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Effects of Dimethyl Sulfoxide, Ethylene Glycol, Propylene Glycol and Glycerol on Cryopreservation of Wild Tree Shrew (*Tupaia belangeri* Chinese) Cauda Epididymal Sperm

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Abstract: The objective was to examine the effect of Permeable Cryoprotectant Agents (CPAs) on sperm cryopreservation of tree shrew. Epididymal sperm were surgically harvested from captured wild male tree shrews and cryopreserved with Tes-Tris-Egg yolk based cryodiluent (TTE) containing either of the four CPAs, Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG), Propylene Glycol (PG) and Glycerol (Gly) at concentrations of 1, 3, 6 and 10%, respectively. Sperm motility, acrosome integrity and fertility were assessed. In Experiment 1, sperm equilibrated at 4°C in TTE containing 1, 3 and 6% DMSO, respectively showed similar motility to that in TTE without CPA ($p>0.05$). Following the increase of concentration of CPAs and equilibration time (30-90 min), the other CPAs reduced sperm motility ($p<0.05$). In Experiment 2, sperm frozen in TTE containing 3% DMSO showed the highest post-thaw motility ($p<0.05$) and recovery rate of motility ($p<0.05$) among groups. In Experiment 3, there were no differences in the fertilization rate of oocytes and the proportion of tree shrews yielding fertilized oocytes inseminated with fresh and thawed sperm frozen in TTE containing 3% DMSO ($p>0.05$). In conclusion, among the permeable CPAs tested, DMSO provided the best cryoprotective ability for captured wild tree shrew epididymal sperm.

Key words: Tree shrew, sperm, cryopreservation, cryoprotectant, DMSO, China

INTRODUCTION

Earlier molecular phylogeny studies suggest that tree shrews are in the same order as primates which include the closest relatives to human beings (Janecka *et al*., 2007). It is important, therefore that the tree shrew has been used as a laboratory animal model for physiology or human diseases such as neurobiology of stress (Kohlhause *et al*., 2011; Remple *et al*., 2007; Rice *et al*., 2011; Wang *et al*., 2011), cancer (Li *et al*., 2008a), human virus infections and immunology (Feng *et al*., 2011; Li *et al*., 2008b; Xu *et al*., 2007), defects of the visual system (Norton *et al*., 2010; Poveda and Kretz, 2009) and reproduction and development (Collins *et al*., 2007). In addition, tree shrews have the potential to be genetically engineered as models of human diseases. Notwithstanding real and potential clinical and laboratory applications, there are no strains that have been domesticated from wild tree shrews (Ping *et al*., 2011). Thus, most tree shrews employed in disease models are still captured from the wild and acclimated in captivity for 3 months or more before use. Sperm cryopreservation provides an economical and effective way to preserve these valuable models and wildlife genetic resource (Hashemi and Nayebpoor, 2008; Memon *et al*., 2012).

The presence of a permeable Cryoprotectant Agent (CPA) can be an important factor that affects sperm survival during cryopreservation. Permeable CPAs such as Glycerol (Gly), Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG) and Propylene Glycol (PG) have been widely

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applied for mammalian sperm cryopreservation (Farshad *et al*., 2009; Fernandez-Santos *et al*., 2006; Kashiwazaki *et al*., 2006; Li *et al*., 2005a; Si *et al*., 2004). To the knowledge, there is no study on the effect of permeable CPAs on sperm cryopreservation of wild tree shrews. Therefore, the objective of the present study was to test their toxicity or prevention on cryopreservation of wild tree shrew sperm and determinate the appropriate types and concentrations of CPAs. Researchers examined motility and acrosome integrity of sperm that were thawed after having been frozen in cryodiluents at four concentrations of DMSO, EG, PG and Gly, respectively. Finally, frozen-thawed sperm were used for Artificial Insemination (AI) to assess the ability to fertilize ova.

MATERIALS AND METHODS

Animals: Twenty five adult male and ten adult female tree shrews (*Tupaia belangeri* Chinese, bodyweight 120-140 g) were employed in this study. The male tree shrews were captured from a village area near Kunming China. They were individually housed in cages (400 mm length, 200 mm width, 300 mm height) in a controlled environment (20-25°C, humidity 40-60%, 08:00-20:00 h light). Epididymal sperm were retrieved after being captured. Female tree shrews were captive for >1 year. All of the animals were fed with special softened forage, eggs, milk and fresh fruits. All experiments were conducted during the physiologic breeding season (March and June) and were performed in accordance with the regulations and recommendations of the Animal Care Committee of Kunming University of Science and Technology.

Epididymal sperm collection and processing: Both cauda epididymides were surgically isolated from the testes when wild male tree shrews were under anesthesia (ketamine hydrochloride, 2 mg kg^{-1}, Xingang Co., Shanghai, China). The excised tissues were rinsed with modified Tyrode's solution with albumin, lactate and pyruvate (TALP-HEPES) (Bavister *et al*., 1983) and blood vessels and adipose tissue were carefully removed. The epididymides were then minced with fine scissors and sperm released into pre-warmed (37°C) TALP-HEPES by gently squeezing the tracts. The sperm suspension was transferred into a 5 mL sterile tube and washed once with 1 mL TALP-HEPES by centrifugation at 250×g for 5 min. Then, the sperm pellet was immediately resuspended by gentle mixing in Tes-Tris-based cryodiluent plus egg yolk (TTE) composed of 12.7 mM Tris, 47.8 mM 76 TES, 111.0 mM glucose, 55.5 mM lactose, 3.4 mM raffinose, 0.069 mM streptomycin sulfate salt, 100 IU mL^{-1} Penicillin-G and 20% (v:v) fresh egg yolk (Sankai *et al*., 1994). A 10 μL aliquot of sperm suspension was examined for motility and acrosome integrity. Only samples with motility and acrosome integrity exceeding 60 and 85%, respectively were used.

Sperm cryopreservation and experimental designs

Experiment 1 (Effects of four cryoprotectants on sperm motility during equilibration at 4°C): Sperm from eight males were used to evaluate the the prefreezing toxic effects of DMSO, EG, PG and Gly on sperm motility. The epididymal sperm from each animal was divided into seventeen equal aliquots. Each was diluted in a 5 mL sterile test tube with TTE at room temperature (20-25°C) to obtain a final concentration of approximately 2×10^6 sperm/mL. The tubes were closed and transferred to a beaker containing 200 mL water at 25°C and the sperm samples were equilibrated in a 4°C refrigerator for 2 h to allow the sperm samples being slowly cooled to 4°C. Then, each aliquot was further diluted 1:1 with TTE at 4°C containing 2, 6, 12 and 20% of DMSO, EG, PG and Gly (v:v), respectively using stepwise addition at 6 min intervals to reach the final concentrations of 1, 3, 6 and 10% of each CPA, respectively. These concentrations were chosen because they are commonly used in mammalian sperm cryopreservation studies (Khan *et al*., 2009; Varisli *et al*., 2009). In the control group, the sperm were diluted in TTE again without any CPA. All sperm samples were kept at 4°C for additional 30, 60 and 90 min to assess the negative effect of CPAs on tree shrew sperm motility during equilibration. After each time period, a 10 μL aliquot of sperm suspension from each sample was transferred into a disposable sterile tube and diluted stepwise at 30 sec intervals within 3 min with fifty volumes of prewarmed TALP-HEPES medium containing 0.3% bovine serum albumin to examine sperm motility. Each suspension was washed once with TALP-HEPES by centrifugation at 250×g for 5 min to remove the added CPAs and the sperm pellet was immediately dispersed with TALP-HEPES. Sperm motility was then examined.

Experiment 2 (Effect of different concentrations of the four CPAs on motility and acrosomal integrity of tree shrew sperm after cryopreservation): Twelve males were used to investigate the cryoprotective effect of each CPA on the freezing of epididymal sperm. The procedure for sperm harvesting, equilibrating and testing of pre-freeze motility were the same as described in Experiment 1 except that each sperm sample was exposed to the CPAs for only 30 min at 4°C. Sperm samples were loaded into 0.25 mL pre-cooled (4°C) cryo-straws (IMV, L'Aigle, France) and sealed. The loaded straws were laid horizontally on a 15×10 cm iron frame that was pre-cooled to 4°C. The

straws were then frozen by hanging the frame flatly at 4 cm above the surface of LN_2 in a styrofoam box without a lid. The inside dimensions of the box were 25×21×25 cm and it contained 6 cm of LN_2. After 10 min, the frame containing the straws was finally submerged into the LN_2 for storage (Ping *et al*., 2011). Straws were thawed rapidly by plunging them directly into a 37°C water bath for 1 min after being stored in LN_2 for at least 2 days. Post-thaw sperm motility and acrosomal integrity were examined as described.

Experiment 3 (Assessment of fertilizing ability of post-thaw sperm frozen with optimal freezing conditions): The objective was to compare the fertilizing ability of frozen-thawed and fresh sperm. Sperm collected from five male tree shrews were individually frozen under optimal conditions with TTE containing 3% DMSO. As control, fresh sperm collected from another five male tree shrews were individually used for fertilization. The frozen-thawed, or fresh sperm were immediately washed twice with 1 mL TALP-HEPES and centrifuged at 250×g for 5 min. The pellet was diluted to a concentration of 2.0-2.4×10^7 motile sperm mL^{-1}. Meanwhile in the females as described below, after inducing ovulation, the uterus was exposed and approximately 50 μL of sperm sample was injected into each uterine horn. Frozen-thawed sperm exhibiting motility and acrosome integrity >30 and 75%, respectively were used.

The protocols for ovarian stimulation and AI were used as a previous report (Ping *et al*., 2011). Briefly, ten adult females received 60 IU pregnant mare serum gonadotrophin (PMSG, Ningbo Inc., Zhejiang, China) subcutaneously on any day between 16:00-19:00 h and 24 h later injected with 30 IU hCG (Pregnyl, N.V Organon Oss, Netherlands) and 30 IU PMSG. Just before the injection of hCG, intrauterine AI was performed as described below using frozen-thawed or fresh sperm. Briefly, the animals were anaesthetized with ketamine hydrochloride (2 mg kg^{-1}, Xingang Co., Shanghai, China) and both uterine horns were exposed via a small longitudinal incision (approximately 7 mm) in the middle of the abdomen. A sterile metal 16 gauge needle was used to make a hole in the uterus near the cervix where fewer blood vessels were observed, then a catheter with O.D. 3.8 mm containing the sperm suspension was inserted into each uterine horn through the hole and the semen was ejected into the terminal segment of the oviduct. Finally, the abdominal opening was closed with a standard surgical sutures and 5000 IU penicillin and an analgesic were given to prevent infections and pain. The ovaries and oviducts were removed through a similar operation 48 h after AI and the embryos or ovulated oocytes were flushed from the oviducts. The recovered oocytes were examined on an inverted microscope and the number of two-cell embryos relative to the number of recovered oocytes was expressed as the fertilization rate. Furthermore, the rate of tree shrews yielding fertilized oocytes relative to the number of tree shrews used was analyzed.

Sperm functional assays: The percentage of sperm showing motility was assessed with a light microscope at 37°C (Saragusty *et al*., 2009). Briefly, a 10 μL drop of each sperm sample was deposited onto a pre-warmed Neubauer hemacytometer (BOECO, Hamburg, Germany) and covered with a coverslip. The slides were incubated on a 37°C warming plate for 2 min and then the motility of each sample was evaluated by counting approximately 200 sperm in duplicate. Motility was assessed by an experienced evaluator who examined all of the specimens. Post-thaw sperm motility was evaluated immediately after thawing and washing. To minimize evaluator bias, the evaluator was blinded as to the identity of the samples under evaluation.

Sperm density was determined in triplicate by diluting fresh and frozen-thawed samples of semen with TALP-HEPES and counting sperm cells using a Neubauer hemacytometer at x400 magnification with a light microscope.

Acrosome integrity was assessed by Alexa Fluor-488-peanut agglutinin conjugate (Molecular Probes, Eugene, 144 OR, USA) using the same procedure as earlier described (Varisli *et al*., 2009). Briefly, a 20 μL aliquot of sperm suspension was smeared on a microscopic slide and allowed to dry. The samples were then fixed with methanol and allowed to air-dry in an air-filter hood (Captair, DFS Co., Ltd. Jiangsu, China). After fixation, sperm samples were incubated with 40 μg mL^{-1} Alexa Fluor-488-peanut agglutinin conjugate at 37°C for 30 min and washed with PBS (pH 7.4) followed by counting and analysis under a confocal Laser Scanning System (LSM 510 META; Carl Zeiss, Jena, Germany). The fluorophores were excited with the 488 nm line of the laser and Alexa Fluor-488 emission was collected with a 530 nm band pass filter. The observed images of sperm stained with Alexa Fluor-488-peanut agglutinin conjugate were classified into three groups (Ping *et al*., 2011); sperm displaying intensively uniform apple-green fluorescence of the acrosomal cap indicating an intact acrosome; sperm displaying partially green fluorescence of the acrosomal cap indicating the process of breakdown of the acrosomal cap (partially damaged acrosome) and sperm displaying no fluorescence indicating a complete loss of the outer

acrosomal membrane (damaged acrosome). In each replicate, >200 sperm were counted to determine the percentage of sperm with intact acrosome.

Statistical analysis: All data were expressed as means±standard error of the means. The percentage data for motility and acrosome integrity were subjected to arcsine transformation before statistical analysis. Then analysis of variance and Tukey's test were used to analyze the differences in motility and acrosome integrity among treatment groups. Results of the fertilization rate and the proportion of tree shrews yielding fertilized oocytes with fresh sperm and frozen sperm were analyzed by χ^2-test. Number of oocytes and fertilized oocytes recovered after ovarian stimulation and AI treatment were analyzed by ANOVA and Tukey's test. All data were analyzed using SPSS Version 11.5 Statistical Software (SPSS Inc., Chicago, IL, USA). For all analyses, $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Experiment 1: For each time of equilibrium at 4°C, the lowest sperm motility occurred at the highest concentration, 10% of each CPA in TTE (Table 1). Compared with the control at 30 min of equilibration, sperm motility decreased significantly in 1, 3 and 6% of PG, EG, and Gly, respectively. The loss of motility was greater at longer times of exposure. In contrast, sperm motility in 1, 3 and 6% DMSO did not differ significantly from the control and any time point of equilibration ($p>0.05$).

Experiment 2: The most notable finding was that sperm frozen in TTE + 3% DMSO had the greatest post-thaw motility and recovery rate of motility ($p<0.05$, Table 2). Additionally, sperm frozen in TTE + 1% DMSO and TTE + 3% EG exhibited higher motility recovery rates than did the control ($p<0.05$). However, the recovery of sperm motility for all other CPAs and concentrations was similar to that of the control ($p>0.05$, Table 2). Acrosome integrity was significantly higher in sperm frozen in 6% EG and 10% Gly compared to controls ($p<0.05$, Table 2). All other CPAs and concentrations resulted in acrosome integrities that were similar to that of the control ($p>0.05$).

Table 1: Tree shrew sperm motility after equilibration in cryodilutents with permeable CPAs at 4°C

CPA (%)	Sperm motility before equilibration (fresh)	Sperm motility after equilibration (%)		
		30 min	60 min	90 min
Non-CPA	76.9±0.8	73.1±1.3^{abA}	71.6±1.3^{abA}	68.2±1.1^{aA}
1% DMSO	-	75.4±2.1^{aA}	75.2±2.1^{aA}	71.8±2.4^{aA}
3% DMSO	-	74.3±2.2^{abA}	69.7±2.5^{abA}	65.3±1.7^{abA}
6% DMSO	-	64.8±3.3^{abcdA}	60.9±3.0^{abcA}	57.9±3.0^{abcA}
10% DMSO	-	52.2±2.3^{cdefA}	48.1±1.7^{cdeAB}	39.0±2.1^{defB}
1% PG	-	72.2±1.3^{abA}	71.3±1.3^{abA}	67.9±1.3^{abA}
3% PG	-	67.4±3.0^{abcA}	63.2±3.4^{abcAB}	59.7±3.0^{abcB}
6% PG	-	52.5±4.3^{cdefA}	49.8±4.7^{cdeAB}	45.6±4.8^{cdeB}
10% PG	-	46.3±4.7^{efA}	41.9±4.9^{defAB}	37.3±4.9^{efB}
1% EG	-	71.6±2.4^{abA}	69.2±2.3^{abAB}	64.6±2.3^{abB}
3% EG	-	57.4±2.6^{bcdeA}	54.5±2.6^{bcdAB}	50.4±2.7^{bcdeB}
6% EG	-	45.8±3.6^{efA}	41.7±3.7^{defAB}	33.3±2.6^{efB}
10% EG	-	37.5±4.2^{fA}	31.5±2.9^{efgAB}	25.3±1.7^{fgB}
1% Gly	-	64.8±1.3^{abcdA}	62.6±1.2^{abcAB}	56.4±2.2^{abcdB}
3% Gly	-	48.1±5.0^{defA}	45.1±4.9^{cdefAB}	40.2±4.6^{defB}
6% Gly	-	37.4±4.7^{fA}	30.2±6.7^{fgAB}	26.5±5.1^{fgB}
10% Gly	-	20.6±3.9^{gA}	17.3±3.8^{gAB}	13.0±2.3^{gB}

$^{a-g}$Within a column, values without a common superscript differ ($p<0.05$); $^{A, B}$within a row, values without a common capital superscript differ ($p<0.05$); CPA: Cryoprotectant; DMSO: Dimethyl Sulfoxide; PG: Propylene Glycol; EG: Ethylene Glycol; Gly: Glycerol; n = 8 per condition

Table 2: Pre-freeze and post-thaw motility and acrosome integrity of tree shrew sperm cooled and frozen with permeable CPAs

CPA (%)	Motility (%)			Recovery rate of motility (%)	Acrosome integrity (%)	
	Fresh	Pre-freeze	Post-thaw		Fresh	Post-thaw
Non-CPA	73.4±0.4	69.3±1.8^{ab}	27.7±1.3^{cd}	40.1±1.8^{defgh}	92.5±1.1	73.6±2.5^{c}
1% DMSO	-	70.4±1.9^{a}	35.1±2.3^{b}	50.5±4.0^{bc}	-	77.5±2.7^{bc}
3% DMSO	-	69.3±1.7^{ab}	44.9±1.8^{a}	65.2±3.0^{a}	-	79.8±1.6^{bc}
6% DMSO	-	60.3±1.6^{cd}	28.9±1.5^{cd}	47.9±2.1^{bcd}	-	78.7±1.7^{bc}
10% DMSO	-	48.8±1.3^{efg}	21.8±1.3^{ef}	45.0±2.9^{cde}	-	78.1±1.3^{bc}
1% PG	-	70.7±1.8^{a}	29.2±1.9^{cd}	42.0±3.2^{defg}	-	71.5±1.9^{c}
3% PG	-	66.1±1.7^{abc}	29.9±1.3^{c}	45.9±2.8^{bcd}	-	79.2±2.0^{bc}
6% PG	-	51.5±1.3^{ef}	24.7±1.3^{de}	48.4±2.9^{bcd}	-	80.7±1.6^{bc}
10% PG	-	46.0±1.2^{fg}	16.1±1.5^{g}	35.3±3.6^{fgh}	-	72.1±2.4^{c}
1% EG	-	67.1±1.8^{abc}	25.6±1.8^{cde}	38.6±3.3^{defgh}	-	74.6±2.1^{bc}
3% EG	-	53.8±1.4^{de}	28.7±2.9^{cd}	54.2±5.0^{b}	-	79.3±2.6^{bc}
6% EG	-	42.9±1.1^{gh}	19.5±2.0^{fg}	45.1±4.2^{cde}	-	82.6±1.7^{ab}
10% EG	-	34.8±0.9^{i}	15.6±0.8^{g}	44.7±1.7^{cdef}	-	76.8±3.0^{bc}
1% Gly	-	63.5±1.6^{bc}	22.3±2.3^{ef}	35.6±4.0^{efgh}	-	75.4±2.3^{bc}
3% Gly	-	47.1±1.2^{efg}	16.1±1.6^{g}	34.0±3.1^{gh}	-	79.2±2.7^{bc}
6% Gly	-	36.4±0.9^{hi}	11.4±0.7^{h}	31.4±1.9^{h}	-	76.4±6.2^{bc}
10% Gly	-	20.1±0.5^{j}	9.0±0.9^{h}	44.6±4.2^{cdef}	-	83.0±1.4^{ab}

$^{a-h}$Within a column, values without a common superscript differ ($p<0.05$). CPA: Cryoprotectant; DMSO: Dimethyl Sulfoxide; PG: Propylene Glycol; EG: Ethylene Glycol; Gly: Glycerol; n = 12 for each condition

Experiment 3: As shown in Table 3, there were no differences in the fertilization rate of oocytes and the proportion of tree shrews yielding fertilized oocytes inseminated with fresh and frozen sperm ($p>0.05$).

In this study, researchers evaluated the motility and acrosome integrity of post-thawed tree shrew sperm that were equilibrised and frozen in four concentrations of the CPAs DMSO, EG, PG and Gly, respectively. The result demonstrated that DMSO showed the high cryopretective ability and the least prefreezing toxicity. Furthermore, AI trial showed the same capacity for fertilization of sperm cryoproserved by 3% DMSO as control of fresh sperm. To a certain extent, all CPAs can be harmful for cells because of inherent toxicity, even at relatively low concentrations (Fahy *et al*., 1990; Fernandez-Santos *et al*., 2005). However, the use of CPAs is essential for protection of sperm and ova against freezing damage that arises due to intracellular ice crystal formation (Yang *et al*., 2007). The optimal choice of CPA for each species seems to have been largely a matter of trial and error. This is partly because a complete and satisfactory explanation for the action of CPAs does not exist yet (Holt, 2000).

Permeable CPAs have different cryoprotective efficacies with various mammalian spermatozoa (Jones, 1973; Kashiwazaki *et al*., 2006; Li *et al*., 2005a). Glycerol and EG are always employed for non-human primate sperm cryopreservation (Li *et al*., 2005b; Sadleir, 1966; Seier *et al*., 1993; Si *et al*., 2004). However, in the present study, compared with Gly, EG, PG, DMSO might cause less osmotic stress or prefreezing toxic effects during the addition of the CPA before freezing and removal of CPA after thawing and therefore could provide sufficient cryoprotection and result in fewer cell injuries. Similarly, Gly is also unsuitable for sperm cryopreservation in rodents including mice and rats or rabbits (Kashiwazaki *et al*., 2006; Nakatsukasa *et al*., 2001; Sztein *et al*., 2001). DMSO is more effective than Gly in these species (Kashiwazaki *et al*., 2006; Sztein *et al*., 2001). Thus, permeability coefficient of tree shrew sperm could be more similar to that of rodents not to that of non-human primates which could imply that tree shrew in reproduction aspect, may be close to rodent.

Table 3: Fertilizing ability of fresh and frozen-thawed tree shrew sperm

Fertilizing ability	Fresh sperm	Frozen sperm
Sperm motility (Mean±SEM%) before AI	68.9±5.7	43.5±3.3
Sperm acrosome integrity (Mean±SEM%) before AI	87.8±3.4	78.7±2.1
No. of female tree shrews used	5	5
No. of tree shrews yielding fertilized oocytes (%)*	5 (100)	5 (100)
No. of oocytes recovered (Mean±SEM per female)	24 (4.8±0.8)	21 (4.2±1.5)
No. of fertilized oocytes **(Mean±SEM per female)	17 (3.4±1.1)	12 (2.4±1.1)
Fertilizition rate of oocytes (%)	69.7±14.2	55.3±7.8

*Relative to the number of tree shrews used; **At least two-cell embryos when recovered

One surprising finding is that the CPA had almost no effect on acrosome integrity. This indicates that the acrosome of tree shrew sperm is more resistant to freezing compared with motility. Thus, sperm motility is a better parameter for evaluation of tree shrew post-thaw sperm function (Ping *et al*., 2011).

Artificial insemination with frozen-thawed sperm always results in lower fertility rates than with freshly harvested sperm (O'Meara *et al*., 2005). Therefore, researcher examined the fertilizing ability of fresh and frozen sperm by comparing the fertilization but not pregnancy rate because the breeding management and ART protocols in tree shrews have not been reported yet. However, the *in vivo* fertilization rate (57.1%) of sperm frozen with the addition of 3% DMSO into TTE in the present study was slightly high to that (56.5%) of sperm frozen only with TTE in the earlier study (Ping *et al*., 2011) even though the addition of 3% DMSO into TTE did improve sperm motility compared with that frozen without any penetrating CPA in the present study. One reason could be that male tree shrews used in this study were wild, not domesticated animals. These animals did not have the opportunity for rich nutrition supplementation that could improve sperm quality. Another reason may be due to small number of animals used in this study, inappropriate ovarian stimulation or both.

CONCLUSION

The study shows that the toxicity and cryoprotective efficacy of penetrating cryoprotectants on tree shrew sperm survival. The results showed that epididymal sperm of wild tree shrews can be cryopreserved in TTE + 3% DMSO and that the frozen/thawed sperm have the ability to fertilize oocytes *in vivo* by intrauterine AI.

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