Study on Cryopreservation of Epididymal and Ejaculated Semen in Korean Native Canine and Subsequent Pregnancy Rate after Artificial Insemination

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ABSTRACT

This study was carried out to investigate the general characteristics and viability of sperm after freezing and thawing and the pregnancy rates after artificial insemination with thawed semen. The rates of viable sperm after slow and rapid freezing were 87.4±3.85% and 70.8±4.45%, respectively which were significantly lower than that of fresh semen control (91.7±3.45%). The mean concentration of epididymal sperm after dilution in 1.0 ml saline and 3.0 ml extender in a various concentrations of cryoprotectants was $124.5\pm48.3\times10^6$ (range of 45×10^6 to 280×10^6 /ml). There was a significant difference not in the percentage of acrosome-reacted sperm, but in the percentage of capacitated sperm, between fresh and frozen-thawed epididymal semen. When frozen-thawed after diluting with tris-buffer extender containing glycerol, DMSO and ethylene glycol with concentration of 2 to 6%, the rates of epididymal sperm exposed to different cryoprotectants ranged from 14.4±4.7% to 20.7±5.8%, 17.8±5.2% to 36.5±4.9%, and 14.4± 4.6% to 18.5±5.3%, respectively which were lower compare to fresh semen control. The pregnancy rate after artificial insemination with frozen semen was 70.6%, whereas that with fresh semen was 90.0% in dogs with naturally induced estrus.

(Key words : Canine, Semen, Survival rate, AI, Pregnancy rate)

INTRODUCTION

The reproduction of small pet dogs is mainly completed by natural copulation and the proliferation of bad genes has caused concern. The use of artificial insemination has therefore been investigated urgently, but canine semen has a large physiological difference with that of common domestic animals the viability and the fertility rate of frozen semen was quite low. So artificial insemination utilizing frozen sperm is not used commonly and a freezing method to increase the viability is urgently needed. Studies to investigate the sperm capacitation rate *in vitro* have been performed in the dog by several researchers (Hewitt and England, 1997; Hewitt and England, 1998; Mahi and Yanagamachi, 1978; Nickson et al., 1993; Shimazu et al., 1992; Yamada and Yamada, 1993).

The canine seminal plasma ingredient contains a spermatozoon harmful enzyme and the cytoplasmic droplets in seminal plasma contain lysosomal enzyme. Therefore, if it is not eliminated and preserved under this condition, it will be harmful to the spermatozoa's survivability. It was reported that the pregnancy rate was not decreased when artificial inseminated with fresh semen of the 2nd fraction of canine semen, but that the viability was decreased when the sperm was preserved *in vitro* (Maule, 1960; Arthur, 1975). Seager and Fletcher (1973) reported that when fertilized with semen preserved for 1~4 days, the pregnancy rate was 53%. Province et al. (1984), Davis et al. (1963) and Foote (1964) reported that the viability rate was maintained at 50% when the canine semen was cryopreserved with 20% egg yolk extender for 2~4 days, or 4~8 days. Harrop (1962) reported that the survival rate with post-thaw sperm was 40~50%. Seager et al. (1975) were the first to gain success in pregnancy and parturition of canine utilized with frozen semen. Dog epididymal sperm collected after euthanasia has been frozen and successfully used after storage for 3.5 months, although, the recovery sperm was poor, the study involved only one animal and the apparent fertility was low (Marks et al., 1994). To summarize these reports, the majority has focused on short-term preservation research and there are few reports on the pregnancy rate after fertilization using frozen seminal fluid in domestic animals. Therefore, a

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report was urgently needed on the production of high viability frozen seminal fluid and the pregnancy rate after artificial insemination.

This study investigated the general characteristics of the small canine elimination of seminal plasma, sperm viability of post-freezing preservation and pregnancy rate after artificial insemination using fresh, frozen seminal fluid and epididymal semen.

MATERIALS & METHODS

Donor Animals

Twenty-three heads of small male Korean dogs and 80 heads of female Korean dogs were cleaned of parasites and vaccinated for use in this experiment.

Freezing of Semen and Viability Examination

The semen was collected separately by hand massage method. The highest concentration of 2nd fractional semen was used. The cauda epididymis and vas deferens was dissected from each testis and placed in a clean, dry Petri dish. Sperm samples were collected by repeating dicing of the cauda epididymides and proximal vas deferens. A 1.0 ml volume of saline was added to the sperm suspension, and then a 4.0 ml extender buffer modified with varying concentrations of cryoprotectants was mixed with the sperm-saline suspension. Epididymal semen was frozen-thawed after diluting with tris-buffer extender containing 2, 4 and 6% concentrations of glycerol, DMSO and ethylene glycol. The number of live and dead sperm and their morphological characteristics were examined on Nigrosin and Eosin stained smears using the classification of Christiansen (1984). Whole semen and epididymal semen were diluted with physiological saline and tris-buffer (1:3) and centrifuged for 6 min with 700 g and the supernatant was removed. Spermatozoa pellets were diluted in two stages with the frozen diluents. Semen was supplemented with 1.5 ml extender-1 at room temperature and diluted solution was cooled for 45min at 4°C. Following cooling for 25 min, 1.5 ml extender-2 and extender-2' were supplemented at 4°C and then mixed. Freezing was carried out by the method of Kim (2001). The straw semen preserved after being frozen for 1 month was placed for 30 min at room temperature and then dissolved in a water bath at 37°C. In order to remove cryoprotectants, frozen semen was shaken upside down and tris-buffer was removed and dissolved. The dissolved semen sample was moved to a slide glass and observed by microscope and the viability, survival rate, and morphological test were examined by using sperm analyzer imaging system (SAIS Si-100).

Artificial Insemination

Sixty-five heads of dogs expressing normal estrus symptoms were used in this experiment. Non-estrus dogs were treated with 20 mg of $PGF_{2\alpha}$ and then with 2,000 IU of PMSG and 2,000 IU of HCG. Estrus induced dogs and the dogs with natural estrus symptoms were carried out artificial insemination. Frozen straw semen (0.5 ml) was kept at room temperature for 30 s and thawed in water-bath at 37°C in order to remove cryoprotectants, shaken upside down for 10 min, transferred to tris-buffer and thawed. Artificial insemination was carried out with catheter to transcervical catheterization visualize passage of the catheter through the into the uterine body and into a uterine horn non-surgically at 1~4 days before and after ovulation. About 10 ml of semen was injected 2 times in order to prevent the adverse current of the semen fluid, which was elevated for several minutes.

Pregnancy Diagnosis

After artificial insemination, the dogs with normal estrus were stopped and the abdomen was expanded to examine for pregnancy symptoms. Within 50~70 days after fertilization, pregnancy was determined by ultrasound test.

Table 1. Component of semen extender

Composition	Extender-1	Extender-2	Extender-2'	Tris-buffer
Tris-buffer(g)	2.4	2.4	2.4	2.4
Citric acid monohydrate(g)	1.4	1.4	1.4	1.4
Fructose(g)	0.8	0.8	0.8	0.8
Na-benzylpenicillin(g)	0.06	0.06	0.06	0.06
Streptomycin sulphate(g)	0.1	0.1	0.1	0.1
Egg-yolk(ml)	20	20	20	
Glycerol(ml)	3	7	7	
Equex STM paste(ml)	0	0	1	
D.W.	100	100	100	100
pH	6.53	6.56	6.48	6.6
Osmotic pressure	760	1400	1380	260

Statistical Analysis

Data were expressed as mean SD. For comparison of means, Duncan's multiple verification was performed using SAS package of General Linear Model (GLM) procedures(SAS Institute, 1996).

RESULTS

Viability of Frozen-Thawed Semen

The 2nd fractional semen was selected and diluted with tris-buffer solution and centrifuged to remove seminal plasma. After equilibrium was achieved with extender-1 and 2 solutions, the mixture was cooled at 4°C. Table 2 presents the semen viability when frozen-thawed by slow and rapid freezing. The viability rates when the fractional semen was pretreated and then frozen-thawed and the epididymal semen by slow and rapid freezing were 87.4±3.85%, 70.8±4.45%, 52.5±5.82%, and 34.5±4.64%, respectively. These were lower than that of the control group (91.7±3.45%).

Assessment of Epididymal Sperm Prior to Freezing and after Thawing

The characteristics and survival rates of various concentrations of cryoprotectants of epididymal semen are shown in Tables 3. The rates of live sperm of fresh and frozen-thawed epididymal semen at 0 h were 47.5±8.7% and 10.5±3.6%, respectively. There was a significant difference between the percentage of capacitated sperm, but not of acrosome-reacted sperm, between the fresh and frozen-thawed epididymal semen. There was also a significant difference in the survival of sperm detected with Hoechest staining between the fresh and frozen-thawed epididymal semen.

The survival rates of epididymal semen at various concentrations of cryoprotectants are shown in Table 4.

Table 2. Survival rates of frozen-thawed semen by slow and rapid freezing

Treatment		Sperm motility of frozen-thawed semen	
		Survival sperm(%)	Dead sperms(%)
Control(fresh)[a]		91.7 ± 3.45	8.3 ± 1.45
Frozen semen[b]	Slow-freezing	82.4 ± 3.85	12.6 ± 3.72
	Rapid-freezing	70.8 ± 4.45	29.2 ± 3.84
Epidiymal semen	Slow freezing	52.5 ± 5.82	47.5 ± 4.88
	Rapid freezing	34.5 ± 4.64	65.5 ± 7.45

* Values with different letters within the same rows differ significant ($p<0.05$).

Table 3. Mean semen characteristics(±) prior to culture for fresh and frozen-thawed epididymal sperm

Parameter	Fresh semen	Frozen-thawed semen
Capacitated sperm(%)	40.0 ± 7.8	9.3 ± 3.0
Acrosome-reacted sperm(%)	0.5 ± 0.3	1.6 ± 1.0
Live sperm(%)	47.5 ± 8.7	10.5 ± 3.6

Table 4. The survival rate of epididymal sperm observed following dilution with extender's buffer at four different concentrations of cryoprotectants

Cryoprotectants(%)	Fresh semen	Frozen-thawed straw semen
Glycerol		
2.0	37.5 ± 5.9	18.4 ± 4.5
4.0	55.5 ± 9.3	20.7 ± 5.8
6.0	42.2 ± 8.2	14.4 ± 4.7
DMSO		
2.0	38.2 ± 5.5	20.4 ± 3.8
4.0	64.7 ± 8.9	36.5 ± 4.9
6.0	40.0 ± 6.7	17.8 ± 5.2
Ethylene glycol		
2.0	32.5 ± 4.9	16.1 ± 4.0
4.0	52.0 ± 7.2	18.5 ± 5.3
6.0	38.3 ± 6.5	14.4 ± 4.6

The survival rates of epididymal semen when frozen-thawed after diluting with tris-buffer extender containing 2, 4 and 6% concentrations of glycerol, DMSO and ethylene glycol were 14.4±4.7%~20.7±5.8%, 17.8± 5.2%~36.5±4.9%, and 14.4±4.6%~18.5±5.3%, respectively. These rates were lower than the fresh semen survival rate of 32.5±4.9%~64.7±8.9%.

Pregnancy Rate after AI

Table 5 presents the pregnancy rates when using artificial insemination with frozen-thawed 2nd fractional semen and epididymal semen in dogs with naturally induced estrus.

The pregnancy rate (70.6% and 23.8%) was lower than

Table 5. Pregnancy rates of frozen-thawed canine semen and epididymal semen

Treatment	No. of dogs examined	No. of dogs AI	No. of dogs pregnant(%)
Control	10	10	9/10(90.0)
Frozen semen	40	34	24/34(70.6)
Epididymal semen	25	21	5/21(23.8)

that of fresh semen(90.0%) when using artificial insemination with frozen semen in dogs with naturally induced estrus.

DISCUSSION

This study carried out to investigate the general characteristics of fractional semen, the survival rate of cryopreserved sperm and the pregnancy rate after artificial insemination using frozen semen of small Korean dogs. The seminal plasma and cytoplasmic droplets in canine semen contain harmful enzyme and many lysosomal enzymes which, if not eliminated and preserved, may be harmful to spermatozoa's survivability(Dott and Dingle, 1968; Allison and Hartree, 1970; Allen and England, 1992).

The 2nd fractional semen was selected and diluted with tris-buffer solution, centrifuged to remove the seminal plasma, equilibrated with extender-1 and -2 solutions and cooled at 4°C. The survival of semen when frozen-thawed by rapid freezing is shown in Table 2. The survival rates of semen when frozen-thawed by slow and rapid freezing after pre-treatment of the 2nd fractional semen were 87.4±3.85% and 70.8±4.45%, respectively, which were lower than that of the control group(91.7±3.45%). The above results were higher than those of Takeishi et al.(1975), who reported that the survival rate of whole semen after cryopreservation was 40~65%. Takeishi et al.(1975) reported that semen volume increased in spring and summer, but that the number of sperm was decreased. They found that the reverse phenomenon occurred in autumn and winter. The 2nd fractional frozen semen was recommended to be utilized in artificial insemination.

The characteristics and survival rates of various concentrations of cryoprotectants of epididymal semen are shown in Tables 3 and 4. The survival rates of epididymal semen when frozen-thawed after diluting with tris-buffer extender containing 2, 4 and 6% concentrations of glycerol, DMSO and ethylene glycol, at 14.4±4.7%~20.7±5.8%, 17.8±5.2%~36.5±4.9%, and 14.4±4.6%~18.5±5.3%, respectively, were lower than that of fresh semen at 32.5±4.9%~64.7±8.9%. This result was a little higher than that of Heitt et al. (2001) who reported that when using glycerol the motility rates were 6.2±9.0%~17.5±10.4% and the normal spermatozoa ratios were 6.5±1.7~14.8±8.5% (fresh semen 27.5±9.6%~35.0 ±5.7% and 32.5±5.3%~ 53.5±11.5%.

The pregnancy rate using artificial insemination with frozen semen and epididymal semen, in dogs with naturally induced estrus, was 70.6% and 23.8%, respectively, which was lower than that for fresh semen (90.0%). In addition, the pregnancy rate was similar with the 91% reported by Tsutsui (1975) when using artificial inseminated with fresh semen in the period of 54~108 hrs before and after ovulation. Generally, the pregnancy rate increased when artificial insemination was executed 1~2 days at the 1st fraction, and 3~4 days at the 2nd fraction after estrus, there is no respective comparison here (Maule, 1960; Arthur, 1975). This result was a little lower than that of Seager and Fletcher (1973) who reported pregnancy rate of 53% when inseminated with semen preserved for 1~4 days. The pregnancy rate did not decrease when inseminated with the 2nd fractional fresh semen but the survival rate decreased when preserved *in vitro*(Maule, 1960; Arthur, 1975). Province et al. (1984), Davis et al. (1963) and Foote (1964) reported a survival rate of 50% when the semen was cryopreserved with 20% egg yolk extender for 2~4 days or 4~8 days. Harrop (1962) reported that the survival rate with freezing-thawed canine semen was 45~50%. Seager et al. (1975) were the first to gain success in pregnancy and parturition by using canine frozen-thawed semen.

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