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Effects of Extenders and Glutamine on the Cooled Storage of Semen of Thai Native Crossbred and Full-Size Purebred Horses

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ABSTRACT

The effects of four different extenders on semen storage of Thai, native, crossbred (T; n = 5) and full-size, purebred (F; n = 4) horses were investigated. The extenders used were Kenney (E1), Kenney with 50 mM glutamine (E2), INRA-82Y (E3) and INRA-82Y with 50 mM glutamine (E4). The semen was collected through the use of an artificial vagina. It was then washed and the seminal plasma removed and maintained at 5°C in each extender for up to 96 h. The cooled samples were evaluated for viability using eosin-nigrosin staining and the hypo-osmotic swelling test (HOST) and then submitted to CASA for the analysis of total motility (TMOT), progressive motility (PMOT) and velocity parameters. It was found that there was a significant difference at (P<0.05) between T and F stallions for: the mean percentage of TMOT (77.8 %, 73.0 %); PMOT (55.4 %, 46.8 %); sperm concentration (309 × 10^6 ml^{-1}, 374.5 × 10^6 ml^{-1}); and osmolarity of fresh semen (329.3 mOsm, 314.6 mOsm). All the characteristics of the semen collected from the T and F stallions significantly decreased (P<0.05) after being stored for 24 h to 48 h in all extenders. The E3 tended to maintain sperm motility and membrane integrity for a longer period of time than the other extenders that were tested. Supplementing the extenders with 50 mM glutamine had adverse effects on the sperm motility and membrane integrity in cooled storage.

Key words: cooled semen, extender, glutamine, horses

INTRODUCTION

In Thailand, there are two main horse groups: sport, full-size, purebred and Thai, crossbred, native horses, with a total population of 2,327 horses (Statistics of Livestock in Thailand: 2006, Department of Livestock Development, Ministry of Agriculture and Cooperatives). The sport, full-size horse group includes breeds such as: Arabian, Warmblood and Thoroughbred and originally these were all imported. The Thai, crossbred, native horse is a pony horse and may originally have originated from a breed of the Burmese pony (Panasophonnkul *et al.*, 2007), although its scientific origin is still obscure. A recent study indicated that only one Y chromosome lineage has been found in a large number of horse breeds

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including the Thai, native breed (Lindgren *et al*., 2004) and the microsatellite markers also showed a highly-polymorphic, genetic characteristic (Tawatsin *et al*., 2005). Nowadays, this breed is generally used in religious ceremonies, for recreation and some transportation in highland areas. Natural breeding with a stallion is a common method used to increase the numbers of this breed.

Horse reproduction in recent times has been based on the use of artificial insemination (AI), the widespread use of which has accelerated genetic progress by making selected stallions available to breeders outside the region where each stallion is located. The advantages conferred by the use of AI with cooled, transported semen (Pickett, 1995) have stimulated more interest in this technology, specifically the technique's ability to maintain equal fertility between stored and fresh semen for a period of up to 24 hours (Francl *et al*., 1987). However, the motility and velocity of sperm (Jasko *et al*., 1992) and fertilization rates (Pickett *et al*., 1989) tend to decrease, with fertility after AI using cooled, stored semen remaining dependent on the inherent fertility of the mares and stallions involved. In addition, fertility is also affected by the services provided by laboratories (Katila, 1997), particularly the storage temperature, the extender components, the number of sperm that are inseminated, cooling rates and the storage time (Shore *et al*., 1998).

To preserve sperm fertility for a long period, extenders must be used to provide an environment that is metabolically and physiologically encouraging to the survival of sperm cells. The extender also protects sperm from shock due to the cold temperature, the negative effects of seminal plasma and inhibits bacterial growth. A large variety of extenders combining assorted ingredients (sugar, electrolytes, buffers, egg yolk, milk and milk products) have been proposed for cooling sperm. Most extenders for the dilution, centrifugation and cooled storage of equine semen are based either on milk or egg yolk, and maintain the motility and fertilizing ability of semen for about 24 h at 5°C (Batellier *et al*., 1997).

The positive effects of milk or egg yolk on sperm protection may be due to their ability to reduce the deleterious effects of seminal plasma proteins on the sperm membrane (Bergeron and Manjunath, 2006). Accordingly, skim milk-based extenders added to glucose and antibiotics and ultimately added to salts and other sugars, are now in worldwide use (Shore *et al*., 1998). Furthermore, extenders containing low concentration of egg yolk (2 % to 4 %) are thought to improve the motility of stallion spermatozoa (Rota *et al*., 2004).

It has been reported that supplementing extenders with taurine could improve stallion sperm viability and motility after 24 h of storage (Ijaz and Ducharme, 1995), but the effect was not observed when cysteine was added (Pagl *et al*., 2006). Adding taurine may also improve sperm survival during cooled storage in rabbits (Alvarez *et al*., 1983) and rams (Bucak *et al*., 2007). In specific cell types (such as enterocytes, cells of the immune system and cancer cells), glutamine has been shown to have a role in cell survival and in eliciting and modulating cellular stress responses (Fuchs and Bode, 2006). Additionally, it has been reported that supplementing cryopreservation extenders with glutamine could increase frozen-thaw sperm motility in humans (Renard *et al*., 1996), poitou jackasses (Trimeche *et al*., 1996) and stallions (Trimeche *et al*., 1999). This methodology may also have the same effect in cynomolgus monkeys, with the possible additional effect of improving membrane and acrosome integrity (Li *et al*., 2003). Consequently, supplementation of cooled semen extenders with this amino acid was included in this study. The amount of glutamine supplementation was 50 mm, as this had been demonstrated to have a beneficial effect on sperm motility in frozen stallion sperm after thawing (Khlifaoui *et al*., 2005).

This study aimed to: investigate the effect of different extenders on the semen characteristics of Thai, native crossbred stallions after storage at 5°C; compare the results with the characteristics of full-size, purebred stallion semen; examine whether supplementation of the extenders with L-glutamine could improve semen quality after storage; and study the semen characteristics of Thai, native, crossbred and full-size, purebred horses.

MATERIALS AND METHODS

Experimental animals

Nine horses consisting of Thai, native, crossbred stallions (T; n = 5) and full-size, purebred stallions (F; n = 4; 1 Warmblood, 1 Standard bred and 2 Thoroughbreds) aged between 5 and 12 years were used in the study. The average body weight of T was 210 kg and for F was 500 kg. The full-size, purebred stallions were active breeding sires used in artificial insemination programs, but the T stallions had no breeding record. Physical examination showed that all stallions had normal reproductive tracts (i.e. no cryptorchids were present). The testicular size of each horse was measured with calipers.

Experimental procedures

Preparation of extenders

The ingredients for each extender were: 1) Kenney extender (E1) (Kenney *et al*., 1975) which consisted of glucose monohydrate (40 g/l); skim milk (24 g/l); penicillin G sodium salt 150000 IU/l; and streptomycin (crystalline) (0.15 g/l); 2) Kenney extender supplemented with L-glutamine (50 mm/l) (E2); 3) INRA-82Y extender (E3) (Vidament *et al*., 2000) which consisted of glucose monohydrate (25 g/l); lactose monohydrate (1.5 g/l); raffinose pentahydrate (1.5 g/l); sodium citrate dehydrate (0.25 g/l); potassium citrate monohydrate (0.41 g/l); ticarcillin (0.1 g/l); skim milk (55.75 g/l); HEPES (7.14 g/l); and egg yolk 2 % (v/v); and 4) INRA-82Y extender supplemented with L-glutamine (50 mm) (E4). After adding all the ingredients, the extender and egg yolk were centrifuged at 1×10^5 m s^{-2} for 20 min to remove any insoluble, egg yolk droplets prior to use. The pH and osmolarity of each extender is presented in Table 1.

Semen collection

Six ejaculates from each stallion were collected from January to June 2007 using the standard procedure and a Missouri model, artificial vagina on an estrous mare. Immediately after collection, the gel fraction of semen was removed by filtering through sterile gauze. Gross appearance and volume of the gel-free ejaculate were recorded and aliquots of raw semen were analyzed for ejaculate traits. In all treatments, E1 was added as a washing media and diluted semen was centrifuged at 3922 m s^{-2} for 15 min. The supernatant was removed and the pellet was diluted to a concentration of 200×10^6 sperm/ml in each extender, which was then placed in storage at 5°C. The semen was transported to the laboratory within 3 h after collection.

Table 1 pH and osmolarity of extenders.

Extender	pH (mean ± SEM; range)	Osmolarity (mOsm) (mean ± SEM; range)
E1	7.10 ± 0.04 (6.80 – 7.20)a	367.4 ± 3.58 (350 – 380) a
E2	7.38 ± 0.04 (7.20 – 7.60) b	424.5 ± 8.79 (380 – 490) b
E3	7.18 ± 0.10 (6.60 – 7.70) a,b	352.4 ± 5.56 (320 – 375) c
E4	7.14 ± 0.12 (6.80 – 7.20) a,b	412.7 ± 7.78 (370 – 450) b

Significant differences (P<0.05) in column are indicated by different letters (a, b, c).

Semen analysis

Ejaculate traits of the sperm concentrate (using a hematocytometer) and subjective motility were evaluated. Total motility and the progressive motility of fresh semen were assessed subjectively under a phase contrast microscope (x100 and x400 magnification, respectively). The morphological abnormalities of the spermatozoa were studied in wet preparations made from the formal-saline fixed samples (Hancock, 1957) under a phase-contrast microscope at a 1000x magnification. Altogether, 200 spermatozoa were examined for normal and abnormal morphology. The defect sperm with abnormalities of the head (for example pyriform, tapered, giant, round, narrow, loose head or detached), the midpiece (proximal or distal cytoplasmic droplets) and the tail (simple bent, coiled or double-folded) were classified. The membrane integrity of the spermatozoa was determined using the hypo-osmotic swelling test (HOST) (Neild *et al*., 1999), while live sperm was analysed using the eosin-nigrosin staining test (William, 2003).

After transportation, cooled semen were evaluated for motility and sperm velocity, using a Computer-Assisted Sperm Analysis (CASA) system (IVOS version 12, Hamilton Thorne Research, MA, USA) at 0 h, 24 h, 48 h, 72 h and 96 h. The parameters evaluated by CASA were: percentage of total motile spermatozoa (TMOT); percentage of progressively motile spermatozoa (PMOT); curvilinear velocity in mm/s (VCL); linear velocity in mm/s (VSL); and average path velocity in mm/s (VAP). The system parameters and their values were: minimum contrast (70); minimum cell size (5 pixels); VAP cut-off (10 mm/s); VAP cut-off for progressive cells (15 mm/s); VSL cut-off (0 mm/s); and straightness (60 %). A 3-µl drop of each sample was placed on a preheated (37°C) 2X cell chamber (20 mm depth). To select cells from the debris, the camera recognized the position of the sperm heads in successive frames. At least five fields or 400 spermatozoa per sample, with approximately 100 cells per field were evaluated. Sperm tracks with a straightness value of less than 60 % were considered non-progressive, while sperm tracks with VAP less than 20µm/s were considered non-motile.

Statistical analysis

Statistical comparisons were made using the SPSS/PC+ statistics package (version 11.5 for Windows, SPSS Inc, Chicago, IL, USA). The percentage of live sperm, motility, velocity and pH between the different extenders during a 96 h period were analyzed using analysis of variance (ANOVA). Pair-wise comparisons between the groups were made based on the LSD test, with the probability threshold set at 5% ($P<0.05$) considered as a significant difference. Data were reported using mean values ± the standard error of mean (SEM).

RESULTS

Testicular size

Mean testicular size (length × width × height) for T of the left and right testes were 8.9 cm × 5.2 cm × 5.3 cm and 8.3 cm × 5.3 cm × 5.1 cm, respectively, while the same measurements of testicular size of F were 12.9 cm × 10.3 cm × 6.7 cm and 12.7 cm × 10.1 cm × 6.7 cm, respectively. Mean testicular volume (± SEM) of the left and right testes in T was 131.3 cm^3 ± 14.5 cm^3 and 119.8 cm^3 ± 14.5 cm^3 and in F was 266.6 cm^3 ± 32.6 cm^3 and 260.0 cm^3 ± 35.7 cm^3, respectively. The testicular sizes of the left and right testes were not different ($P>0.05$) in both T and F. However, both the left and right testicular sizes and volumes of T were significantly smaller ($P<0.05$) than those of F.

Semen characteristics

The raw semen characteristics of T and F stallions are presented in Table 2. The ejaculate traits of T were different ($P<0.05$) from F, except

with regard to gel-free volume, percentage of live sperm, percentage of normal morphological sperm, percentage of positive HOST sperm, and semen pH. In T and F, respectively sperm concentrations were from 55 × 10^6 cells/ml to 2,655 × 10^6 cells/ml and from 105 × 10^6 cells/ml to 740 × 10^6 cells/ml, with the percentage of live sperm 77.2 % ± 2.2 % to 81.7 % ± 2.0 % and 73.5 % ± 3.35 % to 76.4 % ± 3.0 %.

Effect of extenders on cooled storage semen characteristics

Comparisons between extenders

The mean percentages of TMOT, PMOT, HOST positive and live sperm after dilution (0 h) did not differ between any of the extenders for either T or F (P> 0.05, Figure 1). In T, a difference in TMOT between the four extenders was clearly observed (P<0.05, Figure 1A). From 48 h compared to 96 h of storage, E3 tended to maintain TMOT better than E1, E2 or E4. However, at 96 h of storage, E1 had more TMOT than the others (*P*<0.05). In F, there was no difference in TMOT between any of the extenders in any period of storage (*P*>0.05, Figure 1B). Differences in both the percentage of PMOT and of HOST positive sperm in T between the extenders were observed at 24 h to 96 h of storage (*P*<0.05, Figures 1C, 1E).

In a similar manner to that described above for TMOT, the diluted T semen with E3 tended to maintain PMOT better than E1, E2 or E4. However, semen diluted in E1 at 96 h of storage had more PMOT (P<0.05). The percentage of PMOT and of HOST positive sperm in F (Figures 1D, 1F) was not different for any of the extenders in any period (P>0.05). The percentage of live sperm in T was different (P <0.05, Figure 1G) at 48 h compared to 96 h of storage, and E3 and E4 tended to be higher than E1 and E2, while in F there was no difference between the extenders (P>0.05; Figure 1H).

The sperm velocity parameters, VCL, VSL and VAP, did not differ at 0 h with extender in both T and F (Table 3). In T, VAP of diluted semen was different between extenders (P<0.05) at 24 h compared to 96 h of storage, and E1 and E3 had more VAP (P<0.05) after storage for 24 h, 48 h and 72 h. However, E1 had more VAP at 96 h of storage than E3 (P<0.05).

For VAP in F, diluted semen did not differ between the extenders (P>0.05) up to 48 h of storage, while at 72 h and 96 h, E4 tended to be lower than E1, E2 and E3. For VCL and VSL in T, there was also a difference between extenders (P<0.05) at 24 h of storage. Semen samples diluted with E1 and E3 tended to be higher than E2 and E4 at 24 h compared to 72 h of storage, but at 96

Table 2 Ejaculate traits of Thai native crossbred (T) and full-size purebred (F) stallions (mean ± SEM).

Parameter	T	F
Total volume (ml)	64.0 ± 5.2a	48.6 ± 3.2b
Gel-free volume (ml)	44.0 ± 2.1	47.0 ± 3.2
Sperm concentration/ml (x106sperm)	309.0 ± 30.7a	374.5 ± 28.4b
Total sperm/ejaculate (x109sperm)	10.5 ± 0.7a	17.3 ± 1.7b
Total motility (%)	77.8 ± 1.3a	73.0 ± 2.0b
Progressive motility (%)	55.4 ± 1.3a	46.8 ± 1.7b
Live sperm (%)	75.5 ± 1.3	73.9 ± 1.6
Normal morphologically sperm (%)	49.7 ± 1.3	48.1 ± 2.8
HOST positive sperm (%)	58.7 ± 1.9	57.8 ± 1.7
pH	7.6 ± 0.03	7.6 ± 0.04
Osmolarity (mOsm)	329.3 ± 3.8a	314.6 ± 2.2b

Significant differences (P<0.05) in line are indicated by different letters (a, b).

h, E1 had more VCL and VSL than E3 (P<0.05). In F, for VCL there was no difference at 24 h compared to 72 h of storage (P>0.05) between the extenders, while at 96 h, E4 tended to be lower than the others. The VSL of F was not different at 24 h compared to 48 h of storage (P>0.05) among extenders, and at 72 h and 96 h, E4 tended to be lower than E1, E2 or E3.

Comparisons within extenders

Table 4 illustrates the period of cooled storage in which a significant decrease in the semen characteristics in each extender was observed. TMOT and live sperm in both T and F stallions decreased (P<0.05) at 48 h in all the extenders examined. PMOT of T semen in E1 and E4 decreased (P<0.05) at 48 h, but only at 24

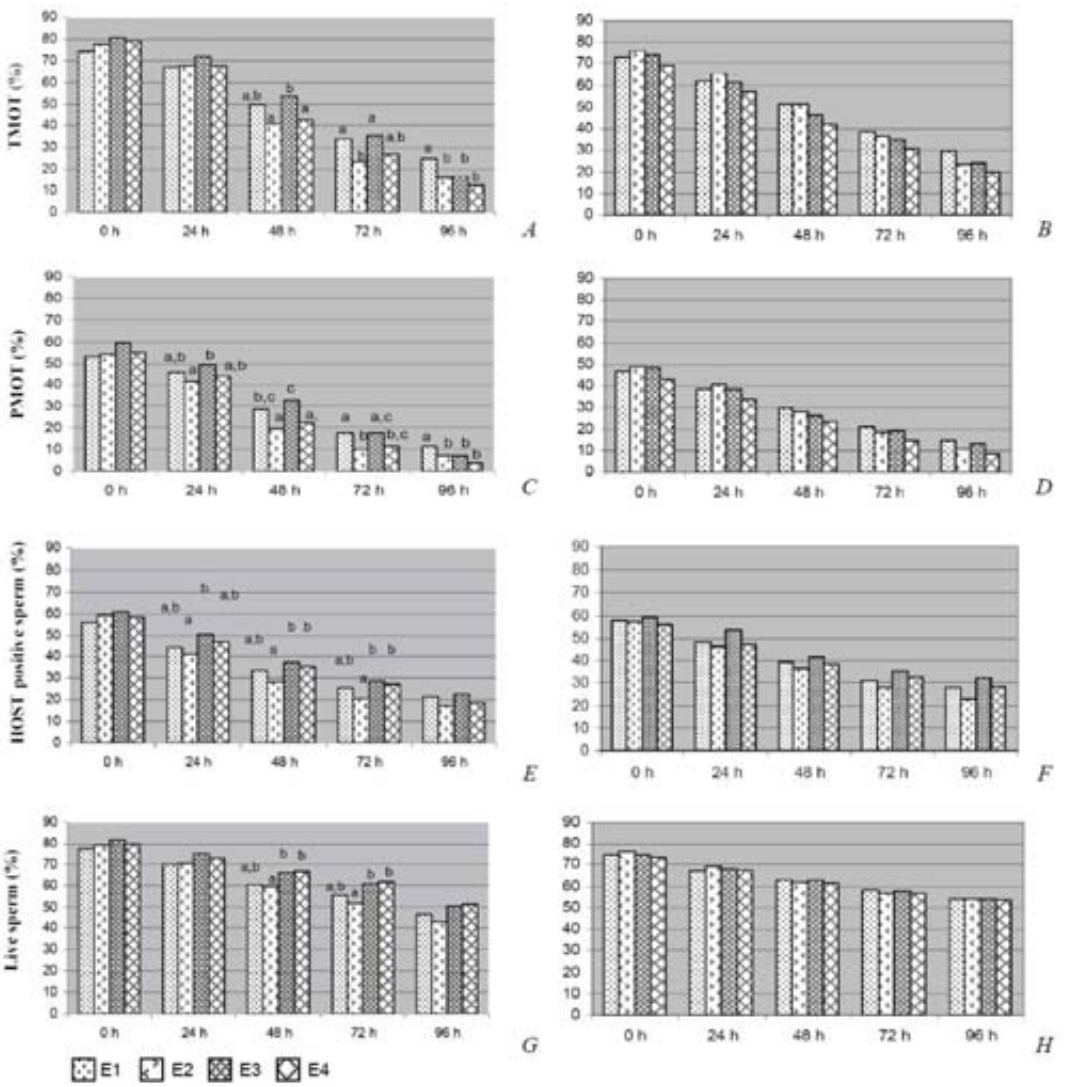

Figure 1 Semen characteristics of Thai native crossbred (*A, C, E* and *G*) and full-size purebred stallions (*B, D, F* and *H*) after storage at 5°C in different extenders for up to 96 h. Within the same time interval, significant differences (*P*< 0.05) among extenders are indicated by different letters (a, b, c).

Table 3 Average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL) of Thai, native, crossbred (T) and full-size, purebred stallion (F) sperm after storage at 5°C in different extenders.

Sperm velocity	Storage time (h)*	T				F			
		E1	E2	E3	E4	E1	E2	E3	E4
VAP	0	81.1 ± 2.7	80.1 ± 2.3	86.3 ± 2.6	80.1 ± 2.5	77.3 ± 4.0	81.6 ± 3.6	79.1 ± 3.4	77.8 ± 4.7
	24	78.0 ± 2.7 a	68.2 ± 1.9 b	77.5 ± 2.2 a	67.4 ± 2.6 b	69.9 ± 4.0	77. 9 ± 3. 9	71.9 ± 3.8	67.3 ± 4.9
	48	63.8 ± 2.0 a	52.8 ± 2.6 b	63.0 ± 2.6 a	53.1 ± 2.6 b	60.8 ± 3.0	65.0 ± 3.7	62.0 ± 4.0	57.4 ± 3.6
	72	56.0 ± 2.5 a	44.1 ± 2.5 b	49.5 ± 2.7a	42.5 ± 2.6 a,b	59.0 ± 3.7 a,b	55.6 ± 2.5 a	59.5 ± 4.0 b	49.3 ± 3.3 a,c
	96	47.8 ± 3.1 a,b	45.1 ± 1.9 a	41.3 ± 1.9 c	35.5 ± 2.1 b,c	57.4 ± 2.8 a,b	50.0 ± 1.9 a	54.7 ± 3.5 b	44.8 ± 2.6 a,c
VCL	0	165.3 ± 5.1	170.4 ± 5.2	175.5 ± 4.2	169.2 ± 4.9	156.1 ± 5.6	166.1 ± 6.3	159.7 ± 5.0	155.4 ± 11.4
	24	161.7 ± 5.6 a,b	150.1 ± 3.8 a	161.7 ± 3.7 b	181.2 ± 4.3 a	144.2 ± 5.5	160.5 ± 6.1	148.5 ± 5.4	143.3 ± 8.4
	48	143.7 ± 3.8 a,b	125.5 ± 4.9 c	142.7 ± 4.4 a	125.0 ± 4.5 c	134.2 ± 4.3	141.5 ± 6.5	137.5 ± 6.5	124.7 ± 8.8
	72	130.9 ± 4.8 a,b	114.5 ± 4.1 c	119.0 ± 4.9 b	102.5 ± 5.6 a,c	125.1 ± 8.6	125.4 ± 5.3	126.3 ± 9.0	116.0 ± 6.4
	96	117.7 ± 5.1 a,b	110.4 ± 3.9 a	101.8 ± 4.6 c	93.0 ± 3.8 b,c	130.5 ± 4.0 a,b	119.2 ± 3.3 a	127.7 ± 5.5 b	110.5 ± 4.8a,c
VSL	0	49.8 ± 2.0	48.0 ± 1.8	51.3 ± 2.4	47.2 ± 2.2	49.0 ± 11.5	50.3 ± 11.0	49.9 ± 12.5	48.0 ± 12.3
	24	46.1 ± 1.5 a	39.5 ± 1.4 b	44.4 ± 2.8 a,b	40.6 ± 2.5 a,b	45.4 ± 3.3	48.3 ± 2.8	45.7 ± 3.50	42.5 ± 3.8
	48	35.6 ± 1.2 a	28.6 ± 1.6 b	35.4 ± 2.1 a	30.2 ± 2.0 b	37.3 ± 2.2	43.8 ± 2.3	37.1 ± 3.03	33.2 ± 2.8
	72	29.6 ± 1.5 a	23.7 ± 1.4 b	26.6 ± 1.9 a	22.6 ± 1.7 a,b	35.1 ± 2.6 a,b	33.0 ± 1.6 a	35.1 ± 2.8 b	27.9 ± 2.6 a,c
	96	25.0 ± 1.7 a,b	22.6 ± 1.1 a	21.7 ± 1.3 b	19.6 ± 1.3 a,b	32.5 ± 2.2 a,b	29.1 ±1.6 a,b	35.0 ± 2.6 a	24.4 ± 1.6 b

* Within the same time interval and within breed, significant differences ($P<0.05$) among extenders are indicated by different letters (a, b, c).

h when diluted in E2 and E4, while PMOT of F diluted semen decreased (P<0.05) at 48 h in all extenders. The percentage of HOST positive sperm decreased (P<0.05) at 24 h, except for those samples diluted with E3 for both T and F, which also decreased (P<0.05) at 48 h. T sperm velocities (VAP, VSL and VCL) decreased (P<0.05) from 48 h to 72 h in E1 and from 24 h to 48 h in the other extenders. The sperm velocities of F decreased (P<0.05) from 24 h to 48 h in E1, from 48 h to 96 h in E2, from 48 h to 72 h in E3 and from 24 h to 96 h in E4. Considering all parameters, the semen characteristics of both T and F after cooled storage were maintained by E3 for longer than any other extender.

DISCUSSION

The raw semen quality of T stallions was better than that of F stallions in terms of TMOT and PMOT. The total number of sperm per ejaculate depends on weight and testicular volume (Thompson *et al*., 1979; Love *et al*., 1991) and thus T produced less sperm per ejaculate than F. The mean semen pH of both T and F was in the normal range (pH 7.35 to pH 7.7) (Davies-Morel, 1999). The higher osmolarity of T semen might have been due to the greater volume of gel in this breed's semen when compared with F. The mean osmolarity values of T and F semen in this study were lower than those reported by Griggers *et al*. (2001) (331.5 mOsm) but higher than the presumptive normal range (290 mOsm to 310 mOsm) (Davies-Morel, 1999).

The percentage of morphologically normal sperm was 49.7 % and 48.1 % for T and F stallions, respectively. These mean values corresponded to earlier findings by Long *et al*. (1993) (47.5 %) and Pickett (1993) (51 %), but are lower than the findings of Parlevliet *et al*. (1994) (Dutch Warmblood; 66 %), Dowset and Knott (1996) (Standardbreds; 67.8 %) and Kavak *et al*. (2004) (Estonian; 74.4 %). Nevertheless, this study showed that in horses, as in other species such as rams (Abdel-Rahman *et al*., 2000) and bulls (Brito *et al*., 2004), native breeds produce semen of a better quality than that ejaculated by imported or maladapted breeds.

During cooled storage, the quality of T semen varied between extenders after 24 h of storage, which differed from the experience with F semen. The characteristics of F semen were not significantly different between the extenders during the period leading up to 96 h. This may indicate that the T sperm was more sensitive or less tolerant to various categories of extenders than

Table 4 Periods of cooled storage in which semen characteristics of Thai native crossbred (T) and full-size purebred (F) stallion were significantly decreased (p<0.05), compared to fresh, diluted semen.

Parameter	T				F			
	E1	E2	E3	E4	E1	E2	E3	E4
TMOT	48	48	48	48	48	48	48	48
PMOT	48	24	48	24	48	48	48	48
Live sperm	48	48	48	48	48	48	48	48
HOST positive sperm	24	24	48	24	24	24	48	24
VAP	48	24	48	24	48	48	48	96
VSL	48	24	24	24	24	48	72	24
VCL	72	48	48	48	24	96	72	48
pH*	NS	NS	NS	NS	NS	NS	NS	NS

* NS – not significant

the F sperm. E3 (INRA82-Y) tended to provide a better quality of stored semen than the other extenders for both T and F. The superior results of INRA82-Y, compared to EZ-mixin, the ingredients of which are similar to E1 (Kenney), have also been reported in the study by Rota *et al*. (2004). This may be due to the more complex composition of the extender (Brito *et al.,* 2004) and the beneficial effects of egg yolk (low-density lipoproteins and/or phosphatidylcholines) on the preservation of sperm membrane integrity (Bergeron and Manjunath, 2006).

The percentage of HOST positive sperm persisted longer in E3 than in E1 (Kenney) in both T and F semen (Table 4), which could be evidence of the superiority of INRA82-Y. However, the extender pH and osmolarity also affected the semen characteristics. It has been suggested that to optimize sperm motility and viability, the extender pH should be in the range from 6.6 to 7.2 (Wendt *et al*., 2002), and the osmolarity from 300 mOsm to 325 mOsm (Pommer *et al*., 2002). The higher osmolarity of E1 may be a factor in this inferior result (Table 1).

Furthermore, high osmolarity could also be responsible for the adverse effect of the extenders supplemented with glutamine (E2 and E4) on PMOT, HOST positive sperm and sperm velocities of T semen and on the HOST positive sperm of F semen (Table 4). Thus, supplementing extenders with 50 mM glutamine did not increase sperm viability, even though it has been described as a cellular survival factor in enterocytes, immune system-derived cells and cancer cells (Fuchs and Bode, 2006). Also, in contradiction to studies of frozen-thaw semen, supplementation had adverse effects on sperm motility and membrane integrity. This may be due to the prolonged exposure of sperm to the high osmolarity of the extenders.

Similar to the results reported in earlier studies, a long period of cooled storage diminished sperm motility and viability, particularly when the sperm were stored for longer than 48 h (Moran *et al*., 1992; Dawson *et al*., 2000). While the cause was unclear, some studies have considered that the rising pH during storage correlates with the lower sperm motility (Vyt *et al*., 2007). However, the pH in the present study did not increase during the experimental period, but TMOT and PMOT declined. Therefore, other factors such as lipid peroxidation of the plasma membrane or a decrease in the energy supply by the mitochondria due to ATP depletion (Aurich, 2005) may have been the main cause of this reduced motility.

CONCLUSION

The data from this study indicated that the semen quality of T is better than F stallions in terms of TMOT and PMOT. INRA82-Y tended to provide a better quality of stored semen than Kenney extenders in both T and F. Overall, cooled storing of semen maintained the sperm characteristics for less than 48 h. Furthermore, supplementing cooled storage extenders with 50 mm of glutamine had adverse effects on sperm motility and membrane integrity, which may have been caused by an increase in the osmolarity of the extenders.

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