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Effects of cold storage on plasma membrane, DNA integrity and fertilizing ability of feline testicular spermatozoa

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ABSTRACT

This study examined the effects of cold storage on plasma membrane, DNA integrity, and fertilizing ability of domestic cat spermatozoa. Intact cat testes were stored at 4 °C in Dulbecco's phosphate buffered saline (DPBS) for 7 days. Membrane integrity (experiment 1) and DNA integrity (experiment 2) of extracted spermatozoa were assessed over time during storage. Testicular spermatozoa were also tested for their fertilizing ability *via* intracytoplasmic sperm injection (ICSI) in term of gamete activation and early embryonic development at 18 h (experiment 3).

The membrane integrity of testicular spermatozoa was well preserved in DPBS for 4 days compared to non-preserved control (Day 0) ($P < 0.05$). The incidence of testicular sperm DNA fragmentation was <1% after 7 days of cold storage and was not significantly affected by the duration of cold storage ($P > 0.05$). Finally, testicular spermatozoa could form pronuclei and sustain embryo development following ICSI regardless of the storage time ($P > 0.05$). In conclusion, cat testicular spermatozoa can be preserved at 4 °C for up to 7 days without severely compromising of plasma membrane and DNA integrity while retaining a normal fertilizing ability.

1. Introduction

Assisted reproductive technologies (ARTs) such as artificial insemination (AI), and *in vitro* fertilization (IVF) combined with gamete preservation can be used to support the management of wild felid populations (Pukazhenthi et al., 2006a) that rapidly decrease because of many threatening factors. These include illegal hunting, loss of habitats and inbreeding-related problems, such as immunodepletion-related illness (Peña et al., 2006) and infertility due to teratospermia (Pukazhenthi et al., 2006b). Spermatozoa used for these ARTs in domestic and wild cats are normally collected by electroejaculation (Zambelli and Cunto, 2006). Intracytoplasmic sperm injection (ICSI) with testicular spermatozoa offers many advantages. This technique allows the study and use of immotile intragonadal gametes from genetically valuable: (1) wild animals that die unexpectedly or are castrated for medical reasons, (2) adult males with obstructive and nonobstructive azoospermia (Levine et al., 2003) and (3) prepubertal males after xenografting of testis tissue in immunodeficient mice (Nakai et al., 2010). In humans, testicular spermatozoa yield similar percentages of fertilization, cleavage and pregnancy compared to epididymal spermatozoa (Silber et al., 1995) but these were reported to be slightly lower than ejaculated spermatozoa (Nagy et al., 1995; Qian et al., 2005). Until recently, only a few studies have used testicular spermatozoa for producing feline embryos (Comizzoli et al., 2006a,b).

Cold storage technique is a useful tool for 'short-term' preservation of sperm viability, particularly when transportation was delayed or in conditions that immediate use

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of testicular spermatozoa (*e.g.* embryo production and cryopreservation) cannot be performed. Besides, cold storage is more suitable for temporary storage than cryopreservation because it provided less effect on sperm qualities than cryopreservation (Zavos et al., 1980; England and Ponzio, 1996; Dondero et al., 2006; Siemieniuch and Dubiel, 2007). However, prolonged cold storage and suboptimal cold storage conditions also induce sperm damage of critical sperm components, such as plasma membrane, acrosome and chromatin (Shahiduzzaman and Linde-Forsberg, 2007). Although techniques for cold storage of testicular tissue have been developed, the success has been variable between techniques and across species employed (Jahnukainen et al., 2007; Zeng et al., 2009; Yang and Honaramooz, 2010). In domestic cats, testicular spermatozoa recovered from chilled testes (4 °C) for approximately 6 h have been successfully used to fertilize matured oocytes *via* intracytoplasmic sperm injection (ICSI) (Comizzoli et al., 2006a,b). However, the effects of prolonged cold storage on quality of feline testicular spermatozoa have not been examined. The objectives of this study were to examine the effects of cold storage on membrane and DNA integrity of domestic cat testicular spermatozoa, and also to assess the fertilizing ability of preserved testicular spermatozoa.

2. Materials and methods

All chemicals used in the experiments were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.1. Collection and cold storage of testes

Testes were obtained from adult domestic cats (approximately 1–5 yrs) sterilized at the Fertility and Neutering Clinic, Small Animal Teaching Hospital, the Faculty of Veterinary Science, Chulalongkorn University and The Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand. They were maintained in 0.9% (w/v) saline (NaCl) solution and transported to the laboratory within 3 h at ambient temperature. Upon arrival, external tissues were removed and only the testes (weighing between 1 and 1.8 g) encapsulated within the tunica albuginea were used in this study. The testes were washed and maintained at 4 °C in Dulbecco's phosphate buffered saline (DPBS) in a 15-ml conical tube (BD Falcon, Bedford, MA, USA).

2.2. Testicular sperm extraction

To extract the spermatozoa, testes were first decapsulated and then mechanically minced with sharp-ended scissors in DPBS at room temperature (25–27 °C). Spermatozoa were further extracted from minced tissue by gentle pipetting. The sperm suspension was finally filtered through 100 and 40 μm cell strainer (BD Falcon, Bedford, MA, USA), respectively. A total of 100–200 spermatozoa per sample were examined for each sperm quality test.

2.3. Assessment of sperm plasma membrane integrity

Extracted sperm samples were centrifuged and then stained with 2 μM ethidium homodimer-1, a membrane impermeable DNA stain (EthD-1: Invitrogen, Oregon, USA). Hoechst-33342 (3.5 μg/ml) was used as a counterstaining. The fluorescently labeled testicular spermatozoa were smeared onto a glass slide and examined with an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan) at 1000× magnification. Bright red sperm head (EthD-1 positive sperm) indicated the loss of plasma membrane integrity (dead spermatozoa, Fig. 1E), while spermatozoa negative to the EthD-1 were classified as viable spermatozoa (Fig. 1F).

2.4. Assessment of DNA integrity

Detection of DNA fragmentation was performed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (*In Situ* Cell Death Detection Kit; Roche, Mannheim, Germany) following the manufacturer's instructions. Because the late apoptosis (DNA fragmentation) occurs in a specific stage prior to necrosis (cell death), the EthD-1 staining was used to discriminate the dead sperm cells from apoptotic spermatozoa. After staining the extracted spermatozoa with 2 μM EthD-1 for 15 min, the EthD-1 was neutralized by adding excessive amount of salmon sperm DNA. The testicular spermatozoa were subsequently smeared onto a glass microscopic slide coated with aminopropyltriethoxysilane and air dried. The slides containing testicular spermatozoa were fixed with 4% (w/v) paraformaldehyde for 30 min. After a brief wash with phosphate buffered saline (PBS) supplemented with 0.1% (w/v) BSA (PBS–BSA), the sperm plasma membrane was permeabilized on ice with 0.1% (v/v) Triton X-100 in PBS for 5 min. To perform TUNEL assay, the slides were washed in PBS and incubated with a mixture of TUNEL reaction mix (TdT enzyme and nucleotide) for 1 h at 37 °C in a humidified chamber. Slides were counter stained with 4′6′ diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/ml). The antifade mounting medium (Vectashield™, Vector labs, Burlingame, USA) was used to mitigate the photobleaching. Spermatozoa positive to TUNEL displayed a bright green head under an excitation and emission wavelength of 460–490 nm and 520 nm, respectively (Fig. 1G). Spermatozoa positive to EthD-1 (dead spermatozoa) exhibited bright red sperm head, while live spermatozoa were negative to EthD-1 (Fig. 1F). The percentage of DNA fragmented spermatozoa was defined as the number of TUNEL positive spermatozoa in relation to the total number of live spermatozoa (*i.e.* EthD-1 negative spermatozoa).

2.5. Oocyte collection and in vitro maturation

Cumulus oocyte complexes (COCs) were recovered after mincing cat ovaries in holding medium (HM) consisted of HEPES-buffered M199, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin and 0.1% (w/v) bovine serum albumin (BSA, embryo tested grade). Only oocytes completely surrounded with more