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Transported Equine Semen (17-Apr-2000)

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Introduction

Most breed registries now authorize registration of foals conceived through use of transported cooled semen. Interest among mare and stallion owners in this technology has increased because of some distinct advantages gained by breeding with transported semen [1, 2]:

- convenience of maintaining the broodmare at home;
- avoids the stress and cost of shipping a broodmare and foal to a distant breeding farm;
- reduces the costs for broodmare care at other facilities;
- decreases chance of injury to broodmares and foals during transport to, and maintenance at, other facilities;
- reduces likelihood for transmission of diseases between horse farms;
- access to superior stallions which otherwise are not available to mare owners;
- increases the genetic pool by permitting wider use of stallions;
- allows scrutiny of spermatozoal quality and numbers used for insemination.

There are also some disadvantages to breeding horses with transported semen, which should be considered when deciding to use this technology:

- fertility of cooled spermatozoa from some stallions is reduced;
- the insemination dose (total spermatozoal number) must be increased with transported cooled semen to maximize the likelihood of achieving pregnancies;
- expenses and time commitment associated with shipping semen and breeding mares with transported semen can be high, particularly if multiple breedings are required per estrous cycle;
- increased intensity of mare management is required to ensure transported semen is ordered and inseminated at an optimal time during estrus;
- increased technical demands for transported semen and the potential effect of poor technique on fertility requires heightened knowledge and skill of the stallion manager and attendant veterinarian due to the susceptibility of stallion spermatozoa to environmental injury.

Registry and Health Regulations

Not all equine breed registries allow registration of foals produced by artificial insemination and, of those that do, not all permit the use of cooled, transported semen. To insure full compliance with the breed registry involved, the breed registry should be contacted prior to becoming involved in breeding with transported semen, so that all requirements for registration of foals can be met.

Regulations regarding shipment of semen are not well defined. Semen is often transported throughout the United States via passenger or express-mail air carriers without accompanying health certificates. It is imperative that appropriate regulations be met to avoid unlawful shipment of semen. Some states and countries have general health certificate requirements, while others require special permits for transported semen. Therefore, individuals planning either interstate or international shipment of semen should meet the requirements specified by the state or country of destination for the semen. Regulatory veterinarians should be contacted to obtain information regarding specific regulations. Authorized shipment of semen outside the United States requires that specific regulations of the importing country be met. For most stallions, cooled semen must be inseminated within 24-48 hours following collection to achieve acceptable fertility, so cooled semen may be less amenable to intercontinental shipment, depending on its destination.

Mare Management Considerations

Before the mare is to be bred, she should undergo a complete reproductive examination so that any abnormalities which may adversely affect her reproductive potential can be recognized and corrected. Common correctable conditions include poor body condition, endometritis and poor perineal conformation. Optimally, the mare's breeding records should be examined or she should be monitored through one or two estrous cycles prior to breeding to establish the dynamics of her cycle and the size of the follicle that she typically ovulates.

When planning to breed with cooled transported semen, the mare owner and/or their veterinarian should obtain information regarding the level of the stud farm's experience with processing semen for cooling and transport, the quality of the stallions' semen after cooling, and the pregnancy rates he has achieved with cooled transported semen. This type of information will help one decide whether or not it is wise to invest the time, effort and expense required to breed to a particular stallion. Many stud farms advertise the availability of transported semen without documenting the suitability of the stallion's semen for cooling and transport, nor do they differentiate pregnancy rates obtained by transported semen versus those obtained by insemination with fresh semen or natural cover. Fertility trials using cooled transported equine semen have yielded pregnancy rates varying from 0% to over 70% per cycle, emphasizing that there is great variability among stallions in spermatozoal ability to maintain fertilizing capacity following cooled storage [3-7]. Experienced, reputable stud farms maintain good records on their stallions and should be willing to provide this information.

Communication among mare owners, veterinarians and stallion managers is imperative for success. Once the stallion is selected, the stallion manager should be contacted as soon as possible to inform them of the anticipated breeding plans for the mare and to obtain information regarding the semen collection and shipping schedules for the stallion. Pertinent information above and beyond the stud fee should include:

- additional fees for semen collection and shipment;
- the days of the week that the stallion is collected and whether or not there is any flexibility in this schedule;
- how much notice the stallion manager needs prior to shipment;
- whether there is a seasonal limit to the total number of doses that will be shipped for a mare;
- whether the semen will arrive the next day or if the semen can be shipped to arrive the same day if needed;
- how quickly the shipping container needs to be returned, and if there are any penalties associated with a delay in return.

Ordering Semen

The optimal time to order semen depends on a number of factors including, how quickly the mare typically develops and ovulates her follicles and thus the size of the follicle, whether or not ovulation will be induced and when the semen will be available for insemination. When the mare comes into estrus on the cycle that she is to be bred, the stud farm should be contacted to inform them that the mare is in heat and when semen is expected to be needed. Close monitoring of the mare's follicular status is essential for ordering semen at the right time. For the majority of lighthorse breeds, the dominant follicle grows approximately 3 mm per day and ovulation occurs when follicles are > 35 mm in diameter [8]. Therefore, semen is usually ordered when the dominant follicle reaches 30 to 35 mm in diameter. The use of ovulation-inducing agents greatly enhances the synchrony between insemination and ovulation. When the dominant preovulatory follicle is at least 35 mm in diameter, ovulation will occur in the majority of mares within 36 to 48 h after the administration of either hCG (1500 to 3000 units, IV or IM) [9] or an Ovuplant® implant [10]. Therefore, if it can be confirmed that the semen will arrive the next day, an ovulation-inducing agent is administered when the follicle reaches 35 mm in diameter and the semen should arrive and be ready for insemination within 12 to 24 h before ovulation. If there is any doubt that the semen will arrive by the next day, it is prudent to delay the administration of ovulation-inducing agents until the semen is in hand to reduce the chances that the mare will ovulate before the semen arrives.

Semen-Handling Protocol and Insemination Technique

When the semen arrives, the shipping container should be opened and the identity of the stallion providing the semen should be confirmed. Ideally, transported semen should be accompanied by a form containing the name of the stallion and farm providing the semen as well as the name of the mare for which insemination is intended. This is most important when there are a number of different mares to breed and multiple semen shipments are received from different farms or stallions from the same farm. Once the identity of the semen has been confirmed, it should be removed from the shipping container, gently mixed and aspirated into a syringe to which an insemination pipette is attached.

The mare is prepared for insemination using aseptic technique and inseminated, just as one would for insemination with fresh semen. The semen does not need to be warmed prior to insemination unless a cream-gel extender has been used. Cream-gel extenders are too viscous for aspiration and insemination at refrigerator temperatures. These extenders are typically warmed

in an incubator or water bath at 37 - 38°C until the sample become fluid enough for use. As with any semen sample, care must be taken not to contaminate the semen with water.

A small aliquot of the semen sample should be retained and warmed to 37°C to assess spermatozoal motility for the purpose of quality control. Ideally a phase-contrast microscope with a temperature controlled stage should be used. If such a microscope is not available, the stage of a conventional microscope should be preheated and the condenser and light intensity adjusted to maximize visualization of the spermatozoa. All equipment that will come in contact with the semen including the microscope slides and transfer pipettes should be clean and pre-warmed to prevent cold shock of the semen, which can lead to an erroneous assessment of semen quality. If the spermatozoal motility is extremely poor, the stud farm should be contacted to arrange for an additional semen shipment as soon as possible. It is not uncommon for mares to become pregnant with semen demonstrating 25% to 30% progressive motility, provided adequate ($\geq 1 \times 10^9$) sperm numbers are present. However, a review of breeding records at Texas A&M University found that pregnancy rates were significantly higher ($P < 0.02$) in mares bred with cooled transported semen that contained $\geq 500 \times 10^6$ progressively motile sperm than those bred with samples that contained $< 500 \times 10^6$ progressively motile sperm. Additional quality control measures include determining the sperm concentration and performing an assessment of sperm morphology of the sample. The concentration of the sample can be readily obtained by performing a hemacytometer count [11]. Spermatozoal motility is optimized with cooled transported semen when the sample contains 25 - 50 $\times 10^6$ sperm/ml [12, 13]. Spermatozoal motility and fertility can be compromised if the semen is too concentrated or too dilute. If semen quality problems are apparent, the stallion manager should be contacted and enquiries made into the semen handling and packaging procedures employed. If there is a high percentage of morphologic abnormalities present or semen quality is consistently poor, despite the use of proper technique, then it is likely that the stallion's semen is not suitable for use in a cooled transported breeding program.

Double Inseminations

Semen shipments often arrive containing two insemination doses. If the semen quality is good then a single insemination dose on the day of arrival should be sufficient. The second dose can be maintained refrigerated at $< 10^\circ\text{C}$ in an insulated container and used the following day if the mare has not ovulated. If the mare has ovulated, a second insemination is unnecessary and only serves to introduce additional contaminants into the mare's uterus. It should be noted that for most stallions, sperm motility declines with continued storage. Although semen quality may be acceptable after 24 hours of storage it may be unacceptable by 48 hours. This is most notable for some disposable containers which only maintain semen below 10°C for less than 36 h, even when unopened [14]. If sperm motility is poor on arrival, the motility will be even lower the following day. In such cases, putting both doses in the mare at the same time will help maximize the number of motile sperm in the insemination dose. This may provide the best chance for obtaining a pregnancy on that cycle, especially if a second semen shipment cannot be expedited to arrive prior to ovulation.

When a second insemination is used, optimal timing of the second insemination must also be considered. Recommendations for the interval between inseminations have ranged anywhere from 6 to 24 hours. However, no controlled breeding trials have been performed to address this question. Some degree of inflammatory response will occur in virtually every mare after insemination. This inflammatory response (as measured by PMN influx) begins within 0.5 to 1 h, peaks at 6 to 12 h, persists for 24 h and while still present, is greatly diminished by 48 h after insemination [15]. Although sperm can survive in an inflamed uterus, *in vitro* studies have demonstrated that uterine secretions from the inflamed uterus adversely affects sperm motility [16]. This detrimental effect appears as early as 6 h, is most pronounced in the presence of PMNs at 12 h and persists for at least 24 h after insemination.

Fertility trials done at two different laboratories resulted in disparate results when the question of whether breeding with a single dose of cooled semen after 24 h of storage, a double dose of semen after 24 h of storage or a dose after 24 h and a dose after 48 h of storage affected pregnancy rates. One study found no difference in pregnancy rate [17], while the other found a slight improvement in pregnancy rate with the two-dose, two-day method [18]. A noticeable stallion effect is apparent with these studies and serves to emphasize that it is important to consider the quality of semen after cooling and the inherent fertility of the stallion when deciding how to best utilize the semen when two doses are sent. If the semen quality is marginal at 24 h or if it is known that the stallion's spermatozoal motility will decline significantly with additional storage, both doses should be inseminated as soon as possible after the semen arrives.

Insemination Volume

Whether or not to breed with two doses of semen also raises the question of the effect of inseminate volume on fertility. The number of normal motile spermatozoa in an insemination dose appears to be more critical to fertility than the volume of the inseminate. Although smaller or larger volumes can be used successfully, typical insemination volumes for cooled transported equine semen range from 30 to 120 ml. Excessively large insemination volumes of fresh semen are not recommended because much of this volume may be lost through the mare's dilated cervix following insemination. However, one report suggested that insemination volumes of up to 170 ml using chilled transported semen do not adversely affect

pregnancy rates (a pregnancy rate of 12/13, or 92%, was reported for these mares). When pony mares were bred with either 30 or 120 ml of cooled semen extended at 50 million spermatozoa per ml, pregnancy rates did not differ between the two groups (7/9; 78% and 10/10; 100%). The authors concluded that insemination volumes as large as 120 ml of chilled semen do not adversely affect fertility as long as a sufficient number of progressively motile spermatozoa is inseminated [19].

Pregnancy Diagnosis

Mares bred with cooled transported semen should be examined for pregnancy using transrectal ultrasonography 14-15 days after ovulation. This accomplishes a number of important functions. First, it allows early detection of pregnancy. Second, it helps establish if the conceptus and uterus appear normal for the stage of gestation. Third, it provides adequate time to contact the stallion manager and arrange for semen at the next estrus if the mare has failed to become pregnant. Fourth, it allows early contact with the stallion manager to inform them of the breeding results so that they can monitor pregnancy rates. And finally, it allows detection of twins before they become fixed in the uterus.

Testing the Stallion's Semen for Cooling and Storage

Not all stallions produce spermatozoa that survive the cooling process. Stallions for which breeding with cooled transported semen is planned should be tested before transported semen from these stallions is advertized. Evaluation of the ability of each stallion's spermatozoa to survive the process will help ensure that spermatozoal motility will be acceptable when mares are bred with cooled semen. If a stallion has been sexually rested, semen should be collected a few times prior to cooling a sample for analysis in order to remove spermatozoa which may have been stored in the excurrent duct system for a prolonged period. If spermatozoal quality is acceptable after 24 hours of cooling, it is reasonable to assume that the stallion's semen will survive the cooling process and can be used for breeding after cooling and transport. This testing procedure also provides information regarding the total number of spermatozoa that should be packaged in order to provide a cooled insemination dose that should maximize pregnancy rates.

The most accurate method for determining the insemination dose required for breeding after transport, is to conduct semen cooling trials for each individual stallion. The semen is appropriately diluted in extender, and then cooled for 24 hours. Spermatozoal motility is evaluated after warming to 37°C, and the percentage of progressively motile sperm following storage is used to help ensure that shipments will provide a minimum of 500 million progressively motile sperm after 24 hours of cooling. For example, if after 24 hours of cooling the percentage of progressively motile spermatozoa is 50%, 1 billion total spermatozoa would need to be prepared for shipment to ensure that an insemination dose of 500 million progressively motile spermatozoa is available after cooling and transport.

Procedures for Processing Cooled Transported Semen

Extended semen from fertile stallions can oftentimes be stored in a cooled state for hours to days prior to insemination without a significant reduction in pregnancy rate. Longevity of spermatozoal viability may following cooled storage can be maximized by following these guidelines:

Evaluate initial semen quality - Good initial semen quality is imperative to successful transport of cooled semen. Only stallions with normal fertility utilizing fresh semen should be considered for use in a cooled-transported semen program.

Dilute semen with a high quality seminal extender - Semen extenders contain protective ingredients which permit spermatozoal survival outside the reproductive tract. Lipoproteins, such as those contained in milk or egg yolk, protect spermatozoa against cold shock. Metabolizable substrates, such as glucose, provide a plentiful source of energy for spermatozoa. Antibiotics are added to extenders to retard or eliminate growth of bacterial organisms. A comparison of antibiotics commonly used in semen extenders, demonstrated that a combination of potassium penicillin and amikacin sulfate maintained the highest level of spermatozoal motility, while providing the broadest spectrum of antibacterial activity [20]. However, the most appropriate antibiotic(s) to use can vary among stallions, depending on the bacterial flora in their ejaculates and the effect of the antibiotic(s) on their spermatozoal motility after cooling and storage.

Semen should be mixed with a prewarmed (37°C) seminal extender within a few minutes after ejaculation. Best results are obtained when semen is diluted at least 3:1 (v/v), extender:semen, to a final concentration of 25 - 50 x 10⁶ spermatozoa/ml prior to storage [12, 13]. Alternatively, seminal plasma volume can be reduced to only 5 - 20% by centrifugation and resuspension in extender [13].

Cool extended semen to refrigerated temperature for storage and transport - Extended semen should be promptly removed from the incubator for storage because extensive damage to spermatozoa will occur within a few hours at this temperature [21, 22]. Both cooling rate and storage temperature affect spermatozoal survival following storage. A storage temperature of 4-6°C is considered preferable as long as it is achieved with a relatively slow cooling rate [23]. Studies have shown that spermatozoa are most sensitive to rapid cooling between 20°C and 5°C. Spermatozoa can be rapidly cooled from 37°C to 20°C, but may require a cooling rate of -0.05 to -0.1°C/minute from 20 to 5°C to maximize spermatozoal motility [24]. Cooling rate and storage temperature are affected by the type of container that is being used to transport the semen [25].

Factors Influencing Success Rates When Breeding With Transported Cooled Semen

Numerous factors influence pregnancy rates achieved in mares bred with cooled stallion semen. In controlled laboratory experiments, factors which influence viability of cooled stallion semen include the number of spermatozoa inseminated, frequency of insemination, concentration of spermatozoa in extender, type of extender utilized (including antibiotics), cooling rate of extended semen, storage time and storage temperature prior to breeding, response of spermatozoa to cooling, and inherent fertility of the stallion and mare [3-7]. If the quality of the stallion's fresh semen is poor or pregnancy rates achieved by breeding with fresh semen are low, it is highly unlikely that breeding with cooled transported semen will be successful.

Other factors which undoubtedly impact fertility using this technology include semen collection technique, semen collection-extension interval, semen packaging technique, insemination-ovulation interval, and insemination technique. Spermatozoa are very sensitive to many environmental factors, including temperature, light, physical trauma, and a variety of chemicals. Therefore, any factor that negatively impacts the ability of spermatozoa to resist environmentally-induced damage will adversely affect fertility achieved when using cooled transported semen for breeding.

Pregnancy rates per cycle achieved by breeding with semen cooled for 24 hours at 5°C can reach 60 - 70%, so one can expect near normal pregnancy rates when semen is used for breeding after short term (≤ 24 hours) storage, provided semen quality is good following this cooling period [3, 5-7]. Breeding with equine semen cooled for 48 hours at 5°C usually reduces pregnancy rates to approximately half that achieved with fresh semen [3].

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