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Short Communication

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Abstract

Two semen samples of Siamese Eld's deer (*Cervus eldii siamensis*: SED) and seven semen samples of Thamin Eld's deer (*Cervus eldii thamin*: TED) were collected by using electroejaculation. Each ejaculate was diluted and cryopreserved with 2 extenders; 1) TRIS; and 2) modified BF5F supplemented with 5% final glycerol concentration. The movement patterns of frozen-thawed spermatozoa were assessed using computer-assisted sperm analysis (CASA). In TED, the results revealed a significantly higher ($p< 0.05$) percentage of total motility (%TM), percentage of progressive motility (%PM), track speed (VCL) and lateral amplitude (ALH) in TRIS extender. The results also revealed a significantly low ($p< 0.05$) progressive velocity (VSL), straightness (STR) and linearity (LIN) in TRIS extender. However, for path velocity (VAP) and beat frequency (BCF), there were no differences between both extenders. In SED, sperm motility and sperm movement parameters did not show differences between TRIS and modified BF5F extenders. Findings from this study will aid in Eld's deer semen banking and artificial insemination success in the future.

Keywords: Eld's deer, frozen semen, motility, movement patterns, semen extender

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บทคัดย่อ

ผลของสารละลายน้ำเชื้อต่อการเคลื่อนที่และรูปแบบการเคลื่อนที่ของน้ำเชื้อละมั่ง (*Cervus eldii*) แช่แข็งหลังการอุ่นละลาย

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ทำการเก็บตัวอย่างน้ำเชื้อจำนวนสองตัวอย่างจากละมั่งพันธุ์ไทย (Siamese Eld's deer *or Cervus eldii siamensis* or SED) และตัวอย่างน้ำเชื้อจำนวนเจ็ดตัวอย่างจากละมั่งพันธุ์พม่า (Thamin Eld's deer *or Cervus eldii thamin* or TED) ด้วยวิธีการกระตุ้นด้วยไฟฟ้าผ่านทางทวารหนัก นำตัวอย่างน้ำเชื้อที่เก็บได้ไปประเมินคุณภาพเบื้องต้น จากนั้นนำไปเจือจางด้วยสารละลายน้ำเชื้อสำหรับแช่แข็ง 2 ชนิด ได้แก่ 1) TRIS และ 2) modified BF5F โดยสารละลายที่เจือจางน้ำเชื้อแล้วทั้งสองชนิดมีกลีเซอรอลความเข้มข้นสุดท้ายที่ 5% ก่อนนำไปแช่แข็ง จากนั้นทำการอุ่นละลายแล้วนำไปตรวจลักษณะการเคลื่อนที่ด้วยเครื่อง computer-assisted sperm analysis (CASA) จากการทดลองพบว่า ในละมั่งพันธุ์พม่า ค่า percentage of total motility (%TM), percentage of progressive motility (%PM), track speed (VCL) และ lateral amplitude (ALH) ของน้ำเชื้อในสารละลาย TRIS มีค่าสูงกว่าอย่างมีนัยสำคัญ อย่างไรก็ตามค่า progressive velocity (VSL), straightness (STR) และ linearity (LIN) ของน้ำเชื้อในสารละลาย TRIS มีค่าต่ำกว่าอย่างมีนัยสำคัญเช่นกัน นอกจากนี้ยังพบว่าค่า path velocity (VAP) และ beat frequency (BCF) ของน้ำเชื้อในสารละลายทั้งสองชนิดนั้นไม่มีความแตกต่างกัน สำหรับละมั่งพันธุ์ไทย ทุกลักษณะการเคลื่อนที่ของน้ำเชื้อแช่แข็งหลังการอุ่นละลายในสารละลายทั้งสองชนิดนั้นไม่มีความแตกต่างกัน ผลการทดลองที่ได้จากการศึกษานี้จะเป็นประโยชน์ต่อการสร้างธนาคารน้ำเชื้อและการผสมเทียมละมั่งในอนาคต

คำสำคัญ: ละมั่ง น้ำเชื้อแช่แข็ง การเคลื่อนที่ รูปแบบการเคลื่อนที่ สารละลายน้ำเชื้อ

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Introduction

Eld's deer or brow-antlered deer (*Cervus eldii*) is listed as an endangered (EN) species in Red List by World Conservation Union: IUCN (Lynam et al., 2011). It is 1 of 15 national reserved species of Thailand under Wildlife Preservation and Protection Act (1992). There are 4 subspecies of Eld's deer in the world including 1) Manipur Eld's deer (*Cervus eldii eldii*), 2) Thamin Eld's deer (*C. e. thamin*), 3) Siamese Eld's deer (*C. e. siamensis*) and 4) Hainan Eld's deer (*C. e. hainanus*). Their distribution range is from India and along the dry forests of South-East Asia and Hainan Island. There are 2 subspecies of Eld's deer in Thailand including Siamese Eld's deer or SED and Thamin Eld's deer or TED, both extinct in the wild for over 4 decades due to habitat loss and poaching. At present, small numbers of both subspecies are kept in captivity (approximately 1000 TEDs and 50 SEDs). Due to the few numbers of captive founders, the small populations are facing with an increased inbreeding pressure. Management for enhancing their genetic diversity of both species has become widely concern. Assisted Reproductive Technology (ART) such as artificial insemination (AI) with frozen semen is one of the tools to overcome infertility and achieve offspring production. Frozen sperm is beneficial to preservation of their genes and breeding program management (Hammerstedt et al., 1990; Fickel et al., 2007). The first successful artificial insemination using frozen-thawed spermatozoa of TED was performed in USA (Monfort et al., 1993) and the latest or second successful AI in TED was done in 2010. However, the conception rate was low. Furthermore, there has been no report of successful AI in SED. For cryopreservation study, extenders that were used for

energy source of spermatozoa, maintaining pH and protecting cell and plasma membrane from crystal ice formation during freezing were important factors for saving the quality and viability of frozen-thawed spermatozoa (Hammerstedt et al., 1990). Post-thaw semen quality is one of the important factors that affect AI success rate. In general, deer semen extender was developed from the commonly used sugar-based tris and/or citrate-buffered (Asher et al., 2000). It is similar to those developed for other small ruminants (Cheng et al., 2004). For deer semen cryopreservation, egg yolk is commonly used for protection against cold shock and glycerol is also commonly used as cryoprotectant (Asher et al., 2000). Despite, the acceptance of existing diluents, research focusing on particular deer species is required. However, limited information on Eld's deer semen cryopreservation and evaluation is available. BF5F extender was successfully used to preserve Eld's deer spermatozoa (Monfort et al., 1993). However, in that report, details about semen cryopreservation were not available. The objective of this study was to compare the survival of frozen-thawed semen that is cryopreserved by using BF5F and TRIS extenders. Semen evaluation was carried out by computer-assisted sperm analysis or CASA, system diminishing human error and bias. Sperm movement patterns have already been reported on the correlation with various fertility factors in some species such as human (Larsen et al., 2000; Fréour et al., 2012) and boar (Broekhuijse et al., 2012). In this present study, the effects of freezing extenders on sperm motility and movement patterns were compared using CASA.

Materials and Methods

Chemicals: All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO) unless stated otherwise.

Animals and semen collection: Two SED and 7 TED were used for semen collection and cryopreservation study. The deer semen samples were collected by using electroejaculation during March to July 2009. Adult male Eld's deer (more than 2 years of age) were used in this study. The SED (n= 2) were housed at Banglamung Wildlife Breeding Station in Chonburi province. The TED were housed at Kao Kheow Open Zoo (n= 4) and Nakhonratchasima Zoo (n=3). The Eld's deer were anesthetized by using a combination of ketamine hydrochloride (3-5 mg/kg) (Calypsol®,Gedeon Richter Ltd., Budapest, Hungary) and xylazine hydrochloride (0.5 mg/kg) (Ilium Xylazil-100®, Troy laboratories PTY Limited, Smithfield NSW, Australia) (Noimoon et al., 2011). They were mixed in a single dart and administered intramuscularly via a dart (DanInject®, Denmark). Ejaculations were stimulated by a sine-wave electrostimulator (AC, 60-150 Hz, PT Electronics, Boring, Oregon). The diameter of a teflon rectal probe with three longitudinal electrodes was 3 cm. A total of 3 series of 10 stimuli were performed (the first series were 3, 4 and 5 volts; the second series were 4, 5 and 6 volts; and the third series were 5, 6 and 7 volts, respectively). Between each series, the animals rested for 5 min and their vital signs were evaluated. The protocols used with animals in this study were approved by Kasetsart University Animal Ethic Committee.

Semen evaluation and cryopreservation: Each ejaculation was immediately evaluated for volume, color, pH, sperm concentration, sperm motility and sperm viability. Sperm concentration was assessed using a hemocytometer (Anzar et al., 2009). Due to the unavailability of CASA in the field, progressive motility was visually assessed by using light microscopy (Nikon, Eclipse E200, Tokyo, Japan) by two independent investigators. Sperm viability was assessed by using eosin-nigrosin staining. The ejaculate with more than 60 percentage of progressive motility and more than $200x10^6$ sperm/ml of sperm concentration were selected for cryopreservation study as previously described in Asian elephant (Thongtip et al., 2004).

The semen sample was diluted with 2 kinds of extenders: modified BF5F and TRIS. Diluent BF5F is a modification of diluent BF5 which was developed for freezing boar semen (Pursel and Johnson, 1975). It has successfully been used in African elephant sperm cryopreservation (Howard et al., 1986). For this study, BF5F was modified for using in deer semen cryopreservation. Modified BF5F extender consists of fructose 1.6%, glucose 1.6%, Tes 1.2%, Tris 0.2%, sodium lauryl sulfate 0.5%, penicillin G 0.006%, streptomycin 0.1%, egg yolk 20% and glycerol 5% (Howard et al., 1986). TRIS extender consisted of fructose 0.5%, citric acid 1.99%, Tris 3.36%, penicillin G 0.006%, streptomycin 0.1%, egg yolk 15% and glycerol 5% (Evans and Maxwell, 1987). Two-steps cryopreservation method was used. Each semen sample was split into 2 aliquots, diluted with the extenders, and adjusted to a final concentration of $100x10^6$ sperm/ml. The extenders were divided into two parts. The first part extender contained no glycerol or 0% glycerol while the second part contained double amount of glycerol or 10% glycerol. Initially, part I extender was slowly added to a semen sample (1:1) at room temperature. The semen mixture was cooled from room temperature (28–32^{0}C) to 5^{0}C on ice in a Styrofoam box. Thereafter, equal volume of part II extender containing 10% glycerol was divided into four parts and each part was added to the semen mixture every 15 min. Then, the semen mixture was further equilibrated at 5^{0}C for 1 hour. The equilibrated semen was packed in a 0.25 ml labeled plastic straw container (Kruuse, Ltd., Leeds, UK) and sealed by the sealing powder (ARS™, Chino, CA). The straws were held at 2.5 cm above liquid nitrogen level for 10 min and then were plunged into liquid nitrogen and kept in the canister of liquid nitrogen tank at least 7 days before thawing.

Thawing and post-thaw evaluation: The frozen samples were rapidly thawed by placing the straws in a 37^{0}C water bath for 30 sec. The semen samples were expelled into a 1 ml microtube and kept on a 37^{0}C warm plate. Five µl aliquot of the thawed semen was placed on a pre-warmed microscopic slide and the progressive motility of post-thaw spermatozoa was assessed under light microscopy. The remaining thawed semen was subjected for sperm motility and

movement pattern assessment by using computer-assisted sperm analysis (CASA).

Sperm motility and movement pattern analysis: The computer-assisted sperm analysis or CASA (TOX IVOS version 12.0 Hamilton Thorne Biosciences, Beverly MA, USA) was used for sperm motility and movement pattern assessment. The machine set up followed goat semen guideline recommended by the manufacturing company (Davis and Katz, 1993). At least 800 sperms were counted in one time with 60x magnification. Playback checking was always done and error was corrected before starting new assessment. Sperm motility was assessed for percentage of total motility (%TM) and percentage of progressive motility (%PM). Sperm movement patterns were assessed for path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR) and linearity (LIN).

Statistical analysis: The comparison of mean±SEM of fresh semen qualities of SED and TED were conducted using *t*-Test. The comparison of mean±SEM of sperm motility and movement patterns of frozen-thawed spermatozoa between TRIS and modified BF5F extenders were done by using paired sample *t*-test.

Results and Discussion

The fresh semen characteristics are summarized and shown in Table 1. Ejaculates were obtained from all deer. During electrical stimuli, the physical responses of the deer and the characteristics of obtained ejaculates differed among each series. Series I, the semen appearance was thick and milky color. Occasionally, some deer was not ejaculated in this series. Series II, semen appearance was still thick and milky color. However, the color was clearer than the first ejaculation and almost all deer ejaculated in this series. Series III, semen appearance was clear and slightly sticky. The ejaculate volume (SED: 2.1±0.5 ml; TED: 2.1±0.4 ml), pH (SED: 7.4±0.2; TED: 7.2±0.2), sperm concentration (SED: 1079.6±138.0x10^6/ml; TED: 1007.5±177.5x10^6/ml), percentage of progressive motility (SED: 80.0±3.1%; TED: 80.0±0%) and sperm viability (SED: 89.1±2.3%; TED: 88.5±4.5%) between the two subspecies were not significantly different (p< 0.05).

The comparison of mean±SEM of the percentage of sperm motility and sperm movement parameters of frozen-thawed samples between TRIS and BF5F extender is shown in Table 2. For TED, mean±SEM of movement parameters in TRIS extender were as follows: TM 56.4±8.3%, PM 30.6±4.3%, VAP 152.2±7.6 µm/sec, VSL 91.7±3.0 µm/sec, VCL 284.6±14.1 µm/sec, ALH 16.0±1.0 µm, BCF 27.6±1.2 Hz, STR 62.1±2.4 % and LIN 34.4±1.7%. Mean±SEM of movement parameters in modified BF5F extender were as follow: TM 36.1±8.7%, PM 23.0±4.1%, VAP 138.3±8.4 µm/sec, VSL 107.4±5.0 µm/sec, VCL 226.7±18.6 µm/sec, ALH 11.8±1.6 µm, BCF 30.5±0.9 Hz, STR 77.3±4.1% and LIN 50.1±4.6 %. The results revealed the significantly higher (p< 0.05) of %TM, %PM, VCL, and ALH in samples diluted with TRIS extender. The results also revealed the significantly lower (p< 0.05) VSL, STR and LIN in TRIS extender. However, there were no differences between extenders for VAP and BCF. For SED, mean±SEM of movement parameters in TRIS extender were as follows: TM 34.0±9.0, PM 23.0±5.0, VAP 140.3±26.2 µm/sec, VSL 101.3±9.7 µm/sec, VCL 241.0±51.0 µm/sec, ALH 12.5±1.6 µm, BCF 26.4±1.2 Hz, STR 73.0±3.5% and LIN 45.5±2.5%. Mean±SEM of movement parameters in modified BF5F extender were as follows: TM 29.5±22.5%, PM 23.0±17.0 %, VAP 146.5±28.4 µm/sec, VSL 126.5±20.0 µm/sec, VCL 211.6±60.4 µm/sec, ALH 8.8±2.3 µm, BCF 31.55±0.8 Hz, STR 86.0±5.0% and LIN 66.0±9.0%. Sperm motility and sperm movement parameters of SED did not show any differences between TRIS and modified BF5F extender.

Table 1 Fresh semen characteristics of SED (*C. e. siamensis*) and TED (*C. e. thamin*)

Semen parameters	TED (n= 7)	SED (n= 2)
Volume (ml)	2.1±0.5	2.1±0.4
pH	7.4±0.2	7.2±0.2
Concentration (x10^6/ml)	1079.6±138.0	1007.5±177.5
Progressive motility (%)	80.0±3.1	80.0+0
Viable sperm (%)	89.1±2.3	88.5±4.5

Values are mean±SEM

In this study, semen samples were successfully collected from all males. All fresh ejaculates showed the high sperm motility (80-90%) visually evaluated by two independent researchers. Fresh semen characteristics of Eld›s deer in this study were consistent with red deer (*Cervus elaphus*) (Giżejewski, 2004). For previous deer semen cryopreservation studies, the diluents used in the studies were based on Citrate-based diluent and Tris-based diluent (Asher et al., 2000). Reports showed acceptable post-thawed motility in deer species such as Pe`re David›s deer (*Elaphurus davidianus*) (40-55%) (Asher et al., 1988), White-tailed deer (*Odocoileus virginianus*) (> 50%) (Jacobson et al., 1989), Fallow deer (*Dama dama*) (70%) (Asher et al., 1993) and Red deer (*Cervus elaphus*) (30-70%) (Asher et al., 1988; Fennessy et al., 1990; Zomborszky et al., 1999). For Eld›s deer semen cryopreservation study, by using BF5F extender, Monfort et al. (1993) reported high post-thaw motility (65-70%) with high proportion of morphologically normal sperm. However, in this study, TRIS extender showed the better quality of frozen-thawed semen than modified BF5F extender. The frozen-thawed semen cryopreserved with TRIS extender showed significantly higher (p< 0.05) percentage of TM and PM. Furthermore, VCL and ALH levels in semen samples with TRIS extender were also significantly higher than those of modified BF5F. Therefore, TRIS extender can be one alternative choice for Eld›s deer semen cryopreservation. In this present study, we found lower percentage of post-thaw motility in frozen-thawed semen cryopreserved with BF5F than that previously reported by Monfort et al. (1993). Some chemicals in our modified BF5F

Table 2 Comparison of frozen-thawed sperm motility and movement parameters (means±SEM) between TRIS and modified BF5F extender assessed by CASA

Parameter	TED (n=7)		SED (n=2)	
	TRIS	BF5F	TRIS	BF5F
TM (%)	56.4±8.3[a]	36.1±8.7[b]	34.0±9.0	29.5±22.5
PM (%)	30.6±4.3 [a]	23.0±4.1 [b]	23.0±5.0	23.0±17.0
VAP (μm/sec)	152.2±7.6	138.3±8.4	140.3±26.2	146.5±28.4
VSL (μm/sec)	91.7±3.0 [a]	107.4±5.0 [b]	101.3±9.7	126.5±20.0
VCL (μm/sec)	284.6±14.1 [a]	226.7±18.6 [b]	241.0±51.0	211.6±60.4
ALH (μm)	16.0±1.0 [a]	11.8±1.6 [b]	12.5±1.6	8.8±2.3
BCF (Hz)	27.6±1.2	30.5±0.9	26.4±1.2	31.55±0.8
STR (%)	62.1±2.4 [a]	77.3±4.1 [b]	73.0±3.5	86.0±5.0
LIN (%)	34.4±1.7 [a]	50.1±4.6 [b]	45.5±2.5	66.0±9.0

Values are mean±SEM, Different superscripts within the same parameter indicate significant different ($p< 0.05$).

differed from the original formula. Two substances in the extender were changed. Sodium and triethanolamine lauryl sulfate or Equex was replaced by sodium lauryl sulfate or SLS. SLS has been proved to enhance post-thaw quality of canine (Hori et al., 2006) and feline (Mizutani et al., 2010) frozen semen. However, in this study, the efficiency of freezing with and without SLS was not assessed. The effects of using SLS in Eld›s deer semen remain unclear. In addition, the final concentration of glycerol in this study was only about 5%, which is lower than 8% glycerol used in Monfort et al. (1993). Therefore, glycerol in modified BF5F extender (this study) may not be optimized to protect Eld›s deer sperm during cryopreservation protocol. The effect of SLS and Equex and the percentage of final concentration of glycerol should be studied and optimized in the future. The evaluation of sperm motility and movement patterns using CASA is an alternative method. It is repeatable, objective and helps reduce manner bias or human error (Krause and Viethen, 1999). Although this study used the set up of goat semen, CASA successfully assessed Eld›s deer sperm quality. However, the calibration of the specific deer sperm set up need to be done in the future.

Results from this study indicate that TRIS extender could be an alternative choice for Eld›s deer spermatozoa cryopreservation. This result will assist in Eld's deer artificial insemination, ART research and the establishment of Eld's deer Genome Resource Bank in the future.

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