

Optimizing the use of frozen–thawed equine semen

C.D. Miller*

Equine Medical Center of Ocala, 7107 West Highway 326, Ocala, FL 34482, United States

Abstract

This manuscript is a review of current protocols, advantages, and disadvantages of breeding mares with frozen–thawed equine semen. Issues affecting pregnancy rates are discussed, including proper mare selection, induction of ovulation, insemination dose, timing of insemination (single-dose versus multiple-dose insemination), methods of insemination (transrectal-guided deep-horn versus hysteroscopic insemination), and post-insemination mare management procedures. In a retrospective analysis of breeding records, a single-dose of frozen–thawed semen was inseminated within 6 h post-ovulation; the pregnancy rate (14–16 days after AI) was 67 of 149 (45%). These results were comparable to those previously achieved under commercial conditions, as well as previous studies using multiple doses of frozen–thawed semen per estrous cycle. In conclusion, these data provided evidence that, with appropriate breeding management, an acceptable pregnancy rate can be achieved in mares with a single-dose of frozen–thawed semen (per-cycle) inseminated within 6 h after ovulation.

© 2008 Elsevier Inc. All rights reserved.

Keywords: Insemination; Spermatozoa; Stallion; Cryopreserved semen; Frozen semen

1. Introduction

The first foal was born using frozen–thawed semen in 1957 [1]. Since then, scientific advances have made cryopreservation of equine semen an international success. Every year, more and more mares are being bred with cryopreserved semen within the USA and abroad. Although breeding with frozen–thawed semen has tremendous advantages to the mare and stallion owner, there are also considerable challenges for breeding management of mares. This article will address the advantages and disadvantages of frozen–thawed semen, and discuss current protocols, including suggestions to improve success.

2. Advantages of frozen semen

Frozen semen adds a new dimension to the horse breeding industry by enabling long-term preservation of spermatozoa from stallions and permitting distribution of this semen to breeding farms worldwide. This advantage maximizes utilization of premiere stallions and greatly reduces mare shipping and boarding costs, as well as the potential for transmission of contagious diseases. Geographical constraints are abolished; semen can be selected from a much larger pool of stallions, including deceased stallions. Additionally, frozen semen can be used to breed mares even when the stallion is at performance events, ill, or recovering from an injury. Frozen semen can also be stored as insurance against injury to the stallion or his untimely death. A final advantage is that it can eliminate collecting and shipping issues, because frozen semen can be shipped and stored where the mare will be bred prior to her coming into estrus. By having the semen stored at the

* Tel.: +1 352 873 7830; fax: +1 352 873 7700.

E-mail address: CMi1047690@aol.com.

facility, mare owners no longer have to worry about “missing” the mare due to a stallion’s collection schedule or delays in priority overnight shipping of cooled semen.

3. Disadvantages of frozen semen

There are several disadvantages to using frozen–thawed semen. Most stallions will have a decreased pregnancy rate when using frozen–thawed semen. This disadvantage, in many cases, can be overcome by meticulous breeding management and can be insignificant when considering the numerous advantages of frozen semen. Another disadvantage is the higher cost for semen processing (freezing and storage) versus breeding with cooled shipped semen, which are frequently passed on to the mare owner in a higher or no-guarantee breeding fee. When these higher expenses are combined with the more detailed and time-consuming mare management expenses, breeding with frozen semen can become expensive.

4. Mare selection

The past reproductive history of a mare and her body condition play vital roles in determining her inherent fertility; however, they are often overlooked. When a client expresses an interest in breeding a mare with frozen semen, a thorough breeding soundness examination should be performed. The breeding soundness examination should include evaluation of the health and body condition of the mare; the perineum for a sunken anus, vulvar tip, or poor apposition of the vulvar labia; transrectal palpation; transrectal ultrasonography to determine fluid accumulation within the uterus, endometrial cysts and ovarian structures; and vaginal examination by digital palpation and speculum to detect evidence of urine pooling, cervical tears or adhesions, or irritation of the vaginal mucosa due to windsucking or urine pooling. Any abnormalities should be corrected well in advance of the breeding season. If the subject is an older mare (>15 years), barren mare, or one with a history of subfertility, an endometrial culture and biopsy are warranted. The mare should have a uterine biopsy with either a Kenney grade I or IIA; those with Kenney grade biopsies of IIB or III have a lower probability of carrying a pregnancy to term [2]. If the endometrial grade is the result of a condition amenable to treatment (i.e., not periglandular fibrosis), the condition can be treated, with a potential for improvement in fertility.

In 1995, Metcalf reported that first-cycle pregnancy rates with frozen semen were highest in maiden mares

(9 out of 9, 100%), then foaling mares (18 out of 21, 86%), and finally barren mares (6 out of 15, 40%) [3]. A more recent study reported a significant effect of mare status on pregnancy rate, with older maiden mares having a significantly reduced pregnancy rate (pregnancy rates were 27.7% in maiden mares >8 years versus 43.6% in maiden mares <8 years) [4].

5. Induction of ovulation

Appropriate mare management includes the use of an ovulatory agent to hasten ovulation within a predictable time frame. Ovulatory agents are most effective in mares with obvious endometrial edema, an ovarian follicle ≥ 35 mm in diameter, and a relaxed cervix. There are three products currently available: human chorionic gonadotrophin (hCG), deslorelin (GnRH analogue), and rLH (recombinant equine LH). This author uses deslorelin the majority of the time and typically administers it between 16:00 and 20:00. Thereafter, the mare is examined (transrectal palpation and ultrasonography) 24 h later, and again approximately 07:00 the following morning. This timing induces ovulation in the majority of the mares between 07:00 and 13:00 the second day after the injection, allowing a single post-ovulation breeding to occur during regular working hours (with few exceptions).

6. Insemination dose

There is not a standard insemination dose for frozen–thawed semen that has been thoroughly evaluated in the horse. Post-thaw progressive motility varies significantly among stallions and, frequently, among ejaculates. Each laboratory generally has a unique freezing method, so that the number of sperm packaged per straw, as well as the number of straws per insemination dose, varies greatly, not only by stallion, but also by freezing center. In an effort to increase uniformity, the World Breeding Federation for Sport Horses has established a minimum standard for frozen semen that is to be transferred between member countries, where an insemination dose should have a minimum of 250×10^6 progressively motile spermatozoa, with a post-thaw progressive motility of at least 35% (www.wbfs.org). There has been increased interest and research into reducing the number of spermatozoa in an insemination dose. Most of the factors influencing this demand are commercial in nature and include: increasing the number of mares bred per ejaculate; optimizing the usage of frozen semen from deceased stallions; reducing the occurrence of post-breeding

endometritis; utilizing stallions with poor semen quality; and using sex-sorted spermatozoa [4].

7. Timing of insemination

Frozen–thawed spermatozoa appear to have shorter longevity, perhaps due to “capacitation-like” changes [5]; therefore, breeding close to ovulation should increase fertility for most stallions. There are two methods of timing the insemination(s) currently used in the United States: single-dose post-ovulation insemination and multiple-dose inseminations.

7.1. Single-dose, post-ovulation insemination

Single-dose, post-ovulation insemination is the conventional method used to breed with frozen semen. This is the primary method currently used when the amount of frozen semen is limited or expensive, such as frozen semen from deceased stallions or imported from a foreign country. With this method, the use of an ovulation-inducing agent is necessary to minimize the cost and time involved. This agent should be given when the mare is in estrus and has a large (diameter, 35–40 mm) preovulatory follicle and uterine edema detectable ultrasonographically. The majority of mares (94%) ovulated 36–42 h after administration of either hCG or deslorelin [6]. Regular examinations of the mare (transrectal palpation and ultrasonography) should begin 12–24 h after treatment, and continue every 6 h until ovulation is detected, at which point a single-dose of semen is inseminated. Researchers have looked at alternate methods of breeding with a single-dose of semen to reduce the costs and time involved. One study inseminated a low-dose of frozen semen hysteroscopically on one occasion, 32 h post-hCG treatment, and obtained a pregnancy rate of 67% [7]. This author uses a protocol whereby mares with a 38–45 mm follicle are given deslorelin between 16:00 and 18:00. The mares are then examined 24 h later to determine ultrasonographic changes of imminent ovulation (decreased uterine edema, increased luteinization of the follicular wall, and changes in the shape of the follicle from round to conical). Mares with changes indicative of impending ovulation are re-examined 6 h later, whereas mares with only slight changes are examined in 12 h. This process continues until ovulation occurs, at which point they are bred via transrectal-guided deep-horn insemination. Following these guidelines reduces the need for many of the overnight examinations, and reduces the number of examinations required. The majority of the mares

ovulated between 36 and 42 h after the administration of deslorelin, and received an ultrasonographic examination an average of two or three times after deslorelin administration.

7.2. Multiple-dose insemination

It is becoming more common in the USA for veterinarians to use an insemination protocol that requires at least two doses of semen per-cycle. This method of insemination is a viable alternative when multiple doses of semen are available (domestic stallions) and/or there are limitations to the number of times a mare can be examined. A minimum of two doses should be available per-cycle, and, depending upon the method used, the client should be made aware that a third dose may be required if ovulation is delayed. Follicular development is monitored (ultrasonographically) on a daily basis until a large (diameter, 35–40 mm) preovulatory follicle is detected, at which point an ovulation-inducing agent is administered. The mare is inseminated with a single-dose of frozen semen 24 h later. The mare is examined by ultrasound 18 h after the first insemination, and a second insemination with another dose of frozen semen is performed, even if the mare has ovulated. If ovulation has not occurred, the mare is re-examined 12 h after the second insemination. If ovulation has not occurred by this point, a third dose of semen would be inseminated. Use of this protocol places viable semen within the uterus within 12 h before ovulation and up to 6 h after ovulation [8].

Another protocol for two-dose insemination combines ideas from each of the first two protocols discussed above. In this protocol, the mare is given an ovulatory agent in the morning (between 08:00 and 09:00), and an ultrasonographic examination is done the following afternoon (between 16:00 and 17:00), at which time the mare is inseminated with the first dose of semen. The mare is re-examined 16 h later (09:00); if ovulation has occurred, she is inseminated again. If ovulation has not occurred and only two doses of semen were available, the mare goes on a schedule whereby she is examined every 6 h and bred post-ovulation. If a third dose of semen is available, the mare is inseminated and checked again 12 h later to confirm ovulation and/or inseminated a third time (if ovulation has not occurred).

There were no differences in pregnancy rates for mares inseminated once within 6 h after ovulation versus mares bred with multiple doses in a given cycle [8]. For domestic stallions (of average fertility) that

have an abundance of readily available frozen semen, breeding with multiple doses of semen is a viable alternative for mare owners who wish to reduce their mare management expenses. It should be noted, however, that this method is not recommended for older mares, that are frequently more susceptible to uterine inflammation, as well as mares with delayed uterine clearance.

8. Methods of insemination

Standard practice for AI has been to deposit the semen within the uterine body. In recent years, researchers have examined whether deep-horn insemination (guided with either transrectal manipulation or a hysteroscope) can increase pregnancy rates for sub-fertile stallions or for low-dose insemination of fertile semen. Rigby et al. reported that depositing semen at the uterotubal junction increased the number of spermatozoa in the ipsilateral oviduct, which would presumably increase pregnancy rate [9]. In another study using cooled semen, there was no significant difference in pregnancy rates between transrectal-guided deep-horn insemination (50%) and hysteroscopic insemination (62%) [10]. Consideration should be given to the age and status of the mare when deciding upon the method of insemination. Sieme et al. reported that problem mares (with a history of barrenness or pregnancy failure), had significantly lower pregnancy rates when inseminated hysteroscopically versus conventional uterine body insemination (33.3% versus 84.2%) [11].

8.1. Transrectal-guided deep-horn insemination

Deep-horn insemination has been used intermittently since the 1930s [12]. Deep-horn insemination using a transrectal-guided technique is relatively inexpensive, utilizes disposable material, and is very effective and quick for an experienced practitioner. The procedure requires that a flexible pipette be passed through the cervix, at which point it is maneuvered via transrectal manipulation towards the tip of the horn ipsilateral to the ovary where the dominant follicle was (or is) located. After the tip of the pipette is confirmed to be at the top of the uterine horn, the semen is deposited close to the uterotubal papilla. The primary disadvantage with this procedure is the potential trauma to the endometrium caused by an inexperienced technician. With experience, however, it reduces the time and expense required for breeding, compared to hysteroscopic insemination.

8.2. Hysteroscopic insemination

Hysteroscopic insemination with frozen-thawed semen is more expensive and time-consuming than rectally guided deep-horn insemination. Perhaps the best usage for this procedure is when dealing with very low volume of semen (<0.5 mL). The procedure requires tranquilization, enabling the uterus to be partially inflated with air (to facilitate visualization). The videoendoscope is then passed to the tip of the uterine horn, ipsilateral to the ovary with the dominant follicle. The semen is then advanced through the biopsy channel of the videoendoscope, and deposited on and around the uterotubal papilla. One study indicated that the minimum number of sperm needed to achieve a normal pregnancy rate can be significantly decreased by changing the site of deposition (i.e., placing the semen at the oviductal papilla via hysteroscopic AI) [13]. In the author's opinion, hysteroscopic insemination is only preferable to deep-horn insemination when the volume of a breeding dose <0.5 mL.

9. Post-insemination procedures

Post-insemination management of the mare is vitally important when breeding with frozen-thawed semen. Transient inflammation is a physiologically normal response to breeding; this response is exacerbated by the glycerol and egg yolk used in the frozen semen extender. Normal mares typically clear any fluid from the uterus within 12 h after insemination [14]. Mares that cannot evacuate their uterus normally, or who suffer from prolonged uterine inflammation, may experience reduced pregnancy rates, even with aggressive therapeutic protocols.

Post-insemination uterine inflammation needs to be addressed in a timely manner after insemination (as early as 4 h), with additional follow-up as necessary. Standard treatments range from a few injections of oxytocin to multiple uterine lavages with oxytocin and/or infusion(s) of antibiotics. The expense of breeding with frozen semen, and, in particular imported frozen semen or frozen semen from a deceased stallion, surely warrants aggressive mare management to ensure the best environment for embryonic development. In these situations, a uterine lavage with 1–3 L of sterile-saline and administration of oxytocin should be performed as early as 4 h after insemination. It is useful to note that the uterine inflammatory response in reproductively normal mares peaks at 8 h after breeding [14]. The lavage effluent should be collected and evaluated for color, debris, and other abnormalities. If there is

excessive debris or discoloration, then a second uterine lavage should be performed the next day, and an appropriate intrauterine antibiotic should be infused. If necessary, a uterine lavage can be performed as late as 3 days after ovulation. Oxytocin can be given at 12-h intervals after the uterine lavage to ensure clearance of any remaining lavage fluid. If a mare shows persistent signs of post-insemination endometritis, a recommendation should be made to the mare owner that the mare is perhaps not the best candidate for breeding with frozen–thawed semen.

10. Retrospective study of pregnancy rates with frozen semen

10.1. Methods

A retrospective study of the 2006 and 2007 breeding seasons was conducted at the Equine Medical Center of Ocala to determine pregnancy rates after breeding with frozen–thawed semen. Medical records of mares that were presented to be bred with frozen–thawed semen were reviewed. Mares that were bred with frozen semen with a single-dose post-ovulation by transrectal-guided, deep-horn insemination, and with a record of pregnancy examination, were included in the study. Mares flushed for embryo recovery or that were bred with multiple doses of frozen semen were excluded. There were 149 estrous cycles included in this study. Mares with a dominant follicle ≥ 35 mm were given deslorelin (1.5 mg i.m.). Mares were examined (transrectal palpation and ultrasonography) 24 h after administration of deslorelin, and subsequently re-examined every 6 h until ovulation was confirmed. The mares were then inseminated with a single-dose of frozen semen via transrectal-guided, deep-horn insemination. Four to 12 h after insemination, a 2 L, sterile-saline uterine lavage was performed and oxytocin was administered (15 IU given IV and 15 IU given i.m.). Pregnancy diagnosis was done 14–16 days after AI.

10.2. Results and discussion

There were 149 estrous cycles included in this study, with 67 confirmed pregnancies (45% per-cycle pregnancy rate). It was noteworthy that this study was conducted in a commercial setting with many uncontrolled variables, including mare reproductive status, mare fertility, stallion fertility, post-thaw progressive motility of semen, insemination dose, and variations in freezing and thawing methods. This per-cycle pregnancy rate seemed comparable to findings in other studies in similar, commercial settings [8,15–20].

Furthermore, these data provided evidence that, with appropriate breeding management, an acceptable pregnancy rate can be achieved in mares with a single-dose of frozen–thawed semen (per-cycle) inseminated within 6 h after ovulation.

11. Conclusion

The goal of any breeding program, including one that includes the use of frozen–thawed semen, is to be cost-effective and produce high-quality foals. By incorporating recent advances in the field of cryopreserved equine semen, this economic efficiency can be achieved. Driven by this improved economic efficiency, more mares are being bred with cryopreserved semen from the highest quality stallions, thereby producing more valuable, higher-quality foals. Stallion owners utilize frozen semen to market their stallions to a wider range of mare owners, by offering more flexibility to breeders with regard to their mares' global location and ovulation schedule. Additionally, having a bank of cryopreserved semen allows the stallion to continue a career as a competition horse without the distractions of collecting during competitions. However, semen cryopreservation also provides additional challenges for the veterinarian and requires attention to details.

Mare management becomes vitally important when breeding with frozen–thawed semen. Use of ovulation-inducing agents is necessary to encourage a reliable time frame around which to schedule examinations. Protocols exist for breeding with single and multiple doses of frozen–thawed semen, with equal success rates, which allows for some flexibility in planning mare management and associated costs. Post-insemination management is required to resolve any uterine inflammatory response caused by the frozen–thawed semen; this treatment can vary from a few injections of oxytocin to multiple uterine lavages with several liters of fluid, and is dependent upon the mare and her individual response to the semen and the treatments provided.

Demands for breeding with frozen–thawed equine semen will undoubtedly increase as the industry continues to grow. Future advances and standardization in freezing techniques, when combined with improving mare management protocols, provide the best opportunity for future success when breeding with frozen–thawed semen.

References

- [1] Barker CAV, Gandier JCC. Pregnancy in a mare from frozen epididymal spermatozoa. *Can J Comp Med Vet Sci* 1957;21:47.

- [2] Kenney RM, Doig PA. Equine endometrial biopsy. In: Morrow DA, editor. *Current therapy in theriogenology*. Philadelphia: WB Saunders; 1986. p. 723–9.
- [3] Metcalf L. Maximizing reproductive efficiency in private practice: the management of mares and the use of cryopreserved semen. In: *Proceedings of the Society for Theriogenology*; 1995. p. 155–9.
- [4] Samper JC. Methods of inseminating with frozen semen. In: *Proceedings of the Florida Association of Equine Practitioners*; 2007. p. 118–21.
- [5] Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000;60/61:481–92.
- [6] Samper JC. Ultrasonographic appearance and the pattern of uterine edema to time of ovulation in mares. In: *Proceedings of the 45th Annual Convention of the American Association of Equine Practitioners*; 1997. p. 189–91.
- [7] Morris LH, Tiplady C, Allen WR. Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen–thawed spermatozoa onto the uterotubal junction. *Equine Vet J* 2003;35:197–201.
- [8] Loomis PR, Squires EL. Frozen semen management in equine breeding programs. *Theriogenology* 2005;64:480–91.
- [9] Rigby SL, Derczo S, Brinsko S, Blanchard T, Taylor T, Forrest D, et al. Oviductal sperm numbers following proximal uterine horn or uterine body insemination. In: *Proceedings of the American Association of Equine Practitioners*, vol. 46; 2000. p. 332–4.
- [10] Rigby SL, Lindsey AC, Brinsko SP, Blanchard TL, Love CC, Varner DD. Pregnancy rates in mares following hysteroscopic or rectally guided utero-tubal insemination with low sperm numbers. In: *Proceedings of the Third International Symposium on Stallion Reproduction*. Fort Collins, CO: Colorado State University; 2001. Abstr., pp. 49.
- [11] Sieme H, Bonk A, Hamann H, Klug E, Katila T. Effects of different artificial insemination techniques and sperm doses of fertility of normal mares and mares with abnormal reproductive history. *Theriogenology* 2004;62: 915–28.
- [12] Salzman AA. The injection of sperm into the horns of the uterus in the artificial insemination of mares. *Probl Zivotn* 1937;5: 164–5.
- [13] Morris LH, Hunter RH, Allen WR. Hysteroscopic insemination of small numbers of spermatozoa at the utero-tubal junction of preovulatory mares. *J Reprod Fertil* 2000;118:95–100.
- [14] LeBlanc M. Management and treatment of the infertile mare: what would you do? In: *Proceedings of the Florida Association of Equine Practitioners*; 2007. p. 31–43.
- [15] Metcalf ES. Optimizing pregnancy rates using frozen–thawed equine semen. *Anim Reprod Sci* 2005;89:297–9.
- [16] Hemberg E, Lundeheim N, Einarsson S. Successful timing of ovulation using deslorelin (Ovuplant) is labour-saving in mares aimed for single AI with frozen semen. *Reprod Domest Anim* 2006;41:535–7.
- [17] Barbacini S. Management of mares for frozen semen. In: *Proceedings of the 14th International Congress Animal Reproduction AI*; 2000. p. 308.
- [18] Vidament M, Écot P, Noue P, Bourgeois C, Magistrini M, Palmer E. Centrifugation and addition of glycerol at 22 °C instead of 4 °C improves post-thaw motility and fertility of stallion spermatozoa. *Theriogenology* 2000;54:907–19.
- [19] Loomis PR. The equine frozen semen industry. *Anim Reprod Sci* 2001;68:191–200.
- [20] Samper JC. Management and fertility of mares bred with frozen semen. *Anim Reprod Sci* 2001;68:219–28.