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The development of cat testicular sperm cryopreservation protocols: Effects of tissue fragments or sperm cell suspension

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ABSTRACT

In endangered animals that have been found dead or sterilized for medical reasons, testis is the ultimate source of haploid DNA or sperm. Thus, preservation of testicular sperm may be performed to rescue their genetics. The aim of this study was to evaluate protocols for testicular sperm freezing: as tissue fragments or cell suspension in domestic cats as a model. A pair of testes from each cat (n = 9) were cut into eight equal pieces. Four randomly selected pieces were cryopreserved as: (1) tissue pieces using two-step freezing; (2) tissue pieces using a slow passive cooling device (CoolCell); (3) sperm suspension after single-layer centrifugation (SLC) through colloids; and (4) sperm suspension without being processed through SLC. A testicular piece from each cat served as fresh control. Testicular sperm membrane and DNA integrity were evaluated before, and after, the cryopreservation process. In addition, spermatogenic cell types (testicular sperm, spermatogonia, spermatocyte, and spermatid) present in the suspension samples were counted before and after SLC. The results found that testicular sperm membrane integrity in the suspension after SLC process was higher than that in the fragment form neither using the two-step nor CoolCell freezing, both before and after freezing (before freezing: 92.3 ± 3.4 vs. 81 ± 4.5 and 80.0 ± 7.0; after freezing: 84.5 ± 4.6 vs. 71.2 ± 12 and 76.2 ± 4.6; $P \leq 0.05$). Testicular sperm DNA integrity was, however, not different among groups. Furthermore, the samples processed through the SLC had higher ration of sperm cells: other spermatogenic cells than those were not processed through the SLC (88.9 ± 3.8 vs. 30 ± 7.9; $P \leq 0.05$). In summary, testicular sperm cryopreserved as a minced suspension is considered suitable in terms of preventing sperm membrane integrity, and SLC is considered a selection tool for enriching haploid sperm cells from castrated or postmortem cats.

1. Introduction

Apart from ejaculates and epididymides, testes are considered the organ upholding plenty number of genetic materials e.g., in infertile human patients (such as oligoasthenozoospermia, obstructive azoospermia) [1,2], and castrated or postmortem endangered animal species [3]. Together with intracytoplasmic sperm injection technique, testicular sperm extraction (TESE) after cryopreservation can increase a pregnancy outcome in several infertility couples [4,5] and animal species [6,7]. Testicular sperm can generally be cryopreserved as a small tubular piece (testicular tissue biopsy) or as a minced suspension [8–11]. Testicular tissue biopsy has been frozen using either a slow freezing by utilization of programmable control rate freezer, or vitrification (rapid and ultrarapid freezing rate) [10,12]. The slow freezing procedure requires a lower

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concentration of cryoprotective agents compared with vitrification, thus, decreasing the potential toxicity of these agents to the cells [13]. An expensive programmable freezer is, however, required. A dry ice locker or a slow passive cooling device (i.e., CoolCell, Mr. Frosty) was applied recently for germ cell and ovarian cortex cryopreservation [14,15], which is inexpensive and practical. This equipment provides a freezing rate of 1 °C/min, thus, considered an alternative to the programmable freezer [15].

In our previous study, freezing testicular tissue above liquid nitrogen or the two-step freezing method (rapid freezing rate) yielded higher survival rate of post-thawed cat testicular sperm compared with the other procedures (−80 °C and direct freezing in liquid nitrogen or ultrarapid vitrification) [16]. Hence, this freezing procedure is suggested for practical use in remote areas [16]. Although optimizing procedure for testicular tissue cryopreservation is challenging and complex due to the presence of various cell types and sizes, many research groups paid more attention to testicular piece cryopreservation than minced suspension [11,12]. It has been reported that seminiferous tubules can protect testicular sperm, whereas toxins from cell lyses compromise testicular sperm viability during mincing [17]. However, in felids, testicular sperm cryopreservation in the minced suspension form is interesting because a similar program for semen cryopreservation can be used, resulting in 55 ± 2.7% sperm with intact membrane [18]. A test-yolk extender with optimal cryoprotective agent has been reported to maintain human testicular sperm membrane integrity [8]. In felids, egg-yolk tris (EYT) extender supplemented with Equex STM paste can protect post-thawed epididymal sperm membrane integrity [19]. Furthermore, an epididymal cat sperm selection technique using single-layer centrifugation (SLC) through silica colloids was recently developed, which improved normal sperm morphology, membrane, and DNA integrity [20]. The contaminated cells e.g., red blood cells, were decreased after SLC was used [20]. Thus, this technique may be a useful alternative method to maintain testicular sperm quality and avoid toxins from cell lyses, which can compromise cat testicular sperm viability.

Of the 37 felid species, domestic cat is the only species that is not classified as threatened or endangered. Thus, the domestic cat has been widely accepted as a suitable research model for wild felids. Because of the difficulties in *ex situ* breeding management of wild felids, gamete preservation or gene banking for conservation is necessarily established. Apart from ejaculated sperm, testicular sperm are genetic materials that can be salvaged in castrated and dead animals. However, testicular sperm cryopreservation study in felid species is limited. The objectives of this study were to optimize and evaluate the efficiency of four different cryopreservation protocols to protect cat testicular sperm from cryoinjury. The cryopreservation protocols were as follows: (1) rapid freezing of testicular tissue above liquid nitrogen vapor (two-step freezing); (2) slow freezing of testicular tissue using a slow passive cooling device (CoolCell); (3) minced testicular suspension and cryopreserved in an EYT extender after processed by SLC; and (4) minced testicular suspension and cryopreserved in EYT without being processed through SLC.

2. Materials and methods

2.1. Experimental design

Testes from adult male domestic cats with unknown age (n = 9) were obtained from routine castrations at the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Thailand. They were kept in an isotonic normal saline solution supplemented with 1% penicillin–streptomycin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and transported to the laboratory at room temperature within 4 hours. The testes were cleaned of blood and washed in sterile saline. After the removal of the attached epididymides, each testis was weighed on a digital balance. A pair of testes from each cat was cut into eight equal pieces. Five randomly selected pieces from each cat were allocated to be (1) fresh control; (2) testicular tissue cryopreserved above liquid nitrogen vapor (two-step freezing); (3) testicular tissue cryopreserved in a slow passive cooling device (CoolCell; BioCision, Mill Valley, CA, USA); (4) extracted testicular sperm in a suspension form cryopreserved in suspension with the EYT extender after being processed by SLC through colloid (SLC or Androcoll-F); and (5) extracted testicular sperm in a suspension form cryopreserved in EYT without being processed through SLC. Fresh, cold-stored, and cryopreserved testicular sperm were evaluated for integrity of membrane and DNA. In addition, types of spermatogenic cell in minced testicular tissue suspension were differentiated before and after SLC process. The experimental design flow chart is shown in Figure 1.

2.2. Media

Testicular tissue storage medium was isotonic normal saline solution supplemented with 1% penicillin–streptomycin (Sigma–Aldrich Chemie GmbH). Cryopreservation media used in this study are discussed in the following sections.

2.2.1. Two-step freezing media

Two-step freezing media comprised holding, equilibration, vitrification and warming solutions. The holding solution, used for TESE, was composed of TCM 199 containing 25 mM of HEPES (modified TCM 1999; Sigma Chemical Co., St Louis, MO, USA) and 10% fetal calf serum (FCS; Invitromex, Winchester, VA, USA). The equilibration solution was 15 mL mTCM 199, 20% FCS (Invitromex), and a combination of 7.5% dimethyl sulfoxide (DMSO; Sigma) and 7.5% ethylene glycol (EG, Sigma). The vitrification medium was prepared by the addition of 0.5 M sucrose (Sigma), 20% FCS (Invitromex) and a combination of 15% DMSO (Sigma) and 15% EG (Sigma) in 15 mL mTCM 199 (Sigma). The warming solution consisted of 15 mL mTCM 199 (Sigma), 20% FCS (Invitromex), and 1 M sucrose (Sigma) [16]. All media were freshly prepared before laboratory processing.

2.2.2. Slow passive cooling media

A protein-free buffer; PBS (holding solution) was used for TESE. The cryopreservation medium was composed of 0.1-M sucrose (Sigma) and 11% DMSO (Sigma) in 5-mL PBS. The thawing solution was composed of two media: (1)