Original Articles

The Effect of Cold Storage on the Quality of Stallion Semen and Pregnancy Rate after Artificial Insemination

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Abstract

While the use of frozen-thawed semen has been hampered by poor cryopreservability of equine sperm, the technique of choice in equine practice remains artificial insemination (AI) using cold storage semen. This study examined the effect of cold storage on semen quality and pregnancy rate after AI. In experiment I, ejaculates were collected from 3 stallions of proven fertility and then diluted to a final concentration of 25-50 x 10^6 sperm/ml. After transportation, the semen was then divided into two aliquots. The first part of semen was centrifuged to partially remove the seminal plasma, while the remaining was left intact with seminal plasma. Subsequently, the semen was slowly cooled and maintained at 4 ^oC for 24, 48, 72, 96 and 120 h where quality of semen was examined. At 6 h and 72 h post cold storage, spermatozoa were also morphologically analyzed. Experiment II aimed to examine fertilizability of equine sperm *in vivo*. Collected semen was stored at 4 ^oC for 6, 24 and 48 h and then used for AI. Fresh semen extended with semen extender without cooling (0 h) served as a control.

Overall, viability and progressive motility of equine sperm were dramatically decreased during cold storage, while partial removal of seminal plasma improved sperm viability. Of stallions used, two were classified as "cold tolerant" stallions, judged by progressive motility and viability that were greater than 50% after cold storage for 48 h. When cold semen (6, 24 and 48 h storage times) was inseminated to estrus mares (n=21), fourteen (66.7%) mares were pregnant, and this did not significantly differ from controls (7/12: 58.3% pregnancy rate). We concluded that stallion semen can be cooled to 4 ^oC while sperm retain their viability and fertilizability. It is suggested that stallion semen to be used as cold-transported semen should be tested prior to use. Preserving stallion semen at 4 ^oC for longer than 48 h is likely to be detrimental.

Keywords : stallion, sperm, cold storage, viability

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บทคัดย่อ

ผลของการแช่เย็นต่อคุณภาพน้ำเชื้อม้าและอัตราการตั้งท้องภายหลังการผสมเทียม

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เนื่องจากน้ำเชื้อม้ามีความคงทนต่อการแช่แข็งต่ำ การผสมเทียมม้าในภาคสนามจึงนิยมใช้น้ำเชื้อแช่เย็น การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบผลของการแช่เย็นต่อคุณภาพน้ำเชื้อและอัตราการผสมติดภายหลังการผสมเทียม ทำการรีดเก็บน้ำเชื้อจากม้าสมบูรณ์พันธุ์จำนวน 3 ตัว จากนั้นนำมาเจือจางจนได้ความเข้มข้นของอสุจิ 25-50 x 10^6 ตัว/มล. สำหรับการทดลองที่ 1 นั้น น้ำเชื้อจะถูกแบ่งออกเป็น 2 ส่วน คือ ส่วนที่ถูกปั่นเหวี่ยงเพื่อแยกน้ำเลี้ยงอสุจิออกและส่วนที่ยังมีน้ำเลี้ยงอสุจิอยู่ นำน้ำเชื้อทั้ง 2 ส่วนแช่เย็นที่ 4 องศาเซลเซียส เป็นเวลานาน 24, 48, 72, 96 และ 120 ชั่วโมง เพื่อตรวจคุณภาพอสุจิ (การเคลื่อนที่ไปข้างหน้าและอัตราอสุจิมีชีวิต) และทำการตรวจความปกติของอสุจิส่วนหัวและหางภายหลังการแช่เย็นน้ำเชื้อที่ 6 และ 72 ชั่วโมง การทดลองที่ 2 มีวัตถุประสงค์เพื่อตรวจอัตราการตั้งท้องภายหลังการผสมเทียมด้วยน้ำเชื้อแช่เย็นนาน 6, 24 และ 48 ชั่วโมง โดยใช้น้ำเชื้อสดที่ผสมน้ำยาเจือจางน้ำเชื้อโดยไม่ผ่านการแช่เย็นเป็นกลุ่มควบคุม

การเคลื่อนที่ไปข้างหน้าและอัตราอสุจิมีชีวิตลดลงเมื่อทำการแช่เย็นน้ำเชื้อ ในขณะที่การปั่นเหวี่ยงเพื่อแยกน้ำเลี้ยงอสุจิออกก่อนการเก็บรักษาน้ำเชื้อแช่เย็นจะช่วยเพิ่มคุณภาพอสุจิ จากการศึกษาพ่อม้าจำนวน 2 ตัวถือว่าเป็นพ่อม้าที่ให้น้ำเชื้อที่ทนต่อความเย็น โดยตัดสินจากร้อยละของอสุจิที่เคลื่อนที่ไปข้างหน้าและอัตราอสุจิมีชีวิตที่ค่ามากกว่า 50 ภายหลังการแช่เย็นน้ำเชื้อนาน 48 ชั่วโมง เมื่อนำน้ำเชื้อที่แช่เย็นนาน 6, 24 และ 48 ชั่วโมง ไปผสมเทียม จากการผสมเทียมทั้งหมด 21 ตัว แม่ม้าจำนวน 14 ตัวตั้งท้อง (66.7%) ซึ่งอัตราการตั้งท้องนี้ไม่มีความแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม (7/12: 58.3%, P > 0.05) การศึกษาในครั้งนี้สรุปได้ว่า สามารถทำการแช่เย็นน้ำเชื้อม้าที่ 4 องศาเซลเซียสโดยที่อสุจิยังคงมีชีวิตและมีความสามารถในการปฏิสนธิ ควรทำการทดสอบประสิทธิภาพของการเก็บรักษาน้ำเชื้อแช่เย็นก่อนนำน้ำเชื้อไปใช้ในภาคสนาม และการแช่เย็นน้ำเชื้อนานมากกว่า 48 ชั่วโมงอาจมีผลเสียต่อคุณภาพอสุจิ

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Introduction

Assisted reproduction techniques (ART) have been well established in both human and animals, aimed specifically to increase reproductive efficiency and to treat some fertility problems. Although many ART techniques such as artificial insemination (AI) and embryo transfer have recently been applied in equine industry, these techniques have met only limited success (Squires, 2005). AI is a method of choice to allow genetic improvement of valuable stallion. Semen using for AI can be broadly divided into three categories: fresh, cold and frozen semen. It is important to note that viability of raw or fresh semen decreases rapidly, and this semen must be used within 30-60 min (Brinsko and Varner, 1992). However, when stallion semen is extended with suitable semen extender and cooled slowly to 4-6 ^{o}C, sperm viability was acceptably maintained for up to 72 h (Heiskanen et al., 1994). Indeed, it would be the best to cryopreserve-thaw stallion semen and use when required as it is routinely employed in bovine. Cryopreservation of stallion semen has, however, been considerably difficult because stallion spermatozoa are fairly sensitive to cryopreservation (% progressive motility <40 %) and pregnancy rate of mares inseminated with frozen-thawed semen is critically low (Samper, 2001). This is likely because cryopreservation induces capacitation-like reaction,

thereby reducing sperm longevity (Watson, 2000). In addition, mare exhibits estrus for approximate 1 week, and it is relatively difficult to inseminate the mare close to the time of an ovulation.

To date, the use of cooled semen for AI is increasing because many stallions (20-40%) respond poorly to cryopreservation (Vidament et al., 1997). Unfortunately, pregnancy rate after insemination with cold semen has also been variable (Katila, 1997), and the reason remains unclear. Several factors contributing to the success of cold storage of stallion sperm have been studied. For example, a number of studies have demonstrated that suitable temperature for maintaining viability of equine semen should be at 4-6 °C (Varner et al., 1988; 1989, Moran et al., 1992) or at 15-20 °C. However, a latter storage temperature seemed suitable only for some stallions (Francl et al., 1987, Magistrini et al., 1992).

Among a large variety of extenders currently used, milk and milk based extender have intensively employed because it is easy to prepare and less expensive. Kenny's (non-fat dry milk glucose-based extender) and E-Z mixin® semen extenders maintain sperm viability (Batellier et al., 1997) and result in 60-70% pregnancy rate similar to non-cooled semen (Squires et al., 1998), although biological properties of milk may be variable between bathes (Batellier et al., 2001). In addition, ejaculated semen *per se* can also be detrimental by unknown substances in seminal plasma. It has been shown that centrifugation and partial removal of seminal plasma from sperm prior to cold storage improved longevity of equine sperm especially for poor cooler stallion (Brinsko et al., 2000).

It is therefore very important to standardize and to optimize techniques for preserving stallion semen especially in Thailand, where the efficiency of cooled semen in practice has not yet been tested. This study aimed to examine the effect of cold-storage techniques on viability of equine sperm in terms of sperm quality and pregnancy rate after AI.

Materials and methods

Semen collection and handling

The stallions used in this study were selected by previous fertility records and semen quality, such that the percentages of progressive motility and normal sperm morphology (sperm head, midpiece and tail morphology) must be greater than 50 % (Table 1). The ejaculates from 3 fertility proven stallions, aged from 6-10 years old, were collected using artificial vagina (AV, Hanover, Germany). The AV was filled with 45-50 °C water and the pressure was also adjusted to be optimal for each individual stallion. The stallions were then allowed to mount either on phantom or on estrus mares. Soon after semen collection, the semen was filtered through sterile gauze to remove gel fraction which may decrease sperm motility. The semen was then examined for basic semen characteristics that included color, pH, volume, sperm motility and concentration. Subsequently, semen was then diluted with non-fat dry milk glucose based extender (Modified Kenny's extender, pH 7.3, 350 mOSm) in order to prolong the longevity of sperm before cooling and to adjust the sperm concentration to 25-50 x 10^6 cells/ml. The extended sperm was transported in an insulating box at approximately 4 °C to the laboratory within 6 h.

Assessing sperm quality in vitro

When required, cooled semen was removed from refrigerator (4 °C) and maintained at room temperature for 10 min. A 10 µl droplet of semen was then placed on a pre-warmed glass microscopic slide and subjectively examined for progressive motility using a phase contrast microscope (CH2, Olympus, Japan). To assess sperm viability, the semen sample was stained with aniline-blue eosin, smeared on a glass microscopic slide and examined at x 100 magnification. A total of 200 sperm cells from each sample were counted. The sperm positive with aniline-blue eosin (red color) were classified as damaged plasma membrane or dead sperm, while "non-stained" sperm indicated the intactness of sperm plasma

membrane or viable sperm.

Additionally, sperm prior to or after 72 h of cold storage were morphologically examined for head and tail abnormality using William's staining and wet smear with formal saline solution, respectively.

Artificial insemination and pregnancy determination

This part of the study was performed to examine the fertilizability of stallion semen after cold storage. Semen from fertility-proven stallion was collected and cooled to 4 °C as described previously. The semen were then divided and inseminated to fertile mares at 0, 6, 24 and 48 of cold storage. The AI technique has been described elsewhere. In brief, mare's ovaries were examined by rectal palpation and real-time ultrasonography equipped with a 5 MHz array transrectal probe (Aloka, Japan). If corpus luteum was present in the ovaries, the mares were then injected with 526 μg of cloprosternol (Estrumate, Schering-Plough Animal Health) to induce luteolysis. The ovulation was either estimated by an experience surgeon or induced by 1500-3000 IU human chorionic gonadotrophin (hCG, Intervet, Thailand). For artificial insemination, cold semen containing with ≥ 500 progressively motile sperm were loaded into a 20 ml syringe and artificial insemination was performed by depositing semen at uterine body through a 22-inch plastic pipette. The ovulation was determined daily after insemination. The mares that did not ovulate by 2 days after insemination were excluded from the experiment. The pregnancy tests were performed on 14-35 days post-ovulation. The pregnancy rates of the first cycle of insemination were only recorded and used.

Experimental design

Experiment I: effect of cold storage on *in vitro* semen quality

The experiment was conducted during November to December to avoid the effect of season on semen characteristics and quality. The semen collected from 3 fertility proven stallions was diluted with semen extender and transported at 4 °C to the laboratory. Within 6 h post-semen collection, the semen from each stallion was divided into two parts. The first part of semen was centrifuged at 300 g for 6 min to remove approximately 90% of seminal plasma, while remaining part of semen was left intact with seminal plasma (as during transportation). The centrifuged and non-centrifuged semen were maintained at 4 °C. The semen were sampled every 24 h for 5 days to examine the motility and viability of spermatozoa.

Experiment II: fertility assessment of cold storage semen

This study was performed to test fertility of cooled semen by means of pregnancy rate after artificial insemination. Because it is not practical in stud farm to remove seminal plasma from the semen prior to cold storage, and experiment I demonstrated that if semen is not preserved for longer than 48 h, centrifugation to partially remove seminal plasma may not be necessary. Fertility data (pregnancy rate) in this study was only obtained from mares inseminated with non-centrifuged cold semen (semen from stallion No. 2 and 3). After estimation of ovulation time, mares were inseminated with cooled semen that had been stored at 4 °C for 6, 24 and 48 h. Fresh ejaculates extended with semen extender without cooling (0 h) served as controls.

Statistical analysis

In experiment I, semen were collected from 3 fertility proven stallions and used for semen quality analysis. Data of each stallion was obtained from 3 independent replications (ejaculations). Progressive motility and viability of cooled sperm within and between stallions were compared using either Student's t or one-way ANOVA test. For fertility result (experiment II), pregnancy rates and storage times were compared by Chi-square statistical test. In all cases, statistical analysis was performed using SPSS for windows (SPSS Inc, Chicago, IL, USA) and differences were considered

statistically significant when $p<0.05$.

Result

After semen collection, basic semen characteristics including volume, pH, semen concentration and % normal morphology sperm were examined (Table 1). The mean percentage of progressive motile and viable sperm did not differ among the stallions (motility ~75 % and 70 % for motility and viability, respectively). In general, the motility and viability of sperm decreased dramatically during cold storage.

Experimental I

The motility and viability of equine sperm decreased rapidly during 24 h of storage (Figures 1, 2, 3 and 4), this was unlikely related to the time acquired during semen transportation since the viability and progressive motility did not significantly differ between before and after 6h transportation ($p>0.05$). With regard to the progressively motile sperm, the semen from stallion No. 2 and 3 gave similar results and could be preserved at 4 °C for 48 h with acceptable sperm motility of 44% (Figure 1), while progressive motility of semen from stallion No.1 was poor (% motility = 11.2). Indeed, removal of semen extender by centrifugation improved the success of cold storage (Figure 2). For instance, semen from No. 2 and 3 stallions remained at high motility and viability after 48 h storage (61% and 72% respectively; Figures 2 and 4) while survival of sperm from stallion No.1 was improved when the semen was stored at 4 °C with minimal amount of seminal plasma (centrifugation group, progressively motile = 31.7% compared favorably to 11.2% of non-centrifugation group). In practice, semen used for artificial insemination should contain with high percentage of progressively motile sperm (usually ~50 %), preserving sperm for more than 48 h without centrifugation treatment would not therefore be acceptable. Only semen from centrifugation group maintained motility and viability above 50 % at 72 h post cold storage, regardless the "cool-intolerant" stallion (stallion No. 1). To examine the effect of cold storage on sperm morphology, sperm morphology was compared between before and after 72 h of storage (both with centrifuged- and non-centrifuged groups). Cooling semen up to 72 h at 4 °C did not significantly affect the percentage of normal morphology sperm (Figure 5).

Experimental II

A total of 33 mares were inseminated with semen that had been cooled for 0, 6, 24 and 48 h. Of 21 inseminated with cold semen, fourteen mares (66.7%) were pregnant on day 14-35 (Figure 6), and this did not significantly differ from controls (7/12: 58.3%; $p>0.05$). Preserving stallion semen by means of cold storage at 4 °C for up to 48 h did not significantly affect pregnancy rate of mares ($p>0.05$, Table 2).

Discussion

This study examined the effect of short-term storage of stallion semen by means of cold storage for up to 120 h. Indeed, sperm survival rate in vitro (% progressive motility and % alive sperm) decreased dramatically over storage times. Although mechanism underlying cold shock is not well understood, losing of phospholipids in sperm plasma membrane during cold storage is hypothesized to be detrimental to sperm viability. Plasma membrane of equine sperm is composed of a large amount of unsaturated fatty acids, equine sperm is therefore prone to suffer from oxidative stress (Kodama et al., 1996; Neild et al., 2005) that may induce poor sperm viability. In accordance with other publications, it is clearly evident that there is an effect of individual stallion in preserving viability of cold storage sperm (Katila, 1997; Aurich and Spergser 2007), albeit a small number of stallions were used in the current study. Soon after ejaculation, sperm plasma membrane undergoes a series of changes in order to modify its plasma membrane suitable for normal fertilization such as a depletion of cholesterol/phospholipid ratio (Gadella et al., 2001). It is worth to note that cholesterols are not evenly distributed

Table 1 Semen characteristics of stallion semen. Data (mean ± SD) of each stallion was collected and pooled from 3 independent ejaculations.

Horse no.	Volume	pH	%Motility	%Viability	Concentration	% Normal morphology	
					x 10^6 sperm/ml (range)	Head	Tail
1	45.0±8.7	7.3±0.2	81.7±2.9	77.1±4.8	432 (207-517)	84.1±9.2	55.7±9.5
2	36.0 ± 7.9	7.6±0.4	75.0±5	71.0±6.1	361.7 (350-367)	79.7±6.8	65.7±7.9
3	37.7±10.8	7.6±0.5	75.0±10	74.2±5.3	365.7 (268-461)	80.7±6.7	72.5±5.3

Table 2 Pregnancy rates of mares inseminated with semen that had been cooled at 4°C for 0, 6, 24 and 48 h.

Storage times	No. of inseminated mares	No. of pregnant mares (%)
0h	12	7 (58.3)
6h	3	2 (66.7)
24h	8	3 (37.5)
48h	10	9 (90)
Total	**33**	**21(63.6)**

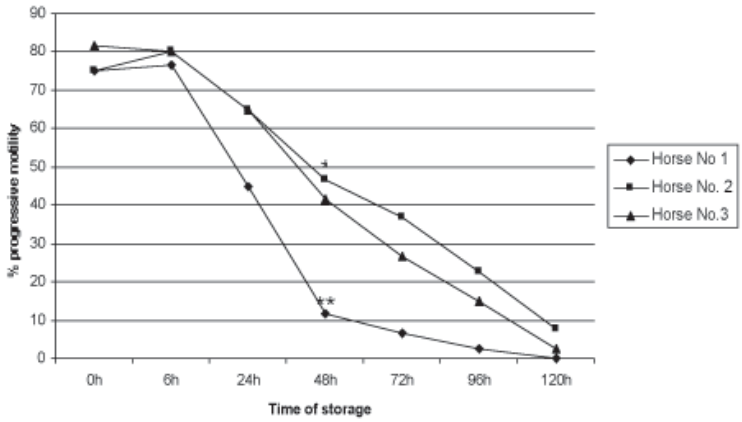

Figure 1 Means of progressive motility during 5 days of cold storage (non-centrifugation). The seminal plasma was left intact with sperm. *, ** Within storage time, different superscripts denote values that differ significantly (p<0.05).

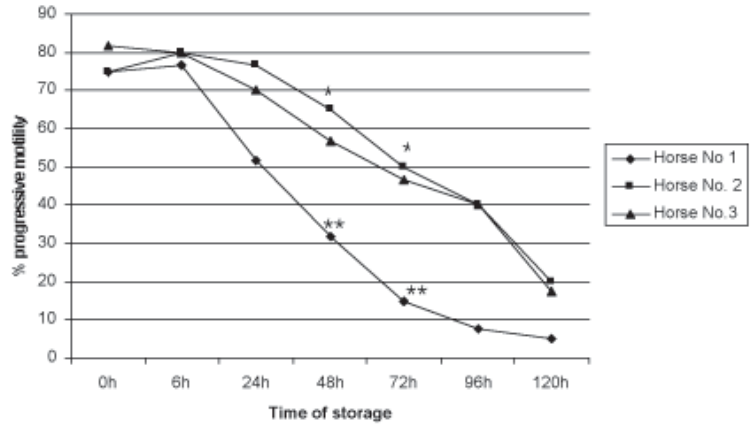

Figure 2 Means of progressive motility during 5 days of cold storage (seminal plasma was removed prior to cold storage). *, ** Within storage time, different superscripts denote values that differ significantly (p<0.05).

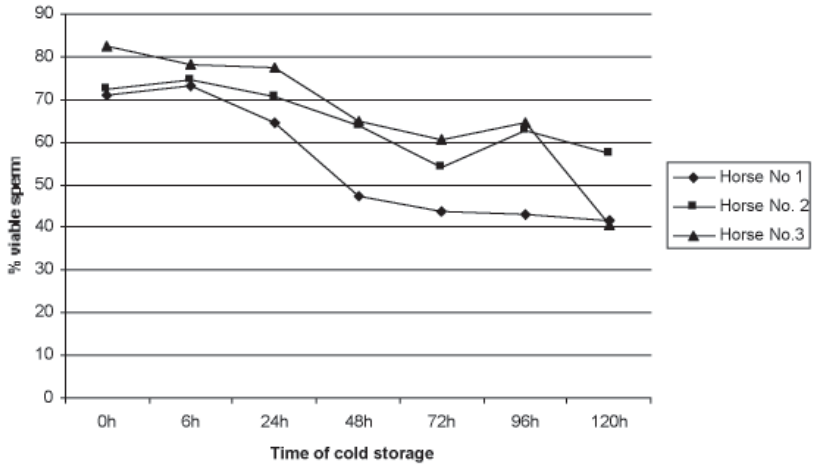

Figure 3 Viability of equine sperm during 5 days of cold storage (non-centrifugation).

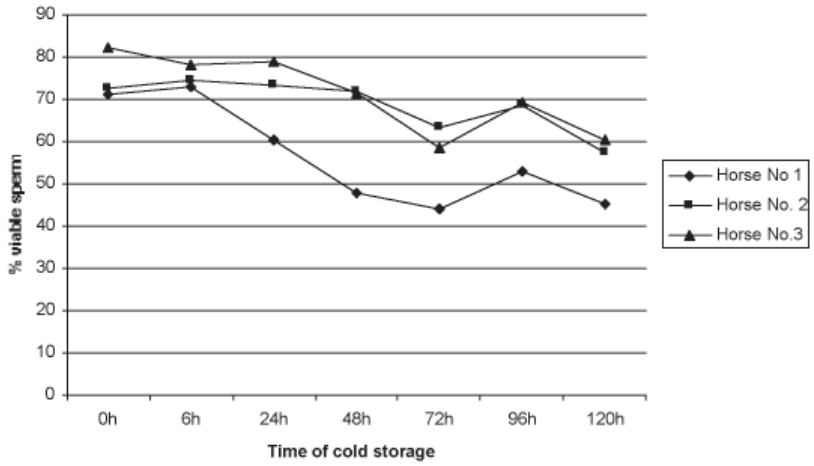

Figure 4 Viability of equine sperm during 5 days of cold storage (seminal plasma was removed prior to cold storage).

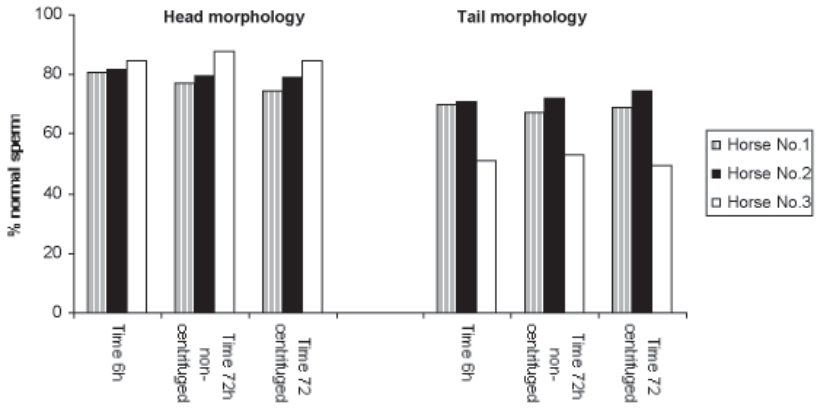

Figure 5 Microscopic morphology of equine sperm before and after cold storage.

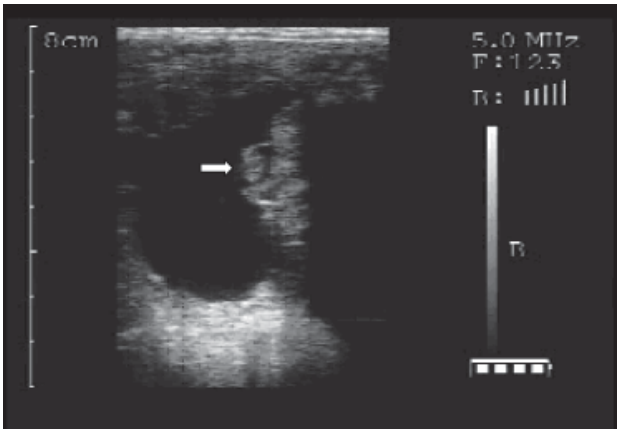

Figure 6 Ultrasound image demonstrating a pregnant uterus (day 35) filled with embryonic sac with a viable embryo (arrow).

and amount of cholesterols may differ among individual stallion. This appears to result in different sensitivity to cold stress among stallions. It is therefore advisable for "poor cooler" stallion that the semen can alternatively be stored at 15 °C rather than 4 °C (Batellier et al., 2001) or use for insemination soon after semen collection.

Moreover, because seminal plasma blocks cholesterol efflux from the sperm plasma membrane by cholesterol rich vesicles originating from the prostate glands (Minelli et al., 1998), removal of seminal plasma has been used as a strategy to improve viability of equine sperm during cold storage. While viability and motility of a "poor cooler" stallion (stallion No 1.) was markedly reduced when stored at 4 °C, partial removal of seminal plasma prior to cold storage decreased cold sensitivity, and thereby improving sperm survival rates. Removal of all seminal plasma from ejaculate stallion semen is, however, detrimental to sperm viability during cold storage (Jasko et al., 1992), suggesting that a little amount of seminal plasma is required.

Until recently, although cold storage of stallion semen has been widely used in equine practice, its efficiency remains variable between laboratories (Katila, 1997) due principally to several factors such as cooling rate (Moran et al., 1992), type of semen extender (Rigby et al., 2001) and antimicrobial agents used (Varner et al., 1998; Aurich and Spergser, 2007). Moran et al. (1992) examined the effect of cooling rates on sperm viability and reported that the temperature of semen should be reduced slowly through critical temperature zone (8 °C to 19 °C) at a rate of 0.05 °C/min. However, reducing temperature of stallion semen at this rate is only achieved by active cooling system. Passive cooling system as used in this study also provided acceptable sperm viability and pregnancy rates (progressive motility and pregnancy rate > 50% during 48 h of cold storage), although the rates of heat transfer during cold storage in the current study were not examined. To our knowledge, effect of cold storage on stallion's semen quality has not yet been demonstrated in Thailand. As semen is a potential source of bacterial contamination of the mare's reproductive tract and type of antibiotic employed affects sperm viability during cold storage (Aurich and Spergser, 2007), it is also essential to examine whether antibiotics used in this study (penicillin and streptomycin) is suitable for preserving stallion semen in terms of its effectiveness to sufficiently inhibit bacterial growth.

In experiment II, we examined the effect of cold storage on fertilizability by means of pregnancy rate after artificial insemination. Although experiment I demonstrated that viability and progressive motility of spermatozoa remained approximately 50% (centrifugation group) after cold storage for 72 h, equine semen is usually stored for only 48 h in practice and preserving semen for longer than 48 h may be detrimental. The ideal insemination would be the mares that ovulate soon before or after ovulation, although stallion sperm survive in female reproductive tract for a few days (Troedsson et al., 1998). The 48 h storage time is therefore sufficient, in practice, to allow insemination to be performed every 2 days. Pregnancy rates (~60%) obtained from cold semen in this study were similar to other reports (Shore et al., 1998, Squires et al., 1998) except that pregnancy rate of mares inseminated with 24 h stored semen was unfortunately low (37.5%). This could be explained by intrinsic factors such as susceptibility of mare's uterus to inflammation/ infection and poor sperm transport since semen quality

prior to insemination was satisfactory (% progressive motility > 50%) and high pregnancy rates could be achieved in 48 h cold storage group.

In conclusion, "cool tolerant" stallion semen can be stored at 4 °C for up to 48 h, while viability and fertility are in an acceptable range. Centrifugation of semen to partially remove the seminal plasma improves longevity and quality of stallion spermatozoa, especially when long-term storage (48-72 h) is required.

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