Computer-assisted sperm analysis of fresh epididymal cat spermatozoa and the impact of cool storage (4 °C) on sperm quality

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Abstract

Epididymal cat sperm is commonly used for in vitro fertilization. Because of the high variability in preparation protocols and methods of evaluation, sperm quality may vary considerably between experiments and laboratories. The aims of the present study were (1) to describe an epididymal sperm preparation protocol to produce clean, highly motile samples using density gradient centrifugation, (2) to provide reference values of computer-assisted semen analysis (CASA) parameters of fresh epididymal cat sperm after density gradient centrifugation and (3) to investigate the effect of cool storage on various spermatozoa characteristics. After slicing the epididymides, viable and motile sperm cells were isolated using Percoll® centrifugation. Sperm motility parameters were subsequently assessed using CASA in experiment 1. In experiment 2, fresh (day 0) sperm samples were evaluated for motility parameters (HTR) and stained for assessment of acrosomal status (FITC-PSA), morphology (eosin/nigrosin (E/N)), membrane integrity (E/N and SYBR®-14-PI) and DNA fragmentation (TUNEL). After addition of a Tris–glucose-citrate diluent containing 20% egg yolk, samples were cooled to 4 °C and reassessed on d1, d3, d5, d7 and d10. Cool storage impaired most motility and velocity parameters: MOT, PMOT, VAP, VSL, VCL, BCF, RAPID and the percentage of normal spermatozoa showed a decrease over time (P < 0.05) as compared to fresh samples. In contrast, STR, ALH, membrane integrity, DNA fragmentation and the percentage of acrosome intact spermatozoa were not affected by cool storage. However, the influence of cool storage of cat spermatozoa on subsequent in vitro embryo development and quality after IVF requires further investigation.

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1. Introduction

There are several methods to collect semen of a tomcat. A frequently used technique is electro-ejaculation [1–3]. This well-studied and reliable technique causes no apparent distress and can be used on any male that can be safely anesthetized [4]. A second method for obtaining cat semen samples is by use of an artificial vagina [5,6]. Using this method, no physical or chemical restraint is necessary. Nonetheless, for successful collection, a period of training is required, and not all males show a positive response [4]. For laboratories without access to the equipment or animals requisite for these two collection techniques, excised epididymides represent a practical alternative approach. Recovery of domestic cat spermatozoa from epididymides after routine orchietomy is a commonly used technique [7], especially in laboratories developing
techniques for assisted reproduction, such as in vitro fertilization [8] and artificial insemination [9]. Additionally, it opens the opportunity to conserve potentially valuable genetic material from postmortem males [4,10,11]. Epididymal sperm quality parameters, especially motility, vary considerably among laboratories [12–16], mainly due to the different methods for epididymal sperm processing and the subjectivity of the standard techniques currently used for microscopic analysis of feline semen [10,12,17–22]. Such variability makes it difficult to compare and interpret results, implicating the need to develop more objective and standardized methods for assessing cat spermatozoa.

The first validated system for computerized evaluation of sperm motility was developed three decades ago [23,24]. Nowadays, computer-assisted semen analysis (CASA) is widely used in human and veterinary andrology laboratories [25,26]. Stacecki et al. [17] were the first to describe the use of CASA for the domestic cat. The Hamilton-Thorne computer-based semen analyzer (HTR) objectively assesses various sample characteristics simultaneously and rapidly evaluates the motion of individual spermatozoa by processing digital images of the sperm cell tracks [25,26]. Consequently, subtle changes in motion characteristics can be detected which cannot be discovered by the use of conventional microscopy. The capability of detecting even the slightest alterations in movement is critically important when monitoring the effect of environmental or occupational stress on sperm [27]. Additionally, subtle changes in sperm motility and velocity patterns have been correlated with fertilizing ability in vitro and in vivo in several species, including rat, bull, man and dog [28–31].

Density gradient centrifugation has been used as a preparatory technique for processing ejaculated sperm samples in many species [32,33] by separating motile and potentially fertile spermatozoa from immotile sperm cells, tissue debris, blood cells and seminal plasma, creating a soft pellet containing a homogeneous population of motile spermatozoa [34–37]. Even though density gradient centrifugation has been shown to be beneficial for survival of spermatozoa in liquid storage [38], there are few reports describing the benefits of combining the two techniques. The enhanced survival of spermatozoa after gradient density centrifugation may be attributable to the elimination of bacteria [39] and/or reactive oxygen species arising from cell debris and dead spermatozoa [40].

For laboratories without the capability of collecting semen samples, cat testes must be obtainable consistently to conduct in vitro fertilization experiments on a regular basis. Since a steady supply of cat epididymides is not usually available to most laboratories, sperm samples are often stored temporal for later use. Cooling of spermatozoa prolongs the survival by lowering their metabolism and facilitates storage of important germplasm [41]. However, the effects of cooling and prolonged storage at 4 °C on several motility characteristics or on DNA status has not been studied in cat spermatozoa. Therefore, the objectives of the present study were (1) to describe an epididymal sperm preparation protocol to produce clean, highly motile samples using density gradient centrifugation, (2) to provide a set of reference values for CASA parameters of fresh epididymal cat sperm after density gradient centrifugation and (3) to investigate the effect of prolonged cool storage (4 °C) on various cat spermatozoa characteristics.

2. Materials and methods

2.1. Media

All chemicals and media were obtained from Sigma–Aldrich (Bornem, Belgium) and Life Technologies, Gibco BRL® products (Merelbeke, Belgium). Spermatozoa were released passively from the sliced epididymides, into Hapes-TALP medium (114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH2PO4, 2.1 mM CaCl2, 0.4 mM MgCl2, 2 mM NaHCO3, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 μg/ml gentamicin sulphate, 10 mM Hapes and 3 mg/ml bovine serum albumin) [42]. The composition of the Tris–glucose-citrate semen extender has been described by Iguer-ouada and Verstegen [43].

2.2. Collection and preparation of semen

Testes were recovered from tomcats subjected to routine orchiectomy at the Department of Small Animal Medicine and Clinical Biology, Ghent University and at several local veterinary clinics over a 4-month period (October to January). For each replicate, three to four pair of testes was used. The testes were placed immediately in a sterile 0.9% sodium chloride solution supplemented with 50 μg/ml gentamicin, and stored for up to 24 h at 4 °C. From each testis, the cauda epididymis and part of the vas deferens were dissected, sliced repetitively in a glass Petri-dish containing Hapes-TALP and placed in a conventional incubator at 39 °C for 20 min to allow for movement of the passively liberated spermatozoa into the surrounded medium. Sperm motility was checked with a phase contrast
microscope (magnification 100×) and the sample was centrifuged (5 min, 500 × g). After disposal of the supernatant, the sperm pellet was resuspended in 1 ml of Hepes-TALP, gently placed on top of a two-layer gradient (45:90) (Percoll™, GE Healthcare, Uppsala) and centrifuged (20 min, 500 × g). By carefully inserting a pipette tip through the gradient, the sperm pellet (150–250 μl) was recovered and placed into a sterile 1.5 ml micro-centrifuge tube for assessment of sperm quality immediately after retrieval (d0). Subsequently, the pellet was diluted (1:2) using Tris–glucose-citrate extender containing 20% egg yolk, allowed to cool gradually to 4 °C and cool stored for 10 days.

2.3. Epididymal sperm quality assessment

2.3.1. Experiment 1: establishing reference CASA parameters for fresh epididymal cat spermatozoa after density gradient centrifugation

In experiment 1, several sperm motility parameters were assessed immediately after retrieval (d0) using a computer-assisted semen analyzer (CASA), the HTR Ceros 12.1 (Hamilton-Thorne Research, Beverly, MA, USA). This computerized measuring device includes a phase contrast microscope, a camera, a minitherm stage warmer, an image digitizer and a computer to store and analyze data [26]. The software settings of the HTR Ceros 12.1 used in the present study are summarized in Table 1. For each measurement, an aliquot (8 μl) of fresh undiluted sperm sample was mounted on a prewarmed Leja® counting chamber (Orange Medical, Brussels, Belgium) and immediately placed in the minitherm stage warmer. Five randomly selected microscopic fields were scanned five times each. Thus, each sample was scanned 25 times. After every scan, we used the playback function to ensure that all spermatozoa were identified correctly and that their trajectory could be reconstructed properly [26]. The resultant measurements were used to calculate concentration (CONC, ×10⁶/ml), motility (MOT, %), progressive motility (PMOT, %), the velocity average pathway (VAP: the average velocity of the smoothed cell path (μm/s)), the velocity straight line (VSL: the average velocity measured in a straight line from the beginning to the end of track (μm/s)), the velocity curved line (VCL: the average velocity measured over the actual point-to-point track of the cell (μm/s)), the amplitude lateral head (ALH: amplitude of lateral head displacement (μm)), the beat cross-frequency (BCF: frequency of sperm head crossing the sperm average path (Hz)), the straightness (STR: the average value of the ratio VSL/VAP (%)), and the linearity (LIN: the average value of the ratio VSL/VCL (%)) [25]. Consistent with the low VSL cut-off value and the low (LVV) and medium (MVV) VAP cut-off values, spermatozoa were categorized in three groups of movement: rapid (RAP; with VAP > MVV), slow (SLOW; with VAP < LVV) and static (STATIC). This experiment was replicated 20 times. Descriptive data are presented as mean ± 1.96 × S.D. For seven additional sperm samples, motility and concentration were also evaluated by means of light microscopy and a Bürker counting chamber (Merck, Leuven, Belgium), respectively, in order to compare the CASA results with conventional semen analysis methods. Pearson’s correlations between the results obtained for motility, progressive motility and concentration by the HTR Ceros 12.1 and by subjective evaluation were established. The mean differences between the subjective and objective measurements and their 95% CI were computed.

2.3.2. Experiment 2: effect of prolonged cooled storage (4 °C) on motility, morphology, plasma membrane integrity, acrosome status and DNA fragmentation

In experiment 2, sperm quality was determined before cooling (d0) and after 1 (d1), 3 (d3), 5 (d5), 7 (d7) and 10 days (d10) of storage. The whole experiment was replicated four times. In addition to CASA assessment of sperm motility characteristics, sperm morphology and plasma membrane integrity were evaluated using eosin/nigrosin staining. Additionally, sperm plasma membrane integrity was determined after samples were stained with SYBR-14 and propidium iodide (PI) (LIVE/DEAD® Sperm Viability Kit, Molecular Probes, Leiden, The Netherlands) and evaluated by use of Leica DMR fluorescent microscopy (magnification 400×, immersion oil). Briefly, a stock solution of 1 mmolL⁻¹ SYBR-14 reagent was diluted (1:50) in Hepes-TALP, stored

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Table 1
Set up of the Hamilton-Thorne Ceros 12.1 used in the present study for the motility assessment of epididymal cat sperm

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frame rate (Hz)</td>
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<tr>
<td>Frames acquired</td>
<td>30</td>
</tr>
<tr>
<td>Minimum contrast</td>
<td>25</td>
</tr>
<tr>
<td>Minimum cell size (pixels)</td>
<td>3</td>
</tr>
<tr>
<td>Non-motile head size (pixels)</td>
<td>15</td>
</tr>
<tr>
<td>Non-motile head intensity</td>
<td>80</td>
</tr>
<tr>
<td>Medium VAP cut-off (μm/s)</td>
<td>50</td>
</tr>
<tr>
<td>Straightness cut-off (%)</td>
<td>70</td>
</tr>
<tr>
<td>Low VAP cut-off (μm/s)</td>
<td>30</td>
</tr>
<tr>
<td>Low VSL cut-off (μm/s)</td>
<td>15</td>
</tr>
</tbody>
</table>
frozen at −20 °C and thawed just before use. A mixture of 25 μl sperm suspension and 200 μl Hapes-TALP was incubated with 1.25 μl SYBR-14 and 1.25 μl PI, a 10 μl aliquot was removed, placed on a microslide and mounted under a cover-slip. Spermatozoa with an intact plasma membrane stain fluorescent green with SYBR-14, while those with a damaged membrane exhibit red fluorescence. Moribund or slightly damaged spermatozoa exhibit a dual-staining pattern [44] and were counted as cells with a damaged membrane. For each sample, at least 200 spermatozoa were assessed in duplicate.

Furthermore, acrosome integrity was evaluated after staining with Pismus Sativum Agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) using fluorescence microscopy (magnification 400×, immersion oil). In brief, 25 μl of sperm suspension was washed in 200 μl of Hapes-TALP and centrifuged (2 min, 500 × g). After removal of the supernatant, the sperm pellet was resuspended in 50 μl absolute ethyl alcohol (EtOH; Vel cat no.: 1115, Haasrode, Belgium), cooled for 30 min in the refrigerator, and then 50 μl of EtOH was added again to the suspension. A 20 μl aliquot of the resultant suspension was smeared onto a glass microslide and allowed to air-dry. Then, 20 μl of FITC-PSA (2 mg PSA-FITC diluted in 2 ml phosphate-buffered saline) was added and held for 15 min at 4 °C. Subsequently, the spermatozoa on the glass slide were washed with fresh water, type 2 (B60, B. Braun Medical N.V/S.A., Diegem, Belgium). At least 200 spermatozoa were evaluated. Acrosome intact (AI) spermatozoa were characterized by intense green fluorescence in the acrosomal area, while for acrosome reacted spermatozoa, fluorescence was restricted to the equatorial area.

Additionally, we conducted a Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Boehringer, Mannheim, Germany) to detect the presence of free 3′-OH termini in single and double-stranded sperm DNA at different time points: d0, d1, d5, d7 and d10. Only sperm samples (1–2 pairs/replicate) with >75% motility were used. In short, sperm samples were diluted with PVP solution (1 mg/ml in PBS) to a final concentration of 10 × 10^6 sperm/ml from which a 10 μl aliquot was smeared onto a poly-L-lysine-coated microslide. After fixation with 4% paraformaldehyde in PVP solution (pH 7.4) and permeabilisation with 0.5% (v/v) Triton X-100 in PBS, the spermatozoa were incubated with TUNEL-mixture (fluorescein-dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37 °C in the dark. Both positive (1 mg/ml DNAse I) and negative controls (nucleotide mixture in the absence of transerase) were included in each replicate. Hoechst 33342 was used to counter stain sperm DNA. Samples were examined by fluorescence microscopy (Leica DMR; magnification 400×, oil immersion). At least 200 spermatozoa from each sample were analyzed randomly to evaluate the percentage of TUNEL-positive sperm cells (bright green nuclear fluorescence) (see De Pauw et al. [42] for further details).

The software packs S-Plus (Version 7.0) and MLwiN (Version 2.02) were used for the statistical analyses. The effect of prolonged cooled storage on the different sperm quality parameters was investigated in a linear mixed effect model, with ‘replicate’ (n = 4) as a random factor and ‘day’ as a fixed factor. The effect of prolonged cooled storage on apoptosis was investigated using binary logistic regression, with ‘apoptosis’ as the binary outcome variable (0 = not apoptotic; 1 = apoptotic), ‘replicate’ (n = 4) as a random factor and ‘day’ as a fixed factor. The level of significance was set at P < 0.05.

3. Results

3.1. Experiment 1

The results of the HTR analysis are summarized in Table 2. The reference values for the different HTR-parameters of fresh epididymal cat sperm obtained by density gradient centrifugation (Percoll) are presented as mean ± 1.96 × S.D. Separation of motile and non-motile sperm by density gradient centrifugation produced samples in which both velocity and motility values were relatively high. The high STR (89.6 ± 6.6) corresponded to a high percentage of progressively motile spermatozoa (69.9 ± 23.2). Sperm concentration

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAP (μm/s)</td>
<td>98.7 (24.2)</td>
</tr>
<tr>
<td>VSL (μm/s)</td>
<td>89.3 (25.4)</td>
</tr>
<tr>
<td>VCL (μm/s)</td>
<td>134.8 (31.9)</td>
</tr>
<tr>
<td>ALH (μm)</td>
<td>4.3 (2.0)</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>34.6 (7.0)</td>
</tr>
<tr>
<td>STR (%)</td>
<td>89.6 (6.6)</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>68.0 (18.6)</td>
</tr>
<tr>
<td>MOT (%)</td>
<td>80.8 (23.5)</td>
</tr>
<tr>
<td>PMOT (%)</td>
<td>69.9 (23.2)</td>
</tr>
<tr>
<td>RAPID (%)</td>
<td>75.9 (25.1)</td>
</tr>
<tr>
<td>SLOW (%)</td>
<td>10.7 (15.0)</td>
</tr>
<tr>
<td>STATIC (%)</td>
<td>7.3 (15.0)</td>
</tr>
<tr>
<td>CONC (×10^6/ml)</td>
<td>41.2 (64.8)</td>
</tr>
</tbody>
</table>
varied widely among the samples. Pearson’s correlations between MOT, PMOT and CONC using the two assessment methods (i.e. HTR Ceros 12.1 and conventional semen analysis) were 0.82, 0.82 and 0.97, respectively ($P < 0.05$). The subjective assessment of MOT and PMOT resulted in a mean overestimation of 16.0% (95% CI: 6.5–25.5) and 16.6% (95% CI: 7.6–25.6), respectively, compared to the objective assessment. The subjective assessment of CONC resulted in a mean difference of −1.1% (95% CI: −5.5–3.2).

3.2. Experiment 2

The results of Experiment 2 are summarized in Table 3. Most motility and velocity parameters – VAP, VSL, VCL, BCF, MOT, PMOT, RAPID and STATIC – showed a significant decrease over time ($P < 0.05$) compared to fresh semen samples and the values became statistically different beginning on days 3, 5, 3, 7, 1, 1, 1 and 1, respectively. In contrast, STR, LIN and ALH of cooled samples did not differ significantly over time compared to fresh samples. A quarter and one fifth of the spermatozoa were still motile or progressively motile after 10 days of preservation (24.7% ± 9.7 and 19.3% ± 9.3, respectively). The percentage of live (intact plasma membrane) sperm cells as assessed by eosin/nigrosin staining did not decrease ($P=0.11$) during 10 days of cool storage, an observation which was confirmed by SYBR<sup>H</sup>-14-PI staining. However, consistently lower percentages of intact spermatozoa were found using SYBR<sup>H</sup>-14-PI staining as compared to eosin/nigrosin staining. Storage at 4 °C significantly decreased the percentage of normal spermatozoa ($P < 0.0001$) between d0 and the time points (d1, d3, d5, d7 and d10) after cooling. At all time points examined from day 1 to 10, the percentage of normal spermatozoa was significantly lower after storage at 4 °C. The percentage of spermatozoa with abnormal heads ($P < 0.0001$) and abnormal tails ($P=0.0034$) was higher after 3 days of storage than after 1 day. The occurrence of spermatozoa with proximal and distal protoplasm droplets was unaffected by cool storage. Although acrosomal status was not significantly affected by storage at 4 °C for 10 days, there was a tendency towards an increase in the percentage of acrosome reacted spermatozoa ($P = 0.14$). Prolonged cool storage did not have a significant effect ($P = 0.91$) on the proportion of apoptotic sperm cells.

4. Discussion

In the present study, we have provided a sperm preparation protocol and presented values of motility parameters which could serve as standard references for CASA analysis of fresh epididymal cat spermatozoa. By the use of a gradient centrifugation technique (Percoll<sup>E</sup>), we obtained fresh epididymal sperm samples with consistently higher motility values than...
has been reported in previous studies [12,15,16,21,45]. This can be explained by a number of reasons. First, the high motility characteristics of spermatozoa are a result of the Percoll® centrifugation we have applied to all fresh sperm samples, leading to the isolation of high quality spermatozoa from the sperm sample. In addition to density gradient centrifugation [37], a variety of techniques for sperm separation have been described that create a higher yield of motile, viable and non-apoptotic spermatozoa in different species [32,33,36,46,47]. In cats, swim-up procedures have been used to recover a high proportion of motile, structurally normal and viable sperm cells from teratozoospermic ejaculates [48–50] and are considered to be a suitable approach to compensate for the drop in motility after cryopreservation [10]. Density centrifugation, while widely used in bovine IVF [38], has not been applied previously for cat IVF, except for zona pellucida binding [51]. Our intention in the present study was to produce clean, highly motile epididymal sperm samples that, after temporary storage at 4 °C, would result in optimal fertilization capacity when used subsequently for IVF. However, it was usually necessary to pool three to four sets of testes to obtain a sufficient number of spermatozoa after filtration and separation by gradient density centrifugation. In a preliminary experiment (data not shown), we found out that after Percoll® centrifugation, the concentration of the sperm sample was approximately half of the original concentration. Although this procedure enhances the percentage of motile and normal spermatozoa, Percoll® centrifugation has the disadvantage of producing a lower final yield of sperm cells. While the primary reason for combining multiple sets of testes was therefore quantitative, a side-benefit of the pooling was that individual variation was reduced. Another important advantage of density centrifugation is the separation of the potentially fertile spermatozoa from tissue debris, seminal plasma, blood cells, reactive oxygen species and pathogens [37,38]. Removal of seminal plasma before cool storage of domestic cat semen had no effect on motility, viability and acrosomal status [13]. Although the presence of blood and serum has no immediate effect on several sperm parameters of different species during storage, it appears to have a negative influence on cryopreserved sperm, possibly due to the release of hemoglobin after freezing/thawing [52]. Additionally, blood and serum seem to have a negative effect on the in vitro fertilizing capacity of bovine sperm [53], a consequence which should be considered in the preparation of epididymal sperm for IVF.

Another explanation for the disparity in motility rates that have been reported from different laboratories may be related to differences in the method of assessment. Usually, motility of cat sperm samples is estimated subjectively by visual observation with phase contrast microscopy [12,14,18,21,45]. In the present study, computer-assisted sperm analysis (CASA) was used for the assessment of several motility and velocity patterns. Diminutive differences in sperm motion patterns can be discriminated by CASA, making the technique suitable for monitoring the effects of environmental factors on sperm motion patterns [27]. Although this technique has proven to be rapid, objective and useful for evaluation of sperm motility in several species, there are few reports that describe the motility characteristics of cat spermatozoa as determined by CASA [17,54–56]. Our goal of establishing reference values for cat epididymal sperm samples obtained by density gradient centrifugation can therefore facilitate comparisons of sperm quality and in vitro fertilization among laboratories. The perceptible variation in sperm parameters among replicates may partly be attributable to the small differences in storage time between orchietomy and sperm collection in the present study.

High and positive correlations were found between microscopical and computerized determination of MOT, PMOT and CONC. The values obtained for MOT and PMOT by the CASA system were lower in comparison with the results of the subjective assessment. This overestimation of the subjectively evaluated motility is in agreement with Davis and Katz [57], who proposed several possible explanations for this finding.

In experiment 2, a gradual decrease in most motility and velocity parameters was observed during cool storage, although motile sperm cells were still present after up to 10 days. The chronology of cat sperm motility during cool storage has been examined previously [13,14,16,58,59]. A decrease in mean motility and vigor, attributable to cooled storage, was found in all of the studies; however, the rate at which motility decreased over time was variable. The temporal inconsistencies are probably a result of the type of extender, the presence of egg yolk and differences in sperm preparation protocols or evaluation methods [13,14].

Our results are in agreement with a previous report [14] in which the percentage of morphologically normal spermatozoa significantly decreased during extended cool storage. While Villaverde et al. [14] found that one half (52.7%) of the epididymal spermatozoa were morphologically abnormal, we observed a much lower rate of 10.7%. The explanation for this incongruity is undoubtedly our use of density gradient centrifugation
in the sperm preparation protocol. In our study, the percentage of abnormal heads and abnormal tails increased significantly during cool storage, possibly due to repeated pipetting and cold shock, respectively.

An important indicator of sperm viability is the integrity of the plasmalemma. Mammalian spermatozoa are known to contain high amounts of unsaturated fatty acids, making them susceptible to oxidative stress [60]. In our study, cool storage of sperm cells during 10 days in a Tris–glucose-citrate semen extender had no significant influence on the plasmalemma integrity as determined by SYBR®-14-PI or eosin/nigrosin staining. Although the motility declined rather quickly, the plasmalemma integrity was preserved quite well during cold storage. We hypothesize that this phenomenon is probably caused by irreversible damage of the motility apparatus and of mitochondrial DNA [42]. We found consistently lower percentages of membrane-intact spermatozoa using dual fluorescent SYBR®-14-PI staining than we did with eosin/nigrosin staining. Our observation is in agreement with several reports in other species, in which the proportion of membrane intact spermatozoa detected by E/N and trypan-blue vital stains was significantly higher than the proportion of membrane intact spermatozoa detected by PI-based fluorescence [42,61,62]. Possible explanations for this phenomenon can be the superior sensitivity and rapidity with which fluorescent probes can detect minor membrane defects [61] or differences in exposure time to the stain [63]. Siemieniuch and Dubiel [59], however, found no difference between these two methods of viability assessment for cat spermatozoa.

The acrosome reaction is a crucial step during gamete interaction which occurs as the sperm approaches the zona pellucida. Cryopreservation has been shown to induce loss of acrosomal integrity in cat epididymal spermatozoa [10]. Similarly, cooling of cat spermatozoa was shown to cause extensive damage to acrosomal membranes [64] with gradual deterioration of acrosomal integrity over time [16,55,65]. In the present study, acrosomal status did not change during time in storage and cooling as such did not appear to damage the acrosomes. Still, a tendency towards a decrease in the percentage of acrosome intact sperm cells was observed during cool storage at 4 °C, but this decrease seemed to be more a result of time than of cold shock.

Routine semen analysis in cats does not include evaluation of DNA fragmentation. However, DNA assessment is important since spermatozoa with DNA damage may be able to fertilize an oocyte, which potentially could disturb (epi)genetic regulation of the early embryo and block further development [66,67]. Under the conditions of the present study, we observed no significant influence of extended cool storage on DNA integrity status of epididymal cat spermatozoa. Fraser and Strzezek [68] used the Comet assay to detect double-stranded DNA-breaks during liquid storage of boar semen and found a gradual increase in the percentage of spermatozoa with damaged DNA during storage for 96 h at 5 and 16 °C. Boe-Hansen et al. [69] used a sperm chromatin structure assay to show an increase in DNA fragmentation of extended boar semen during storage at 18 °C for 72 h, although there was variation between animals. Individual differences in sperm DNA fragmentation have been previously reported in the stallion. Love et al. [70] found that degradation of chromatin quality occurred at different rates, and was dependent on storage temperature and fertility status. The authors suggested that the variation could be related to the inherent chromatin quality or to environmental factors. Recently, Shahiduzzaman and Linde-Forsberg [65] stored pooled dog semen at 5 °C for 23 days and found no disintegration of DNA until day 14, which is in agreement with our results. In the present study we found no significant influence of cool storage on DNA integrity status of epididymal cat sperm in samples that had ≥70% motility. In contrast, DNA fragmentation increased significantly over time in sperm samples with an initial motility of <40% (data not shown). Mota and Ramalho-Santos [15] reported that DNA fragmentation was negatively correlated with motility parameters of fresh epididymal cat spermatozoa. In the previous study, the percentage of DNA damaged sperm with DNA damage (13% ± 12) was higher than that observed in the present study (4% ± 3.4). Most likely, the discrepancy was a result of our use of density gradient centrifugation to isolate a highly motile fraction of structurally normal, non-apoptotic spermatozoa [46,71].

To summarize, we have described a method for recovery of cat epididymal spermatozoa that uses gradient density centrifugation to produce clean, highly motile samples. Additionally, we are presenting a set of CASA-derived motility parameters that will provide reference values for future studies. We subsequently determined that cool storage (4 °C) of epididymal samples for 10 days impaired most motility and velocity parameters and lowered the percentage of morphologically normal spermatozoa, but did not influence membrane integrity, acrosomal status or DNA fragmentation. The consequential influence of temporary cool storage of cat spermatozoa on in vitro embryo development and quality after in vitro or in vivo insemination requires further investigation.
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