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Freezing of epididymal spermatozoa from dogs after cool storage for 2 or 4 days

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Abstract

An experiment was conducted to investigate the freezing ability of canine epididymal spermatozoa after cool storage at 5 °C for 2 or 4 days. Spermatozoa were collected from the caudae epididymidis from 16 dogs. Total motility, plasma membrane integrity and acrosome integrity were evaluated immediately on harvesting, and after 2 and 4 days of storage at 5 °C, and at 0 and 2 h post-thaw at 37 °C. Sperm motility decreased significantly during cold storage, compared to freshly harvested spermatozoa ($P < 0.001$). Although there was no significant effect of pre-freeze storage time on post-thaw motility, there was a tendency towards decreased motility in spermatozoa that had been stored for 4 days, compared to spermatozoa that were frozen immediately after collection ($P = 0.09$). The number of post-thaw spermatozoa with an intact plasma membrane was decreased in spermatozoa cold-stored for 4 days ($P < 0.001$). There was no significant effect of pre-freeze storage time on the acrosomal status of post-thaw spermatozoa. In conclusion, canine epididymal spermatozoa were stored at 5 °C for up to 4 days without a clear detrimental effect on post-thaw motility and acrosome integrity, but storage may have decreased post-thaw motility. Results were, however, generally low.

Keywords: Epididymal spermatozoa; Cryopreservation; Dog

1. Introduction

Cryopreservation of spermatozoa is an important tool for preserving genetic material and for maintaining genetic diversity in endangered wild species, including canids. Successful in vivo use of frozen canine epididymal spermatozoa has been reported [1,2]. It would be advantageous if spermatozoa could be harvested from animals that die unexpectedly. This would entail harvesting testicles or epididymal spermatozoa in the field and transporting the material chilled and in a suitable medium to a cryopreservation center [3]. Interestingly, ejaculated dog spermatozoa stored at 5 °C in an egg yolk Tris–fructose extender retain their quality for up to 8 days of storage [4]. The aim of our experiment was to determine if canine epididymal spermatozoa stored in an egg yolk Tris–fructose extender at 5 °C for 2 or 4 days would retain viability, motility and plasma membrane and acrosome integrity following freezing and thawing at the same level as epididymal spermatozoa frozen immediately after collection.

2. Materials and methods

2.1. Experimental design

Epididymal spermatozoa were collected into a Tris–fructose citrate solution. Freshly collected spermatozoa

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were allocated into three equal portions. Cryopreservation of the epididymal spermatozoa was done immediately after harvesting (Day 0), or after 2 or 4 days of cool storage at 5 °C in an egg yolk Tris–fructose extender. Sperm motility, plasma membrane integrity and acrosome integrity were evaluated after collection, after 2 and 4 days of cool storage, and at 0 or 2 h post-thaw at 37 °C.

2.2. Media used for harvesting and freezing of epididymal spermatozoa

The cryopreservation medium was developed by Rota et al. [5] for freezing dog spermatozoa. Extender I contained 2.4% (w/v) Tris (BDH Chemical, England), 1.4% (w/v) citric acid (BDH Chemical), 0.8% (w/v) fructose (BDH Chemical), 3% (v/v) glycerol (BDH Chemical), 20% (v/v) egg yolk, 0.06% (w/v) Na-benzylpenicillin (M&H Manufacturing, Thailand) and 0.1% (w/v) streptomycin sulphate (M&H Manufacturing) in distilled water. Extender II had the same composition as Extender I, except that it also contained 7% glycerol (v/v). The Tris–fructose citrate solution used for harvesting and thawing had the composition like Extender I, except that it did not contain egg yolk or glycerol.

2.3. Collection of epididymal spermatozoa

Testes were obtained from 20 healthy dogs between 2.5 and 6 years of age that were undergoing routine orchidectomy at the Small Animal Hospital, Chulalongkorn University. Within 15 min after orchidectomy, the caudae epididymides and vasa deferentia were dissected from the testes. Spermatozoa were collected by retrograde flushing of the two caudae epididymidis from each dog; a blunted 25-gauge needle, connected to a 3-mL sterile plastic syringe, was inserted into the vas deferens. A total of 1.5 mL of the Tris–fructose citrate solution at 37 °C was then flushed through the cauda epididymis and the fluid collected into a tube at 37 °C. For the samples to be included in the study, the percentage of total motility had to exceed 70%. Sperm samples from 16 dogs met this criterion and were included in the study.

2.4. Freezing and thawing of epididymal spermatozoa

Epididymal spermatozoa were cryopreserved immediately after harvesting (Day 0), or after 2 or 4 days of cool storage at 5 °C in Extender I. The sperm concentration was determined, and the sperm suspension from each dog was allocated into three equal portions and placed in three sterile plastic tubes. The three aliquots were centrifuged at $300 \times g$ for 10 min and the supernatants were discarded. The sperm pellets were re-suspended in Extender I to obtain a sperm concentration of 300×10^6 sperma-spermatozoa/mL. The sperm samples were placed in a cooler at room temperature, programmed to cool to a temperature of 5 °C in 45 min. Prior to freezing, a 300-μL aliquot of each sample was taken to evaluate sperm motility, plasma membrane integrity and acrosome integrity, and the same volume of Extender II as previously of Extender I was then added to the cooled sperm aliquots, resulting in a final sperm concentration of 150×10^6 spermatozoa/mL. One sample was loaded in 0.5-mL straws and frozen [6]. The two remaining aliquots of the chilled, extended semen samples were then stored at 5 °C in a refrigerator to be frozen after 2 or 4 days of storage, as described above. Thawing was done in a water bath at 39 °C for 30 s. After thawing the contents of each straw were emptied in 1 mL of the Tris–fructose citrate solution at 37 °C.

2.5. Evaluation of spermatozoa

Sperm characteristics were evaluated after collection (i.e. before freezing), at 2 and 4 days after cooling, and at 0 and 2 h post-thaw. Post-thaw spermatozoa were incubated at 37 °C. The percentage of motile spermatozoa (total motility) was assessed subjectively on a warmed glass-slide; a minimum of four fields was evaluated using phase-contrast microscopy. The integrity of the plasma membrane was determined using 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) staining [7]. A fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) was used to evaluate acrosome integrity [8]. For each sample, 200 sperm cells were assessed for membrane integrity and acrosome integrity.

2.6. Statistical analyses

Data on post-thaw motility, plasma membrane integrity and acrosome integrity were analyzed using analysis of variance (PROC MIXED; SAS Institute Inc., Cary, NC, USA). A pair-wise *t*-test was applied to compare differences between the means of sperm characteristics before freezing in fresh and cooled samples. All data are presented as mean ± S.D. A value of $P < 0.05$ was considered statistically significant.