NOTE *Theriogenology*

Influence of the Time between Removal and Cooling of the Canine Epididymis on Post-Thaw Caudal Epididymal Sperm Quality

Tatsuya HORI[1)], Yuusuke UEHARA[1)], Eiichi KAWAKAMI[1)] and Toshihiko TSUTSUI[1)]

[1)]*Department of Reproduction, Nippon Veterinary and Life Science University, 7–1 Kyonan-cho, 1 chome, Musashino-shi, Tokyo 180–8602, Japan*

(Received 20 August 2008/Accepted 15 January 2009)

ABSTRACT. The effects of the time period between canine epididymis removal and cooling on post-thaw caudal epididymal sperm quality were investigated. Sperm recovered from the epididymis stored for 6 hr at 4 or 20°C exhibited similar motility. However, when the epididymis was stored for 12 hr or longer at 20°C, sperm motility was significantly lower than that at 4°C ($p<0.01$). The post-thawed qualities of sperm recovered from the caudal epididymides that had been stored at 20°C for 0 or 6 hr and then at 4°C for 24 hr after removal were not significantly different. Therefore, leaving the canine epididymis at 20°C for up to 6 hr after its removal may have little effect on the post-thaw quality of recovered caudal epididymal sperm.

KEY WORDS: canine, caudal epididymal sperm, frozen semen.

J. Vet. Med. Sci. 71(6): 811–815, 2009

Many wild animals including canids are endangered. As artificial reproductive techniques to preserve these species, methods of gamete preservation have been investigated [1, 5, 7]. Gametes from male animals are generally collected by ejaculation. However, when animals die accidentally, sperm are recovered from the cauda epididymis, the sperm storage site [3, 4, 8].

In a previous study [3], unilateral intrauterine insemination with sperm that was recovered from canine caudal epididymis in prostatic fluid (PF) and cryopreserved resulted in a high conception rate, 80.0%. We also showed that when caudal epididymal sperm were recovered after storage of the epididymis in sterile physiological saline at 4°C for 48 hr, sperm motility was reduced, but intrauterine insemination with this post-thaw sperm again led to a high conception rate, 80.0% [4]. In these two experiments, the epididymis was kept at 4°C immediately after removal, and the time period until the start of cooling after removal was not considered. However, when the bodies of male animals, such as wild animals, that have died accidentally are discovered, it is likely that several time have already passed under various temperature conditions, and so the epididymis can rarely be cooled immediately. This suggests that the time until cooling needs to be investigated.

It has been reported that mouse caudal epididymal sperm shows a sperm motility of approximately 30% after 10 days of postmortem storage at 4°C [6], but only shows a sperm motility of 10–15% after 24 hr of storage at room temperature [12]. Hishinuma *et al.* [2] reported that when the sika deer (*Cervus nippon*) epididymis is removed 4 hr postmortem and stored at 4°C for 1–4 days, the caudal epididymal sperm motility is high, at approximately 50%, but, when removed 8–12 hr postmortem and stored at 4°C for 1–4 days, the sperm motility is as low as 6.4%. These reports suggest that, in order to recover highly motile caudal epididymal sperm, it is important to store dead male animals immediately after death at 4°C or to remove the epididymis as soon as possible and rapidly store it at 4°C, thereby reducing the time of exposure to room temperature. However, in all the reported studies concerning dog caudal epididymal sperm, the epididymis was processed immediately after excision or stored immediately at 4°C, and no studies have reported the effect of leaving the epididymis at room temperature on semen quality.

Therefore, in Experiment 1 of this study, we compared the quality of caudal epididymal sperm recovered from uni- and contralateral epididymides that had been stored at 4 and 20°C, respectively, for 6, 12 and 24 hr after removal in order to investigate the effect of leaving the epididymis at room temperature (20°C) on caudal epididymal sperm. Based on the results of Experiment 1, in Experiment 2, we stored epididymides at 20°C for the time periods in which no significant difference in sperm motility was found compared with the caudal epididymal sperm recovered from epididymides that were cooled for the same amounts of time at 4°C immediately after removal. After storage, epididymal sperm were cryopreserved, and their post-thawed quality was examined to assess the effect of the time between epididymis removal and cooling on post-thaw caudal epididymal sperm quality.

Animals: The animals were 18 male dogs castrated at local animal hospitals or the veterinary medical teaching hospital at our University. The breeds, ages and body weights are shown in Table 1.

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

Epididymal excision and preservation methods: After ligation of the vas deferens and pampiniform venous plexus,

* CORRESPONDENCE TO: HORI, T., Department of Reproduction, Nippon Veterinary and Life Science University, 7–1 Kyonan-cho, 1 chome, Musashino-shi, Tokyo 180–8602, Japan.
e-mail: t-hori@nvlu.ac.jp

Table 1. Weights of the testes and epididymides of the dogs used in this study

Dog No.	Breeds	Age (yrs)	Body Weigt (kg)	Testis (g)		Epididymis(g)	
				L	R	L	R
1	Mongrel	1.1	2.2	1.7	1.9	0.5	0.6
2	Chihuahua	1.5	2.0	1.8	1.7	0.6	0.6
3	Chihuahua	3.0	1.6	1.6	1.7	0.5	3.8
4	Miniture Dachshund	1.2	4.3	4.6	4.3	1.9	1.5
5	Golden Retriever	1.0	30.0	16.5	14.9	4.1	0.5
6	Beagle	1.0	10.0	4.9	4.0	1.3	1.3
7	Beagle	1.0	10.0	6.9	6.8	2.0	1.8
8	Miniture Dachshund	0.8	4.0	6.5	6.0	1.9	1.5
9	Mongrel	1.0	5.0	2.8	2.4	1.2	1.2
10	Shih Tzu	9.9	5.3	3.6	3.7	1.3	1.6
11	Cavalier King Charles Spaniel	9.0	8.0	8.9	8.0	2.6	2.4
12	Cavalier King Charles Spaniel	1.3	8.7	4.2	4.6	1.4	1.4
13	Cavalier King Charles Spaniel	3.0	8.0	4.8	4.9	1.1	1.4
14	Shiba	1.0	10.9	6.4	6.4	1.3	1.1
15	Shetland Sheepdog	4.0	9.0	4.8	4.9	1.1	1.4
16	Welsh Corgi Pembroke	3.6	14.0	11.8	10.1	3.3	2.8
17	Wire Fox Terrier	1.0	6.7	8.5	9.8	1.5	1.6
18	Mongrel	0.8	10.0	9.0	9.1	1.8	1.6
Mean ± SD		2.5 ± 2.7	8.3 ± 6.4	6.1 ± 3.8	5.8 ± 3.5	1.9 ± 1.0	1.6 ± 0.8

the removed testis and epididymis were stored in sterile physiological saline to avoid drying as follows. In Experiment 1, one testis with epididymis from each dog was stored at 20°C (room temperature storage group: RTS group), and the other was stored at 4°C (low temperature storage group: LTS group; both groups were stored for 6 (n=3), 12 (n=4) or 24 (n=3) hr. In Experiment 2, one testis with epididymis from each dog was left at 20°C for 6 hr and then stored at 4°C for 18 hr (6-hr room temperature storage group: 6-hr RTS group), and the other was stored at 4°C for 24 hr (0-hr room temperature storage group: 0-hr RTS group;n=8).

Transmigration of sperm from caudal epididymis: Sperm were recovered from the caudal epididymides at room temperature (22 to 23°C) by the mincing method, as previously reported [3, 4]. The excised testes and epididymides were separated and then weighed The blood vessel on the surface of the epididymis was removed, and the epididymis was then cut at the corpus near the cauda. PF was used as the solution for recovery of sperm. PF was previously collected from 6 other beagles and centrifuged at 600 × g for 5 min, and the supernatant was stored at –40°C.

Semen quality test: Sperm recovered from caudal epididymides were subjected to a semen quality test by microscopic examination [3, 4]. The sperm concentration was determined by hematocytometer counts, sperm motility was determined as the percentage of actively motile sperm using a semen quality examination plate and a warm plate and the percentages of viable, morphologically abnormal and immature sperm were assessed by eosin-nigrosin staining. Sperm demonstrating cytoplasmic droplets on their mid-pieces were judged as immature.

Cryopreservation of sperm: Caudal epididymal sperm were frozen according to the method previously reported [4]. The semen was centrifuged (600 × g) for 5 min to remove the PF. Egg yolk Tris-fructose citrate (EYT-FC) solution was used as the extender for frozen canine semen. 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 with stirring 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). The qualities of sperm were also examined after glycerol equilibration for 1 hr. The semen was then loaded into a 0.5-m*l* straw in a 4°C thermostat. For freezing, LN_2 was poured into a styrene foam box (20 × 27 × 12 cm, 1.8 cm thick) to a level 5 cm deep, and the straws were maintained horizontally at 7-cm heights from the LN_2 surface for 10 min for sensitization with LN_2 vapor. The straws were then plunged into LN_2.

Semen quality test after thawing: Semen straws were thawed in warm water at 37°C for 45 sec, at least 1 week after freezing. After the general semen quality tests described above, the semen was stored at 20°C, and the time courses of sperm motility and sperm viability of semen preparations from the two groups were examined after 1, 2, 4 and 6 hr.

Statistical analysis: All data are presented as means ± SD. Differences in sperm parameters between the groups were analyzed using one-way or two-way analysis of variance (ANOVA;storage time × storage temperature), and post hoc multiple comparisons between groups were made by the Tukey-Kramer test using StatView 5.0 (Abacus Concepts

Table 2. Qualities of the sperm recovered from the caudal epididymides that had been stored at 4 (LTS group) or 20°C (RTS group) for various periods

Storage Time (hr)	Dog No.	Sperm motility (%)		Sperm viability (%)		Sperm abnormality (%)		Immature sperm (%)	
		LTS	RTS	LTS	RTS	LTS	RTS	LTS	RTS
6	1	70	60	85.3	81.0	6.6	10.7	1.8	0.6
	2	80	75	95.8	87.3	5.1	3.9	5.7	3.6
	3	30	30	87.4	84.8	12.1	10.8	0.0	0.6
	Mean ± SD	60.0 ± 26.5	55.0 ± 22.9	89.5 ± 5.6	82.7 ± 3.2	7.9 ± 3.7	8.5 ± 4.0	2.5 ± 2.9	1.6 ± 1.7
12	4	75	15	76.3	66.8	8.0	4.5	2.7	9.9
	5	60	0	80.7	83.3	10.4	8.5	13.0	11.7
	6	85	5	90.1	91.3	4.2	4.0	0.0	0.0
	7	85	30	92.7	89.1	11.7	12.5	0.3	0.2
	Mean ± SD	76.3 ± 11.8[a)]	12.5 ± 13.2[b)]	85.0 ± 7.7	82.6 ± 11.1	8.6 ± 3.3	7.4 ± 4.0	4.0 ± 6.1	5.5 ± 6.2
24	8	85	0	86.3	87.7	7.5	6.6	0.0	1.2
	9	50	0	79.6	67.3	12.6	6.0	2.1	14.7
	10	40	30	88.8	52.7	6.6	6.6	5.1	0.5
	Mean ± SD	58.3 ± 23.6[a)]	10.0 ± 17.3[b)]	84.9 ± 4.8	69.2 ± 17.6	8.9 ± 3.2	6.4 ± 0.3	2.4 ± 2.6	5.5 ± 8.0

a) Significantly different from the RTS group (P<0.01).
b) Signifcantly different from the 6-hr RTS group (P<0.01).

Table 3. Qualities (%) immediately after removal, after equilibration with glycerol or after freeze-thawing of sperm recovered from the caudal epididymides that had been stored at 20°C for 0 (0-hr RTS group) or 6 (6-hr RTS group) hr and then at 4°C for 24 hr after removal

Dog No.	Immediately after removal								After equilibration with glycerol				After freeze-thawing					
	Sperm motility		Sperm viability		Sperm abnormality		Immature sperm		Sperm motility		Sperm viability		Sperm motility		Sperm viability		Sperm abnormality	
	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr	0-hr	6-hr
11	80	75	89.2	83.8	11.4	9.0	5.1	3.0	70	70	84.9	68.9	30	5	66.1	30.2	9.5	10.8
12	60	60	69.3	83.8	5.1	4.2	1.5	8.7	50	40	68.0	82.1	20	35	35.7	41.7	6.6	3.6
13	50	30	71.7	80.2	4.8	4.2	5.4	6.3	30	30	55.2	30.4	25	5	54.1	46.0	3.5	4.5
14	80	70	65.4	88.8	4.2	5.7	4.2	20.1	70	30	86.1	70.5	35	25	56.4	39.0	6.2	4.5
15	40	70	63.9	65.4	11.4	3.6	2.1	1.8	20	45	-[a)]	-	10	10	19.5	19.8	19.6	9.1
16	60	50	85.0	66.6	9.5	7.2	0.0	0.3	40	50	66.6	66.6	15	30	33.6	44.1	11.3	9.6
17	95	80	86.2	93.9	8.1	8.1	0.3	0.6	65	60	51.1	59.5	20	30	42.8	41.4	10.4	12.2
18	80	50	79.2	63.7	7.2	5.1	7.2	2.4	75	45	53.0	62.9	15	20	35.7	52.8	11.9	6.3
Mean	68.1	60.6	76.2	78.3	7.7	5.9	3.2	5.4	52.5	46.3	66.4	63.0	21.3	20.0	43.0	39.4	9.9	7.6
± SD	18.5	16.6	9.9	11.6	2.9	2.0	2.6	6.6	20.7	13.8	14.6	16.0	8.3	12.0	15.0	10.2	4.9	3.3

a) Not observed.

Inc., Berkely, CA, U.S.A.). A significance level of less than 5% was regarded as significant.

The weights of the testes and epididymides of the dogs used in this study are shown in Table 1. Since the left and right testes and epididymides did not significantly differ in weight, they were considered to be similar to each other.

Experiment 1: The qualities of sperm recovered from the caudal epididymides that had been stored at 4 or 20°C for various periods are shown in Table 2. Two-way ANOVA revealed that there was a significant interaction between the effect of storage period and the effect of storage temperature on the sperm motility ($p<0.05$). Sperm recovered from epididymides stored for 6 hr at 4 or 20°C exhibited similar motility. However, when epididymides were stored for 12 or 24 hr, sperm motility was significantly lower after storage at 20°C than after storage at 4°C ($p<0.01$). No significant differences were noted in the motilities of sperm recovered from caudal epididymides that had been stored at 4°C for 6, 12 or 24 hr, whereas the motility of sperm after storage for 6 hr at 20°C was higher than those of sperm stored for 12 and 24 hr. No significant differences were noted in the rates of viable, morphologically abnormal, and immature sperm between the storage temperatures and times.

Experiment 2: The qualities of sperm recovered from the caudal epididymides that had been stored at 20°C for 0 or 6 hr and then at 4°C for 24 hr after removal and of sperm after equilibration with glycerol or freeze-thawing are shown in Table 3. In all dogs except No. 15, sperm motility was higher, but not significantly, in the 0-hr RTS group compared with the 6-hr RTS group. The mean sperm viability,

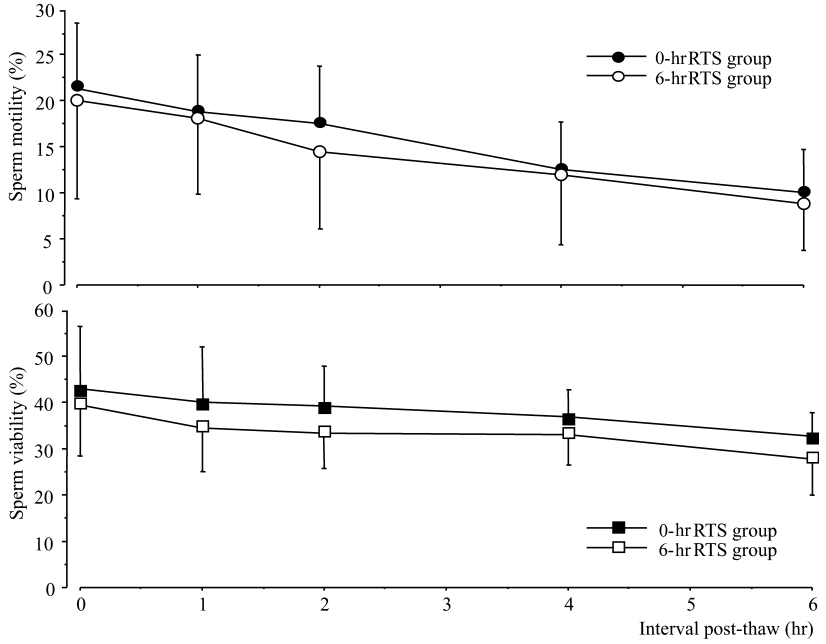

Fig. 1. The time-dependent changes in sperm motility and viability for the two groups when sperm were left at 20°C after thawing (Mean ± SD).

sperm abnormality and rates of immature sperm did not differ between the two groups.

After equilibration with glycerol and immediately after thawing, no differences in sperm qualities were noted between the 0-hr and 6-hr RTS groups.

The time-dependent changes in sperm motility and viability in the two groups when sperm were left at 20°C after thawing are shown in Fig. 1. No significant difference was noted in sperm motility or viability between the two groups at any time point until 6 hr after thawing.

The method of recovering and cryopreserving sperm from the caudal epididymis, the sperm storage site, is an effective artificial reproductive technique in male animals and has been reported by several researchers [3, 4, 8–10, 13, 15]. We previously reported that intrauterine insemination with caudal sperm recovered from the dog epididymis, which had been stored at 4°C for 48 hr immediately after removal, resulted in conception [4]. However, it is not always possible to remove epididymides from dead animals and recover or store sperm at 4°C immediately after death. Therefore, in this study, we investigated the effect of leaving the epididymis at room temperature immediately after removal on the recovered sperm. As a result, we found that when the dog epididymis was stored at 20°C for 6 hr or longer, the caudal epididymal sperm recovered did not differ in sperm viability from that recovered from the epididymis stored at a low temperature (4°C) for the same length of time, but it did clearly exhibit reduced sperm motility. These results are in agreement with a report showing that the motility of sika deer (*Cervus nippon*) sperm recovered from the epididymis removed 8–12 hr postmortem and then stored at 4°C for 1–4 days was as low as 6.4% [2]. Sperm are very sensitive to temperature change, which easily affects sperm metabolism, motility and viability. High temperatures (around 38°C) increase sperm metabolism and shorten survival, whereas, at 4°C, sperm are able to remain viable for a long period of time by halting their movements [14]. The epididymides of animals are normally anaerobic, have low pH and do not contain carbohydrates as a source of energy; therefore, respiration and glycolysis are suppressed in sperm, and they remain viable for approximately one month [11]. However, since blood flow stops and postmortem autolysis occurs after the death of a male animal or epididymis removal [2], sperm quality is considered to deteriorate markedly. The results of this study also suggest that these changes become marked after epididymis storage at room temperature for 6 hr or longer, resulting in loss of motility in the recovered sperm. To reduce this effect, it is necessary to store the epididymis at 4°C as soon as possible.

Similarly, in some studies using mice, it has been shown that caudal sperm recovered from the epididymis and left at 4°C after death exhibit higher motility than that left at room temperature (22°C) [6,12]. If male animals die accidentally, they are probably left under conditions different from those of the present experiment, such as epididymis storage in physiological saline. Since experiments similar to those in mice cannot be performed in dogs, the difference between leaving the intact dead dog and the removed epididymis untouched remains unclear.

In this study, the motility of sperm recovered from the epididymis stored at room temperature (20°C) for 6 hr and then at a low temperature (4°C) for 18 hr varied from 30 to

80%, depending on the individual dog, but it did not significantly differ from that (20–95%) of the caudal sperm recovered from the epididymis stored at a low temperature for 24 hr after removal. Sperm motility was high in all (7) dogs in the 0-hr RTS group except for one (Dog No. 15). The cause of this was unclear because the left and right epididymides did not differ in appearance or morphology.

In this study, the rate of sperm abnormality, particularly of the tail (data not shown), was high in some dogs in the 0-hr and 6-hr RTS groups. This was presumably due to changes associated with cold storage of the epididymis, as previously reported by Hori *et al.* [4]. The rate of immature sperm was relatively low, at 0–8.7%, except in Dog No. 14, whose epididymis was stored at room temperature for 6 hr. As shown in our previous study [4], this was probably due to the effect of addition of PF; hypothermic storage of the epididymis is associated with difficulty in cytoplasmic droplet removal, and this may have caused the somewhat high rate of immature sperm in Dog No. 14.

Post-thaw sperm motility was slightly lower, but not significantly, in the 6-hr RTS group compared with the 0-hr RTS group. However, there was individual variation; sperm motility was higher or the same in 4 of the 8 dogs in the 0-hr RTS group and in the remaining 4 dogs in the 6-hr RTS group. Similar individual variation was observed in sperm viability. Although the cause of this is unclear, it seems difficult to predict post-thaw sperm quality based on that before freezing. In regard to the post-thaw changes in sperm motility, sperm motility was higher in the 0-hr RTS group immediately after thawing, but was higher in the 6-hr RTS group 6 hr after thawing. However, no significant difference was observed between the two groups, suggesting that the time-course changes in sperm motility are similar in the two groups.

The present results showed wide individual variation, and all measured variables were lower than those previously reported by Hori *et al.* [4]. We speculate that this was due to the use of other breeds in addition to beagles and to the variation in their ages.

In conclusion, leaving the dog epididymis at 20°C for up to 6 hr after removal had little effect on the post-thaw quality of the recovered caudal epididymal sperm. Further studies are needed to investigate the fertilizing capacity of these sperm in artificial insemination.

REFERENCES

1. Farstad, W. 2000. Current state in biotechnology in canine and feline reproduction. *Anim. Reprod. Sci.* **60–61**: 375–387.
2. Hishinuma, M., Suzuki, K. and Sekine, J. 2003. Recovery and cryopreservation of sika deer (*Cervus nippon*) spermatozoa from epididymides stored at 4 degrees C. *Theriogenology* **59**: 813–820.
3. Hori, T., Hagiuda, K., Kawakami, E. and Tsutsui, T. 2005. Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. *Theriogenology* **63**: 1573–1583.
4. Hori, T., Hagiuda, K., Endo, S., Hayama, A., Kawakami, E. and Tsutsui, T. 2005. Unilateral intrautenine insemination with cryopreserved caudal epididymal sperm recoverd from refrigerated canine epididymides. *J. Vet. Med. Sci.* **67**: 1141–1147.
5. Howard, J. G. 1999. Assisted reproductive techniques in non-domestic carnivores. pp. 449–457. *In*: Zoo and Wild Animal Medicine (Fowler, M. E., Miller, R. E. eds.), Saunders, Tronto,
6. Kishikawa, H., Tateno, H. and Yanagimachi, R. 1999. Fertility of mouse spermatozoa retrieved from cadavers and maintained at 4 degrees C. *J. Reprod. Fertil.* **116**: 217–222.
7. Leibo, S. P. and Songsasen, N. 2002. Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology* **57**: 303–326.
8. Marks, S. L., Dupuis, J., Mickelsen, W. D., Memon, M. A. and Platz, C. C. Jr. 1994. Conception by use of postmortem epididymal semen extraction in a dog. *J. Am. Vet. Med. Assoc.* **204**: 1639–1640.
9. Nöthling, J. O., Gerber, D., Colenbrander, B., Dijkstra, M., Bakker, T. and De Cramer, K. 2007. The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed. *Theriogenology* **67**: 264–275.
10. Ponglowhapan, S. and Chatdarong, K. 2008. Effects of Equex STM Paste on the quality of frozen-thawed epididymal dog spermatozoa. *Theriogenology* **69**: 666–672.
11. Salisbury, G.W. and Lodge, J.R. 1962. Metabolism of spermatozoa. *Adv. Enzyme Regul.* **24**: 35–104.
12. Songsasen, N., Tong, J. and Leibo, S. P. 1998. Birth of live mice derived by in vitro fertilization with spermatozoa retrieved up to twenty-four hours after death. *J. Exp. Zool.* **280**: 189–196.
13. Tittarelli, C., Savignone, C. A., Arnaudín, E., Stornelli, M. C., Stornelli, M. A. and de la Sota, R. L. 2006. Effect of storage media and storage time on survival of spermatozoa recovered from canine and feline epididymides. *Theriogenology* **66**: 1637–1640.
14. Tsutsui, T., Tesuka, T., Mikasa, Y., Sugisawa, H., Kirihara, N., Hori, T. and Kawakami, E. 2003. Artificial insemination with canine semen stored at a low temperature. *J. Vet. Med. Sci.* **65**: 307–312.
15. Yu, I., Songsasen, N., Godke, R. A. and Leibo, S. P. 2002. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. *Cryobiology* **44**: 62–78.