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Effects of epididymis cold storage on frozen-thawed epididymal sperm quality in tomcats (*Felis catus*)

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ABSTRACT: The effect of cold storage of testes and epididymides at 4 °C for 12 h on the cryopreservation capacity of epididymal feline sperm was evaluated. Ten domestic cats were castrated, and testes and epididymides collected. Specimens were randomly assigned to two groups: in Group A, epididymal samples were immediately processed and frozen in 0.25-ml straws; in Group B, both testes and epididymides were maintained in saline at 4 °C for 12 h and sperm was then processed and frozen. Motility, morphology, acrosome status, sperm viability and DNA integrity were assessed in epididymal sperm samples before freezing (baseline), at thawing (0 h) and 6 h post-thawing (6 h). Although values were lower in Group B, no significant intergroup difference was observed for any of the parameters tested either at baseline or at 0 h. However, significantly higher values ($P < 0.05$) were observed in Group A at 6 h for total sperm motility (29.0 ± 2.4% vs 13.0 ± 4.3%), sperm viability (35.2 ± 5.4% vs 15.4 ± 1.4%) and normal morphology (47.6 ± 0.8% vs 40.0 ± 2.1%). It was observed that motility and acrosome status of epididymal sperm are the most sensitive parameters when both types of sperm samples (from fresh epididymis or from 12 h cold-stored epididymis) are frozen-thawed. When sperm quality was assessed 6 h after thawing, spermatozoa precooled in the epididymides showed significantly lower values for motility, viability and morphology than spermatozoa from fresh epididymal samples.

Keywords: feline; spermatozoa; cryopreservation; cooling

Sperm freezing is a potential tool for preserving the genetic material of endangered feline species. Sometimes, however, the sudden death of animals can hinder the recovery and cryopreservation of sperm. In such cases, the refrigeration of samples for transport to specialized laboratories and subsequent sperm freezing could enable satisfactory cryopreservation of this genetic material, which might otherwise be lost.

Research suggests that cat sperm is highly resistant, and can be kept for several days in cold storage with no impairment of quality (Harris et al. 2001), and that storage of cat spermatozoa within the epididymis gives rise to no changes in motility, although it does reduce sperm freezability (Hay and Goodrove 1993). Toyonaga et al. (2010) reported that extragonadal storage of epididymal spermatozoa at 4 °C for 24 h did not significantly impair motility, viability or morphology, while Chatdarong et al. (2009) found that the cold storage of epididymides for four days prior to sperm freezing could be used for the rescue of male gametes in cat, and that such storage gave results comparable to those when epididymal sperm was collected, diluted and refrigerated after castration. However, these authors demonstrated that sperm quality was significantly lower when the cold storage time was longer. Findings regarding the deposition of sperm cooled for 24 h before freezing suggest that unilateral intratubal artificial insemination is more effective than unilateral intrauterine artificial insemination (Toyonaga et al. 2011).

It was hypothesised that delayed sperm collection from epididymis could affect sperm quality and also influence its subsequent freezability. The present exploratory study evaluated the effect of cold storage of testes and epididymides at 4 °C for 12 h on cat sperm freezability, with a view to enhancing sperm preservation in wild and domestic felines suffering sudden accidents or death.

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MATERIAL AND METHODS

Animals. Ten mature European shorthair tomcats aged 12–16 months and weighing 3.1 ± 1.7 kg, with epididymal total sperm motility values of greater than or equal to 50% post-castration, were assigned to two experimental groups by simple random sampling. Five cats were excluded from the study because their sperm quality failed to meet inclusion criteria. The study was conducted in Cordoba (Spain) from 2009 to 2010, with the collaboration of the municipal pet control centre and private veterinary clinics. Experimental procedures were performed in accordance with Spanish Animal Protection Law RD 1201/2005, implementing Directive 86/609 of the European Union concerning the protection of animals used in scientific experimentation.

For orchiectomy, cats were pre-medicated with 80 µg/kg *i.m.* medetomidine (Domtor, Pfizer, Spain) and anaesthesia was maintained with isoflurane (Isoflo, Abbott Laboratories, UK) in oxygen at a total gas flow rate of 1.5 l/min delivered to a circle rebreathing system. Postoperative analgesia consisted of a subcutaneous injection of 0.30 mg/kg meloxicam (Metacam, Boehringer Ingelheim, Spain).

Sperm recovery. After orchiectomy, vasa deferentia were tied, and testes and epididymides were transported to the Animal Reproduction Laboratory (University of Cordoba) in a sterile 0.9% NaCl solution (saline) at 22 °C. Samples were randomly assigned to two groups. In Group A (n = 5 toms), epididymal samples were immediately processed and frozen, while in Group B (n = 5 toms), testes and epididymides were kept in saline at 4 °C for 12 h, and sperm was subsequently collected and cryopreserved.

In Group A, epididymides and proximal vasa deferentia were isolated within 1 h of collection. Then, right and left cauda epididymides were minced and immersed in 3 ml Dulbecco's phosphate buffered saline (dPBS) (Oxoid, Hampshire, England; osmolality, 260–290 mOsm) at 38 °C. Ten minutes later, this mixture was washed through a non-woven tissue and non-absorbing filter (Minitub Iberica, Tarragona, Spain) to obtain spermatozoa, and the tissue was rewashed with 3 ml dPBS. Baseline evaluations of sperm motility, morphology, acrosome status, viability and DNA integrity were carried out as described below. In Group B, gonads were similarly processed after being kept for 12 h in saline at 4 °C.

After initial evaluation in dPBS medium, sperm samples were centrifuged at 300 × g for 8 min. The sperm pellet was resuspended with 700 ml Triladyl (Minitub Iberica, Tarragona, Spain) supplemented with 20% egg yolk at 20 °C (T-1 extender; 1325 ± 14 mOsm). Sperm was then cooled to 4 °C (at a rate of 0.3 °C/min) using a controlled refrigerator with a digital thermometer. Finally, 700 ml of the initial extender (T-1) plus Equex STM paste (1340 ± 14 mOsm) were added to the sperm sample. Equex STM paste (Minitub Iberica, Tarragona, Spain) was added after cooling to a final dilution of 0.5% as a protection for sperm membranes.

Sperm doses were diluted at a final concentration of approximately 5 × 10^6 spermatozoa in 0.25 ml straws. After 15 min, they were suspended horizontally 4 cm above the liquid nitrogen level for 10 min and subsequently immersed in liquid nitrogen.

For thawing, straws were placed in a 38 °C water bath for 30 s and diluted 1 : 1 in egg yolk-supplemented extender.

Sperm assessment. Sperm assessment was carried out in fresh samples and after freezing (0 and 6 h after thawing). Thawed sperm samples were diluted 1 : 1 in extender (Table 1) and kept in the incubator at 38 °C until evaluation.

Freshly diluted and frozen-thawed sperm samples were evaluated for motility, morphology, acrosome status and viability (live cells); DNA integrity was evaluated in frozen-thawed sperm samples. Baseline values were obtained after epididymal sperm recovery using dPBS medium (in both groups), and frozen-thawed sperm were assessed after thawing.

Before sperm assessment, samples were incubated at 38 °C for 5 min. Then, 10 µl of epididymal sperm samples were placed on a warm glass slide and the percentage of total motility was evaluated subjectively (magnification × 100). Sperm morphology was studied in smears stained with Romanowski stain (fixative, eosin and blue solutions for fast staining; Panreac, Spain). A total of 200 spermatozoa in each smear were analysed under a light microscope (magnification × 1000, immersion oil). The percentages of normal and abnormal spermatozoa were determined (Howard et al. 1990).

Acrosome integrity was evaluated after staining with *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC) (Sigma Chemical Co., St Louis, USA), using epifluorescent microscopy (magnification × 400, immersion oil) (Filliers et al. 2008). Briefly, 25 ml of sperm suspension were washed in 200 ml of Hepes-TALP and centrifuged (2 min, 500 g). The sperm pellet was

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then resuspended in 50 ml absolute ethyl alcohol, cooled for 30 min, and 50 ml of absolute ethyl alcohol was again added to the suspension. A 20 ml aliquot of the resulting suspension was smeared onto a glass microslide and allowed to air-dry. Then, 20 ml of PSA-FITC (2 mg PSA-FITC diluted in 2 ml dPBS) were added followed by incubation at 4 °C for 15 min. Afterwards, the spermatozoa on the glass slide were washed with fresh water and two hundred spermatozoa were evaluated. Acrosome-intact spermatozoa were characterised by intense green fluorescence in the acrosomal area.

Sperm viability was determined using SYBR-14 and propidium iodine (Live/dead Sperm Viability Kit, Molecular Probes, Leiden, The Netherlands) and evaluated using epifluorescent microscopy (magnification × 400, immersion oil). Briefly, a stock solution of 1 mmol/l SYBR-14 reagent was diluted (1 : 50) in Hepes-TALP, stored frozen at –20 °C and thawed just before use. A mixture of 2 ml sperm suspension and 200 ml Hepes-TALP was incubated with 1.25 ml SYBR-14 and 1.25 ml propidium iodine, and a 10-ml aliquot was removed, placed on a microslide, mounted under a cover slip and incubated for 10 min at 38 °C in darkness. Spermatozoa with an intact plasma membrane stain fluorescent-green with SYBR-14, while those with a damaged membrane exhibit red fluorescence. Slightly damaged spermatozoa exhibit a dual-staining pattern and were counted as cells with damaged membranes. For each sample, at least 200 sperm were assessed in duplicate.

An adapted method for evaluating sperm DNA integrity using acridine orange was used (Thuwanut et al. 2008). Briefly, a 10-ml sperm sample previously fixed in methanol-glacial acetic acid (Carnoy's solution, 3 : 1, v/v) was dipped in acridine orange staining solution (Sigma Chemical Co., St Louis, USA) for 5 min and evaluated under epifluorescent microscopy. Two hundred spermatozoa were evaluated and classified based on the fluorescence emitted, i.e. spermatozoa with normal DNA integrity emitted green fluorescence and denatured or single-stranded DNA exhibited orange, yellow or red fluorescence.

Statistical analysis. To test normality, data were analysed using the Kolmogorov-Smirnov test. The lack of significant values revealed that the data were adjusted to a normal curve. Data for motility, morphology, percentage of intact acrosomes, viability, and DNA integrity obtained for baseline and frozen-thawed (0 and 6 h) epididymal sperm samples were analysed using repeated measures ANOVA (GLM), and the Bonferroni test was used for comparison of different cryopreservation times. ANOVA was used to evaluate differences between groups (A and B) at each time.

The SPSS 15.0 package (SPSS, Chicago, USA) was used for statistical analysis. All results are presented as mean ± SEM. The significance level was set at $P < 0.05$.

RESULTS

Significant intergroup differences were observed at baseline and 0 h post-thawing for sperm motility. However, assessment at 6 h post-thawing revealed significantly lower values in Group B ($n = 5$) vs Group A ($n = 5$) for total sperm motility (29.0 ± 2.4% vs 13.0 ± 4.6%, $P < 0.05$), sperm viability (35.2 ± 5.4 vs 15.4 ± 1.4; $P < 0.01$) and normal morphology (47.6 ± 0.8% vs 40.0 ± 2.1%, $P < 0.05$; Table 1).

Table 1. Fresh and post-thaw assessment of freezability of epididymal sperm samples (in percents) processed immediately after castration (fresh, $n = 5$) or after storage for 12 h at 4 °C (cold, $n = 5$; mean ± SEM)

Sperm parameter	Groups	Assessment time		
		baseline	at thawing	6 h after thawing
Motility	fresh	74.0 ± 2.4^{aA}	54.0 ± 4.0^{b}	29.0 ± 2.4^{cA}
	cold	60.0 ± 4.4^{aB}	40.0 ± 6.3^{a}	13.0 ± 4.3^{bB}
	P	0.025	0.098	0.013
Sperm viability	fresh	71.6 ± 8.0^{a}	65.6 ± 6.6^{a}	35.2 ± 5.4^{bA}
	cold	63.4 ± 9.8^{a}	47.4 ± 5.7^{a}	15.4 ± 1.4^{bB}
	P	0.54	0.07	0.01
Normal morphology	fresh	55.4 ± 3.3	53.4 ± 2.2	47.6 ± 0.8^{A}
	cold	59.6 ± 2.8^{a}	49.6 ± 2.6^{a}	40.0 ± 2.1^{bB}
	P	0.37	0.31	0.01
Intact acrosome	fresh	55.2 ± 1.5^{a}	37.0 ± 1.2^{b}	18.4 ± 0.9^{c}
	cold	55.4 ± 2.3^{a}	36.8 ± 2.2^{b}	20.8 ± 0.9^{c}
	P	0.95	0.94	0.12
DNA integrity	fresh	na	87.8 ± 0.6^{a}	78.4 ± 0.4^{b}
	cold	na	87.6 ± 1.3^{a}	79.0 ± 1.2^{b}
	P		0.89	0.66

na = not assessed

^{a,b,c}Values in the same row are significantly different ($P < 0.05$)

^{A,B}Values in the same column within each sperm parameter are significantly different ($P < 0.05$)

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Table 2. Percentages (mean ± SEM) of abnormal spermatozoa from immediately processed (fresh) and 12 h-cooled (cold) epididymal sperm samples at collection and after freeze-thawing

Sperm abnormalities	Groups	Assessment time			*P*
		baseline	at thawing	6 h after thawing	
Head	fresh	5.8 ± 0.9[a]	1.2 ± 0.5[b]	0.3 ± 0.1[b]	**
	cold	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	ns
Midpiece	fresh	19.2 ± 1.5	16.6 ± 1.1	15.2 ± 2.0	ns
	cold	18.2 ± 1.2	14.2 ± 1.9	15.0 ± 1.7	ns
Tail	fresh	19.6 ± 0.9[a]	28.8 ± 1.3[b]	37.2 ± 1.2[c]	***
	cold	21.8 ± 1.4[a]	36.0 ± 1.5[b]	45.0 ± 1.7[c]	***

[a,b,c]Values in the same row are significantly different

**$P < 0.01$, ***$P < 0.001$

The percentage of tail abnormalities significantly increased after the freeze-thawing process (Table 2). The percentage of sperm head defects was significantly higher in samples assessed after collection than in cooled samples, although differences disappeared after freezing. After cooling, there was no increase in midpiece defects, thus confirming resistance to cold storage.

DISCUSSION

In the present study, we explored the effect of epididymal cold storage on freezability at up to after thawing. We hope that the gained insights will contribute to optimising insemination practices and in determining which sperm characteristics are significantly impaired with thawing time in fresh and cooled epididymides sperm samples. Studies in cats have shown that refrigeration of epididymides and testes after death enables sperm to retain fertilising ability for extended periods of time and to be successfully cryopreserved (Goodrove and Hay 1993; Chatdarong et al. 2009; Thuwanut and Chatdarong 2012). Toyonaga et al. (2010) reported that the quality of sperm from epididymides stored at 4 °C for 24 h was similar after the freeze-thawing process to that of fresh samples. It has also been shown that the viability of sperm recovered from dead animals can be maintained for a short period, depending on environmental temperature and time until processing (Kishikawa et al. 1999). Although sperm retains adequate quality after freeze-thawing, few studies have analyzed the effect of thawing time on sperm characteristics.

Chatdarong et al. (2006) noted that cat sperm is highly resistant to refrigeration, observing that sperm maintained at 4 °C within or outside the epididimydes over different periods of time retains its quality. However, the present study revealed that the motility of cat sperm from epididymides pre-cooled for 12 h displays significant impairments at 6 h post-thawing, compared with epididymal samples frozen immediately after castration. As also reported in deer (Soler et al. 2005), post-thawed sperm motility falls more markedly than other sperm parameters after gonad storage. It has been suggested that changes occur in the environment surrounding the epididymal spermatozoa as a consequence of death, and that the effect of those changes on post-thaw motility is more pronounced than on other sperm characteristics.

Morphology of sperm from epididymal samples processed after castration was similar to that of samples from epididymides kept at 4 °C for 12 h, a finding also reported elsewhere (Axner et al. 2004; Ganan et al. 2009). Other authors, by contrast, suggest that sperm morphological abnormalities are more frequent in epididymides stored for 24 h (Hay and Goodrowe 1993) or 48 h (Toyonaga et al. 2010) than in fresh samples frozen immediately after collection. The difference between these findings may be attributable to the storage medium, evaluation method and freezing protocol used, and particularly to extender composition. Evaluation of sperm samples at 6 h post-thawing revealed a significant increase in abnormalities in cold-stored samples. These abnormalities were linked to tail defects, suggesting that pre-cooling led to cold shock and therefore to a larger number of abnormalities in this region (Chatdarong et al. 2009).

After thawing (0 and 6 h), no intergroup difference was observed in the percentage of normal acrosomes, as assessed using the PSA-FITC fluorescent dye. Thus, refrigeration of genital tissues did not reduce the percentage of intact acrosomes, as also reported by Axner et al. (2004), who found that the cytoplasmic membrane maintained its integrity and function after cooling. Equex is known to negatively impact sperm longevity, and this parameter may have been less affected if Equex had not been included in the media; however, Equex paste seems to effectively protect membranes and acrosomes against potential damage due to chilling and freezing (Axner et al. 2004).

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No significant differences in DNA integrity were observed between study groups, although significant differences have been reported as a function of incubation time after thawing. Although DNA integrity is essential for the accurate transmission of genetic information, it has not been widely studied in cat sperm. Sperm DNA fragmentation has been found to increase during freeze-thawing, when endogenous generation of reactive oxygen species occurs (Baumber et al. 2003).

Assessment at 6 h post-thawing revealed a significant decline in sperm quality compared with values obtained at 0 h post-thawing, suggesting that this sperm should be used as soon as possible. Toyonaga et al. (2011), reporting on the use of frozen-thawed sperm collected from the caudal epididymides and previously stored at a low temperature for 24 h, noted that better pregnancy rates were obtained when intratubal rather than intrauterine insemination was used. The present findings may in part account for these results, since sperm characteristics were significantly impaired at 6 h post-thawing, and unilateral intratubal artificial insemination shortens the distance that spermatozoa must travel to reach the fertilization site.

Acceptable sperm values were obtained after 12 h of refrigeration at 4 °C. However, epididymal sperm samples kept in cold storage before freezing showed significantly reduced motility, viability and morphology when assessed at 6 h after thawing. These findings support the view that, while sperm samples should be collected as soon as possible from epididymides (cauda segment), they can be kept for at least 12 h with no loss of sperm quality. However, sperm should be utilised rapidly after thawing, since some sperm characteristics were found to be significantly impaired after 6 h. This would support the view that artificial insemination techniques need to be improved, and that sperm must be deposited near the oviduct, in order to avoid a major reduction in sperm motility.

REFERENCES

Axner E, Hermansson U, Linde-Forsberg C (2004): The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Animal Reproduction Science 84, 179–191.

Baumber J, Ball BA, Linfor JJ, Meyers SA (2003): Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. Journal of Andrology 24, 621–628.

Chatdarong K, Boonyen T, Rungsuk K, Chowtrakul C, Lohachit C (2006): Freezing of cat epididymal spermatozoa after cold storage. In: Proceedings of the 2nd symposium of the Asian zoo and wildlife medicine and the 1st workshop on the Asian zoo and wildlife pathology, Bangkok, Thailand. 20 pp.

Chatdarong K, Thuwanut P, Suksamai P, Patanatiradaj S, Sangwornrachasup A (2009): Survival of frozen-thawed cat spermatozoa pre-cooled in the epididymides. Reproduction in Domestic Animals 44, 377–380.

Filliers M, Rijsselaere T, Bossaert P, De Causmaecker V, Dewulf J, Pope CE, Van Soom A (2008): Computer-assisted sperm analysis of fresh epididymal cat spermatozoa and the impact of cool storage (4 °C) on sperm quality. Theriogenology 70, 1550–1559.

Ganan N, Gomendio M, Roldan ERS (2009): Effect of storage of domestic cat (Felis catus) epididymides at 5 °C on sperm quality and cryopreservation. Theriogenology 72, 1268–1277.

Goodrowe KL, Hay M (1993): Characteristics and zona binding ability of fresh and cooled domestic cat epididymal spermatozoa. Theriogenology 40, 967–975.

Harris RF, Pope CE, Gomez MC, Leibo SP, Dresser BL (2001): Storage of domestic cat spermatozoa for extended periods at 4 °C. Theriogenology 55, 308.

Hay MA, Goodrowe KL (1993): Comparative cryopreservation and capacitation of spermatozoa from epiddidymis and vasa deferentia of domestic cat. Journal of Reproduction and Fertility 47, 297–305.

Howard JG, Brown JL, Bush M, Wildt DE (1990): Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. Journal of Andrology 11, 204–215.

Kishikawa H, Tatetno H, Yanagimachi R (1999): Fertility of mouse spermatozoa retrieved from cadavers and maintained at 4 °C. Journal of Reproduction and Fertility 116, 217–222.

Soler AJ, Esteso MC, Fernandez-Santos MR, Garde JJ (2005): Characteristics of Iberian red deer (Cervus elephus hispanicus) spermatozoa cryopreserved after storage at 5 °C in the epididymis for several days. Theriogenology 64, 1503–1517.

Thuwanut P, Chatdarong K (2012): Cryopreservation of cat testicular tissue: effects of storage temperature, freezing protocols and cryoprotective agents. Reproduction in Domestic Animals 47, 777–781.

Thuwanut P, Chatdarong K, Techakumphu M, Axner E (2008): The effect of antioxidants on motility, viability,

doi: 10.17221/253/2015-VETMED

acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology 70, 233–240.

Toyonaga M, Sato Y, Morita M, Watanabe M, Oba H, Mizutani T, Hori T, Tsutsui T (2010): The qualities of cryopreserved epididymal sperm collected from feline epididymides stored at low temperature. Journal of Veterinary Medical Science 72, 777–780.

Toyonaga M, Sato Y, Sasaki A, Kaihara A, Tsutsui T (2011): Artificial insemination with cryopreserved sperm from feline epididymides stored at 4 °C. Theriogenology 76, 532–537.

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