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Cryopreservation and storage of cat epididymal sperm using –75 °C freezer *vs* liquid nitrogen

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ABSTRACT

The quality of cat epididymal sperm cryopreserved and stored by four methods was assessed. Epididymal sperm were suspended in Tris-glucose-citrate egg yolk extender, loaded in 0.25 mL straws and then cryopreserved. The samples in a standard protocol (LN) were cryopreserved and stored in liquid nitrogen (LN_2). The sperm straws in the LN-Fr-LN group were cryopreserved in LN_2 and stored in a −75 °C freezer; the straws were returned to LN_2 prior to thawing. The loaded straws in the Fr group were transferred directly from 4 °C to the freezer and maintained in the freezer until thawing. The Fr-LN samples were cryopreserved and stored in the freezer and were introduced into LN_2 before thawing. The sperm thawing was conducted on days 30, 60, 90 and 120 of cryopreservation. The sperm motility, viability, membrane integrity and acrosome integrity were evaluated at 15 and 180 min after thawing. The quality of post-thaw sperm in all three modified protocols was comparable ($P > 0.05$) and did not differ from that in the standard protocol except the membrane integrity of the 60 days stored samples evaluated at 15 min after thawing, which was significantly higher for the LN-Fr-LN than the Fr-LN groups ($P = 0.04$). The length of cryopreservation time had no effect ($P > 0.05$) on the sperm parameters assessed at 15 min after thawing. The sperm motility was significantly greater ($P = 0.01$ to $P = 0.02$) for the 15 min than the 180 min incubation. In conclusion, cat epididymal sperm could alternatively be cryopreserved and/or stored by using the −75 °C freezer for 120 days. To use, the cryopreserved sperm in the freezer could be thawed immediately or after being transferred to LN_2. This was useful for the application of the −75 °C cryopreserved sperm in remote areas.

1. Introduction

One of the reasons for production of cryopreserved semen in any species is to preserve the male reproductive cell for an indefinitely long time even after the death of sperm donors (Squires, 2013). This is especially useful for highly proven genetic merit animals and/or animals threatened with extinction. The risk of extinction is the problematic issue for felids with the exception of the domestic cat. According to the IUCN Red List, 18 feline species such as flat-headed cat, Iberian lynx, cheetah and tiger are classified as imperiled (critically endangered, endangered, or vulnerable) species (Baillie et al., 2004). Therefore, the domestic cat deemed as an appropriate agent for its wild relatives is most frequently used to study various aspects including the development of sperm cryopreservation (Farstad, 2000).

In general, production and storage of cryopreserved semen is dependent on the existence of liquid nitrogen, LN_2 (Sherman, 1973; Watson, 1990; England, 1993). However, a consistent supply of LN_2 is not ensured in some geographical area and in some situation. Depletion of LN_2 in a semen tank during storage, which results in increased temperatures, can be harmful to cryopreserved sperm.

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Such thermal injury to sperm is permanent and cannot be corrected by refilling the storage tank with LN_2 (O'Connor, 2017). Processing and storage of cryopreserved semen without use of LN_2 is, thereby, an interesting alternative for feline and other species. In previous studies, dry ice-alcohol (−79 °C) was used for cryopreservation and storage of bovine semen, in comparison with LN_2. Results showed that the motility and metabolic activity of frozen-thawed sperm were significantly greater for semen stored at −196 °C than at −79 °C over 18 months of storage (Sullivan and Mixner, 1963). Similar finding was also investigated in other studies where bull semen was stored for 2 wk and 2 months (Larson and Graham, 1958), 8 wk (Pickett et al., 1959) and 18 wk (Pickett et al., 1960). In contrary, a recent study demonstrated that cryopreserved bull semen stored at −80 °C in a mechanical freezer had post-thaw sperm quality similar to the semen preserved in LN_2. This result was found throughout 1-month of storage (Buranaamnuay et al., 2016). According to *in vivo* fertility test, bull semen maintained at −196 °C and −79 °C revealed no difference in conception rate after artificial insemination in female cattle (Larson and Graham, 1958; Macpherson, 1960). In dogs, it has been reported that a −152 °C ultra-low temperature freezer was a viable alternative to LN_2 to cryopreserve and store semen. At 120 days after cryopreservation, the percentages of sperm motility, viability and normal morphology were comparable between samples cryopreserved and stored in LN_2, samples cryopreserved in LN_2 and stored in the freezers and samples cryopreserved and stored in the freezers (Alamo et al., 2005). Likewise, Medrano et al. (2002) conducted a study in male goats which found that, at 2 months of cryopreservation, the quality of semen after having been cryopreserved using LN_2 did not differ from that after having been cryopreserved and stored by ultra-low temperature freezers of −150 °C. On the other hand, the post-thaw sperm motility was found to be lower when human semen was maintained in a mechanical freezer at −70 °C compared with that maintained in LN_2. The decline in motility was more pronounced in 3-month than in 7-day stored samples. Difference in post-thaw sperm morphology was nonetheless not detected among sperm preserved at −70 °C *versus* −196 °C (Trummer et al., 1998). In domestic cats, to the author knowledge, no studies have been carried out evaluating the use of a mechanical freezer to cryopreserve and store sperm samples.

The aim of this experiment was to appraise the use of a −75 °C mechanical freezer to cryopreserve and store cat epididymal sperm, for a maximum period of 120 days. The efficiency of this technique was also compared with the use of LN_2 to cryopreserve and store sperm samples.

2. Materials and methods

2.1. Chemicals

Chemical agents purchased from Sigma-Aldrich (St. Louis, MO, USA) were used in the present study unless stated otherwise.

2.2. Specimen acquisition and handling

The present study received the approval of the Institutional Animal Care and Use Committee at the Institute of Molecular Biosciences, Mahidol University (Protocol code COA.NO.IMB-ACUC 2017/011). Mixed breed tom cats aged between 1 and 3.5 years were brought to the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Bangkok for castration. A total of 110 testes were acquired, with 7 repeats of collection. All testes collected (14–18 testes/replicate) were pooled and rinsed initially with a sterile 0.9% sodium chloride solution plus a combination of antibiotics [0.065% (w/v) Penicillin G sodium, 0.056% (w/v) Streptomycin sulfate and 0.004% (w/v) Gentamycin sulfate (A.N.B. Laboratories Co., Ltd., Bangkok, Thailand)]. The testes soaked in the solution were kept warm (approximately 37 °C) while being transported to the laboratory for further processes. The maximum transportation period did not exceed 90 min after castration.

2.3. Sperm harvesting

At the laboratory, the testes were rinsed thoroughly with the same type of pre-warmed (37 °C) solution to remove remaining

Table 1
The composition of semen extenders prepared and used in the present study.

	Type of extender		
	Tris buffered solution	Freezing extender I	Freezing extender II
Tris[a] (g)	3.025	3.025	3.025
Citric acid (g)	1.4	1.4	1.4
Glucose (g)	0.8	0.8	0.8
Benzyl penicillin (g)	0.06	0.06	0.06
Streptomycin sulfate (g)	0.1	0.1	0.1
Glycerol (mL)	–	3	7
Egg yolk (mL)	–	20	20
Equex STM[b] (mL)		–	1
Distilled water added to (mL)	100	100	100

[a] Tris(hydroxymethyl) aminomethane.
[b] Nova Chemical Sales Inc., Scituate, MA, USA.