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ScienceDirect
Theriogenology 70 (2008) 1550–1559
Theriogenology
www.theriojournal.com

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

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Received 13 February 2008; received in revised form 26 June 2008; accepted 1 July 2008

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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Keywords: Domestic cat; CASA; Cooled storage; Epididymal sperm; Percoll®

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 orchiectomy is a commonly used technique [7], especially in laboratories developing

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0093-691X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.07.004

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]. Stachecki 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 Hepes-TALP medium (114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 μg/ml gentamicin sulphate, 10 mM Hepes and 3 mg/ml bovine serum albumin) [42]. The composition of the Tris–glucose-citrate semen extender has been described by Iguerouada 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 Hepes-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

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