

# Intrauterine and Intravaginal Insemination with Frozen Canine Semen Using an Extender Consisting of Orvus ES Paste-Supplemented Egg Yolk Tris-Fructose Citrate

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**ABSTRACT.** Our previous report indicated that addition of Orvus ES Paste (OEP) to the extender of frozen canine semen protected acrosomes and maintained sperm motility after thawing. In this study, artificial insemination (AI) using the frozen semen was carried out. The frozen semen was prepared using egg yolk Tris-fructose citrate, and the final concentrations of glycerol and OEP were 7% (v/v) and 0.75% (v/v), respectively. AI was performed during the optimal mating period predicted from the peripheral plasma progesterone level. In intrauterine insemination (IUI), the bitches were laparotomized and  $1 \times 10^8$  spermatozoa were infused into one of the uterine horns. In insemination of non-OEP supplemented semen,  $3 \times 10^8$  spermatozoa were inseminated. In intravaginal insemination (IVI),  $10\text{--}40 \times 10^8$  spermatozoa were inseminated. Conception was obtained in nine of 10 bitches (90.0%) that underwent IUI. The number of newborns was from 1 to 7 (mean  $3.6 \pm 0.9$ ). The mean ratio of the number of puppies to the number of ovulations in the inseminated uterine horn was 71.8%. The number of puppies did not exceed the number of ovulation in the inseminated uterine horn. Conception using non-OEP supplemented frozen semen was unsuccessful in all four bitches. In IVI, conception was not obtained in any of the six bitches that received insemination of  $10 \times 10^8$  or  $40 \times 10^8$  spermatozoa, but two of three bitches that received insemination of  $20 \times 10^8$  spermatozoa were fertilized. It was shown that a high conception rate can be obtained by IUI using OEP-supplemented frozen canine semen. Development of a non-surgical method of IUI and a method of freezing canine sperm applicable to IVI is necessary.—**KEY WORDS:** artificial insemination, canine, frozen semen, Orvus ES Paste.

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Conception using frozen canine semen was initially achieved by Seager and Platz using pellet form semen in 1969 [14]. Following this, conceptions by the straw method were reported by Andersen in 1972 [1]. Thereafter, conceptions using frozen semen have been obtained at a small number of research institutions, using egg yolk Tris-fructose citrate (EYT-FC) [1–4, 11–13, 17, 18, 24], Triladyl [8, 10], laiciphos [15, 16], biciphos [16], and CLONE [17] as extenders. The composition of some of these extenders has not been revealed. With regard to the insemination site, both intravaginal [2, 4, 8–13, 15] and intrauterine [3, 4, 6, 12, 13, 15, 16, 24] insemination (IVI, IUI) have been performed. Moreover, the numbers of sperm and modes of insemination vary among researchers. A high conception rate has been documented in many of these reports. However, it is generally recognized that currently frozen canine semen is not practical for clinical use, contradicting the research reports.

We have previously reported [20] that the quality of frozen semen prepared using EYT-FC, which is used as an extender of frozen canine semen, is reasonable, however, the sperm motility rapidly decreased in the early phase after thawing, and was reduced to 0% after three hours. Referring to the report by Mahi and Yanagimachi [7] that capacitation of canine sperm required seven hours in *in vitro* experiments, it appears difficult to achieve conception by artificial insemination (AI) using such frozen semen. However, we have shown that by adding Orvus ES Paste (OEP) to the extender, acrosomes are protected and the

duration of maintaining sperm motility after thawing is prolonged [21]. Therefore, in this study, we performed IUI and IVI using frozen semen prepared with an extender of OEP-supplemented EYT-FC. We also performed IUI using an extender containing no OEP in a small number of animals. IUI and IVI was performed referring to our results previously obtained using fresh semen [19, 22, 23].

## MATERIALS AND METHODS

**Animals:** Thirteen male beagles aged 2 to 6 years with copulation ability and fertility were used. For AI, 23 female beagles aged 2 to 4 years were used.

**Frozen semen:** Semen collection and preparation of frozen semen were performed as previously reported [20]. Primary and secondary dilutions were performed at 20°C using EYT-FC. The final glycerol concentration was 7% (v/v), and the semen solution was equilibrated with of glycerol for one hour. OEP was added to a final concentration of 0.75% (v/v). For straws, 1.0 ml straws were used, and the sperm concentration was adjusted to  $1 \times 10^8$ /ml, and frozen using a conventional freezer.

**AI:** As previously reported [5], AI was performed during the optimal mating period, i.e. three or four days after the ovulation day predicted from the peripheral plasma progesterone level [5]. The semen used for AI had been kept frozen for from 1 to 15 months. Frozen semen was thawed in warm water at 37°C for 1 min. AI was performed once per bitch during the optimal mating period.

IUI was performed by a method previously reported [22]. The animals were laparotomized under general anesthesia, and semen was introduced into one of the uterine horns. For insemination, one straw of semen in extender with OEP ( $1 \times 10^8$  spermatozoa, 10 dogs) was thawed, and centrifuged (230 g, five minutes). The supernatant was then removed, and the precipitate, approximately 100  $\mu$ l, was used. For AI using non-OEP supplemented semen, three straws ( $3 \times 10^8$  spermatozoa, four dogs) were centrifuged and the precipitate (approximately 200  $\mu$ l) used. The number of ovulations was obtained by counting the corpora luteum in bilateral ovaries.

For IVI, frozen semen was centrifuged after thawing followed by removal of the supernatant, and the precipitate (approximately 2 ml) used. As we previously reported [19, 23], bitches were made to stand on their forelegs, and the semen was inseminated into the deep vagina. The bitches were kept in this position for 15 min. Three bitches each received insemination of  $10 \times 10^8$ ,  $20 \times 10^8$ , and  $40 \times 10^8$  spermatozoa, establishing three groups. As large amounts of semen were used for IVI, semen frozen on different days and from a multiple number of male dogs was used.

**Diagnosis of pregnancy:** Pregnancy was determined on 25 days after ovulation using an ultrasonic imaging diagnosis system (ECHOVISION SSD-500EV, Aloka Co., Japan). Pregnant bitches were observed every five days after determination of pregnancy to confirm normal embryonic development until delivery. The numbers of newborns were counted on the delivery days.

## RESULTS

**Conception by IUI:** The results of IUI using OEP-supplemented frozen semen in 10 bitches are shown in Table 1.

Conception was obtained in nine of 10 bitches (90%) that underwent IUI. The numbers of newborns were 1–7 puppies ( $3.6 \pm 0.9$ ). One animal (No. 2467) experienced spontaneous abortion of two fetuses on day 30 of gestation. The ratio of number of puppies to the number of ovulations in the inseminated uterine horn was 100% in five bitches, and 66.7, 40.0 (aborted), 25.0, and 14.3% in the other bitches (mean 71.8%). The number of puppies in the inseminated uterine horn did not surpass the number of ovulations in any of the bitches. Puppies were normal except for one stillborn puppy of animal No. 1548. The semen used for AI had been kept frozen for 1–10 months (mean  $4.4 \pm 1.1$  months). Regarding the semen quality after thawing, the sperm motility was 25–30% (mean  $28.0 \pm 0.9\%$ ), and the sperm viability was 47.2–79.6% (mean  $60.3 \pm 3.5\%$ ).

The results of conception by insemination of non-OEP supplemented frozen semen in four bitches are shown in Table 2.

Conception was unsuccessful in all four bitches. Regarding the quality of frozen semen used for AI, the sperm motility was 30% in all four bitches, and the sperm viability was 50.5–69.8% (mean  $64.9 \pm 5.5\%$ ).

**Conception by IVI:** The results of conception obtained by IVI of  $10$ – $40 \times 10^8$  spermatozoa including dead cells in nine bitches are shown in Table 3.

Conception was unsuccessful in all six bitches that underwent insemination of  $10 \times 10^8$  and  $40 \times 10^8$  spermatozoa. However, conception was obtained in two of three bitches that were inseminated with  $20 \times 10^8$  spermatozoa. One of the fertilized bitches delivered one normal puppy, but the other bitch experienced spontaneous abortion of one fetus on day 30 of gestation. The frozen semen used had been kept frozen for 1–15 months. Semen quality was assessed after thawing, and sperm motility was

Table 1. Conception by intrauterine insemination using frozen canine semen in Orvus ES Paste-supplemented extender

Bitch No.	Male No.	Interval between freezing and AI (month)	Semen quality (%)		No. of ovulations		No. of pups
			Motility	Viability	L	R	
289	266	1	30	60.8	5 <sup>a)</sup>	4	5
316	273	1	30	79.6	1	6 <sup>a)</sup>	— <sup>b)</sup>
301	271	1	25	50.4	2	6 <sup>a)</sup>	4
298	273	1	30	72.0	3	6 <sup>a)</sup>	6
322	253	4	25	47.2	1 <sup>a)</sup>	4	1
2467	266	10	25	62.1	4	5 <sup>a)</sup>	2 (abortion)
5204	269	9	30	66.4	3	4 <sup>a)</sup>	4
1607	271	6	30	51.7	7 <sup>a)</sup>	1	7
1548	267	6	25	61.4	7 <sup>a)</sup>	3	1 (stillbirth)
5197	276	5	30	51.5	3	4 <sup>a)</sup>	1
Mean		4.4	28.0	60.3	3.6	4.3	3.6
± SE		1.1	0.9	3.5	0.7	0.5	0.9

a) Inseminated uterine horn ( $1 \times 10^8$  sperm/100  $\mu$ l).

b) Sterile.

Table 2. Conception by intrauterine insemination using frozen canine semen in extender without Orvus ES Paste

Bitch No.	Male No.	Interval between freezing and AI (month)	Semen quality (%)		No. of ovulations		No. of pups
			Motility	Viability	L	R	
288	231	4	30	69.4	4 <sup>a)</sup>	3	— <sup>b)</sup>
297	253	3	30	69.8	3	4 <sup>a)</sup>	—
303	239	6	30	69.7	3 <sup>a)</sup>	2	—
293	230	2	30	50.5	3	4 <sup>a)</sup>	—
Mean		3.8	30.0	60.3	3.3	3.3	3.6
± SE		1.0	0.0	3.5	0.3	0.6	0.9

a) Inseminated uterine horn ( $3 \times 10^8$  sperm/200  $\mu$ l).

b) Sterile.

Table 3. Conception by intravaginal insemination using frozen canine semen in extender with Orvus ES Paste

Inseminated sperm count ( $\times 10^8/2$ ml)	Bitch No.	Interval between freezing and AI (month)	Semen quality			No. of pups
			Male No.	Motility (%)	Viability (%)	
10	2536	9	266	30	51.2	— <sup>a)</sup>
	1550	4–10	267	30	48.8	—
	5200	10	269	15	48.9	—
20	1609	4–10	273	20	73.6	1
	5202	5–7	276	20	69.4	—
	2560	1–12	275	20	76.4	1 (abortion)
40	332	2–12	253 266	25	66.6	—
	308	2–14	267 269 271	25	56.8	—
	321	3–15	276 266 272	25	52.1	—
Mean				23.3	60.4	
± SE				5.0	3.9	

a) Sterile.

15–30% (mean  $23.3 \pm 5.0\%$ ) and the sperm viability was 48.8–76.4% (mean  $60.4 \pm 3.9\%$ ).

## DISCUSSION

Although the number of animals used in this study was small, no conceptions were obtained in bitches that received IUI of frozen canine semen prepared in the extender without OEP, despite the large number of spermatozoa. In contrast, a high conception rate (90.0%) was obtained in bitches that underwent IUI of frozen canine semen prepared in the OEP-supplemented extender. However, the number of newborns did not exceed the number of ovulations in the inseminated horn. The number of inseminated spermatozoa including dead cells was  $1 \times 10^8$ , and the mean sperm motility was 28%. Therefore,  $2.8 \times 10^7$  motile sperm were inseminated. In a previous study of IUI using fresh semen,

we showed that when  $2 \times 10^7$  spermatozoa were inseminated into one of the uterine horns, oocytes ovulated in both the semen-inseminated horn and the other horn were fertilized [22]. Based on these data, the sperm motility may not have been maintained at a degree sufficient to allow the sperm to move from the inseminated uterine horn to the other horn via the corpus uteri. Therefore, to increase fertility, insemination into bilateral uterine horns is necessary. We previously reported that IVI of fresh semen requires  $2 \times 10^8$  spermatozoa [23]. Therefore, setting the base to  $10 \times 10^8$  spermatozoa for IVI of frozen semen, we inseminated using  $20 \times 10^8$  and  $40 \times 10^8$  spermatozoa. Although the number of animals was small, the conception rate was low (22.2%). It was considered that in IVI, the sperm motility was not maintained at a level sufficient for reaching the oviduct, which is the fertilization site. Therefore, direct insemination into the uterus was necessary

to obtain conception using OEP-supplemented frozen canine semen. Development of a non-surgical method of IUI and a method of freezing canine sperm applicable to IVI is necessary.

OEP (Equex STM Paste) was initially applied to frozen canine semen by Nöthling and Volkmann in 1993 [10]. However, they did not describe the significance of the addition of OEP to frozen canine semen. Thereafter, Rota *et al.* showed the effectiveness of OEP addition to frozen canine semen similar to our previous results [12, 13].

We consider that the addition of OEP is essential for preparation of frozen canine semen using EYT-FC as the extender. However, a high conception rate was obtained when using frozen semen prepared in the same extender without OEP as previously described [2–4, 13, 24]. Further studies are required to help elucidate the reasons for these differences.

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