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Research Article

Freezing of Cat Semen in Straws with Different Glycerol Levels Containing Tris Extender*

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Abstract: Cat semen was extended with 3% and 4% glycerol containing Tris extender and frozen in (0.25 ml) straws. Spermatological characteristics were examined in post-thaw semen samples.

Five tom cats aged 2-3 were used. Semen was collected by electro-ejaculator under general anesthesia (xylazine-ketamine HCl combination). On a weekly basis a total of 8 ejaculates were collected from each tom cat. Spermatozoon concentration (x10^6/ml) was determined by the hemocytometric method. Morphologic examination was done by light microscope with Spermac® stain (x1000 magnification, immersion objective) and progressive motility was examined by hot-plate phase-contrast microscope (x200 magnification). Semen samples chosen as suitable for being frozen were extended 1:1 (semen/extender) by Tris extender containing 20% egg yolk without glycerol [Tris (hydroxymethyl-aminomethane) (2.40 g) – fructose (1.00 g) - citric acid (1.30 g) osmolarity: 285 mOsmol/kg] at +26 ºC. Extended semen was cooled to +5 ºC in 1 h and divided into 2 parts. Each volume was re-extended with glycerol containing Tris extender to obtain 3% and 4% final glycerol levels. The semen samples were left under equilibration for 30 min after glycerolization. Semen was filled into 0.25 ml straws and frozen in liquid nitrogen vapor. Frozen semen samples were stored in liquid nitrogen at -196 ºC. The application was repeated 8 times for the tom cats and 40 straws were frozen for each group (n=40). The frozen straws were thawed for 30 s at 37 ºC. During the study, examinations of motility (%) and morphology (%) (acrosome, other and total) were done at collection, dilution at +26 ºC, chilling at +5 ºC, equilibration and post-thaw points.

The mean post-thaw motility, acrosome, other and total morphologic defect rates with 3% glycerol containing Tris extender were 53.00 ± 10.85%, 26.35 ± 8.55%, 14.43 ± 3.99% and 40.53 ± 11.00%, respectively. These values were 50.50 ± 13.90%, 25.45 ± 7.08%, 15.20 ± 4.36% and 40.40 ± 10.22%, respectively, with 4% glycerol containing Tris extender. The differences between the values of the 2 groups were not statistically significant (P > 0.05).

At the end of the study, it was observed that 3% and 4% glycerol containing Tris extender could be used to preserve the post-thaw motility and morphology of cat semen frozen in straws.

Key Words: Domestic cat, semen freezing, glycerol, abnormal spermatozoa

Kedi Spermasının Farklı Oranlarda Gliserol İçeren Tris Sulandırıcısı İle Payetlerde Dondurulması

Özet: Bu çalışmada, kedi sperması % 3 ve % 4 gliserol içeren Tris sulandırıcısı ile sulandırıldı ve payetlerde (0,25 ml) donduruldu. Spermatolojik özellikler eritme sonrası sperma örneklerinde incelendi.

Çalışmada, 2-3 yaşlı beş erkek kedi kullanıldı. Sperma genel anestezi altında (xylazine-ketamine HCl kombinasyonu) elektro-ejakülatör yardımıyla alındı. Her erkek kediden haftada 1 kez olmak üzere toplam 8 kez sperma alındı. Spermatozoon yoğunluğu (x10^6/ml) hemositometrik yöntemle belirlendi. Morfolojik muayene Spermac® boya ile ışık mikroskobunda (x1000 büyütme, immersion objektif) ve ileri yönlü motilite ısıtma tablalı faz kontrast mikroskopta (x200 büyütmede) incelendi. Donma işlemine elverişli olduğu saptanan sperma örnekleri +26 ºC'de 1:1 (sperma / sulandırıcı) oranında % 20 yumurta sarılı gliserol içermeyen Tris sulandırıcısı [Tris (hydroxymethyl-aminomethane) (2,40 gr.) – fruktoz (1,00 gr.) - sitrik asit (1,30 gr.) ozmolarite: 285 mOsmol/kg] ile sulandırıldı. Sulandırılmış sperma 1 saat içerisinde +5 ºC'ye soğutuldu ve iki eşit hacime ayrıldı. Her bir hacim final gliserol oranı % 3 ve % 4 olacak şekilde gliserollü Tris sulandırıcısı ile tekrar sulandırıldı. Sperma örnekleri gliserolizasyon sonrası 30 dakika ekilibrasyona bırakıldı. Sperma 0,25 ml'lik payetlere çekildi ve sıvı azot buharında dondurulduu. Donmuş sperma numuneleri sıvı azot içerisinde -196 ºC'de saklandı. Uygulama kediler için 8 kez tekrarlandı ve her bir grup için 40 payet donduruldu (n=40).

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Dondurulmuş payetler 37 ºC'de 30 saniye sürede eritildi. Çalışma süresince; sperma alınması, +26 ºC'de sulandırma, +5 ºC'ye soğutma, ekilibrasyon ve eritme sonrası aşamalarda, ileri yönlü motilite (%) ve anormal spermatozoon oranlarının (%) muayeneleri (akrozom, diğer ve toplam) yapıldı.

Eritme sonrası % 3 gliserollü Tris sulandırıcısı ile ortalama motilite, akrozom, diğer ve toplam anormal spermatozoon oranları sırasıyla; % 53,00 ± 10,85, % 26,35 ± 8,55, % 14,43 ± 3,99 ve % 40,53 ± 11,00 olarak bulundu. Bu değerler % 4 gliserol içeren Tris sulandırıcısı ile sırasıyla; % 50,50 ± 13,90, % 25,45 ± 7,08, % 15,20 ± 4,36 ve % 40,40 ± 10,22 olarak bulundu. İki grubun değerleri arasındaki fark istatistiksel olarak önemli bulunmadı (P > 0,05).

Çalışmanın sonucunda, % 3 ve % 4 gliserollü Tris sulandırıcısı ile payet yöntemine göre dondurulmuş kedi spermasının, eritme sonrası motilite ve morfolojik bütünlüğünün başarıyla korunabildiği gözlendi.

Anahtar Sözcükler: Evcil kedi, sperma dondurma, gliserol, anormal spermatozoa

Introduction

Reports on the use of frozen semen for the exchange of feline genetic material have been limited (1-4). Much of the research that has generated knowledge about assisted reproduction in the cat has been developed using the domestic cat as a model for the wildcat species that are threatened by extinction (5). There are limited studies on the artificial insemination or semen freezing in domestic cats in Turkey (6,7). Studies in this field will be beneficial in protecting native Ankara and Van cats from extinction.

Tris (hydroxymethyl-aminomethane) and TesT (N-Trishydroxymethyl-methyl-2-aminomethane-sulphonic acid + Tris) extenders are used to freeze cat semen in straws and the glycerol rate in these extenders is between 3% and 8%. Axner and Linde-Forsberg (5) suggested the use of 5% glycerol containing Uppsala Equex, 4% glycerol containing egg yolk-lactose and 7% glycerol containing TesT-egg yolk extender in the freezing of cat semen.

Currently, cat semen is frozen with 3% glycerol containing egg yolk-lactose extender in pellets (3,4,8-11), and with 4-8% glycerol containing Tris-fructose-citric acid extender in straws. However, the optimal glycerol level for frozen cat semen is obscure (8). Nelson et al. (12) reported the deleterious effect of excess amounts of DMSO and glycerol (i.e. 8%) in the freezing of cat semen. The toxicity of glycerol varies greatly among species and the optimal concentration is a compromise between its cryoprotective and toxic effects (12).

Investigation of the effects of 3% and 4% glycerol addition to the extender for freezing of cat semen in straws and its effects on post-thaw motility and morphology was the aim in this study.

Materials and Methods

Five stray cats aged 2-3 (average 3.0-4.5 kg live weight each) served as semen donors in this study. The cats were kept in 60 x 90 x 120 (h) cm stainless steel cages. The cats consumed 65-70 g commercial cat food (IAMS Company, Ohio, USA) and drinking water ad libitum daily.

Semen Collection by Electro-Ejaculator

Semen was collected from the tom cats by means of a specially adapted electro-ejaculator (P-T Electronics, Model 302, Boring, Oregon, USA). A xylazine (2 mg/kg body weight, s.c., Rompun®, Bayer, İstanbul, Turkey) and ketamine HCl (5 mg/kg body weight, i.m., Ketalar®, Eczacıbaşı, İstanbul, Turkey) combination was employed in the anesthesia of the tom cats. Anesthetized tom cats were fixed horizontally and the lubricated rectal probe (P-T Electronics, Boring, Oregon, USA), which was 1 cm thick, 12 cm long and had 3 electrodes (1.5 mm thick and 3 cm long each), was placed into the rectum (average 9 cm) (Figure 1). Electric pulses were administered following the Platz and Seager (13) procedure (3 s pulse, 2 s interval). The set of administrations was between 2 and 7 volts and each set contained 60 pulses, with a total of 180 electric pulses. Before the electrical stimulation, an Eppendorf tube was placed over the penis (Figure 2).

Spermatological Examinations

Semen volume was determined by adjustable automatic pipette (10-1000 µl) and the value was recorded as µl. Motility was estimated by a hot plate phase-contrast microscope at x200 magnification by viewing at least 3 fields and estimating the % rate of processing spermatozoa. Spermatozoon concentration was calculated by the hemocytometric method and was

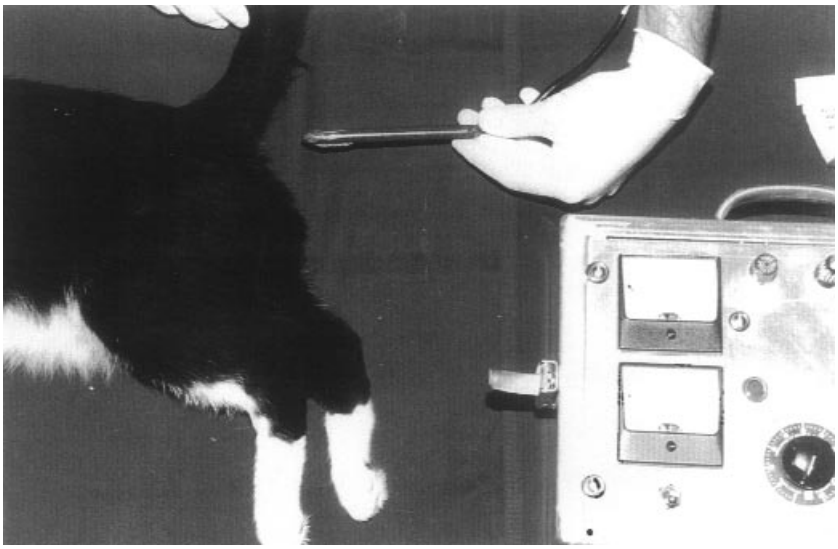

Figure 1. Anesthetized tom cat and administration of rectal probe. (P-T Electronics, Oregon, USA).

Figure 2. Semen collection in domestic tom cat with electro-ejaculator.

recorded as $x10^6$/ml. A Spermac® stain kit (Stain Enterprises, Onderstepoort, Republic of South Africa) was employed in morphologic observations. The preparation was examined by light microscope at x1000 magnification by counting 200 cells [acrosome, other (head, mid-piece and tail) and total] (Figure 3).

Dilution and Freezing of Semen

Semen samples that were decided to be suitable for freezing (>75% motility and <25% total abnormal spermatozoa) were extended 1:1 (semen/extender) with 20% egg yolk containing Tris extender without glycerol [Tris (hydroxymethyl-aminomethane) (2.40 g) – fructose (1.00 g) - citric acid (1.30 g) osmolarity: 285 mOsmol/kg] at +26 ºC (Table 1). The semen was then divided into 2 parts and chilled to +5 ºC in 1 h by measurement with a digital thermometer. Each sample was diluted with 6% and 8% glycerol containing Tris extender at the same volume (v/v) to accomplish the glycerolization stage (final glycerol rates of 3% and 4%).

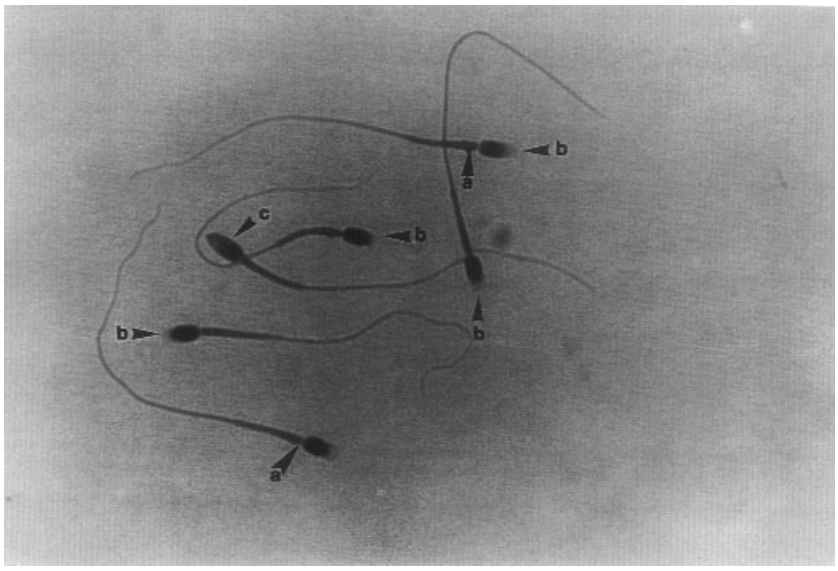

Figure 3. Various morphological defect types observed in post-thaw cat semen.
a) Mid-piece defect b) Swelling acrosome c) Acrosomal defect

Table 1. Preparation of 3% and 4% glycerol containing Tris extender.

Ingredients	Tris extender (without glycerol)	Tris (with 3% glycerol)	Tris (with 4% glycerol)
Tris (hydroxymethyl-aminomethane)	2.40 g	2.40 g	2.40 g
Citric acid	1.30 g	1.30 g	1.30 g
Fructose	1.00 g	1.00 g	1.00 g
Streptomycin sulfate	1000 µg/ml	1000 µg/ml	1000 µg/ml
Penicillin G	1000 IU/ml	1000 IU/ml	1000 IU/ml
Egg yolk	20	20	20
Glycerol	-	6	8
Distilled water	to 100 ml	to 100 ml	to 100 ml

Glycerolization was completed in 10 equal portions at 6 min intervals and equilibration lasted for 30 min. Equilibrated semen samples were filled into 0.25 straws and frozen in liquid nitrogen vapor at -110 ºC for 7 min. Frozen semen samples were stored in liquid nitrogen at -196 ºC. The procedure was repeated 8 times for each tom and 40 straws were frozen for each group (n = 40). Frozen straws were stored for a month and thawed for examination in a water bath at 37 ºC for 30 sec (1,3,9).

Motility and abnormal spermatozoa rates [acrosome, other (head, mid-piece and tail) and total] were examined during the study at the collection, dilution at +26 ºC, chilling to +5 ºC, equilibration and post-thaw stages.

Statistical Analysis

Statistical analysis of the spermatological characteristics of fresh semen was performed by the SPSS program and one-way ANOVA. In the meantime the analysis of post-equilibration and post-thaw spermatological characteristics was performed by Student's t-test.

Results

No difference was observed among the tom cats with regard to semen volume, spermatozoa concentration, motility and morphology ($P > 0.05$). The results for fresh semen are presented in Table 2, and motility and

Table 2. Spermatological characteristics in fresh semen.

Cat No:	no.	Volume (ml)	Motility (%)	Sperm concentration (x10^6/ml)	Morphologic defects (%)		
					Acrosome	Other	Total
1	8	198.13 ± 36.15	89.38 ± 6.23	147.50 ± 60.89	8.25 ± 2.25	8.25 ± 2.49	16.50 ± 2.51
2	8	165.63 ± 31.10	86.25 ± 9.16	176.25 ± 16.37	8.63 ± 2.92	10.88 ± 4.42	19.50 ± 5.93
3	8	183.75 ± 23.26	89.38 ± 7.29	123.75 ± 20.66	7.50 ± 2.83	8.38 ± 3.20	15.88 ± 5.41
4	8	195.00 ± 64.37	89.38 ± 6.78	132.50 ± 45.90	9.75 ± 2.71	7.50 ± 3.16	17.25 ± 4.13
5	8	191.25 ± 24.16	85.00 ± 4.63	185.00 ± 71.51	7.88 ± 3.44	9.50 ± 2.07	17.38 ± 3.66
Means	40	186.75 ± 38.62	87.87 ± 6.88	153.00 ± 71.12	8.40 ± 2.82	8.90 ± 3.23	17.30 ± 4.44

Means ± SD: Standard deviation, P > 0.05

morphologic defect rate results after dilution at +26 ºC and chilling to +5 ºC are presented in Table 3.

After equilibration motility and morphologic defect rates with 3% and 4% glycerol containing Tris extenders were 77.88 ± 9.99% and 77.00 ± 9.99% and 29.03 ± 7.42% and 30.08 ± 7.56%, respectively (Table 4). These values were 53.00 ± 10.85% and 50.50 ± 13.90% and 40.53 ± 11.00% and 40.40 ± 10.22%, respectively, for post-thaw semen samples (Table 5). Neither post-equilibration nor post-thaw spermatological characteristics were affected by varying glycerol levels (P > 0.05).

Discussion

Electro-ejaculator and artificial vagina techniques are both used for semen collection in the domestic tom cat. Researchers (5,7,13-19) reported that in ejaculates collected by electro-ejaculator semen volume was 57-233 µl, spermatozoon concentration was 168-361 x 10^6/ml, motility was 50-80%, and total morphologic defect rate was 2-38%. According to the results of our study, electro-ejaculator collected cat semen can be of good quality.

Motility and morphologic integrity of the spermatozoa in semen diluted 1:1 (v/v) with Tris without glycerol and

Table 3. Motility and morphologic defect values at dilution at +26 ºC and after chilling at +5 ºC.

Stage	Motility (%)	Morphologic defects (%)		
		Acrosome	Other	Total
Dilution (n = 40)	84.25 ± 10.04	11.40 ± 3.37	11.33 ± 3.66	22.73 ± 4.81
Chilling +5 ºC (n = 40)	80.87 ± 09.47	13.75 ± 3.64	13.85 ± 3.78	27.60 ± 4.97

Means ± SD: Standard deviation

Table 4. Post-equilibration motility and morphologic defects with 3% and 4% glycerol containing Tris extender.

Extender	Motility (%)	Morphologic defects (%)		
		Acrosome	Other	Total
Tris with 3% glycerol (n = 40)	77.88 ± 9.99	17.25 ± 5.97	11.78 ± 3.56	29.03 ± 7.42
Tris with 4% glycerol (n = 40)	77.00 ± 9.99	18.23 ± 5.81	11.85 ± 3.50	30.08 ± 7.56

Means ± SD: Standard deviation, P > 0.05

Table 5. Post-thaw motility and morphologic defects with 3% and 4% glycerol containing Tris extender.

Extender	Motility (%)	Morphologic defects (%)		
		Acrosome	Other	Total
Tris with 3% glycerol (n = 40)	53.00 ± 10.85	26.35 ± 8.55	14.43 ± 3.99	40.53 ± 11.00
Tris with 4% glycerol (n = 40)	50.50 ± 13.90	25.45 ± 7.08	15.20 ± 4.36	40.40 ± 10.22

Means ± SD: Standard deviation, P > 0.05

cooled to +5 ºC in 1 h were not affected at all. The motility value of 84.25 ± 10.04% after dilution was similar to the 87.5% value reported by Tsutsui et al. (1) with the same extender.

The motility and morphologic defect rates after cooling to +5 ºC can be considered suitable for freezing. Glover and Watson (20) compared the effects of various osmolarities of extenders in their study and scored the highest motility value of 51% after cooling with 325 mOsmol/kg TesT extender. This value was rather lower than the 80.87 ± 09.47% value in our study. Harris et al. (21) diluted epidydimal and ejaculated semen samples with TesT extender and reported the after cooling motility rates as 62.2% and 79.3% for ejaculated and epididymal semen samples, respectively. It has been observed that 285 mOsmol/kg Tris extender can be used successfully to dilute cat semen and store it at +5 ºC for shorter periods.

There was no difference between the 3% and 4% glycerol (final) containing 2 semen samples in motility and morphologic defects after glycerolization and equilibration (P > 0.05). The motility rates of the 3% and 4% glycerol containing Tris extender groups in the present study were higher than the 69.7 ± 3.2% rate reported by Nelson et al. (12) with 4% glycerol containing Tris extender.

The post-thaw motility of this study was 53.00 ± 10.85% and 50.50 ± 13.90% for the 3% and 4% glycerol containing extender groups, respectively; and there was no statistical significance between these values (P > 0.05). Luvoni et al. (22) collected semen from the epididymis, extended with taurine added 3% glycerol containing Tris-citric acid-glucose extender and froze it in straws. The post-thaw motility rate of 52-53% in that study resembled the results obtained in both glycerol level groups in our study. Nelson et al. (12) collected semen from the cauda epididymis of tom cats, diluted it with 8% glycerol containing Tris extender, froze it in straws and obtained a post-thaw motility level of 43.0 ± 2.5%. Hay and Goodrowe (23) used the same technique and the same extender (3% glycerol) and observed a 37% post-thaw motility. Tsutsui et al. (1) and Pope et al. (18) reported 30.0 ± 9.7% and 44% post-thaw motility, respectively, in straw frozen semen with 7% glycerol containing Tris extender. In a study in Turkey (7), cat semen was frozen with 8% glycerol containing Tris extender and a 35% post-thaw motility rate was reported. According to the above studies, the researchers used 7-8% glycerol levels in Tris extender and obtained various post-thaw motility rates. The highest motility rates in the present study can be attributed to the lower glycerol levels used. In addition, some researchers claimed that higher glycerol rates (e.g., 8%, v/v) could be detrimental to cat semen (8,12).

Thus researchers have used glycerol levels as high as 7-8% and achieved various post-thaw motility rates. The higher motility rates in the present study can be attributed to the lower glycerol levels used. Therefore, high glycerol levels in extender (i.e. 8%) can be detrimental to cat semen.

No difference was determined between the motility and morphologic defect rates of the semen samples in 3% and 4% glycerol (final) containing groups after glycerolization and equilibration (P > 0.05). The motility values of our study in 3% and 4% glycerol containing groups after equilibration were a little higher than the 69.7 ± 3.2% motility value reported by Nelson et al. (12) in 4% glycerol containing Tris extender.

The post-thaw acrosomal defect rates of semen samples extended with 3% and 4% glycerol containing Tris extender were 26.35 ± 8.55% and 25.45 ± 7.08%, respectively. This value is higher than the value (4.97 ±

0.34%) reported by Kaya (7). On the other hand, high levels such as 8% of glycerol containing Tris extender can reduce seminal motility but preserve morphologic integrity. Meantime, the staining techniques and fixating solutions used for morphologic evaluations play an important role. Schäfer and Holzman (24) suggested that the Spermac staining technique was more successful in post-thaw morphologic evaluation.

In semen samples extended with 3% and 4% glycerol containing Tris extender, post-thaw total morphologic defect rates were 40.53 ± 11.00% and 40.40 ± 10.22%, respectively. These values are similar (mean 38%) to the value reported by Kaya (7) with 8% glycerol containing extender. Schäfer and Holzman (24) reported a 57 ± 12% post-thaw abnormal spermatozoa rate with 7.5% glycerol containing TesT extender, and this value was higher than the total morphological defect rates in both glycerol level groups in our study. The presumptive reason for this difference is the collection of semen from the epididymidis and the use of high glycerol rates by these researchers.

In conclusion, no difference was observed between the post-thaw motility and morphologic defect rates in 3% and 4% glycerol containing Tris extenders ($P > 0.05$), and Tris extender can be used for freezing semen with both glycerol levels. However, more studies are needed regarding artificial insemination with frozen semen and to determine pregnancy rates.

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