Original Article

Freezability of Thai Native Crossbred Horse Semen in Different Extenders

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Abstract

The aim of this study was to investigate the freezability of Thai native crossbred (T) and purebred horse (F) sperm using different extenders. Semen samples were collected monthly from five T and four F stallions between 5 and 12 years of age. The extenders used were INRA-Y (E1), INRA-Y supplemented with 50 mM L-glutamine (E2), lactose-EDTA (E3) and lactose-EDTA supplemented with 50 mM L-glutamine (E4). Sperm motility, membrane integrity and acrosome integrity were assessed in fresh and frozen samples by computer-assisted sperm analyses (CASA; IVOS, Hamilton Thorne), HOS tests and combined FITC-PNA and PI techniques respectively. Total motility and progressive motility of post-thaw T semen were significantly higher in E3. However, no significant differences between intact membrane-acrosome and positive membrane integrity of post-thaw T semen were observed between extenders. All characteristics of post-thawed semen in extenders supplemented with 50 mM L-glutamine tended to be lower than those without L-glutamine, and semen from one F and two T stallions could be used for cryopreservation. In conclusion, semen from T and F stallions in our study displayed poor overall freezability. Cryopreservation of stallion semen in lactose-EDTA with 4% glycerol exhibited better post-thaw semen quality. Supplementing glutamine to extenders did not produce beneficial effects on any post-thaw semen parameters examined due to poor initial freezability.

Keywords : extender, freezability, native horses, semen

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บทคัดย่อ

ความสามารถในการทนต่อการแช่แข็งของน้ำเชื้อพ่อม้าพื้นเมืองไทยในสารละลายเจือจางน้ำเชื้อต่างชนิด

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การศึกษาครั้งนี้เป็นการศึกษาความสามารถในการทนต่อการแช่แข็งของน้ำเชื้อพ่อม้าพื้นเมืองไทยและม้าสายพันธุ์แท้ในสารละลายเจือจางน้ำเชื้อต่างชนิด น้ำเชื้อถูกรีดเก็บเดือนละครั้งจากพ่อม้าพื้นเมืองไทย 5 ตัวและม้าสายพันธุ์แท้ 4 ตัวอายุระหว่าง 5-12 ปี นำมาทดสอบในสารละลายเจือจางน้ำเชื้อที่ใช้สำหรับการแช่แข็ง 4 ชนิด คือ อินรา-วาย, อินรา-วายที่เติม 50 มิลลิโมลของ แอลกลูตามีน แลคโตส อีดีทีเอ และ แลคโตส อีอีทีเอ ที่เติม 50 มิลลิโมลของ แอลกลูตามีน โดยเปรียบเทียบอัตราการเคลื่อนที่ของอสุจิ, ความสมบูรณ์ของเยื่อหุ้มเซลล์และอโครโซม และ การทำหน้าที่ของเยื่อหุ้มเซลล์ในน้ำเชื้อก่อนและหลังการแช่แข็ง พบว่าอัตราการเคลื่อนที่โดยรวม การเคลื่อนที่ไปด้านหน้าของน้ำเชื้อม้าพื้นเมืองไทยในสารละลายเจือจางน้ำเชื้อชนิดแลคโตส-อีดีทีเอมีค่าสูงแตกต่างอย่างมีนัยสำคัญ อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของค่าความสมบูรณ์ของเยื่อหุ้มเซลล์และอโครโซม รวมทั้งค่าการทดสอบการทำหน้าที่ของเยื่อหุ้มเซลล์อสุจิม้าพื้นเมืองไทยในสารละลายเจือจางน้ำเชื้อชนิดต่างๆ คุณภาพน้ำเชื้อแช่แข็งของม้าพื้นเมืองไทยและม้าพันธุ์แท้ในสารละลายเจือจางน้ำเชื้อที่เติมแอลกลูตามีนต่ำกว่าน้ำเชื้อในสารละลายเจือจางน้ำเชื้อไม่เติมแอลกลูตามีน และน้ำเชื้อพ่อม้าพื้นเมืองไทย 2 ตัวและพ่อม้าสายพันธุ์แท้ 1 ตัวที่สามารถเก็บรักษาด้วยการแช่แข็ง สรุปผลการศึกษาในครั้งนี้พบว่าน้ำเชื้อพ่อม้าพื้นเมืองไทย และพ่อม้าสายพันธุ์แท้มีความสามารถในการทนต่อการแช่แข็งต่ำ การแช่เข็งน้ำเชื้อพ่อม้าในสารละลายเจือจางน้ำเชื้อชนิดแลคโตส-อีดีทีเอ ที่มีกลีเซอรอลร้อยละ 4 ให้คุณภาพน้ำเชื้อภายหลังการแช่แข็งดีที่สุด การเติมแอล-กลูตามีนในสารละลายเจือจางน้ำเชื้อไม่ก่อให้เกิดผลดีต่อคุณภาพน้ำเชื้อภายหลังการแช่แข็ง ซึ่งอาจเนื่องมาจากความสามารถในการทนต่อการแช่แข็งของน้ำเชื้อต่ำตั้งแต่ต้น

คำสำคัญ : สารละลายเจือจางน้ำเชื้อ ความสามารถในการแช่แข็ง ม้าพื้นเมืองไทย น้ำเชื้อ

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Introduction

Sport purebreds and Thai native crossbreds are the two most prevalent horse groups in Thailand, comprising a total of 5,629 horses nationwide (2007 Statistics of Livestock in Thailand, Department of Livestock Development, Ministry of Agriculture and Cooperative). Sport horses were originally imported into Thailand from foreign countries and included breeds such as Arabian, Standardbred, Warmblood and Thoroughbred. The Thai native crossbred horse is a pony horse, possibly originating from Burmese ponies (Panasophonkul et al., 2007), although its exact etiology still remains unknown. Recent studies indicate the presence of only one Y chromosomal lineage and a high number of genetic polymorphisms by microsatellite marker analysis in a large majority of horse breeds, including Thai native crossbreds (Tawatsin et al., 2005). Today, Thai native crossbreds are generally used for religious ceremonies, recreational activities and occasionally as vehicles for transportation in highland areas. Natural breeding with a stallion is a common method to increase the numbers of this horse. As the Thai native crossbred horse population (particularly of purebred stallions) is very small, long-term semen preservation is important for increasing horse

numbers and improving existing horse breeds.

Semen preservation and storage would provide major advances in breeding animals with high genetic potentials. Genetic material preserved from animals permanently or temporarily unable to reproduce is invaluable, as semen can be used even after death of the stallion. In addition, cryopreservation will allow for long-distance shipment of semen, reducing any risks and inconveniences associated with transport of high-value stallions to breeding centers.

However, the efficiency of equine semen preservation varies significantly (Samper and Morris, 1998). In general, success rates are lower compared to those of other farm animals, as only 30-40% of stallions can produce semen suitable for cryopreservation (Brinsko and Varner, 1992), and consistent variations in sperm freezability have been observed among different breeds (Alvarenga et al., 2003). High initial semen quality is one of the most important for successful preservation of stallion semen, which can vary considerably between individual animals. Furthermore, variation in freezability of stallion semen is one of the major factors determining success of AI with frozen spermatozoa (Vidament et al., 1997).

Ideal semen extenders used for cryopreservation of equine semen would minimize damage resulting from multiple freeze-thaw cycles and maximize recovery of motile and viable spermatozoa. Many studies have sought to improve the quality of freeze-thawed equine semen using different cryoprotective agents (Alvarenga et al., 2003), various freeze-thaw protocols (Nunes et al., 2008) and extenders containing skim milk/glucose (Kenney et al., 1975), purified milk fractions (Pagl et al., 2006), lactose-egg yolk (Martin et al., 1979) or powered coconut water (Sampaio et al., 2002).

Furthermore, some amino acids have been reported to protect mammalian cells against freeze-thaw damage (Kruuv and Glofcheski, 1992). Accordingly, proline and glycine betaine in sperm cells appeared to preserve motility of post-thaw ram spermatozoa (Sanchez-Partida et al., 1992). Trimeche et al. (1999) found that the addition of glutamine or proline to the INRA freezing extender did significantly enhance motility of stallion spermatozoa after multiple freeze/thaw cycles. Additionally, L-glutamine increased motility in post-thaw Poitou Jackass, human and stallion spermatozoa (Renard et al., 1996; Trimeche et al., 1996; Khlifaoui et al., 2005).

The aim of the study was to investigate the freezability of semen from Thai native crossbred and purebred stallions in INRA and lactose-EDTA extenders, using glycerol as a cryoprotectant either with or without 50mM L-glutamine. Evaluation was based on parameters of sperm motility and membrane function relevant to the process of fertilization.

Materials and Methods

Experimental animals: Five Thai native crossbred stallions (T) between 5 and 12 years of age were studied. All animals had no prior breeding records and were housed under standard husbandry conditions at a private farm in Nakhon Ratchasrima, Thailand. Stallions were fed a diet of hay, Pangola grass and mineral-supplemented pellets. Concurrently, we also collected semen from four purebred stallions (F; one Warmblood, one Standard bred and two Thoroughbreds) and compared those data with Thai native crossbred horses. F stallions were housed under standard husbandry conditions at the Department of Veterinary and Remount, Royal Thai Army, Kanchanaburi, Thailand. Physical examinations showed that all stallions had normal reproductive tracts (i.e., no cryptorchids were observed).

Experimental Procedures

Preparation of extenders: One liter of four different extenders was prepared as follows: 1) INRA extender (Vidament et al., 2000) consisted of glucose monohydrate (25 g), lactose monohydrate (1.5 g), raffinose pentahydrate (1.5 g), sodium citrate dehydrate (0.25 g), potassium citrate monohydrate (0.41 g), ticarcillin (0.1 g), skim milk (55.75 g), HEPES (7.14 g) and egg yolk (2 % v/v); 2)

INRA extender supplemented with 50 mM L-glutamine (INRA-G); 3) lactose-EDTA extender (Martin et al., 1979) consisted of 25 mL of glucose-EDTA [glucose (0.3 g), EDTA (0.185 g), $NaHCO_3$ (0.06 g), ticarcillin (0.1 g) in 50 ml of distilled water], 50 ml of 11% lactose solution, SDS (0.1 g) and egg yolk (20% v/v); and 4) lactose-EDTA extender supplemented with 50 mM L-glutamine (lactose-EDTA-G). Extenders were centrifuged at 10,000 x g for 20 min to remove insoluble egg yolk droplets prior to use. Then 3.5% and 4% glycerol base cryoprotectant were added to the INRA and lactose-EDTA extenders, respectively. Osmolarities of each cryopreservation extender are shown in Table 1.

Semen collection: Six ejaculates from each stallion were collected between March 2007-August 2007 with a Missouri Artificial Vagina (AV) on a jump mare once a month for a total of six ejaculates/ stallion. Immediately after dismount, the ejaculates were initially evaluated for total volume, gel-free volume, osmolarity, concentration and morphology. The osmolality of raw semen from each stallion was routinely determined with a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Germany). Total number of spermatozoa was measured using a Neubauer Hemocytometer. After evaluation, semen were diluted at a ratio of 1:3 (semen: extender) in a Kenney extender (Kenney et al., 1975) and subjected to centrifugation at 400 x g at room temperature (30°C) for 10 min to remove seminal plasma. Spermatozoa pellets were resuspended in four different freezing extenders (see above) to final concentration of approximately 200 x 106 sperm/ml and then placed in a mobile refrigerator (5°C) for equilibration during transport to the laboratory for further processing. All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St Louis, MO, USA) unless stated otherwise.

Cryopreservation: Semen sample was equilibrated at 5°C for 3 h, and then loaded into 0.5 ml polyvinylchloride straws. Prior to freezing, straws were sat in nitrogen vapor 3 cm above liquid nitrogen for 10 min, and then submerged in liquid nitrogen, finally stored in liquid nitrogen (-196°C). Before freezing (BF), samples were evaluated for motility, motion velocity, viability and membrane integrity. All samples were required to have an original motility of at least 50% in order to be considered acceptable for freezing in our study. Frozen semen was thawed in a 37°C water bath for 30 sec. Evaluations were made 5 to 10 min after thawing the semen.

Semen evaluation

Percentages of motile spermatozoa: Experimental endpoints included total spermatozoa motility (TMOT; %), progressive spermatozoa motility (PMOT; %), curvilinear velocity (VCL; μm/s), linear velocity (VSL, μm/s) and average path velocity (VAP, μm/s), as measured by computer-assisted sperm analysis (CASA; HTM-IVOS 12; Hamilton Thorne Research, Beverly, MA); five fields were selected for each sample. System parameters for CASA were 30 frames acquired at 60 frames/second, a minimum contrast of 70, a minimum cell size of 5 pixels, VAP cut-off of 10 μm/s, progressive cell cut-off of 15 μm/s, VSL cut-off of 0 μm/s and 60% straightness. Slow cells were considered static. A droplet of each sample (3 μL) was placed on a preheated (37°C) 2X cell chamber 20 mm thick.

Acrosomal state and plasma membrane integrity: To distinguish between different acrosomal conditions, the acrosome was visualized by staining with FITC-conjugated peanut agglutinin (*Arachis hypogaea,* PNA) and propidium iodide (Cheng et al., 1996). Microscopic examination (Olympus BX50, Japan) was conducted with an oil immersion objective at 1000X magnification.

Table 1 Osmolarity of freezing extenders.

Extender	Osmolarity (mOsm) mean ± SEM (range)
INRA	835.33 ± 35.69 (680-914)[a]
INRA-G	877.67 ± 37.83 (776-989)[ab]
Lactose-EDTA	967.67 ± 43.04 (806-1101)[bc]
Lactose-EDTA-G	1023.33 ± 47.64 (833-1189)[c]

Functional plasma membrane integrity of freeze-thawed semen was determined with lactose 50 mOsm hypoosmotic swelling tests (HOS tests; Neild et al., 1999). A minimum of 200 spermatozoa were individually observed at 400X magnification and classified by the presence or absence of a swollen tail (curled/coiled principal or end piece). The percentage of HOS-positive spermatozoa (number of spermatozoa with swollen tails per total number of spermatozoa x 100) was recorded for each sample (Nie and Wenzel, 2001).

Semen freezability: A post-thaw motility ≥30% is a generally accepted criterion for decent freezability of stallion semen production (Boyle, 1999). Freezability of stallion semen was calculated by the number of ejaculates selected after freeze-thaw over the total number of ejaculates (Vidament et al., 1997).

Statistical analysis: Data were analyzed statistically by ANOVA followed by Fisher's Least Significant Difference (LSD) tests to identify statistically significant differences between extenders. Sperm parameters in fresh and frozen spermatozoa were compared using t-tests for two related samples with a p-value<0.05 considered significant. Data are reported as mean ± SEM.

Results

Ejaculate color and texture ranged from milky white to opalescent white. Raw semen characteristics of T and F stallions were presented in Table 2. Total motility, progressive motility, intact membrane-acrosome and positive membrane integrity parameters before freezing (BF) and post-thawed (PT) in T and F semen samples are listed in Table 3. With the exception of total motility in T semen, no other significant difference in BF semen parameters was observed when using the INRA extenders (E1 and E2) compared to the lactose-EDTA extenders (E3 and E4). Motility, intact membrane-acrosome and positive membrane integrity of post-thawed semen were all reduced after thawing (p<0.05) in both T and F semen samples. Total motility and progressive motility of post-thawed T semen were significantly higher in E3 than in E1 extenders, as well as in E2 versus E4 extenders. Although intact membrane-acrosome and positive membrane integrity of post-thawed T semen and motility, intact membrane-acrosome and positive membrane integrity of post-thaw F semen were not significantly difference between extender. Motion velocities (VAP, VSL and VCL) of T and F semen before freezing and post-thawed are listed in Table 4. Before freezing parameters of VAP, VCL for T semen and VCL for F semen were significantly different (p<0.05) in the INRA extenders (E1 and E2) compared with those in the lactose-EDTA extenders (E3 and E4). However, no significant differences among the parameters before freezing (p>0.05) were observed between extenders supplemented with glutamine (E2 and E4) compared to extenders without glutamine (E1 and E3). The VAP, VSL and VCL of post-thawed semen were reduced (p<0.05) in both T and F stallion sperm. The post-thawed VAP and VCL of F semen were statistically different (p<0.05) in the INRA extenders (E1 and E2) compared to those in the lactose-EDTA extenders (E3 and E4).

Adding 50 mM L-glutamine to extenders had no adverse effects on motility, velocity parameters, membrane integrity and acrosome integrity. Moreover, all characteristics of post-thawed semen in extenders supplemented with 50 mM L-glutamine tended to be lower than those in extenders without 50 mM L-glutamine.

The number of suitable ejaculates for freezing is shown in Table 5. Only one purebred stallion and two Thai native crossbred stallions gave ejaculates suitable for freezing, containing sperm motility >50% and post-thawed motility ≥30%. One T preferred both E1 and E3 (3/5 and 2/5 ejaculates passed), and one T preferred E3 (2/5 ejaculates passed). Only one of four F stallions preferred E3 (2/5 ejaculates passed). Freezability of T semen samples was 40% (8/20) and that of the F semen sample was 25% (5/20). We found that the number of good ejaculates was higher when using E3 and E1 extenders.

Discussion

Our study revealed that using glycerol as a cryoprotectant for T semen cryopreservation, the lactose-EDTA extender gave better post-thaw percentages of total motile and progressive motile sperm ($p<0.05$) than did INRA extenders. Supplementing glutamine into the extenders did not produce any beneficial effects on post-thaw semen quality, as post-thaw F semen quality was not significantly different between either extender.

T semen quality after a 3 h equilibration in glycerol-containing extenders was significant different between INRA and lactose-EDTA extenders with regard to the number of total motile sperm; No difference was seen in F semen quality parameters, indicating a higher sensitivity of T stallion sperm to extender composition and/or osmolarity (see Materials and Methods; Table 1) (Phetudomsinsuk et al., 2008[a]). The morphology of T sperm heads was larger and rounder than F sperm heads

Table 2 Ejaculate traits of Thai native crossbred (T; n=30) and purebred (F; n=24) stallions (mean ± SEM).

Parameters	T	F
Gel free volume (ml)	44.0 ± 2.1	47.0 ± 3.2
Sperm concentration (x10^6/ml)	309.0 ± 30.7[a]	374.5 ± 28.4[b]
Total motility (%)	77.8 ± 1.3[a]	73.0 ± 2.0[b]
Progressive motility (%)	55.4 ± 1.3[a]	46.8 ± 1.7[b]
Viability (%)	75.5 ± 1.3	73.9 ± 1.6
Host test positive (%)	58.7 ± 1.9	57.8 ± 1.7
Osmolarity (mOsm/kg)	329.3 ± 3.8[a]	314.6 ± 2.2[b]

Significant differences ($p<0.05$) between stallion groups are indicated with lower-case letters (a,b).

Table 3 Total motility, progressive motility, plasma membrane integrity and acrosome integrity of Thai native crossbred (T) and purebred stallion sperm (F) in different extenders before freezing (BF) and post-thawed (PT).

		Thai Native Crossbred (mean ± SEM)		Full size purebred (mean ± SEM)	
Parameters	Extenders	BF	PT	BF	PT
MOTILE (%)	INRA	80.70 ± 2.05[a]	15.87 ± 2.27[a]	75.84 ± 3.90	15.56 ± 2.16
	INRA-G	83.02 ± 1.87[a,b]	15.78 ± 2.16[a]	78.58 ± 3.72	14.11 ± 2.26
	L-EDTA	76.38 ± 2.30[b,c]	22.55 ± 2.15[b]	70.42 ± 4.65	17.71 ± 2.44
	L-EDTA-G	75.47 ± 2.34[c]	17.65 ± 2.33[a,b]	70.26 ± 4.40	15.76 ± 2.46
PMOT (%)	INRA	51.07 ± 2.05	10.64 ± 1.67[a]	47.26 ± 3.46	9.06 ± 5.24
	INRA-G	50.12 ± 1.99	10.82 ± 1.45[a]	47.16 ± 3.46	8.33 ± 5.38
	L-EDTA	53.05 ± 2.32	17.86 ± 1.59[b]	45.89 ± 3.16	9.41 ± 5.20
	L-EDTA-G	49.79 ± 2.04	12.82 ± 1.54[a]	44.00 ± 3.70	9.06 ± 6.43
Lived/intact	INRA	61.91 ± 3.33	38.64 ± 2.82	59.00 ± 3.45	30.29 ± 3.70
(%)	INRA-G	67.82 ± 2.34	40.93 ± 2.42	63.33 ± 2.67	29.00 ± 2.90
	L-EDTA	61.69 ± 2.65	41.00 ± 2.27	59.00 ± 2.34	32.56 ± 2.92
	L-EDTA-G	69.00 ± 3.2	37.14 ± 2.86	59.43 ± 3.75	33.19 ± 3.12
Positive	INRA	61.07 ± 2.05	20.1 ± 2.88	57.43 ± 0.43	14.00 ± 2.05
membrane	INRA-G	60.82 ± 1.99	18.6 ± 2.29	57.16 ± 0.38	13.20 ± 2.70
integrity (%)	L-EDTA	63.05 ± 0.82	24.3 ± 3.34	58.33 ± 0.39	21.30 ± 2.43
	L-EDTA-G	59.69 ± 0.71	19.1 ± 2.67	56.00 ± 0.41	17.20 ± 2.87

Within the same time interval, significant differences ($p<0.05$) between extenders are indicated with lower-case letters (a, b, c).

(Phetudomsinsuk et al., 2008[b]), suggesting a possible reason for differences in sensitivity to extenders.

In general, the average percentage of post-thaw motile sperm in the INRA extender (E1) was considerably lower in both T (15.9%) and F (15.6%) sperm compared to previously observed results (38.3%, Khlifaoui et al., 2005). This difference could be due to the glycerol concentrations (3.5%, 2.5% and 2.5%) and freezing methods (liquid nitrogen vapours versus programmable freezing machines) utilized between the various experiments. However, post-thaw motility in comparative studies between 2.5% VS 3.4% glycerol (28.3% and 26.0%; Ecot et al., 2000) and concentration of glycerol ranged from 1.7% to 3.7% (46-49%; Vidament et al., 2001)

Table 4 Semen motion characteristics of Thai native crossbred (T) and purebred stallion sperm (F) in different extenders before freezing (BF) and post-thawed (PT).

		Thai Native Crossbred		Full size Purebred	
Motion Velocity	**Extenders**	**Mean BF**	**Mean PT**	**Mean BF**	**Mean PT**
VAP	INRA	85.7 ± 2.5[a]	49.9 ± 2.5	79.0 ± 4.4	46.7 ± 2.6[a]
(μm/s)	INRA-G	82.0 ± 2.4[a,c]	46.4 ± 2.1	78.4 ± 4.2	44.3 ± 1.9[a]
	Lactose-EDTA	77.1 ± 2.0[b,c]	47.3 ± 1.3	71.4 ± 3.7	41.1 ± 3.2[a,b]
	Lactose-EDTA-G	73.8 ± 2.1[b]	45.8 ± 1.9	70.3 ± 3.7	36.4 ± 2.8[b]
VSL	INRA	49.6 ± 1.6	40.5 ± 3.1	48.4 ± 3.2	35.8 ± 2.5
(μm/s)	INRA-G	47.9 ± 1.6	36.6 ± 3.0	47.1 ± 3.1	33.7 ± 2.0
	Lactose-EDTA	53.1 ± 1.3	42.4 ± 2.9	49.4 ± 2.8	32.5 ± 2.8
	Lactose-EDTA-G	51.4 ± 1.6	40.0 ± 2.8	46.3 ± 3.2	29.1 ± 2.4
VCL	INRA	174.4 ± 5.6[a]	100.2 ± 6.6	162.1 ± 6.3[a]	98.0 ± 4.2[a]
(μm/s)	INRA-G	170.1 ± 4.9[a,b]	96.2 ± 5.9	160.8 ± 8.4[a,b]	92.9 ± 4.2[a,b]
	Lactose-EDTA	165.3 ± 3.0[b,c]	93.1 ± 4.7	142.1 ± 7.0[b,c]	82.1 ± 5.5[b,c]
	Lactose-EDTA-G	162.7 ± 3.7[c]	91.4 ± 5.0	140.6 ± 7.7[c]	72.4 ± 4.4[c]

Within the same time interval, significant differences ($p<0.05$) between extenders are indicated with lower-case letters (a, b, c).

Table 5 Number of acceptable ejaculates (>50% total sperm motility) and semen freezability (≥30% post-thawed motility) of Thai native crossbred (T1 - T5) and purebred stallion sperm (F1 - F4) in different extenders.

Stallion	**Number of ejaculates**	**Number of acceptable ejaculates for freezing**	**Number of ≥30% motility post-thawed ejaculates**			
			INRA	**INRA -G**	**L-EDTA**	**L-EDTA-G**
T1	6	5	0/5	0/5	2/5	0/5
T2	6	5	1/5	0/5	1/5	0/5
T3	6	5	1/5	0/5	1/5	1/5
T4	6	5	3/5	2/5	2/5	1/5
T5	6	3	0/3	0/3	0/3	0/3
Total	**30**	**23**	**5/23**	**2/25**	**6/23**	**2/23**
F1	6	5	1/5	1/5	2/5	1/5
F2	6	2	0/2	0/2	0/2	0/2
F3	6	5	1/5	0/5	0/5	0/5
F4	5	4	0/4	0/4	0/4	0/4
Total	**23**	**16**	**2/16**	**1/16**	**2/16**	**1/16**

resulted in no significant difference. Thus, the lower percentage observed in our study may not necessarily be due to the glycerol concentration used. Clulow et al. (2008) recently demonstrated good sperm freezability in selected stallions, with a significantly higher post-thaw progressive motility after freezing in a programmable freezer. The programmable freezer also provided more consistent and reliable freezing rates than the use of liquid nitrogen vapours. Therefore, freezing methods may affect recovery of total and progressive sperm motility after thawing. Under the same conditions, the average percentage of post-thaw sperm motility in the lactose-EDTA with 4% glycerol (E3) extender was lower for both T (22.5%) and F (17.7%) sperm compared to the previously observed 38-41% (Cochran et al., 1984).

We found that supplementing glutamine into the INRA and lactose-EDTA extenders did not produce any beneficial effects on post-thaw semen parameters of both T and F sperm. In previous studies, beneficial effects of glutamine on post-thaw motility were observed when INRA with 2.5% glycerol was used as a freezing extender (Khlifaoui et al., 2005). The beneficial effect of 50 mM L-glutamine appeared to be limited in 2.5% glycerol (Khlifaoui et al., 2005). Additionally, this beneficial effect on sperm freezability was demonstrated only for average (30-35% post-thaw motility) to good sperm (>35% post-thaw motility), but not in sperm with poor post-thaw motility (<30%) (Khlifaoui et al., 2005). The various glycerol concentrations used in this study in addition to poor sperm freezability may explain why no beneficial effects were observed.

Comparison of different freezing extenders showed that post-thaw motility was higher in the INRA than in the lactose-EDTA extender, even though both contained 4% egg yolk and 4% glycerol (Heitland et al., 1996). However, the lactose-EDTA extender yielded better motility than INRA-82 for epididymal sperm freezing; both extenders contained 5% glycerol (Papa et al., 2008). Numerous factors can affect the outcome of post-thaw sperm motility including individual sample variations in freezability, stallion breeds (Boyle, 1999), types of cryoprotectant (i.e. dimethyl-formamide/glycerol; Alvarenga et al., 2005) and freezing protocols utilized (liquid nitrogen vapours / programmable freezing machine, Clulow et al., 2008; conventional/directional freezing, Saragusty et al., 2007). Thus, the most effective freezing extender for stallion semen remains inconclusive. However, we found that the lactose-EDTA extender yielded higher percentages of total and progressive sperm motility ($p<0.05$) than did INRA extenders for T sperm. These differences were not observed in F sperm, possibly due to differences in the head size and shape of T and F sperm.

Post-thaw percentages of HOS-positive sperm with intact acrosomes were not significantly different among extenders in both T and F semen samples. As reported by Papa et al. (2008), no difference in the number of sperm with intact plasma membranes between INRA (47.9%) and lactose-EDTA (49.3%) extenders was observed. The percentages of live sperm with intact acrosomes ranged from 37.1-40.9% in T sperm and 29.0-33.2% in F sperm, comparable to previous studies (37-42% using skim milkñegg yolk with 3% glycerol, Wilhelm et al., 1996; and 40% using either skim milkñegg yolk or lactose-EDTA with 4% glycerol, Kirk et al., 2005).

Post-thaw percentages of HOS-positive sperm were 18.6-20.3% for T samples and 13.2-21.3% for F samples, which were both lower than previously-reported values (lactose-EDTA with 4% glycerol: 30.2%, Neild et al., 1999; 26%, Saragusty et al., 2007). The low recovery rate of post-thaw motility in this study may be respond for these inferior results, since there was significant correlation between HOS and progressive motility (0.57, Neild et al., 1999; 0.77, Saragusty et al., 2007). Thus, the less adverse effect of cryopreservation on sperm plasma membrane integrity (live sperm with intact acrosomes) than motility and membrane function (HOS-positive) was observed in this study. Sperm motility was also affected more by semen handling factors (Neild et al., 1999) and osmotic stress (Ball and Vo, 2001) than from issues of plasma membrane integrity.

Characteristics of motile sperm such as VAP and VCL in T sperm as well as VCL in F sperm displayed significant differences between the various extenders before freezing, and VAP and VCL in F after thawing (Table 4). The lactose-EDTA-G extender had the highest osmolarity and lowest motion velocity values (Table 1). Thus, osmolarity of the extender could affect not only the percentage of sperm motility (Ball and Vo, 2001), but also its motion characteristics. Nonetheless, all parameters

displayed significant decreases after a freeze-thaw cycle, similar to previous reports (Wilhelm et al., 1996).

The freezability (>30% post-thaw motility) results listed in Table 5 indicated that only two of five T stallion sperm samples could be used for semen cryopreservation. One T preferred both INRA82 and lactose-EDTA (3/5 and 2/5 ejaculates passed), and one T preferred lactose-EDTA (2/5 ejaculates passed). For F stallions, only one from 4 stallions could be used for semen cryopreservation, which preferred lactose-EDTA (2/5 ejaculates passed). However, the use of other freezing protocols and cryoprotectants may improve post-thaw semen quality and should be further investigated.

In conclusion, cryopreservation of stallion semen using lactose-EDTA with 4% glycerol gives a higher probability of good post-thaw semen quality. Supplementing glutamine to extenders, however, did not produce any beneficial effects due to the initial poor freezability of stallion sperm.

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