Unilateral Intrauterine Horn Insemination of Frozen Semen in Cats

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(Received 4 February 2000/Accepted 10 August 2000)

ABSTRACT. Frozen feline semen was prepared using two types of extenders, egg yolk Tris-fructose citric acid (EYT-FC) and egg yolk sodium citrate solution (EYC), and the semen qualities after thawing and the conception rates obtained by unilateral intrauterine horn insemination (UIUI) were investigated. Cats used in the experiment were six males and 11 females aged 2–12 years (the number of experimental cases was 17). For preparation of frozen semen, semen collected by the artificial vagina method was adjusted to 1×10^8 sperm/ml and 7% glycerol, put in 250 μ l straws, and then frozen using a cell freezer. The mean sperm motility after thawing was 30.0 ± 9.7 (SE) % in the semen prepared with EYT-FC and $30.0 \pm 3.3\%$ in the semen prepared with EYC. Four of seven animals were fertilized by UIUI using two straws in both extenders, and the conception rate was 57.1%. The mean ratios of number of kits to the number of ovulations in the inseminated side were $61.1 \pm 24.5\%$ and $30.5 \pm 3.4\%$ for EYT-FC and EYC, respectively, showing that the ratio tended to be higher in the semen prepared with EYT-FC. The above findings, comparing the two extenders for preparation of frozen feline semen, showed that EYT-FC is slightly superior to EYC. To increase conception and fertility rates, it may be important to increase the sperm count for insemination and to inseminate both uterine horns.

KEY WORDS: feline, frozen semen, intrauterine insemination, straw method.

J. Vet. Med. Sci. 62(12): 1247–1251, 2000

Feline artificial insemination (AI) using frozen semen has been reported only by Platz *et al.* in 1978 [3]. In their study, $50\text{--}100 \times 10^6$ viable sperms were inseminated into the vagina. Although only six of 56 animals were fertilized, obtaining a conception rate of 10.7%, these were the first cases of fertilization by frozen semen in cats. We previously performed intravaginal insemination (IVI) of fresh semen and clarified that a number of sperms, 80×10^6 , are required for fertilization [6]. This sperm count is equal to the number of sperm in two ejaculations in cats [5]. We also clarified the sperm count required for fertilization by unilateral intrauterine horn insemination (UIUI) of fresh semen to be 8×10^6 in cats, which is 1/10 of that by IVI [7]. Freezing markedly reduces motility and viability of sperms. Accordingly, it is important to develop a technique of fertilization using a small number of sperm. Therefore, intrauterine insemination (IUI) may be appropriate for feline AI using frozen semen rather than IVI.

The method using frozen feline semen reported by Platz *et al.* was the pellet method, which may pose difficulties for storage and use [3]. Therefore, we investigated freezing of feline semen by the straw method.

Platz *et al.* used egg yolk lactose as the extender for frozen feline semen [3]. However, the semen qualities after thawing were not reported, and thus, we could not evaluate the extender used in their study. Therefore, we prepared frozen feline semen using egg yolk fructose citric acid (EYT-FC) [6,7], which we use as an extender for fresh feline semen, and egg yolk sodium citrate (EYC), which is an extender of bovine semen [4], and compared the semen quality after thawing and the conception rate obtained by UIUI. To ensure uniform conditions for the freezing method, a cell

freezer (Cell Freezer R204, England) was used. For AI, UIUI was performed, as reported [7] for insemination of fresh semen, and we investigated the fertilization of ova in the non-inseminated uterine horn by the inseminated sperms. Because a higher conception rate was previously obtained by insemination before ovulation than insemination after ovulation, AI was performed 15 or 20 hr after hCG administration, a time point predicted to be prior to ovulation [7].

MATERIALS AND METHODS

Animals: Six males cats aged 2–12 years with normal semen qualities, copulation capability, and fertility, from which semen were collected by the artificial vagina (AV) method, were used for the experiment. For females subjected to AI, 11 female cats aged 2–7 years bred and maintained in our colony were repeatedly used, and the number of experimental cases was 17. All these female cats had experienced pregnancy and delivery 1–3 times. The animals were maintained in an animal room in which the temperature was kept at $23 \pm 2^\circ\text{C}$. Male cats were individually kept in cages measuring $60 \times 90 \times 120$ (h) cm, and female cats were kept in a group in a room measuring 4×7 m. The animals were supplied dry food (Hill's Feline Maintenance, U.S.A.) and drinking water *ad libitum*. Females were placed with males every day in the morning and evening to observe estrus.

Semen collection and examination of semen quality: Semen was collected using an AV for cats that was previously reported [5].

While a male cat mounted an estrous female cat and thrust, the penis was advanced into an AV held between the forefinger and middle finger, and ejaculation was induced. Using

Table 1. Composition of extenders used for preparation of frozen semen in cats

Ingredients	EYC		EYT-FC	
	1st extender	2nd extender	1st extender	2nd extender
Sodium citrate dihydrate	1.45 g	1.21 g	—	—
Citric acid	—	—	1.30 g	1.30 g
Disodium hydrogenphosphate 12-water	0.17 g	0.14 g	—	—
Tris (hydroxymethyl) aminomethane	—	—	2.40 g	2.40 g
Glucose	1.70 g	1.42 g	—	—
Lactose	0.26 g	0.21 g	—	—
Fructose	—	—	1.00 g	1.00 g
Penicillin potassium	100,00 U	100,000 U	100,000 U	100,000 U
Streptomycin sulfate	0.10 g	0.10 g	0.10 g	0.10 g
Glycerol	—	14 ml	—	14 ml
Egg yolk	20 ml	20 ml	20 ml	20 ml
Distilled water to final volume	100 ml	100 ml	100 ml	100ml

the same AV, a second semen collection was performed. To avoid drying, 100 μ l of the extender (primary extender) was added to the semen, and a previously determined general test for semen quality was performed as we previously reported [5]. Frozen semen was prepared using semen with a sperm motility of 80% or greater.

Frozen semen: Two types of extenders, EYT-FC and EYC shown in Table 1, were used as the extender of frozen semen. After the semen quality test, semen was subjected to the 1st dilution at 20°C to adjust the sperm count to 2×10^8 /ml, and was then kept in a water bath at 4°C for one hour for the 1st cooling. After the 1st cooling, using the secondary extender supplemented with 14% glycerol, the 2nd dilution of the semen was performed by dripping at 4°C, taking approximately 10 min with stirring using a stirrer. The semen was diluted with an equal volume of the extender, and thus, the final sperm concentration was 1×10^8 /ml, and the final glycerol concentration was 7%. After the 2nd dilution, the semen was put in 250 μ l straws, and equilibrated with glycerol for one hour. Then, the semen was frozen using Cell Freezer R204. The temperature conditions for freezing were $-1.0^\circ\text{C}/\text{min}$ from 4°C to -1.0°C , $-33.0^\circ\text{C}/\text{min}$ from -1.0°C to -50°C , and $-58.4^\circ\text{C}/\text{min}$ from -50°C to -196°C . When the temperature reached the maximal cooling temperature, -196°C , the semen was transferred to a liquid nitrogen container.

Examination of semen qualities after thawing: The feline semen qualities after thawing were compared at each dilution among four cases. The semen preparations frozen for one week or more were thawed in a water bath at 37°C for 30 sec, and the qualities were examined. In the semen quality test, the motility, viability, and abnormality of the sperm were evaluated.

Ovulation induction method: Ovulation was induced by administration of 100 iu $\times$ 2 hCG into the jugular vein on days 2–4 of estrus. Cats that were before ovulation at insemination received a second administration immediately after surgery. To evaluate the success of induction of ovulation in the animals that were before ovulation at insemination excluding pregnant animals, when the peripheral blood progesterone (P) level 15 days after hCG administration was increased, induction of ovulation was judged to have been

successful. The P level was measured by EIA [2].

AI: In UIUI of frozen feline semen prepared using two extenders, semen stored for 1–19 months was used. AI was performed 15 or 20 hr after hCG administration.

UIUI was performed under general anesthesia. For anesthesia, after pretreatment with atropine sulfate (0.05 mg/kg) and acepromazine maleate (0.025 mg/kg), ketamine (5 mg/kg) was administered for induction of anesthesia, then the anesthesia was maintained by halothane [6, 7].

The semen of two straws (50×10^6 sperms) was inseminated. The semen was thawed, and after semen quality test, the semen was centrifuged at 230G for 5 min, and approximately 30 μ l after removing the supernatant was used. The animals were laparotomized along the median line, and semen was inseminated into the uterine horn with a higher number of mature ovarian follicles (or ovulation).

An 18G needle was inserted into the central region of the uterine horn, and semen was inseminated into the tip of the uterine horn using an injector consisting of a 20G indwelling needle connected to a 1 ml syringe. After insemination, the injury made by inserting the needle was one-stitched with a soluble suture. Follicles with a diameter of 4 mm or greater were regarded as mature follicles, and all these mature follicles were supposed to have been ovulated by hCG administration.

Diagnosis of pregnancy: As we previously reported, pregnancy of artificially inseminated cats was evaluated 15 days after insemination using an ultrasonic imaging diagnosis system (ALOKA ECHO VISION SSD-500EV) [1]. In pregnant animals, the development of the embryo was observed every five days after the confirmation of pregnancy. The number of kits was counted on the delivery day.

Statistical analysis: The results of this experiment were analyzed by Student's *t*-test and χ^2 test.

RESULT

Quality of frozen semen: The qualities of frozen feline semen diluted with EYT-FC and EYC after thawing are shown in Table 2.

The sperm motility was 80–90% at semen collection. The

Table 2. The quality of frozen semen in cats

Extender	Tom No.	Age (years)	Initial						Post thaw					
			Motility (%)	Viability (%)	Abnormality (%)				Motility (%)	Viability (%)	Abnormality (%)			
					Head	Mid-piece	Tail	Total			Head	Mid-piece	Tail	Total
EYT-FC	T4	12	90	93.1	0	0	3.0	3.0	25	45.4	0	3.0	21.0	24.0
	T32	4	90	93.8	2.0	0	1.0	3.0	20	45.6	2.0	2.0	23.0	27.0
	T40	2	90	94.9	0	0	1.0	1.0	55	68.4	0	5.0	10.0	15.0
	T40	2	80	78.7	4.0	2.0	2.0	8.0	20	52.9	2.0	3.0	4.0	9.0
	Mean		87.5	90.1	1.5	0.5	1.8	3.8**	30.0	53.1	1.0	3.3	15.0	18.8
	± SE		2.8	4.4	1.1	0.6	0.6	1.7	9.7	6.2	0.7	0.7	5.2	4.8
EYC	T4	12	80	94.0	1.0	1.0	3.0	5.0	35	38.1	1.0	3.0	27.7	31.7
	T32	4	80	82.0	0.9	4.3	2.6	7.8	25	57.0	2.0	0	19.0	21.0
	T36	2	85	90.8	1.0	0	5.3	6.3	35	48.9	0	0	32.0	32.0
	T40	2	90	93.0	0	4.0	1.0	5.0	25	58.7	1.0	0	30.0	31.0
	Mean		83.8	90.0	0.7	1.7	3.0	6.0	30.0	50.7	1.0	0.8	27.2	28.9
	± SE		2.8	3.2	0.3	0.8	1.0	0.8	3.3	5.4	0.5	0.9	3.3	3.1

** : Significantly different from initial abnormality at $p < 0.01$.

Table 3. Conception by UIUI of frozen semen in cats (EYT-FC)

Queen No.	Tom No.	Period of storage (month)	Semen quality (%)				Interval from hCG admin. to AI (hr)	No. of mature follicles		No. of kits	Gestation period (day)	
			Initial		Post thaw			L	R			
			Motility	Viability	Motility	Viability						
116	T36	1	90	92.7	20	64.1		2	4*	No ovulation	65	
D6	T32	1	85	92.5	30	51.8		1	4*	2		
97	T36	1	85	82.4	40	65.9		2*	2	No ovulation		
113	T40	1	85	91.6	30	66.1		2*	1	Ab. (48)**	63	
134	T32	1	90	92.4	30	66.2		3	6*	—		
D6–2	T31	9	85	90.1	20	43.0		4*	2	4		
116–2	T32	10	80	94.2	15	65.2		3	3*	1	68	
128	T32	9	85	89.3	20	51.6		3*	3	—	68	
122	T36	1	90	92.7	30	57.2		2	2*	—		
Mean		3.8	86.1	90.9	26.1	59.0			2.6±0.4	3.0±0.7	2.3	65.3
±SE		1.5	1.2	1.2	2.8	3.0				5.6±0.8	1.1	1.8

*: Inseminated side **: Abortion.

sperm motility and viability of the semen diluted with EYT-FC after thawing were 20–50% (Mean $\pm$ SE: $30.0 \pm 9.7\%$) and 45.4–68.4% ($53.1 \pm 6.2\%$), respectively. The sperm abnormality was $3.8 \pm 1.7\%$ in the fresh semen, but had increased to $18.8 \pm 4.8\%$ after thawing. In the EYC group, the sperm motility and viability after thawing were 25–35% ($30.0 \pm 3.3\%$) and 38.1–58.7% ($50.7 \pm 5.4\%$), respectively. The mean sperm abnormality was $6.0 \pm 0.8\%$ in the fresh semen, but had increased to $28.9 \pm 3.1\%$ after thawing. The abnormality was clearly increased especially in the tail region by freezing ($P < 0.01$).

These observation indicate there were no differences in the semen qualities after thawing between the semen prepared with the two extenders.

Conception results of UIUI of frozen feline semen diluted with EYT-FC: The conception results of UIUI of frozen feline semen prepared with EYT-FC are shown in Table 3. AI was performed 20 hr after hCG administration.

Semen frozen for 1–10 months after preparation (3.8 ± 1.5 months) was used in AI. The sperm motility and viability

after thawing were 15–40% ($26.1 \pm 2.8\%$) and 43.0–66.2% ($59.0 \pm 3.0\%$), respectively. Excluding two cats in which ovulation did not occur, four of seven animals (57.1%) became pregnant by UIUI. In all of these animals, AI was performed before ovulation. Among the pregnant animals, abortion occurred in No. 113 on day 48 of gestation, but the other three animals delivered 1–4 kits (2.3 ± 1.1 kits) on days 63–68 of gestation.

Conception results of UIUI of frozen feline semen diluted with EYC: The results of UIUI of frozen feline semen prepared with EYC are shown in Table 4. AI was performed on four animals at 15 and 20 hr after hCG administration.

The semen frozen for 6–19 months after preparation (8.4 ± 1.6 months) was used in AI. The sperm motility and viability after thawing were 20–35% ($28.1 \pm 2.2\%$) and 46.6–77.5% ($61.2 \pm 3.6\%$), respectively. Excluding one cat in which ovulation did not occur, four of seven animals (57.1%) were fertilized by UIUI. Among the pregnant animals, early embryonic death occurred in No. 137 on day 20 of gestation, but each of the other three cats delivered one kit on days 66–

Table 4. Conception by UIUI of frozen semen in cats (EYC)

Queen No.	Tom No.	Period of storage (month)	Semen quality (%)				Interval from hCG admin. to AI (hr)	No.of mature follicles		No. of kits	Gestation period (day)
			Initial		Post thaw			(No. of ovulations)			
			Motility	Viability	Motility	Viability		L	R		
131	T4	7	80	88.9	30	64.3	15	3*	0	1	66
128-2	T32	6	90	97.0	30	46.6		3*	2	—	
D12	T44	8	90	93.8	35	77.5		2	3*	1	69
122-2	T36	6	90	94.9	25	60.0		5*	3	—	
131-2	T44	7	90	93.8	35	56.2	20	2*	1	No ovulation EED (20)**	
137	T32	19	90	92.7	30	69.7		4*	1		
114	T36	7	90	95.8	20	59.6		4*	1		1
97-2	T44	7	90	92.4	20	55.3		(2)	(3)*	—	
Mean		8.4	88.8	93.7	28.1	61.2		33±0.5	1.9±0.5	1.0	67.3
±32		1.6	1.3	0.9	2.2	3.6		5.1±0.6		0	1.1

*: Inseminated side. **: Early embryonic death.

Table 5. The number of kits compared to the number of ovulations in the inseminated side in unilateral IUI of frozen feline semen

Extender	Queen No.	No. of mature follicles		No. of kits (a)	a/No. of ovulations (%)
		L	R		
EYT-FC	D6	1	4*	2	2/4 (50.0)
	D6-2	4*	2	4**	4/4 (100)
	116	3	3*	1	1/3 (33.3)
	M±SE	2.6±1.1	3.0±0.7	2.3±1.1	61.1±24.5
EYC	131	3*	0	1	1/3 (33.3)
	D12	2	3*	1	1/3 (33.3)
	114	4*	1	1**	1/4 (25.0)
	M±SE	3.0±0.7	1.3±1.1	1.0±0	30.5±3.4

*: Inseminated side.

69 of gestation.

Ovulation induction: Ovulation was induced by hCG administration in 14 of 17 animals (ovulation induction rate: 82.4%). Excluding three cases in which ovulation did not occur, the ovulation induction rate at each time point after hCG administration was 0% (0/4) after 15 hr and 10.0% (1/10) after 20 hr.

The number of kits compared to the number of ovulations in the inseminated side in UIUI of frozen feline semen: The ratio of the number of kits to the number of ovulations in the inseminated side obtained by UIUI of frozen feline semen is shown in Table 5. One case each of abortion and early embryonic death were excluded from the data.

The number of kits (fertility rate) compared to the number of ovulations in the inseminated side was 33.3–100% (61.1 ± 24.5%) in three pregnant animals in the EYT-FC group. The fertility rate in the three pregnant animals in the EYC group was 25.0% or 33.3%, and the mean rate was 30.5 ± 3.4%. Therefore, the fertility rate tended to be higher in the EYT-FC group than the EYC group. However, the number of kits did not exceed the number of ovulations in the inseminated side in any of the animals in either group.

Time of UIUI after hCG and conception rate: The relationship between the time of UIUI after hCG and the concep-

Table 6. Time of IUI after hCG and conception rate in cats

Extender	Interval from hCG admin. to AI (hr)		Conception (%)
	15	20	
EYT-FC	—	4/7 (57.1)	4/7 (57.1)
EYC	2/4 (50.0)	2/3 (66.7)	4/7 (57.1)
Total	2/4 (50.0)	6/10 (60.0)	8/14 (57.1)

tion rate is shown in Table 6.

In the EYT-FC group, AI was performed 20 hr after hCG administration in all cases, and four of seven animals (57.1%) were fertilized. In the EYC group, the conception rates were 50.0% (2/4) and 66.7% (2/3) 15 and 20 hr after hCG administration, respectively, showing, no difference in the conception rate between AI performed at 15 hr and 20 hr after hCG administration.

DISCUSSION

Because Platz *et al.* [3] did not clarify the qualities of frozen feline semen after thawing, we could not compare our

results with their results. There were no differences in the semen qualities after thawing and the conception rate between the two extenders in our study. However, the time-course changes in the sperm motility after thawing were slightly higher in the semen diluted with EYT-FC than the semen diluted with EYC.

In our previous study of UIUI of fresh semen, the conception rates were 31.3% and 80.0% at sperm counts of 4×10^6 and 8×10^6 , respectively [7]. In this study, 50×10^6 sperm were used and the motility was 15–40% (approximately 25%). Therefore, the mean number of viable sperms was 12.5×10^6 . However, the conception rate (57.1%) was lower than that (80.0%) obtained by insemination of fresh semen at a sperm count of 8×10^6 . Regarding the number of kits compared to the number of ovulations in the inseminated side, ova in the non-inseminated uterine horn were fertilized in approximately half of the fertilized cases by insemination of fresh semen [7]. In contrast, in this study using frozen semen, such cases were not observed, suggesting that although the sperm are motile after thawing, frozen semen is greatly inferior in fertility to fresh semen. Therefore, to increase the fertility and conception rates of IUI of frozen semen, insemination into both uterine horns may be important. It is also considered important to investigate a non-surgical method of artificial insemination.

Because a constant number of sperms, 50×10^6 , was inseminated, the sperm count required for fertilization could not be determined in this study. The primary cause of the low conception rate of 57.1% in this study may have been the small number of sperm inseminated, and it will be necessary to investigate the sperm count required for fertilization by

insemination of frozen semen. The duration of storage used in the eight fertilized cases was 1–19 months (Mean: 7–8 months). Since conception was obtained using long-term stored semen in this study, the semen qualities may not be affected by duration of storage. In this study, we used a Cell Freezer to ensure uniform freezing conditions. However, it may be necessary to compare its usefulness with those of other freezing methods.

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