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Feline spermatozoa from fresh and cryopreserved testicular tissues have comparable ability to fertilize matured oocytes and sustain the embryo development after intracytoplasmic sperm injection

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ABSTRACT

Cryopreservation of testicular tissue associated with intracytoplasmic sperm injection (ICSI) is a critical tool that still needs to be explored for preserving the fertility of endangered species. Using the domestic cat as a model for wild felids, the study aimed at determining the effect of different cryoprotectants and freezing techniques (two-step freezing vs. controlled slow freezing) on the sperm quality (membrane and DNA integrity). Then, spermatozoa were extracted from frozen-thawed testicular tissues and used for ICSI to assess early gamete activation or developmental competence *in vitro*. The percentage of spermatozoa with intact plasma membrane was not different ($P > 0.05$) among nonfrozen control, glycerol-, and ethylene glycol-frozen tissues (63.2 ± 2%, 58.2 ± 2.6%, 53.3 ± 2.3%, respectively). However, these percentages were significantly lower ($P < 0.05$) in groups of dimethyl sulfoxide (46.3 ± 3.3%) and 1,2 propanediol (44.3 ± 2.9%) when compared with control. Conventional freezing combined with 5% (vol/vol) glycerol best preserved sperm membrane integrity (55.0 ± 2.7%) when compared with other freezing techniques. The incidence of DNA fragmentation was found to be low (0.2%–1.1%) in all freezing protocols. After ICSI with frozen testicular spermatozoa, male and female gametes were asynchronously activated and the percentages of normal fertilization at 6, 12, and 18 hours were 11.2%, 20.6%, and 22.1%, respectively. Metaphase II-arrested oocytes containing or not a decondensed sperm head were predominantly found after ICSI with frozen testicular spermatozoa. Although two-pronucleus formation could be observed as soon as 6 hours post ICSI (10%), the rate increased dramatically after 12 and 18 hours post ICSI (17.2% and 19.5%, respectively). ICSI using frozen-thawed testicular spermatozoa yielded cleavage (32.7%), morula (6.5%), and blastocyst (4.4%) percentages similar to nonfrozen control ($P > 0.05$). It is concluded that conventional freezing technique with glycerol as a principle cryoprotectant is simplified and applicable for cat testicular tissue cryopreservation. We also demonstrate for the first time that feline spermatozoa derived from frozen-thawed testicular tissues retain their fertilizing ability and can be used to produce ICSI-derived embryos.

1. Introduction

Most wild felid species are currently classified as threatened or endangered species, because their population rapidly declines primarily because of many threatening factors including illegal hunting, loss of habitats, and inbreeding-related problems, such as immunodepletion-related illness and teratospermic-induced infertility [1]. To propagate and preserve the genetic potential of these wild felids, gamete preservation techniques associated with assisted reproductive technologies such as artificial

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insemination, *in vitro* fertilization, and intracytoplasmic sperm injection (ICSI) have been developed in domestic and in wild cats [1]. Although ejaculated spermatozoa collected by electrostimulation are normally used for assisted reproductive technologies in domestic and wild cats [2], the use of these spermatozoa is not applicable for valuable animals that would unexpectedly die. Therefore, only spermatozoa from epididymis and testicular tissue recovered from sudden death animals are meaningfully used for embryo production via *in vitro* fertilization technique [3–6]. In addition, these sperm sources also increase the number of sperm that can be collected from a given individual.

In vitro embryo production using cryopreserved-thawed testicular spermatozoa has been successfully performed via ICSI in human [7–9] and mouse [10]. Though successful application of these techniques, in terms of embryo production and live offspring has not been established in domestic and endangered wild felids, we hypothesized that frozen feline testicular spermatozoa were probable to maintain ability to activate oocytes similar to other species. However, the ICSI outcome was affected by sperm qualities.

Because sperm obtained from testicular tissue are not fully matured and most of them usually show only a slowly twitching movement or are completely immotile, sperm motility is therefore not to be used to assess the postthaw quality of testicular spermatozoa. Although plasma membrane integrity of spermatozoa used for ICSI is not essential for fertilization and embryo development [11], prolonged exposure of plasma membrane-disrupted spermatozoa to unfavorable environment appeared to increase the incidence of sperm DNA damage [12]. The damaged DNA impairs ICSI outcomes in terms of fertilization and embryo developmental rate [13]. Suboptimal freezing and thawing potentially damages plasma membrane and DNA integrity of spermatozoa [4,13–19]. To improve sperm quality after freezing and thawing, several techniques have been applied. Testicular spermatozoa can be frozen as cell suspension, or as a piece of testicular tissue [20–23] using different freezing regimens. Several cryoprotectants (CPAs) have been used to protect testicular tissue against cryoinjury including penetrating and nonpenetrating CPAs such as sucrose, trehalose, glycerol, ethylene glycol (EG), 1, 2-propaneodiol (PrOH), and dimethyl sulphoxide (DMSO) [7,19,24]. Nevertheless, the efficacy of these CPAs depends on several factors such as CPA concentration, cooling rate, and also species studied. For example, though DMSO has been demonstrated to be a promising CPA for preserving human testicular tissue [25–27], this CPA was inferior to protect porcine testicular cells when compared with glycerol and EG [28]. Until recently, data on types of CPA and also freezing techniques that affect postthaw testicular sperm quality including the fertilizing ability in terms of successful embryo production via ICSI in domestic cats has been limited. Moreover, the efficient and simplified freezing technique for cat testicular tissue cryopreservation is still required.

The objective of this study was to determine (1) the effects of CPAs and freezing protocols on testicular sperm plasma membrane and DNA integrity, and (2) the fertilizing ability (first cell cycle and early embryo development) after ICSI.

2. Materials and methods

All chemicals used in this experiment were purchased from Sigma Aldrich, St. Louis, MO, USA, unless otherwise specified.

2.1. Collection of testes

Testes were obtained from adult cats submitted for castration 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. After castration, testes were maintained in 0.9% (vol/wt) normal saline solution and transported to the laboratory within 3 hours at ambient temperature. Testicular tissues were then prepared at room temperature (approximately 25 °C) before freezing procedures. The extraneous tissues were removed from these testes, later they were washed once in normal saline solution supplemented with antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) and dried with clean gauze. Only the testes that weighed between 1 and 2 g were used in this study.

2.2. Cryopreservation

The freezing extender including extender1 (EXI) and extender2 (EXII) were prepared according to Axnér et al. [15]. The EXI and EXII contained 3% (vol/vol) and 7% (vol/vol) CPA (glycerol, EG, PrOH, or DMSO), respectively.

Testicular tissue from individual testis was cut into 20 equal portions (approximately 2 × 3 × 5 mm in size). They were equilibrated in EXI at 5 °C for 1 hour before adding an equal volume of EXII. The equilibrated testicular tissues were subsequently loaded into a 0.5 mL polyvinyl straw (5 pieces per straw). For two-step freezing, the straws containing testicular tissues were horizontally placed on a rack, 4 cm above liquid nitrogen surface, for 10 minutes before plunging into liquid nitrogen for long-term storage. Controlled-rate slow freezing was performed using a programmable CL863 freezer (Cryologic PL, Victoria, Australia). The freezing rate used in this study was performed as previously described by Keros et al. [25] with minor modifications. The initial temperature was set at 4 °C. After holding the straw at this temperature for 5 minutes, the temperature was then slowly reduced at a rate of 0.5 °C/min to −8 °C. The straws were held at this temperature for 5 minutes before seeding by touching the wall of the freezing straw with forceps precooled in liquid nitrogen. The temperature was subsequently reduced from −8 °C to −40 °C at a rate of 0.5 °C/min and then to −70 °C at a rate of 6 °C/min. The straws were finally plunged into liquid nitrogen and stored in liquid nitrogen until analysis.

2.3. Thawing of cryopreserved testicular tissues

The straws containing testicular tissue were thawed in air for 10 seconds, followed by immersion in warm water (37 °C) for 30 seconds. The testicular tissues was then