EVALUATION OF SEMEN EXTENDERS AND ANTLER STAGES ON CHARACTERISTIC OF FRESH AND POST-THAWED SPERMATOZOA IN HOG DEER (*AXIS PORCINUS*)

Angsumalin Jaisawang[1,2], Apichaya Sudsukh[1,2], Manakorn Sukmak[3], Sirinart Chaichnathong[1,2], Ampika Thongphakdee[4], Worawidh Wajjwalku[5] & Nikorn Thongtip[1,2,6,*]

ABSTRACT

The objective of this study was to evaluate fresh and post-thawed semen quality in hog deer (*Axis porcinus*). Fourteen ejaculates (*n*=14) from two hog deer bucks were collected by electro-ejaculation during breeding (hard antlered stage, *n*=10) and non-breeding season (velvet antlers, *n*=4). The fresh semen of hard antlered stage revealed higher volume and progressive motility than those in velvet antlered stage 2.6 times. The width of testicular size of hard antlered stage was significantly higher than those in velvet antlered stage. Ejaculated semen samples were diluted with three semen extenders included of TRIS, TEST and modified BF5F. The diluted samples were cryopreserved. After thawing, post-thawed semen was analyzed. The highest percentage of motility (39.30±7.24%) and progressive motility (22.70±5.95%) were found in hard antlers. In hard antlers, values of movement pattern were also higher than those in velvet antlers. Moreover, percentages of total motility (33.21±4.99%) and progressive motility (18.14±3.07%) were highest when semen was diluted with TRIS extender (P<0.05). For movement patterns, only VAP and VCL were highest values in TRIS extender. TRIS extender also provided more high value of movement pattern parameters. Thus, the results indicated the hog deer semen collection during hard antlers and dilution with TRIS extender were preferred for maintaining hog deer frozen semen. These results fulfilled our basal knowledge for development of assisted reproductive technologies (ARTs) in this species

Keywords: Antlers, extender, hog deer, semen cryopreservation

INTRODUCTION

Hog deer (*Axis porcinus*) is classified as a protected species according to the National Wildlife Protection and Preservation Act of 1992, as well as endangered species listed according to IUCN (2011). It is a member of wild deer in Thailand. However, habitat loss and poaching in wild population

[1] Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
[2] Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Department Commission on Higher Education, Ministry of Education (AG-BIO/PERDO-CHE)
[3] Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen, Nakorn Pathom, 73140, Thailand
[4] Wildlife Reproductive Innovation Center, Bureau of Conservation and Research, Zoological Park Organization under the Royal Patronage H.M. the King, Bangkok, 10300, Thailand
[5] Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
[6] Department of Large Animal and Wildlife Clinical Science, Faculty of Veterinary Medicine, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
* Corresponding author: fvetnit@kku.ac.th

and decreasing of genetic diversity in captivity are leading hog deer to high risk of extinction. Semen cryopreservation is the one of assisted reproductive technologies (ARTs) that has been used to increase animal number and genetic diversity via artificial insemination in wildlife species (Wildt, 2009).

Research on the hog deer has a few studies in Thailand. However, Cervidae family has several literatures available on aspects of reproductive physiology. Previous studies revealed the relation to the seasonality of reproduction in cervids (Monfort *et a*l., 1993*b*; Phraluk *et al*., 2014; Sudsukh *et al*., 2017). During mating season, the reproductive organ of the male deer revealed a maximum gonad size and spermatogenesis (Scanlon & Lenker, 1983). This breeding season is related to fertility (Asher *et al*., 1987). In addition, the reports revealed that artificial photoperiodic regimens affected the timing of the annual antler and testicular growth cycle of males in red deer (Bubenik & Bubenik, 2012) and Sika deer (Goss, 1983). Then, all reports were proved about seasonality of male deer reproductive physiology.

ART is the one of alternative technique for wildlife conservation. It has been introduced into the animal breeding for increasing animal number and genetic diversity that might be solved inbreeding problem. Cryopreservation is a technique for long-term semen storage. Though, freezing and thawing can be affected to spermatozoa quality. The methods for processing and freezing of spermatozoa have been developed to reduce cryogenic injures to spermatozoa. Extender is liquid diluents to preserve semen, which is supported to protect spermatozoa, provide energy source and prevent the effects of changed temperature (Anel *et al*., 2010). Nevertheless, deer species required various extender diluents and storage techniques on a cryopreservation by species basis (Asher *et al*., 2000). Previous study reported that the most common diluents used successfully for deer species are sodium citrate-egg yolk-glycerol (Krzywinski & Jaczewski, 1978) and tris-glucose-citrate-egg yolk-glycerol (Evans & Maxwell, 1987). In Addition, Monfort et al. (1993a) suggested that an extender used for Eld's deer semen freezing (BF5F with 8% final concentration of glycerol) provided good results for Eld's deer semen. Garde *et al*. (2008) reported that TEST could be used in Cuvier's gazelle (*Gazella cuvieri*).

Therefore, the aims of this study were to find out the suitable period for semen collection and to compare the ability of three extenders to maintain Hog deer spermatozoa after cryopreservation between hard antlers and velvet antlers. And, Computer-Assisted Sperm Analysis (CASA) was used to assess semen quality.

MATERIALS AND METHODS

Two hog deer bucks housed at Faculty of Veterinary Medicine Kamphaengsaen Campus, Kasetsart University were used in study. Sexually mature and healthy bucks, weighing around 35-40 kg, average aging 5 years were selected for semen collection during December 2012 to November 2013. Anesthesia was done in differences antler stages (Figure 1). Hard antlered stage (Figure 1a) was during June to March and velvet antlered stage (Figure 1b) was during February to May.

Animals were anesthetized, measured for width, length and circumference of testes and collected semen by electro-ejaculator. After collection, semen sample was immediately evaluated.

All ejaculates were evaluated for color, volume, pH, sperm concentration and total sperm motility. The volume was estimated with micropipette. Concentration was measured using a hemocytometer. The pH was measured by pH paper. Semen samples that having with ≥ 60% progressive motility, ≥ 50x106 sperm/ml and 6-8 pH were used for cryopreservation studies (Thongtip *et al*., 2004).

Figure 1 Sexually mature and healthy hog deer bucks with hard antlered stage (a) and velvet antlered stage (b)

This study, we used three extenders for cryopreservation study including TRIS, TEST and modified BF5F (with 5% glycerol final concentration). The following three extenders were prepared for semen cryopreservation; 1) Tris-egg yolk consisted of 1% fructose, 1.4% citric acid, 2.7% Tris, 20% egg yolk, 1000 i.u/ml penicillin G and 1000 µg/ml streptomycin (Fernández-Santos *et al*., 2006*a*), 2) TEST consisted 4.83% Tes and 1.15% Tris, 0.4% glucose, 5% egg yolk, 1000 i.u/ml penicillin G and 1000 µg/ml streptomycin (Garde *et al*., 2003) and 3) modified BF5F consisted 1.6% glucose, 1.6% fructose, 1.2% Tes, 0.2% Tris, 20% egg yolk, 0.5% surfactant mixture of sodium lauryl sulfate, 1000 i.u/ml penicillin G and 1000 µg/ml streptomycin (Monfort *et al*., 1993*a,b*).

The extenders were divided into two parts. The first part contained no glycerol while the second part contained 10% glycerol. Firstly, the first extender was slowly added to a semen sample (1:1) at room temperature. The semen mixture was equilibrated at 5°C in styrofoam box (Fernández-Santos et al., 2006*a*). Next, half of second part of extender was slowly added to the semen mixture every 30 minutes and further equilibrated at 5°C for 1 h (Thongtip *et al*., 2004). Therefore, the final concentration of glycerol was 5%.

The mixture was packed in the 0.5 ml labeled plastic straw container (Krusse, Ltd., Leeds, UK) and sealed by the sealing powder. The straws were placed on a rack in a Styrofoam box containing liquid nitrogen and held at 4 cm above liquid nitrogen level for 10 min (Garde *et al*., 2008) and then plunged into liquid nitrogen and kept in the canister of liquid nitrogen tank at least 7 days before thawing (Thongtip *et al*., 2004).

Frozen sample was quickly thawed by placed at 37°C water bath for 30s and evaluated immediately. The comparison of post-thaw semen quality included percentages of motility, progressive motility, movement patterns, sperm morphology, acrosome integrity and functionally sperm membrane integrity. Percentages of total motility, progressive motility and movement patterns were analyzed by CASA (TOX IVOS version 12.0, Hamilton Thorne Bio- sciences, Beverly MA, USA). Morphology and acrosome integrity assessments were evaluated by Diff-Quick and Coomassie blue staining, respectively. Intact acrosome spermatozoa had a blue head with coomassie blue stained whereas non-intact spermatozoa showed colorless head. Functionally sperm membrane integrity was evaluated by hypo-osmotic swelling test (HOST). The resultant swelling of the tail means an intact membrane and presumably normally functioning spermatozoa whereas dead or abnormally functioning spermatozoa presents no tail swelling (Hossain *et al*., 1998).

An analysis of data was performed by SPSS. Data was presented as mean±S.E.M. The values were analyzed using one-way ANOVA to evaluate the effect of extenders on semen characteristic. Significant differences between periods of semen collection, depended on antlers cycle were determined by Pair *t*-test. The values were considered statically significant when $P<0.05$.

Table 1 Testicular volume of hog deer in hard and velvet antler stages.

	Width (cm)		Length (cm)		Testicular volume (cm^3)
	Left	Right	Left	Right	
Hard antlered deer (*n*=10)	3.64±0.21[a]	3.75±0.28[a]	7.1±0.48	6.99±0.40	55.52±11.30
Velvet antlered deer (*n*=4)	2.67±0.20[b]	2.53±0.25[b]	5.58±0.43	5.52±0.40	20.53±4.71

n = number of ejaculates

Values are presented as Mean±S.E.M.

[ab] Different superscripts between means within rows indicate significant differences ($P<0.05$)

Table 2 Fresh semen characteristics of hog deer collected by electro-ejaculation.

	Volume (ml)	Concentration (x 10^6/ml)	Motility (%)
Hard antlered deer (*n*=10)	1431.30±141.35[a]	2075.30±286.19[a]	91.60±1.05[a]
Velvet antlered deer (*n*=4)	535.25±163.48[b]	1788.75±835.28[a]	61.66±7.99[b]

n = number of ejaculates

Values are presented as Mean±S.E.M.

[ab] Different superscripts between means within rows indicate significant differences ($P<0.05$)

RESULTS AND DISCUSSION

Testicular volume of hog deer in hard and velvet antler stages were not significant different (Table 1). However, the width of testis was significant different between the stages.

Semen was collected successfully by electro-ejaculation from two hog deer bucks. A total of 14 ejaculates (hard antlers, n = 10; velvet antlers, n = 4) were immediately assessed. There were significant differences between hard antlers and velvet antlers. The values of volume and motility during hard antlered stage (1,431.30±141.35 µl and 91.60±1.05%) were higher than those of velvet antlered stage (535.25±163.48 µl and 61.66±7.99%) (Table 2). The semen volume of hard antlered stage was 2.6 times greater than those of velvet antlered stage.

Although, the testicular volume of both velvet and hard antler stages had no significant differences, the semen volume during hard antler stage was greater 2.6 times than velvet antler stage. Likewise, previously study has been suggested that testicular size was important in the breeding ability and it was an indicator of reproductive function (Dama dama; (Gosch & Fischer, 1989). The study demonstrated that collection of hog deer semen during hard antler stage provides a good result in term of semen volume, semen concentration and sperm motility. Similarity, there was previous report the high values of testicular volume, semen volume, sperm concentration and sperm motility during hard antler stage (Umapathy *et al*., 2007).

After cryopreservation at least seven days, straws were thawed and immediately estimated by CASA. Post-thawed semen was compared between hard and velvet antler stages to compare the seasonal effect of spermatozoa quality (Table 3). The results were showed that the percentage of total motility (39.30±7.24%) and the value of progressive motility (22.70±5.95%) during hard antlered stage in TRIS extender was significantly higher than those in hard antlered stage (P<0.05). The TRIS extender in hard antlered stage were also shown the highest values of movement pattern parameters and the value of VCL (142.63±8.33µm/s) was significantly higher than TEST and BF5F (Table 3). The membrane functionality, sperm morphology and acrosome integrity were examined under light microscope. Comparison among three extenders in hard antlered stage revealed that the percentage of normal membrane functionality (21.20±5.34%) was significant higher (P<0.05) than those in of TEST (6.15±1.67%) and BF5F (10.03±1.87%) (Table 3). However, in term of percentage of morphology and acrosome integrity were not significantly different. In velvet antlered stage, all values among three extenders were not significantly different. Considered to the factor of antler stages, only poor post-thawed sperm motility was found when the bucks were in velvet antler stage. The result indicated that hard antlered stage is suitable period not only for a semen collection but also cryopreservation. During hard antlered stage, TRIS provided more significant effective preservation in term of total and progressive motility.

Moreover, post-thawed semen was compared among three extenders to compare the extender effect of spermatozoa quality (Table 4). The percentage of sperm motility and progressive motility in TRIS (33.21±4.99% and 18.14±3.07%) were significant higher (P<0.05) than those of TEST (18.07±4.99% and 7.93±3.07%) and BF5F (12.00±4.99% and 4.57±3.07%). The movement patterns of spermatozoa were shown in Table 4 that the values of average path velocity (VAP) (79.95±8.52µm/s) in TRIS was significant higher (P<0.05) than those of TEST (54.95±8.52 µm/s) and BF5F (51.14±8.52

Table 3 Characteristics and movement patterns of hog deer post-thawed spermatozoa in hard and velvet antlered stages.

	Hard antler deer (n=10)			Velvet antler deer (n=4)		
	TRIS	TEST	BF5F	TRIS	TEST	BF5F
Total Motility (%)	39.30±7.24[a]	19.90±4.58[b]	15.20±5.12[b]	18.00±9.55[a]	13.50±11.89[a]	4.00±2.31[a]
Progressive motility (%)	22.70±5.95[a]	9.00±2.57[b]	5.70±1.37[b]	6.75±3.50[a]	5.25±4.31[a]	1.75±1.03[a]
Movement pattern						
VAP (μm/s)	83.91±4.98[a]	64.06±8.26[a]	58.45±10.84[a]	70.05±23.39[a]	32.20±19.14[a]	32.88±19.20[a]
VSL (μm/s)	61.67±3.99[a]	49.38±5.92[a]	49.88±9.51[a]	51.50±18.57[a]	25.18±14.62[a]	26.58±15.43[a]
VCL (μm/s)	142.63±8.33[a]	117.36±16.06[b]	93.45±17.20[b]	108.60±38.03[a]	54.13±33.01[a]	56.65±32.90[a]
ALH (μm/s)	6.35±0.45[a]	5.95±0.79[a]	4.93±0.88[a]	3.63±1.35[a]	2.73±1.78[a]	2.55±1.47[a]
BCF (Hz)	25.52±1.21[a]	21.60±3.48[a]	19.63±3.52[a]	24.25±8.61[a]	13.25±7.73[a]	11.75±7.75[a]
STR (%)	74.60±1.83[a]	69.30±7.96[a]	67.70±11.68[a]	56.75±19.99[a]	36.75±21.34[a]	40.00±23.13[a]
LIN (%)	46.10±1.69[a]	40.20±4.82[a]	45.30±8.20[a]	39.25±15.13[a]	23.25±13.77[a]	25.75±14.87[a]
Elongation (%)	56.40±3.05[a]	46.70±5.63[a]	48.10±9.41[a]	34.75±11.60[a]	21.00±12.37[a]	23.50±13.62[a]
Normal Membrane functionality (%)	21.20±5.34[a]	6.15±1.67[b]	10.03±1.87[b]	16.44±8.83[a]	22.38±10.34[a]	12.50±4.72[a]
Normal morphology (%)	92.55±1.58[a]	92.53±2.09[a]	93.88±1.52[a]	95.38±0.23[a]	92.75±1.16[a]	94.94±1.93[a]
Acrosome integrity (%)	95.75±2.18[a]	90.50±3.50[a]	95.13±1.22[a]	97.63±0.51[a]	97.00±1.83[a]	95.13±1.53[a]

n = number of ejeculates

Values are presented as Mean±S.E.M.

[ab] Different superscripts between means within column indicate significant differences (P<0.05)

μm/s). The values of curvilinear velocity (VCL) in TRIS (132.91±14.71 μm/s) was also significant higher (P<0.05) than BF5F (82.93±14.71 μm/s), whereas trend to higher than TEST (99.29±14.71 μm/s) (P>0.05).

Table 4 Characteristics and movement patterns of hog deer post-thawed spermatozoa.

Parameters	TRIS	TEST	BF5F
Total motility (%)	33.21±4.99[a]	18.07±4.99[b]	12.00±4.99[b]
Progressive motility (%)	18.14±3.07[a]	7.93±3.07[b]	4.57±3.07[b]
Movement pattern			
VAP (μm/s)	79.95±8.52[a]	54.95±8.52[b]	51.14±8.52[b]
VSL (μm/s)	59.47±6.88[a]	42.46±6.88[a]	43.22±6.88[a]
VCL (μm/s)	132.91±14.71[a]	99.29±14.71[ab]	82.93±14.71[b]
ALH (μm/s)	5.57±0.73[a]	5.03±0.73[a]	4.25±0.73[a]
BCF (Hz)	25.15±3.04[a]	19.21±3.04[a]	17.37±3.04[a]
STR (%)	69.50±8.67[a]	60.00±8.67[a]	59.78±8.67[a]
LIN (%)	44.14±5.76[a]	35.35±5.76[a]	39.71±5.76[a]
Elongation (%)	50.21±6.38[a]	39.35±6.38[a]	41.07±6.38[a]
Membrane functionality (%)	19.84±3.43[a]	11.18±3.43[a]	10.73±3.43[a]
Normal morphology (%)	93.36±1.30[a]	92.59±1.30[a]	94.18±1.30[a]
Acrosome integrity (%)	96.29±1.85[a]	92.35±1.85[a]	95.13±1.85[a]

Critical velocity or track speed, ALH: Amplitude of lateral head displacement, BCF: Beat cross frequency, VAP: Average path velocity, VSL: Straight line velocity, STR: Straightness (Average value of the ratio VSL/VAP), LIN: linearity (Average value of the ratio VSL/VCL)
Values are presented as Mean±S.E.M.
[ab] Different superscripts between means within column indicate significant differences (P<0.05)

Sperm cryopreservation is an alternative technique for breeding of endangered animals in captivity. An earlier study suggested cryopreservation such as freezing steps, equilibration periods, cooling rate and various complex cryoprotective media can be affect the maintenance of spermatozoa function during freezing and thawing (Salamon & Maxwell, 1995; Watson, 2000). In this present study, we found that TRIS extender provided the most efficiency maintaining of total motility, progressive motility and velocity parameter (average path velocity) including VAP, VSL, VCL, ALH, BCF, STR, LIN and Elongation after freezing compared to TEST and modified BF5F extenders. In according to previous studies revealed that the most diluents successfully used in deer species is sugar-based Tris and/or citrate-buffered (Salamon & Maxwell, 1995). Indeed, percentage of egg-yolk and glycerol was suggested about their relation to interaction in cryopreservation process. Acceptable results generally have been obtained in 4 - 8 % of glycerol in wild ruminates (Holt & Pickard, 1999; Leibo & Songsasen, 2002). Our results confirmed that Tris, glucose, citrate, egg yolk and glycerol are suitable

to be common diluents used for freezing deer semen (Evans & Maxwell, 1987). Egg yolk and glycerol could help to resist cold shock and cryoinjury (Salamon & Maxwell, 1995).

It is generally accepted that sperm motility is a central component of normal male fertility, good sperm motility increases male fertile ability (Turner, 2005). Sperm motility parameters were accessed by CASA (Wilson-Leedy & Ingermann, 2007). Ramón *et al*. (2013) pointed out that male spermatozoa with a low fertility were characterized by spermatozoa with slow movement, which is lower VCL and VAP. On the other hands, high fertility rates exhibited an increase in high VCL spermatozoa. Accordingly, in term of movement pattern parameter, VAP, VCL and VSL has been assumed to be good indicators of sperm freezability and closely related with thawed sperm fertility in red deer (Ros-Santaella *et al*., 2014). These parameters are all measures of sperm velocity over specified paths. Thus, these parameters can indicate that the high mobility sperm swam faster than lower mobility sperm (King *et al*., 2000). However, our results revealed the percentage of total motility, progressive motility and sperm velocity parameters (only VAP and VCL) in TRIS extender significantly higher than those in TEST and BF5F extenders. Moreover, the results of sperm movement pattern in hard antler stage was shown significant higher values than velvet antler stage. This showed similarly as previous study, the better quality of post-thawed semen in TRIS extender was showed than BF5F (Rittem *et al*., 2012).

Cryoprocess seem to affect to sperm membrane and acrosome integrity (Watson, 1976; White, 1993). However, sperm morphology, membrane and acrosome integrity among 3 extenders used in the study were similar. Interestingly, acrosome integrity of post-thawed sperm was over 90%, which refluxed the tolerant of hog deer sperm to cryoprocess after diluted in TRIS, TEST or BF5F. In conclusion, the results in this study indicates that collection and cryopreservation of hog deer semen during antlered stage provided a better of semen quality in both fresh and post-thawed than velvet one. Semen extender, TRIS can be a good choice for hog deer semen cryopreservation.

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