Effect of storage of domestic cat (*Felis catus*) epididymides at 5 °C on sperm quality and cryopreservation

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Abstract

Postmortem sperm recovery from the epididymides may constitute a powerful tool for the conservation of valuable genetic material. The domestic cat (*Felis catus*) is a good model for wild felids and, using this model, we have explored the effect of epididymides storage time on sperm motility and percentage of intact acrosomes upon sperm recovery and after cryopreservation. We also examined the effect of time of sperm equilibration with glycerol before freezing on sperm motility and the percentage of intact acrosomes. Motility varied between sperm recovered from epididymides that were stored for different times. Significant differences were seen in the sperm motility index (SMI) before freezing (55.91 ± 2.02, 48.21 ± 1.47, and 43.03 ± 1.32) and after thawing (51.81 ± 3.02, 41.90 ± 2.14, and 42.35 ± 1.95) of sperm recovered from epididymides stored for 0, 48, or 72 h, respectively. The percentage of intact acrosomes did not vary significantly with storage time (average 60.33 ± 1.38% before and 52.50 ± 1.91% after freezing, respectively). The percentage of normal sperm after different storage times did not differ (average 19.22 ± 1.25% normal sperm after recovery). When epididymides were stored for 72 h, time of sperm equilibration with glycerol (30 vs. 120 min) resulted in significant differences in both motility (SMI = 39.17 ± 2.76 and 45.00 ± 2.65, respectively) and the percentage of intact acrosomes (45.76 ± 4.91% and 60.67 ± 3.64%, respectively) after thawing. In conclusion, best results are achieved when sperm are recovered from epididymides within 24 h of cool storage and when they are equilibrated with glycerol during 120 min before freezing. The current results should be useful in the further development of techniques for the rescue and cryostorage of epididymal spermatozoa of endangered felids.

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1. Introduction

All 37 feline species, with the exception of the domestic cat (*Felis catus*), are threatened or endangered [1]. Reproductive research on nondomestic felids is necessary to develop more effective genetic and conservation management programs [2]. However, such research is usually difficult to accomplish in endangered species due to a limited access to individuals in the wild or in captivity. Thus, research on feline reproduction benefits from the use of model species [3,4]. The domestic cat has often been used as model for wild felids, and, therefore, the improvement of knowledge on reproductive biology and technology in this species could improve conservation efforts for its wild relatives [5].

Cryopreserved spermatozoa could be stored virtually indefinitely in genome resource banks (GRBs) as a reserve of species genetic diversity [6]. The collection
and preservation of sperm cells, in combination with assisted reproductive techniques (ARTs) such as artificial insemination, in vitro fertilization, and intracytoplasmic sperm injection, could play a critical role in sustaining the future of the earth’s threatened animal biodiversity [7]. Assisted reproductive techniques have tremendous potential in conservation programs of endangered feline populations to allow adequate genetic management within or between wild or captive populations [8,9]. The development of sperm cryopreservation techniques is thus imperative to realize an adequate preservation of genetic diversity in feline species [10].

Spermatozoa can be recovered after ejaculation using electrostimulation or recovered postmortem from the epididymides and/or ductus deferens [6,11,12]. No major differences appear to exist between ejaculated and epididymal spermatozoa with regard to motility, plasma membrane integrity, or morphology either before or after cryopreservation [13,14]. The timing of capacitation for epididymal and ejaculated sperm does not appear to differ [15]. Both epididymal and ejaculated frozen-thawed spermatozoa are able to fertilize oocytes in vitro [16–19] or by means of intracytoplasmic sperm injection (ICSI) [20]. Ideally, to ensure storage of good-quality samples of epididymal spermatozoa, collection and processing should be carried out immediately after the animal’s death. Sperm cells can survive for some time in the epididymides of dead animals, but deterioration in quality follows changes related to body decomposition [21] and increases as the postmortem interval increases. In wild species, death may occur unpredictably as a result of accidents (e.g., road kills) or disease and, most likely, will occur far away from the laboratory. Cool storage (5 °C) prolongs the survival of spermatozoa in the epididymides and allows extra time for sperm recovery and processing. Postmortem sperm recovery may provide an opportunity for males that have not reproduced or the possibility for a final sperm collection after death to preserve valuable genetic resources that otherwise would be lost [22].

Spermatozoa have been collected from the epididymides of several species [e.g., 23–25] including the domestic cat [e.g., 18,19,26]. To date, the results of studies that have examined sperm quality upon recovery after various days of epididymal storage at 5 °C have shown that motility decreases as storage interval increases [26]. Few studies have examined the integrity of the acrosome after storage [26,27]. Furthermore, even fewer studies have examined the ability of spermatozoa to withstand cryopreservation after recovery from epididymides stored for several days at 5 °C [18].

Cryopreservation causes a decrease in sperm motility and viability [28]. In addition, freezing and thawing may result in high percentages of damaged acrosomal membranes [29] that may compromise the sperm’s ability to participate in fertilization. The structural normality and integrity of the sperm cell is an important predictor of sperm survival after cryopreservation [7]. On the other hand, sperm from different species vary in their cryosensitivity (i.e., differences in endurance to cold-shock) [30–32], and, consequently, optimal cryopreservation protocols are usually species-specific. One factor that may affect sperm cryosurvival is the time of exposure to the cryoprotectant agent (usually glycerol). Long-term, extended exposure to cryoprotectant may be detrimental; therefore, it is necessary to assess whether exposure of spermatozoa stored in the epididymides for varying periods of time affects the time needed to equilibrate with glycerol.

The aims of the current study, using domestic cat epididymal spermatozoa, were to test (1) the effect of epididymides storage time at 5 °C on sperm quality upon recovery and after cryopreservation and (2) the effect of time of cryoprotector (glycerol) exposure before freezing (equilibration time) on sperm quality after thawing.

2. Materials and methods

2.1. Animals

Testes and epididymides from a total of 126 sexually mature domestic cat (Felis catus) males were used. Testes were obtained throughout the year from veterinary clinics and animal shelters located in Madrid. Testes and epididymides were placed in a resealable plastic bag (11 × 7 cm), and the bag was placed in a polystyrene container (25 × 17 × 11.5 cm) for transport to the laboratory at ambient temperature within 2 to 12 h after castration.

2.2. Epididymal sperm collection

Samples were randomly allocated to one of eight experimental groups: four storage times (0, 24, 48, and 72 h at 5 °C) and two glycerol equilibration times (30 or 120 min). Control (nonstored) epididymides were handled at room temperature, whereas all others were stored in a cold room (5 °C). Epididymides were dissected and cut transversally with a scalpel blade, and the caudae epididymides (distal portion) placed in 0.4 mL of cryodiluent (see later) in a Petri dish.

[Note: The rest of the text is not visible in the image.]
and coverslip (prewarmed to 37°C) to a 1.5-mL sterile microtube. An aliquot of each sperm sample was placed between a microslide and Petri dish to a 1.5-mL sterile microtube. An aliquot of volume (with a micropipette) and transferred from the final concentration of glycerol.

2.3. Sperm evaluation

Sperm suspensions were evaluated immediately for volume (with a micropipette) and transferred from the Petri dish to a 1.5-mL sterile microtube. An aliquot of each sperm sample was placed between a microslide and coverslip (prewarmed to 37°C) and examined using phase-contrast optics to assess the percentage of motile spermatozoa and the quality (vigor) of motility (using a scale from 0 to 5, where 0 is no movement and 5 represents rapid, linear forward movement [33]). A sperm motility index (SMI) was calculated using the formula: (% motile sperm + [quality × 20])/2 [34]. One sperm aliquot (5 μL) was fixed with 45 μL 1% glutaraldehyde solution in 0.165 M cacodylate buffer (pH 7.3) and used to estimate sperm concentration using a hemocytometer. Another 10 μL sperm subsample was fixed in 250 μL 4% paraformaldehyde in 110 mM Na₂HPO₄ and 2.5 mM NaH₂PO₄ buffer solution (pH 7.4), stored at 4°C, and later stained with Coomassie blue [35] to assess acrosome integrity and sperm morphology. For sperm morphology assessment, a total of 100 sperm cells were examined using bright-light microscopy and classified as normal or with abnormalities in the head, midpiece, or principal piece (i.e., rest of the flagellum) [36]. The percentage of intact acrosomes was quantified in each sample by counting another set of 100 spermatozoa at ×1000; acrosomes were classified as intact, damaged, or missing [37].

2.4. Sperm cryopreservation

Spermatozoa collected from epididymides stored at 5°C for 24, 48, or 72 h were suspended in cryodiluent at room temperature, loaded into straws, cooled for 30 or 120 min until they reached 5°C, and frozen. In another set, spermatozoa in cryodiluent were cooled for 120 min to 5°C, loaded into straws at 5°C, and frozen. In all cases, 50 μL sperm suspension was loaded into short straws. Before loading, 0.25-mL straws (Minitüb) were cut in half with a pair of scissors, and the half of the straw with the cotton-sealed end (7 cm long) was used. After loading, straws were closed with a thermal sealer (ERSA; Minitüb). Straws were frozen on nitrogen vapors using a two-step freezing method [7] and transferred into liquid nitrogen for storage.

2.5. Sperm thawing and assessment

Straws were thawed by exposing them to air (at room temperature) for 10 s and then by immersing them for 30 s in a bath containing sterile 0.9% (vol/vol) NaCl at 37°C. The straw contents were poured into a sterile 1.5-mL microtube prewarmed to 37°C. Then, spermatozoa were diluted (1:3 v:v) dropwise with a HEPES-containing modified Ham’s F-10 medium (Irvine Scientific cat. no. 99168; Izasa, Barcelona, Spain) supplemented with modified Ham’s F-10-Gentamicin-50X (Irvine Scientific cat. no. 1290127; Izasa), which is supplied lyophilized and results in a final concentration (when diluted 1:50) of 1 mM glutamine, 1 mM pyruvate, and 10 μg gentamicin/mL. The medium was further supplemented with L-glutamine (Sigma, Madrid, Spain; final concentration 2 mM), pyruvate (Sigma; final concentration 1 mM), and triple antibiotic (Sigma; final concentrations: penicillin, 130 IU/mL; streptomycin, 130 μg/mL; neomycin, 260 μg/mL) and 5% inactivated fetal bovine serum (Gibco, Invitrogen, Barcelona, Spain). Subsamples were taken to assess motility and the percentage of intact acrosomes postthaw. The remaining sperm suspension was incubated at 37°C under air, and sperm subsamples were evaluated for motility and acrosome integrity at 1.5, 2.5, and 3.5 h postthaw.

2.6. Experimental design and statistical analysis

A mixed (split plot) design was employed to investigate the effects of epididymal storage time and glycerol equilibration during the collection/cryopreservation process. Analyses were carried out using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Data were examined for normality and homoscedasticity, and both were verified. Results are presented as mean ± SEM. A value of P < 0.05 was regarded as statistically
significant. Possible differences in sperm parameters between and within years were analyzed using one-way ANOVA and post hoc Tukey test. Effects of epididymides storage time and equilibration time on SMI and percentage of intact acrosomes were analyzed by a General Linear Model (GLM) where cryopreservation time was the repeated measures factor, and epididymal storage time and equilibration time with glycerol were between-subjects measures. Subsequently, post hoc pairwise comparisons were carried out. Differences between control samples loaded into the straws before or after cooling were evaluated using a t-test for two independent samples. Relationships between sperm parameters were analyzed by nonparametric Spearman rank correlation.

3. Results

3.1. Spermatozoa collected from stored domestic cat epididymides

Sperm samples were collected from epididymides during a period of 3 yr (2005 to 2007). Comparisons of sperm traits between years showed no significant differences. Similarly, no significant differences were observed throughout each year. Therefore, data were pooled for analyses.

Significant differences (P < 0.01) due to storage time were found in the percentage of motile sperm, quality of motility, and, consequently, SMI in recently collected spermatozoa (Table 1). The SMI diminished significantly after 48 h (48.21 ± 1.47) and 72 h storage (43.03 ± 1.32) with respect to control nonstored epididymides (55.91 ± 2.02) (Table 1). In contrast, the percentage of morphologically normal spermatozoa and percentage of spermatozoa with intact acrosomes were similar in the control and after storing for 24, 48, or 72 h (Table 1).

3.2. Sperm cryosurvival

Comparison of different sets of control samples (i.e., sperm collected from nonstored epididymides, loaded in straws before or after cooling to 5 °C, and frozen in nitrogen vapors) revealed no significant differences between them in SMI or percentage of intact acrosomes during the cryopreservation process and incubation postthaw (not shown). There was a significant decrease in SMI values after thawing in comparison with fresh samples when spermatozoa from nonstored epididymides were refrigerated/equilibrated for 30 min (Fig. 1). No such decrease was observed in spermatozoa refrigerated/equilibrated for 120 min. On the other hand, there was a significant decrease in the percentage of intact acrosomes after thawing in relation to values recorded in fresh samples for spermatozoa refrigerated/equilibrated for 30 or 120 min (Fig. 1C, D).

Spermatozoa collected from epididymides stored for 24, 48, or 72 h showed no significant decreases in SMI values within each experimental group due to cryopreservation; that is, SMI values after thawing within each group were not significantly different from those before freezing (Fig. 1A, B). Nevertheless, differences in SMI values between spermatozoa collected from epididymides stored for various times (with highest SMI values for sperm from epididymides stored for 24 h and lowest for sperm from epididymides stored for 72 h) were also present after thawing, and this was evident for both equilibration times (Fig. 1A, B). With an equilibration time of 120 min, the highest SMI value after thawing was observed in the control group (SMI 56.94 ± 3.33), significantly different from samples that were stored for 24 h (SMI 48.93 ± 1.90), 48 h (SMI 42.5 ± 3.69), or 72 h (SMI 45.00 ± 2.65).

The percentage of spermatozoa with intact acrosomes at recovery was not different among groups, regardless of the length of the storage of epididymides.
Fig. 1. Cryosurvival of domestic cat spermatozoa after storage of epididymides for 0, 24, 48, and 72 h at 5°C. Spermatozoa were collected in a Tris-based diluent (Biladyl) containing 20% egg yolk and 4% glycerol. Sperm-glycerol equilibration time was (A, C) 30 min or (B, D) 120 min. (A, B) Sperm motility index (SMI) and (C, D) % sperm with intact acrosomes. Steps in cryopreservation: 1, upon addition of cryodiluent (just after collection); 2, after equilibration with glycerol (just before freezing); 3, after thawing and dilution in Ham’s F-10 medium containing 5% fetal bovine serum. White bars, control; no storage; light gray bars, epididymides stored for 24 h; dark gray bars, epididymides stored for 48 h; black bars, epididymides stored for 72 h. Within cryopreservation steps (1, 2, or 3), different letters indicate significant differences between epididymides storage times. Results are means ± SEM. N = 100 males distributed randomly between groups.

Fig. 2. Postthaw incubation of spermatozoa that were cryopreserved after storage of epididymides for 0, 24, 48, and 72 h at 5°C. Sperm motility index (SMI) of spermatozoa during incubation postthaw at 37°C in Ham’s F-10 medium with 5% fetal bovine serum. Spermatozoa equilibrated with glycerol for (A) 30 min or (B) 120 min before freezing. White bars, control, no storage; light gray bars, epididymides stored for 24 h; dark gray bars, epididymides stored for 48 h; black bars, epididymides stored for 72 h. Within incubation times, different letters indicate significant differences between epididymides storage times. Results are means ± SEM. N = 100 males distributed randomly between groups (same males as in Fig. 1).
A significant decrease in the percentage of intact acrosomes was seen between samples examined after collection and just before freezing when samples were equilibrated with glycerol for 30 min, but no further significant decrease was detected after thawing. No significant decrease in the percentage of intact acrosomes was recorded in spermatozoa equilibrated with glycerol for 120 min.

When thawed spermatozoa were incubated for 3.5 h in F-10 medium containing 5% FBS, a decline in SMI values was observed in sperm samples regardless of the time of epididymides storage interval or time of equilibration with cryoprotectant (Fig. 2A, B). The significant differences in SMI values seen upon thawing in spermatozoa from various storage intervals remained during incubation postthaw. This was particularly evident for spermatozoa equilibrated with glycerol for 30 min (Fig. 2A) and less so for spermatozoa equilibrated for 120 min (Fig. 2B).

3.3. Equilibration time with cryoprotectant

In sperm samples collected from epididymides stored for 24 or 48 h and equilibrated with glycerol for 30 or 120 min, there were no significant differences between samples in either SMI values or percentage of intact acrosomes after thawing. On the other hand, when epididymides were stored for 72 h before sperm collection and cryopreservation, there were differences between the two equilibration times with glycerol (30 vs. 120 min). After equilibration (before freezing), no significant differences were observed in SMI (Fig. 3A), but a significantly higher percentage of sperm with intact acrosomes was noted after equilibration for 120 min (Fig. 3B). After thawing, there were significant differences in both SMI (Fig. 3A) and the percentage of intact acrosomes (Fig. 3B), with higher values for samples equilibrated with glycerol during 120 min. Incubation after thawing of sperm equilibrated for 30 or 120 min in glycerol (from epididymides stored for 72 h before sperm collection) revealed that differences in SMI seen after thawing remained after 3.5 h of incubation, with significantly higher values for sperm.
equilibrated for 120 min than for those equilibrated during 30 min (Fig. 4).

No significant interactions between epididymides storage time and equilibration time in cryoprotectant (glycerol) were found.

3.4. Correlation between sperm morphology and storage- or cryopreservation-induced sperm damage

No significant relationship was found between the proportion of normal spermatozoa, or the proportion of sperm with intact acrosomes, and sperm motility (percentage of motile sperm or SMI) among samples at collection (not shown).

No significant correlations were found between percentages of sperm with normal morphology or intact acrosomes at collection and SMI values or intact acrosomes after thawing, in the groups with epidydymal storage intervals of 0 or 24 h (not shown). Significant relationships were detected between percentages of intact acrosomes at collection and postthaw in samples from epididymides stored for 48 h ($r_s = 0.40; P = 0.05$) or 72 h ($r_s = 0.77; P < 0.01$).

After thawing, a significant correlation ($r_s$ range, 0.36–0.57; $P < 0.05$) was found between the percentage of intact acrosomes and the percentage of motile sperm in all groups.

4. Discussion

This study addressed the effects of duration of storage of epididymides at 5°C before sperm collection and time of equilibration of spermatozoa with cryoprotectant (glycerol) at 5°C on motility and acrosome integrity and morphology before and after cryopreservation. The results showed that sperm motility at collection decreases as the duration of the epidydidymal storage interval increased, that this reduction in sperm motility is also seen after cryopreservation, and that 120 min equilibration with glycerol rendered better results than did exposure for 30 min.

Studies on storage of epididymides for various times before retrieval of viable spermatozoa have been reported in the mouse [24], dog [25], and various domestic (boar [23], ram [38], bull [39]) and wild (sika deer [40], Iberian red deer [41], buffalo [42,43], eland [43,44]) ungulates. Studies in domestic cat [26], mouse [24], sika deer [40], and red deer [45] have shown that sperm viability is more resilient to storage time than is sperm motility and that viability was not affected by duration of storage interval between the animal’s death and recovery of spermatozoa. Normal live mouse fetuses have been obtained using ICSI with immotile spermatozoa retrieved 20 days after death [24].

We found that motility of epididymal domestic cat spermatozoa was affected by the time of epididymides storage. Motility of sperm collected after 24 h of storage was not different from that of sperm recovered from freshly collected, nonstored epididymides, an observation that is in agreement with a previous report [18]. On the other hand, longer storage times of 48 or 72 h resulted in a significant reduction in motility, in agreement with studies in which epididymides were stored for up to 72 h [26]. In the latter study, the proportion of motile spermatozoa was numerically lower than that seen in the current study. Autolytic changes within epididymides could be a reason for the reduction in sperm motility during storage in the epididymides before collection [26]. The decline in sperm motility associated with epididymal storage time was similar to that seen when domestic cat epididymal spermatozoa were recovered just after castration, cooled to 5°C, and then incubated for 72 h [46]. In one report, domestic cat epididymal sperm stored at 4 to 5°C for more than 14 days still had ~50% motile sperm and ~85% viable sperm [47]. Despite the decrease in sperm motility during storage at 4 to 5°C, some authors have concluded that the storage of domestic cat sperm at refrigeration temperatures for up to 2 to 3 days may be a preferable option because of its convenience and procedural simplicity [48].

In nonstored cat epididymides, the percentages of intact sperm acrosomes (average ~60%) were not different from that seen in some earlier studies (~67% [27]) but were numerically lower than that reported by others (~80% [26]). We did not observe a considerable decrease in the percentage of intact acrosomes during storage for up to 72 h, although occasionally there were slight decreases. This agrees with previous work that also found high proportions of intact acrosomes during storage for 24 h [18] or for up to 72 h [26].

Spermatozoa recovered from nonstored epididymides experienced a decrease in SMI values and percentage of intact acrosomes due to cryopreservation when sperm cells were cooled/equilibrated during 30 min; a decrease in percentage of intact acrosomes was also seen during cryopreservation in spermatozoa from nonstored epididymides that were equilibrated for 120 min. On the other hand, sperm recovered from epididymides stored at 5°C for 24, 48, or 72 h showed no differences in SMI values or percentage of intact acrosomes after thawing with regards to values seen in recently collected sperm. These results were seen
regardless of whether sperm were equilibrated with glycerol for 30 or 120 min before freezing. Although there were no apparent differences between groups in cryosurvival, differences in SMI values noted upon sperm collection (i.e., highest values in nonstored samples and lowest values in samples from epididymides stored for 72 h) remained after thawing and were also maintained during the postthaw incubation interval. Thus, our results suggest that for the rescue of epididymal sperm from wild felids, collection and cryopreservation should be done as soon as possible after recovery to maximize sperm quality and subsequent use in assisted reproductive techniques. In contrast with previous studies [18,19], we found that acrosome integrity was largely maintained after thawing even in spermatozoa collected from epididymides stored for up to 72 h.

Comparison of the two equilibration times revealed significantly higher SMI values and percentage of intact acrosomes upon thawing with a long (120 min) rather than with a short (30 min) period of equilibration. It is possible that the cryoprotectant needs a longer period of time to stabilize sperm membranes. This agrees with results showing that, in domestic cat spermatozoa recovered from nonstored epididymides, cooling from room temperature (20 °C) to 5 °C over a period of 120 min is better than a shorter period of time (N. Gañán, M. Gomendio, E.R.S. Roldan, unpublished results). Because significant differences in SMI values were observed before freezing and after thawing when epididymides were stored for 72 h before sperm collection, it seems that sensitivity of spermatozoa to the extreme osmotic changes associated with cryopreservation increased with increasing duration of epididymal storage. During incubation after thawing, SMI values decreased only slightly within each group suggesting that there was no major damaging effect for postthaw survival due to cryopreservation. In the current study, sperm motility and percentages of intact acrosomes during postthaw incubation were higher than in a previous study in which another cryodiluent was used [27]. Differences in sperm characteristics between storage groups seen after sperm collection and immediately postthaw remained during the postthaw incubation period, suggesting that the main effect was due to epididymides storage time.

No significant correlations were found between percentages of normal sperm among recently collected spermatozoa and sperm motility or acrosome integrity after thawing. This agrees with an earlier report on domestic cat epididymal spermatozoa [27] but differs from the suggestion that there is a link between sperm morphology in fresh semen and survival during cryopreservation [7]. Ejaculated spermatozoa from normozoospermic and teratozoospermic cats have been reported to differ in their susceptibility to damage after cooling and after osmotic stress [49,50]. The lack of correlation between sperm morphology and cryopreservation-induced damage in studies on epididymal spermatozoa may be due to differences in membrane characteristics between epididymal and ejaculated spermatozoa.

In conclusion, we (1) successfully cryopreserved domestic cat spermatozoa from epididymides after storage at 5 °C for up to 3 days; (2) showed that sperm motility decreased as duration of epididymal storage interval increased, highlighting the importance of celerity in the recovery of epididymides after an animal’s death for the rescue and cryopreservation of spermatozoa from wild felids; (3) identified that a cryoprotectant equilibrium period of 120 min was significantly better for preservation of motility and acrosome integrity during cryopreservation than a short period of 30 min; (4) found no relation between abnormalities in sperm collected from the epididymis and sperm motility or acrosome integrity after cryopreservation; and (5) did not identify significant interactions between epididymal storage time and the time of equilibration with glycerol on sperm motility or acrosome integrity after thawing.

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