NOTE *Theriogenology*

Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality

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ABSTRACT. Canine epididymal sperm was collected from the cauda epididymis using 2 different methods (flushing and mincing) to compare the qualities (the percentage of progressively motile, viable, morphologically abnormal, immature and intact acrosomes) before and after freezing and thawing. No significant difference was noted in the quality of the cauda epididymal sperm immediately after collection and after freezing-thawing between the collection methods, although the mean levels of sperm quality with the flushing method were slightly better than that of the mincing method. The flushing method is simple and free of blood contamination, although the vas deferens was too small to be perfused in only 1 dog, and our results suggest that the flushing method is preferable to the mincing method for collecting sperm from the canine cauda epididymis.

KEY WORDS: canine, collection method, epididymal sperm, frozen semen

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Cryopreservation of sperm cells is an important assisted reproductive technology that has been investigated in various animal species [1, 2, 11]. Sperm is routinely collected as semen from ejaculate: if a donor male animal accidentally dies, sperm can still be collected from the cauda epididymis, a sperm storage site. We have conducted a series of studies on the cryopreservation of the canine cauda epididymal sperm [4–6, 8]. Our results have indicated that sperm motility and viability are better maintained after freezing and thawing by sensitizing sperm from the canine cauda epididymis to canine prostatic fluid (PF) during collection. A high conception rate (90%) could be achieved by surgical intrauterine insemination with 2×10^8 sperm [6]. In the above experiment, cauda epididymal sperm was collected immediately after removal of testes and epididymides. However, if a donor male unexpectedly dies, sperm cannot be collected in this way. Thus, we examined the cryopreservation of sperm collected from the cauda epididymides stored for 48 hr in physiological saline at 4°C. As a result, the motility of these sperm was better maintained after thawing. A high conception rate (80%) could be achieved by intrauterine insemination with 2×10^8 sperm [8].

In our previous experiments [4–6, 8], a widely utilized mincing method [3, 18, 19] was employed to collect canine epididymal sperm. Specifically, a canine cauda epididymis was minced with a scalpel in a collection solution to collect transmigrated sperm. Other collection methods include flushing the epididymis, vas deferens and epididymal duct in an ascending manner (flushing method) [12–14] and squeezing the epididymis and deferential ducts with an anatomic clamp [9, 10]. While the mincing method allows sperm collection from dogs of any size, the collected sperm is frequently contaminated with small amounts of blood and tissue. The flushung method, conversely, is free from blood and tissue contamination. However, the flushing method requires an epididymal duct of an appropriate thickness, precluding its application in some dog breeds, especially in smaller dogs, such as the kaninchen dachshund or chihuahua. It is also generally thought that collection of sufficient numbers of sperm is difficult with the squeezing method in comparison with the 2 other methods. Thus, various methods are being employed to collect sperm from the canine cauda epididymis, and each method includes an advantage and a problem. However, few studies have investigated the effects of the different collection methods on the quality of the cauda epididymal sperm after freezing and thawing.

In the present study, sperm was collected from the left and right cauda epididymides using 2 different methods (flushing and mincing) and cryopreserved. The effects of the different collection methods on the quality of the canine cauda epididymal sperm immediately after collection and after freezing and thawing were examined to compare these methods.

A total of 10 male beagles ranging from 1.0 to 3.5 years of age (mean ± S.E.: 1.7 ± 0.3 years) and from 9.4 to 13.9 kg of body weight (mean: 12.3 ± 0.6 kg) were used in this experiment. These male dogs were kept in 160 × 75 × 65 cm cages in each 2 dogs or 80 × 75 × 65 cm cages in 1 dog. Commercial dog food (Health Nutrition, Royal Canin Japon, Inc., Tokyo, Japan) was given once daily, and drinking water was given three times daily (morning, afternoon and evening). This study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Life Science University.

Testes and epididymides were surgically removed under

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general anesthesia. Animals were pretreated with 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.), and anesthesia was induced with propofol (4 mg/kg/i.v.; Fuji Pharma Co., Ltd., Tokyo, Japan) and maintained with isoflurane (ISOFLU®, Dainippon pharmaceutical Co., Ltd., Osaka, Japan). The scrotal upper skin was incised, and the epididymis was excised with the testis. Surgically excised testes and epididymides were immediately weighed, and we observed no differences in size between the right and left sides. The blood vessels on the surface of the epididymis were removed, and the epididimis was cut at the end of caudal part.

Sperm was collected from the left and right cauda epididymides using the mincing or flushing method at random. In the mincing method [4–6, 8], cauda epididymides were placed in 35-mm plastic culture dishes (NUNCLON, Nalge Nunc International Co., Ltd., Tokyo, Japan), into which a small mount of PF was added, followed by mincing with surgical scalpels (SURGICAL BLADES No.23, FUTABA Co., Ltd., Tokyo, Japan). Tissue pieces were subsequently removed by filtration through 80-μm metal meshes, and the sperm suspensions were collected in conical tubes. In the flushing method, a trocar with 24-G indwelling needles (Terumo) was inserted into the vas deferens in 35-mm plastic culture dishes, followed by flushing with PF using 2.5-m*l* syringes. The sperm suspensions were collected in conical tubes. All procedures were carried out at room temperature. PF that was used for the recovery of the sperm was previously collected from 3 beagles (3 to 6 years old) and centrifuged at 600 × *g* for 5 min, and the supernatant was stored at −40°C. All procedures from excision of the epididymis to sperm collection were completed in less than one hr.

Sperm recovered from cauda epididymides using the 2 collection methods were examined by a general semen quality test using microscopy. The sperm concentration was determined based on hematocytometer counts. Sperm motility was subjectively assessed with a semen quality examination plate and a warm plate at 37°C. Sperm that showed active forward movements were regarded as (+), those that moved slowly as (±), and those that were motionless as (−), and only the percentage of (+) sperm was calculated as sperm motility (the percentage of progressively motile sperm). The percentages of viable, morphologically abnormal and immature sperm were assessed by counting 300 to 400 sperm using eosin-nigrosin staining. Sperm that had cytoplasmic droplets on their mid-piece were judged as immature. The percentage of sperm with intact acrosome was evaluated in a smear after staining with Spermac® stain (FertiPro NV, Beernem, Belgium) by counting approximately 100 sperm and classifying them into normal or abnormal groups. Sperm with normally appearing acrosomes were considered as normal, and sperm with vesiculated, swollen, lost or ruptured acrosomes were considered as acrosome reacted or abnormal.

Each cauda epididymal sperm sample recovered using the 2 methods was frozen according to a previously reported method [7]. The semen was centrifuged (600 × *g*) for 5 min to remove PF. Egg yolk Tris-fructose citrate (EYT-FC) solution was used as an extender. Primary and secondary dilutions with EYT-FC were performed at room temperature. By primary dilution, sperm concentration was adjusted to 2×10^8/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 with stirring for approximately 10 min. The final concentrations of sperm, OEP and glycerol were 1×10^8/m*l*, 0.75% and 7%, respectively. The semen was subjected to the first refrigeration at 4°C for 1 hr using a programmable cooling system (MIR-153, Fujihira Industry Co., Ltd., Tokyo, Japan) and then loaded into a 0.5-m*l* straw in a 4°C thermostat. The straws were frozen by first maintaining them horizontally at 7 cm above the surface of liquid nitrogen (LN_2) in a styrene foam box for 10 min for sensitization with LN_2 vapor and then plunging into LN_2 (the second refrigeration) [7].

Semen straws were simultaneously thawed in warm water at 37°C for 45 sec, at least 1 week after freezing. After sperm motility, viability, abnormality and acrosome status of the thawed semen from the 2 groups were evaluated, the semen was kept at 20°C and the time course of changes in sperm motility and viability after 1, 2, 4 and 6 hr and sperm acrosome status after 6 hr were examined.

Data obtained in this study were analyzed using one-way ANOVA, and the significance of differences was analyzed by the paired Student's *t*-test using STATVIEW 5.0 (Abacus Concepts Inc., Berkely, CA, U.S.A.). A significance level lower than 5% was regarded as significant.

The weight of the left and right testes and epididymides and the sperm counts of 10 dogs are shown in Table 1. No significant difference was noted in the weights of the left and right testes and epididymides among the 10 dogs. One of the 10 dogs (Dog No. 10) was excluded, because the vas deferens was too small to be inserted with a trocar with a 24-G indwelling needle and to be perfused. A small amount of blood-contaminated semen was observed during mincing (less than 2% of the total volume when calculated using a hematocrit tube), but little during flushing. No significant difference was noted in the time from epididymis removal to sperm collection, although the flushing method was slightly simpler. Mean total sperm counts were slightly higher for the flushing method ($595.8 \pm 90.2 \times 10^6$) than for the mincing method ($494.6 \pm 67.7 \times 10^6$), although no significant difference was noted.

Semen qualities immediately after collection from the cauda epididymides of 9 dogs are shown in Table 2. Specifically, sperm motility was the same between the 2 collection methods or slightly higher for the flushing method than for the mincing method, except for in 1 dog (Dog No. 3). The mean sperm motilities were 92.2 ± 0.9 and 87.2 ± 3.9% for the flushing and mincing methods, respectively, showing no significant difference. No significant difference was noted in the percentages of viable sperm, morphologically abnormal sperm and immature sperm. No significant difference was noted in mean percentage of sperm with intact acrosome (95.0 ± 1.3 and 95.8 ± 1.9%, respectively).

The qualities of sperm collected from the cauda epididy-

Table 1. The weight of the left and right testes and epididymides and sperm counts of 10 beagle dogs

Collection methods	Dog No.	Testis weight (g)	Epididymis weight (g)	Number of sperm ($\times 10^6$)
Mincing method	1-R[a)]	9.9	2.8	384.0
	2-R	4.7	1.4	259.6
	3-R	9.1	2.2	880.0
	4-R	7.7	1.9	687.5
	5-L	8.1	2.1	450.0
	6-R	7.7	1.5	455.5
	7-L	8.5	2.3	502.5
	8-L	5.3	1.9	306.0
	9-L	8.2	2.1	527.0
	10-L	7.9	1.4	(396.5)
	Mean	7.7	2.0	494.6[b)]
	±S.E.	0.5	0.1	67.7
Flushing method	1-L	11.0	3.0	694.4
	2-L	5.1	1.5	224.0
	3-L	9.5	2.5	1040.0
	4-L	7.7	1.9	646.0
	5-R	6.0	1.8	720.0
	6-L	6.7	1.8	567.0
	7-R	8.6	2.3	675.0
	8-R	6.1	2.0	208.0
	9-R	8.1	2.2	587.6
	10-R	7.8	1.3	–[c)]
	Mean	7.7	2.0	595.8[b)]
	±S.E.	0.6	0.2	90.2

a) R: Right testis and epididymis L: Left testis and epididymis. b) Data of Dog No. 10 were excluded. c) Not capable of being collected.

Table 2. Semen quality immediately after collection using different methods from the cauda epididymides of 9 beagle dogs

Collection methods	Dog No.	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Immature sperm (%)	Sperm with an intact acrosome (%)
Mincing method	1-R[a)]	70	81.3	5.7	0.3	96
	2-R	80	93.3	10.5	1.5	95
	3-R	95	98.9	3.7	4.2	96
	4-R	95	95.0	3.3	1.8	90
	5-L	95	95.2	3.9	0.9	99
	6-R	95	94.2	5.7	1.5	93
	7-L	95	93.5	3.0	0.3	97
	8-L	70	72.1	9.6	2.4	89
	9-L	90	96.7	5.3	5.0	100
	Mean	87.2	91.1	5.6	2.0	95.0
	±S.E.	3.9	3.1	1.0	0.6	1.3
Flushing method	1-L	90	84.1	4.8	0.3	99
	2-L	90	87.7	10.2	2.0	84
	3-L	90	89.8	10.5	4.2	98
	4-L	95	92.2	6.3	0.0	96
	5-R	95	97.6	4.2	0.6	98
	6-L	95	95.6	3.3	0.3	98
	7-R	95	94.3	5.9	0.6	98
	8-R	90	88.4	6.9	1.5	99
	9-R	90	93.1	8.1	0.9	100
	Mean	92.2	91.4	6.7	1.2	95.8
	±S.E.	0.9	1.5	0.9	0.5	1.9

a) R: Right epididymis L: Left epididymis.

Table 3. Semen quality after freezing and thawing of sperm collected using different methods from the cauda epididymides of 9 beagle dogs

Collection methods	Dog No.	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Immature sperm (%)
Mincing method	1-R[a)]	10	47.1	17.4	0.0
	2-R	20	50.8	11.1	4.5
	3-R	15	37.8	3.3	1.6
	4-R	25	53.0	6.6	0.3
	5-L	30	47.1	9.3	2.1
	6-R	30	57.2	6.3	1.5
	7-L	35	46.1	3.9	0.9
	8-L	10	55.8	9.4	2.7
	9-L	5	64.7	6.0	0.0
	Mean	20.0	51.1	8.1	1.5
	±S.E.	3.8	2.7	1.5	0.5
Flushing method	1-L	25	64.5	7.2	0.3
	2-L	10	60.7	9.0	0.9
	3-L	25	38.0	9.0	0.0
	4-L	30	56.0	10.5	0.3
	5-R	30	52.0	6.9	0.3
	6-L	35	43.4	9.6	0.0
	7-R	35	58.4	3.9	0.3
	8-R	25	67.1	12.0	0.0
	9-R	25	70.4	7.0	1.4
	Mean	26.7	56.7	6.7	0.4
	±S.E.	2.7	3.8	0.9	0.2

a) R: Right epididymis L: Left epididymis.

mides using the different methods, after freezing and thawing are shown in Table 3. Sperm motility immediately after thawing was the same between the 2 methods or slightly higher for the flushing method than for the mincing method, except for in 1 dog (Dog No. 2). The mean sperm motilities immediately after thawing were 26.7 ± 2.7 and 20.0 ± 3.8% for the flushing and mincing methods, respectively, showing no significant difference. No significant difference was noted in the percentages of viable sperm, morphologically abnormal sperm and immature sperm.

The time courses of changes in sperm motility and viability when the semen was left at 20°C after thawing are shown in Fig. 1. The time courses of sperm motility and viability after freezing and thawing showed slightly higher values for the flushing method, although no significant difference was noted between the two methods for up to 6 hr after thawing.

The percentages of sperm with intact acrosome significantly decreased during the time between collection and freezing-thawing, as shown in Fig. 2. No significant difference was noted in the mean percentages between the mincing (61.4 ± 4.9%) and flushing (65.0 ± 3.7%) methods at 6 hr after thawing.

In the present study, sperm was collected from canine cauda epididymides using the flushing and mincing methods to compare semen qualities before and after freezing and thawing. As a result, no significant difference was noted in the qualities of the cauda epididymal sperm immediately after collection between these 2 methods, although sperm counts were slightly higher for the flushing method. As we previously reported [5], the sperm counts, determined using the mincing method, were slightly different between the left and right cauda epididymides, although no significant difference was noted. In the present study, the differences between the 2 methods were considered to be caused by residual sperm on the metal meshes used for the mincing method and epididymal tissue sections. The semen quality after freezing and thawing was compared, demonstrating slightly higher sperm motility for the flushing method. No significant difference was noted in the semen quality after freezing and thawing between the sperm-collection methods. It has been shown that the acrosomes are easily damaged by mechanical stimulation (e.g. centrifugation, freezing and thawing processes) [3, 4, 15]. Thus, it has been proposed that the relatively high pressure produced in the cauda epididymal duct by the flushing method can damage acrosomes, when it cut off cauda epididymal site near corpus epididymis, affecting the percentage of sperm with intact acrosome after freezing and thawing. However, we did not observe such effects in the present study. Our results are inconsistent with those reported by Sirivaidyapong *et al.* [17] who reported that sperm motility was significantly higher for the flushing method than for the mincing method, but are consistent in that no significant difference was noted in the semen qualities after freezing and thawing. Sirivaidyapong *et al.* [17] did not report the percentage of sperm with intact acrosome. The present study demonstrated that both methods similarly damaged the sperm acrosomes before and after freezing and thawing.

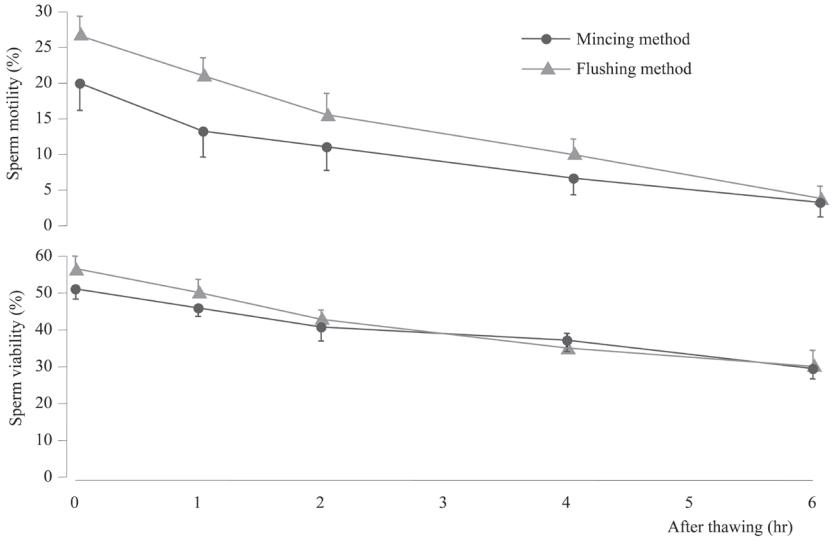

Fig. 1. The time-dependent changes in the sperm motility and viability in the 2 groups when sperm were left at 20°C after thawing (Mean ± S.E.).

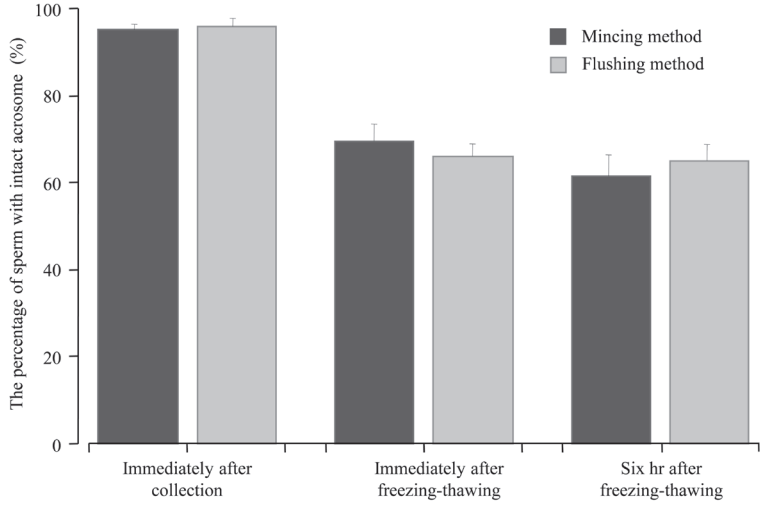

Fig. 2. The percentages of sperm with intact acrosome before and after freezing and thawing in the 2 groups.

In the flushing method, a trocar with a 24-G indwelling needle could not be inserted into the vas deferens in 1 of the 10 dogs, precluding flushing and sperm collection. As only beagles that met the requirements for age, body weight and testis weight, and epididymides weight were used in the present study, these were excluded as plausible causes. The testes and epididymides of the excluded dog appeared normal, although the vas deferens was small. In our other experiments, when sperm was collected using the flushing method from the cauda epididymides of smaller dogs, such as chihuahuas and pomeranians, a trocar could be readily inserted into the vas deferens (personal data). Therefore, it remains unclear why a trocar could not be inserted in this dog. Thus, the flushing method may be applicable to all dog breeds as long as they show no morphological abnormalities. However, the flushing method cannot be employed, if the vas deferens is shortened during orchiectomy or if the vas deferens or epididymal duct is perforated during blood vessel removal from the epididymal surface.

Our comparison demonstrated that the mincing method caused a small amount of blood contamination (less than 2%) even if the blood vessels were removed from the surface, while the flushing method caused no blood contamination. Rijsselaere *et al.* [16] demonstrated that blood contamination of less than 10% had no negative effects on the sperm motility and acrosomal caps after storage at 4°C. In our previous

study, a conception rate of 80% was achieved for intrauterine insemination with semen that was collected by the mincing method and frozen. Thus, a small amount of blood contamination caused no problem. However, blood contamination may have negative effects on sperm and should therefore be avoided. The mincing method requires specialized equipment (e.g., 80-μm metal meshes and glass funnels), while the flushing method requires only simple equipment (e.g., 24-G indwelling needles and 2.5-m*l* syringes). Hence, the flushing method is more useful than the mincing method for collecting sperm from the canine cauda epididymis.

In summary, the flushing method is simple and does not affect semen quality after freezing and thawing, and is therefore a useful method for collecting sperm from the canine cauda epididymis. However, our data suggest that the mincing method shoud be employed when flushing is impossible.

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