ejaculated group ($p<0.01$), and the percentage in the 48-hr group was lower than the other three groups ($p<0.01$). Mean sperm viability did not show any marked differences among the 4 groups (93.3, 88.9, 89.6, and 92.9% in the 0-hr storage, 24-hr storage, 48-hr storage and ejaculated groups, respectively). The mean levels of sperm abnormalities were 5.6 and 4.9% in the 0-hr storage and ejaculated groups, respectively; there was no significant differences between the 2 groups. The mean levels of sperm abnormalities were 19.3 and 27.7% in the 24-hr and 48-hr storage groups. The percentage in the 24-hr storage group was higher than that in the 0-hr storage and ejaculated group ($p<0.01$), and the percentage in the 48-hr storage group was higher than that in the 24-hr storage group ($p<0.05$). Most of the morphological abnormalities of the sperm involved a bent or coiled tail region. The mean incidences of immature sperm were 4.7 and 3.4% in the 0-hr storage and ejaculated groups, respectively; there were no significant differences between the two groups. The mean incidences of immature sperm were 17.9 and 18.4% in the 24-hr and 48-hr storage groups, respectively. The percentages in the 24-hr and 48-hr storage groups were higher than those in the 0-hr storage and ejaculated group ($p<0.05$).

Semen qualities after freezing-thawing: The semen qualities after freeze-thawing in the 0-hr, 24-hr, and 48-hr storage groups are shown in Fig. 2. Sperm motility, sperm viability and incidence of immature sperm after freeze-thawing in the four groups, including the ejaculated group, were lower than those before freezing ($p<0.01$), but there were no significant differences among the four groups. The mean levels of sperm abnormalities in the 48-hr storage group (25.1%) was higher than those in the 0-hr (9.0%) and 24-hr (12.0%) storage and ejaculated (14.1%) groups. However, the percentage of morphologically abnormal sperm after freeze-thawing in all four groups did not significantly differ from

Table 1. The total number of ejaculated and caudal epididymal sperm in 12 dogs

Dog No.	Age (year)	B.W. (kg)	Preservation time (hr)			Days from ejaculation to excison	Numbers of sperm ($\times 10^8$)			Ejaculated sperm/ total epididymal sperm rate (%)#
							Epididymal sperm		Ejaculated sperm	
			0	24	48		L	R		
1	1.6	11.1	R*	L**		7	5.4	7.0	6.9	55.8
2	1.6	11.5	R	L		7	4.0	5.0	8.5	94.7
3	1.6	12.6	R	L		8	4.3	9.4	5.2	38.3
4	2.6	10.5	R	L		251	6.4	6.0	4.6	36.8
5	2.0	15.2	R	L		146	9.0	6.1	8.0	53.1
6	0.9	8.8	R		L	34	2.1	2.6	5.1	109.1
7	1.8	12.5	R		L	34	3.6	4.6	7.0	85.0
8	1.4	10.0		R	L	8	3.7	7.2	11.1	102.1
9	1.4	8.5		R	L	7	1.8	1.6	5.8	171.9
10	1.6	11.0			L,R	87	6.2	6.2	2.7	21.7
11	1.7	9.0			L,R	72	2.4	2.8	4.0	78.3
12	1.8	10.0			L,R	101	4.4	3.8	6.2	75.4
Mean	1.7	10.9				63.5	4.4	5.2	6.2	76.8
± SE	0.1	0.6				22.5	0.6	0.7	0.7	12.3

#: The numbers of ejaculated sperm/the combined number of left and right epididymal sperm.
*: Right cauda epididymis. **: Left cauda epididymis.

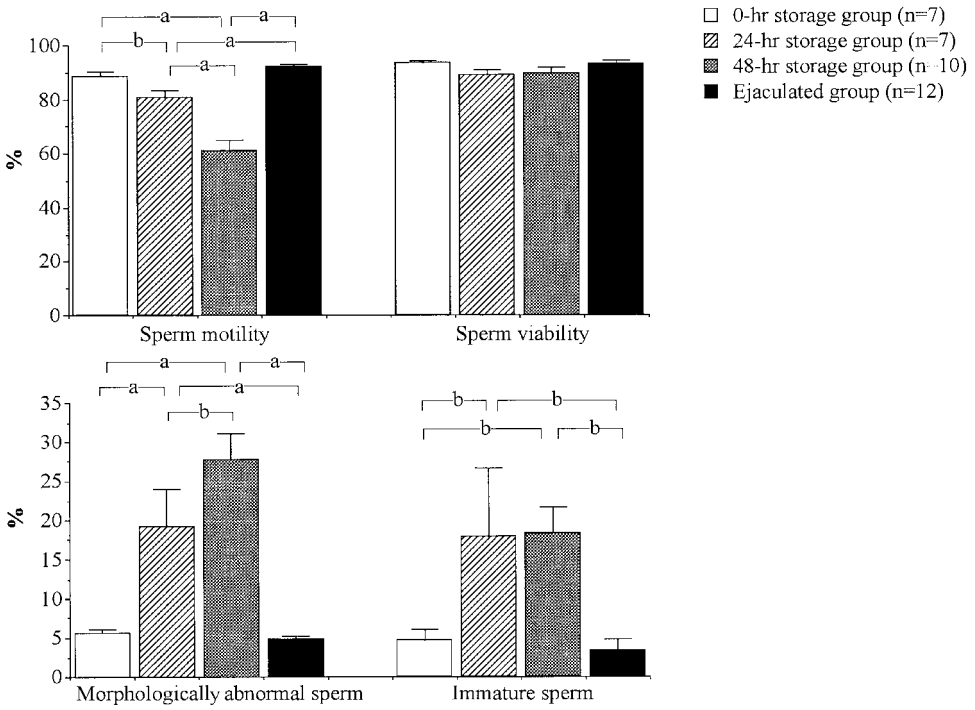

Fig. 1. The qualities of caudal epididymal sperm recovered from epididymides stored at 4°C for 0, 24, and 48 hr (mean ± SE). Significantly different between two groups at $p<0.01$ (a) and $p<0.05$ (b).

that before freezing.

The sperm motility and viability during incubation at 20°C were lowered with time in the four groups, but there were no marked differences at any time point among the four groups.

State of acrosomes in sperm immediately after recovery and after thawing: The state of the sperm acrosomes immediately after recovery from the epididymis, immediately after thawing, and 6 hr after thawing in the 0-hr, 24-hr, and 48-hr groups is shown in Fig. 3. The percentage of sperm with acrosomes among viable sperm did not significantly differ among all 4 groups (0-hr, 24-hr, and 48-hr storage groups and the ejaculated groups) immediately after recovery or ejaculation. Immediately after thawing and 6 hr after thawing, the percentage of sperm with acrosomes among viable sperm did not significantly differ among all 4 groups.

Artificial unilateral intrauterine insemination of frozen-thawed sperm: The results of intrauterine insemination of caudal epididymal sperm recovered from epididymides stored at 4°C for 48 hr and frozen-thawed are shown in Table 2. Intrauterine insemination resulted in conception in 4 of the 5 bitches (80%). The number of ovulations on the inseminated side was between 3 and 6 (mean: 4.4 ± 0.6), and the number of puppies was between 1 and 4 (mean: 2.3 ± 0.9). The ratio of the number of newborns to the number of ovulations on the inseminated side was between 25.0 and 75.0% (mean: 50.0 ± 14.2%). The interval between ovulation and delivery in the 4 pregnant bitches ranged from 63 to 68 days (mean: 64.5 ± 1.4 days).

DISCUSSION

After storage of canine epididymides at 4°C, there were no significant differences in sperm viability or the ratio of sperm with intact acrosomal caps. However, sperm motility decreased with the duration of storage, and the incidence of abnormal sperm and immature sperm increased. These findings were similar to those in other animal species in which the epididymis was stored at a low temperature and epididymal sperm was later recovered [2, 9, 13]. In contrast, although the sperm motility in the groups with the epididymis stored at a low temperature (4°C) was significantly lower than those in the 0-hr storage group and ejaculated sperm group, there was no significant difference in sperm motility among the groups after freeze-thawing. Cold sensitization-vulnerable epididymal sperm lost activity due to exposure to a temperature of 4°C before freezing, decreasing the sperm motility. But, cold sensitization-resistant sperm may have been motile after freeze-thawing, resulting in the absence of a difference in sperm motility.

As a reason for the decrease in the qualities of caudal sperm recovered from the epididymis after storage at 4°C, Songsasen *et al.* [21] and Hishinuma *et al.* [9] suggested that detachment and pyknosis occurred in epithelial cells of the duct of the epididymis during storage, using degeneration of the epididymal tissue in mice and sika deer (*Cervus Nippon*), respectively. Based on these findings, similar changes in the qualities of epididymal fluid may have occurred in the dogs, worsening the semen qualities, such as decreasing the

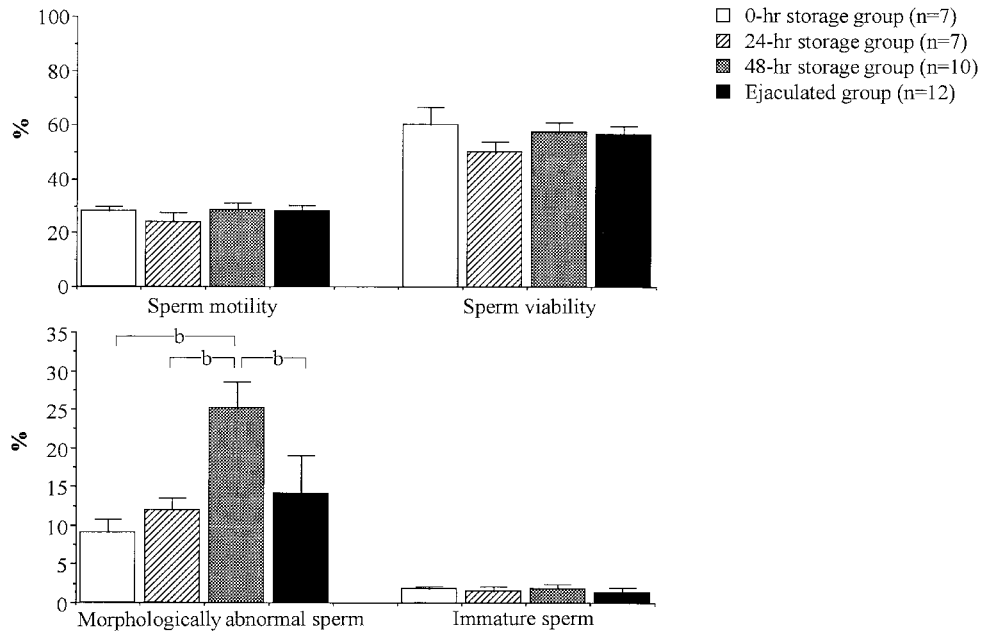

Fig. 2. The qualities of frozen-thawed caudal epididymal sperm recovered from epididymides stored at 4°C for 0, 24, and 48 hr (mean ± SE). Significantly different between two groups at $p<0.05$ (b).

sperm motility and increasing the incidence of abnormal sperm.

It is known that the osmotic pressure of epididymal fluid is higher than those of seminal plasma and fluid in the female genital duct [12]. Thus, when sperm are added to seminal plasma during ejaculation, the sperm are shocked by the low osmotic pressure [3]. Sperm is capable of reversing low osmotic pressure-induced changes in volume, and can adapt to the pressure [3, 19, 20]. However, it has been reported that in cattle and pigs, increasing and decreasing the sodium and potassium concentrations in the epididymal fluid changed the osmotic pressure and increased the incidence of abnormal tails [5, 8]. Based on these reports, changes in the osmotic pressure of the epididymal fluid during storage at a low temperature may have been a cause of the decreased sperm motility and increased incidence of abnormal sperm, particularly the increased incidence of abnormal tails in the caudal epididymal sperm. However, the osmotic pressure of epididymal fluid could not be measured in this study. To clarify the cause of the decrease in semen qualities, it is necessary to measure the osmotic pressure and sodium and potassium concentrations of epididymal fluid, and investigate their effects on the storage of the epididymis in a solution that has the same osmotic pressure and sodium and potassium concentrations as epididymal fluid.

Regarding changes in cytoplasmic droplets, we previously reported that transmigration using EYT-FC did not detach cytoplasmic droplets from sperm, although the cytoplasmic droplet removal rate was high when PF was used for transmigration [11]. However, in this study, removal of cytoplasmic droplets became difficult as the duration of low-temperature storage increased. We suspected that there was some reason other than changes in osmotic pressure for the removal of cytoplasmic droplets since the osmotic pressures of PF (311 ± 7.0 mOsm/l) and EYT-FC (301 ± 5.1 mOsm/*l*) were similar, although slightly higher in PF. To confirm whether this phenomenon was induced by the change of osmotic pressure, measurement of the osmotic pressure in the epididymis after storage at 4°C is necessary.

In this study, the qualities of ejaculated sperm and epididymal sperm from the same animal were compared after freeze-thawing. Hewitt *et al.* [7] have described that epididymal sperm is more sensitive to low temperatures than ejaculated sperm. However, in this study, the semen qualities of the caudal epididymal sperm immediately after recovery from the epididymis and the ejaculated sperm did not significantly differ. Sperm motility after thawing was different for caudal epididymal sperm and ejaculated sperm between different animals in our previous study [10], but there was no difference in the comparison over time of sperm obtained from the same animal in this study. This suggested that differences in the qualities resulted from individual differences in sensitivity to low temperatures, as Yu *et al.* noted [28]. It was clarified that in dogs, resistance to freezing did not differ between caudal epididymal sperm and ejaculated sperm from the same animal.

When the number of caudal sperm stored in the epididymis and the number of sperm ejaculated each time were compared in the same animals, although individual variation ranging from 58.2 to 460.7% (mean: 76.8 ± 12.3%) was noted, about 80% of the sperm stored in the caudal epididymis was ejaculated each time. Although the interval

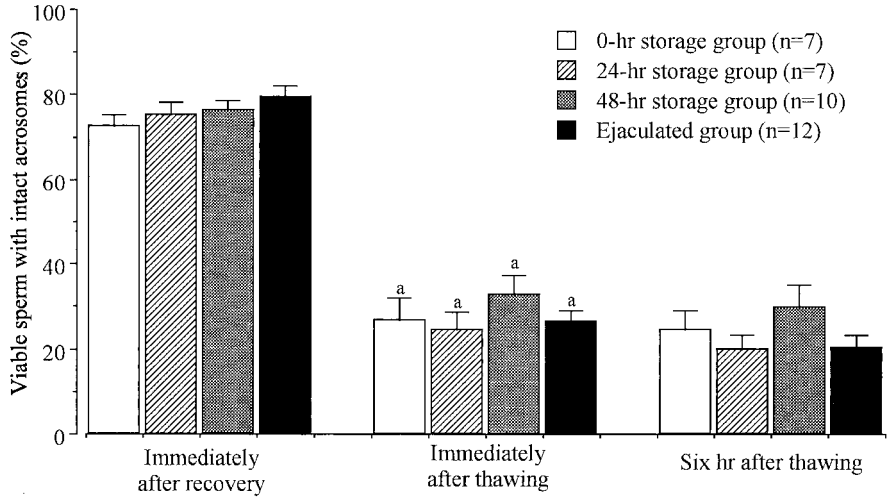

Fig. 3. The percentage of viable sperm with intact acrosomes in caudal epididymal sperm immediately recovered from epididymides preserved at 4°C for 0, 24, and 48 hr, immediately after thawing, and 6 hr after thawing (mean ± SE). Significantly different from levels immediately after recovery at $p<0.01$ (a).

Table 2. The results of intrauterine insemination of frozen-thawed caudal epididymal sperm recovered from epididymides preserved at 4°C for 48 hr

Dog No.	Sperm motility (%)	Sperm viability (%)	Bitch No.	No. of ovulations L	No. of ovulations R	No. of pups	No. of pups/No. of ovulations (%)	Days from ovulation to delivery
7	25	68.5	A	3	* 2	1	1/3 (33.3)	64
8	30	56.2	B	6	3	4	4/6 (66.7)	63
10	30	69.9	C	4	5	3	3/4 (75.0)	63
11	20	51.5	D	4	5	–[#]	–	–
12	25	55.9	E	3	4	1	1/4 (25.0)	68
Mean	26.0	60.4		3.8	4.0	2.3	50.0	64.5
± SE	2.1	4.1		0.7	0.6	0.9	14.2	1.4

*: Inseminated uterine horn. [#]: Not pregnant.

between semen collection and excision of the epididymis varied from 7 to 251 days, there was no significant correlation between the interval and the number of epididymal sperm recovered.

Several factors are considered to have an effect on the qualities of sperm when epididymal sperm are stored. The first factor is storage temperature. It is known that spermatic metabolism is inhibited in an environment at 4°C, maintaining sperm motility [24]. Since many researchers have noted that better semen qualities could be obtained when sperm was stored at 4°C than when it was stored at room temperature [9, 13, 14], it is suggested that storage of the epididymis at 4°C is appropriate. The second factor is the interval between excision of the epididymis (after death of the animal) and storage at 4°C. Since the epididymis was stored at 4°C immediately after excision, this issue was not investigated in this study. Yu and Leibo [27] investigated recovery times between 1 and 6 hr after excision, and found that there were no significant changes in the acrosomal integrity or zona pellucida-binding ability of the sperm. However, Hishinuma *et al.* [9] found that, in sika deer, the sperm motility was low when the epididymis was excised 8 to 12 hr after death and stored at 4°C for 1 to 4 days, but the motility was higher when the epididymis was excised within 4 hr after death. Based on these findings, the epididymis excised after death should be stored at 4°C as early as possible because storage at room temperature decreases the qualities of recovered caudal epididymal sperm. The third factor is the age of the animal. Yu and Leibo [27] reported that there were no age-related differences in the motility of epididymal sperm after recovery or acrosomal integrity in dogs, but the zona pellucida-binding ability of sperm recovered from dogs aged 1 to 5 years was higher than that of sperm recovered from dogs aged 6 years or older. However, they did not investigate the influence of cryopreservation. Age-related changes in the qualities of caudal epididymal sperm could not be investigated in this study because young dogs aged 1–2 years were used. A detailed investigation of aged-related differences in cryopreservability is necessary as recovery of sperm from the epididymis is more often

applied to older dogs and sperm qualities decrease with age.

As a result of intrauterine insemination, conception was obtained in 4 of 5 bitches (80%). This finding was the first report of parturition of puppies delivered from intrauterine insemination with caudal epididymal sperm recovered from epididymides stored at 4°C. However, the mean ratio of the number of newborns to the number of ovulation on the inseminated side was low, 50.0 ± 14.2%, showing that the number of fertilized ova did not exceed the number of ovulations on the inseminated side. To increase the number of newborns, it may be necessary to increase the number of inseminated sperm or inseminate sperm into the bilateral uterine horns.

In conclusion, in frozen canine caudal epididymal sperm recovered after storage of the epididymis at a low temperature for 48 hr, there was no effect on sperm motility, sperm viability, or acrosomal integrity. Although the incidence of abnormal sperm was increased, a high conception rate was obtained by intrauterine insemination of the sperm. These techniques may be applicable to the preservation of caudal epididymal sperm of wild animals that are on the verge of extinction, including animals of the canine family that unexpectedly die in accidents.

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