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Intrauterine insemination with cat semen frozen with various extenders

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Abstract: The aim of this study was to investigate the post-thaw motility and morphological defect rates of cat semen frozen with various extenders, and pregnancy rates after intrauterine insemination (IUI). Cats, 2 males and 14 queens, 2 to 3 years of age, and under the same managemental conditions were used. Semen was collected by means of an electro-ejaculator under general anesthesia. Based on spermatological traits, semen samples presumed suitable for freezing were extended with either tris-fructose-citric acid (EY-TFC) containing 20% egg yolk, or skimmed milk-glucose-taurine (EY-SMGT) containing 10% egg yolk extender, and frozen in 0.25 mL straws. For the extenders, post-thaw motility and morphological abnormality values were found to be efficient for IUI and no statistical significance was observed between the values of the extender groups. To achieve ovulation, queens showing behavioral estrus received 250 IU of hCG via IM route, 30 h prior to IUI. Under general anesthesia, queens were laparatomised on the medial line and the left and right uterine horn were taken out. Into both uterine horns, 2 straws containing 25×10^6 sperm each were thawed at 37 °C for 30 s and equally injected. Pregnancy was diagnosed using an ultrasonography device on the 19th day after IUI by detecting the embryonic vesicles. Of the 3 EY-TFC group queens found to be pregnant, 1 aborted and other 2 had early embryonic death (EED); the pregnancy rate was 42.90%. In a queen in the EY-SMGT group, no ovulation took place after the hCG injection. In 2 of the queens found to be pregnant in this group, EED occurred and the pregnancy rate was 33.30%. In conclusion, cat semen extended and frozen with EY-TFC and EY-SMGT extenders was found to be successful in post-thaw motility and morphological defect rates. Therefore, surgical IUI may be more effective for feline AI using frozen semen rather than intravaginal insemination.

Key words: Cat semen, extender, frozen semen, queen, intrauterine insemination

Farklı sulandırıcılar ile dondurulmuş kedi sperması ile intrauterin tohumlama

Özet: Bu çalışmada, farklı sulandırıcılarla dondurulmuş kedi spermasının, eritme sonrası spermatolojik özellikleri ve intrauterin suni tohumlama sonrası gebelik oranları üzerine etkilerini araştırmak amaçlandı. Çalışmada aynı bakım ve beslenme şartlarında barındırılan 2-3 yaşlarında 2 adet erkek ve 14 adet dişi kedi kullanıldı. Sperma erkek kedilerden genel anestezi altında elektro-ejakülatör cihazı kullanılarak alındı. Spermatolojik özellikler yönünden donmaya elverişli bulunan sperma örnekleri, % 20 yumurta sarılı Tris-Fruktoz-Sitrik Asit (TFS) ve % 10 yumurta sarılı Yağsız Süt-Glukoz-Taurin (YSG-T) sulandırıcıları ile 0,25 mL payetlerde dondurulduk. Her 2 sulandırıcı için eritme sonrası

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elde edilen motilite ve morfolojik bozukluk değerleri intrauterin tohumlama için yeterli bulundu ve her 2 sulandırıcı grubu arasındaki fark istatistiksel olarak önemli değildi. Davranışsal östrus belirtileri gösteren dişi kedilere ovulasyonun gerçekleşmesi için intrauterin tohumlamadan 30 saat önce 250 İÜ dozda hCG hormonu i.m. olarak uygulandı. Genel anesteziye alınan dişi kediler median hattan laparotomize edilerek sağ ve sol kornuları dışarıya çıkarıldı. Tohumlama için 25×10^6 spermatozoon içeren 2 payet sperma 37 °C de 30 saniyede eritildi ve her 2 kornuya 20G iğne ile eşit olarak verildi. Gebelik muayenesi intrauterin suni tohumlama sonrası 19. günde ultrasonografik olarak, yavru keselerinin görülmesi ile saptandı. TFS grubunda gebe kaldığı saptanan üç dişi kedinin gebeliklerinden biri abort ve ikisi ise erken embriyonik ölümle sonuçlandı. Elde edilen gebelik oranı ise % 42,9 olarak saptandı. YSG-T grubunda ise 1 dişi kedide hCG enjeksiyonu sonrası ovulasyon gerçekleşmedi. Bu grupta gebe kaldığı saptanan 2 dişinin de gebeliği erken embriyonik ölümle sonuçlandı. Elde edilen gebelik oranı ise % 33,3 olarak saptandı. Sonuç olarak, TFS ve YSG-T sulandırıcısı ile dondurulmuş kedi sperması, eritme sonrası motilite ve morfolojik bozukluklar yönünden başarılı bulundu. Bununla beraber, kedilerde donmuş sperma ile intrauterin tohumlamanın, intravajinal tohumlamadan daha fazla etkili olabileceği düşünüldü.

Anahtar sözcükler: Kedi sperması, sulandırıcı, donmuş sperma, dişi kedi, intrauterin tohumlama

Introduction

The domestic cat is a convenient research model for wild felids threatened by extinction (1). Furthermore, there is an increasing demand for assisted reproduction among breeders of domestic cats due to the small genetic variation in their breeds (2).

The use of AI with frozen-thawed semen has been limited in cats (3,4); the availability of good quality frozen-thawed semen could improve the use of artificial insemination (AI) with new techniques of intrauterine insemination (IUI) by transcervical catheterization (5,6). Although frozen-thawed semen results in lower pregnancy rates compared with fresh samples (3,4,7,8), it shows higher applicability when considering conservation programs.

Feline AI using frozen-thawed semen with intravaginal insemination (IVI) has only been reported by Platz et al. (3). They reported a conception rate of 10.6% with IVI of frozen-thawed cat semen. There are few other reports on IUI of frozen-thawed semen in cats. Conception rates of 0% (0 of 12 queens) (9), 0% (0 of 8 queens) (10), 41.7% (5 of 12 queens) (9), and 75% (6 of 8) (10), respectively, were achieved with IVI and transcervical IUI with frozen semen. There is only one report on surgical IUI with frozen cat semen in queens. Conception rates of 57% (8 of 14 queens) (8) were achieved with unilateral IUI with frozen semen. To achieve conception rate with IUI, the number of sperm required was 50×10^6 (8). When frozen-thawed cat semen was used, the site of intrauterine semen deposition and optimal timing of the AI in relation to the ovulation was even more important, due to the short lifespan of frozen thawed sperm. Mature ovarian follicles ovulate approximately 25-27 h after the administration of hCG (11).

In this study, 2 extender models were used. The first extender was a widely used tris-fructose-citric acid extender (EY-TFC) (7,8,12). The other was a skimmed milk-glucose-taurine (EY-SMGT) extender, which had been used before in short term cold storage of cat semen (13). Added to this extender was taurine, an antioxidant that controls lipid peroxidation and ROS (reactive oxygen species) at definite concentrations (50-75 mM) (14). Because of this, it was thought as an alternative extender for freezing cat semen.

The main purpose of this study was to improve the procedure for freezing cat semen. We focused on 4 factors affecting the cryopreservation success: (i) type of extender, (ii) post-thaw spermatological characteristics of cat semen frozen with the extenders, (iii) comparison of pregnancy rates, and (iv) efficiency of surgical IUI with frozen semen.

Materials and methods

For this study, 14 mixed breed female cats (No. 1-14), aged 2 to 4 years, and 2 male cats aged, 2 to 3 years, with normal semen quality (≥80% sperm motility and ≤30% morphologically abnormal sperm), copulation capability, and fertility were used. The female cats previously experienced pregnancy and delivery 1 or 2 times. The animals were

maintained in animal rooms with the temperature adjusted to 23 ± 2 °C. Males and females were kept individually in cages measuring 60 × 90 × 120 (h) cm. The female cats were maintained under natural lighting. The experiments were performed during the breeding season between March and April. The animals were supplied with dry food (Eagle Pack Pet Foods, Mishawaka, IN, USA) and drinking water ad libitum. Daily, in the mornings and evenings, the females were placed with males to observe estrus. This experiment was approved by the Ethical Committee for Experimental Animal Uses of the College of Veterinary Medicine.

Semen collection was performed from February and March using an electro-ejaculator (P-T Electronics, Boring, OR, USA). The male cats were anesthetized with xylazine (Alfazyne®, Alfasan Int. Netherlands; 2 mg/kg bw) in combination with ketamine-HCl (Alfamine® 10%, Alfasan Int. Netherlands; 10 mg/kg bw). The anesthetized male cats were fixed horizontally and a lubricated rectal probe was placed into the rectum. Electro-ejaculation was performed in the male cats using a previously described protocol (15). Briefly, a total of 80 stimuli (2-5 V) divided into 3 series were delivered through a rectal probe (1.3 cm diameter, 12 cm long) and an electro-stimulator. Before the electrical stimulation, an Eppendorf tube was placed over the penis.

The volume of the semen was measured using an adjustable automatic micropipette (10-1000 µL) and the value was recorded in microliters. Sperm motility was assessed subjectively (3 µL sample) using a phase-contrast microscope at ×200 magnification by viewing at least 3 fields. An aliquot (5 µL) of semen was diluted with 10% formol saline and evaluated for total number of sperm using a hemocytometer chamber (Neubauer, Boeco, Hamburg, Germany). A Spermac® stain kit (Stain enterprises, Onderstepoort, Republic of South Africa) was employed in morphologic observations. The preparation was examined by light microscope at ×1000 magnification by counting 200 cells (acrosome, and total abnormal spermatozoa rate), as described by Baran et al. (16), and the values expressed as percentages.

Extenders, one with 20% egg yolk bearing tris-fructose-citric acid (EY-TFC) and the other with 10% egg yolk and skimmed milk-glucose-taurine (EY-SMGT), shown in Table 1, were used when freezing the semen. Semen samples that were suitable for freezing (≥80% sperm motility and ≤30% total abnormal spermatozoa) were divided into 2 parts and extended 1:1 (semen/extender) with EY-TFC

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

Ingredients	EY-TFC		EY-SMGT	
	1st extender	2nd extender	1st extender	2nd extender
Tris-(Hydroxymethyl) aminomethane	2.40 g	2.40 g	-	-
Glucose	-	-	1.00 g	1.00 g
Taurine	-	-	75 mM	75 mM
Fructose	1.00 g	1.00 g	-	-
Citric acid	1.30 g	1.30 g	-	-
Egg yolk	20 ml	20 ml	10 ml	10 ml
Glycerol	-	8 ml	-	8 ml
Skim milk to final volume	-	-	90 ml	90 ml
Distilled water to final volume	100 ml	100 ml	-	-
Penicillin potassium	100.000 U	100.000 U	100.000 U	100.000 U
Streptomycin sulfate	0.10 g	0.10 g	0.10 g	0.10 g

and EY-SMGT extender to a final concentration of 2 × 10^8 motile spermatozoa/mL. The extended semen was then cooled to 5 °C in 1 h (the temperature was measured with a digital thermometer). An equal volume of EY-TFC and EY-SMGT containing 8% glycerol was added to the sample, drop by drop, with gentle agitation, to obtain a final concentration of 1 × 10^8 motile spermatozoa/mL and 4% glycerol. Glycerolization was completed in 10 equal portions at 6 min intervals and equilibration lasted for 30 min. Equilibrated semen samples were loaded into 0.25 mL straws at 5 °C and placed 4 cm above liquid nitrogen (LN_2) for 7 min and plunged into LN_2. All straws were stored at –196 °C for at least 2 weeks prior to use.

For each IUI procedure, 2 straws (50 × 10^6 sperms) from the same male cat were thawed in a water bath at 37 °C for 30 s and the contents were emptied into a centrifuge tube and centrifuged (200 × *g* for 5 min). The supernatant was discarded and the sperm pellet was resuspended gently with the remaining liquid, approximately 100 µL. Samples were then assessed for sperm motility and morphology as described above. Thawed samples were maintained at 37 °C for no longer than 5 min prior to IUI.

Queens (not showing estrus behavior and <60% of superficial cells in vaginal cytology with Giemsa stain) received a single injection of PMSG (100 IU; im) (Folligon®, Intervet, Boxmeer, The Netherlands) to induce estrus and, after 90 h, an injection of hCG (250 IU; IM) (Chorulon®, Intervet, Boxmeer, The Netherlands) to induce ovulation. Follicular growth was confirmed by visulation of pre-ovulatory follicles (2 to 4 mm in diameter, clear in appearance, and generally flattened or only slightly raised above the ovarian surface) or post-ovulatory corpora lutea (each approximately 4 mm in diameter, dark red and prominently raised 2-3 mm above the ovarian surface) as described Wildt and Seager (17) when IUI was performed. Queens presenting more than 60% of superficial cells were considered in estrus. Females exhibiting at least one CL were classified as post-ovulatory regardless of the number of follicles present.

Intrauterine inseminations were performed during March and April. Females were randomly separated into 2 groups; the first group was given cat semen frozen with the EY-TFC extender, with male A (n = 3) or B (n = 4) and the second was given cat semen frozen with the EY-SMGT extender, with male A (n = 3) or B (n = 3). It was observed during IUI that ovulation did not occur in 1 of the queens in the second group (n7), and IUI was not performed.

IUI was performed under general anesthesia 28 h after hCG administration for ovulation induction. For laparotomy of females, a surgical plane of anesthesia was induced with a combination of ketamin-HCl (Alfamine® 10%, Alfasan Int. Netherlands; 15 mg/kg; IM) and xylazine (Alfazyne®, Alfasan Int. Netherlands; 1 mg/kg; IM). Surgical plane anesthesia was maintained with halothane gas. Food and water were withheld from the females for 12 h and 2 h, respectively, before anesthesia.

Each female was subjected to laparotomy. Briefly, an incision was made along the medium line and the uterine horns were exposed. An 18G needle was inserted into the central region of the uterine lumen, 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. All queens were inseminated in both uterine horns as described above.

Pregnancy was confirmed 19 days after IUI by visulation of embryo vesicles using transabdominal ultrasonography (7.5 MHz; Medison Sonovet SA600V). Queens determined to be pregnant were examined by ultrasonography at weekly intervals for early embryonic death (EED).

Data are expressed as means ± SD. Pregnancy rate was compared using a chi-square test. Level of significance was set at $P < 0.05$ for all analysis.

Results

Ejaculate quality parameters for fresh, equilibration, and frozen-thawed samples from both male cats are shown in Table 2. For extenders, post-thaw motility, and morphological defect rate, values have been efficient for IUI and no statistical significance was observed between the values of both extender groups ($P > 0.05$).

After PMSG treatment, 92.85% (13 of 14) of the queens exhibited follicular activity. In the EY-TFC extender group, all females were inseminated

Table 2. Motility, acrosomal and morphological defect rates in native, and post-equilibration and thawing.

Extender	Stage	Motility (%)	Morphological defects (%)	
			Acrosome	Total
EY-TFC	Initial	88.4 ± 5.4	8.2 ± 1.2	16.2 ± 2.6
	Post-equilibration	72.1 ± 3.2	12.4 ± 1.4	25.4 ± 2.8
	Post-thaw	42.8 ± 2.7	26.4 ± 6.9	36.4 ± 2.2
EY-SMGT	Initial	86.2 ± 7.2	7.9 ± 2.2	15.2 ± 1.6
	Post-equilibration	82.8 ± 4.2	13.6 ± 2.8	20.6 ± 2.1
	Post-thaw	45.7 ± 3.0	23.7 ± 5.6	30.7 ± 2.6

Mean ± SD

after ovulation, which occurred within 30 h after hCG administration. In 1 queen (n7) in the EY-SMGT group, no ovulation took place after the hCG injection.

The results obtained for EY-TFC and EY-SMGT extender groups, and the number of embryo vesicles 19 days after intrauterine inseminations are presented in Table 3. Using sperm samples from male cat A and B, respectively, pregnancy rates of 50% (3 of 6) and 28.60% (2 of 7) were obtained when performing IUI, resulting in a total of 38.46% (5 of 13) pregnancy. One (n6) of the queens which had IUI with EY-TFC

Table 3. Pregnancy results, number of embryo vesicles observed at 19 days after surgical IUI, and post-thaw sperm quality.

Extender	Male cat	Queen	Pregnancy (+/–)	Number of embryo vesicles	Motility (%)	Morphological defects rate (%)	
						Acrosome	Total
EY-TFC	A	n1	–	-	45	22	32
		n2	+**	2	45	15	37
		n3	+**	1	40	32	36
	B	n4	–	-	45	28	38
		n5	–	-	45	26	37
		n6	+*	3	40	26	36
		n7	–	-	40	36	39
Mean ± SD				2.0 ± 1.0	42.8 ± 2.7	26.4 ± 6.9	36.4 ± 2.2
EY-SMGT	A	n1	+**	1	40	34	36
		n2	–	-	45	23	28
		n3	–	-	45	29	32
	B	n4	+**	2	50	21	29
		n5	–	-	50	18	30
		n6	–	-	45	20	29
		n7	NO	-	45	21	31
Mean ± SD				1.5 ± 0.7	45.7 ± 3.0	23.7 ± 5.6	30.7 ± 2.6

*: 35 day abort
**: 26 day early embryonic death
NO: No ovulation

extender aborted at the 35th day, but the embryonic vesicle was determined in other 2 queens (n2 and n3) at the 19th day. However, at ultrasound examinations on the 26th day, embryonic vesicles were not visible and early embryonic death (EED) was diagnosed. Considering these results in this group a 42.90% pregnancy rate and evaluation (100%) in all the queens treated with hCG was achieved. In 1 of the queens in the EY-SMGT group (n7), AI had not carried out because follicular growth was not sufficient (<3 mm) and there was no corpus luteum. In 2 (n1 and n4) of the remaining 6 queens, EED was diagnosed because embryonic vesicles which have been observed at the first ultrasound examination were absent at the 26th day. In this group, a 33.30% pregnancy rate was determined and in the hCG treated queens a 85.71% ovulation rate were determined. Data about the pregnancy rates of queens which had IUI with male cats semen frozen with EY-TFC and EY-SMGT extenders are presented in Table 4.

Table 4. Comparison of conception rates of queens between the extender groups.

Extender	Pregnancy rate (%)
EY-TFC (n = 7)	(3/7) 42.9
EY-SMGT (n = 6)	(2/6) 33.3
Mean ± SD	(5/13) (38.1)

P > 0.05

Discussion

In order not to affect the pregnancy rates, the semen of 2 male cats which were known to have semen resistant to the freezing-thawing processes was used in the study. The motility and total morphologic defected rates (Table 2) of these male cats were in accordance with most researchers' values (8,12).

In our study, 2 different extender models were employed and 1 of them was widely used and successful as a EY-TFC extender (6,8,9,18,19). The other was an EY-SMGT extender, which was modified by us, and had not been used before. The mean values of motility and morphological defected spermatozoa after equilibration were parallel with the results of researchers with an EY-TFC extender (2,8,12). These mean values for the EY-SMGT extender have been slightly superior to those of the EY-TFC extender however; comparison was not possible since no research dealing with this extender could be found.

Tsutsui et al. (8) used a 20% egg yolk bearing TFC extender with a 7% final glycerol rate and frozen cat semen in straws. They reported means of 87.5% fresh and 30% post-thaw motility. From the control group in our study, a mean of 88.38 ± 5.45% motility was obtained from the EY-TFC extender with fresh semen and 42.85 ± 2.64% post-thaw motility. Considering these results, the fresh semen results from our study were similar and post-thaw results were slightly higher than those of other researchers.

We observed successful estrus induction in 92.85% (13 of 14) of the females treated with 100 IU of PMSG, which was slightly lower than that reported by Howard et al. (20) using an identical dose (98.1%). Furthermore, all females in the EY-TFC and EY-SMGT extender groups exhibited at least 1 CL after hCG (250 IU) administration and were classified as ovulated. Using 2 injections of 100 IU of hCG, with a 24 h interval, Tsutsui et al. (4,8) obtained ovulation in 95.5% and 82.4% of the natural estrus females. Comparing these results, the use of a single high hCG dose seems to be equally efficient for ovulation induction in queens (21,22). In all females IUI, assessment of ovaries at the time of insemination confirmed that ovulation occurred within 30 h after hCG administration, prior to insemination. In accordance, Howard et al. (20) reported ovulation 30 h after hCG treatment. According to Howard et al. (20), when anesthesia is performed prior to ovulation it can negatively affect ovulation triggers and cause a reduction in fertility. Unlike these findings, Tsutsui et al. (8) obtained a better conception rate when insemination was performed before ovulation. The difference between the 2 studies might be due to the fact that Tsutsui et al. (8) used hCG at a higher dosage than Howard et al. (20) and natural estrus queens.

Chatdarong et al. (9) achieved a 41.7% pregnancy rate (5/12) from queens inseminated with frozen-thawed semen by non-chirurgical technique via the cervix (transcervically), after 28 h of hCG treatment, and these results were similar to our results from the EY-TFC extender. These researchers did not achieve pregnancy by intravaginal inseminations. Tsutsui et al. (8) had a 57% pregnancy rate by chirurgical unilateral IUI with the EY-TFC extender, which was higher than our results (42.9%). The reason for this could be the hCG treatment time and the dose used to induce ovulation. The presumptive cause of early embryonic death in the pregnancies in this study was estradiol/progesterone hormone imbalance due to ovulation induction (23). Fontbonne and Malandain (24) stated that embryos need effective development of endometrial cells and glandular secretions for growth and implantation. Roth et al. (25) reported impaired CL functions after induction of queens by exogenous gonadotrophins treatments and abnormal uterine structure of EED.

In conclusion, cat semen extended and frozen with EY-TFC and EY-SMGT extenders has been found to be successful in post-thaw motility and morphological defect rates. Therefore, surgical IUI may be appropriate for feline AI using frozen semen rather than IVI. In future studies, it has been suggested that, non-surgical IUI techniques need to be tried in queens.

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