FULL PAPER *Theriogenology*

Effects of Final Dilution Rate, Sperm Concentration and Times for Cooling and Glycerol Equilibration on Post-Thaw Characteristics of Canine Spermatozoa

Tsukasa OKANO[1)], Tetsuma MURASE[1,2)]*, Makoto ASANO[1)] and Toshio TSUBOTA[1,3)]

[1)]The United Graduate School of Veterinary Sciences, [2)]Laboratory of Veterinary Theriogenology and [3)]Laboratory of Zoo and Wildlife Medicine, Faculty of Applied Biological Sciences, Gifu University, Yanagido 1–1, Gifu 501–1193, Japan

(Received 20 February 2004/Accepted 23 June 2004)

ABSTRACT. This study re-evaluated a protocol for cryopreservation of canine semen. Semen from 4 beagle dogs was pooled, concentrated by centrifugation and adjusted to increasing sperm concentrations by adding back seminal plasma. The prepared or original semen was diluted with an extender (Egg yolk-Tris-citrate-glucose) and cooled to 4°C (cooling), followed by a second dilution with the same extender including glycerol, equilibrated at 4°C (equilibration), then stored in liquid nitrogen. The semen was diluted for frozen samples having a fixed sperm concentration with increasing dilution rates or for those having the reverse combinations. Various dilution rates of 2.5–10 folds or sperm concentrations of $0.25–2.5 \times 10^8$/m*l* had no significant effect on post-thaw sperm characteristics. When cooling was done for different times (0–26 hr) with glycerol equilibration for 1 hr, post-thaw characteristics were better at 2 and 3 hr of cooling, while various times for equilibration (0–4 hr) with cooling for 3 hr had no effect. These results suggest that different dilution rates and sperm concentrations within the ranges tested may not affect the post-thaw sperm characterisitics and that sufficient time for cooling may be essential but a specific equilibration time may not necessarily be required.

KEY WORDS: artificial insemination, canine, cryopreservation, semen, spermatozoa.

J. Vet. Med. Sci. 66(11): 1359–1364, 2004

Biotechnological studies may contribute much to the conservation of endangered wildlife species, and assisted reproduction techniques in domestic dogs may be applicable to non-domesticated canid species [11, 14]. Indeed, techniques of sperm cryopreservation developed for domestic dogs have been applied to captive red wolves [10]. On the other hand, if semen were collected from wild animals in the field, manipulation of the ejaculate would have to be carried out under unfavorable conditions. Thus, modifications would be needed for the application of techniques developed for dogs to wild animals.

Successful artificial insemination with frozen canine semen has been well documented [18, 22, 23, 25, 27] since the first conception was reported by Seager [21], and recently, the freezing protocol for canine semen has been improved [1, 3, 20, 28–30]. If semen is diluted to a constant final sperm concentration, the dilution rates may differ. Alternatively, if the dilution rate is fixed, the final sperm concentration might fluctuate according to the sperm concentration measured in the collected semen. Systematic studies examining the effects of the final dilution rate and the final sperm concentration on post-thaw sperm characteristics are lacking, except for limited information provided by Peña and Linde-Forsberg [17].

In most cases, canine semen is diluted, equilibrated with a cryoprotectant such as glycerol, and stored frozen in liquid nitrogen. In some reports, collected semen is directly diluted with an extender including glycerol and then equilibrated for hours before freezing [1, 7, 9, 13, 18, 20, 23, 26]. In other reports, semen is first cooled following an initial dilution with an extender not including glycerol, then diluted again with the same extender supplemented with glycerol, and equilibrated [3, 16]. This study, in which the latter method is employed, re-evaluates the protocol for freezing canine semen. Utilization of a system where the sperm concentration of semen was adjusted with its own seminal plasma before any processing for freezing made it possible to independently examine the effects of the final dilution rate and the final sperm concentration after dilution on post-thaw sperm characteristics. The present study also investigates the optimal times required for cooling without glycerol and for equilibration with the cryoprotectant before freezing.

* CORRESPONDENCE TO: MURASE, T., Laboratory of Veterinary Theriogenology, Faculty of Applied Biological Sciences, Gifu University, Yanagido 1–1, Gifu 501–1193, Japan.

MATERIALS AND METHODS

Semen collection: Semen was collected by digital manipulation from 4 healthy beagle dogs of unknown age (presumed around 6 years), kept in a reasonably spacious area and fed with ordinary pet food. For Experiments 1 and 2, the first (seminal plasma), second (sperm-rich fraction) and third (seminal plasma) fractions of the ejaculate were collected for a total of about 2 m*l* and a similar volume of the third fraction was also recovered in a separate tube from 1–4 animals, depending on volumes obtained, and pooled separately (Experiments 1 and 2). For Experiments 3 and 4, mainly the second fraction was collected with a minimun inclusion of the first and third fractions from 1–4 animals.

Dilution, cooling, equilibration, and freezing of spermatozoa: The pooled semen and seminal plasma were centrifuged at 700 × g for 10 min, and the supernatant was removed. The pelleted spermatozoa were resuspended by

gentle mixing, and according to the sperm concentration, the suspension was diluted with the recovered seminal plasma to the desired sperm concentrations of 2.5, 3, 4, 6, and 10×10^8/m*l* (for Experiment 1), or 1.5, 3, 6, 9, 12, and 15×10^8/m*l* (for Experiment 2). For Experiments 3 and 4, sperm concentration was not adjusted by seminal plasma but the collected semen was directly diluted with the extender to have 1×10^8/m*l* after the first dilution as described below.

Semen prepared as above was diluted at room temperature with an egg yolk-TRIS-citrate-glucose extender [4, 20] supplemented with 0.52 mg potassium penicillin G/m*l* and 0.8 mg streptomycin sulphate/m*l* (first extender). The diluted semen was then cooled down to 4°C in a refrigerator for the indicated times (cooling). An equal volume of a second extender, consisting of the first extender supplemented with 16 v/v% glycerol, was then added at 4°C and samples were kept at the same temperature for the indicated times (equilibration). Thus, the final concentration of glycerol was always 8 v/v% [1, 3].

The prepared semen samples with increasing sperm concentrations were diluted at increasing dilution rates (2.5, 3, 4, 5, 6, and 10 folds) to a constant final sperm concentration of 1×10^8/m*l* after the second dilution (Experiment 1). In Experiment 2, the samples with increasing sperm concentrations were diluted 6 folds to have increasing final sperm concentrations (0.25, 0.5, 1, 1.5, 2, and 2.5×10^8/m*l*) after the second dilution. In Experiments 3 and 4, the final dilution rate varied according to the sperm concentration in the collected semen, with a constant final sperm concentration being 0.5×10^8/m*l* after the second dilution.

After equilibration, the semen was loaded into 0.5 m*l* straws at about 5°C in an atmosphere of liquid nitrogen vapor, and placed horizontally 6 cm above the surface of the liquid nitrogen with a height of 8 cm contained in a styrofoam container (17.5 cm × 24.5 cm × 17.5 cm) [3]. The straws were kept thus for 15 min, then immediately plunged into the liquid nitrogen and stored until examination.

Semen evaluation: Characteristics of fresh (concentrated semen before adjustment of sperm concentration by seminal plasma in Experiments 1 and 2 and original semen in Experiments 3 and 4) or frozen-thawed spermatozoa were analyzed as follows. Straws were allowed to thaw at 38°C for a few minutes and used immediately for examination. Sperm motility was examined under a light microscope at a magnification × 100 and was subjectively classified into the following 5 grades: +++, progressively motile at a high speed; ++, progressively motile at a moderate speed; +, progressively motile at a low speed; ±, motile without progression; -, immotile. Each category was presented in percentage, and the results are shown as the percentage of spermatozoa. The proportion of spermatozoa exhibiting a motility grade of +++ (designated as percentage of spermatozoa with a +++ grade) or that of spermatozoa with ± or higher motility grades (percentage of motile spermatozoa) were used as parameters for sperm motility. Semen was diluted with physiological saline, and mixed with an equal volume of 2% glutaraldehyde in 0.165 M sodium cacodylate buffer (pH 7.0). The fixed samples were examined under a light microscope and spermatozoa with a dense apical ridge of the head were considered acrosome-intact [12, 15, 24]. Proportions of spermatozoa with intact acrosomes were obtained (percentage of acrosome-intact spermatozoa) (except for Experiment 1). The diluted semen was smeared onto a glass slide and sperm morphology was examined by staining with Giemsa (7.5 v/v% commercial Giemsa solution in 5 mM phosphate buffer, pH 7.0) for 2 hr after fixation of the smeared sample with methanol for 10 min. The proportion of morphologically abnormal spermatozoa were determined (percentage of morphologically abnormal spermatozoa).

Statistical analyses: Data are presented as mean ± standard error of the mean (SEM). The difference between each pair of treatments was tested by Student's *t*-test. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Evaluation of fresh semen revealed that percentages of spermatozoa with a +++ grade, of motile spermatozoa, of acrosome-intact spermatozoa and of morphologically abnormal spermatozoa were 7.5 ± 9.6, 80.0 ± 8.2, 98.5 ± 0.6 and 27.7 ± 9.8 in Experiment 1, 60.0 ± 7.1, 82.5 ± 4.8, 97.8 ± 0.9 and 17.0 ± 3.1 in Experiment 2, 48.3 ± 17.6, 95.0 ± 0.0, 98.3 ± 0.6 and 9.3 ± 0.3 in Experiment 3 and 76.7 ± 3.0, 95.0 ± 0.0, 95.7 ± 2.5 and 11.3 ± 2.1 in Experiment 4, respectively.

Experiment 1: *Effects of dilution rates*: The diluted semen was cooled down for 1 hr, then equilibrated for another 1 hr before freezing. Effects of different dilution rates (2.5, 3, 4, 6, and 10 folds) were examined with the final sperm concentration being constant at 1×10^8/m*l* (Table 1). None of the parameters examined, i.e., percentages of spermatozoa with a +++ grade, of motile spermatozoa and of morphologically abnormal spermatozoa, was statistically different among the final dilution rates tested.

Experiment 2: *Effects of final sperm concentrations*: Semen was frozen at different final sperm concentrations with a 6-fold constant dilution rate. Times for cooling and equilibration were both 1 hr. After thawing, there were no significant differences in any of the parameters tested within a range from 0.25 to 2.5×10^8/m*l* (Table 2).

Experiment 3: Effects of different cooling times: The above 2 experiments showed that final dilution rate or sperm concentration did not affect sperm characteristics after freezing and thawing. Therefore, in the later experiments, the final dilution rate was not fixed but for convenience, samples were diluted to have a final sperm concentration of 0.5×10^8/m*l*. In this experiment, the diluted semen was cooled down for varying times, and following the second dilution, semen was equilibrated for a fixed time of 1 hr before freezing. When the semen was cooled for 1 to 3 hr, percentage of spermatozoa with a +++ grade was significantly higher than when the time for cooling was 0 hr (Table 3). Although percentage of motile spermatozoa had a peak at 2 to 3 hr of cooling, the difference was not statistically

Table 1. Characteristics (mean ± SEM) of canine spermatozoa after freezing and thawing at different final dilution rates

Final dilution rate	Percentage of spermatozoa with a +++ grade	Percentage of motile spermatozoa	Percentage of morphologically abnormal spermatozoa
2.5	10.0 ± 0.0	46.7 ± 3.3	30.7 ± 1.8
3.0	10.0 ± 0.0	46.7 ± 3.3	27.5 ± 1.2
4.0	10.0 ± 2.9	50.0 ± 10.0	32.2 ± 1.2
6.0	10.0 ± 0.0	46.7 ± 8.8	28.5 ± 3.1
10.0	10.0 ± 0.0	50.0 ± 5.8	35.5 ± 3.2

Final sperm concentration was constant, 1×10^8/m*l*. Times for cooling and equilibration were both 1 hr. Results are averages of three replicates.

Table 2. Characteristics (mean ± SEM) of canine spermatozoa after freezing and thawing at different final sperm concentrations

Final sperm concentration ($\times 10^8$ cells/m*l*)	Percentage of spermatozoa with a +++ grade	Percentage of motile spermatozoa	Percentage of acrosome-intact spermatozoa	Percentage of morphologically abnormal spermatozoa
0.25	15.0 ± 6.1	53.8 ± 5.5	48.9 ± 4.6	35.7 ± 6.1
0.5	16.3 ± 6.6	60.0 ± 0.0	51.5 ± 5.1	35.3 ± 6.2
1.0	15.0 ± 6.1	56.3 ± 2.4	55.8 ± 6.9	30.3 ± 1.2
1.5	15.0 ± 6.1	51.3 ± 4.3	51.3 ± 7.4	37.0 ± 7.1
2.0	16.3 ± 6.6	56.3 ± 2.4	55.0 ± 4.6	44.3 ± 8.7
2.5	15.0 ± 6.1	55.0 ± 2.9	56.7 ± 4.5	39.7 ± 7.9

Final dilution rate was constant, 6 times. Times for cooling and equilibration were both 1 hr. Results are averages of four replicates except for percentage of morphologically abnormal spermatozoa (number of replicates was three).

Table 3. Effects of different times for cooling on canine sperm characteristics (mean ± SEM) after freezing and thawing

Time (hr) for cooling	Percentage of spermatozoa with a +++ grade	Percentage of motile spermatozoa	Percentage of acrosome-intact spermatozoa	Percentage of morphologically abnormal spermatozoa
0	10.8 ± 0.8	51.7 ± 4.4	44.5 ± 0.5	73.7 ± 5.5
1	23.3 ± 3.3[a)]	57.5 ± 4.3	51.3 ± 2.9	49.3 ± 3.2[c)]
2	25.0 ± 2.9[a)]	61.7 ± 4.4	55.8 ± 3.4[b)]	45.0 ± 2.6[c)]
3	21.7 ± 1.7[a)]	60.8 ± 5.8	62.3 ± 4.2[b)]	36.3 ± 0.7[c,d)]
6	20.8 ± 3.6	56.7 ± 1.7	66.0 ± 7.0[b)]	36.0 ± 5.5[c)]
16	21.7 ± 4.4	60.0 ± 0.0	54.3 ± 10.3	38.3 ± 2.4[c)]
26	21.7 ± 4.4	58.3 ± 1.7	49.7 ± 3.6	38.0 ± 3.0[c)]

Final sperm concentration was 0.5×10^8/m*l*. The time for equilibration was constant, 1 hr. Results are averages of three replicates.
a, b, c) Significantly different from 0 hr.
d) Significantly different from 1 and 2 hr.

significant. The percentage of acrosome-intact spermatozoa were significantly higher at 2, 3, and 6 hr of cooling than at 0 hr. On the other hand, percentage of morphologically abnormal spermatozoa was significantly reduced by cooling for 1 hr or longer, compared with 0 hr of cooling and was significantly lower at 3 hr than at either 1 or 2 hr. The abnormal morphology was mainly bent tail (the proportion to the number of the total morphologically abnormal spermatozoa: 90.3 ± 1.6% at 0 hr, 88.7 ± 1.7% at 1 hr, 88.8 ± 4.7% at 2 hr, 86.9 ± 6.4% at 3 hr, 75.0 ± 4.5% at 6 hr, 75.5 ± 6.7% at 16 hr and 79.4 ± 2.2% at 26 hr). The proportions did not significantly change among the different cooling times. The earliest time for cooling at which the percentage of spermatozoa with a +++ grade and that of acrosome-intact spermatozoa were significantly higher and the percentage of morphologically abnormal spermatozoa was significantly lower than at 0 hr was 3 hr in all cases.

Experiment 4: *Effects of different equilibration times*: In

Table 4. Effects of different times for equilibration with glycerol on canine sperm characteristics (mean ± SEM) after freezing and thawing

Time (hr) for equilibration	Percentage of spermatozoa with a +++ grade	Percentage of motile spermatozoa	Percentage of acrosome-intact spermatozoa	Percentage of morphologically abnormal spermatozoa
0.0	29.2 ± 2.2	69.2 ± 0.8	52.8 ± 4.5	39.8 ± 7.3
0.5	27.5 ± 5.0	68.3 ± 1.7	49.5 ± 6.0	36.3 ± 13.8
1.0	27.5 ± 5.2	67.5 ± 2.5	46.0 ± 4.5	36.8 ± 7.8
2.0	30.0 ± 2.9	69.2 ± 0.8	50.5 ± 7.4	35.0 ± 6.5
4.0	28.3 ± 3.0	67.5 ± 0.0	44.8 ± 7.6	30.0 ± 5.0
16.0	17.5 ± 2.5[a)]	58.3 ± 2.2[b)]	39.7 ± 3.8	34.8 ± 2.8

The time for cooling was constantly 3 hr. Final sperm concentration was 0.5×10^8/m*l*. Results are averages of three replicates except for percentage of morphologically abnormal spermatozoa (number of replicates was two).
a) Significantly different from 0 and 2 hr.
b) Significantly different from 0, 0.5, 2 and 4 hr.

this experiment, the initially diluted semen was cooled for a fixed time of 3 hr, then equilibrated with glycerol for different times ranging from 0 to 16 hr after the second dilution. Although equilibration for 0 to 4 hr did not significantly affect the post-thaw sperm characteristics tested, the percentages of spermatozoa with a +++ grade and of motile spermatozoa were significantly decreased at 16 hr than at 0 or 2 hr and than at 0, 0.5, 2 and 4 hr of equilibration, respectively (Table 4). No significant changes in percentage of morphologically abnormal spermatozoa were observed.

DISCUSSION

Different dilution rates make the final concentrations of chemicals and spermatozoa vary in the final mixture of semen and extender, and strong dilution may reduce sperm longevity, as pointed out by England [5]. Sperm concentration and the dilution rate might be interrelated so as to affect the post-thaw characteristics of spermatozoa. Adjustment of sperm concentration by seminal plasma before dilution allowed experiments to separately test these two factors, and the results suggest that a dilution rate within a range from 2.5- to 10-fold may not be important when egg yolk-TRIS-citrate-glucose extender is utilized. The fact that the sperm motility (percentages of spermatozoa with a +++ grade and of motile spermatozoa) after thawing was similar even with as low a dilution rate as 2.5 folds (Table 1) suggests that an energy source may have been sufficiently supplied at this rate.

It was also assumed that different final sperm concentrations might affect post-thaw sperm characteristics and that glucose as an energy source may be consumed more rapidly at higher sperm concentrations. However, within the ranges used, the sperm motility (percentages of spermatozoa with a +++ grade and of motile spermatozoa) was not different after freezing and thawing. This suggests that the energy source may have been sufficient at a sperm concentration of 2.5×10^8/m*l* with a final dilution rate of 6 folds. It was not clear from this study whether the sperm motility might be decreased at a higher sperm concentration with a lower dilution rate. However, because semen is usually diluted at a ratio of approximately 1:3 to 1:5 (4- to 6-fold dilution) and the final sperm concentration is about 0.5 to 2×10^8/m*l* [3, 19, 20, 25], this range may cover the practical availability.

The results show that it takes about 3 hr to obtain higher sperm motility, better acrosomal integrity, and lower numbers of morphologically abnormal spermatozoa (particularly bent tail) after freezing and thawing. This study, which employed two steps of dilution before freezing, shows that if spermatozoa are first cooled for a sufficient time without glycerol, equilibration may not necessarily take a long time. When spermatozoa were not cooled before glycerol equilibration for 1 hr at 4°C, a very high percentage of morphologically abnormal spermatozoa (73.7% on average), most (90.3% on average) of which had bent tails, was observed and it decreased with time for cooling. This implies that addition of glycerol at 4°C might be preferable to that at room temperature. Similar results were obtained in ram spermatozoa, where addition of glycerol at 4°C was better than 15°C in terms of post-thaw sperm parameters [8]. Cooling for 0 hr and glycerol equilibration for 1 hr (refrigeration for only 1 hr in total) might have been insufficient to decrease the temperature of samples to 4°C before freezing manipulation, because many spermatozoa with bent tails were observed and this may have resulted from abrupt changes in temperature [2].

Canine semen was diluted with an extender without glycerol followed by a second dilution with glycerol, both at 20°C, and then frozen in liquid nitrogen [28–30]. The method corresponds to cooling for 0 hr and equilibration for 1 hr in this study (Table 3), and the percentage of acrosome-intact spermatozoa after thawing was increased by the addition of Orvus ES paste [29] whereas in this study, the prolonged cooling time increased acrosomal integrity (Table 3). It appears that canine spermatozoa might acquire tolerance well after sufficient cooling time (2–3 hr in this study).

The observation that time for equilbration up to 4 hr did not affect the post-thaw sperm characterisitics, when sperm

were prior-cooled for 3 hr, and resulted in better post-thaw sperm characteristics agrees with the description that penetration of glycerol into the cell may not take long and that, instead, the time may be required for membrane changes or ion fluxes to take place [31]. This is further supported by the findings that bull spermatozoa cooled slowly for a longer period had a lower requirement for equilibration with glycerol before freezing than those cooled rapidly [6]. In fact, offspring were obtained by insemination with canine semen frozen after cooling followed by no particular period of glycerol equilibration; indeed, the time corresponded to that required for loading the semen into straws (about 15–30 min) [27].

The concentration of glycerol used here was 8% whereas other studies employed less [28–30]. Since at this concentration, the post-thaw sperm characteristics were generally acceptable, no further investigations were carried out. However, further studies will be necessary in order to find out an optimal concentration for the freezing protocol improved by the present results.

The present study suggests that if canine semen is processed for freezing using two steps of dilution (a final dilution rate of 2.5 to 10 folds, or a final sperm concentration within a range of 0.25 to 2.5×10^8 cell/m*l*), and the cooling time without glycerol is at least for 1 hr, with the optimum duration time being 3 hr, then a specific time for equilibration with glycerol may not be necessary.

Japanese black bears have only the prostate as an accessory sex gland (unpublished data), which is anatomically similar to domestic dogs and both species belong taxonomically to the Canoidea superfamily. Thus, it is speculated that the dog might be a good model for studies on semen processing of Japanese black bears.

Manipulation of ejaculates collected from wild animals in the field might be effectively carried out by ignoring estimation of sperm concentration in the ejaculate and calculation of a dilution rate at collection in the field, and postponing the second dilution by about 3 hr. Studies investigating whether or not this method might be applicable to semen from free-ranging Japanese black bears are currently being conducted by our laboratories.

ACKNOWLEDGMENT. This study was supported in part by a Grant-in-Aid for Scientific Research (The 21st Century Center-of-Excellence Program) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (E-1).

REFERENCES

1. Andersen, K. 1975. Insemination with frozen dog semen based on a new insemination technique. *Zuchthyg* **10**: 1–4.
2. Arthur, G. H., Noakes, D. E. and Person, H. 1982. Reproductive abnormalities of male animals. pp. 431–468. *In*: Veterinary Reproduction and Obstetrics, 5th ed., Baillière Tindall, London.
3. Christiansen, I. J. and Schmidt, M. 1980. Deepfreezing of dog semen. *Klg. Vetog. Landbohjsk., Inst. Sterilitetsforskin., Arsberetn.* **23**: 69–75.
4. Davis, I. S., Bratton, R. W. and Foote, R. H. 1963. Livability of bovine spermatozoa at 50, –25 and –85°C in Tris-buffered and citrate buffered yolk-glycerol extenders. *J. Dairy Sci.* **46**: 333–336.
5. England, G. C. W. 1993. Cryopreservation of dog semen: a review. *J. Reprod. Fertil. (Suppl.)* **47**: 243–255.
6. Ennen, B. D., Berndtson, W. E., Mortimer, R. G. and Pickett, B. W. 1976. Effect of processing procedures on motility of bovine spermatozoa frozen in 0.25 m*l* straws. *J. Anim. Sci.* **43**: 651–656.
7. Foote, R. H. 1964. The influence of frequency of semen collections, fractionation of the ejaculate, and dilution rate on the survival of stored dog sperm. *Cornell Vet.* **54**: 89–97.
8. Gil, J., Lundeheim, N., Söderquist, L. and Rodríguez-Martínez, H. 2003. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology* **59**: 1241–1255.
9. Gill, H. P., Kaufman, C. F., Foote, R. H. and Kirk, R. W. 1970. Artificial insemination of beagle bitches with freshly collected, liquid-stored, and frozen-stored semen. *Am. J. Vet. Res.* **31**: 1807–1813.
10. Goodrowe, K. L., Hay, M. A., Platz, C. C., Behrns, S. K., Jones, M. H. and Waddell, W. T. 1988. Characteristics of fresh and frozen-thawed red wolf (*Canis rufus*) spermatozoa. *Anim. Reprod. Sci.* **53**: 299–308.
11. Goodrowe, K. L., Walker, S. L., Ryckman, D. P., Mastromonaco, G. F., Hay, M. A., Bateman, H. L. and Waddell, W. T. 2000. Piecing together the puzzle of carnivore reproduction. *Anim. Reprod. Sci.* **60–61**: 389–403.
12. Kojima, E., Tsuruga, H., Komatsu, T., Murase, T., Tsubota, T. and Kita, I. 2001. Characterization of semen collected from beagles and captive Japanese black bears (*Ursus thibetanus japonicus*). *Theriogenology* **55**: 717–731.
13. Koutsarova, N., Todorov, P. and Koutsarov, G. 1997. Effect of pentoxifylline on motility and longevity of fresh and thawed dog spermatozoa. *J. Reprod. Fertil. (Suppl.)* **51**: 117–121.
14. Luvoni, G. C. 2000. Current progress on assisted reproduction in dogs and cats: *in vitro* embryo production. *Reprod. Nutr. Dev.* **40**: 505–512.
15. Mahi, C. A. and Yanagimachi, R. 1978. Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. *Gamete Res.* **1**: 101–109.
16. Nizanski, W., Dubiel, A., Birger, M. and Bielas, W. 1997. *In vitro* evaluation of dog semen extended in two Tris-buffered diluents and frozen in pellets. *Reprod. Dom. Anim.* **32**: 102.
17. Peña, A. and Linde-Forsberg, C. 2000. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology* **54**: 703–718.
18. Rota, A., Iguer-Ouada, M., Verstegen, J. and Linde-Forsberg, C. 1999. Fertility after vaginal or uterine deposition of dog semen frozen in a Tris extender with or without Equex STM paste. *Theriogenology* **51**: 1045–1058.
19. Rota, A., Linde-Forsberg, C., Vannozzi, J., Romagnoli, S. and Rodriguez-Martinez, H. 1998. Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reprod. Dom. Anim.* **33**: 355–361.
20. Rota, A., Strom, B., Linde-Forsberg, C. and Rodriguez-Martinez, H. 1997. Effects of Equex STM paste on viability of frozen-thawed dog spermatozoa during *in vitro* incubation at 38°C. *Theriogenology* **47**: 1093–1101.
21. Seager, S. W. J. 1969. Successful pregnancies utilizing frozen dog semen. *AI Digest* **17**: 6–16.

22. Seager, S. W. J. and Fletcher, W. S. 1973. Progress on the use of frozen semen in the dog. *Vet. Rec*. **92**: 6–10.
23. Seager, S. W. J., Platz, C. C. and Fletcher, W. S. 1975. Conception rates and related data using frozen dog semen. *J. Reprod. Fertil.* **45**: 189–192.
24. Shams-Borhan, G. and Harrison, R. A. P. 1981. Production, characterization, and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete Res.* **4**: 407–432.
25. Silva, L. D., Onclin, K., Lejeune, B. and Verstegen, J. P. 1996. Comparisons of intravaginal and intrauterine insemination of bitches with fresh or frozen semen. *Vet. Rec*. **138**: 154–157.
26. Ström, B., Rota, A. and Linde-Forsberg, C. 1997. *In vitro* characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology* **48**: 247–256.
27. Takeishi, M., Mikami, T., Kodama, Y., Tsunekane, T. and Iwaki, T. 1976. Studies on the reproduction in the dog. VIII. Artificial insemination using the frozen semen. *Jpn. J. Anim. Reprod.* **22**: 28–33.
28. Tsutsui, T., Hase, M., Hori, T., Ito, T. and Kawakami, E. 2000. Effects of Orvus ES Paste on canine spermatozoal longevity after freezing and thawing. *J. Vet. Med. Sci.* **62**: 533–535.
29. Tsutsui, T., Hase, M., Hori, T., Komoriya, K., Shimizu, M., Nagakubo, K. and Kawakami, E. 2000. Effect of addition of Orvus ES Paste to frozen canine semen extender on sperm acrosome. *J. Vet. Med. Sci*. **62**: 537–538.
30. Tsutsui, T., Hase, M., Tanaka, A., Fujimura, N., Hori, T. and Kawakami, E. 2000. Intrauterine and intravaginal insemination with frozen canine semen using an extender consisting of Orvus ES Paste-supplemented egg yolk Tris-Fructose citrate. *J. Vet. Med. Sci.* **62**: 603–606.
31. Watson, P. F. 1979. The preservation of semen in mammals. Oxford Rev. *Reprod. Biol.* **1**: 283–350.