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Short Communication

Cryopreservation of Siberian tiger (*Panthera tigris altaica*) epididymal spermatozoa: pilot study of post-thaw sperm characteristics

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ABSTRACT Epididymal sperm cryopreservation provides a potential method for preserving genetic material from males of endangered species. This pilot study was conducted to develop a freezing method for tiger epididymal sperm. We evaluated post-thaw sperm condition using testes with intact epididymides obtained from a Siberian tiger (*Panthera tigris altaica*) after castration. The epididymis was chopped in Tyrode's albumin-lactate-pyruvate 1x and incubated at 5% CO_2, 95% air for 10 min. The Percoll separation density gradient method was used for selective recovery of motile spermatozoa after sperm collection using a cell strainer. The spermatozoa were diluted with modified Norwegian extender supplemented with 20 mM trehalose (extender 1) and subsequent extender 2 (extender 1 with 10% glycerol) and frozen using LN_2 vapor. After thawing at 37℃ for 25 s, Isolate® solution was used for more effective recovery of live sperm. Sperm motility (computerized assisted sperm analysis, CASA), viability (SYBR-14 and Propidium Iodide) and acrosome integrity (*Pisum sativum* agglutinin with FITC) were evaluated. The motility of tiger epididymal spermatozoa was 40.1 ± 2.0%, and progressively motile sperm comprised 32.7 ± 2.3%. Viability was 56.3 ± 1.6% and acrosome integrity was 62.3 ± 4.4%. Cryopreservation of tiger epididymal sperm using a modified Norwegian extender and density gradient method could be effective to obtain functional spermatozoa for future assisted reproductive practices in endangered species.

Keywords: CASA, cryopreservation, epididymal sperm, Siberian tiger

INTRODUCTION

According to the International Union for the Conservation of Nature and Natural Resources, the Siberian tiger (*Panthera tigris altaica*) is a critically endangered species and the wild population was estimated to be approximately 360 individuals worldwide in 2011 (Mathers et al., 2017). A significant limiting factor affecting reproductive studies is the low availability of biological material, which has impeded the development and use of assisted

reproductive technology (ART) (Filliers et al., 2008). Cryopreservation of sperm is the most common method of ART used in combination with artificial insemination, *in vitro* fertilization, or intracytoplasmatic sperm injection into oocytes. Sperm cryopreservation allows epididymal sperm from dead or injured males of endangered species to be preserved and provides material for future use (Schleh and Leoni, 2013). We conducted a pilot study to develop a method to collect and freeze epididymal sperm. In addition, we determined kinematic motility parameters using computerized assisted sperm analysis (CASA), viability, and acrosome integrity of epididymal spermatozoa of the Siberian tiger after freezing and thawing.

MATERIALS AND METHODS

Chemicals

All reagents used were purchased from Sigma Chemical Co (St. Louis, MO, USA), unless otherwise indicated.

Animals

Testes with intact epididymides were obtained from a Siberian tiger (*Panthera tigris altaica*) after castration at Cheongju Zoo, Cheongju, Republic of Korea. The tiger was between 13 and 14 years old and weighed 165 kg. To prevent inbreeding, it was surgically castrated after being anesthetized with medetomidine (0.04 mg/kg) and ketamine (3 mg/kg) with additional sevoflurane inhalation; post-castration, the tiger was awakened by an injection of atipamezole (0.08 mg/kg). The testes were placed in 0.9% normal saline and transported from Cheongju Zoo to the laboratory within 2 h. Upon arrival at the laboratory, the testicular weight (50-51 g) and size (60-70 mm length and 40-50 mm width) were recorded (Fig. 1).

Epididymal sperm collection

The testes with epididymides were first washed three times with 0.9% normal saline. After that, the epididymis was dissected from the testis with sterilized scissors and forceps and rinsed with 0.9% normal saline several times. We then added 2 mL of Tyrode's albumin-lactate-pyruvate (TALP 1×) to a Petri dish with the dissected epididymis. The epididymis was chopped repeatedly using a sterile scalpel blade and surgical scissors and a drop of TALP (1×) was added repeatedly to prevent from drying. The chopped epididymis was incubated in the petri-dish with TALP (1×) at 5% CO_2 and 95% air for 10 min to allow the sperm to swim out. Then, the suspension of spermatozoa was filtered using a 40-μm cell strainer (Cat. #93040, SPL Life Sciences Co., Ltd., Gyeonggi, Korea) to remove tissue debris. The sperm filtrate was transferred into a 15 mL centrifuge with a sterile pipette. Percoll® (density gradient solution) was used for selective recovery of motile spermatozoa according to Yu et al. (2002). The filtrate was layered on top of the Percoll gradient solution [45% Percoll on the top (1.5 mL) and 90% Percoll on the bottom (1.5 mL) in a 15 mL centrifuge tube]. The gradient column was then centrifuged for 20 min at 300 g at room temperature and the pellet was recovered after discarding the supernatant. The sperm pellet was washed using 4 mL TALP (1×) by centrifugation at 300 × *g* for 5 min and then the sperm were frozen.

Sperm freezing and thawing

Sperm freezing was performed following (Nabeel et al., 2019). Briefly, spermatozoa (10×10^6 sperm/mL) were first suspended in extender 1 (20 mM trehalose supplemented Norwegian extender: 3.025 g Tris, 1.7 g citric acid, 1.25 g fructose, 0.06 g penicillin, 0.1 g streptomycin, 20% egg yolk (EY) in 100 mL distilled water) and incubated in 4℃ for 1 h. After that, extender 2 (extender 1 with 10% glycerol) was added to the extended sperm (final concentration: 5×10^6 sperm/mL). The extended sperm were loaded in 0.5 mL straws and incubated at 4℃ for 30 min. Then, the straws were aligned 7 cm over the top of LN_2 vapor for 20 min and then plunged directly into LN_2.

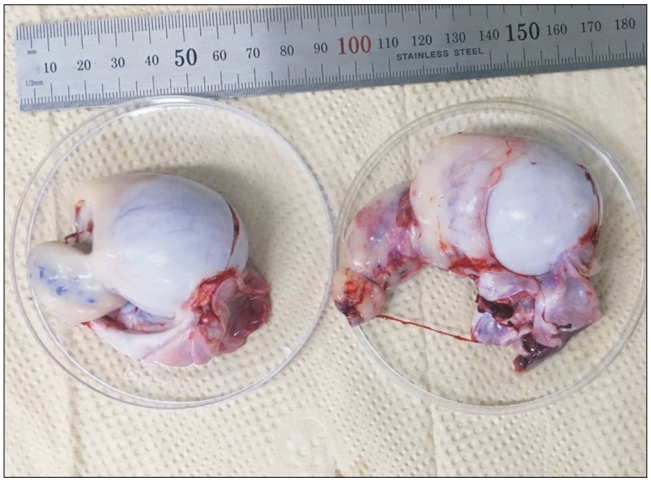

Fig. 1. Testes of Siberian tiger.

Straws were then kept in the LN_2 container until further evaluation. Straws with spermatozoa were thawed in a water bath at 37℃ for 25 s. After thawing, the spermatozoa were overlaid on 1.5 mL layers of Isolate® gradient solutions (FUJIFILM Irvine Scientific, Inc., Santa Ana, CA, USA) and centrifuged at 300 × *g* for 15 min to remove remaining red blood cells. The supernatant was discarded and approximately 100 μL bottom solution including the sperm pellet was resuspended in 100 μL TALP (1×).

Sperm motility

In this study, objective assessments of sperm motility parameters were carried out by using a Sperm Class Analyzer (SCA®) CASA System version 6.3.0.32 (Microptic S.L., Barcelona, Spain). The CASA system was combined with Nikon Eclipse E-200 microscope (Nikon Corporation, Kanagawa, Japan), a Basler camera (aCA1300, 200 uc) and an attached heating stage set (Tokai Hit Co., Ltd., Shizuoka-ken, Japan) at 37℃. Then, 3 μL of thawed spermatozoa was placed into a preheated (37℃) Leja standard count 8-chambered slide (20 μm; Leja Products B.V., NieuwVennep, The Netherlands). The percentage of motile sperm, percentage of progressively motile sperm, and motility parameters of spermatozoa were tested including curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), straightness (STR), and linearity (LIN) of the path. At least two fields, including a minimum of 200 motile spermatozoa, were captured for each sample and analyzed.

Sperm viability

Viability was assessed based on sperm plasma membrane integrity using the LIVE/DEAD™ Sperm Viability Kit (Thermo Fisher Scientific, OR, USA) as previously described by Yu et al. (2002). Briefly, 5 μL of SYBR-14 was added to 50 μL of spermatozoa and the mixture was incubated for 5 min at room temperature in the dark. Then, 5 μL of propidium iodide (PI) was added and the mixture was incubated for an additional 5 min. For each sample, two slides were examined using a fluorescence microscope (Axio, Carl Zeiss, Göttingen, Germany). Live sperm cells appear intense green and dead sperm appears red. At least 200 spermatozoa were counted and the result was expressed as the percentage of live sperm.

Acrosome integrity

Sperm acrosome integrity was assessed as previously described by Yu (2014). Briefly, spermatozoa were stained with *Pisum sativum* agglutinin labeled with FITC (FITC-PSA). For each sample, two slides were examined using a fluorescence microscope. Approximately 200 spermatozoa were counted per slide. The percentage of spermatozoa with intact acrosomal membranes (intense green fluorescence at the anterior acrosomal region of the spermatozoa) was calculated.

Statistics

In this pilot experiment, epididymal spermatozoa from one individual animal were used. Three replicates (straws) were prepared and used to evaluate the assays. The data are represented as mean value and standard error (mean ± SE).

RESULTS

Table 1 shows kinematic parameters of motility. The motility of frozen-thawed tiger epididymal spermatozoa was 40.1 ± 2.0%. Progressive motility was 32.7 ± 2.3%. The percentage of curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness index (STR), and linearity index (LIN) were 82.8 ± 4.3%, 64.5 ± 5.5%, 60.1 ± 6.7%, 90.1 ± 3.5%, and 69.8 ± 5.4%, respectively.

Fig. 2A and 2B shows the viability of spermatozoa stained by SYBR-14 (A) and PI (B), whereas Fig. 2C shows spermatozoa stained with FITC-PSA to assess acrosome integrity of post-thaw spermatozoa under a fluorescent microscope. Fig. 2D shows the results of viability and acrosome integrity tests. The viability was 56.3 ± 1.6% and acrosome integrity was 62.3 ± 4.4%, respectively.

Table 1. Post-thaw motility of Siberian tiger epidydimal spermatozoa cryopreserved with modified Norwegian extender

Parameters	MOT (%)	PM (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)
Results	40.1 ± 2.0	32.7 ± 2.3	82.8 ± 4.3	64.5 ± 5.5	60.1 ± 6.7	90.1 ± 3.5	69.8 ± 5.4

MOT, motility; PM, progressive motility; VCL, curvilinear velocity; VAP, Average path velocity; VSL, straight-line velocity; STR, straightness; LIN, linearity. Values are expressed as mean ± SE.

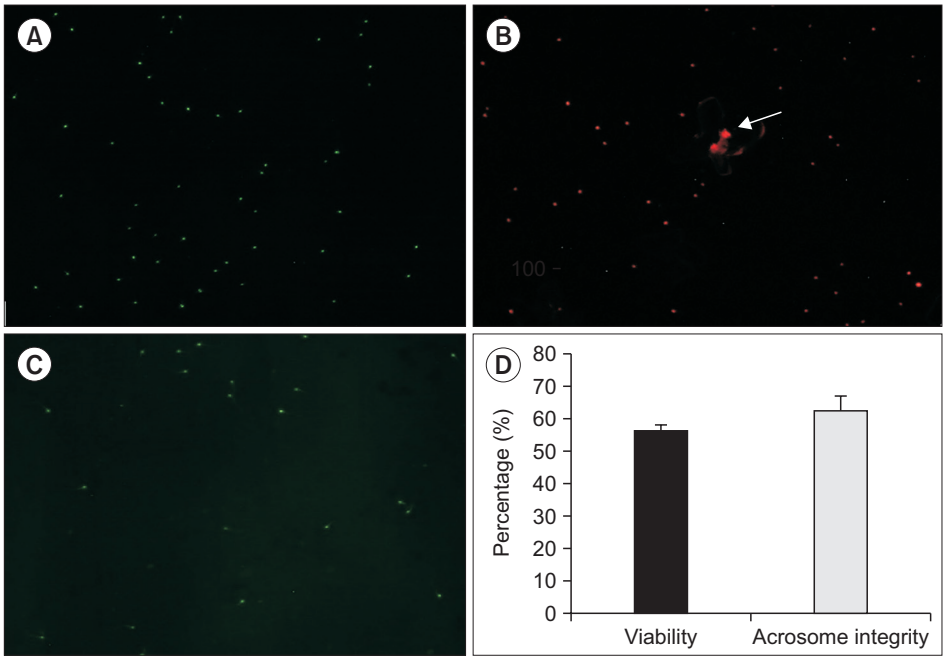

Fig. 2. Post-thaw viability and acrosome integrity of Siberian tiger epididymal spermatozoa cryopreserved with modified Norwegian extender. (A) Live spermatozoa stained with SYBR® 14. Magnification at 100x. (B) Dead spermatozoa stained with propidium iodide. Magnification at 100x. Arrow indicates artifacts. (C) Intact acrosome of spermatozoa stained with Pisum Sativum Agglutinin labelled with FITC. (D) A graph shows the results of viability and acrosome integrity. Values are expressed as mean ± SE.

DISCUSSION

Previous studies have tried to find the best way for cryopreservation protocol in several domestic animals including cow, pig and chicken (Kang et al., 2020; Almubarak et al., 2021; Assumpção et al., 2021; Kang et al., 2021; Kim et al., 2022). However, the information for wild animals is still unclear. Most spermatozoa are collected from live animals by electro-ejaculation, although epididymal spermatozoa can be used to preserve genetic material from valuable and endangered species in the case of injury or sudden death. There have been few studies examining epididymal sperm freezing in tigers. In this study, we tried to develop a freezing method for the preparation of Siberian tiger epididymal sperm and evaluated post-thaw sperm characteristics.

In a previous study of epididymal sperm retrieval from Bengal tigers, Dulbecco's PBS was used to wash the epididymis and then the epididymides were suspended in 2 mL Tris extender without EY (Cocchia et al., 2010). In this study, we used 0.9% normal saline to wash the epididymis and TALP (1×) for the collection of sperm. We then used 20 mM trehalose supplemented Norwegian extender (modified Tris extender) for sperm freezing. In a previous study by Donoghue et al. (1992), a Tris extender containing 20% EY, 11% lactose, and 4% glycerol was used. Cocchia et al. (2010) used a Tris extender containing 20% EY with a final concentration of 4% glycerol, while Byers et al. (1989) used TEST-yolk extender [N-Tris (hydroxymethyl) methyl-2-amino ethanesulfonic acid (TES) with tris(hydroxymethyl)aminomethane (Tris)] with 7.5% glycerol for ejaculated sperm cryopreservation. Therefore, Tris EY with glycerol is commonly used for cryopreservation of tiger sperm (Byers et al., 1989).

However, the presence of RBCs in the sperm freezing extender has been shown to affect spermatozoa fertilization ability (Coccia et al., 2010). Coccia et al. (2010) removed visible blood vessels. In this study, we used the Percoll gradient method to remove blood cells but it was difficult to extract all blood cells using this method. Therefore, Isolate® solution was used for more effective density separation after sperm thawing.

To evaluate sperm motility after thawing, we used CASA for the modern, rapid and comprehensive objective analysis of motility with high reproducibility using identical instrument settings (Patil et al., 1998). To our knowledge, there have been no previous studies incorporating CASA for analyzing tiger epididymal sperm. Coccia et al. (2010) evaluated the characteristics of frozen-thawed Bengal tiger epididymal sperm and found that motility based on subjective microscopic examination was 21.5 ± 16.8%. The results of the present study (40.1 ± 2.0%) were similar to the results of Byers et al. (1989). Their results showed that motility of electro-ejaculated and frozen-thawed

Siberian tiger sperm was 40.4 ± 11.3%. The kinematic parameters were higher compared to the results for frozen tiger sperm presented by Patil et al. (1998), who used CASA to assess electro-ejaculated sperm motility, which was 24.12 ± 8.39% after thawing. They also observed decreases in VAP, VSL, STR, and LIN compared to our results.

Our findings included viability of 56 ± 31.6%, which was higher than that observed by Coccia et al. (2010) but lower than that of Karja et al. (2016). They found that frozen-thawed epididymal and ejaculated sperm had viabilities of 33.5 ± 12.9% and 78.5 ± 3.6%, respectively. The viability of electro-ejaculated sperm was 58 ± 12.9% in another investigation by Byers et al. (1989). The percentage of intact acrosomes in the current study was 62.3 ± 4.4%, close to that of Byers et al. (1989), which was 63.1 ± 12.6%.

CONCLUSION

In conclusion, the freezing system for tiger epididymal sperm used in the present study could be effective to obtain functional spermatozoa for future assisted reproductive practices. However, more replicates are needed to confirm the efficacy of the freezing method examined in this study, including the sperm collection method used. Moreover, different extenders and freezing methods should be compared to develop more useful methods of tiger epididymal sperm cryopreservation for future use in assisted reproductive technology studies.

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