韓國受精卵移植學會誌(2006) 第 21 卷 第 1 號
Korean J. Emb. Trans. (2006) Vol. 21, No. 1, pp. 77~83

Effects of Straw Size and Thawing Rate on Post-thaw Quality of Dog Semen

J. M. Son, Y. S. Kim, Y. J. Shin, Y. H. Lim, K. Y. Yoon[1], D. S. Lee, S. T. Shin and J. K. Cho[†]

College of Veterinary Medicine, Chungnam National University

개의 동결 정액 제조시 Straw의 크기와 융해온도가 정자의 생존율에 미치는 영향

손정민 · 김영실 · 신영지 · 임영환 · 윤기영[1] · 이두수 · 신상태, · 조종기[†]
충남대학교 수의과대학

SUMMARY

본 연구는 개 동결 정액 융해 시 straw 크기 및 융해 속도가 융해 정자의 질(quality)에 미치는 영향을 조사하고 최적의 융해 조건을 조사하는데 그 목적이 있다. 정상적인 번식능을 가진 비글 수컷 5마리에서 정액을 채취하여 원심 분리하여 정장을 버리고 남은 정자에 동결보호제인 glycerol이 첨가된 tris-glucose-egg yolk extender를 첨가하여 동결하고 액체질소에 보관한 후 융해하였다. 동결 융해 조건에 따른 효과를 알아보기 위해 straw는 0.25 ml과 0.5 ml 크기를 사용하였고 융해 조건은 75℃에 10초, 55℃에 12초 및 37℃에서 120초로 하여 융해 후 정자의 활력도(vigor), 운동성(motility), Hypo-osmotic test(HOS test)를 이용한 생존성(viability) 및 SperMac® 염색을 하여 정자의 membrane integrity를 비교 조사하였다. 조사 결과 0.5 ml 크기의 straw를 사용한 경우 37℃ 융해가 55℃, 75℃ 융해보다, 0.25 ml 크기의 straw를 사용한 경우에는 37℃, 55℃ 융해가 75℃ 융해보다 유의적으로 높은 활력지수 및 생존성을 보였다($P<0.05$). Straw 크기에 따라 비교하였을 경우 0.5 ml 군에서 유의적으로 높은 활력도, 생존성 및 membrane integrity를 보였다($P<0.05$).

결론적으로 개 정액이 동결 및 융해 시 0.5ml straw를 이용하여 동결한 후 37℃에서 120초 동안 융해하는 것이 최적의 조건임이 사료된다.

(Key words : frozen-thawed dog semen, straw size, thawing rate)

INTRODUCTION

Cryopreservation of canine semen like that of other animal species has been used for artificial insemination and storage of semen samples from valuable dog breeds (Yu *et al.*, 2002). Compared with fresh semen, frozen-thawed semen suffers from decrease in motility and morphological integrity, accompanied by decline in the survival in the female reproductive tract and reduction in fertility (Paulenz *et al.*, 2002). Using frozen-thawed semen in bitches, large numbers of spermatozoa have to

*This study was supported by the High-Technology Development Project for Agriculture and Forestry (2003-6628).
[1]Department of Animal Science, Shingu College
[†] Correspondence: cjki@cnu.ac.kr

be inseminated repeatedly during an estrus period to optimize fertility (Linde-Forsberg *et al.*, 1999). Therefore, it is necessary to optimize the freeze-thaw processes in order to increase the number of insemination doses obtained from an ejaculate.

Thawing rates (Ivanova-Kicheza *et al.*, 1995; Olar *et al.*, 1989; Battista *et al.*, 1988), freezing rates (Dobrinski *et al.*, 1993; Olar *et al.*, 1989), extenders (Rota *et al.*, 1997; Olar *et al.*, 1989; Battista *et al.*, 1988), cryoprotectants (Battista *et al.*, 1988), packing systems (Thomas *et al.*, 1993) and straw sizes (Nothling and Shuttleworth, 2005) have all been shown to affect the quality of frozen-thawed dog semen. In the previous studies, when using an extender that contains tris, citric acid and glycerol results in better post-thaw quality of dog semen compared to using other extender components including with lactose, PIPES and dextrose (Thomas *et al.*, 1993; Olar *et al.*, 1989; Battista *et al.*, 1988; Froman *et al.*, 1984). The post-thaw quality of dog semen is also better when thawed at higher temperatures such as 70℃ to 75℃ or 55℃, compared to 37℃, with no interaction between freezing and thawing rate (Rota *et al.*, 1998; Olar *et al.*, 1989). When using 0.5 ml French straws, sperm motility were better, compared that using the both 2.5 ml minitubes and 0.25 ml straws (Nizanski *et al.*, 2000; Thomas *et al.*, 1993).

The objectives of the study were to determine the effects of straw sizes and thawing temperatures on the quality of frozen-thawed canine semen with respect to vigor, viability and sperm membrane integrity. Additionally, investigation of most efficient combination of straw size and thawing rates was conducted.

MATERIALS and METHODS

1. Dogs

Clinically healthy five mature male dogs were used: beagles, aged 2～3 years, 6～11 kg. The animals were housed individually in kennels and were fed on a dry commercial food twice a day with free access to water.

2. Semen Collection

The semen was collected by digital manipulation using an artificial vagina. This procedure was performed in the presence of an estrous bitch. The sperm-rich fraction of each ejaculate was collected into a artificial vagina.

3. Evaluation of Fresh Semen

After collection, the ejaculates were immediately transferred to the laboratory for semen evaluation. Semen volume was recorded and the sperm concentration was determined using a hemocytometer (Superior, Germany) and 10 μl of semen diluted with 190 μl of 0.1 N HCl. Vigor (status of the sperm motility) was assessed by placing a 5 μl of semen on a Markler chamber and examining it under the microscope at 100 and 200 magnification. The status of the sperm vigor was scored on a scale from 0 to 5 (Table 1) (Seager *et al.*, 1975).

Sperm viability was evaluated by Hypo-osmotic swelling (HOS) test. The HOS test was performed by mixing 180 μl of 60 mOsm fructose in a test tube with 20 μl of ejaculate and incubating the mixture for 45 min at 37℃ in water bath. After incu-

Table 1. Classification on vigor of canine spermatozoa by its characteristics

Ranges	Characteristics
5	Very rapid and vigorous forward motion
4	Rapid progressive motion
3	Steady progressive motion
2	Slow progression, including stop and start motion
1	Weak undulation or oscillatory motion
0	No discernable motility

bation, 5 μl of the mixture was placed on a slide, in thin slide-coverslip preparation, and immediately examined under the microscope at 200 and 400 magnification. One hundred spermatozoa per slide were counted, and the percentage of spermatozoa exhibiting curling was determined. Curling was the indicative of sperm response to the HOS test (Fig. 1).

4. Semen Freezing Extenders

The first extender consisted of 200 mM Tris, 73 mM citric acid, 44 mM glucose, 1% (v/v) penicillin/streptomycin and 20% (v/v) egg yolk dissolved in the distilled water. And, in the second extender, 12% (v/v) glycerol was added to the same as the first solution (Table 2).

5. Freezing Procedure

Each ejaculate was centrifuged at 750 rpm (MF 550, Hanil, Korea) for 10 min and seminal plasma was discarded. The spermatozoal-pellets were resuspended to 200×10^6 sperm/ml with the first extender at 37℃. The diluted semen was then cooled down to 4℃ for 1 h (cooling). An equal volume of a second extender was then added at 4℃ and sample was kept at the same temperature for 1 h (equilibration). Thus, the final concentration of glycerol was 6 % (v/v) and sperm concentration was 100×10^6 sperm/ml. After equilibration, three 0.25 and 0.5 ml straws, destined to be thawed at 37, 55 and 75℃, were filled extended sperm and the end was powder-sealed. This procedure was performed at 4℃.

Fig. 1. Morphological changes of canine frozen-thawed semen after hypo-osmotic swelling (HOS) test. A sperm with curing tail, meaning alive (closed arrow). Spermatozoa with straight tail represent dead (open arrow).

Table 2. Composition of canine semen freezing buffer

	First buffer	Second buffer
Tris (g)	2.4	2.4
Ctric acid (g)	1.4	1.4
Glucose (g)	0.8	0.8
Penicillin/streptomycin (ml)	1	1
Egg yolk (ml)	20	20
Glycero l(ml)		12
Distilled water (ml)	79	67

A styrofoam box (24.7 cm × 24.7 cm × 24.2 cm with 2.0 cm depth) was filled to a depth of about 5 cm with liquid nitrogen. The freezing rack was placed 7 cm above the surface of the liquid nitrogen in the styrofoam box. The straws were loaded on the freezing rack for 10 min. Ten min later, the straws were allowed to drop from the rack into the liquid nitrogen. And then the straws were transferred to a liquid nitrogen container and were stored for at least three months.

6. Thawing Procedure

Straws were removed from the liquid nitrogen and thawed respectively. Each 0.25 and 0.5 ml straw was thawed in condition at 37℃ for 120 sec, 55℃ for 12 sec, or 75℃ for 10 sec. Thawed straws were dried with Kimwipes®, and the contents were released into a tube.

7. Evaluation of Frozen-thawed Semen

After thawing, vigor, viability and integrity of acrosomes were investigated. Evaluation of vigor

and viability were performed in the same way performed in the fresh semen. Sperm morphology was evaluated in a dry smear after staining with Spermac® (Fertility Technologies, Natick, MA, USA). Spermac® smears were examined under the microscope, at the magnification × 1,000 (Fig. 2). The acrosomal status of 100 spermatozoa on each smear was evaluated (Baran *et al.*, 2004).

8. Statistical Analysis

The effects of straw size and thawing rate on sperm vigor were analyzed by the Chi-square test using SPSS program (Release 11.0. Chicago, IL). Differences in the mean percentage of sperm viability and membrane integrity among the treatments were analyzed by the one-way ANOVA using the SPSS program. A probability of $P<0.05$ was considered to be statistically significant.

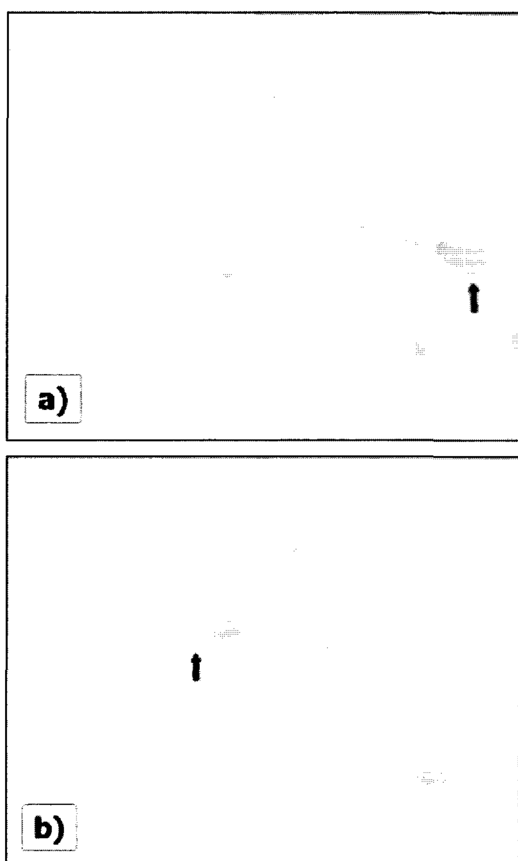

Fig. 2. Canine spermatozoa stained with Spermac®. a) Normal acrosome; acrosome was stained green and the head red (arrow). b) Acrosomal abnormality; lost acrosomal membrane (arrow).

RESULTS

1. Fresh Semen Quality

Ejaculate characteristics varied among dogs, with means and standard deviations for volume, concentration, vigor and hypo-osmotic swelling test being 7.30±2.97 ml, 76.0±67.25×10^6 spermatozoa/ml and grade 4.09±0.54 and 83.82±9.68%, respectively.

2. Effect of Straw Sizes

Straw sizes significantly affected the sperm parameters after thawing. As compared with semen characteristics in each straw size, sperm vigor, viability and intact acrosome of 0.5 ml straws resulted in better than those of 0.25 ml straws ($P<0.05$) (Table 3).

3. Effects of Thawing Rate in 0.5 ml Straw

Sperm vigor, viability and intact acrosome according to the effect of thawing rate are obtained (Table 4). The results indicated that thawing procedure in 0.5 ml straw had no significant differences on sperm viability and membrane integrity among the three groups of sample ($P<0.05$). However, vigor was higher for semen thawing in water

Table 3. The effect of straw sizes on post-thawing sperm characteristics in canine

Size of straw (ml)	Vigor	Viability (%)	Intact acrosome (%)
0.5	2.75±0.81[a]	48.92±11.58	79.45±9.73
0.25	1.97±1.42[b]	39.33±13.43	76.53±12.80

Values are means±SD.

[ab] Values with different superscript in the same column are significantly different ($P<0.05$).

at 37℃ for 120 sec, compared with 55℃ for 12 sec and 75℃ for 10 sec (P<0.05).

4. Effects of Thawing Rate in 0.25 ml Straw

Sperm vigor, viability and intact acrosome according to the effect of thawing rate are obtained (Table 5). Thawing rate significantly affected the sperm vigor, percentage of viability and intact acrosome. The results showed that thawing at 55℃ for 10 sec and 37℃ for 120 sec were significantly higher for sperm parameter than that at 75℃ for 10 sec (P< 0.05).

DISCUSSION

The thawing rate is important to improve post-thaw vigor and survival of spermatozoa. An excessively slow thawing rate can result in recrystallization of intracellular ice crystals, resulting in a reduction in cell survival (Olar *et al.*, 1989). On the other hand, rapid warming can induce osmotic stress on the spermatozoa because of the abrupt melting of the extracellular solution, that can cause unbalance rates of water influx and cryoprotectant egress, and can lead to swelling and lysis of cells (Griffiths *et al.*, 1979). In the present study, the significant effects of thawing rate on post-thawing sperm characteristics were shown. When using 0.25 ml straws, vigor, viability and morphology of sperm was greater with thawing rate of 55℃ for 12 sec and 37℃ for 120 sec, compared with that of 75℃ for 10 sec. When using the 0.5 ml straws, post-thaw vigor was higher at the 37℃ for 120 sec, compared to that at 55℃ for 12 sec and 75℃ for 10 sec. Based on the results of this study, the dog semen should not be thawed in a water bath at 75 ℃ for 10 sec with 0.25 ml straws. Several studies were reported about effects of thawing rates on the sperm characteristics in domestic dog semen. Out of these studies, some studies were shown similar results with present study when using thawing condition of 37℃ for 15 sec (Ström *et al.*, 1997; Yubi *et al.*, 1987) and 37℃ for 30 sec (Yu *et al.*, 2002; Yildiz *et al.*, 2000), which were comparable

Table 4. The effect of thawing temperature on post-thawing sperm characteristics using 0.5 ml straws in canine

Thawing temperature	Vigor	Viability (%)	Intact acrosome (%)
75℃ for 10 sec	2.55±0.69^{a}	49.00±14.14	78.82±11.94
55℃ for 12 sec	3.00±0.63^{ab}	48.36±11.17	77.82±11.46
37℃ for 120 sec	3.09±0.54^{b}	47.09±10.77	80.73± 6.97

Values are means±SD.
^{ab} Values with different superscript in the same column are significantly different (P<0.05).

Table 5. The effect of thawing temperature on post-thawing sperm characteristics using 0.25 ml straws in canine

Thawing temperature	Vigor	Viability (%)	Intact acrosome (%)
75℃ for 10 sec	0.27±0.47^{a}	26.36± 7.37^{a}	64.36± 7.37^{a}
55℃ for 12 sec	3.00±0.77^{b}	44.64±12.24^{b}	81.45±12.75^{b}
37℃ for 120 sec	3.00±0.45^{b}	43.73± 9.29^{b}	82.45±10.34^{b}

Values are means±SD.
^{ab} Values with different superscript in the same column are significantly different (P<0.05).

to conditions utilized in this study. However, significant higher post-thaw motility were shown in the fast thawing rate groups when thawed at 70 or 75°C water bath (Peña *et al.*, 2000; Peña *et al.*, 1998; Hay *et al.*, 1997; Olar *et al.*, 1989).

The results of this study suggest that dog semen be frozen in 0.5 ml straws rather than in 0.25 ml straws, because the vigor and viability after thawing were higher and the incidence of abnormal acrosomes was lower for semen frozen in 0.5 ml straws. The previous studies support the results that 0.5 ml straws resulted in a higher post-thaw semen quality than 0.25 ml straws (Nothling and Shuttleworth 2005).

In conclusion, freezing of dog semen using 0.5 ml straw container and thawing in 37℃ water for 120 sec was the most efficient combination in the canine semen freezing technique. A more in-depth study cryopreservation is needed to gain further understanding of where and when damage occurs in canine spermatozoa before these techniques can be effectively utilized.

CONCLUSION

The objectives of this study were to compare the effects of two straw sizes (0.25 ml and 0.5 ml) and three thawing rates (in water 37, 55 and 75℃) on post-thaw quality of dog semen, and to determine the most efficient treatment combination. Eleven ejaculates from five dogs collected by digital manipulation assessed by macroscopic and microscopic criteria. Each ejaculate was centrifuged, and the seminal plasma was discarded. Each sperm pellet was diluted with Tris-glucose-egg yolk extender with glycerol (final concentration of spermatozoa 100×10^6 spermatozoa/ml, 6% glycerol) and frozen and stored in the liquid nitrogen. Spermatozoa with extender was filled into 0.25 ml and 0.5 ml straws, respectively. The frozen semen was thawed at 75℃ for 10 sec, 55℃ for 12 sec or 37℃ for 120 sec, respectively. Then the post-thawed semen was evaluated on sperm vigor, viability using HOS test, morphology and sperm membrane integrity using Spermac® stain. Significant differences were shown in the evaluated sperm characteristics among the three thawing rates. When using 0.5 ml straws, vigor of spermatoza thawed was higher at 37℃ water compared with 55℃ and 75℃ (P<0.05). Quality of semen thawed at 37℃ and 55℃ were better than those at 75℃ using 0.25 ml straws (P< 0.05). As compared with semen characteristics in each straw size, sperm vigor, viability and morphology of 0.5 ml straws resulted in better than those of 0.25 ml straws (P<0.05).

In conclusion, freezing of dog semen using 0.5 ml straw container and thawing in 37℃ water for 120 sec was the most efficient combination in the canine semen freezing technique.

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(접수일: 2006. 2. 28 / 채택일: 2006. 3. 21)