

Frozen semen management in equine breeding programs

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Abstract

Success with frozen semen requires attention to detail and a basic understanding of the techniques for properly handling and thawing and inseminating frozen semen. Practitioners should also be familiar with strategies used for managing mares for insemination with thawed semen. This manuscript will review those techniques and also present fertility data collected in a commercial setting. Factors that affect pregnancy rates for mares inseminated with frozen-thawed semen such as timing and frequency of insemination were examined for two separate data sets consisting of 332 and 536 mare cycles collected during the 2002 and 2003 breeding seasons. There were no differences observed in pregnancy rates for mares inseminated once or multiple times in a given cycle (51.5% versus 51.7% for data set 1 and 47.1% versus 46.1% for data set 2). Mares inseminated twice on a cycle, once before and once after ovulation, became pregnant at a rate similar to mares inseminated once within 6 h post-ovulation (48.1% versus 47.3%).

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1. Introduction

In 2000, the American Quarter Horse Association (AQHA) and the American Paint Horse Association (APHA) voted to allow the registration of foals conceived by frozen semen AI. The AQHA and APHA boast a combined 5.1 million registered horses with approximately 300,000 mares bred each year. Add this to the number of mares bred from

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all the other major breeds that now allow frozen semen and it becomes clear that frozen semen AI as a mainstream method for breeding horses is here to stay. Breeders have embraced the use of transported cooled semen for all the benefits associated with shipping semen to mares as apposed to shipping valuable mares and foals to stallions for live cover or on-farm AI. As the fertility of cryopreserved stallion semen has improved and simple AI protocols have evolved, breeders more often opt to use transported frozen semen for the additional benefits realized. Access to semen from stallions standing abroad, competition stallions, stallions that become ill, injured or overbooked during the breeding season and the ability to have semen on hand and available for use when the mare is at the optimum time for breeding are among the added benefits of frozen semen. Success with frozen semen however requires that the practitioner be familiar with the techniques for properly thawing, evaluating and handling of frozen semen as well as the breeding strategies employed to maximize fertility. Presented herein are recommendations for frozen semen handling and data collected by Select Breeders Service on the fertility of frozen semen in a commercial setting.

2. Materials and methods

2.1. Semen handling

2.1.1. Shipping and storage

Frozen spermatozoa lose fertilizing capacity when exposed to fluctuations in storage temperature. Improper storage or transfer of straws from storage to a shipping container and again into storage can cause severe damage if careful attention is not paid to minimizing exposure to increased temperatures. Frozen semen is typically transported in a nitrogen vapor container. These cryogenic containers maintain near liquid nitrogen temperatures (typically around -190°C) for days or weeks without the use of hazardous liquid nitrogen. Vapor phase containers work by absorbing liquid nitrogen into a thick layer of absorbent material that surrounds the inner cavity of the container where the semen is stored (Fig. 1). A long holding time is achieved through the superior insulation afforded by the double-walled aluminum shell that is filled with insulating foil and vacuum sealed. Vapor phase containers must be properly “charged” by filling with liquid nitrogen to the point of saturation of the absorbent material and then pouring off the excess liquid nitrogen to provide maximum holding time. Occasionally, semen is shipped in liquid shipping containers that maintain semen for a longer period of time provided adequate liquid nitrogen level is present. There is no absorbent material in these containers therefore if they are tipped and the liquid spills out, the container warms quickly to room temperature and semen thaws. Vapor shippers can be used to store semen for longer than the typical holding time. If the practitioner receives semen in a vapor shipper in anticipation of inseminating a mare and she does not develop a pre-ovulatory follicle as anticipated or she ovulates before the semen arrives, the vapor container may be used to store the semen for an extended time provided the vapor container is filled with liquid nitrogen. If this occurs, the practitioner should contact the semen supplier as soon as possible to discuss the shipper’s policy regarding tank returns.

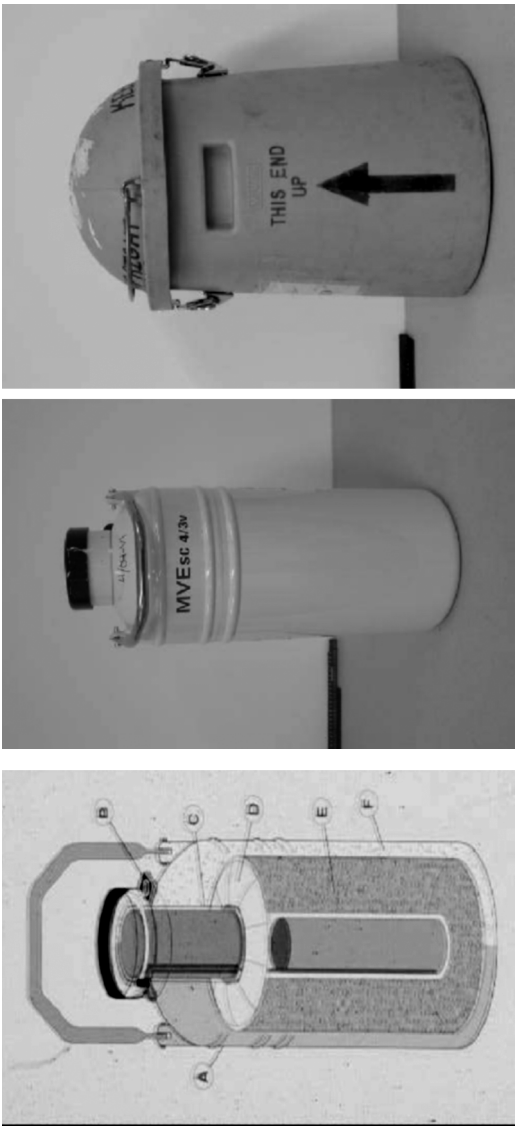


Fig. 1. MVE vapor shipping container used for transport of frozen semen.

One of the main benefits of using frozen semen is that it can be shipped to the veterinarian or mare owner in advance thus eliminating the anxiety and logistical headaches associated with cooled semen breeding. Ideally, semen can be ordered in advance of the mare's estrus and transferred upon arrival to a storage container at the farm or clinic. All too often, practitioners trapped in the cooled semen mind set order semen the day before the insemination is planned resulting in the same scheduling problems seen for cooled semen. Even if long-term storage is not available, ordering semen on the first day of estrus and keeping it in the shipping tank until needed is better than waiting until the last minute and missing the mare because she ovulated before the semen arrives.

When transferring frozen semen, the technician should take care not to expose the straws to room temperature by lifting them into the warm neck of the tank or transferring across any significant distance between the shipper and permanent storage. Proper transfer of 0.5 mL straws from a vapor shipper to permanent storage is illustrated in Fig. 2. Lift the canister slightly into the neck of the shipper and then, using pre-cooled hemostats or tweezers gently grasp the individual straws or the plastic goblet and lower the canister back into the container (Fig. 2a). Never lift the canister or straws above the frost line (visible about 5 cm from the top of the container neck) until it is time to transfer the semen. While holding the semen well inside the shipper, lift the canister from the storage container slightly up into the neck, again never above the frost line (Fig. 2b). As quickly as possible transfer the straws into the liquid storage canister and lower back down into the liquid nitrogen (Fig. 2c and d).

The following are recommended guidelines for the proper storage of frozen semen:

1. Keep nitrogen containers in a clean, dry, well ventilated room.
2. Do not store aluminum containers directly on concrete floors as this will erode the aluminum.
3. Keep containers in an area that allows daily visual inspection. A container that has lost its vacuum will display frosting around the neck on the outside, quickly lose nitrogen and warm to room temperature.
4. Check liquid nitrogen levels weekly and record to determine evaporation rates of individual containers.
5. Top off containers when the liquid level reaches 1/2 capacity.
6. Inspect the neck cork regularly for damage and replace if necessary to maintain maximum holding time.

2.1.2. Thawing and insemination

During cooling and freezing, spermatozoa undergo a series of chemical and physical changes that include; partial dehydration, cryoprotectant penetration of cells, reorganization of membrane lipids and proteins, exposure to high salt concentrations and exposure to inter and intracellular ice crystals. Cryopreservation protocols are designed to minimize the negative effects of these stresses. Thawing spermatozoa exposes the cells to these same types of stresses but in reverse. Cells rehydrate as water moves back across the plasma membrane to balance the osmotic imbalance created when extracellular ice melts. Plasma membrane proteins and lipids reorganize and cryoprotectants diffuse out of the cells.

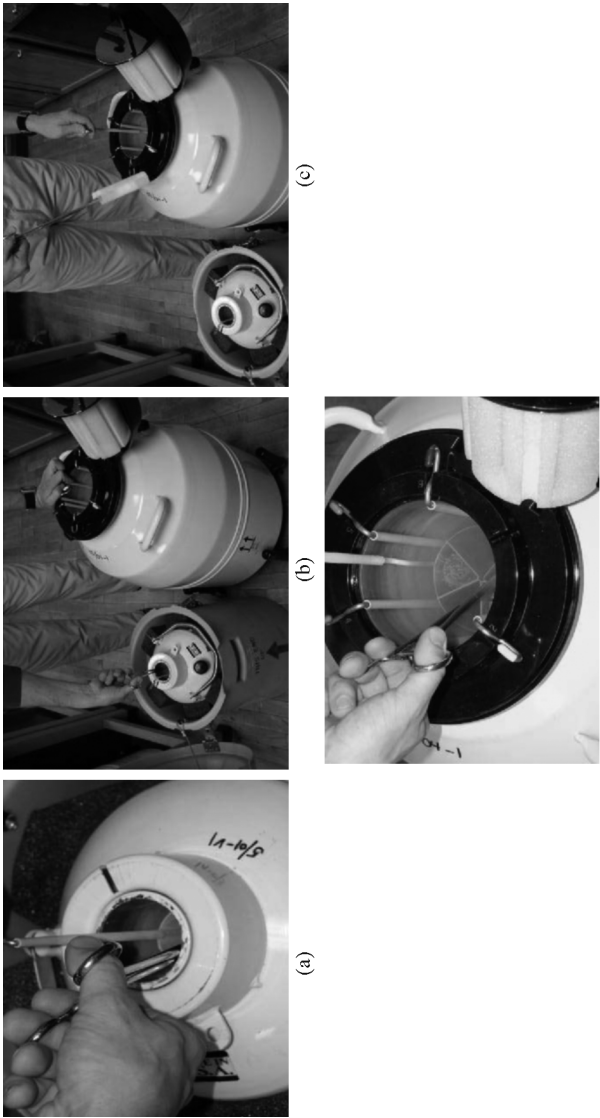


Fig. 2. Proper technique for transfer of frozen semen straws from a transport container to permanent liquid nitrogen storage.

Thawing semen improperly, i.e. too fast or too slow for the freezing protocol employed, reduces the viability of the spermatozoa and decreases the chance for obtaining a pregnancy. All frozen semen should be shipped with detailed instructions on the recommended procedure for thawing. Presumably the laboratory that processed the frozen semen should know the best protocol for thawing. Generally, semen frozen in 0.5 mL straws is thawed at 37 °C. The duration that the straw is kept at that temperature is not critical as long as it is left in the water bath for at least 20 s to allow the semen to fully thaw. An accurate thermometer is essential and it is always better to err on the low side of 37 °C. Thawed spermatozoa dies rapidly if kept at 39 or 40 °C whereas they survive quite well at 35 or 36 °C. Some freezing laboratories recommend thawing semen frozen in 0.5 mL straws at 75 °C for 7 s. While this technique works well for recovering sperm motility when performed correctly, the risk of damaging the spermatozoa from exposure to 75 °C temperature for more than exactly 7 s is great, especially in a commercial setting.

The number of straws that make up a single insemination dose varies depending on the freezing laboratory and stallion. Generally, an insemination dose consists of 4 or 8 (0.5 mL) straws. When using a 37 °C thawing temperature, the straws can be placed in the water bath one after another and left there until the last straw has been at 37 °C for 30 s. All of the straws can then be removed, dried and prepared for insemination. Once the semen is thawed and the straws are dried, the contents can be emptied into a sterile, pre-warmed container such as a centrifuge tube or red top test tube. The semen can then be drawn into a standard insemination pipette to inseminate the mare. The series of pictures in Fig. 3 illustrates this technique. Although most commercial laboratories now freeze semen in 0.5 mL straws, some semen is still frozen in 4.0 or 5.0 mL Macrotubes. These straws are generally thawed at 50 °C for 45 s. With these straws it is critical that they do not remain in the 50 °C water bath for more than 45 s to prevent damage from exposure to elevated temperatures.

2.2. Management of mares bred with frozen semen

2.2.1. Strategies for AI with frozen semen: effects of timing and frequency of insemination

A major limiting factor to the widespread application of frozen semen is the cost associated with the intense management of mares being inseminated. It is generally recommended that frozen stallion semen be inseminated within 12 h prior to, or up to 6 h after ovulation. The presumed shortened life-span for frozen-thawed stallion spermatozoa in the mare reproductive tract combined with the “by the dose, no guarantee” system of marketing semen has led to the practice of three or four times per day examinations of mares inseminated with frozen semen. Currently, Select Breeders Service recommends use of a timed insemination protocol for cost-effective management of mares bred with frozen semen. This protocol involves daily ultrasonographic examinations during estrus, induction of ovulation using hCG or deslorelin following detection of a >35-mm follicle and insemination at 24 and 40 h post-injection. Using this insemination schedule, mares that ovulate 18–52 h after administration of the ovulatory agent will have had spermatozoa deposited in the reproductive tract within 12 h prior to ovulation or within 6 h after ovulation or both. In a clinical trial in Italy [1], 26 of 34 mares conceived (76%) after two

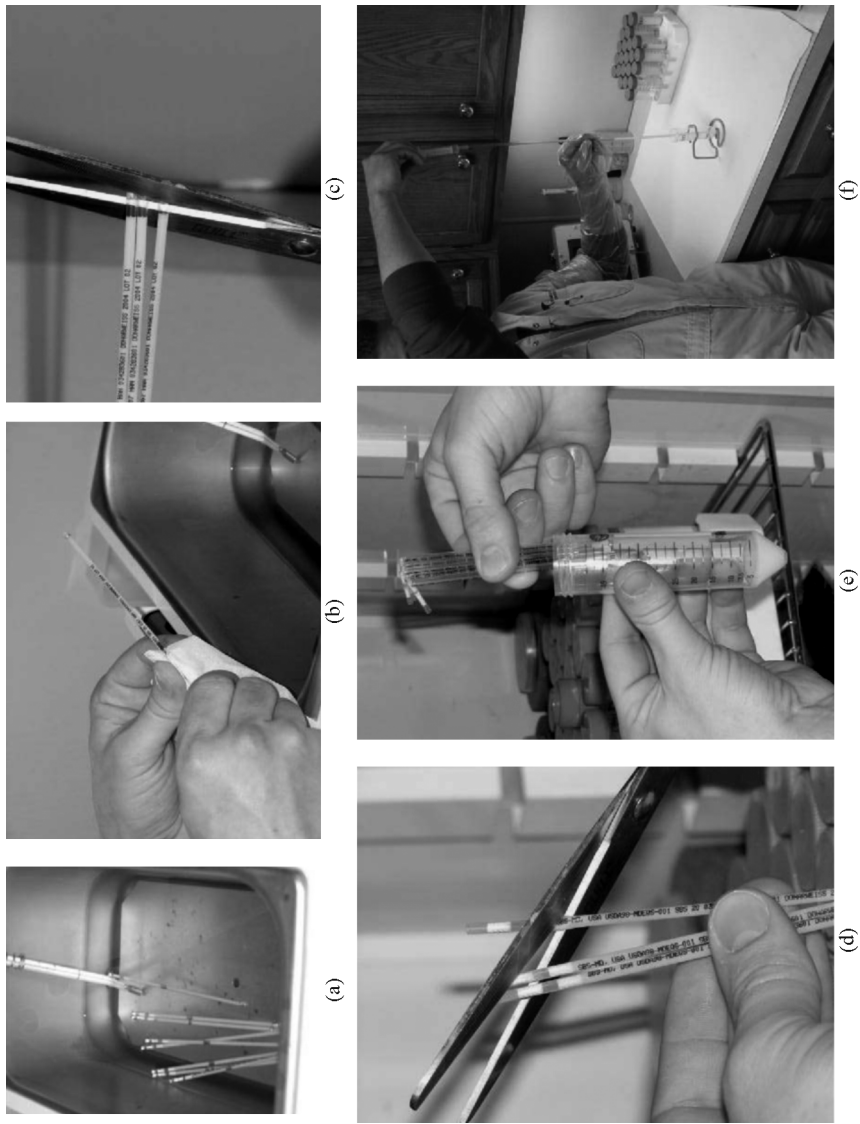


Fig. 3. Recommended technique for thawing and insemination of stallion semen packaged in 0.5 mL straws when a single insemination dose consists of multiple straws.

timed inseminations versus 15 of 21 (71%) conceiving following a single insemination within 6 h post-ovulation. In a separate controlled study in Colorado [1], Reger et al., reported no difference in embryo recovery rates for mares inseminated once within 6 h post-ovulation with 800×10^6 total frozen-thawed spermatozoa (60%) versus mares inseminated twice at 24 and 40 h post-deslorelin with 400×10^6 total spermatozoa per insemination (55%). Matthews (unpublished) reported a per-cycle pregnancy rate of 46% (157 cycles) for mares inseminated twice with 200×10^6 total frozen-thawed spermatozoa from one stallion using a video endoscope and two timed inseminations.

2.3. 2003 Survey of frozen semen shipped from SBS Maryland

2.3.1. Methods

A retrospective study was conducted at the conclusion of the 2003 breeding season to examine pregnancy rates and the effects of insemination strategies for frozen semen distributed commercially throughout the U.S. from our Maryland laboratory. Surveys were included along with each of the 535 shipments of frozen semen sent in 2003. We requested information on each of the mares as well as details concerning insemination dates and times, use of ovulating drugs, time of ovulation and fertility results. Surveys for 217 mares and 332 cycles were returned. These mares were inseminated with semen of varying quality from 54 different stallions of numerous breeds. Generally, insemination doses contained 800 million to 1 billion total spermatozoa and exhibited a minimum post-thaw progressive motility of 30%. The number of progressively motile spermatozoa per insemination dose ranged from approximately 240 to 600 million. Obviously, mare management varied tremendously. Complete survey data for all 332 cycles was not reported. Therefore, we have presented the various factors affecting pregnancy rates based only on the cycles for which all pertinent data was available. For this study a positive result was recorded if an embryo was recovered or an embryonic vesicle was observed using ultrasonography >13 days post-ovulation.

2.3.2. Results

Although not different ($P > 0.05$), mares >16 year of age tended to have a lower per cycle pregnancy rate (48.4% for 31 cycles) than young mares aged 3–16 year (55.2% for 201 cycles). Of the 217 mares bred, 126 (58.1%) conceived on the first cycle of breeding. Overall the pregnancy rate per cycle was 52.7%. There was no effect of the number of inseminations on the fertility of frozen semen from 283 cycles for which data were available (Table 1). The pregnancy rates for seven different insemination strategies are given in Table 2. Three of the strategies used a single insemination and four used multiple inseminations. The vast majority of mares were inseminated either with a single post-ovulation insemination or with two

Table 1
Effects of single versus multiple AI on conception rates for frozen-thawed stallion semen

	Cycles	Conceptions	Conception rate (%)
One AI	132	68	51.5
Multiple AI ^a	151	78	51.7

^a Of the 151 multiple AI cycles, 128 were inseminated twice.

Table 2

Effect of insemination strategy on conception rates for frozen-thawed stallion semen

	AI Strategy ^a						
	One AI			Multiple AI			
	A	F	G	B	C	D	E
Cycles	93	11	12	104	9	6	10
Conceptions	44	8	7	50	5	1	8
Conception rate (%)	47.3	72.7	58.3	48.1	55.6	16.7	80.0

^a A: One AI, post-ovulation; F: one AI, pre-ovulation within 12 h of ovulation; G: one AI, pre-ovulation within 24 h of ovulation; B: two AI's, one pre- and one post-ovulation; C: multiple AI's pre-ovulation, last within 12 h of ovulation; D: multiple AI's pre-ovulation, last within 24 h of ovulation; E: multiple AI's pre-ovulation and one AI post-ovulation.

inseminations, one pre- and one post-ovulation. There was no difference in pregnancy rates for mares inseminated with these two common techniques.

2.4. Data for mares managed at Select Breeders Service affiliate laboratory facilities

2.4.1. Methods

Data were obtained from six different facilities in 2002 and 2003. Facilities were located in Maryland, Kentucky, Florida, Texas, Italy and Germany. All of these facilities were affiliates of Select Breeders Service, Inc. (Colora, MD, USA). Data were available for mares inseminated with frozen semen for 259 mare cycles in 2002 and 277 mare cycles in 2003. Mares were inseminated with semen from a variety of sources; not all semen was processed by Select Breeders Service. Some of the information sent from the facilities was not complete for all parameters, and thus the number of cycles varied depending upon the parameter analyzed. Endpoints included: age, number of inseminations per cycle, timing of AI in relation to ovulation, incidence of uterine fluid and pregnancy rates 12–16 days after ovulation. Where appropriate, statistical analysis was performed using Chi square analyses.

2.4.2. Results

There was no significant effect of mare age on pregnancy rates with frozen semen. Although, a trend similar to that seen in the 2003 survey data towards lower fertility for mares >16-year-old was also observed for the SBS Affiliate managed mares. Pregnancy rates were 49.6% for 403 cycles from mares aged 3 to 16 years and 41.7% for 36 cycles from mares >16-year-old. The effect of frequency of insemination on pregnancy rates is presented in Table 3. Mares were categorized as being inseminated once during an estrous

Table 3

Effect of frequency of insemination on conception rate in mares

No. AI	Cycles	Conceptions	Conception rate (%)
One AI	255	120	47.1
Multiple AI	280	129	46.1

Combined for 2002 and 2003.

Table 4
Effect of insemination timing on pregnancy rate in mares

	Single AI		Multiple AI		
	Pre	Post	Pre/pre	Pre/post 1	Pre/post 2
Cycles	20	221	32	182	29
Conceptions	9	106	13	84	15
Conception rate (%)	45.0	48.0	40.6	46.2	51.7

Combined for 2002 and 2003.

(1) One insemination the day before ovulation and one insemination on the day ovulation was detected.

(2) One insemination before ovulation and one insemination after ovulation, both on the day ovulation was detected.

Table 5
Effect of frequency of AI on incidence of post-AI uterine fluid in mares

No. AI	Total cycles	Cycles with fluid	(%)
Once	244	56	23.0
Multiple	255	59	23.1

Table 6
Effect of post-breeding uterine fluid on pregnancy rate in mares

	Cycles	Conceptions	Conception rate (%)
Fluid	115	44	38.3
No fluid	387	194	50.1

cycle or two or three times. Over 90% of mares bred multiple times were bred only twice, generally at 24 and 40 h after hCG. One-cycle pregnancy rates for mares inseminated with frozen semen were not affected by frequency of insemination.

Presented in Table 4 are data on both the effect of frequency of AI and timing of insemination on pregnancy rates. For mares inseminated only one time, the data was divided into pre-ovulatory insemination and postovulatory insemination. For those inseminated multiple times during a cycle, the data were divided into mares inseminated twice pre-ovulation (Pre/pre), once pre-ovulation and once on the day of ovulation or the day after (Pre/post 1), or twice on the day of ovulation (Pre/post 2). For these mares, there was no effect of timing or frequency of insemination on pregnancy rate.

The overall incidence of uterine fluid for mares inseminated with frozen semen was 23% and was not different for mares inseminated once or multiple times within a given heat cycle (Table 5). Presence of uterine fluid decreased pregnancy rates of mares inseminated with frozen semen (Table 6).

3. Discussion

These data indicate that acceptable pregnancy rates with frozen semen can be obtained in a commercial setting. It is important to point out that the data reported herein was

collected under field conditions and therefore one must use caution when drawing conclusions. Ideally, experiments would be designed so that large numbers of mares were randomly assigned to be inseminated with a constant number of motile spermatozoa using one of several pre-determined insemination schedules with semen from the same stallions. Ideally, the management of the mares would also be constant across experimental groups. Obviously, this is unrealistic in a commercial setting. The commercial pregnancy rates obtained in these studies were similar to those reported by others [2–5].

The purpose of recommending a timed insemination protocol employing two inseminations per cycle is to provide a simple and effective way to manage mares being bred with frozen semen. It is a commonly held belief that mares bred with frozen semen need to be examined 3–4 times a day during the periovulatory period so that a single dose of frozen semen can be inseminated within 6–8 h after ovulation. This usually requires that mares are boarded at a clinic or that late night farm calls must be made by the practitioner to insure that the post-ovulation insemination is performed within the critical 6 h window after ovulation. The cost in veterinary care to the mare owner is substantial and often discourages them from using frozen semen. Stallion owners who sell semen by the dose for hundreds or even thousands of dollars are forcing the mare owners to utilize this type of protocol because the cost of the veterinary care is less than the cost of the additional semen required for a two-dose timed protocol. However, many stallion owners provide multiple doses per cycle and are paid per pregnancy, in this case using frozen semen as just another mechanism to achieve a pregnancy. Even in this situation many practitioners believe that they can only achieve acceptable pregnancy rates with frozen semen if the mares are managed with multiple daily examinations around the time of anticipated ovulation. These data support the theory that two inseminations timed to occur both before and after ovulation yield comparable conception rates to a single post-ovulation insemination. This is in agreement with data recently published by Sieme, et al. [6]. In that study, mares inseminated twice per cycle at 24 h intervals had a 50% per cycle pregnancy rate and mares inseminated once averaged 42% per cycle pregnancy rate. Pregnancy rates for mares inseminated once within 12 h prior to ovulation or once within 12 h post-ovulation were 41 and 50%, respectively. Samper [5] and Vidament [7] also reported that pregnancy rates with frozen semen were higher when mares were inseminated more than once per cycle.

Often, mares are only inseminated once in a cycle with frozen semen for fear that multiple inseminations may result in an increased incidence of post-breeding uterine fluid. The incidence of post breeding uterine fluid present in this study was 23%. This is higher than reported by others [8,9]. The difference may be due to the fact that in the present study mares were considered to have post breeding uterine fluid even if there were trace amounts of fluid present whereas others [9] only recorded fluid if it measured at least 20 mm in size. There was however, no difference in the incidence of uterine fluid for mares inseminated once or multiple times in a cycle. Regardless of insemination frequency, the presence of post-breeding uterine fluid did appear to have a negative effect on fertility. The fact that multiple inseminations with frozen semen did not increase the incidence of uterine fluid is in agreement with Regar et al. [1] however; their study did not show any effect of the presence of uterine fluid on pregnancy rates.

A two-dose timed insemination protocol allows a practitioner to examine mares once daily during normal hours without compromising fertility. Multiple inseminations with frozen semen during a single estrus did not increase the incidence of uterine fluid. However, use of this protocol may not be appropriate for all breeding situations. For example, mares that are susceptible to post breeding endometritis such as older or barren mares may require a more intense management scheme in order to minimize invasion of the susceptible uterus. Older mares may also be less responsive to ovulatory agents such as hCG [2].

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