FULL PAPER *Theriogenology*

Artificial Insemination of Frozen Epididymal Sperm in Beagle Dogs

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ABSTRACT. Freeze-storage of epididymal sperm is an important technique for the preservation of gametes in animals, including those becoming extinct. We froze canine sperm recovered from the cauda epididymis and investigated the fertility. The qualities of sperm from the cauda epididymis before freezing were: mean sperm motility, 89.4 ± 1.6 (SE) %; sperm viability, 89.1 ± 1.1%; and these were significantly higher than those of sperm from the caput-corpus epididymis ($P<0.01$, $P<0.05$). The number of sperm recovered from both cauda epididymides varied among animals: 6.3–122.3 $\times 10^7$, mean 61.5 ± 10.0 $\times 10^7$. Freezing was used only for sperm recovered from the cauda epididymis. The sperm motility and viability after thawing were 19.5 ± 2.5% and 53.1 ± 3.3%, respectively. These were slightly lower than those of frozen-thawed ejaculated sperm, but the differences were not significant. When 2×10^8, 3×10^8, or 4×10^8 sperm were inseminated in the unilateral uterus, only one animal inseminated with 3×10^8 sperm was fertilized (1/16, 6.3%). When 1×10^8 sperm were inseminated in the bilateral uterine tubes, one of six animals (16.7%) was fertilized. Therefore, although the qualities of epididymal sperm after thawing were similar to those of ejaculated sperm, the conception rate obtained with frozen-thawed epididymal sperm was low in beagle dogs. It is necessary to investigate the differences in damage between epididymal sperm after thawing and ejaculated sperm and to develop a method for improving the conception rate.

KEY WORDS: canine, epididymal sperm, frozen, intratubal insemination, intrauterine insemination.

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Sperm reach maturation during migration from the caput to the cauda of the epididymis and are retained in the cauda epididymis until ejaculation. It has been demonstrated in many species that sperm in the cauda epididymis exhibited fertility in *in vitro* fertilization and artificial insemination [1, 4, 7–9, 14]. For animals that die unexpectedly, transmigration of epididymal sperm, and its freeze-storage are important techniques for gamete preservation. These techniques are considered important for preventing species from becoming extinct. Conception obtained by artificial insemination of frozen epididymal semen has been reported in one dog [7], pigs [8], goats [1], and cats [14]. Marks *et al.* [7] successfully obtained delivery of one pup after intrauterine insemination despite poor semen quality after thawing. There is only one study about the quality and resistance to freezing of canine epididymal sperm, reported by Hewitt *et al.* [5]. Their study showed that the number of sperm that reached maturation was lower in the epididymis than in frozen ejaculated sperm, but there was no difference in oocyte-penetrating ability [5]. In the method of epididymal sperm recovery, Marks *et al.* [7] reported that the epididymis was perfused from the seminal duct to the epididymis in boxer dogs and Hewitt *et al.* [5] used the mincing method. Sirivaidyapong [11] compared the quality of semen collected by the two methods before and after freezing/thawing, and sperm motility and viability after freezing/thawing was not significantly different. Since the dogs we used were beagles, perfusion was considered difficult because of the narrow duct of the epididymis, and we employed the mincing method.

We previously reported that the addition of OEP to the extender increased the conception rate of cryopreserved ejaculated semen [13]. The addition of OEP to sperm recovered from the epididymis for storage was speculated to increase the fertility after freezing/thawing.

Thus, we froze canine epididymal sperm by using the same method reported for the preparation of frozen ejaculated sperm and investigated the possibility of fertilization with frozen-thawed epididymal sperm by intrauterine insemination and intratubal insemination.

MATERIALS AND METHODS

Animals: The animals were 17 castrated males aged 2–9 years and six males aged 1–6 years from which frozen ejaculated semen was prepared. For artificial insemination, 15 female dogs aged 1–6 years were used. Since some animals were repeatedly used, the number of cases was 22. All dogs were beagles. The dogs were kept in 160 × 75 × 65 cm cages. Commercial dog food (Hill's Canine Maintenance, U.S.A.) was given once daily and drinking water was given three times daily (morning, afternoon and evening).

Transmigration of sperm from epididymis: Surgically excised testes and epididymides were weighed. The blood vessel on the surface of the epididymis was removed and it was cut at the corpus part near the cauda epididymis and divided into the caput-corpus and caudal epididymides. Each part was minced on a plastic dish with 2 m*l* of extender, egg yolk tris-fructose citrate solution (EYT-FC) [16]. The solution to transmigrate sperm was filtered through a metal mesh (80 μm) to remove tissue fragments, and sperm were recovered at room temperature (22–23°C). The time from excision of the epididymis to transmigration of sperm was about 30 min.

Semen quality test: Sperm recovered from the caput-corpus and caudal right and left epididymides were examined by the general semen quality test [17]. The sperm concentration was determined by hematocytometer counts, sperm motility was examined with a semen quality examination plate and a warm-plate, and the percentage of sperm viability, sperm abnormality and immature sperm were assessed by eosin-nigrosin stain. Sperm which had cytoplasmic droplets on their mid-piece were judged as immature.

Frozen semen: Only sperm recovered from the cauda epididymides were frozen. When the motility, viability and abnormality of sperm from the right and left epididymides did not differ, sperm from the bilateral cauda epididymides were combined. Frozen semen was prepared according to the method we previously reported for ejaculated sperm [12, 13]. EYT-FC was used as the extender, and the concentrations of OEP and glycerol were 0.75% and 7%, respectively. We used 0.5 m*l* straws, and the sperm concentration was adjusted to 1×10^8/m*l*, then frozen with a conventional freezer. As the control for frozen epididymal semen quality, ejaculated semen from 6 beagles was frozen as described above.

Semen quality test after thawing: The semen quality test was performed on epididymal sperm and ejaculated sperm after thawing for 10 and 6 animals, respectively. Semen straws were thawed in warm water at 37°C for 45 sec. After the general semen quality test, semen was kept at 20°C and time-course sperm motility of the two semen preparations after 1, 2, 3, 4 and 6 hr was compared.

Intrauterine insemination: Semen from 13 animals was used for intrauterine insemination. Semen was inseminated in the unilateral uterine horn with more ovulated ova during laparotomy by the method reported previously [13]. Briefly, 2×10^8, 3×10^8 and 4×10^8 sperm were inseminated in 5, 5 and 6 animals, respectively. The semen volume infused was about 200 μl at the tube bottom after centrifuging thawed semen at 1,500 rpm for 5 min. Artificial insemination was performed during the optimal mating period 3–5 days after ovulation, estimated from the peripheral blood progesterone level as reported previously [3].

Intratubal insemination: Semen from 6 animals was inseminated in the uterine tube. A median section was made under general anesthesia during the optimal mating period and the infundibulopelvic ligament was immobilized with a clamp for ovary fixation. Semen was inseminated at about 2 cm deep from the abdominal ostium of the uterine tube with a glass capillary [15]. Semen was inseminated in bilateral uterine tubes. Semen containing 1×10^8 sperm was thawed and centrifuged, and about 20 μl at the tube bottom was inseminated.

Determination of the numbers of ovulated ova and newborns: The number of corpus lutea counted in both ovaries at the time of artificial insemination was regarded as the number of ovulated ova. Newborns were counted on delivery day.

Diagnosis of pregnancy: Pregnancy was determined by the detecting fetal sac 25 days after artificial insemination with an ultrasonograghic diagnostic system (ECHOVISON SSD-500EV 7.5MHz, Aloka Co., Japan). Pregnant animals were examined by ultrasonography every five days until delivery to confirm normal maintenance of pregnancy.

Statistical analyses: Data obtained in this study were analyzed by Student's *t*-test and a significance level lower than 5% was defined as significant.

RESULTS

Size of testis and epididymis: Weights of the testes and epididymides of 17 experimental dogs are shown in Table 1. The left testis was slightly heavier than the right testis but the difference was not significant. The right and left epididymides weighed almost the same. There were no significant relationships among weights of the body, testis and epididymis.

Quality of epididymal sperm: Epididymal semen quality, motility of sperm from the cauda epididymis in particular, varied among animals, but the values were almost the same in the bilateral epididymides in the same animal. Therefore, the results of bilateral epididymides are combined in Table 1. The sperm motility widely varied, 0–75% (mean: 35.9 ± 5.4%), in the caput-corpus epididymis, but the motility was about 80% or significantly higher in the cauda epididymis ($P<0.01$). The viability of sperm did not show marked variations: 64.7–92.1% and 79.2–94.7%, in the caput-corpus and cauda epididymides, respectively, but viability was significantly higher in the cauda epididymis ($P<0.05$). The percentage of immature sperm in the caput-corpus and cauda epididymides was high: 40.0–79.8% and 28.8–78.3%, respectively, showing no significant difference between the two epididymides. The abnormality of sperm was 3.0–14.7% and 1.2–7.3% in the caput-corpus and cauda epididymides, respectively, showing that the rate was slightly higher in the caput-corpus epididymis, but the difference was not significant. The number of recovered sperm in the caput-corpus and cauda epididymides was $0.9–40.8 \times 10^7$ (mean: $8.0 \pm 2.6 \times 10^7$) and $6.3–122.3 \times 10^7$ (mean: $61.5 \pm 10.0 \times 10^7$), respectively, showing a large variation among animals. The ratio of the sperm count in the caput-corpus epididymis to the sperm count in the cauda epididymis was 1.0–67.8% (mean: 14.9%), and the sperm count in the caput-corpus epididymis was significantly lower than that in cauda epididymis ($P<0.01$).

Semen quality after thawing: The characteristics of thawed ejaculated sperm and sperm recovered from the cauda epididymis are shown in Table 2. Time-course changes in the motility of sperm kept at 20°C are shown in Fig. 1.

The sperm motility and viability of caudal epididymal sperm after thawing from 10 animals were 19.5 ± 2.5% and 53.1 ± 4.7%, respectively, and those of ejaculated sperm were 25.8 ± 1.7% and 57.6 ± 4.7%, respectively. The motility and viability were higher in ejaculated sperm (n=6, motility: 94.2 ± 0.9%, viability: 92.7 ± 2.5%), but the differences were not significant. No significant difference in the

Table 1. Quality of epididymal sperm in dogs

Dog Number	Age (year)	Body weight (kg)	Testis (g)		Epididymis (g)		Semen quality (%)									
							Sperm motility		Sperm viability		Sperm abnormality		Immature sperm		Number of sperm ($\times 10^7$)	
			Left	Right	Left	Right	Caput[a] corpus	Cauda[b]	Caput-corpus	Cauda	Caput-corpus	Cauda	Caput-corpus	Cauda	Caput-corpus	Cauda
IQ275	1	10	8.2	7.9	2.6	2.7	0	80.0	64.7	87.5	–	–	–	–	0.9	30.4
W1032	1	10	9.6	10.1	2.9	2.4	17.5	82.5	82.5	88.8	–	–	–	–	4.1	51.5
SOGM3	1	9	5.5	5.3	1.4	1.7	25.0	72.5	85.3	91.4	–	–	–	76.6	5.9	42.8
FRIM2	1	12	9.7	9.3	2.0	2.0	27.5	90.0	85.2	92.8	–	–	–	84.8	7.5	63.2
AH2M4	1	11	6.7	6.7	2.1	2.2	35.0	92.5	82.5	89.5	–	–	–	–	6.3	85.3
HIJAGX	2	10	8.6	7.4	1.9	1.8	15.0	92.5	91.0	85.0	13.1	6.6	54.0	48.3	40.8	122.3
HIJADB	2	8	8.1	8.7	2.3	2.2	5.0	95.0	91.7	93.2	–	3.7	–	50.3	1.0	101.0
6ME296	2	13	9.1	9.2	1.9	2.2	75.0	92.5	92.1	88.8	6.0	5.8	63.8	74.4	6.9	62.2
6ME268	2	12	17.6	13.6	2.8	3.1	30.0	95.0	91.2	94.4	14.7	4.6	79.8	28.8	20.0	120.7
6ME262	2	12	7.9	7.8	1.5	1.7	45.0	95.0	79.8	94.7	5.2	5.7	50.6	44.4	5.4	82.3
6ME301	2	9.5	5.0	5.0	1.6	1.5	45.0	87.5	86.8	93.3	4.5	6.0	41.1	78.3	2.5	24.4
6ME286	2	10	12.3	12.2	2.0	2.0	50.0	95.0	91.6	84.0	3.6	4.5	67.1	56.6	20.9	97.6
6ME437	2	7.5	6.8	6.2	1.5	1.3	40.0	90.0	86.0	89.5	5.6	6.3	44.3	57.2	1.9	12.1
6ME439	2	8	6.7	5.0	1.3	1.2	67.5	95.0	80.1	84.0	10.7	7.2	43.4	45.9	1.4	18.1
Q469	2	11	5.4	4.8	1.5	1.4	70.0	82.5	76.9	79.2	8.1	7.3	40.0	37.2	4.3	6.3
Q493	2	9	9.7	10.0	1.7	1.9	27.5	95.0	91.3	90.8	5.6	1.8	42.8	50.7	4.1	108.5
Q459	2	8	5.6	5.0	1.9	1.9	35.0	87.5	86.5	87.2	3.0	1.2	41.7	50.3	2.3	16.2
Mean ± SE		10.0 ± 0.4	8.4 ± 0.8	7.9 ± 0.7	1.9 ± 0.1	2.0 ± 0.1	35.9 ± 5.4	89.4 ± 1.6	85.0 ± 1.8	89.1 ± 1.1	7.3 ± 1.2	5.0 ± 0.6	51.7 ± 4.2	56.0 ± 4.6	8.0 ± 2.6	61.5 ± 10.0

a) Left and right caput-corpus epididymis. b) Left and right cauda epididymis.

Table 2. Quality of frozen-thawed epididymal and ejaculated sperm in dogs

	Dog Number	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)			
				Head	Mid-piece	Tail	Total
Epididymal sperm	6ME296	35	52.9	0.6	0.3	24.6	25.5
	6ME268	20	60.8	0.3	0.9	17.5	18.7
	6ME262	20	59.7	0.6	0.0	6.3	9.9
	6ME301	15	58.0	0.0	0.0	7.9	7.9
	6ME286	15	52.8	0.3	0.0	22.0	22.3
	6ME437	15	64.0	1.2	0.3	30.9	32.4
	6ME439	15	53.5	1.2	0.9	38.1	40.2
	Q469	10	28.9	0.6	0.6	12.9	14.1
	Q493	30	47.2	0.3	0.3	10.6	11.2
	Q459	20	53.5	0.6	0.3	18.3	19.2
	Mean±SE	19.5±2.5	53.1±3.3	0.6±0.1	0.4±0.1	18.9±3.4	20.1±3.4
Ejaculated sperm[a]	1	20	61.6	0.3	0.9	3.3	4.5
	2	25	54.7	1.2	0.6	2.4	4.2
	3	30	45.9	1.5	1.2	4.8	7.5
	4	25	65.8	0.9	0.9	3.3	5.1
	5	25	71.6	0.3	1.5	2.1	3.9
	6	30	45.8	1.5	0.3	2.1	3.9
	Mean±SE	25.8±1.7	57.6±4.7	1.0±0.2	0.9±0.2	3.0±0.5	4.9±0.6

a) Sperm motility and viability before freezing were 94.2±0.9% and 92.7±2.5%, respectively.

sperm motility was observed between caudal epididymal sperm and ejaculated sperm with time. In contrast, the abnormality of sperm after thawing was significantly higher in caudal epididymal sperm (20.1 ± 3.4%) than in ejaculated sperm (4.9 ± 0.6%) ($P<0.01$). Most abnormalities were seen in the tail.

Conception results of intrauterine insemination: The conception results after unilateral intrauterine insemination of 2×10^8, 3×10^8, and 4×10^8 sperm are shown in Table 3. The mean sperm motility and viability in all animals were 18.4 ± 2.2% and 53.8 ± 2.7%, respectively. One animal inseminated with 3×10^8 sperm out of 16 animals inseminated with $2–4 \times 10^8$ sperm was fertilized (6.3%) and delivered 2 normal puppies on day 63 of gestation.

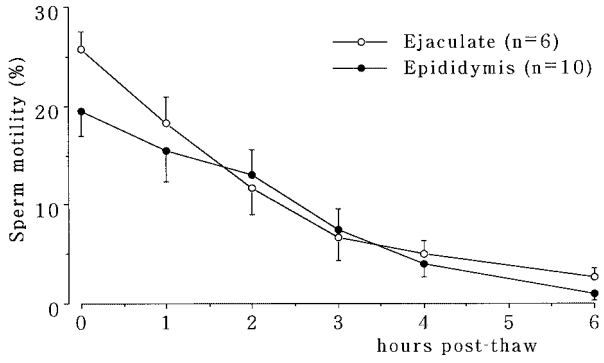

Fig. 1. Time-course changes in motility of post-thaw canine sperm from the cauda epididymis and after ejaculation (Mean ± SE).

Conception results of intratubal insemination: The conception results after inseminating 1×10^8 sperm in each bilateral uterine tube are shown in Table 4. The mean sperm motility and viability were 19.2 ± 2.4% and 59.9 ± 4.1%, respectively. One of 6 animals (16.7%) was fertilized and delivered 3 normal puppies on day 60 of gestation.

DISCUSSION

It is considered that sperm acquires motility during migration from the caput to the cauda of the epididymis, and is stored in the cauda of the epididymis, exhibiting fertility similar to that of ejaculated sperm in cats, as previously demonstrated [4, 6, 9, 14]. Since conception has been achieved with caudal epididymal sperm in dogs [7], the fertility of canine caudal epididymal sperm may be similar to

Table 3. Conception results of intrauterine insemination of frozen epididymal sperm

Inseminated sperm count ($\times 10^8$)	Bitch Number	Semen quality (%)			Number of ovulations		Number of pups	Conception rate (%)
		Dog Number	Sperm motility	Sperm viability	Left	Right		
2	311	IQ275	5	33.0	2	[5] a)	0	0/5 (0)
	330	W1032	10	55.2	3	[4]	0	
	313	SOGM3	15	36.2	[3]	2	0	
	323	FRIM2	30	65.8	3	[4]	0	
	322	AH2M4	15	56.5	3	[4]	0	
Mean±SE			15.0±4.7	49.3±7.1				
3	319	Q493	30	59.7	[2]	2	0	1/5 (20.0)
	320	6ME268	20	55.1	[4]	3	2	
	311	6ME296	35	65.0	3	[4]	0	
	324	6ME262	15	56.8	[4]	4	0	
	305	6ME268	20	52.9	1	[3]	0	
Mean±SE			24.0±4.1	57.9±2.3				
4	310	Q493	30	63.0	[4]	3	0	0/6 (0)
	337	6ME262	20	53.4	[4]	3	0	
	309	6ME301	15	48.1	3	[5]	0	
	316	6ME286	10	47.2	[5]	2	0	
	322	HIJAGX	15	59.5	2	[4]	0	
	317	HIJADB	10	55.2	3	[4]	0	
Mean±SE			16.6±3.4	54.4±2.8				

a) Inseminated site.

Table 4. Conception results of intratubal insemination of frozen epididymal sperm (1×10^8)

Bitch Number	Semen quality (%)			Number of ovulations		Number of pups
	Dog Number	Sperm motility	Sperm viability	Left	Right	
322	6ME296	30	69.0	[4] a)	4	3
337	HIJADB	15	59.8	[4]	4	0
316	6ME262	20	64.0	[5]	3	0
317	HIJAGX	15	53.4	[4]	4	0
320	6ME268	20	68.0	[3]	3	0
305	6ME286	15	45.1	[4]	3	0
Mean±SE		19.2±2.4	59.9±4.1	4.0±0.3	3.5±0.2	
				7.5±0.3		

a) Inseminated site.

that in cats.

Compared to the perfusion recovery method, contamination with blood and tissue fragments is problematic in the mincing method that we used in this study. England and Allen [2] reported that blood in canine semen might negatively affect the sperm motility in culture at 37°C, but the influence of blood on semen quality after freezing/thawing was not clarified. On the other hand, Hewitt *et al.* [5] reported that in cryopreserved epididymal sperm prepared by the mincing method, semen quality including the ability to penetrate the oocytes was good, and epididymal sperm could be stored by the same storage method as that for ejaculated sperm. In this study, the motility of caudal epididymal sperm was slightly lower than that of ejaculated sperm, but the difference was not significant, suggesting the usefulness of this recovery method. Moreover, since dogs bleed from the uterus at estrus and may bleed during mating, blood may not affect fertilization.

Semen quality after freezing/thawing was lower in epididymal sperm than in ejaculated sperm. In particular, the incidence of abnormal spermatozoa was four-fold higher, and the incidence of immature sperm was also high. In cryopreservation of ejaculated sperm, we previously reported a conception rate of 90% after unilateral intrauterine insemination of 1×10^8 sperm [13]. Therefore, in this study, we increased the number of inseminated sperm to 2–4-fold, but the conception rate was low. The major difference between ejaculated sperm and caudal epididymal sperm is that ejaculated sperm is sensitized by prostate fluid. Although changes in sperm caused by sensitization with prostate fluid are unknown, it is possible that a decapacitation factor was coated and acrosomes were protected. Nöthling *et al.* [10] reported that the addition of prostate fluid to frozen-thawed sperm increased the conception rate, although the sperm was ejaculated sperm. Damage caused by acrosomes and the influence of the addition of prostate fluid should be investigated in the future.

The results of this study clarified the fertility of canine frozen epididymal sperm as reported by Marks *et al.* [7]. Nevertheless, the conception rate was low, suggesting that it is necessary to investigate differences in damage between ejaculated sperm and epididymal sperm after thawing and to develop a method for improving the conception rate.

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