Cryobiology 67 (2013) 244–247

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Brief Communication

Sperm quality and the morphology of cryopreserved testicular tissues recovered post-mortem from diverse wild species ☆

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ARTICLE INFO

Article history:
Received 7 May 2013
Accepted 9 July 2013
Available online 18 July 2013

Keywords:
Bovid
Cervid
Felid
Freezing
Testis

ABSTRACT

This study compared the effects of slow and fast freezing of testicular tissue of wild animals collected at post-mortem on testicular structure and testicular sperm. The testes of seven animals that had died in captivity; three felids (jungle cat, lion and leopard), two cervids (rusa deer and fea's muntjac) and two bovids (Sumatran serows) were cryopreserved using slow- and fast-freezing protocols. There were greater reductions in the integrity of the sperm membrane and DNA in tissues cryopreserved using slow freezing compared to fast freezing (membrane integrity reduced by 21.5 ± 12.4% vs. 13.0 ± 6.9%, $P = 0.11$ and DNA integrity reduced by 22.7 ± 16.3% vs. 6.6 ± 6.3%, $P = 0.13$). Histologically, there were similar degrees of detachment and shrinkage of the seminiferous tubules whereas, TUNEL assay revealed a tendency towards more apoptotic changes in the intra-tubular cells of tissues frozen using fast freezing compared to slow freezing ($P = 0.09$). In conclusion, fast freezing tended to cause less damage to testicular sperm but its protective effect on intra-tubular cells was likely compromised. This is the first report of gamete recovery in the wild and of the comparison in various wildlife species, between testicular tissues cryopreserved using different protocols.

Introduction

Cryopreservation of testicular sperm from testicular tissue collected post-mortem is a potential means of preserving male genetic material in endangered animals that die unexpectedly. Offspring have been produced using frozen-thawed testicular sperm following intra-cytoplasmic sperm injection (ICSI) and also following xenotransplantation of testicular tissue [8,10]. These promising results have stimulated further investigation to optimize protocols for the cryopreservation of testicular tissue, that preserve the integrity of DNA and of the testicular sperm membrane both of which are important for ICSI and to maintain the structure of testicular tissue which is important for successful for xenotransplantation. Another goal for the successful cryopreservation of gametes from the gonadal tissues of diverse species of wildlife is to understand the causes of the species variability that leads to unequal abilities among the species, to withstand damage caused by the cryoprotectant and by the rate of cooling. In addition, the development of a simple and cost-effective method for use under field conditions is an equally important goal [3]. To date, there are two common freezing protocols for the cryopreservation of testicular tissue; they are slow freezing and fast freezing. In a previous study we introduced a two-step freezing protocol which had an advantage over fast freezing in the cryopreservation of feline testicular tissue [11]. This study, therefore, aimed to preserve sperm and the structure of testicular tissue from a diverse range of wild species utilizing a passive cooling device to carry out slow freezing rate and a two-step freezing to perform fast freezing rate.

After post-mortem, the testes of three felids (a jungle cat; *Felis chaus*, a lion; *Panthera leo* and a leopard; *Panthera pardus*) two cervids (a rusa deer; *Rusa timorensis* and a fea's muntjac; *Muntiacus feae*) and two bovids (two Sumatran serows; *Capricornis sumatraensis*) ($n = 7$) were harvested. General information of the individual animals; age, cause of death, the time the testes were removed after post-mortem and the time taken to transport the specimens to our laboratory are presented in Table 1. The tissue was transported in sterile normal saline (NSS) supplemented with 1% penicillin–streptomycin (Sigma–Aldrich Chemie GmbH, Steinheim,

☆ *Statement of fundings:* This study was funded by National Research Council of Thailand and Research Unit of Obstetrics and Reproduction in Animals at Chulalongkorn University. The first author was granted a Post-doctoral Fellowship, Chulalongkorn University, Bangkok, Thailand. The authors are grateful for Assc. Prof. Padet Tummaruk for the statistical analysis and Prof. (Em.) Rex Scaramuzzi for linguistic correction.

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http://dx.doi.org/10.1016/j.cryobiol.2013.07.002

Table 1
General information of individual animals defined by species, age at death, the cause of death, the time elapsed between death and necropsy and the time between necropsy and arrival in the laboratory.

Animals	Age	Cause of death	Death to necropsy (h)	Necropsy to laboratory (h)
Jungle cat	10 Years	Anesthetic complication	2	25
Lion	6 Months	Pericarditis	56	7
Leopard	10 years	Aging	2	7
Rusa deer	Unknown (Adult)	Euthanasia	1	4
Fea's muntjac	Unknown (Adult)	Septicemia	24	0.5
Sumatran serow[a]	1–2 Years	Bone fracture	1	6
Sumatran serow[a]	1 Year	Septicemia	38	12

[a] The first and second Sumatran serows.

Germany) and at a temperature of 4–5 °C. Blood surrounding tissue was removed and the testes were then washed in NSS supplemented with 1% penicillin–streptomycin. After removal of the attached epididymides, the testes from each animal were divided into three equal pieces and allocated to one of three groups; (I) fresh control, (II) cryopreserved using a passive method (Coolcell, BioCision, Mill Valley, CA, USA) of slow freezing (slow freezing) [4], and (III) cryopreserved using a two-step freezing protocol where the tissue was suspended above liquid nitrogen vapor 10 min in a styrofoam box and then plunged in liquid nitrogen (fast freezing) [11]. Each piece of testicular tissue was cut into two small pieces (approximately 0.3 × 0.3 × 0.3 cm); the first was used for to extract testicular sperm for evaluation and the others were used for histology and the estimation of apoptosis. The protocols for freezing testicular tissue are as follow; (I) slow freezing: testicular tissues were placed in a 2 ml cryogenic tube filled with 0.5 ml of the ice-cold PBS used in the cooling device (0.1 M sucrose (Sigma) and 11% DMSO (Sigma) in 5 ml of protein free phosphate buffered saline). The cryogenic tube was agitated every 5 min. After 15 min, the tissues were transferred to new cryogenic tubes filled with the same ice-cold media. After 30 min, the tissues were placed into a new ice-cold cryogenic tube and then put into the pre-cooled, passive cooling device, and kept at −80 °C for 24 h. The cryogenic tube containing the tissues was then plunged into liquid nitrogen and kept in a liquid nitrogen tank (−196 °C) until evaluation. The freezing rate between 2 and −80 °C was 1 °C/min and between −80 and −196 °C it was 259 °C/min; (II) fast freezing; testicular tissues were placed in 0.5 ml freezing medium I at room temperature for 10 min and then in 0.5 ml freezing medium II at 4 °C for 30 min. After exposure to the freezing media, tissues were placed on the wall-side of pre-cooled (4 °C) cryogenic tubes. The tubes were laid horizontally on a rack 4 cm above liquid nitrogen vapor for 10 min, then plunged into liquid nitrogen and kept in liquid nitrogen tank (−196 °C) until evaluation. The freezing rates were between 4 and −50 °C was 10.8 °C/min, from −50 to −90 °C it was 18 °C/min and from −90 to −196 °C it was 252 °C/min. The media used for fast freezing were freezing media I and II. Freezing medium I consisted of 15 ml mTCM 199 containing 25 mM HEPES (modified TCM 1999; Sigma), 10% fetal calf serum (FCS; Invitromex, Valley Biomedical Industrial Drive, Winchester, VA, USA) and a combination of 7.5% dimethyl sulfoxide (DMSO; Sigma) and 7.5% ethylene glycol (EG, Sigma). Freezing medium II was prepared by the addition of 0.5 M sucrose (Sigma), 20% FCS (Inviromex) and a combination of 15% DMSO (Sigma) and 15% EG (Sigma) to 15 ml mTCM 199 (Sigma). For thawing, testicular tissue frozen by the slow freezing method was placed at room temperature in warming solution I (0.25 M sucrose (Sigma) and 11% DMSO (Sigma) in 5 ml PBS) and then in warming solution II (0.25 M Sucrose (Sigma) in 5-ml PBS) each time for 10 min [4]. The testicular tissue from the fast freezing group was thawed at room temperature in a warming solution (15 ml mTCM 199 (Sigma), 20% FCS (Invitromex) and 1 M sucrose (Sigma)) for 10 min [11]. Testicular sperm were extracted from frozen–thawed tissues by mechanical mincing at 37 °C in PBS (slow freezing) or at 37 °C in holding medium (mTCM 199 (Sigma) and 20% FCS (Invitromex)) (fast freezing) and then in both cases, filtered through a 100 μm nylon mesh). At least one hundred sperm were evaluated by SYBR-14/Ethidium homodimer (Molecular Probes Inc., Eugene, OR, USA) staining for the percentage with an intact membrane and DNA quality using Acridine orange (Sigma) under an epifluorescence microscope (1000× magnification) [11]. The integrity of DNA from testicular sperm was evaluated following the method used for epididymal spermatozoa from the cat [11]. In brief, an air-dried smear was prepared on a glass slide using 10 μL of extracted testicular sperm and fixed overnight in freshly prepared methanol-glacial acetic acid (Carnoy's solution; 3/1 v/v) at room temperature. After fixation, the slide was air-dried, stained with a solution of 1% Acridine orange (AO) at a pH of 2.5 (Solutions of 0.1 M citric acid (Merck, Dramstadt, Germany) in 40 ml distilled water and 0.3 M $Na_2HPO_4.7H_2O$ (Merck) in 2.5 distilled water were added to 100 mg of AO in 10 ml distilled water) for 5 min and rinsed with distilled water. At least one hundred testicular sperm were assessed under the epifluorescence microscope and classified into two categories; head of testicular sperm with normal DNA integrity (double-stranded) fluoresced with a bright green color whereas denatured or single stranded DNA, fluoresced orange, yellow or red. A piece of testicular tissue from each freezing protocol was processed for routine histology. The paraffin embedded sections were deparaffinized, rehydrated, the nuclei stained and cytoplasm counterstained with a solution ofHematoxylin and Eosin (H&E). Five random areas of the testicular sections were assessed for the integrity of the cellular compartments (an intact epithelial lining of the seminiferous tubule and intact nuclei of the spermatogonia and sertoli cells) by light microscopy at a magnification of times 200 [5]. Briefly, integrity of the epithelial lining of seminiferous tubules was scored for degrees of: (I) cell detachment from the basement membrane (0 if there was no detachment, 1 if <75% of the circumference was detached and 2 if >75% of the circumference was detached) and (II) shrinkage of the basement membrane (0 if there was no shrinkage, 2 if there was slight shrinkage and 3 if the shrinkage was obvious). The integrity of the nuclei of spermatogonia and sertoli cells were scored for (I) distinction between spermatogonia and sertoli cells (0 if easily distinguished, 1 if difficult to distinguish and 2 if impossible to distinguish, (II) visibility of nuclei (0 if seen easily (>40% visible) and 1 if indistinguishable from the cytoplasmic background) and, (III) nuclear condensation (0 if pyknotic cells were absent, 1 if there was <40% of pyknotic cells and 2 if there was more than >40% of pyknotic cells). The scores for the epithelial of the seminiferous tubules and for the nuclei of spermatogonia and sertoli cells were combined to give a total score which ranged from 0 to 5 (0 = healthy to 5 = complete degeneration). Apoptosis of intratubular nuclei (DNA fragmentation) was determined by TUNEL assay (Apoptag Peroxidase Kit; Chemicon International Inc., Temecula, CA, USA) [9]. Briefly, after tissue deparaffinization and rehydration, the testicular sections were pretreated in a microwave oven at 750 W for 3 min and then at 500 W for a min with