Effects of Sugar Type on Viability of Frozen-Thawed Canine Spermatozoa

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ABSTRACT

This study was conducted to investigate the effects of type of the sugar supplemented to the extender on the vigor, viability and intact acrosomal rates of frozen-thawed dog spermatozoa. The ejaculated semen was diluted with TRIS-citric acid extender containing 200 mM TRIS, 73 mM citric acid, 6% (v/v) glycerol, 20% (v/v) egg yolk, 1% (v/v) antibiotics (streptomycin/penicillin), 44 mM sugar, which was either glucose, fructose or glucose-fructose combination, and distilled water to make the final volume of 100 ml. Extended semen samples were cooled at 4℃ for an hour, packaged in 0.25 ml straws, equilibrated for 10 minutes in liquid nitrogen vapor, and frozen in liquid nitrogen. Samples were thawed by placing straws into 37℃ water for 120 seconds. After thawing, vigor, viability and intact acrosomal rates of frozen-thawed semen were compared according to type of sugar. No significant differences were observed between glucose and fructose groups. In addition, combination of the 2 sugars also did not show any significant differences in the vigor, viability and intact acrosomal rates.

In conclusion, glucose and fructose were equally efficient as sugar supplements for freezing extender.

(Key words : dog, semen, extender, glucose, fructose, viability)

INTRODUCTION

Semen cryopreservation is used basically for artificial insemination and for storage of samples from valuable dog breeds (Gill *et al.*, 1970; Linde-Forsberg, 1991; Ivanova-Kicheva *et al.*, 1997). Many factors can affect the maintenance of spermatozoa function during freezing and thawing, such as freezing method, equilibration periods and cooling rate. Moreover, composition of the extender may be pivotal relative to fertility of spermatozoa (Morton and Bruce, 1989; Ivanova-Kicheva *et al.*, 1997; Alamo *et al.*, 2005).

Amongst the composition, sugars have several functions in sperm extender, which include providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluent, and acting as a cryoprotectant (Watson, 1979; Garcia and Graham, 1989; Abdelhakeam *et al.*, 1991; Aslam, 1992). Sperm can obtain energy through mitochondrial oxidative phosphorylation and glycolysis by the consumption of glycolysable sugars, such as glucose, fructose, mannose, and maltose (Nagai *et al.*, 1982). Among them, glucose and fructose are two of the most commonly used sugars for canine semen extenders. However, it has not been determined which type of sugar is efficient in the semen freezing is controversial. Hence, there is a need to identify which sugar supplement would be most efficacious in dog semen freezing.

The objective of this study was to determine which of the two sugars, or their combination was most efficient in the dog semen freezing with respect to sperm motility, viability and intact acrosome rates of frozen-thawed semen.

MATERIALS AND METHODS

1. Animals

Clinically healthy stud, 8 Beagle dogs were used: aged 2~3 years, 6~11 kg. The animals were housed individually in boxes and were fed a dry commercial food twice a day with free access to water.

2. Sperm Extenders

Two different sugars and their combination in the extenders were tested. The first extender consisted of 200 mM Tris, 73 mM citric acid, 44 mM sugar (glucose, fructose or combination of glucose and fructose), 1% (v/v) penicillin/streptomycin and 20% (v/v) egg yolk dissolved in the distilled water. In the

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second extender, 12% (v/v) glycerol was added to the first solution (Table 1).

3. Semen Collection

Dogs used in this study had been trained for semen collection for several days prior to the experiment. Semen samples were collected, as usual, by digital manipulation according to the technique described by Christiansen (1984). The whole fraction of the ejaculate was collected into warm tubes, and the volume was recorded before placing in a water bath at 37℃. First or second fraction of ejaculates per dog was collected.

4. Sperm Examination

Individual vigor of semen samples was evaluated using a phase contrast microscope. Sperm concentration was determined using a hemocytometer (Mickelsen *et al.*, 1993). Briefly, 10 μl of sperm diluted with 190 μl of 1 N HCl. Vigor, scored on a scale from 0 to 5, was evaluated by light microscope at × 100 (Table 2).

To assess live/dead sperm percentage, 20 μl of semen was mixed with 180 μl of HOS (hypo-osmotic swelling) solution, 60 mOsm fructose solution, and stored at 37℃ for 45 minutes. A total of 100 sperm cells were counted on each slide at 400 × magnification (Fig. 1). Spermac® solution was used to determine acrosomal abnormality (Baran *et al.*, 2004). The solution can stain sperm, especially head part acrosome (Fig. 2).

5. Semen Freezing

After semen collection and examination, the sperm samples were just diluted with the first extender to make the mixed concentration of 2×10^8/ml and were stored at 4℃ for an hour. Then, the mixed samples were diluted with the second extender to make the concentration of 1×10^8/ml and were stored at 4℃ for an hour again. Afterwards, the equilibrated samples were filled into 0.25 ml straw using a syringe-yellow tip. After sealing the opened tip to pull up solution of samples with pink-colored sealing powder, the filled straws were put on the rack in the box for 10 min, as cooling stage. Beforehand, a Styrofoam box sized 24.7×24.7×24.2 cm was prepared. The rack is set up 10 cm above liquid nitrogen in the box. After 10 minutes, they were picked up, seeding by frozen forceps. Then the cooled straws were frozen and stored in liquid nitrogen until evaluation.

Table1. Composition of canine semen freezing buffer used in this experiment

	Tris buffer	First buffer	Second buffer
Tris (g)	2.4	2.4	2.4
Citric acid (g)	1.4	1.4	1.4
Sugar*(g)	0.8	0.8	0.8
Penicillin/streptomycin (ml)	1	1	1
Egg yolk (ml)		20	20
Glycerol (ml)			12
Distilled water (ml)	99	79	67

*Glucose, fructose or combination of glucose and fructose.

6. Semen Thawing

Thawing was achieved by placing the frozen straws in a water bath at 37℃ for 120 sec. To assess vigor, viability and

Table 2. Classification on motility of canine spermatozoa by its vigor

Grade	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

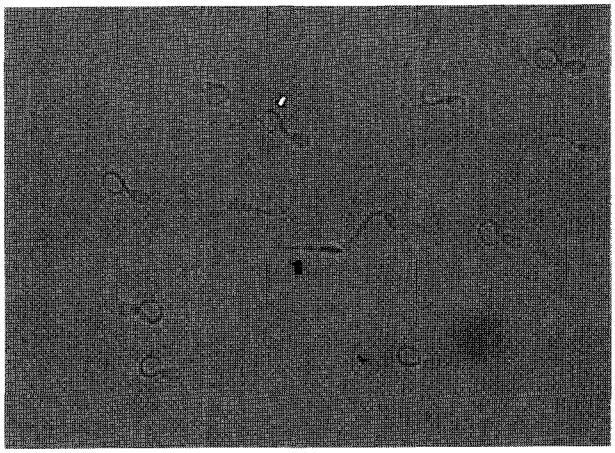

Fig. 1. Morphological changes of canine frozen-thawed semen after hypo-osmotic swelling (HOS) test. Live spermatozoa with curled tails (open arrow) and dead spermatozoa with non-curled tails (closed arrow) (× 400).

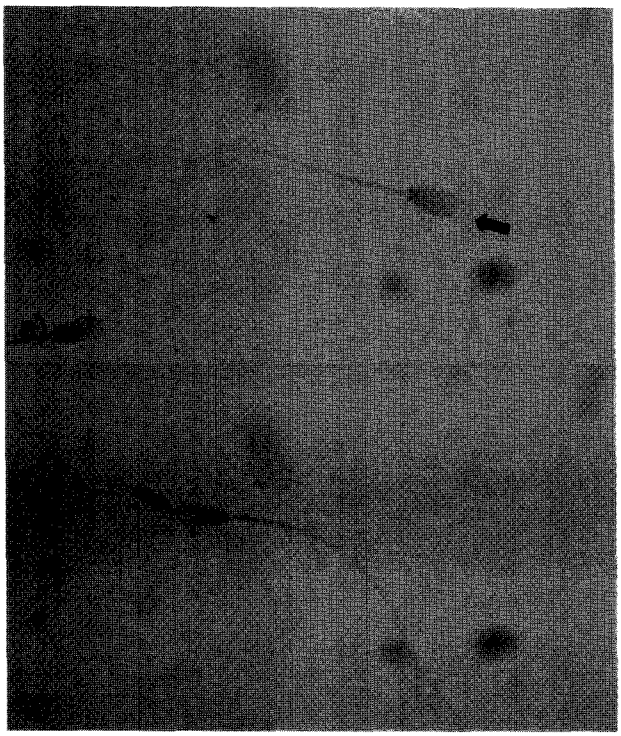

Fig. 2. Canine spermatozoa stained with Spermac®. (a) Normal acrosome; acrosome was stained green and the head red (arrow) (× 1,000). (b) Acrosomal abnormality; lost acrosomal membrane (arrow) (× 1,000).

the percentage of the sperm cells with damaged acrosome sperm smears were prepared after thawing. Processing and evaluation of the smears were conducted as described above.

7. Statistical Analysis

The effects of the sugar supplement on sperm vigor were analyzed using the Chi-square test. Differences in the mean percentage of sperm viability and membrane integrity among the treatments were analyzed using one-way ANOVA. Both statistical tools methods were conducted using the SPSS program (Release 11.0. Chicago, IL). A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

1. Evaluation of Fresh Semen

Normal appearance, white color and variable motility were evaluated. Other characteristics are summarized in Table 3.

2. Evaluation of Frozen-thawed Semen

Mean vigor decreased after thawing, but there was no significant difference among treatments (Table 4). Sperm viability and acrosomal integrity rates did not significantly differ ($p>0.05$) among groups containing different sugar supplements (Table 4).

Table 3. Characteristics of fresh semen (N=13)

Parameters	Mean±S.D
Semen volume (ml)	6.5± 3.2
Sperm concentration (×10^6spermatozoa/ml)	62.2±64.9
Vigor (score: 0~5)	3.8± 0.7
Viability (HOS test, %)	76.8± 17.7

DISCUSSION

In the present study, two types of sugar were added to the extenders and their effects on frozen-thawed sperm characteristics including vigor, viability and acrosomal integrity were evaluated. The major effect of glucose and fructose in semen extenders on frozen canine semen is to support sperm vigor. Vigor is an important indicator of sugar utilization by spermatozoa as the sugars provide the external energy source essential for maintaining vigor (Ponglowhapen *et al.*, 2004). Furthermore, cryoprotective ability of sugars on sperm cells may differ according to molecular weight of sugar (Molinia *et al.*, 1994). It was found out that monosaccharides are more suitable than disaccharides to preserve vigor of spermatozoa frozen in TRIS-citrate extender (Lapwood and Martin, 1966; Molinia *et al.*, 1994).

The results from the present study demonstrated that there were no significant differences between the effects of glucose

Table 4. Effect of sugar included in the extender on vigor, dead sperm and damaged acrosome percentage after thawing

	Type of sugar		
	Glucose	Fructose	Glucose+ fructose
Vigor	2.12± 0.82	2.58± 0.81	2.73± 1.03
Viability (%)	46.23± 7.50	47.00± 7.54	47.38±10.36
Acrosomal integrity (%)	67.23±11.68	69.85±11.73	68.69±12.22

*Data are presented as mean±SEM. All data within each column were not statistically different.

and fructose in the semen extenders on frozen canine semen and combination of 2 type of sugar also had no synergic effect. However, significant differences in the effect of sugar type were reported in previous studies (Ivanova-Kicheva *et al.*, 1997; Yildiz *et al.*, 2000; Ponglowhapan *et al.*, 2004). Ivanova-Kicheva *et al.* (1997) reported that a slight difference between the effects of the Tris-fructose and Tris-glucose based extenders on the post-thaw vigor and viability of semen. Additionally, it was reported that post-thaw motility and viability was significantly higher in a fructose based Tris-citric acid extender when compared to a glucose-based Tris-citric acid extender (Yildiz *et al.*, 2000). It was also noted, in chilled canine semen, that a Tris-fructose extender maintained higher sperm motility than extenders prepared with glucose or a mixture of both sugars (Ponglowhapan *et al.*, 2004). However, this increased beneficial effect of fructose over glucose was not noted in fresh semen where it was reported that there were no significant differences in extenders that were prepared with fructose or glucose on the percentage of motile spermatozoa (Rigau *et al.*, 2001). In this present study, it was considered that the effects of both sugars on sperm vigor, viability and acrosomal integrity are also not different each other since glucose and fructose are monosaccharides and they have similar characteristics.

In conclusion, monosaccharides such as glucose and fructose added to Tris-citric acid extender were equally efficient as cryoprotective agents in the canine semen freezing. Additionally, there are no significant differences among the effects of glucose, fructose and combination of glucose and fructose on sperm vigor, viability and membrane integrity.

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