THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Agricultural Biotechnology)

DEGREE

Agricultural Biotechnology — **FIELD**

Interdisciplinary Graduate Program — **PROGRAM**

TITLE: The Characteristics and Preservation of Thai Native Crossbred and Purebred Horse Semen

NAME: Miss Kanittha Phetudomsinsuk

THIS THESIS HAS BEEN ACCEPTED BY

_____ **THESIS ADVISOR**

(Associate Professor Kaitkanoke Sirinarumitr, Ph.D.)

_____ **COMMITTEE MEMBER**

(Associate Professor Apassara Choothesa, Ph.D.)

_____ **COMMITTEE MEMBER**

(Associate Professor Anuchai Pinyopummin, D.Vet.Med.Sc.)

_____ **GRADUATE COMMITTEE CHAIRMAN**

(Associate Professor Pongthep Akratanakul, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

_____ **DEAN**

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

THE CHARACTERISTICS AND PRESERVATION OF THAI NATIVE CROSSBRED AND PUREBRED HORSE SEMEN

KANITTHA PHETUDOMSINSUK

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Agricultural Biotechnology)
Graduate School, Kasetsart University
2009

Kanittha Phetudomsinsuk 2009: The Characteristics and Preservation of Thai Native Crossbred and Purebred Horse Semen. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Kaitkanoke Sirinarumitr, Ph.D. 100 pages.

The study was aimed to evaluate semen characteristics of Thai native crossbred (T) and purebred (F) stallions after preservation by cooled storage and cryopreservation. The outcome of this study could benefit to the application of artificial insemination technology, which will be an important tool for genetic and reproductive management in this species.

Semen were collected by using artificial vagina, from 5-12 years old T (n=5) and F (n=4) stallions. The semen characteristics examined were color, osmolarity, pH, volume, total motile sperm (TMOT), progressive motile sperm (PMOT), sperm viability, morphology and concentration. The seminal characteristics observed were opalescent white. Some semen parameters were significant different ($P<0.05$) between breeds. Semen quality of T was better than F stallions in terms of TMOT and PMOT. Furthermore, in normal morphologically sperm, head of T sperm were larger and rounder than that of F sperm.

The effects of extenders (Kenney, Kenney+ 50 mM L-glutamine, INRA and INRA+50 mM L-glutamine) on semen characteristics after cooled storage (5 °C) were examined. The cooled samples were evaluated for sperm viability, TMOT, PMOT, and motion velocity, which were significantly decreased ($P<0.05$) after storage for 24 - 48 h in all extenders. However, in both T and F, INRA extender tended to maintain sperm motility and membrane integrity longer than other extenders.

The freezability of semen in different extenders (INRA, INRA+ 50 mM L-glutamine, Lactose-EDTA, Lactose-EDTA+ 50 mM L-glutamine) with glycerol was determined. Lactose-EDTA gave better results ($P<0.05$) than other extenders in post-thaw TMOT and PMOT. Nevertheless, only semen from two T and one F stallions could be preserved by cryopreservation (> 30 % post-thaw TMOT).

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I sincerely thank my major advisor, Associate Professor Dr. Kaitkanoke Sirinarumitr. She always helps, encourage, personal guidance and advises throughout my study. I would like to express my special thanks to the member of my committee; Associate Professor Dr. Apassara Choothesa and Associate Professor Dr. Anuchai Pinyopummin for their kindness, guidances, valuable comments and suggestions, which carry out this thesis successfully.

Special thanks to Associate Professor Dr. Pongthep Akratanakul,, the director of Center for Agricultural Biotechnology, who encouraged me to do research and training abroad. Deep appreciation is likewise extended to the Ministry of Education for education scholarship and partially funded by Center for Agricultural Biotechnology and the Research Development Institute, Kasetsart University through the fund from Subproject Graduated study and Research Development Project, Commission on Higher Education, The Ministry of Education. And also thanks the Faculty of Veterinary Medicine, Kasetsart University for approving and providing the most generous facilities of this research.

In particular, I would like to thank numerous colleagues at the Faculty of Veterinary Medicine, Kasetsart University and Center for Agricultural Biotechnology, Kasetsart University for their assistances, advises and supports me at various times. Special thanks to Ms Piyawan Suthamapinanta, Ms Sudarat Amornsak, Ms Sukuman Prukudom and Assistant Professor Aree Laikul, who helped me with equipment preparation, semen collection and handling the animals. Finally, I express the most gratitude to my parents, my friends and my family for their love, understanding, encouragement, support and affection.

Kanittha Phetudomsinsuk

January 2009

TABLE OF CONTENTS

Page

TABLE OF CONTENTS i
LIST OF TABLES ii
LIST OF FIGURES iv
LIST OF ABBREVIATIONS v
INTRODUCTION 1
OBJECTIVES 4
LITERATURE REVIEW 5
MATERIALS AND METHODS 24
RESULTS AND DISCUSSION 32
Results 32
Discussion 52
CONCLUSION 60
LITERATURE CITED 61
APPENDICES 84
Appendix A General characteristics of Thai native crossbred horses 85
Appendix B Seminal plasma proteins of Thai native crossbred and purebred stallion semen 88
Appendix C A first pregnancy of Thai native crossbred horse resulted from artificial insemination with frozen semen in Thailand 93
CIRRICULUM VITAE 100

LIST OF TABLES

Table		Page
1	pH and osmolarity of cooled extenders	26
2	Settings for the Hamilton Thorne IVOS (CASA, Equine motility software version 12.1, Hamilton Thorne Research, Beverly, MA, USA)	28
3	Osmolarity of freezing extenders	29
4	Ejaculate traits of Thai native crossbred (T) and purebred (F) stallions (mean ± SEM)	33
5	Percentage of sperm morphology of Thai native crossbred (T; T1 – T5) and purebred (F; F1 – Standard-bred; F2 – Warm-blood; F3 and F4 Thoroughbred) stallions (mean ± SEM)	34
6	Normal sperm head morphometry of Thai native crossbred (T; T1 – T5) and purebred (F; F1 – Standard-bred; F2 – Warmblood; F3 and F4 – Thoroughbred) stallions (mean ± SEM)	38
7	Within-animal and between-animal CV of normal sperm head morphometry in Thai native crossbred (T) and purebred (F) stallions	40
8	Average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL) of Thai native crossbred (T) and purebred stallion (F) sperm after storage at 5°C in different extenders.	43
9	Periods of cooled storage in which semen characteristics of Thai native crossbred (T) and purebred (F) horses were significantly decreased ($P<0.05$), compared to fresh diluted semen.	46
10	pH of diluted semen of Thai native crossbred (T) and purebred stallion (F) sperm after storage at 5°C in different extenders.	47

LIST OF TABLES (Continued)

Table		Page
11	Semen motion characteristics of Thai native crossbred (T) and full-sized purebred stallion sperm (F) in different extenders before freezing (BF) and post-thawed (PT).	50
12	Freezability of T and F semen	51

Appendix Table

1	Mean ± SD of Bodyweight (kg), Hieght, Chest Girth, Umbilical Girth, Length from tuber ischium to point of shoulder, and Length from tuber ischium to olecranon (cm) of Thai native crossbred horses (n= 37)	87
2	The total protein concentration in seminal plasma of Thai native crossbred (T) and purebred (F) stallions	91
3	Motility, motility pattern, lived and HOST positive sperm before freezing (BF) and post-thawed (PT) stallion semen	97

LIST OF FIGURES

Figure		Page
1	Anatomy of stallion reproduction.	6
2	Structure of the stallion's penis and prepuce.	7
3	Lateral view of the testis and epididymis of a stallion.	8
4	Light microscopy of Thai native crossbreed stallion sperm after eosin/nigrosin staining	35
5	Scanning electron microscopy of Thai native crossbreed stallion sperm	36
6	Semen characteristics of Thai native crossbred (*A, C, E* and *G*) and purebred horses (*B, D, F* and *H*) after storage at 5°C in different extenders for up to 96 h.	42
7	Total motility, progressive motility and acrosome integrity of Thai native crossbred (T) and purebred stallion (F) sperm in different extenders before freezing and post-thawed.	49

Appendix Figure

1	The criteria of body measurement of Thai native crossbred horse	86
2	The standard curve of Bovine serum albumin protein (Bradford assay, 595 nm)	90
3	Seminal plasma protein profiles of Thai native crossbred (T1 – T5) and purebred (F1 – F4) stallions seminal plasma in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).	92
4	Thai native crossbred embryo after insemination with frozen semen, by using ultrasonography with 5 MHz probe	98

LIST OF ABBREVIATIONS

µg	=	microgram
µl	=	microliter
µm	=	micrometer
AI	=	artificial insemination
ALH	=	amplitude of lateral head displacement
ANOVA	=	analysis of variance
ART	=	assisted reproductive techniques
AV	=	artificial vagina
BCF	=	beat cross frequency
BF	=	before freezing
CASA	=	computer assisted semen analysis
CFDA	=	carboxyfluorescence diacetate
cm	=	centimeter
CRISPs	=	cysteine-rich secretory proteins
CV	=	coefficient of variation
DSO	=	daily sperm output
EDTA	=	ethylene diaminetetraacetic acid
FITC-PNA	=	fluorescein isothiocyanate-conjugated peanut agglutinin
h	=	hour
HOS test	=	hypoosmotic swelling test
i.e.	=	id est
IU	=	international unit

LIST OF ABBREVIATIONS (Continued)

kg	=	kilogram
LN_2	=	Liquid nitrogen
M	=	molar
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
mm^2	=	square millimeters
mOsm	=	milliosmol
p	=	probability of error
pH	=	negative decadic logarithm of the hydrogen ion concentration
PMOT	=	progressive motility
r	=	correlation
SEM	=	standard error of mean
SP	=	seminal plasma
V/V	=	volume to volume
VAP	=	average path velocity
VCL	=	curvilinear velocity
VSL	=	straightline velocity

THE CHARACTERISTICS AND PRESERVATION OF THAI NATIVE CROSSBRED AND PUREBRED HORSE SEMEN

INTRODUCTION

There are two main horse groups in Thailand: full-size purebred and Thai native crossbred pony horses. Full-size purebred horse breeds include Arabians, Standard-bred and Thoroughbred; all of them were originally introduced to Thailand by importation. The Thai native crossbred horse is a pony horse that may have originated from a Burmese pony (Panasophonkul et al., 2007). However, the scientific origin of the breed remains obscure. Nowadays, this native breed is generally used in religious ceremonies, for recreational activities, and occasionally for transportation in highland areas. Natural breeding with stallions is commonly performed to increase horse numbers. The numbers of Thai native crossbred pony is being decrease, by means of crossbreeding with the full-size purebred due to owner's preference for bigger horses. Thus, for conservation of the native horse, preservation of semen and artificial insemination (AI) may be an important method for increasing population numbers, and a detailed study of its semen characteristics was therefore considered necessary.

Horse breeding in recent time has been based on the use of AI, which made selected stallions to be available to breeders outside the regions where the stallions are located. This method could accelerate the usage of native stallion genetic. However, semen needs to be preserved either by cooled storage or cryopreservation. Therefore, a study of cooled storage on semen characteristics of Thai native stallions could provide useful information for implementation of horse AI in the country.

Semen cryopreservation enhances the advantage of AI. Long-term storage facilitates semen transport over distances, permits the quarantine of semen, and

enables extended use of semen, even after the sire's death. Hence, a study for cryopreservation of Thai native stallions' semen was also carried out.

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 cold shock and the negative effects of seminal plasma, and inhibits bacterial growth. A large variety of extenders combining assorted ingredients (sugar, electrolytes, buffers, milk or milk products, and egg yolk) have been proposed for cooled storage of 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). Accordingly, skim milk based extenders added to glucose and antibiotics, and ultimately added to salts and other sugars are now in worldwide used (Shore et al., 1998). Furthermore, extenders containing low concentration of egg yolk (2-4%) are thought to improve the motility of cooled stallion spermatozoa (Rota et al., 2004).

Ideal semen extenders used for cryopreservation of stallion semen would minimize damage resulting from freeze-thaw cycle 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 (Zahn et al., 2002; Alvarenga et al., 2003; Moore et al., 2006), various freeze-thaw protocols (Melo et al., 2007; 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 coconut water (Cardoso et al., 2006). Additionally, it is reported that supplementing cryopreservation extenders with glutamine could increase frozen-thaw equine sperm motility (Trimeche et al., 1999).

The present study was undertaken to examine Thai native crossbred pony stallion semen characteristics and the effects of different extenders with and without

glutamine supplementation on semen characteristics after either cooled storage or cryopreservation, compared to those of full-size purebred stallions.

OBJECTIVES

1. To investigate the semen characteristics of Thai native crossbred compare with those of full-size purebred stallions.

2. To investigate the effect of different extenders with or without glutamine supplementation on semen characteristics after cooled storage and cryopreservation in Thai native crossbred, compare with those of full-size purebred stallions.

LITERATURE REVIEW

1. Overview of horses in Thailand

There are two main horse groups in Thailand: full-size purebred and Thai native crossbred pony horses. For horse population in Thailand, there are about 5,629 horses (Data from Statistics of Livestock in Thailand: 2007, Department of Livestock Development, Ministry of Agriculture and Cooperatives). Full-size purebred horse breeds include Arabians, Standard-bred, Thoroughbred and Warmblood, all of them were originally introduced to Thailand by importation. Thai native crossbred is a pony horse with an average height of 118.03±8.12 cm, an average body weight of 209.54±40.88 kg (Sritrakul et al., in press). It may be originated from a Burmese breed (Panasophonkul et al., 2007). However, the scientific origin of the breed remains obscure. Previous study indicates only one Y chromosome lineage that has been found in a large number of horse breeds including Thai native (Lindgren et al., 2004) and the microsatellite markers also showed high polymorphic genetic characteristic (Tawatsin et al., 2005). Nowadays, this native breed is generally used in religious ceremonies, for recreational activities, and occasionally for transportation in highland areas. Natural breeding is commonly performed to increase the numbers of horses in both groups.

2. Anatomy and physiology of stallion reproductive system

The normal structure of stallion's reproductive system (Figure 1) includes external genitalia and internal genitalia. The external genitalia include scrotum, penis and prepuce that protect internal genitalia, thermo regulate the testes, intromission and ejaculation. The internal genitalia include two testes with attached epididymides, and

four types of accessory sex glands that produce sperm and seminal plasma (Chenier, 2000).

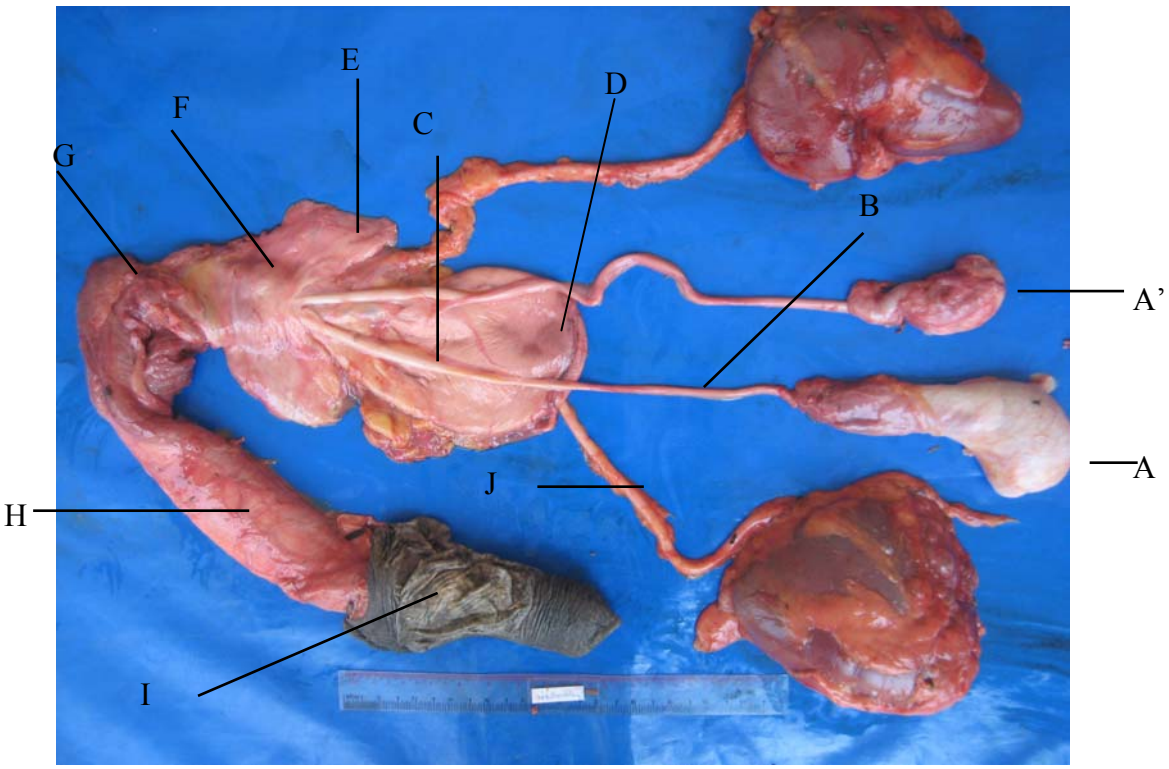


Figure 1 Anatomy of stallion reproduction. A, testis; A', cryptorchid testis; B, vas deferens; C, ampullae; D, urinary bladder; E, seminal vesicle; F, prostate gland; G, bulbouretal gland; H, penis; I, prepuce; J, ureter.

The stallion's penis is classified as a musculocavernous type, and consists of a root, which attaches the penis to the skeleton; the main body (shaft) of the penis; and the glans penis, is the free end of the penis (Figure 2). In the non-erect state, the full size stallion's penis is approximately 50 cm in length, 3 to 5 cm in diameter, and is contained within the sheath or prepuce, which forms two folds around the free end of the penis. During erection, the erectile tissue of the penis (corpus cavernosum and corpus spongiosum) becomes engorged with blood, and the size and diameter of the penis doubles in length and thickness and the glans penis increases by 3 to 4 times.

Examination of the penis and prepuce is most conveniently done at the time of washing (Davies-Morel, 2002).

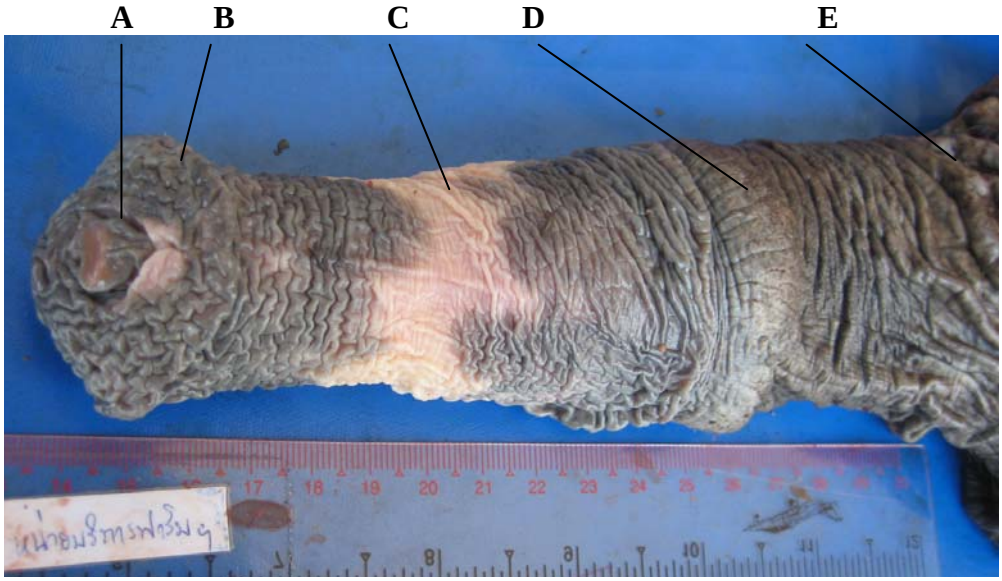


Figure 2 Structure of the stallion's penis and prepuce. A, urethral process; B, glans penis; C, penile body; D, preputial ring; and E, prepuce.

The testes are the site for production of sperm (spermatogenesis, take 57 days in stallion) and the primary male sex hormone, testosterone. The stallion's testes are normally ovoid in shape. Testicular size is related to the stallion's ability to produce spermatozoa. Generally, as testicular size increases, the potential ability to produce spermatozoa increases. In addition to individual differences in testicular size, seasonal and age variations exist as well. The scrotum is a pouch of skin in the inguinal area that encloses the testes. Normally, stallions have two testicles descended in the scrotum. The retention of one (unilateral) or both (bilateral) testicles in the body cavity occurs fairly frequently in stallions and is referred to as cryptorchidism (Figure 1). The stallion's scrotum is not as pendulous as a bull's and is held closer to the abdomen. The scrotum's skin is soft and pliable with a greasy texture due to sebaceous glands. The scrotum is important in thermoregulation of the testicles.

The epididymis (Figure 3) can be divided into three segments; the head (caput), body (corpus) and tail (cauda). Within the epididymis, sperm undergo modifications (maturation) in which they acquire the ability to swim, capable of further development within female reproductive tract (capacition), and to fertilize an egg. Most of these changes are complete by the time sperm reach the end of the corpus. The migration of sperm through the epididymis requires approximately 8 days. Most of the spermatozoa are stored in the tail of the epididymis until ejaculation (Chenier, 2000; Davies-Morel, 2002).

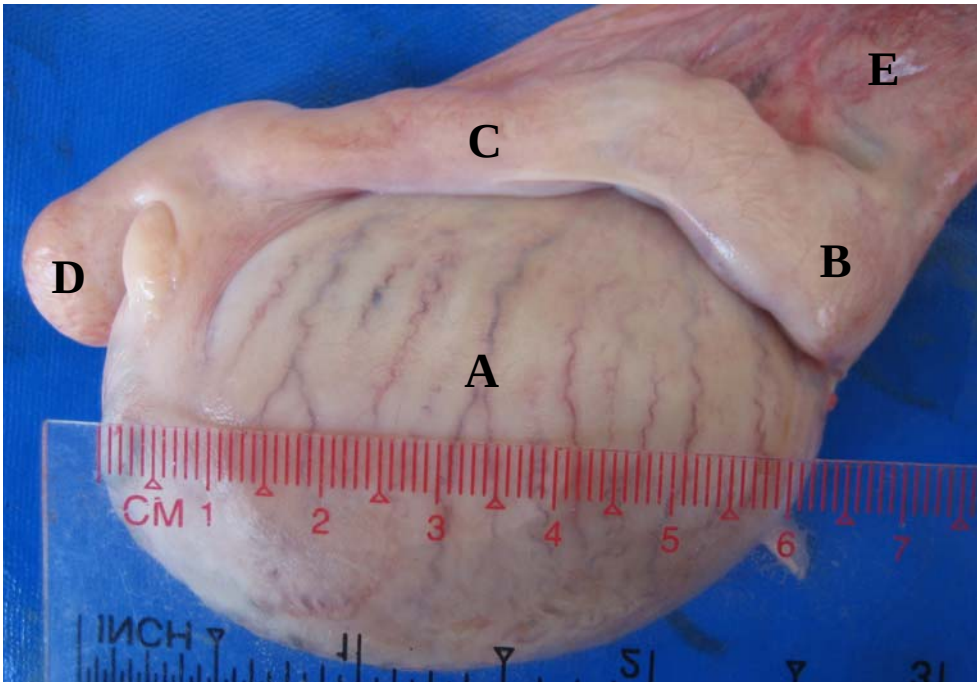


Figure 3 Lateral view of the testis and epididymis of a stallion. A, Testis; B, head of epididymis; C, body of epididymis; D, tail of epididymis; and E, pampiniform plexus

The accessory sex gland (Figure 1) consists of four types of glands: ampullae, seminal vesicles, prostate gland, and bulbourethal glands which were situated between the end of vas deferens and root of penis. These glands are responsible for the secretion of seminal plasma that makes up the majority of the ejaculate volume. Seminal plasma is thought to contain substances beneficial to sperm transport and survival in the female reproductive tract (Killian, 1992). However, seminal plasma

also contains factors that decrease sperm motility and increase sperm death during semen storage (Begley and Quinn, 1982). Identity of motility-reducing factors in seminal plasma is unknown; however, a high molecular weight fraction of seminal plasma has been shown to reduce the motility of bull sperm (Baas et al., 1983). The deleterious effects of seminal plasma can be reduced by collecting and extending only the sperm-rich fraction of the ejaculate or by using high dilution ratios of extender to semen for whole ejaculates (Jasko et al., 1991).

It is well established in many mammalian species, including the stallion, that normal spermatogenesis is dependent upon a functional hypothalamic–pituitary–testicular axis (HPT) axis which involves the classic endocrine actions of gonadotropins, feedback mechanisms of steroids and proteins and, most likely, paracrine/autocrine modulation (Matsumoto, 1989; Skinner, 1991; Amann, 1993; Spiteri-Grech and Nieschlag, 1993; Huhtaniemi and Toppari, 1995; Gnessi et al., 1997). Endocrine control mainly involves the changing patterns of secretion of the hypothalamic hormone, gonadotropin releasing hormone (GnRH), which travels through portal blood vessels to the anterior pituitary. At the anterior pituitary, GnRH stimulates the secretion of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins travel to the testes, where FSH exerts its effect on the Sertoli cells and LH on the Leydig cells and testicular hormones such as the androgens and inhibin (Amann, 1993; Weinbauer and Nieschlag, 1993). Reports in the literature clearly indicate that testosterone is essential for normal sexual behavior, maintenance and restoration of spermatogenesis in the adult testis (Weinbauer and Nieschlag, 1993).

The seasonal effect on stallion reproduction tends to show less than mare. During periods of short daylight, male reproductive function is suppressed due to low testosterone concentrations, causing testicular size and sperm production are reduced, and sexual behavior is also variable.

3. Semen composition

Semen is composed of spermatozoa (sperm), the male gamete produced in the seminiferous tubules of the testis (Johnson et al., 1997), and seminal plasma, which is a mixture of fluid secreted from the testes, epididymis and accessory sex glands. The stallion ejaculates in a series of five to eight jets or fractions. The composition of each fraction varies. The first secretions (presperm) contain no spermatozoa and are primarily from the bulbourethral glands. The next two or three jets contain most spermatozoa (sperm-rich fractions), and a subsequent sperm-poor fraction with the major component, the gel fraction, coming from the seminal vesicles (Mann, 1975; Kosniak, 1975; Magistrini et al., 2000; Katila et al., 2002).

Mammalian spermatozoa consist of head, neck and tail (endpiece), enclosed within plasma and mitochondrial membrane. The head contains the nucleus with the DNA, the male genetic material to be delivered to the ovum, and the acrosome which contains hydrolytic enzymes necessary for penetration of the ovum vestments during fertilization. The neck is a short linking segment between the flagellum and the sperm head. The tail is the longest part of the sperm and consists of midpiece, principal piece, and end piece. The equine spermatozoon (Johnson et al., 1980) is similar in size and shape to the human (Johnson, 1982). Spermatozoa are not mature when released from the seminiferous tubules. Each contains a cytoplasmic droplet on its mid piece and must undergo maturation in the epididymis to gain progressive motility, structural stability, and fertilizing ability (Johnson et al., 1980).

Seminal plasma (SP) is involved in a multitude of sperm functions and events preceding fertilization. The composition of SP varies between fractions as the accessory sex glands release their contents in a specific order. Bulbourethral fluid was the first to be secreted (pre-sperm fluid), and then epididymal and ampullar secretions were appeared in the first fractions of the sperm-rich ejaculate and seminal vesicle fluid was formed as the last ejaculatory fractions (Magistrini et al., 2000). SP consists of proteins, enzymes, electrolytes and trace elements that essential for sperm maturation and fertilization process. Electrophoresis revealed the presence of a variety of protein bands of different molecular weight in seminal plasma. Only seven of these

bands were present in semen from all stallions (Zahn et al., 2006). This illustrates variations in the composition of seminal plasma between individual sires. A considerable variation occurs in cysteine-rich secretory proteins (CRISPs) among different stallions (reviewed by Leeb et al., 2005) and an association of stallion fertility with polymorphism of the CRISP-3 gene (Hamann et al., 2007). However, a relationship between CRISPs and the suitability of semen from individual sires for cooling has not been proven. Further analysis and definition of seminal plasma fractions and proteins responsible for the different cooling ability of individual stallions could have a key role for the development of new extender components.

Season and other environmental parameters, such as age and frequency of ejaculations, may change the properties of equine semen (Johnson et al., 1991; Magistrini et al., 1987; Pickett, 1993; Araujo et al., 1996; Dowsett and Knott, 1996).

4. Semen collection

Sperm collected using an artificial vagina (AV) and manipulated in the laboratory can be damaged by deviations in temperature, osmotic pressure, and exposure to contaminants. Damage can also be caused by prolonged exposure to components of seminal plasma and to byproducts of metabolism during incubation. The goal should be to obtain a complete ejaculate with a single mount and minimal sexual stimulation of the stallion before collection of semen, optimizing the potential to obtain an ejaculate with relatively low volume and high sperm concentration. An increase in the number of mounts resulted in increased seminal volume, decreased sperm concentration, and decreased progressive motility after 24 hours at 5°C (Ionata et al., 1991; Sieme et al., 2002).

5. Semen evaluation

A standard procedure for estimating sperm production in stallions is to collect 2 ejaculates 1 hour apart. Parameters usually included in a conventional evaluation of raw semen quality are physical semen characteristic (volume, seminal pH, and viscosity), sperm concentration, total number of sperm in the ejaculate, percentages of

motile sperm, normal morphology, longevity of sperm motility and bacteriological status. Although, these evaluations provide a lot of information, their correlations with fertility are somewhat conflicting (Mahngren, 1992; Jasko, 1992; Rodriguez-Martinez, 2000). However, it seems likely that the prediction of male fertility could be improved if additional parameters based on the functional characteristics of sperm were used. Several functional tests have been investigated, such as the use of fluorescent stains as a marker for cell membrane integrity (Harrison and Vickers, 1990; Althouse and Hopkins, 1995), sperm-oocyte binding tests (Fazeli et al., 1995) and the hypoosmotic swelling test (England and Plummer, 1993).

5.1 Daily sperm output (DSO)

DSO is defined as the number of sperm that a stallion can produce on a daily basis. In equine breeding, the number of sperm ejaculated is considered an important factor in fertility for predicting the reproductive capacity of stallions. Evaluation of an animal at DSO also can help when estimating book sizes for stallions with very large books or for stallions of marginal fertility whose books must be reduced. There have been numerous schedules recommended to allow a stallion to reach DSO. Originally, it was proposed that a stallion be collected once daily for 10 days before total sperm numbers plateaued at DSO (Gebauer et al., 1974). Thus, it is much more cost and labor efficient compared with, for example, collecting semen once daily for 10 days.

The testicular volume, estimated by testicular measurements, is correlated with sperm production (Thompson et al. 1979; Pickett et al., 1987; Love et al., 1991). Smaller testicles therefore yield lower sperm output than bigger testicles. Based on this, calculation of testicular volume is commonly performed as an additional component of the breeding soundness evaluation (BSE). Testicular volume is calculated by using the formula for the volume of an ellipsoid. Stallions that have normal testicular size but produce low numbers of sperm might be suspected of having a testicular pathology that prevents the testicles from functioning at full capacity.

5.2 Motility

Sperm motility is important because it is readily identifiable and reflects several essential aspects of sperm metabolism. Therefore, motility should be evaluated together with other parameters when estimating the fertilizing potential of spermatozoa. Usually total motility (any type of motility) and progressive motility (spermatozoa moving actively forward) are estimated as percentages. Motility can also be described as circling, oscillating and serpentine (Kenney et al., 1983), and the speed of sperm motion is also assessed. If semen is exposed to low temperatures or it is dried on the slide, motility diminishes rapidly.

Stallion spermatozoa have some species-specific characteristics: an asymmetrical head, an abaxial position of the tail, an acrosome of small volume and the presence of microtubules in the neck (Bielanski and Kaczmarski, 1979). The large, circular motion of normal sperm is due to a high incidence of abaxial connections between the sperm head and neck (Kenney et al., 1983). Estimating only the progressive motility may underestimate good motility in some stallions.

When fresh stallion semen was subjectively evaluated, low correlations were found between fertility and the percentage of motile ($r = 0.40$) and progressively motile ($r = 0.46$) spermatozoa (Jasko, 1992). In a study where 177 mares (an average 19 mares/stallion; min 6, max 51) were inseminated with frozen semen from 9 stallions, the correlation coefficient of the visually estimated percentage of motile cells to the first-cycle pregnancy rate was only 0.32 (Samper et al., 1991). Good motility of frozen-thawed semen was a poor indicator for pregnancy rates in pigs (Hammitt et al. 1989). Similarly, in the horse, the percentage of progressively motile, post-thaw spermatozoa is considered to be a poor predictor of pregnancy rates in mares (Pickett et al., 1987; Squires et al., 1987; Bataille et al., 1990; Wilhelm et al. 1996). A very low motility would probably be an indication not to use the semen, but a good motility does not necessarily indicate that the fertilizing capacity of spermatozoa has been maintained.

Percentages of motile spermatozoa were significantly lower at room temperature (22°C) than at higher temperatures. Maximum percentages of motile spermatozoa were obtained at 37°C or 42°C (Amann and Graham, 1993). Either raw or extended semen can be used, but non-extended semen has disadvantages, such as a tendency to agglutinate, in which case if the sperm concentration is high the estimated percentages of motile spermatozoa may become higher (Van Duijn and Hendriske, 1968). Samples of a higher concentration are usually judged by the human eye as having higher motility (Jasko, 1992). Extending semen prevents the agglutination of spermatozoa and reduces the influences of sperm concentration and seminal pH. Semen should be extended to a constant sperm concentration (25 to 50 x 10^6/ml). Temperature of the slide should be controlled (37°C) by using a stage warmer on a phase-contrast microscope (magnification x 200 to 400), the depth of suspension on the slide should be standardized and multiple fields near the centre of the slide examined. The standardization can be carried out either by taking a certain amount of suspension and using a cover slip of a certain size (10µl drop of semen and a 22- mm^2 cover slip). Multiple fields across at least two suspensions should be examined, and one should be certain that the fields examined are near the center. Motility at the edges declines more rapidly than in the centre as a result of drying and exposure to air (Jasko, 1992). The light microscopic evaluation does not require expensive equipment and is easy to perform. However, the greatest variation is caused by a variation between examiners, since the evaluation is subjective and requires experience.

Repeatability in assessment can also be achieved by Computer-assisted semen analysis (CASA), an objective method that gives extensive information about the kinematic properties of the ejaculate based on measurements of individual spermatozoa motility patterns (Amann and Pickett., 1987; Jasko et al., 1988; Blach et al., 1989). Video images for computerized sperm motion analysis are obtained from viewing fields of motile sperm using a microscope. A set number (usually 20 to 30) of successive video frames is analyzed at a constant rate, typically 30–60 frames per second. When all frames for a given field have been analyzed, computer algorithms are used to distinguish sperm from non-sperm objects and to reconstruct sperm tracks (Jasko, 1992). Each sperm is classified as either motile or immotile, and the concentration of both is calculated. Motility data is further characterized as follows:

mean curvilinear velocity (VCL), path velocity (VAP), mean straight-line velocity (VSL), straightness (STR = VSL/VAP), linearity (LIN = VSL/VCL), percentages of total motility (MOT), progressive motility (PMOT), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Due to the high cost of the instrument, computerized sperm image analysis systems are used primarily for research applications.

The maximum sperm concentration in CASA-systems is usually 50 × 10^6/ml. A dilution of stallion semen to 25 × 10^6/ml has been recommended (Varner et al. 1991a). The recommend was 3 chambers/ejaculate and 3 fields/chamber should be evaluated which would yield a mean spermatozoa number of approximately 500 evaluated per sample.

5.3 Viability

A total of 20 µl of each sample was mixed with 20 µl of 0.5% eosin stain on a glass slide and viewed using light microscopy to determine the percentage of viable sperm. Eosin Nigrosin is a supravital stain; live sperm was unstained whereas dead sperm was stained red, since the integrity of their plasma membranes had been compromised causing an increase in membrane permeability that led to uptake the dye.

Popidium Iodide (PI) is a membrane impermeable, fluorescent nucleic acid stain. Since PI cannot cross intact cell membranes, it gains access to nuclear DNA only when cell membranes are damaged (i.e., only in nonviable cells). Once bound to DNA, PI fluoresces red in response to excitation. Ten microliters of stained spermatozoa was placed on a microscope slide, overlaid with a coverslip and 200 spermatozoa were viewed using combination fluorescent and phase-contrast microscopy. Nonviable sperm was fluorescent red, whereas viable sperm remained unstained and it was visible when viewed using phase-contrast microscopy (Gillan et al., 2007). By combining 5- carboxyfluoroscein diacetate (CFDA) with PI, one can readily and accurately determine the percentage of nonviable (red) cells and the percentage of viable (green) cells either by manually counting a population of sperm

or by using flow cytometry. Alternatively, using SYBR-14 and PI, three populations of cells can be identified: SYBR-14 stained (green, living) cells, PI stained (red, nonviable) cells, and doubly stained (yellow, moribund) cells. Normal values for the percentage of viable sperm in a stallion ejaculate have not yet been established for clinical assays. Therefore, viability assays for a clinical unknown should be performed side by side with a control sample from a known fertile stallion. Most typically, over 70% of the sperm in a fresh ejaculate from a normal stallion are viable; however, the exact percentage can vary from individual to individual.

5.4 Morphological evaluation

Sperm morphology is an important parameter for assessing semen quality. Disturbances in spermatogenesis give rise to morphological sperm abnormalities. However, the effects of specific morphological sperm abnormalities on male fertility have been investigated less thoroughly in horses than in other domestic animals, i.e. swine and cattle.

The relationship between sperm morphology and fertility has been evaluated in several studies. Some studies found no relation between sperm morphology and fertility (Voss et al., 1981; Dowsett and Pattie, 1982), whereas in others increases in sperm abnormalities were associated with decreased fertility (Chevalier et al., 1991; Bielanski, 1975, Jasko et al., 1990). A wide range of morphological deviations may be acceptable for breeding stallions, if the total number of morphologically normal motile spermatozoa in the ejaculate is adequate (Kenney et al., 1983). Sometimes the low pregnancy rates after frozen semen inseminations are simply due to an excessively small number of live morphologically normal post-thaw sperm.

Morphological features are evaluated by light microscope using different sperm stains. The use of fluorescent probes requires epifluorescence optics for the microscope. Scanning and transmission electron microscopic techniques are not in routine use, but have been useful in some abnormal cases and in research. The simplest examination method is to fix sperm cells in buffered formal-saline (Hancock, 1957; Bane, 1961) or buffered glutaraldehyde solution and view unstained cells with

either phase-contrast or differential interference-contrast microscopy (1000x magnification). General-purpose cellular stains (Wright's, Giemsa, haematoxylin-eosin, India ink) can be used (Varner et al. 1991b), but live-dead stains (aniline-eosin, eosin-nigrosin, eosin-fast green) are more widely used for determination cell viability. Integrity of the plasma membrane is shown by the ability of a viable cell to exclude the dye, whereas the dye will diffuse passively into sperm cells with damaged plasma membranes (Colenbrander et al., 1992). Differential stains for morphology of sperm cells are Spermac (Oettle, 1986), William's and Casarett's stains (Kenney et al., 1983), Triple stain, Papanicolau, and Feulgen and Karras among others (Magistrini et al., 1997). It is generally recommended 200 cells be examined, however evaluation of 100 sperm cells probably provides a valid representation of abnormalities (Hermenet et al. 1993). All abnormalities on any given sperm were counted and the overall frequencies were classified according to a system developed by Bane (1961).

Sperm head morphometry assessed by Computer-Assisted Semen Analysis (CASA) shown a relationship to fertility in studies using various species including: horses (Casey et al., 1997), boar (Hirai et al., 2001; Peña ey al., 2005), Iberian red deer (Esteso et al., 2006), and canine (Núñez-Martínez et al., 2007). The different size of sperm head may result in different cooling rates under the same procedure. There was also evidence that sperm head size affects to the sperm’s cryoresistance (Esteso et al., 2006). Thus, the sperm head size or shape may be an aspect to consider for improving cooled storage and cryopreservation protocols.

5.5 Hypoosmotic swelling test

The hypoosomotic swelling test (HOS test) was first developed for human sperm and has since been described for the stallion (Jeyendran et al., 1984; de la Cueva et al., 1997; Nie and Wenzel; 2001; Neild et al., 1999; Pinto and lobo, 1997). The HOS test is designed basically to be a test for functional membrane integrity and, as such, can be considered as an alternative staining for sperm viability determination.

An important property of the sperm cell membrane is its ability to permit selective transport of molecules. When spermatozoa are suspended in a hypoosmotic

solution, water will enter the spermatozoon in an attempt to attain osmotic equilibrium. This inflow of water will increase sperm volume, the initial length of the flagellum and the plasma membrane will bulge (balloon). The influx of water only occurs in the tail region and creates different types of curls. The appearance of a curl in the tail of a sperm is a sign that water has been transported in a physiological manner into the cell to reach osmotic equilibrium. This indicates an intact flagella membrane (Colenbrander et al., 1992) while cells with damaged membranes (i.e., nonviable cells) or cells with weak membranes that rupture in response to the influx of water will not experience the increase in pressure and therefore their flagella will remain unchanged at the light microscopic level. As early as 1966, Drevius and Eriksson (1966) had described the osmotic swelling of mammalian spermatozoa. Since then, several studies have shown that the HOS test may be useful for predicting the spermatozoa's ability to fertilize the ova (Cot-ma and Zavos, 1994; England and Plummer, 1993; Jeyendran et al., 1984; Ktuni-Diaka and Badnam, 1994), although correlations with other semen parameters differ between studies. The percentage of swollen spermatozoa has been reported to be significantly correlated with the percentage of spermatozoa undergoing capacitation, the percentage of penetrating oocytes, and sperm motility (Jeyendran et al., 1984; Ktuni-Diaka and Badnam, 1994). Moreover, a significant positive correlation was obtained between HOS test and fertilization rate (De Albuquerque and Bestimmung, 1995). Also, Rogers and Parker (1991) found a low, but statistically significant, correlation between the results of the HOS test and those of the sperm penetration assay. In contrast, England and Plummer (1993) found no relationship between the percentage of swollen spermatozoa and other measures of semen quality, including motility, morphology, and vital staining (eosin).

The HOS-test applied immediately after fresh semen collection is the best predictive test of the freezability of stallion semen (Vidament et al., 1997). The most frequently osmolality in stallion sperm was between 100 and 150 mOsm (De Albuquerque and Bestimmung, 1995) and between 25 and 100 mOsm (Neild et al., 1999).

The HOS test was conducted as previously described (Revell and Mrode, 1994; Neild et al., 1999) with some modifications. One hundred microliter of stallion semen is added to 1 ml of 100 mOsm fructose solution and incubated at 37°C for 60 min (Nie and Wenzel, 2001). The test solution consisted of fructose and trisodium citrate with double-distilled water, and its osmotic strength was 150 mOsm/ kg for fresh semen and 100 mOsm/ kg for frozen semen. Following incubation, the preparation was thoroughly mixed and ten microliter of semen were dropped on a microscope slide and covered with a cover slip, and evaluated with a 400x phase-contrast microscopy with a 37°C warmed stage. Spermatozoon with intact plasma membranes swelled in response to the treatment. This was manifested by bending, coiling or shortening of the tails.

6. Semen preservation and artificial insemination

The use of artificial insemination (AI) in equine breeding has increased during recent decades and becoming important method in worldwide. AI offers many advantages over natural mating, such as safety for both mare and stallion, reduces risk of infectious disease transmission, and decrease inconvenience of horse transportation. Adequate short- and long-term preservation of stallion semen is a prerequisite for a successful AI procedure.

Success in assisted reproduction technique (ART) is determined mostly by gamete quality. It is therefore essential to use a sperm preparation technique which is known to select normal, functional spermatozoa from the total sperm population presented in the semen sample. In contrast to human ART, where spermatozoa are often prepared on a density gradient (Bolton and Braude, 1984), processing semen for artificial insemination in livestock rarely involves more than extending the semen with a suitable semen extender, either for subsequent cryopreservation (e.g. bull semen), or for liquid storage (e.g. boar semen). Selection is made for donors whose spermatozoa retain motility and fertilizing capacity during storage. However, the situation in other animals, for example in race horses, is similar to that of human ART where it may not be possible to select the donors on the basis of semen quality, nor to

discard ejaculates considered to be of inferior quality in the expectation of obtaining another semen sample.

6.1 Cooled semen

Cooled semen has been routinely used in horse breeding for about 20 years (Brinsko et al., 2003) since its acceptance increases among breed registries. Furthermore, one of the main causes of the increased use of cooled semen is the high proportion of stallions (20–40%) whose sperm responds poorly to cryopreservation (Vidament et al., 1997). Cooled-stored stallion semen is usually kept at 4-6 °C for 24-48h for maintaining both motility (Varner et al., 1988, 1989; Moran et al., 1992; Magistrini et al., 1992) and fertility (Varner et al., 1989, Jasko et al., 1992). As storage time is increased, the fertility of equine semen declines (Pickett, 1995). Fertility rates achieved by breeding with transported, cooled equine semen are remains variable between stallions (Brinsko et al., 2000) and laboratories (Douglas-Hamilton et al., 1984; Katila, 1997; Squires et al., 1988; Brinsko et al., 2003).

The variable between stallions depends on the quality of raw semen, and also on the composition of their seminal plasma and sperm-plasma membranes (Aurich, 2005). The cooled-sensitive semen can be performed only artificial insemination with fresh semen. This reduces major benefits of artificial insemination in a considerable number of valuable breeding sires. A genetic component has to be taken into account, but in horse breeding, the selection of sires for semen traits has not become routine (Leeb et al., 2005). Under natural mating conditions, the semen is ejaculated directly into the uterine lumen and exposure of spermatozoa to seminal plasma is less than after semen collection (Walton, 1960). Greater concentrations of seminal plasma in cooled-stored stallion semen are detrimental to motility and fertility (Jasko et al., 1992). Detrimental effects of seminal plasma on spermatozoa during cooled-storage may be related to action of specific enzymes. Activity of lipase and glycoprotein could be demonstrated in equine seminal plasma and both impair sperm motility (Carver and Ball, 2002). However, completely remove of seminal plasma does not enhance semen longevity. Therefore 5% to 20% of seminal plasma should be retained after centrifugation (Jasko et al., 1992; Loomis, 2006). Beneficial effects of

seminal plasma may be related to its antioxidant properties that are even enhanced by interaction with the semen extender (Kankofer et al., 2005). Results from studies on the effects of different seminal plasma fractions on longevity of semen during cooled-storage are contradictory. Akcay et al. (2006) revealed that fractionated semen collection and selective addition of either seminal plasma from the sperm-rich or the sperm-poor (post-sperm) fraction to cooled-stored semen resulted in decreased semen motility and membrane integrity when seminal plasma from the sperm-rich fraction was added. This result suggests a possible harmful effect of seminal plasma from the sperm-rich fraction on semen longevity. In contrast with Varner et al. (1987), greater sperm motility occurs in sperm-rich fractions in comparison to total ejaculates after storage for 24 h.

Semen extenders are mainly based either on milk or on egg yolk. Extenders with a composition similar to the original recipe published by Kenney et al. (1975) are most popular and used worldwide. These extenders are inexpensive, easy to prepare, and can be stored in frozen form and result in acceptable fertility rates. Extenders based on egg yolk provide comparable results but are more complicated to process and generally do not result in enhanced semen quality or fertility (Malmgren et al., 1994). Recently, more defined media has been developed to diminish detrimental components also possibly included in complex biological substances like milk or egg yolk, and to develop for a constant composition (reviewed by Aurich, 2005). In stallions with poor semen quality after cooled-storage, different extender media should be tested to find the optimal semen extender combination for this individual sire. As semen extenders stabilizing the sperm-plasma membrane, it was hypothesized that this effect might in turn has negative impacts on semen fertility because the ability to undergo the capacitation and acrosome reaction might be decreased (Pommer et al., 2002). *In vitro* induction of the acrosome reaction was significantly more effective in semen incubated in the presence of a skim milk extender than in the presence of TALP medium. This can be concluded that skim milk extender shortens the lifespan and maintenance of fertility of equine semen by induction of the acrosome reaction. The authors suggested the improvement of extender media decreased this effect (Pommer et al., 2002). However, in this study, semen was incubated at 37°C and this condition is certainly not comparable to storage

at 5°C. Components responsible for the induction of capacitation and the acrosome reaction (e.g. progesterone and calcium) are also present in egg yolk extenders (reviewed by Witte and Schäfer-Somi, 2007). However, at least in cooled-stored canine semen, egg yolk in semen extender did not contribute to premature induction of the acrosome reaction, but a stabilizing effect could be confirmed (Witte et al., in press).

6.2 Frozen semen

Preservation of semen is closely connected with the development of artificial insemination (AI). The first report on attempted semen cryopreservation dates back to the Italian priest and physiologist Spallanzani in 1776 who tried to freeze semen with the help of snow (Saragusty et al., 2007b), and who performed the first successful insemination in a dog in 1785 (Pesch and Hoffmann, 2007). There has been a great development since 1789, when frog spermatozoa were able to fertilize eggs after freezing and thawing (Luyet and Gehenio, 1940). Despite this finding, it was not possible to start using cryopreserved semen in breeding programs until accidentally discovered glycerol act as cryoprotectant for spermatozoa (Polge et al., 1949). Horse is the first domestic animal on which artificial insemination was practiced and equine semen was the first animal semen to be submitted to experimental cooling. The first pregnancy from frozen stallion semen is reported in 1957 (Barker and Gandier, 1957). A dramatic increase in the use of cryopreserved stallion spermatozoa has been seen because of large differences among samples (Squires et al., 2003) as to fertilizing capacity and ability to withstand cryopreservation procedures; subfertility is also common in stallions (Casey et al., 1997) and about 1/3 of the stallions is largely unfreezable (Vidament et al., 1997; Pickett and Amann, 1993).

Processing of semen such as freezing and thawing is detrimental to sperm functionality (Watson, 1995) and usually results in the death of large numbers of spermatozoa. Mostly critical time of freezing spermis the period of extracellular ice crystal formation (Pegg, 2002; Rubei et al., 2004). At this stage exposes the cells to osmotic stresses imposed by the water–solute interactions that arise from extracellular

ice crystallization (de la Cueva et al., 1997). Exposure of cells to the hyperosmotic, still unfrozen solutions results in withdrawal of intracellular water and, consequently, to cell shrinkage and, possibly, to influx of ions as well (Holt, 2000). No evident differences in fertility results have been presented between using programmable freezing compared with freezing in the vapors above a liquid nitrogen surface (Clulow et al., 2008). Although the connection between freezing rate and the packaging method is well known, the choice of thawing method has to be carefully considered (Klug et al., 1992). In Thailand, there is no information available on the freezability of semen in the Thai native crossbred horses.

MATERIALS AND METHODS

Chemicals

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

Experimental animals

Nine stallions consisting of Thai native crossbred horses (T; n = 5) and full size purebred horses (F; n = 4; 1 Holstein, 1 Standardbred and 2 Thoroughbreds) aging between 5 and 12 years were used in the study. The average body weight of T was 210 kg, and F was 500 kg. Full size purebred stallions were active breeding sires and they used in artificial insemination programs, however there is no breeding record for T stallions. 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. Semen was collected monthly using a Missouri-type artificial vagina during January to June 2007. A total of six ejaculates were collected from each stallion.

Experimental procedures

1. Semen and sperm head morphometric characters

Testicular size of each stallion was measured with calipers. Immediately after the collection, macroscopic parameters as color and volume of each ejaculate were evaluated. The gel fraction of semen was removed by filtering through sterile gauze. The pH was performed by pH meter. The osmolality of raw semen from each stallion was routinely determined using a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Germany). Evaluation of the ejaculate traits of sperm concentration (using a hemocytometer) and subjective motility was performed. Total motility and

progressive motility of fresh semen were assessed subjectively under a phase contrast microscope (x100 and x400 magnification, respectively). Morphological abnormalities of spermatozoa were studied in wet preparations made from the formal-saline fixed samples (Hancock, 1957) and evaluated under a phase-contrast microscope at a 1000x magnification. Altogether, 200 spermatozoa were examined for normal and abnormal morphology. To measure head size, the sample slides were stained for 40 min with Harris' haematoxylin (Hidalgo et al., 2005), and permanently mounted before measurement of the sperm head by using the IVOS version 12.3 systems (Hamilton Thorne Research, Beverly, MA, USA). The images were evaluated by morphology software (Oval Metrix Version 4.18). Recognition of sperm and the rejection of other cells depended on the hardware and software specifications. The analysis software settings were: minimum contrast 15, minimum size 1 μm^2, erosion level 7.0, camera gain 50, camera contrast 180, and scale 0.147µm/px. The system recommended objective lens for equine sperm was 60x. The number of morphologically normal sperm heads acquired from each replication for analysis was 200 sperm, so a total number of 800 sperm per animal were analyzed. The computer software reported five basic measurements: the sperm head features of length (L; µm), width (W; µm), elongation [(width/length) x 100; %], perimeter (P; µm) and head area (A; μm^2): and also calculated four non-dimensional derived parameters: ellipticity (e) = (L − W) / (L + W); shape factor 1 (Sf1; rugosity) = $4\pi A/ P^2$; Shape factor 2 (Sf2) = Sf1 x (L/W) and shape factor 3 (Sf3; regularity) = π (L/W) / 4A (Buendía et al.,2002). Membrane integrity of spermatozoa was determined with the hypo-osmotic swelling test (HOS test) (Neild et al., 1999), while live sperm was determined using the eosin-nigrosin staining test (William, 2003).

2. Effects of extenders and glutamine on semen characteristics after cooled storage

a. Preparation of Extenders

The ingredients for each extender (per 1 L) were as follows: 1) Kenney extender (Kenney et al., 1975) consisted of glucose monohydrate (40 g), skim milk

(24 g), penicillin G sodium salt 150,000 IU and streptomycin (crystalline) (0.15 g); 2) Kenney extender supplement with 50 mM L-glutamine (Kenney-G); 3) 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) and 4) INRA extender supplement with 50 mM L-glutamine (INRA-G). After adding all the ingredients, the extender were centrifuged at 10,000 x g for 20 min to remove insoluble egg yolk droplets prior to use. The pH and osmolarity of each extender is presented in Table 1.

Table 1 pH and osmolarity of extenders for cooled semen in stallions.

Extenders	pH (mean ±SE; range)	Osmolarity (mOsm) (mean ±SE; range)
Kenney (E1)	7.10±0.04 (6.80-7.20)[a]	367.4±3.58 (350-380)[a]
Kenney-G (E2)	7.38±0.04 (7.20-7.60)[b]	424.5±8.79 (380-490)[b]
INRA (E3)	7.18±0.10 (6.60-7.70)[a,b]	352.4±5.56 (320-375)[c]
INRA-G (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 with lower-case letters (a, b, c).

b. Cooling procedure

Immediately after dismount, all ejaculates were added E1 at a ratio of 1:3 as a washing media and diluted semen was centrifuged at 400 x g for 10 min. The supernatant was removed and the pellet was diluted to a concentration of 200 x 10^6 sperm / ml in each extender, and was then placed in storage in a mobile refrigerator (5°C). The semen was transported to the laboratory within 3 h after semen collection.

c. Semen analysis

After transportation, cooled semen was evaluated for motility and sperm velocity with a Computer-Assisted Sperm Analysis (CASA) system (IVOS version 12, Hamilton Thorne Research, MA, USA) at 0, 24, 48, 72 and 96 h. Parameters evaluated by CASA were total motile spermatozoa (TMOT, %), progressively motile spermatozoa (PMOT, %), curvilinear velocity (VCL, mm/s), linear velocity (VSL, mm/s), and average path velocity (VAP, mm/s). System parameters were set for equine spermatozoa by using equine motility software (Table 2). A 3-µl drop of each sample was placed on a preheated (37ºC) 2X cell chamber (20 µm 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 motile spermatozoa, while sperm tracks with VAP less than 20µm/s were considered non-motile spermatozoa. Assess for viability, membrane integrity were evaluated, as described above.

Table 2 Settings for the Hamilton Thorne IVOS (CASA, Equine motility software version12.1, Hamilton Thorne Research IVOS, Beverly, MA, USA).

Parameters	Setting
Frame rate (Hz)	60
Frames acquired (no.)	30
Minimum contrast	70
Minimum cell size (pixels)	5
Medium VAP cut off (μm/s) (MVV)	20
Medium thredshold straightness (%)	60
Low VAP cut off (μm/s)	100
Low VSL cut off (μm/s)	0.0
Non-motile head size (pixels)	6
Non-motile head intensity	106
Static size limit (min/max)	0.61/3.38
Static intensity limit (min/max)	0.30/1.96
Static elongation limit (min/max)	20/97

3. Freezability of semen in different extenders

a. Preparation of Extenders

One liter of four different extenders were prepared as follows: 1) INRA extender (E1) (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 (E2); 3) lactose-EDTA extender (E3) (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. 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 3.

Table 3 Osmolarity of freezing extenders.

Freezing Extenders	Osmolarity (mOsm/kg) Mean ±SE (range)
INRA	835.33 ± 35.69 (680-914) [a]
INRA-G	877.67 ± 37.83 (776-989) [a,b]
Lactose-EDTA	967.67 ± 43.04 (806-1101) [b,c]
Lactose-EDTA-G	1023.33 ± 47.64 (833-1189) [c]

Significant differences (P<0.05) in column are indicated with lower-case letters (a, b).

b. Freezing procedure

After evaluation, each semen was 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 (30ºC) for 10 min to remove seminal plasma. Spermatozoa pellets were resuspended in four different freezing extenders (E1-E4) and then placed in a mobile refrigerator (5°C) for equilibration during transport to the laboratory for further processing.

Semen samples were equilibrated at 5°C over a 3 h period and stored in liquid nitrogen at -196°C. Before freezing (BF), semen 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 this study. Frozen semen was thawed in a 37°C water bath for 30 s immediately prior to analysis.

c. Semen analysis

Analyses of motility, viability, and membrane integrity were described as above. To distinguish between different acrosomal conditions, the acrosome was visualized by staining with Fluorescein isothiocyanate conjugated peanut agglutinin (*Arachis hypogaea*, FITC-PNA) and propidium iodide (Cheng et al., 1996). Microscopic examination (Olympus BX50, Japan) was conducted with an oil immersion objective at 1000X magnification. Functional plasma membrane integrity of freeze-thawed semen was determined with 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 test positive spermatozoa (number of spermatozoa with swollen tails per total number of spermatozoa × 100) was recorded for each sample (Jeyendran et al., 1984; Nie and Wenzel, 2001).

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

1. Semen and sperm head morphometric characters

Data are reported as mean ± SEM. Data was analyzed statistically by ANOVA (SPSS/PC+ statistics package, version 11.5 for Windows, SPSS Inc, Chicago, IL, USA). For each morphometric parameter, normality and homogeneity of the data variance distribution were checked by the Kolmogorov-Smirnov and Levene's tests. One way ANOVA producing significant F-values was followed by Fisher's Least Significant Difference (LSD) test for multiple comparisons between

animals. An independent-samples T test was used for comparisons between groups of animals. The coefficient of variation (CV) was calculated for within-animal and between-animal groups (Buendía et al., 2002).

2. Effects of extenders and glutamine on semen characteristics after cooled storage

The percentage of live sperm, motility, velocity and pH between the different extenders during a 96 h period were analyzed by ANOVA (SPSS/PC+ statistics package, version 11.5 for Windows, SPSS Inc, Chicago, IL, USA) followed by LSD tests to identify statistically significant differences between extenders.

3. Freezability of semen in different extenders

Data were analysed statistically by ANOVA (SPSS/PC+ statistics package, version 11.5 for Windows, SPSS Inc, Chicago, IL, USA) 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.

RESULTS AND DISCUSSION

Results

1. Semen and sperm head morphometric characters

1.1 Testicular size

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

1.2 Semen Characteristics

Raw semen characteristics of T and F stallions were presented in Table 4. Ejaculate traits of T were significantly 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. Sperm concentrations ranges were 55 - 2,655 x 10^6 cells/ ml and 105 - 740 x 10^6 cells/ml, and percentages of live sperm ranges were 77.2 ± 2.2 to 81.7 ± 2.0% and 73.5 ± 3.35 to 76.4 ± 3.0%, in T and F, respectively.

Table 4 Ejaculate traits of Thai native crossbred (T; n=30) and purebred (F; n=24) stallions (mean ± SEM).

Parameters	T	F
Total volume (ml)	64.0±5.2 [a]	48.6±3.2 [b]
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 sperm ($x10^9$/ejaculate)	10.5±0.7 [a]	17.3±1.7 [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]
Lived 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/kg)	329.3±3.8 [a]	314.6±2.2 [b]

Significant differences ($P<0.05$) in line are indicated with lower-case letters (a, b).

Sperm morphology of individual ejaculate samples of T and F stallions were presented in Table 5. All categories of sperm morphology were varied among stallions. Considering the average of T and F groups, there were not significantly difference ($P>0.05$) in percentage of normal morphology sperm, however, percentages of each types of morphologically abnormal sperm were significantly differences ($P<0.05$). Overall, sperm with an abnormal midpiece had the highest proportion of morphologically abnormal sperm in both T and F stallions. Morphologically normal and abnormal sperm of T stained with eosin/negrosin and detected by light microscopy were presented in Figure 4 and evaluated by scanning electron microscopy were presented in Figure 5.

Table 5 Percentage of sperm morphology of Thai native crossbred (T; T1 – T5) and purebred (F; F1– Standard-bred; F2– Warm-blood; F3 and F4– Thoroughbred) stallions (mean ± SEM).

Stallions	Normal morphology	Abnormal head	Abnormal midpiece	Abnormal tail	Proximal cytoplasmic droplet	Distal cytoplasmic droplet
T1	48.9 ± 1.8 [a]	8.9 ± 0.7 [a]	12.7 ± 0.8 [a]	1.1 ± 0.2 [a]	10.9 ± 0.8 [a]	17.6 ±1.4 [a]
T2	43.8 ± 2.4 [a,c]	14.2 ±1.5 [b]	29.3 ±1.6 [b,c]	1.8 ± 0.4 [a,b]	8.2 ± 1.1 [a,b]	2.9 ± 0.5 [b]
T3	60.8 ± 2.7 [b]	11.5 ± 1.4 [a,b]	16.2 ±1.6 [a,c]	1.4 ± 0.3 [a]	4.9 ±1.2 [b,c]	5.2 ± 1.1 [b,c]
T4	38.3 ± 2.7 [c]	9.2 ±1.1 [a]	10.3 ± 0.8 [a]	2.6 ± 0.5 [b,c]	28.9 ± 2.2 [d]	10.6 ± 1.7 [d]
T5	58.3 ± 2.2 [b]	9.1 ±1.4 [a]	19.8 ± 1.6 [a]	1.6 ± 0.3 [a,c]	3.3 ± 0.5 [c]	7.9 ± 1.1 [c,d]
Mean T	49.7 ± 1.3	10.2 ± 0.5 [*]	16.5 ± 0.8 [*]	1.6 ± 0.1 [*]	11.4 ± 1.0 [*]	10.5 ± 0.8 [*]
(range)	(19 – 75)	(2 – 33)	(3 – 41)	(0 – 7)	(0 – 42)	(0 – 36)
F1	40.1 ± 3.6 [a]	13.8 ± 1.3 [a]	31.6 ± 3.4 [a,b]	5.4 ± 1.3 [a]	3.5 ± 0.8 [a]	5.5 ± 2.0
F2	61.5 ± 2.7 [b]	10.2 ± 2.1 [a]	14.3 ± 2.5 [a]	2.7 ± 1.0 [b]	5.7 ± 1.2 [a,b]	5.5 ± 2.1
F3	35.9 ± 3.9 [a]	17.3 ± 2.8 [b]	29.9 ± 3.2 [b]	0.3 ± 0.2 [c]	9.3 ± 2.0 [b]	7.4 ± 1.5
F4	58.0 ± 5.1 [b]	11.7 ± 1.6 [a]	17.3 ± 2.8 [a]	4.4 ± 1.1 [a,b]	3.9 ± 0.8 [a,b]	4.7 ± 1.3
Mean F	48.1 ± 2.8	13.4 ± 1.1	23.9 ± 2.1	3.3 ± 0.6	5.5 ± 0.7	5.8 ± 0.9
(range)	(21 – 72)	(4 – 23)	(9 – 43)	(0 – 13)	(1 – 17)	(0 – 16)

Significant differences (P<0.05) within T or F are indicated with lower-case letters (a, b, c, d).

Significant differences (P<0.05) between T and F are indicated by * in superscript

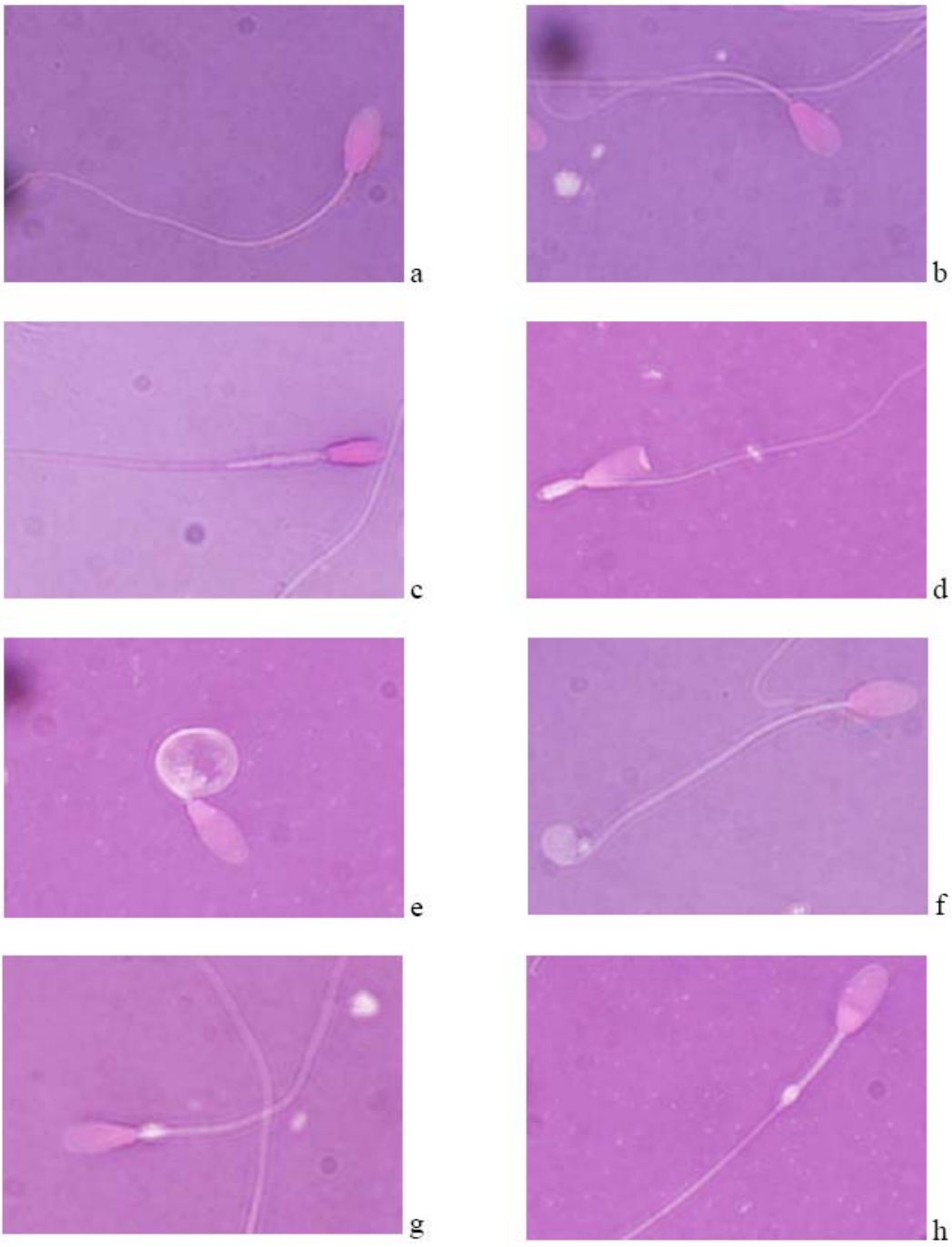


Figure 4 Light microscopy of Thai native crossbred stallion sperm after eosin / nigrosin staining; a – normal sperm; b – pear shaped head; c – narrow head with abnormal midpiece; d – acrosomal defect with abnormal midpiece; e – coiled tail below head; f – terminal coiled tail; g – proximal cytoplasmic droplet and h – distal cytoplasmic droplet (1000x).

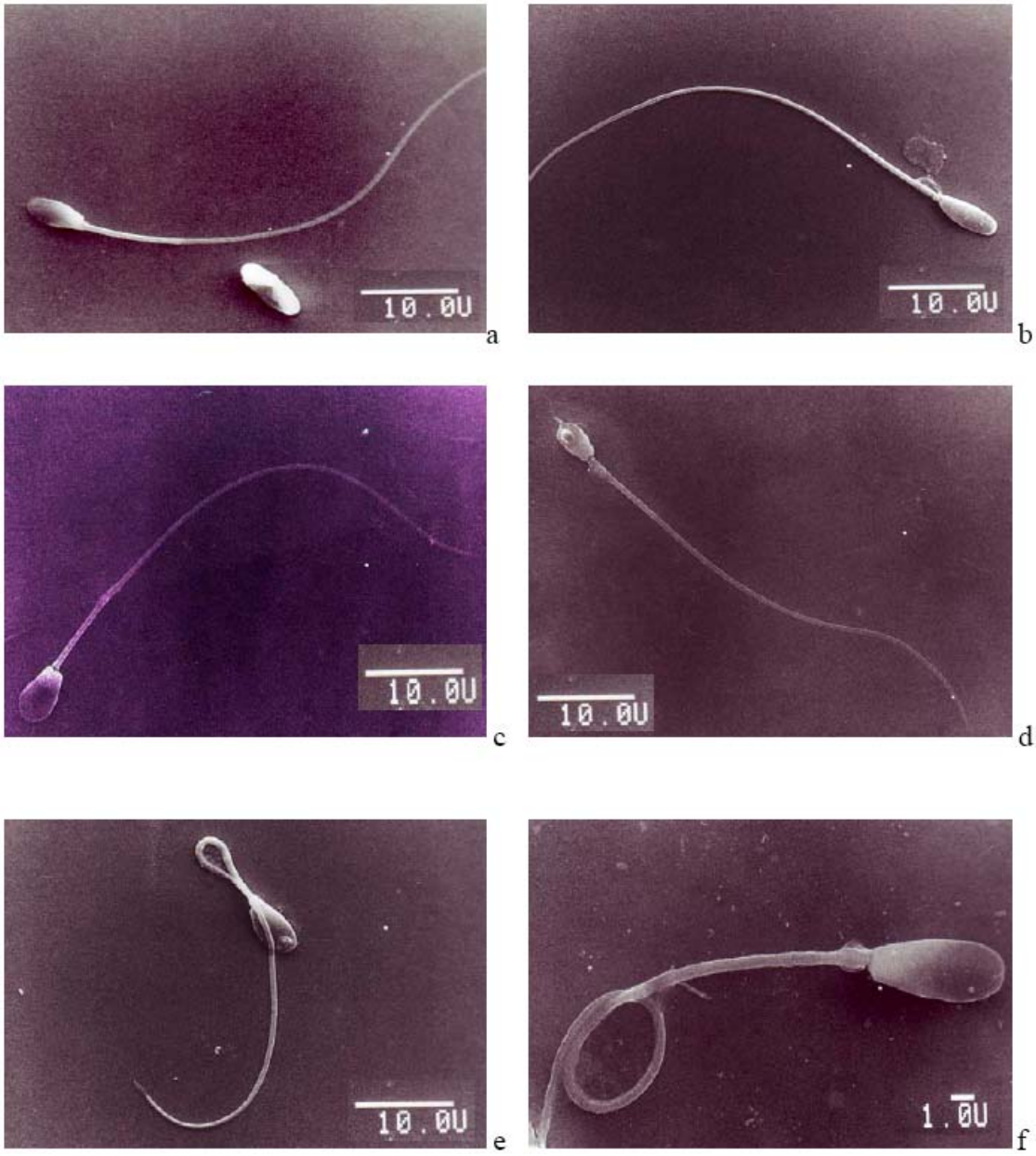


Figure 5 Scanning electron microscopy of Thai native crossbred stallion sperm; a – normal sperm (top) and loose narrow head (below); b – narrow head with proximal cytoplasmic droplet; c – round head; d – acrosomal defect; e – acrosomal defect and bent tail and f – proximal cytoplasmic droplet with coiled tail (a-e – 2000x, bar=10 micrometers; f – 3600x, bar=1 micrometers).

Parameters for the morphometric characteristics of normal sperm heads are summarized in Table 6. There were differences (P<0.05) between individual stallions

in both T and F. Comparisons between the mean value of each characteristic of T and F normal sperm heads found that the dimensions for length, elongation, perimeter and area were higher in T than those of in F ($P<0.05$). The coefficient of variation values (CVs) of normal sperm head morphometric characteristics were quite low, ranging from 3.3 (shape factor 1 and perimeter) to 8.5 (elongation) and 3.4 (shape factor 1) to 8.8 (elongation) in T and F stallions, respectively (in Table 6). Within-stallion group analysis indicated that the CVs in both T and F sperm were also low (Table 7), while analysis between-groups found that the CVs were quite high for perimeter (19.2), area (19.8), length (28.9), shape factor 1 (25.8) and shape factor 3 (32.0) for T stallions, but only shape factor 1 (26.1) for F sperm. The sperm head parameters with a low within-animal CVs and a high between-animal CVs were perimeter (2.9, 19.1), shape factor 1 (2.6, 25.8) and shape factor 3 (3.8, 32.0) for T and only shape factor 1 (2.9, 26.1) for F sperm.

Table 6 Normal sperm head morphometry of Thai native crossbred (T; T1 – T5) and purebred (F; F1 – Standardbred; F2 – Warmblood; F3 and F4 – Thoroughbred) stallions (mean ± SEM).

Stallion	Length (μm)	Width (μm)	Elongation (%)	Perimeter (μm)	Area (μm^2)	Ellipticity	Shape factor (SF1)	Shape factor (Sf2)	Shape factor (Sf3)
T1	6.24±0.03^{a}	2.99±0.02^{a}	0.35±0.002^{a}	15.98±0.04^{a}	15.88±0.09^{a,b}	2.09±0.01^{a}	0.78±0.002^{a}	1.63±0.006^{a}	0.92±0.004^{a}
T2	6.07±0.02^{b}	3.19±0.02^{b}	0.31±0.003^{b}	15.73±0.04^{b}	16.23±0.09^{b}	1.90±0.01^{b}	0.82±0.002^{b}	1.57±0.007^{b}	0.94±0.003^{b}
T3	6.33±0.02^{c}	3.19±0.01^{b}	0.33±0.002^{c}	16.10±0.04^{a}	16.67±0.08^{c}	1.98±0.01^{c}	0.81±0.001^{c}	1.60±0.005^{c}	0.95±0.002^{b}
T4	6.18±0.02^{ab}	3.11±0.02^{c}	0.33±0.003^{c}	15.76±0.04^{b}	15.90±0.09^{a,b}	1.99±0.01^{c}	0.80±0.002^{c}	1.60±0.008^{b,c}	0.95±0.004^{b}
T5	6.18±0.02^{a}	3.08±0.01^{c}	0.33±0.002c	15.71±0.03^{b}	15.77±0.07^{a}	2.02±0.01^{c}	0.80±0.002^{c}	1.61±0.005^{a,c}	0.95±0.002^{b}
Mean T	6.22±0.01*	3.09±0.0*	0.34±0.001*	15.88±0.02*	16.09±0.03*	2.01±0.005*	0.80±0.001*	1.61±0.003*	0.94±0.002*
% CV (T)	4.5	6.2	8.5	3.3	6.6	6.5	3.3	4.6	4.2
Percentile 25/75	6.00/6.40	2.90/3.20	0.31/0.35	15.60/16.20	15.40/16.80	1.94/2.10	0.79/0.82	1.56/1.66	0.92/0.97

Table 6 (Continued)

Stallion	Length (μm)	Width (μm)	Elongation (%)	Perimeter (μm)	Area (μm^2)	Ellipticity	Shape factor (SF1)	Shape factor (Sf2)	Shape factor (Sf3)
F1	5.94±0.02^{a}	2.99±0.01^{a}	0.33±0.002^{a}	15.14±0.03^{a}	14.83±0.07^{a}	1.99±0.07^{a}	0.81±0.001^{a}	1.62±0.004^{a}	0.94±0.002^{a}
F2	5.98±0.01^{a}	2.92±0.01^{b}	0.34±0.002^{b}	14.89±0.03^{b}	13.97±0.05^{b}	2.06±0.01^{b}	0.79±0.002^{b}	1.62±0.004^{a}	0.98±0.001^{b}
F3	6.15±0.03^{b}	2.92±0.01^{b}	0.36±0.002^{c}	15.24±0.05^{a}	14.41±0.09^{c}	2.11±0.01^{c}	0.78±0.002^{c}	1.64±0.006^{b}	0.98±0.003^{bc}
F4	5.85±0.01^{c}	2.80±0.01^{c}	0.35±0.001^{c}	14.55±0.03^{c}	13.20±0.05^{d}	2.09±0.01^{c}	0.79±0.001^{c}	1.64±0.004^{b}	0.98±0.002^{c}
Mean F	5.94±0.08	2.89±0.01	0.35±0.001	14.88±0.02	13.90±0.04	2.06±0.004	0.79±0.001	1.63±0.002	0.97±0.001
% CV (F)	5	6.5	8.8	4	8.5	6.8	3.4	4.7	3.5
Percentile 25/75	5.70/6.20	2.80/3.10	0.33/0.36	14.50/15.20	13.10/14.70	1.97/2.14	0.77/0.81	1.58/1.68	0.95/0.99

Significant differences (P<0.05) within T or F are indicated with lower-case letters (a, b, c, d). Significant differences (P<0.05) between T and F are indicated by * in superscript

Table 7 Within-animal and between-animal coefficient of variation values (CV) of normal sperm head morphometry in Thai native crossbred (T) and purebred (F) stallions.

Stallion	Length	Width	Elongation	Perimeter	Area	Ellipticity	Shape factor (SF1)	Shape factor (Sf2)	Shape factor (Sf3)
T									
Within-animal CV	4.0	5.4	7.5	2.9	5.9	5.6	2.6	4.3	3.8
Between-animal CV	28.9	17.6	11.2	19.2	19.8	13.9	25.8	13.2	32.0
F									
Within-animal CV	4.8	5.8	8.1	3.6	7.2	6.3	2.9	4.5	3.2
Between-animal CV	5.3	11.9	17.6	1.6	4.0	16.0	26.1	14.2	7.2

2. Effects of extenders and glutamine on semen characteristics after cooled storage

2.1 Comparisons between extenders

Mean percentages of TMOT, PMOT, HOST positive and live sperm after dilution (0 h) did not differ among extenders in T and F ($P>0.05$; Figure 6). In T, the difference of TMOT among 4 extenders was clearly observed ($P<0.05$; Figure 6A) at 48 h to 96 h of storage, INRA extender tended to maintain TMOT better than Kenney, Kenney-G and INRA-G extender; however, at 96 h of storage, Kenney extender had more TMOT than the others ($P<0.05$). TMOT in F was not difference among extenders in all periods of storage ($P>0.05$; Figure 6B). The differences of percentages of PMOT and HOST positive sperm in T among extenders were observed at 24 h to 96 h of storage ($P<0.05$; Figure 6C, 6E). Similar to TMOT, T cooled semen in INRA extender tended to maintain PMOT better than Kenney, Kenney-G and INRA -G extenders. However, semen diluted in Kenney extender at 96 h had more PMOT compared to the other extenders ($P<0.05$). The percentages of PMOT and HOST positive sperm in F (Figure 6D, 6F) were not difference among extenders in all periods ($P>0.05$).

The percentage of live sperm in T cooled semen was difference ($P<0.05$; Figure 6G) at 48 h and 96 h of storage, and in INRA and INRA -G tended to be higher than that of Kenney and Kenney-G, while in F there was no difference among extenders ($P>0.05$; Figure 6H). For sperm velocity parameters, VCL, VSL and VAP did not differ (0 h) with extenders in both T and F groups (Table 8). In T, VAP of diluted semen was difference among extenders ($P<0.05$) at 24 h to 96 h of storage, and Kenney and INRA extender had more VAP ($P<0.05$) after storage for 24 h, 48 h and 72 h; however, Kenney extender had more VAP at 96 h of storage than in INRA extender ($P<0.05$).

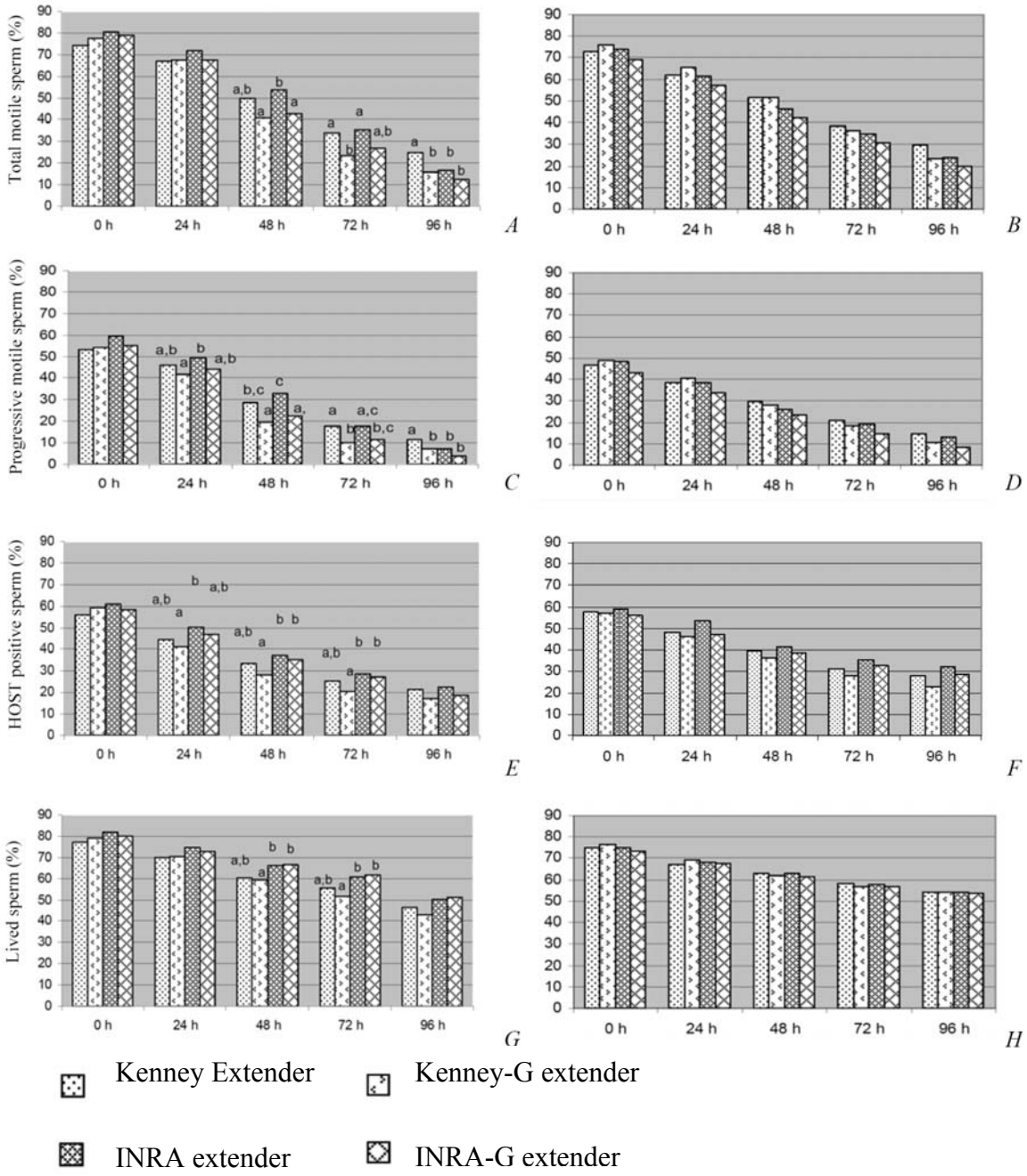


Figure 6 Semen characteristics of Thai native crossbred (*A*, *C*, *E* and *G*) and purebred horses (*B*, *D*, *F* and *H*) after storage at 5°C in different extenders for up to 96 h.Within the same time interval, significant differences ($P<0.05$) among extenders are indicated with lower-case letters (a, b, c).

Table 8 Average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL) of Thai native crossbred (T) and purebred stallion (F) sperm after storage at 5°C in different extenders.

Sperm velocity	Storage time (h)*	T				F			
		Kenney	Kenney-G	INRA	INRA -G	Kenney	Kenney-G	INRA	INRA -G
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 ± 2.7[a]	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 ± 4.8 [a,c]

Table 8 (Continued)

Sperm velocity	Storage time (h)*	T				F			
		Kenney	Kenney-G	INRA	INRA -G	Kenney	Kenney-G	INRA	INRA -G
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 with lower-case letters (a, b, c).

For VAP in F, cooled semen was not difference among extenders (P> 0.05) leading up to 48 h of storage, and at 72 h and 96 h, INRA-G tended to be lower VAP than in Kenney, Kenney-G, and INRA extender. VCL and VSL in T was also difference among extenders (P<0.05) from 24 h of storage. Those diluted with Kenney and INRA tended to be higher VAP in than Kenney-G and INRA-G from 24 h to 72 h of storage, but at 96 h, Kenney extender had more VCL and VSL than in INRA (P<0.05). In F, VCL was not difference at 24 h to 72 h of storage (P>0.05) among extenders, and at 96 h, INRA-G tended to be lower than the others. VSL of F was not difference from 24 h to 48 h of storage (P>0.05) among extenders, and at 72 h and 96 h, INRA-G extender tended to be lower VSL than in Kenney, Kenney-G, and INRA extender.

2.2 Comparisons within cooled extenders

Period of cooled storage significantly decrease in semen characteristics in each extender was observed (Table 9). TMOT and live sperm in both T and F stallion were decreased (P<0.05) at 48 h in all extenders examined. The PMOT of T semen in Kenney and INRA were decreased (P<0.05) at 48 h, but only at 24 h when semen were diluted in Kenney-G and INRA-G, while the PMOT of F diluted semen decreased (P<0.05) at 48 h in all extenders. Percentages of HOST positive sperm decreased (P<0.05) at 24 h, except those diluted in INRA in both T and F, which were decreased (P<0.05) at 48 h. T sperm velocities (VAP, VSL and VCL) were decreased (P<0.05) at 48-72 h in Kenney and 24-48 h in other extenders. Sperm velocities of F were decreased (P<0.05) at 24-48 h in Kenney, 48-96 h in Kenney-G, 48-72 h in INRA, and 24-96 h in INRA-G. Considering all parameters of semen characteristics, INRA maintained for longer than other extenders for both T and F after cooled storage.

Table 9 Periods of cooled storage at serial time in which semen characteristics of Thai native crossbred (T) and purebred (F) horses were significantly decreased (P<0.05), compared to fresh diluted semen.

parameters	T (h)				F (h)			
	Kenney	Kenney-G	INRA	INRA-G	Kenney	Kenney-G	INRA	INRA-G
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 significantly changed

The pH of diluted semen in different extenders were not significantly changed ($p>0.05$) during the period of storage (Table 10).

Table 10 pH of diluted semen of Thai native crossbred (T) and purebred stallion (F) sperm after storage at 5°C in different extenders.

Stallion group	Storage time (h)	Extenders			
		Kenney	Kenney-G	INRA	INRA -G
T	0	8.11±0.07	8.11±0.06	7.48±0.09	7.45±0.10
	24	8.17±0.06	8.13±0.06	7.51±0.09	7.53±0.10
	48	8.21±0.06	8.17±0.06	7.52±0.08	7.54±0.09
	72	8.23±0.06	8.18±0.06	7.52±0.07	7.55±0.09
	96	8.28±0.07	8.11±0.07	7.50±0.08	7.52±0.08
F	0	8.13±0.06	8.07±0.07	7.78±0.11	7.76±0.12
	24	8.15±0.07	8.10±0.07	7.78±0.11	7.78±0.10
	48	8.17±0.05	8.14±0.06	7.79±0.11	7.80±0.11
	72	8.24±0.05	8.21±0.05	7.76±0.10	7.78±0.11

3. Freezability of semen in different extenders

3.1 Effect of extenders on frozen semen characteristics

Motility, progressive motility, intact membrane- acrosome and positive membrane integrity before freezing (BF) and post thawed (PT) in T and F semen were shown in Figure 7. The mean percentage (± SEM.) of total motility, progressive motility, intact membrane-acrosome and positive membrane integrity BF semen in T and F were 78.91±1.09, 73.78±2.09; 50.99±1.05, 46.08±1.70; 63.0±1.65, 59.43±2.34; 61.16±0.70 and 57.23±0.48 respectively. All BF semen parameters were not significantly difference in INRA extender compared with Lactose-EDTA extender,

except total motility of T semen. The motility, intact membrane-acrosome and positive membrane integrity of post-thawed semen were reduced after freezing (P<0.05) in both T and F. Total motility and progressive motility of post-thawed T semen were significantly higher in Lactose-EDTA extender than INRA, INRA with glutamine and Lactose-EDTA with glutamine. Although, intact membrane- acrosome and positive membrane integrity of post-thawed T semen, and motility, intact membrane- acrosome and positive membrane integrity of post-thawed F semen were not significantly difference, Lactose-EDTA extender tended to maintain these characteristics better than other extenders.

Motion velocity (VAP, VSL and VCL) of semen before freezing and post-thawed in T and F were shown in Table11. Before freezing, VAP and VCL of T semen, and VCL of F semen in INRA extender were significant difference (P<0.05) compared with Lactose-EDTA extender, however they were not difference (P>0.05) from extenders added with glutamine compared with extender without glutamine. The VAP, VSL and VCL of post-thawed semen were reduced (P<0.05) in both T and F. The post-thawed VAP and VCL of F semen were different (P<0.05) in INRA extender compared with Lacose-EDTA extender.

Adding 50 mM L-glutamine into extender had no adverse effect on motility, velocity parameters, membrane integrity and acrosome integrity. Moreover, all characteristics of post- thawed semen in extender with 50 mM L-glutamine were tended to lower than extender without 50 mM L-glutamine, such as, VAP, VSL and VCL of post thawed semen in Lactose-EDTA with glutamine or the motility, progressive motility, membrane integrity and acrosome integrity of post thawed semen in INRA added with glutamine.

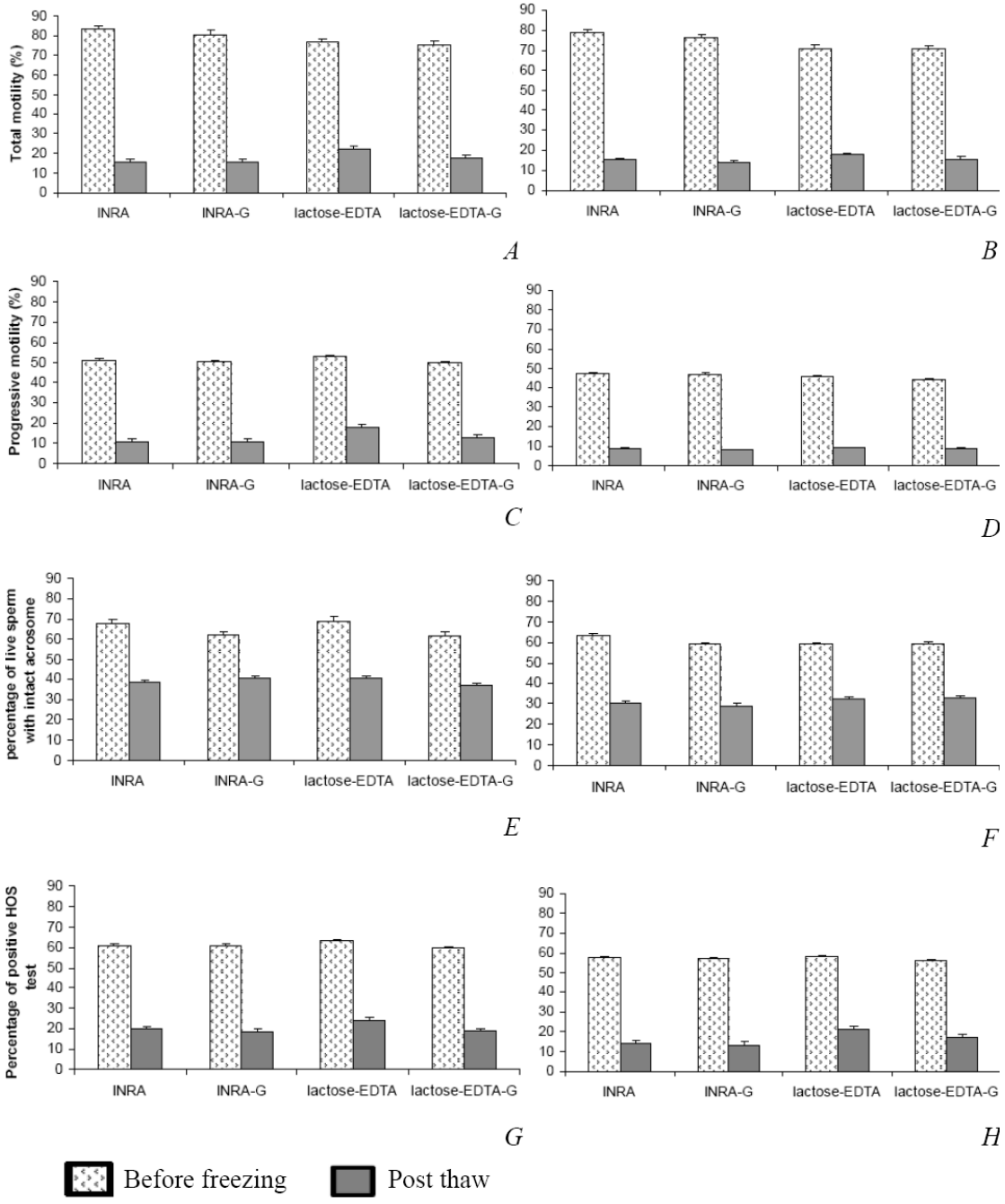


Figure 7 Total motility, progressive motility and acrosome integrity of T and F sperm in different extenders before freezing and post-thawed. Within the post-thawed period, significant differences ($P< 0.05$) between extenders are indicated with lower-case letters (a, b, c).

Table 11 Semen motion characteristics of Thai native crossbred (T) and full-sized purebred stallion sperm (F) in different extenders before freezing (BF) and post-thawed (PT).

Motion Velocity	Extender	T		F	
		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]
	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
	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]
	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).

3.2 freezability of the semen

The number of suitable ejaculates for freezing was shown in Table 12. Only one purebred stallion and two Thai native crossbred stallions were ejaculated suitable semen for freezing, which were contained sperm motility >50% and post-thawed motility ≥30%. One T preferred both extenders INRA and Lactose-EDTA (3/5

and 2/5 ejaculates passed protocol), and one T preferred Lactose-EDTA (2/5 ejaculates passed protocol). Only one of four F stallions preferred Lactose-EDTA (2/5 ejaculates passed protocol). Freezability of T semen samples was 40% (8/20), and that of the F semen sample was 25% (5/20). The numbers of good semen passed freezing protocol were higher when they were frozen by using Lactose-EDTA and INRA extenders.

Table 12 Freezability of T and F semen samples frozen by using INRA, INRA-G, Lactose-EDTA and Lactose-EDTA-G extenders.

Stallions	Number of ejaculates	Number of >50% motility ejaculates	Number of ≥30% motility post-thawed ejaculates				
			INRA	INRA -G	Lactose-EDTA	Lactose-EDTA -G	Total
F1	6	5	1/5	1/5	2/5	1/5	5/20
F2	6	2	0	0	0	0	0/20
F3	6	5	1/5	0	0	0	1/20
F4	5	4	0	0	0	0	0/20
T1	6	5	0	0	2/5	0	2/20
T2	6	5	1/5	0	1/5	0	2/20
T3	6	5	1/5	0	1/5	1/5	3/20
T4	6	5	3/5	2/5	2/5	1/5	8/20
T5	6	3	0	0	0	0	0/20

DISCUSSION

1. Semen and sperm head morphometric characters

Raw semen quality of T was better than that of F stallions in terms of TMOT and PMOT. 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/ejaculate than F. The mean semen pH of both T and F were in normal range (pH 7.35 - 7.7) (Davies-Morel, 1999). The higher osmolarity of T semen might be due to the greater volume of gel in this breed 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 - 310 mOsm) (Davies-Morel, 1999).

The percentage of each type of sperm morphology was variable for individual T stallions and also for F stallions. Percentage variation in semen quality between stallions was found both within breeds (Dowsett and Knott, 1996) and between breeds (Dowsett and Knott, 1996; Pickett, 1993; Kavak et al., 2004). The percentages morphologically normal sperm for T and F stallion were 49.7 % and 48.1 %, respectively. These mean values correspond to earlier findings by Long et al. (1993) (47.5%) and Pickett (1993) (51%), but are lower than the findings by 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. The overall percentage of morphologically normal sperm of T and F stallions were closed to 50% for average normal morphologically normal sperm in stallions (Card, 2005), 43.4% for acceptable fertility (Neild et al., 2000), but lower than the value for fertile stallions (75.5 %; Pesch et al., 2006). For morphologically abnormal sperm, high percentages of abnormal midpiece sperm were observed in both T (16.5 %) and F stallions (23.9 %). The high proportion of sperm with midpiece abnormality (25.3%) was also reported by Voss et al. (1981), however,

the stallions could still give the acceptable pregnancy rates of 62.5 to 91.7% (Voss et al., 1981). The cause of the abnormal midpiece sperm may due to a response to environmental effect as seen in bull scrotal insulation study (Brito et al., 2003; Barth and Oko, 1989). In addition to impaired epididymal function, insults to spermaocytes or spermatids could also result in an increase in cytoplasmic droplet in bulls (Brito et al., 2003). There were higher percentages of both proximal and distal cytoplasmic droplets in T than F stallions. However, these types of abnormality may (Pesch et al., 2006; Jasko et al., 1990) or may not (Voss et al., 1981; Love et al., 2000) affect stallion fertility. A greater impact of sperm abnormality on fertility could be caused by an abnormal head, especially a detached acrosome, as well as by the structural integrity of the plasma membrane and other important organelles, which could be determined under transmission electron microscopy (Pesch et al., 2006; Veeramachaneni et al., 2006).

All stallions studied here had more than one billion morphologically-normal, progressively- motile sperm per ejaculate. On this basis, it might be assumed that all T stallions were fertile, compared to the proven-fertile F stallions. However, their actual fertility or pregnancy rates were required for further studies to be determined.

The morphometric characters of normal sperm heads were significantly differences within individual T or F stallions, and between T and F stallions. Difference in sperm head size within breed was reported in Warm-blood (Ball and Mohammed, 1995) and Spanish thoroughbred stallions (Hildalgo et al., 2008), and the difference sperm head between breed was observed in Arabian, Warm-blood, Thoroughbred and Morgan stallions (Ball and Mohammed, 1995). The results of this study confirm that there is a variation in the normal sperm head characteristics both within- and between- breeds of stallions, including the Thai native crossbred. In general, sperm heads in T group were larger and rounder than that of F group. This characteristic may render T sperm more sensitive to types of extenders employed in cooled storage semen (Phetudomsinsuk et al., 2008a). The cooling rate for stallion sperm can affect sperm motility during cooled storage (Varner et al., 1988; Moran et al., 1992). The different size of sperm may result in different cooling rates under the same procedure. There was also an evidence that the ‘smaller’ and ‘more elongated’

sperm head, the better the sperm's cryoresistance (Esteso et al., 2006). Thus, the sperm head size or shape may be an aspect to consider for improving cooled storage and cryopreservation protocols.

Compared to previous studies of using Harris' hematoxylin staining technique, almost all the morphometric parameters of F sperm head in this study were higher than those of sub-fertile stallions of unmentioned breeds (Casey et al., 1997) and Spanish Thoroughbred stallions (Hildalgo et al., 2005) respectively, namely: length (5.94 μm, 5.77 μm, 5.67 μm); width (2.89 μm, 2.89 μm, 2.85 μm); perimeter (14.88 μm, 14.59 μm, 15.00 μm) and area (13.90 μm^2, 12.66 μm^2, 13.42 μm^2), respectively. Nevertheless, some parameters in this study were lower than the study with unmentioned breed of stallions (Gravance et al., 1997) which reported values of length 6.01 μm, width 2.97 μm, perimeter 15.64 μm and area 13.48 μm^2.

Within-animal group percentage CVs for all head morphometric parameters were low for sperm in both T (from 2.6 for shape factor 1 to 7.5 for elongation) and F (from 2.9 for shape factor 1 to 8.1 for elongation), which reflected a homogeneous sperm population within individuals. The results were similar to those studies with the unmentioned breed of stallion (from 5.8 for length and perimeter to 8.8 for area) (Gravance et al., 1997), ram (from 4.36 for length to 7.33 for shape factor 1) (Sancho et al., 1998), boar (from 2.93 for rugosity or shape factor 1 to 9.38 for elongation) (García-Herreros et al., 2006), but lower than those of cynomolgus monkey (from 2.90 for shape factor 1 to 16.39 for ellipticity) (Gago et al., 1999), or alpaca (from 4.7 for shape factor 1 to 17.8 for ellipticity) (Buendía et al., 2002)

Between-animal group percentage CVs were relatively higher in the sperm of T (from 11.2 for elongation to 32.0 for shape factor 3) than F (from 1.6 for perimeter to 26.1 for shape factor 1). Identification of individual animals could be considered from parameters with low within-animal and high between-animal CVs. Suitable parameters using this process were perimeter (5.42 versus 35.45) and shape factor 1 (7.33 versus 36.98) for ram (Sancho et al, 1998), perimeter (2.69 versus 14.43), shape factor 1 (rugosity; 2.93 versus 26.26) and shape factor 3 (regularity; 2.45 versus 16.31) for boar (García-Herreros et al., 2006). The present study suggested that

perimeter (2.9 versus 19.2), shape factor 1 (2.6 versus 25.8) and shape factor 3 (3.8 versus 32.0) for T and shape factor 1 (2.9 versus 26.1) for F sperm were suitable parameters. The crossbred genetic background may result in more variation of sperm dimensions with between-animal comparisons compared to the purebred.

2. Effects of extenders and glutamine on semen characteristics after cooled storage

During cooled storage, the quality of T semen in the extenders that were examined was divergent from 24 h of storage, which differed from the experience with F semen. The characteristics of F semen were not significantly different among extenders during the period leading up to 96 h. This may indicate that T sperm was more sensitive or less tolerant to various categories of extenders than F sperm. INRA tended to provide a better quality of stored semen than other extenders in both T and F. The superior results of INRA, compared to EZ-mixin, the ingredients of which are similar to Kenney extender, 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 (Kenney et al., 1975).

The percentage of HOS test positive sperm persisted longer in INRA than in Kenney extender in both T and F semen (Table8), which could be an evidence of the superiority of INRA. However, the extender pH and osmolarity also affected semen characteristics. It has been suggested that to optimize sperm motility and viability, the extender pH should be 6.6-7.2 (Wendt et al., 2002), and osmolarity is 300-325 mOsm (Pommer et al., 2002). The higher osmolarity of Kenney extender may be involved in this inferior result (Table1). Furthermore, high osmolarity could also be responsible for the adverse effect of extenders supplemented with glutamine (Kenney-G and INRA-G) on PMOT, HOST positive sperm and sperm velocities of T semen, and on HOS test positive sperm of F semen (Table 8). Thus, supplementing extenders with 50 mM glutamine did not increase sperm viability, even being 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 extenders. Various concentrations of adding glutamine to extenders and also adjusting extender osmolarity closed to seminal osmolarity should be considerd. An appropriate glutamine concentration may have some effects on increasing in sperm motility.

3. Freezability of semen in different extenders

Our study revealed that the use of glycerol as a cryoprotectant improved T semen cryopreservation, and 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 significant difference ($P>0.05$) between either extender. The harmful of adding glutamine in extenders may be due to high osmolarity in extender causing adverse effect on semen quality.

T semen quality after a 3h equilibration in glycerol-containing extenders was significant difference ($P<0.05$) between INRA and Lactose-EDTA extenders with regard to the number of total motile sperm; No difference ($P>0.05$) 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., 2008a). The morphology of T sperm heads was larger and rounder than F sperm heads (Phetudomsinsuk et al., 2008b), suggesting a possible reason for differences in sensitivity to extenders.

In general, the average percentage of post-thaw motile sperm in the INRA extender was considerably lower in both T (15.9%) and F (15.6%) sperm compared to previously observed results (34.7%, Trimeche et al., 1999 and 38.3%, Khlifaoui et al., 2005, respectively). 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) resulting in no significant difference. Thus, the lower percentage observed in our study may not necessarily be due to the glycerol concentration used. Clulow and colleagues (2008) recently demonstrated good sperm freezability in selected stallions, with a significantly higher ($P<0.05$) 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 extender was lower for both T (22.5%) and F (17.7%) sperm compared to the previously observed at 38-41% motility(Cochran et al., 1984; Cristanelli et al., 1984).

In this study 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 (Trimeche et al., 1999; Khlifaoui et al., 2005). The beneficial effect of 50 mM 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 different glycerol concentration 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, and both extenders were 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., 2007a). Thus, the most effective freezing extender for stallion semen remains inconclusive. However, in this study 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 ($P>0.05$). 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 milk–egg yolk with 3% glycerol, Wilhelm et al., 1996; 34.5% using INRA82 with 5% glycerol, Landim-Alvarenga et al., 2005; and 40% using either skim milk–egg 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. Both were lower than previously-reported values (30.2% using Lactose-EDTA with 4% glycerol: 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., 2007a).

The less adverse effect of cryopreservation on sperm plasma membrane integrity (live sperm with intact acrosomes) than motility and membrane function (HOS test positive) was observed in this study. Sperm motility was also more affected 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 11). The Lactose-EDTA-G extender had the highest osmolarity and lowest motion velocity values. Thus, osmolarity of the extender could affect not only the percentage of sperm motility (Ball and Vo, 2001), but also on its motion characteristics. Nonetheless, all parameters displayed significant decreases after a freeze-thaw cycle, similar to previous reports (Blach et al., 1989; Wilhelm et al., 1996).

The freezability (>30% post-thaw motility) results listed in Table 12 indicated that only two of five T stallion sperm samples could be used for semen cryopreservation. One T stallion preferred both INRA and Lactose-EDTA extender (3/5 and 2/5 ejaculates passed), and one T stallion preferred Lactose-EDTA extender (2/5 ejaculates passed). For F stallions, only one out of 4 stallions preferred Lactose-EDTA extender (2/5 ejaculates passed). However, the use of other freezing protocols and cryoprotectants may improve post-thaw semen quality and should be further investigated.

CONCLUSION

The results presented here indicated that the semen quality of T is better than F stallions in term of sperm motility. Variations in percentages of normal and abnormal morphological characteristics of sperm in individual Thai native crossbred stallions were similar to those of purebred stallions. Furthermore, the morphometric characteristics of normal sperm heads were also variable between stallions. Sperm heads of T were larger and rounder than those of purebred stallions. Perimeter, shape factor 1 and shape factor 3 of sperm could be used as parameters for the identification of individual T stallions.

For cooled storage of semen, INRA extender tended to provide better sperm motility and membrane integrity of stored semen than Kenney extender in both T and F semen. Overall, cooled storing of semen maintained 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 be due to increase in the osmolarity of extenders.

Cryopreservation of semen using Lactose-EDTA with 4% glycerol gives a higher probability of good post-thaw sperm motility than did other extenders. Supplementing glutamine to extenders did not produce any beneficial effects on freezability of stallion sperm. However, better extenders and an appropriate freezing protocol for stallion semen should be further studies.

LITERATURE CITED

Abdel-Rahman, H.A., M.S. El-Belely, A.A. Al-Qarawi and S.A. El-Mougy. 2000. The relationship between semen quality and mineral composition of semen in various ram breeds. **Small Rumin Res.** 38: 45-49.

Akcay, E., T. Reilas, M. Andersson and T. Katila. 2006. Effect of seminal plasma fractions on stallion sperm survival after cooled-storage. **J. Vet. Med. A: Physiol. Pathol. Clin. Med.** 53: 481-485.

Althouse, G.C. and S.M. Hopkins. 1995. Assessment of boar sperm viability using a combination of two fluorophores. **Theriogenology.** 43: 595-603.

Alvarenga, M.A., K.M. Leão, F.O. Papa, F.C. Landim-Alvarenga, A.S.L. Medeiros and G.M. Gomes. 2003. The use of alternative cryoprotectors for freezing stallion semen, pp. 74-76. *In* **Proceedings of the Workshop on Transporting Gametes and Embryos.** Brewster, Massachusetts. Newmarket, UK: R & W Publications. (Havemeyer Foundation Monograph Series, 12).

Alvarenga, M.A., F.O. Papa, F.C. Landim-Alvarenga and A.S. Medeiros. 2005. Amides as cryoprotectants for freezing stallion semen: a review. **Anim Reprod Sci.** 89:105-113.

Amann, R.P. 1993. Physiology and endocrinology, pp. 658–685. *In* A.O. McKinnon and J.L. Voss, eds. **Equine Reproduction.** Lea & Febiger, Philadelphia.

Amann, R.P. and J.K. Graham. 1993. Spermatozoal function, pp. 715-745. *In* A.O. McKinnon and J.L. Voss, eds. **Equine Reproduction.** Lea & Febiger, Philadelphia.

Amann, R.P. and B.W. Pickett. 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. **Equine Vet Sci.** 7: 145-173.

Araujo, J.F., A.S.F. Righini, J.J. Fleury, M.C. Caldas, J.B.F. Costa-Neto and N. Marques. 1996. Marques Seasonal Rhythm of Semen Characteristics of a Brazilian Breed ("Mangalarga") Stallion. **Chronobiology International.** 13: 477-485.

Aurich, C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. **Anim Reprod Sci.** 89: 65-75.

Baas, J.W., P.C. Molan and P. Shannon. 1983. Factors in seminal plasma of bulls that affect the viability and motility of spermatozoa**. J Reprod Fertil.** 68: 275-280.

Ball, B.A. and A. Vo. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. **J Androl.** 22: 1061-1069.

Ball, B.A. and H.O. Mohammed. 1995. Morphometry of stallion sperm by computer-assisted image analysis. **Theriogenology.** 44: 367–377.

Bane, A. 1961. Acrosomal abnormality associated with sterility in boar, pp. 810-817. *In* **4th Int. Congr. Anim. Reprod. and Artificial Insemination**.

Barker, C.A.V. and J.C.C. Gandier. 1957. Pregnancy in a mare resulting from frozen epididymal spermatozoa. **Can J Comp. Med Vet Sci.** 21: 41-51.

Barth, A.D. and R.J. Oko. 1989. **Abnormal Morphology of Bovine Sperm.** Iowa State University Press.

Bataille, B., M. Magistrini, and E. Palmer. 1990. Objective determination of sperm motility in frozen-thawed stallion semen. Correlation with fertility. **Anim Breed.** 96-106.

Batellier, F., M. Magistrini, J. Fauquant, and E. Palmer. 1997. Effect of milk fractions on survival of equine spermatozoa. **Theriogenology.** 48: 391-410.

Begley, A.J. and P. Quinn. 1982. Decapacitation factors in semen (Invited review). **Clin Reprod Fertil.** 1: 165-167.

Bielanski, W. 1975. The evaluation of stallion semen in aspects of fertility control and its use for artificial insemination. **J Reprod Fertil.** 23 (Suppl): 19-24.

Bielanski, W. and F. Kaczmarski. 1979. Morphology of spermatozoa in semen from stallion of normal fertility. **J. Reprod Fertil.** 27 (Suppl): 39-45.

Blach, E.L., R.P. Amann, R.A. Bowen and D. Frantz. 1989. Changes in quality of stallion spermatozoa during cryopreservation: Plasma membrane integrity and motion characteristics. **Theriogenology.** 31: 283-298.

Bolton, V.N. and P.R. Braude. 1984. Preparation of human spermatozoa for in vitro fertilisation by isopycnic centrifugation of self-generating density gradients. **Arch Androl.** 13: 167-176.

Boyle, M.S. 1999. Assessing the potential fertility of frozen stallion semen, pp. 13-16. *In* W.R. Allen, and J.F. Wade, eds. **Havemeyer Foundation monograph series no. 1.** R &W Publications Ltd. Newmarket, UK.

Bradford, M.M. 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry.** 72: 248-254.

Brandon, C.I., G.L. Heussner, A.B. Caudle and R.A. Fayrer-Hosken. 1999. Two-dimensional polyacrylamide elctrophoresis of equine seminal plasma proteins and their correlation with fertility. **Theriogenology.** 52: 863–873.

Brinsko, S.P., T.L. Blanchard, S.L. Rigby, C.C. Love and D.D. Varner. 2003. Effect of dead spermatozoa on motion characteristics and membrane integrity of life spermatozoa in fresh and cooled-stored equine semen. **Theriogenology.** 59: 735-742.

Brinsko, S.P., K.R. Rowan, D.D. Varner and T.L. Blanchard. 2000. Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. **Theriogenology.** 53: 1641-1655.

Brito, L.F., A.E. Silva, R.T. Barbosa and J.P. Kastelic. 2004. Testicular thermoregulation in *Bos indicus*, crossbred and *Bos taurus* bulls: relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production. **Theriogenology.** 61: 511-528.

Brito, L.F.C., A.E. Silva, R.T. Barbosa, M.M. Unanian and J.P. Kastelic. 2003. Effects of scrotal insulation on sperm production, semen quality, and testicular echotexture in Bos indicus and Bos indicus x Bos taurus bulls. **Anim Reprod Sci.** 79:1-15.

Buendía P., C. Soler, F. Paolicchi, G. Gago, B. Urquieta , F. Pérez-Sánchez and E. Bustos-Obregón. 2002. Morphometric characterization and classification of alpaca sperm heads using the sperm-class analyzer computer-assisted system. **Theriogenology.** 57: 1207-1218.

Card, C. 2005. Cellular associations and the differential spermiogram: making sense of stallion sperml morphology. **Theriogenology.** 64: 558-567.

Cardoso, R.S., A.R. Silva and L.D. Machado da Silva. 2006. Comparison of two dilution rates on canine semen quality after cryopreservation in a coconut water extender. **Anim Reprod Sci.** 92: 384-391.

Carver, D.A. and B.A. Ball. 2002. Lipase activity in stallion seminal plasma and the effect of lipase on stallion spermatozoa during storage at 5°C. **Theriogenology.** 58: 1587-1595.

Casey, P.J., C.G. Gravance, R.O. Davis, D.D. Chabot and I.K. Liu. 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. **Theriogenology.** 47: 575-582.

Cheng, F.P., A.R. Fazeli, W. Voorhout, M.M. Bevers and B. Colenbrander. 1996. Use of PNA (peanut agglutinin) to assess acrosomal status and the zona pellucida induced acrosome reaction in stallion spermatozoa. **J Androl.** 17: 674–682.

Chenier, T.S. 2000. Anatomy and physical examination of the stallion, pp. 1-23. *In* J.C. Samper eds. **Equine breeding management and artificial insemination.** W.B. Saunders company, Philadelphia.

Chevalier-Clement, F., M.T. Hochereau de Reviers, C. Perreau and M. Magistrini. 1991. Alterations of the semen and genital tract of infertile stallions. **J Reprod Fertil.** 44 (Suppl): 657-658.

Clulow, J.R., L.J. Mansfield, L.H. Morris, G. Evans and W.M. Maxwell. 2008. A comparison between freezing methods for the cryopreservation of stallion spermatozoa. **Anim Reprod Sci.** 108: 298-308.

Cochran, J.D., R.P. Amann, D.P. Froman and B.W. Pickett. 1984. Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on the post-thaw motility of equine sperm. **Theriogenology.** 22: 25-38.

Colenbrander, B., H. Puyk, A.R. Zandee and J. Parlevliet. 1992. Evaluation of the stallion for breeding. **Acta Vet Scand.** 88 (Suppl): 29-38.

Cot-ma, J.R. and P.M. Zavos. 1994. The hypoosmotic swelling test: Its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine spermatozoa. **Theriogenology.** 42: 351-360.

Cristanelli, M.J., E.L. Squires, R.P. Amann and B.W. Pickett. 1984. Fertility of stallion semen processed, frozen and thawed by a new procedure. **Theriogenology.** 22: 39-45.

Davies-Morel, M.C.G. 1999. **Equine Artificial Insemination**. CABI Publishing, New York.

Davies-Morel, M.C.G. 2002. **Equine Reproductive Physiology, Breeding and Stud Management.** 2nd ed. CABI Publishing, New York.

De Albuquerque L. and M. Bestimmung. 1995. **The determination of the osmotic resistance of stallion spermatozoa.** M.S.Thesis, Hannover University.

de la Cueva C.F.I., T. Rigau, S. Bonet, J. Mire, M. Briz and J.E. Rodriguez-Gil. 1997. Subjecting horse spermatozoa to hypoosmotic incubation: effects of ouabain. **Theriogenology.** 47: 765-784.

Douglas-Hamilton, D.H., R. Osol, G. Osol, D.D. Driscoll and H. Noble. 1984. A field study of the fertility of transported equine semen. **Theriogenology.** 22: 291-304.

Dowsett, K.F. and W.A. Pattie. 1982. Characteristics and fertility of stallion semen. **J Reprod Fertil.** 32 (Suppl): l-8.

Dowsett, K.F. and L.M. Knott. 1996. The influence of age and breed on stallion semen. **Theriogenology.** 32: 397-412.

Drevius, L.O. and H. Eriksson. 1966. Osmotic swelling of mammalian spermatozoa. **Expl Cell Res.** 42: 136-156.

Ecot, P., M.Vidament, A. de Mornac, K. Perigault, F. Clément and E. Palmer. 2000. Freezing of stallion semen: interactions among cooling treatments, semen extenders and stallions. **J. Reprod. Fertil.** 56 (Suppl): 141-150.

England, C.C. and J.M. Plummer. 1993. Hypo-osmotic swelling of dog spermatozoa. **J Reprod Fertil.** 47 (Suppl): 261-270.

Esteso, M.C., A.J. Soler, M.R. Fernández-Santos, A.A. Quintero-Moreno and J.J. Garde. 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in iberian red deer (Cervus elaphus hispanicus) epididymal sperm samples**. J Androl.** 27: 662-670.

Fazeli, A.R., W. Steenweg, M.M. Bevers, J. van den Brook, V. Bracher, J. Parlevliet and B. Colenbrander. 1995. Relation between stallion sperm binding to homologous hemizonae and fertility. **Theriogenology.** 44: 751-760.

Fuchs, B.C. and B.P. Bode. 2006. Stressing out over survival: Glutamine as an apoptotic modulator. **J Surgical Res.** 131: 26-40.

Gago, C., F. Pérez-Sánchez, C.H. Yeung, L. Tablado, T.G. Cooper and C. Soler. 1999. Morphological characterization of ejaculated cynomolgus monkey (Macaca fascicularis) sperm. **Am J Primatol.** 47: 105-115.

García-Herreros, M., I.M. Aparicio, F.J. Barón, L.J. García-Marín and M.C. Gil. 2006. Standardization of sample preparation, staining and sampling methods

for automated sperm head morphometry analysis of boar sperm. **Int J Androl.** 29: 553-563.

Gebauer, M.R., B.W. Pickett, J.L. Voss and E.E. Swierstra. 1974. Reproductive physiology of the stallion: daily sperm output and testicular measurements. **J Am Vet Med Assoc.** 165: 711-713.

Gillan , L., T. Kroetsch, W.M.C. Maxwell and G. Evans. 2007. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. **Anim Reprod Sci.** 103: 201-214.

Gnessi, L., A. Fabbri and G. Spera. 1997. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. **Endocrine Rev.** 18: 541-609.

Gravance, C.G., Z. Champion, I.K. Liu and P.J. Casey. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. **Anim Reprod Sci.** 47: 149-155.

Griggers, S., D.L. Paccamonti, R.A. Thompson and B.E. Eilts. 2001. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. **Theriogenology.** 56: 613-622.

Hamann, H., R. Jude, H. Sieme, U. Mertens, E. Töpfer-Petersen, O. Distl and T. Leeb. 2007. A polymorphism within the equine CRISP3 gene is associated with stallion fertility in Hanoveran warmblood horses. **Anim Genet.** 38: 259-264.

Hammitt, D.G., E. Bedia, P.R. Rogers, C.H. Syrop, J.F. Donovan and R.A. Williamson. 1989. Comparison of motility stimulants for cryopreserved human semen. **Fertil Steril.** 52: 495-502.

Hancock, J.L. 1957. The Morphology of Boar Spermatozoa. **J Royal Micr Soc.** 76: 84-97.

Harrison, R.A.P. and S.E. Vickers. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. **J Reprod Fertil.** 88: 343-352.

Heitland, A.V., D.J. Jasko, E.L. Squires, J.K.Graham, B.W. Pickett and C. Hamilton. 1996. Factors affecting motion characteristics of frozen-thawed stallion spermatozoa. **Equine Vet J.** 28: 47–53.

Hermenet, M.J., H.R. Sawyer, B.W. Pickett, R.P. Amann, E.L. Squires and P.L. Long. 1993. Effect of stain, technician, number of spermatozoa evaluated and slide preparation on assessment of spermatozoal viability by light microscopy. **J Equine Vet Sci.** 13: 449-455.

Hidalgo, M., I. Rodrı´guez, J. Dorado and C. Soler. 2005. Effect of sample size and staining methods on stallion sperm morphometry by the Sperm Class Analyzer. **Vet Med-Czech**. 50: 24-32.

Hidalgo, M., I. Rodrı´guez, J. Dorado and C. Soler. 2008. Morphometric classification of Spanish thoroughbred stallion sperm heads. **Anim Reprod Sci.** 103: 374-378.

Hirai, M., A. Boersma, A. Hoeflich, E. Wolf, J. Foll, T.R. Aumüller and J. Braun. 2001. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): relation to fertility and seminal plasma growth factors. **J Androl.** 22**:** 104-110.

Holt, W.V. 2000. Basic aspects of frozen storage of semen. **Anim Reprod Sci.** 62: 3-22.

Huhtaniemi, H. and J. Toppari. 1995. Endocrine, paracrine and autocrine regulation of testicular steroidogenesis, pp. 33–54. *In* A.K. Mukhopadhyay and M.K. Raizada, eds. **Tissue Renin–Angiotensin Systems.** Plenum Press, New York.

Ionata, L.M., T.M. Anderson, B.W. Pickett, J.C. Heird and E.L. Squires. 1991. Effect of supplementary sexual preparation on semen characteristics of stallions. **Theriogenology.** 36: 923–37.

Jasko, D.J., T.V. Little, K. Smith, D.H. Lein and R.H. Foote. 1988. Objective analysis of stallion sperm motility. **Theriogenology.** 30: 1159-1167.

Jasko, D.J., D.H. Lein and R.H. Foote. 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). **J Am Vet Med Assoc.** 197: 389-394.

Jasko, D.J. 1992. Evaluation of stallion semen, pp. 129-148. *In* T.L. Blanchard and D.D. Varner, eds. **The Veterinary Clinics of North America, Equine Practice, Stallion Management.** W.B. Saunders Company, Philadelphia.

Jasko, D.J., J.A. Hathaway, V.L. Schaltenbrand, W.D. Simper and E.L. Squires. 1992. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. **Theriogenology.** 37: 1241-1252.

Jasko, D.J., D.M. Moran, M.E. Farlin and E.L. Squires. 1991. Effect of seminal plasma dilution or removal on spermatozoa1 motion characteristics of cooled stallion semen. **Theriogenology.** 35: 1059 -1068.

Jeyendran, R.S., H.H. Van deVen, M. Perez-Pelaez, B.G. Crab and L.J.D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other Semen characteristics. **J Reprod Fertil.** 70: 219-228.

Jobim, M. I., I. C. B. Filho, C. Trein , V.B. Wald , R.M. Gregory , R.C. Mattos. 2005. Equine seminal plasma proteins related with fertility. **Anim Reprod Sci** 89: 305-308. Cited T. Mann and C. Lutwak-Mann. 1981. **Male Reproductive Function and Semen General Features of the Seminal Plasma**. 2nd ed. Springer-Verlag, New York, pp. 28–34.

Johnson, L. 1982. A re-evaluation of daily sperm output of men. **Fertil. Steril.** 37: 811-816.

Johnson, L., C.S. Petty and W.B. Neaves. 1980. The relationship of biopsy evaluations and testicular measurements to over-all daily sperm production in human testes. **Fertil Steril.** 34: 36-40.

Johnson, L., D.D. Varner and D.L.Jr. Thompson. 1991. Effect of age and season on the establishment of spermatogenesis in the horse. **J Reprod Fertil.** 44 (Suppl): 87-97.

Johnson, L., T.L. Blanchard, D.D. Vamer and W.L. Scrutchtield. 1997. Factors affecting spermatogenesis in the stallion. **Theriogenology.** 48: 1199-1216.

Kankofer, M., G. Kolm, J.E. Aurich and C. Aurich. 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5 °C. **Theriogenology.** 63: 1354-1365.

Katila, T. 1997. Procedures for handling fresh stallion semen. **Theriogenology.** 48: 1217-1227.

Katila, T., M. Anderson, T. Reilas and E. Koskinen. 2002. Post-thaw motility and viability of fractionated and frozen stallion ejaculates. **Theriogenology.** 58: 241-244.

Kavak, A., N. Lundeheim, M. Aidnik and S. Einarsson. 2004. Sperm morphology in Estonian and Tori Breed Stallions. **Acta Vet Scand.** 45: 11-18.

Kenney, R.M., R.V. Bergman, W.L. Cooper and G.W. Morse. 1975. Minimal contamination techniques for breeding mares. **Proc Am Ass Eq Pract.** 21: 327-336.

Kenney, R.M., J.P. Hurtgen, R. Person and J. Simons. 1983. **Society for Theriogenology: Manual for clinical fertility evaluation of the stallion.** Hastings, Nebraska.

Khlifaoui, M., I. Battut, J.F. Bruyas, G. Chatagnon, A. Trimeche and D. Tainturier. 2005. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. **Theriogenology.** 63: 138-149.

Killian, G.J. 1992. Fertility fctors in seminal plasm. *In* **Proc 14th Technical conference on artificial insemination and reproduction, National Association of animal breeders.** Milwaukee, WI.

Kirk, E.S., E.L. Squires and J.K. Graham. 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. **Theriogenology.** 64: 1422-1439.

Klug, E., I. Röbbelen, S. Kneissl and H. Sieme. 1992. Cryopreservation of stallion spermatozoa. **Acta vet scand.** 88 (Suppl): 129-135.

Kosniak, K. 1975. Characteristics of the successive jets of ejaculated semen in stallions. **J. Reprod. Fertil.** 23 (Suppl): 59-61.

Ktuni-Diaka, J. and G. Badnam. 1994. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: in vitro bioassay for canine semen. **Theriogenology.** 41: 1355-1366.

Landim-Alvarenga, F.C., A.S. Medeiros, F.O. Papa and M.A. Alvarenga. 2005. Evaluation of acrosomal integrity of stallions cryopreserved with amides and glycerol. **Anim Reprod Sci.** 89: 288-291.

Leeb, T., H. Sieme and E. Topfer-Petersen. 2005. Genetic markers for stallion fertility-lessons from humans and mice. **Anim Reprod Sci.** 89: 21–29.

Lindgren, G., N. Backstrom, J. Swinburne, L. Hellborg, A. Einarsson, K. Sandberg, G. Cothran, C. Vila, M. Binns and H. Ellegren. 2004. Limited number of patrilines in horse domestication. **Nat Genet.** 36: 335-336.

Long, P.L., B.W. Pickett, H.R. Sawyer, R.K. Shideler, R.P. Amann and E.L. Squires. 1993. Relationship between morphologic and seminal characteristics of equine spermatozoa. **J Equine Vet Sci.** 13: 143-149.

Loomis, P.R., 2006. Advanced methods for handling and preparation of stallion semen. **Vet. Clin. Equine.** 22: 663–676.

Love, C.C., D.D. Varner and J.A. Thompson. 2000. Intra and inter-stallion variation in sperm morphology and their relationship with fertility. **J Reprod Fertil.** 56 (Suppl): 93-100.

Love, C.C., M.C. Garcia, F.L. Riera and R.M. Kenney. 1991. Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume

and predict daily sperm output in the stallion. **J Reprod Fertil.** 44 (Suppl): 99-105.

Luyet, B.J. and P.M. Gehenio. 1940. **Life and death at low temperatures.** Biodynamica, Normandy, MO, USA.

Magistrini, M., I. Couty and E. Palmer. 1992. Interactions between sperm packaging, gas environment, temperature and diluents on fresh stallion sperm survival. **Acta Vet. Scand.** 88 (Suppl): 97–110.

Magistrini, M., P. Chaneloube and E. Palmer. 1987. Influence of season and frequency of ejaculation on production of stallion semen. **J. Reprod. Fertil.** 35 (Suppl)**:** 127–133.

Magistrini, M., E. Guitton, Y. Levern, J.C. Nicolle, M. Vidament, D. Kerboeuf and E. Palmer. 1997. New staining methods for sperm evaluation estimated by microscopy and flow cytometry. **Theriogenology.** 48: 1229-1235.

Magistrini, M., H. Lindeberg, E. Koskinen, P. Beau and F. Seguin. 2000. Biophysical and 1H magnetic resonance spectroscopy characteristics of fractionated stallion ejaculates. **J Reprod Fertil.** 56 (Suppl): 101–110.

Mahngren, L. 1992. Sperm morphology in stallions in relation to fertility. **Acta Vet Scand.** 88 (Suppl): 39-47.

Malmgren, L., B.O. den Kamp, A. Wöckener, M.Boyle and B. Colenbrander. 1994. Motility, velocity and acrosome integrity of equine spermatozoa stored under different conditions. **Reprod. Domest. Anim.** 29: 469-476.

Mann, T. 1975. Biochemistry of stallion semen. **J Reprod Fertil.** 23 (Suppl): 47-52.

Martin, J.C., E. Klug and A. Gunzel. 1979. Centrifugation of stallion semen and its storage in large volume straws. **J. Reprod. Fertil.** 27 (Suppl): 47.

Matsumoto, A. 1989. Hormonal control of spermatogenesis, pp. 181-196. *In* H. Burger and D. de Kretser, eds. **The Testis.** 2nd ed. Raven Press, New York.

Melo, C.M., F.S. Zahn, I. Martin, C. Orlandi, J.A.Jr. Dell'Aqua, M.A. Alvarenga and F.O. Papa. 2007. Influence of Semen Storage and Cryoprotectant on Post-thaw Viability and Fertility of Stallion Spermatozoa. **J Equine Vet Sci.** 27: 171-175.

Moore, A.I., E.L. Squires, J.E. Bruemmer and J.K. Graham. 2006. Effect of cooling rate and cryoprotectant on the cryosurvival of equine spermatozoa. **J Equine Vet Sci.** 26: 215-218.

Moran, D.M., D.J. Jasko, E.L Squires and R.P. Amann. 1992. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. **Theriogenology.** 38: 999-1012.

Neild, D., G. Chaves, M. Flores, N. Mora, M. Beconi and A. Aguero. 1999. Hypoosmotic test in equine spermatozoa. **Theriogenology.** 51: 721-727.

Neild, D.M., M.G. Chaves, M. Flores, M.H. Miragaya, E. Gonzalez and A. Agüero. 2000. The HOS test and its relationship to fertility in the stallion. **Andrologia.** 32: 351-355.

Nie, G.J. and J.G. Wenzel. 2001. Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. **Theriogenology.** 55: 1005-1018.

Núñez-Martínez, I., J.M. Moran and F.J. Peña. 2007. Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. **Int J Androl.** 30**:**182-189.

Nunes, D.B., J.R. Zorzatto, E.V. Costa - Silva and C.E.S.N. Z´uccari. 2008. Efficiency of short-term storage of equine semen in a simple-design cooling system. **Anim Reprod Sci.** 104: 434-439.

Oettlé, E.E. 1986. Using a new acrosome stain to evaluate sperm morphology. **Vet Med.** 3: 263–266.

Pagl, R., J.E. Aurich, F. Muller-Schlosser, M. Kankofer and C. Aurich. 2006. Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5°C. **Theriogenology.** 66: 1115-1122.

Panasophonkul, S., C. Lohachit and S. Sirivaidyapong. 2007. Postpartum Ovarian Activity and Serum Estradiol-17beta Level in Thai Crossbred Native Mares. **Reprod Dom Anim.** 42: 6-10.

Papa, F.O., C.M. Melo, E.G. Fioratti, J.A. Jr. Dell'aqua, F.S. Zahn and M.A. Alvarenga. 2008. Freezing of stallion epididymal sperm. **Anim Reprod Sci.** 107: 293-301.

Parlevliet, J., B. Kemp and B. Colenbrander. 1994. Reproductive characteristics and semen quality in maiden Dutch Warmblood stallions. **J Reprod Fertil.** 101: 183-187.

Pegg, D.E. 2002. The history and principles of cryopreservation. **Semin Reprod Med.** 20: 5-13.

Peña, F.J., F. Saravia, M. García-Herreros, I. Núñez-martínez, J.A. Tapia, A. Johannisson, M. Wallgren and H. Rodríguez-Martínez. 2005. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. **J Androl.** 26**:** 716-723.

Pesch, S., H. Bostedt, K. Failing and M. Bergmann. 2006. Advanced fertility diagnosis in stallion semen using transmission electron microscopy. **Anim Reprod Sci.** 91: 285-298.

Pesch, S. and B.Hoffmann. 2007. Cryopreservation of Spermatozoa in Veterinary Medicine. **J. Reproduktionsmed. Endokrinol**. 4: 101-105. Cited L., Spallanzani. 1785. **Esperimenti che servono nella storio della generazione di animali e piante.** Barthelmi Ciro, Genova.

Phetudomsinsuk, K., K. Sirinarumitr, A. Choothesa, P. Suthanmapinunt, K. Kornkaewrat, A. Laikul, S. Amornsak and A. Pinyopummin. 2008a. Effects of Extenders and Glutamine on Semen Characteristics of Thai Native Crossbred and Full Size Purebred Horses after Cooled Storage. **Kasetsart Journal.** 42: 473-484.

Phetudomsinsuk, K., K. Sirinarumitr, A. Laikul and A. Pinyopummin. 2008b. Morphology and Head Morphometric Characters of Sperm in Thai Native Crossbred Stallions. **ACTA Vet Scan.** In press.

Pickett, B.W. 1993. Factors affecting sperm production and output, pp 689-704. *In* A.O. MCkinnon and J.L. VOSS, eds. **Equine Reproduction.** Lea & Febiger. Philadelphia.

Pickett, B.W. 1995. The stallion: retrospective analyses and opinions. **Biol. Reprod. Monogr.** 1: 547-564.

Pickett, B.W. and R.P. Amann. 1993. Cryopreservation of semen, pp. 769-789. *In* A.O. MCkinnon and J.L. VOSS, eds. **Equine Reproduction.** Lea & Febiger, Philadelphia.

Pickett, B.W., J.L. Voss, R.A. Bowen, E.L. Squires and A.O. McKinnon. 1987. Seminal characteristics and total scrotal width (T.S.W.) of normal and abnormal stallions. pp**.** 487-518. **Proc 33rd Ann Conv AAEP** .

Pinto, N.F. and R.N.B. Lobo. 1997. Use of the HOS-Test to assess plasma membrane integrity in equine semen fresh and cooled for different times. **Rev Brasil Reprod Anim.** 21: 133-135.

Polge, C., A.U. Smith and A.S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature.** 164: 666.

Pommer, A.C., J. Rutllant and S.A. Meyers. 2002. The role of osmotic resistance on equine spermatozoal function. **Theriogenology.** 58: 1373-1384.

Revell, S.G. and R.A. Mrode. 1994. An osmotic resistance test for bovine semen. **Anim Reprod Sci.** 36: 77-86.

Rodriguez-Martinez, H. 2000. Evaluation of frozen semen: traditional and new approaches. *In* P.J. Chenoweth. ed. **Bull Fertility: International Veterinary Information Service.** Available source: http://www.ivis.org.

Rodriguez-Martinez, H. 2006. Can we increase the estimative value of semen assessment? **Reprod Domest Anim.** 41 (Suppl): 2-10.

Rogers, B.J. and R.A. Parker. l991. Relationship between the human sperm hype-osmotic swelling test and sperm penetration assay. **J Androl.** 12: 152-158.

Rota, A., C. Furzi, D. Panzani and F. Camillo. 2004. Studies on Motility and fertility of cooled stallion spermatozoa. **Reprod Dom Anim**. 39: 103-109.

Rubei, M., S. Degl'Innocenti, P.J. De Vries, G. Catone and G. Morini. 2004. Directional freezing (Harmony Cryocare - Multi Thermal Gradient 516): a new tool for equine semen cryopreservation. 2004: **15th International Congress on Animal Reproduction**, Porto Seguro, Brazil, p. 503 Abstract.

Samper, J.C., J.C. Hellander and B.G. Crabo. 1991. Relationship between the fertility of fresh and frozen stallion semen and semen quality. **J Reprod Fertil.** 44 (Suppl): 107-114.

Sancho, M., F. Pérez-Sánchez, L. Tablado, J.J. de Monserrat and C. Soler. 1998. Computer assisted morphometric analysis of ram sperm heads: evaluation of different fixative techniques. **Theriogenology.** 50: 27-37.

Saragusty, J., H. Gacitua, M.T. Pettit and A. Arav. 2007a. Directional freezing of equine semen in large volumes. **Reprod Domest Anim.** 42: 610-615.

Saragusty, J., H. Gacitua, M.T. Pettit and A. Arav. 2007b. Directional freezing of equine semen in large volumes. **Reprod Domest Anim**. 42: 610-615. *Cited* L. Spallanzani. 1776. **Osservazioni e spezienze interno ai vermicelli spermatici dell' uomo e degli animali.** Opusculi di Fisica Animalee Vegetabile, Modena.

Shore, M.D., M.L. Macpherson, G.B. Combes, D.D. Varner and T.L. Blanchard. 1998. Fertility comparison between breeding at 24 hours or at 24 and 48 hours after collection with cooled equine semen. **Theriogenology.** 50: 693-698.

Sieme, H., A. Echte and E. Klug. 2002. Effect of frequency and interval of semen collection on seminal parameters and fertility of stallions. **Theriogenology.** 58: 313-316.

Skinner, M.K. 1991. Cell-cell interactions in the testis. **Endocrine Rev.** 12: 45–77.

Spiteri-Grech, J. and E. Nieschlag. 1993. Paracrine factors relevant to the regulation of spermatogenesis: a review. **J Reprod Fertil.** 98: 1-14.

Squires, E.L., R.P. Amann and BW. Pickett. 1987. Preservation of stallion semen – Colorado experience, pp. 49-54. **Symp Equine Reprod.** Denmark.

Squires, E., R.P. Amann, A.O. McKinnon, and B.W. Pickett. 1988. Fertility of equine spematozoa cooled to 5 or 20°C, pp. 297–299. *In* **Proceedings of the 11th International Congress on Animal Reproduction**, Vol. 3, Dublin.

Squires, E.L., S. Barbacini, D. Necchi, H. Reger and J. Bruemmer. 2003. Simplified strategy for insemination of mares with frozen semen. *In* **AAEP Proceedings.** 49: 353-356.

Sritrakul, T., N. Limsuwan, P. Thamsatit, M. Khiao-in and W. Cherdchutham. 2008. The study of association between measurement of body size value and body weight from electrical weight machine of crossbred native ponies for development of body weight's formula. **Kasetsart Journal.** In press.

Tawatsin, A., P. Poomvises, P. Ruantongdee, C. Grezegorz and C. Sirilerttrakool. 2005. Genetic analysis of Thai native horses using microsatellite markers. *In* **The 4th Chulalongkorn University Veterinary Annual Conference**. Thailand, Abstract 50.

Thompson, D.L., B.W. Pickett, E.L. Squires and R.P. Amann. 1979. Testicular measurements and reproductive characteristics in stallions. **J Reprod Fertil.** 27 (Suppl): 13-17.

Trimeche, A., J.M. Yvon, M. Vidament, E. Palmer and M. Magistrini. 1999. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. **Theriogenology.** 52: 181-191.

Van Duijn, C. and J. Hendriske. 1968. Rational analysis of seminal characteristics of stallions in relation to fertility. **Research Institute for Animal Husbandry, Report No. 97,** Zeist, Netherlands.

Varner, D.D., T.L. Blanchard, C.L. Love, M.C. Garcia and R.M. Kenney. 1987. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. **Theriogenology.** 28: 709-718.

Varner, D.D., T.L. Blanchard, C.L. Love, M.C. Garcia and R.M. Kenney. 1988. Effects of cooling rate and storage temperature on equine sperml motility parameters. **Theriogenology.** 29: 1043-1054.

Varner, D.D., T.L. Blanchard, P.J. Meyers and S.A. Meyers. 1989. Fertilizing capacity of equine spermatozoa stored for 24 h at 5 or 20 °C. **Theriogenology.** 32: 515-525.

Varner, D.D., S.D. Vaughan and L. Johnson. 1991a. Use of a computerized system for evaluation of equine spermatozoal motility. **Am J Vet Res.** 52: 224-230.

Varner, D.D., J. Schumacher, T.L. Blanchard and L. Johnson. 1991b. Management of the breeding stallion, pp. 97-115. *In* P.W. Prat and D.R. Thornwood, eds. **Diseases and Management of Breeding Stallions**. American Veterinary Publications, Goleta, CA.

Veeramachaneni, D.N., C.L. Moeller and H.R. Sawyer. 2006. Sperm morphology in stallions: ultrastructure as a functional and diagnostic tool. **Vet Clin North Am Equine Pract.** 22: 683-692.

Vidament, M., A.M. Dupere, P. Julienne, A. Evain, P. Noue and E. Palmer. 1997. Equine frozen semen: Freezability and fertility field results. **Theriogenology.** 48: 907-917.

Vidament, M., P. Ecot, P. Noue, C. Bourgeois, M. Magistrini and E. Palmer. 2000. Centrifugation and addition of glycerol at 22 °C instead of 4 °C improve post-thaw motility and fertility of stallion spermatozoa. **Theriogenology.** 54: 907-919.

Vidament, M., J.M. Yvon, I. Couty, G. Arnaud, J. Nguekam-Feugang, P. Noue, S. Cottron, A. Le Tellier, F. Noel, E. Palmer and M. Magistrini. 2001. Advances in cryopreservation of stallion semen in modified INRA82. **Anim Reprod Sci.** 68: 201-218.

Voss, J.L., B.W. Pickett and E.L. Squires. 1981. Stallion sperml morphology and motility and their relationships to fertility. **J Am Vet Med Assoc.** 178: 287-289.

Walton, A. 1960. Copulation and natural insemination, pp. 130–160. *In* A.S. Parkes ed. **Marshall's Physiology of Reproduction Part 2.** Longmans, London.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. **Reprod Fertil Dev.** 7: 871-891.

Wendt, K.M., C.C. Love, S.P. Brinsko, J.A. Thompson, T.L. Blanchard and D.D. Varner. 2002. Effect of extender pH on motility characteristics of cool-stored equine spermatozoa. **Theriogenology.** 58: 321-324.

Weinbauer, G.F. and E. Nieschlag. 1993. Hormonal control of spermatogenesis, pp. 99-142. *In* D. de Kretser, ed. **Molecular Biology of Male Reproductive System.** Academic Press, New York.

Wilhelm, K.M., J.K. Graham and E.L. Squires. 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. **Theriogenology.** 46: 559-578.

William, M.B. 2003. Predictive value of hypo-osmotic swelling test to identify viable non-motile sperm. **Asian J Androl.** 5: 209-212.

Witte, T.S., and S. Schäfer-Somi. 2007. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. **Anim Reprod Sci.** 102: 181-193.

Witte, T.S., S. Schäfer-Somi, A. Kuchar, E. Möstl, C. Iben and C. Aurich. 2008. Effect of hen's egg yolk on capacitation and acrosome reaction of diluted canine spermatozoa. **Anim Reprod Sci.** In press.

Zahn, F.S., F.O. Papa and Jr.J.A. dell'Aqua. 2002. Cholesterol incorporation on equine sperm membranes: effects on post-thaw sperm parameters and fertility. **Theriogenology.** 58: 237-240.

Zahn, F.S., F.O. Papa and C.M. Melo. 2006. Blood serum, seminal plasma and sperm membrane protein profiles in stallions: are they correlated to semen freezability? **Anim Reprod Sci.** 94: 64-66.

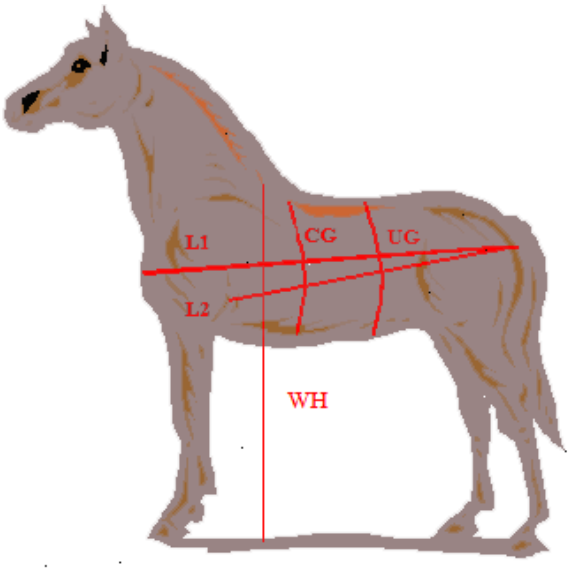
APPENDICES

Appendix A

General characteristics of Thai native crossbred horses

General characteristics of Thai native crossbred horses

Thai native crossbred characteristic were mostly like pony, however some characteristics were different. Thai native crossbred horse's height is below 14.2 hands, with big head, thick body and short legs. It is very strong and resisting. Body sizes of Thai native crossbred horsewere determined in Appendix Figure1 and presented in Appendix Table1.



Appendix Figure 1 The criteria of body measurement of Thai native crossbred horse

CG = Chest Girth

UG = Umbilical Girth

L1 = Length from tuber ischium to point of shoulder

L2 = Length from tuber ischium to olecranon

WH = Height at withers

Source: http://ojas.ucok.edu/01/papers/hapgood01.htm

Appendix Table 1 Mean ± SD of bodyweight (kg), Hieght and Chest Girth, Umbilical Girth, Length from tuber ischium to point of shoulder, and Length from tuber ischium to olecranon (cm) of Thai native crossbred horse (n= 37)

Parameter	Mean ± SD
BW (kg)	209.54±40.88
WH (cm)	118.03±8.12
L1 (cm)	114.77±8.88
L2 (cm)	93.19±7.96
CG (cm)	133.81±8.57
UG (cm)	142.57±11.50

Appendix B

Seminal plasma proteins of Thai native crossbred and purebred stallion semen

Seminal plasma proteins of Thai native crossbred and purebred horses

Equine seminal plasma is a complex mixture of secretions from the testes, duct system and accessory sex glands. This is the natural medium involved in final maturation of spermatozoa and events preceding fertilization through hormonal, enzymatic and surface-modifying events (Jobim et al., 2005). The panel of proteins identified in seminal plasma are various hormones, enzymes, inhibitors of proteinases and other components, growth factors, proteins and glycoproteins which are still unknown nature or function. A possible correlation between seminal plasma proteins and fertility in stallions based on molecular weights and isoelectric points, suggested that the proteins may be related to those found in experiments with bovine semen, which proved to have stimulatory or inhibitory effects on fertility (Brandon et al., 1999). This preliminary study aimed to investigate the profiles of seminal plasma proteins of Thai Native Crossbred and Purebred stallions.

Materials and methods

1. Animals

Seminal plasma was collected from nine stallions consisting of Thai native crossbred horses (T; n = 5) and full size purebred horses (F; n = 4; 1 Holstein, 1 Standardbred and 2 Thoroughbreds) aging between 5 and 12 years old. A total of six ejaculates were collected from each stallion.

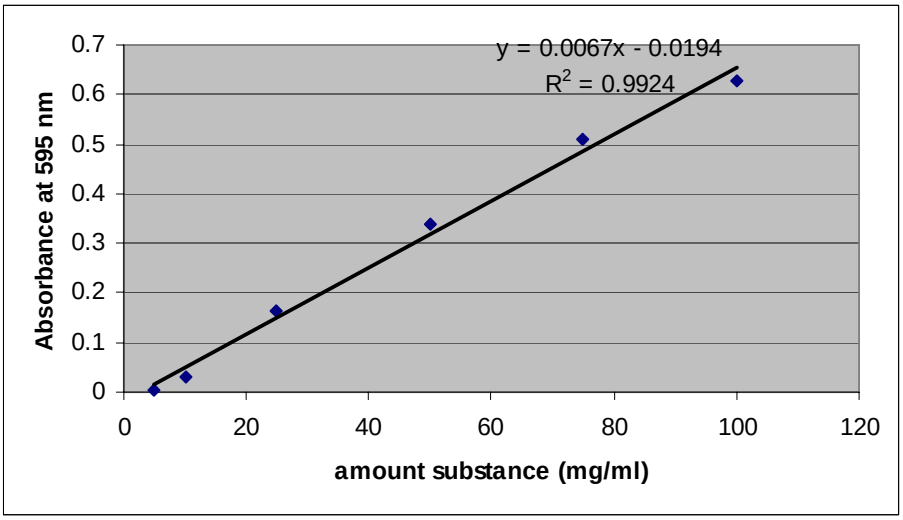
2. Protein assay

Seminal plasma was obtained by two steps of centrifugation at 3000 g for 15 min, changing tubes between them. The resulting supernatant was transferred to cryovials and stored in -20°C freezer until analysis. Total protein concentration was measured using the Bradford assay kit (Bradford, 1976), with bovine serum albumin as a standard and measured the absorbance by using spectrophotometer (Beckman coulter, DU® 530, life Science UV/Vis) at 595 nm. One-dimensional SDS-PAGE was

performed under denaturing condition using 10% polyacrylamide gels containing SDS at 100 volts, 15 mA, for 2 h.

3. Results

A standard BSA curve of absorbance versus micrograms protein of five dilution: 5, 25, 50, 75, 100 µg/µl was shown in Appendix figure 2. There were variation in total seminal plasma proteins concentration among stallions in both T and F (Appendix table 2). The mean concentration in seminal plasma of Thai native crossbred (T) and purebred (F) stallions were 66.23±5.62 and 72.69±7.64 mg/ml, in T and F respectively which were not significantly different (P>0.05). Protein profiles for T and F seminal plasma were shown in Appendix figure 3. In both T and F, total of twelve bands of proteins from 14.4 to 116 kDa were identified, of which four bands were presented in all stallions (band 2-16 kDa; band 3-18 kDa, band 5-22 kDa and band11-60 kDa). The protein compositions and amounts of each protein of seminal plasma were also varied among stallions.

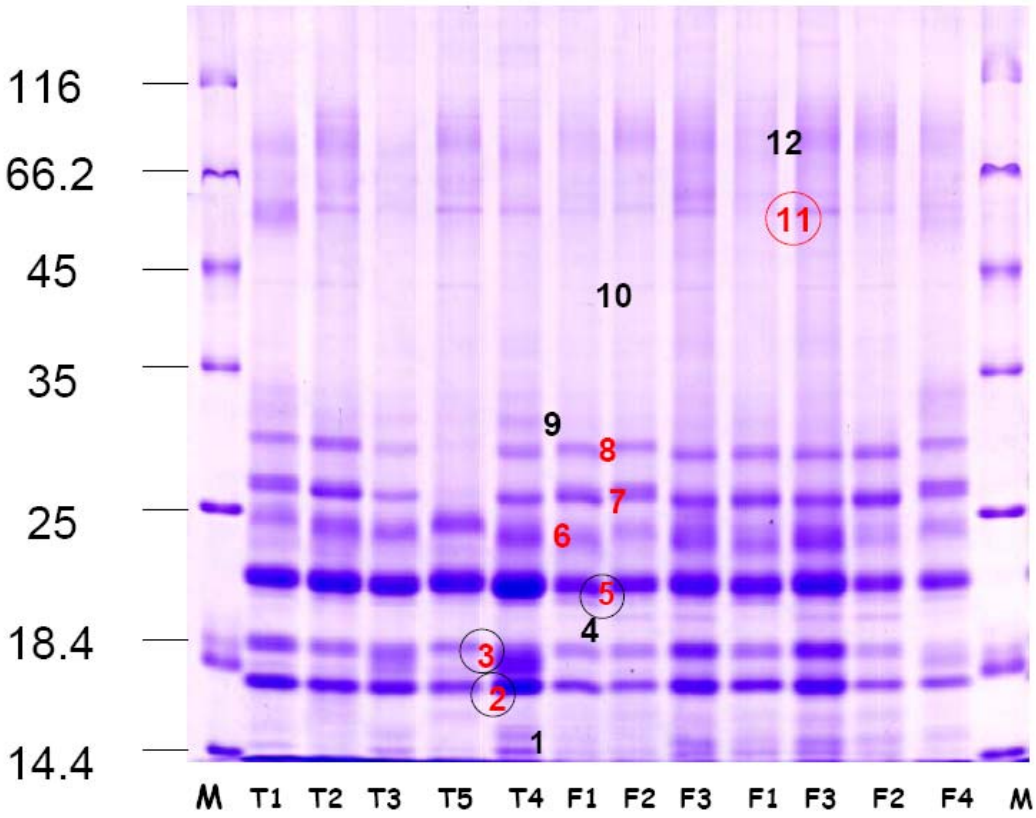


Appendix Figure 2 The standard curve of bovine serum albumin protein (Bradford assay, 595 nm)

Appendix Table 2 The total protein concentration in seminal plasma of Thai native crossbred (T) and purebred (F) stallions

Stallions	Seminal plasma protein concentrations (mg/ml) (mean±SE)
T1	76.19±2.81 [a,b]
T2	71.33±4.2 [a,b,c]
T3	57.84±2.13[a,c]
T4	96.99±1.44 [b]
T5	38.34 ±1.60 [c]
Total T	66.23±5.62
F1	61.81±9.33 [a,b]
F2	49.908±1.30[b]
F3	99.99±2.39 [a]
F4	79.08±2.62 [a,b]
Total F	72.69±7.64

Within the same group of stallion, significant differences ($P<0.05$) between seminal plasma proteins were indicated with lower-case letters (a, b, c).



Appendix Figure 3 Seminal plasma protein profiles of Thai native crossbred (T1 – T5) and purebred (F1 – F4) stallions seminal plasma in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 12 different bands (no. 1 – 12) were detected and 4 of these bands (no. 2, 3, 5 and 11) were presented in all of the samples.

Conclusion

The preliminary results indicated that the mean concentration of T and F total seminal plasma protein were similar. Also, in both T and F, twelve bands of seminal plasma protein profiles were detected and four of these bands were presented in all of the samples. The seminal plasma protein concentration and profile were varied among stallions in both breeds. Its relation with semen quality needs further investigation.

Appendix C

A first pregnancy of Thai native crossbred horse resulted from artificial insemination with frozen semen in Thailand

A first pregnancy of Thai native crossbred horse resulted from artificial insemination with frozen semen in Thailand

This preliminary study was carried out to determine the feasibility of stallion frozen semen usage for artificial insemination (AI). The result obtained could be useful for the development of alternative reproductive management of horses in the country.

Materials and methods

1. Animals

Semen was collected from a Thai native crossbred stallion, aged 7 years old. The stallion was sexually rested for 1 week. A mare approximately 7 years old (judging the age by examining its teeth) was previously foaling in February 2007 and she had regular every 21 days of estrous cycle.

2. Semen collection and cryopreservation

Semen was collected using a Missouri type artificial vagina while the stallion was mounting a teaser mare in oestrus in July 2007. Immediately after dismount the ejaculate was initially evaluated for total volume, gel-free volume, concentration, and motility. The original motility of sample needed to be least 50% to be considered acceptable for freezing in this project. Total number of spermatozoa was measured using a Neubauer counting chamber. After evaluation, semen was diluted at a ratio of 1:3 (semen: extender) in a Kenney extender (Kenney et al., 1975) and subjected to centrifuge at room temperature (30ºC) at 400 x g for 10 min in 15 ml capacity centrifuge tube to remove seminal plasma. Spermatozoa pellets were resuspended in Kenney freezing extenders with 3.5% glycerol to final concentration of approximately 200 x 10^6 sperm / ml and then placed in a passive cooling device (Equitainer^{TM}) (5°C) for equilibration during transport to laboratory room for further processing. Semen sample was equilibrated at 5 °C for 2 h, and then loaded into 0.5 ml polyvinylchloride straws. Before freezing (BF), sample was evaluated motility, motion velocity,

viability and membrane integrity. 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).

3. Semen analysis

Frozen semen was thawed at 37°C water bath for 30 sec immediately prior to processing for analysis.

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

The functional plasma membrane integrity of frozen-thawed semen was performed with the hypoosmotic swelling test (HOS test; Neild et al., 1999). At least 200 spermatozoa were observed at magnification 400× and classified by the presence or absence of a swollen tail (curled/coiled principal or end piece), while live sperm was determined using the eosin-nigrosin staining tests (William, 2003).

4. Artificial insemination (AI)

4.1 Estrus detection

Mare was observed daily for visual signs of standing estrus. The ovaries were scanned by ultrasonography twice daily for at least 35 mm diameter of a preovulatory follicle detection.

4.2 Artificial Insemination technique

According to previously cycle record, ovulation was occurred from follicle with around 40 mm diameter. Thus, AI was performed once when the follicle was approximately 40 mm diameter and the uterus with slightly edematous endometrial gland was observed. The mare was secured in breeding stocks, the tail was wrapped and deflected to one side. One 0.5 ml straw of semen containing approximately 100 x 10^6 spermatozoa was thawed at 37 °C for 30 seconds, and then diluted into 30 ml of Kenney extender. Semen was inseminated using a sterile rigid insemination tube toward the uterine horn, in which ovulation was presumed to occur. Pregnancy diagnosis was performed at 18, 25 and 35 days after ovulation using ultrasonography.

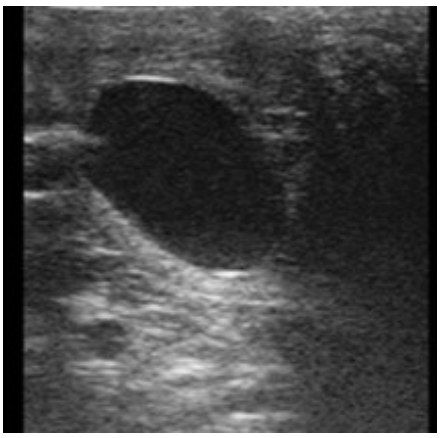
Result

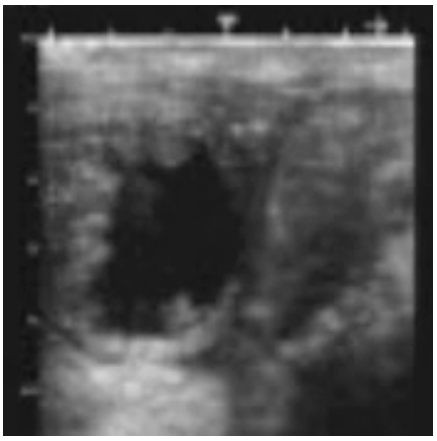
The color of collected semen was milky white. Gel free-volume, total motility (TMOT), progressive motility (PMOT), viability and concentration of fresh semen were 35 ml, 90%, 80%, 94% and 135 x10^6 sperm/ml respectively. The TMOT, PMOT, lived sperm and HOST positive membrane integrity of sperm before freezing (BF) and post-thawed (PT) were shown in Appendix Table 3.

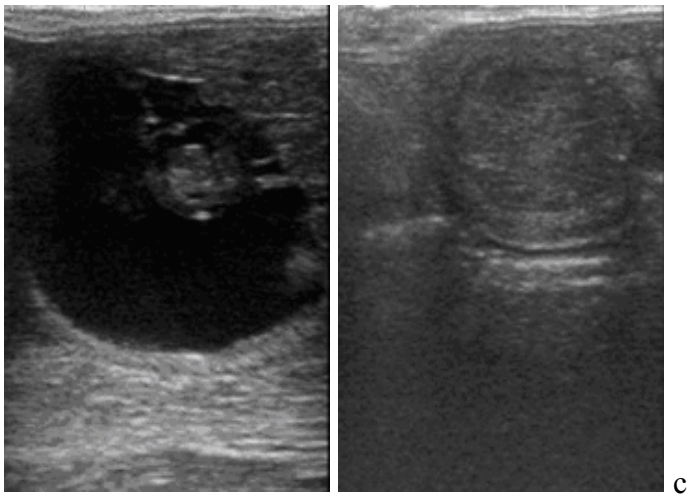
A dominant follicle was observed in the right ovary. Mare was inseminated once and ovulation was occurred within 6 hours after AI. A single embryo sac with embryo was detected in one uterine horn at 18^{th}, 25^{th} and 35^{th} day after ovulation (Appendix figure 4).

Appendix Table 3 Motility, motility pattern, lived and HOS test positive sperm before freezing (BF) and post-thawed (PT) stallion semen

Parameter	BF	PT
TMOT (%)	90	48
PMOT (%)	85	32
VAP (μm/s)	129.1	88.6
VSL(μm/s)	81.5	72.9
VCL(μm/s)	240.6	164.0
ALH	9.4	7.2
BCF (Hz)	38.7	41.5
STR (%)	60	76
LIN (%)	33	41
Area (μm^2)	5.5	5.4
Rapid cell (%)	86	40
Static cell (%)	1	51
Lived sperm (%)	94	47
HOST positive sperm (%)	78	40


a


b


c

Appendix Figure 4 Thai native crossbred embryo after insemination with frozen semen a) at 18 days; b) at 25 days; and c) at 35 days old and non-gravid uterine horn

Conclusion

In conclusion, the results of the present study demonstrated that Thai native crossbred stallion sperm could be frozen in Kenney extender with 3.5% glycerol. And resulted in pregnancy after inseminated with low insemination sperm number as 200 $x10^6$ sperm/ml in 0.5 ml straw at uterine horn with rigid tube in appropriate time. The pregnant mare is anticipated to parturient a foal around June, 2009. This will be the first report of a success using frozen semen for artificial insemination in a mare in Thailand.

CIRRICULUM VITAE

NAME	: Ms. Kanittha Phetudomsinsuk
BIRTH DATE	: August 1, 1977
BIRTH PLACE	: Bangkok, Thailand
EDUCATION	: **YEAR INSTITUTE DEGREE/DIPLOMA**
	2000 Kasetsart Univ. Bachelor's Degree
POSITION/TITLE	Lecturer
WORK PLACE	Department of Large Animal and Wildlife Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University
SCOLARSHIP/AWARDS	Kasetsart University