

Birth of the First Thai Native Cross-bred Foal Through Artificial Insemination with Frozen Semen

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ABSTRACT

Horse breeding has benefited widely from the use of frozen semen. This study investigated the usefulness of artificial insemination in the Thai native crossbred horse breed in order to increase reproductive success and improve breeding management and the genetics of the equine population in Thailand. Semen, collected from a 7-year-old Thai native crossbred stallion was frozen using the liquid nitrogen vapor freezing method. An insemination dose of 50×10^6 motile cells frozen semen was used in one intrauterine artificial insemination and resulted in the pregnancy and birth of the first healthy Thai native crossbred offspring.

Keywords: artificial insemination, frozen semen, foal, Thailand

INTRODUCTION

The Thai native crossbred horse is a pony-sized horse breed that may have originated from a Burmese pony (Panasophonkul *et al.*, 2007). However, the scientific origin of the breed remains obscure. The Thai native crossbred horse is generally used in religious ceremonies, especially in the central part of Thailand, such as in Suphanburi, Ratchaburi and Kanchanaburi provinces. The Thai native crossbred horse is used for recreational activities and occasionally for transportation in highland areas, such as in Chiang Rai, Chiang Mai and Lampang provinces. The number of Thai native crossbred ponies is

decreasing, as a result of crossbreeding with full-size purebreds, due to many owners preferring bigger horses.

Artificial insemination (AI) offers many advantages over natural mating, including increased safety for both mare and stallion, reduced risk of infectious disease transmission and decreased inconvenience of horse transportation. 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. The horse was the first domestic animal on which artificial insemination was practised and the first pregnancy from frozen

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stallion semen was reported in 1957 (Barker and Gandier, 1957). However the use of AI in equine breeding has increased slowly over the decades, due to issues associated with fertilizing capacity and the ability of semen to withstand cryopreservation procedures; subfertility is also common in stallions (Casey *et al.*, 1997) and about one third of stallion semen is largely unfreezable (Pickett and Amann, 1993; Vidament *et al.*, 1997).

A variety of semen extenders have been used for sperm suspension. Semen extenders are mainly based on either milk or egg yolk. Extenders with a composition similar to the original recipe published by Kenney *et al.* (1975) are most popular and are used worldwide. These extenders are inexpensive, easy to prepare, 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). An artificial insemination study in native crossbred horses is a useful method to improve equine breeding management in Thailand and other developing countries.

MATERIALS AND METHODS

Animals

Semen was collected from a Thai native crossbred stallion, aged 7 y that had been sexually rested for 1 week. A Thai native mare, approximately aged 7 y (based on an examination of the teeth) with a regular (21 d) estrous cycle was used; she had previously foaled in February 2007, five months before the commencement of the current study (Figure 1).

Semen collection and cryopreservation

Semen was collected using a Missouri-type artificial vagina while the stallion was mounting an estrous teaser mare. Immediately after dismount the ejaculate was initially evaluated for total volume, gel-free volume, concentration, and motility. The original motility of the sample needed to be least 50% to be considered acceptable for freezing in this project. The total number of spermatozoa was measured using a Neubauer counting chamber. After evaluation, semen was initially diluted at a ratio of 1:3 (semen:extender) in Kenney extender (Kenney *et al.*, 1975) and centrifuged at room temperature (30°C) at 400 x g for 10 min. The seminal plasma was removed and the spermatozoa pellets were resuspended in Kenney freezing extenders with 3.5% glycerol to

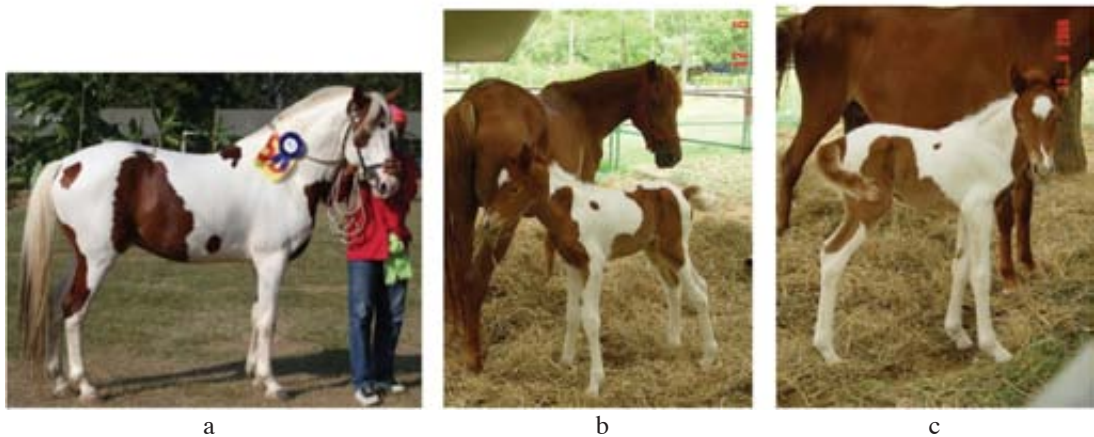


Figure 1 (a) Thai native crossbred stallion; (b) Thai native crossbred mare; and (c) the newborn colt.

a final concentration of approximately 200×10^6 sperm/mL. The diluted semen sample was placed at 5°C in a passive cooling device (Equitainer™) during transport to the laboratory (within 1 h of collection). The semen sample was equilibrated at 5°C for 2 h and then loaded into 0.5 ml polyvinylchloride straws. A diluted sample was taken for evaluation before freezing (BF). The sample was evaluated for motility, motion velocity, viability and membrane integrity. The straws were frozen in liquid nitrogen vapor, 3 cm above the liquid nitrogen level for 10 min and then submerged into liquid nitrogen (-196°C) for storage.

Semen analysis

Frozen semen was thawed in a water bath at 37°C for 30 sec. Experimental endpoints included total sperm motility (TMOT; %), progressive motility (PMOT; %), curvilinear velocity (VCL; $\mu\text{m/s}$), linear velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %) 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, 10 $\mu\text{m/s}$; cut-off for progressive cells, 15 $\mu\text{m/s}$; VSL cut-off, 0 $\mu\text{m/s}$; and straightness, 60%. The slow cells were considered static. A 3- μL drop of each sample was placed on a preheated (37°C) 2X cell chamber (20 mm depth). The functional plasma membrane integrity of frozen-thawed semen was evaluated with the hypoosmotic swelling test (HOS test; Neild *et al.*, 1999). Eosin-nigrosin staining was used to evaluate live and dead sperm (William, 2003).

Estrus detection

The mare was observed once daily for visual signs of standing estrus with a teaser. The behavioral signs of estrus include winking of the vulva, urination, squatting and seeking the stallion. After the mare showed estrus signs, ultrasonography was utilized for estrus detection and determination of ovulation. The ovaries were monitored rectally using an ultrasound scanner (Aloka, SSD 500, Japan). The scanner was equipped with a 5 MHz sector transducer. Both ovaries were scanned to determine in which ovary the preovulatory follicle was located and scanned continuously every 12 h until follicles reached a diameter of 40 mm. Continuous scanning was carried out for 6 h until the disappearance of the follicle, which marked the ovulation time. Based on the previous scanning record, the follicle reached a diameter of 40 mm, and then softened and the uterine endometrial gland was slightly edematous, with ovulation following within 6 h. Thus, the mare was inseminated when the follicle reached 40 mm in diameter.

Artificial insemination technique and pregnancy confirmation

AI was performed once when the follicle was approximately 40 mm diameter and the uterus was observed to have a slightly edematous endometrial gland. The mare was secured in breeding stocks and the tail was wrapped and deflected to one side. Frozen samples were kept under liquid nitrogen pending insemination. For thawing, one 0.5 ml straw of semen containing approximately 100×10^6 spermatozoa was plunged into a water bath at 37°C for 30 s, and then diluted into 30 ml of Kenney extender. Following thawing, the diluted sample was kept in the water bath at 37°C pending evaluation and artificial insemination. Semen were inseminated using a sterile 65 cm insemination pipette (universal pipette, Minitube, USA) toward the uterine horn, in which ovulation was presumed to occur.

Pregnancy diagnosis was evaluated with the aid of ultrasound at 18, 25 and 35 d after ovulation.

RESULTS

The color of the collected semen was milky white. The gel free-volume (mL), total motility (TMOT, %), progressive motility (PMOT, %), viability (%) and spermatozoa concentration ($\times 10^6/\text{ml}$) were 35, 90, 80, 94 and 135, respectively. The TMOT, PMOT, live sperm and HOST positive membrane integrity of sperm before freezing (fresh semen) and frozen-thawed are shown in Table 1.

A dominant follicle (40 mm in a diameter) was observed in the right ovary. The mare was inseminated once and ovulation occurred within 6 h after AI. A single embryo sac with embryo was detected in the left uterine horn at day 18, 25 and 35 after ovulation.

After a gestation period of 319 d, the pregnant mare foaled a colt (male foal) on 17 June 2009. The newborn colt was healthy and stood within 10 min. The colt was white-brown in color and had a bodyweight of 37 kg and a height of 83 cm (Figure 1).

DISCUSSION

The variation in the freezability of semen between stallions depends on the quality of the raw semen, the freezing extender and the freeze-thaw process used with the semen (Watson, 1995). Accordingly, in the current study, the main ingredient of the semen extender was milk, with a composition similar to the original recipe published by Kenney *et al.* (1975). This extender is the most popular and is used worldwide, because it is inexpensive, easy to prepare, can be stored under frozen conditions and results in an acceptable fertility rate. The initial motility of spermatozoa needed to be at least 50% to be considered acceptable for freezing in the current project in order that motility after thawing could produce average (30-35% post-thaw motility) to good (>35% post-thaw motility) sperm (Khelifaoui *et al.*, 2005).

The minimum insemination dose of 500 million progressively motile spermatozoa (PMS) established in these studies has been widely used as an industry standard. Clinical observations suggest that this insemination dose provided a conservative and effective insemination dose for

Table 1 Quality of fresh and frozen-thawed semen from the stallion used in the study.

Parameter	Fresh semen	Frozen-thawed semen
Total sperm motility (TMOT; %)	90	48
Progressive motility (PMOT; %)	85	32
Average path velocity (VAP; $\mu\text{m/s}$)	129.1	88.6
Linear velocity (VSL; $\mu\text{m/s}$)	81.5	72.9
Curvilinear velocity (VCL; $\mu\text{m/s}$)	240.6	164.0
Amplitude of lateral head displacement (ALH)	9.4	7.2
Beat cross frequency (BCF; Hz)	38.7	41.5
Straightness (STR; %)	60	76
Linearity (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

the mare. Other authors have suggested that individual stallions may require fewer sperm for maximal fertility (100 to 250 million PMS) and that adjustment for the percentage of morphologically normal sperm may be desirable (Troedsson *et al.*, 1998). Low-dose insemination techniques in the mare are based upon both hysteroscopic and deep intrauterine techniques for the deposition of fresh, chilled, cryopreserved as well as sex-sorted sperm. In a retrospective analysis of breeding records, 50×10^6 progressive motile cell frozen semen inseminated after 12 and 24 h human chorionic gonadotropin administration resulted in a success rate of 63% (7/11) (Petersen *et al.*, 2002). Based on the results of the current study, the pregnancy was successful after only one insemination with 50×10^6 motile cells of frozen semen that was performed pre-ovulation.

For artificial insemination with frozen semen, ovulation timing is highly desirable in order to reduce the interval between breeding and ovulation. To increase accuracy in the timing of breeding, the insemination time was examined by transrectal palpation and ultrasonography every 8 h when the mare was displaying behavioral estrus and had a follicle size greater than 35 mm. However, ovulation-inducing agents, such as human chorionic gonadotropin (hCG) or the gonadotropin releasing hormone (GnRH) analogue, deslorelin, are considered critical components required to accurately time insemination with frozen semen (Samper, 2001).

In a retrospective analysis of breeding records, a single dose of frozen-thawed semen inseminated within 6 h post-ovulation resulted in a pregnancy rate of 45% (67/149) detected at 14–16 d after AI (Miller, 2008). Sieme *et al.* (2004) reported a pregnancy rate of 37.2% (16/43) resulting from AI using >35% motility of frozen-thawed semen once per cycle, and insemination at 30 h after hCG administration. Based on the result of the current study, pregnancy was successful from only one insemination at 6 h

before ovulation and without using any ovulation inducing agent. However, further study on the pregnancy rate should be considered.

The gestation period in the current study of AI in a Thai native crossbred mare was shorter than the average gestation length produced by natural breeding. However, the gestation period of a pony mare is always shorter than for a full-size mare (Michelle *et al.*, 2000).

Foal birth weights are perceived to be important within the commercial Thoroughbred industry of Australia, as foal birth weight is commonly thought to be associated positively with size as a yearling. There is a significant positive correlation between birth height and mature height, from which it was concluded that birth height could be used to predict mature height accurately (Reed and Dunn, 1977). However, birth weight and birth height measurement, and the relationship between these two measurements have not been investigated in horses in Thailand. The birth weight of AI derived colt in the current study was lower than for Thoroughbred colts, but this might be attributed to different breeds and nutritional conditions.

CONCLUSION

The results of the present study demonstrate that a normal foal can be produced from sperm that was collected from a Thai native crossbred stallion, frozen and then used via AI to insert low-dose motile spermatozoa into the uterine horn of the mare, where ovulation was presumed to occur. This is the first report of a successful foaling from artificial insemination using frozen semen in an equine species in Thailand.

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LITERATURE CITED

- 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.
- 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.
- 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.
- 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.
- 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.
- Michelle, M.L., R.J. Rose and D.R. Hodgson. 2000. Reproduction, pp. 341-380. *In* R.J. Rose, and D.R. Hodgson (eds.). **Manual of Equine Practice**. 2nd eds. W.B. Saunders company. Philadelphia, USA.
- Miller, C.D. 2008. Optimizing the use of frozen-thawed equine semen. **Theriogenology** 70: 463-468.
- Neild, D., G. Chaves, M. Flores, N. Mora, M. Beconi and A. Aguero. 1999. Hypoosmotic test in equine spermatozoa. **Theriogenology** 51: 721-727.
- 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.
- 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.
- Petersen M.M., M.T. Wessel, M.A. Scott, I.K.M. Liu and B.A. Ball. 2002. Embryo recovery rates in mares after deep intrauterine insemination with low numbers of cryopreserved equine spermatozoa. **Theriogenology** 58: 663-665.
- Reed, R. and N. Dunn. 1977. Growth and development of the Arabian horse, pp. 76-98. *In* **Proceedings of the Fifth Equine Nutrition Physiology Symposium**.
- Samper, J.C. 2001. Management and fertility of mares bred with frozen semen. **Anim Reprod Sci.** 68: 219-228.
- Sieme, H., A. Bonk, H. Hamann, E. Klug and T. Katila. 2004. Effects of different artificial insemination techniques and sperm doses on fertility of normal mares and mares with abnormal reproductive history. **Theriogenology** 62: 915-928.
- Troedsson, M.H.T., I.K.M. Liu and B.G. Crabo. 1998. Sperm transport and survival in the mare. **Theriogenology** 49: 905-915.
- 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.
- 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.
- William, M.B. 2003. Predictive value of hypoosmotic swelling test to identify viable non-motile sperm. **Asian. J. Androl.** 5: 209-212.