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**ARTIFICIAL INSEMINATION (AI) USING COOLED AND FROZEN SEMEN**

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### ***Introduction***

Only recently have nearly all equine breed registries allowed the use of shipped cooled or frozen equine semen, so economic interest and therefore funds for research in this field have been restricted and scientific progress has been slow.

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 artificial insemination (AI). As the fertility of cryopreserved stallion semen has improved and simple AI protocol have evolved, breeders are more often opting to use transported frozen semen for the additional benefits realised. Access to semen from stallions standing abroad, competition stallions, stallions that become ill, injured or overbook 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 cooled and frozen semen however requires that the practitioner be familiar with the techniques for properly handling of it as well as the breeding strategies that are being employed to maximise fertility. Additionally, practitioners need to be informed of the limitations of any technology so as to better assist their clients in making decisions about what is appropriate for their particular situation.

### ***What is the Expected Fertility when using Cooled and Frozen Semen?***

Although cooled semen is a well-established method for insemination of mares, the fertility of mares inseminated with cooled semen is difficult to determine. Few studies have provided a comparison of fertility from cooled and frozen semen. Jasko *et al.* (1992) reported a one-cycle pregnancy rate of 65% and 56% for cooled and frozen semen, respectively. These mares were all inseminated with semen from the same stallions and processed by skilled technicians. Loomis (2001) reported similar one-cycle pregnancy rates for mares inseminated with cooled (59%) and frozen (51%) semen in a commercial setting. A retrospective study (Squires *et al.*, submitted for publication) conducted during the 2002 and 2003 breeding seasons in six different AI centres throughout the United States and Europe showed a per-cycle pregnancy rate of 44% and 46% for cooled and frozen semen, respectively. Several recent studies have reported the fertility of frozen-thawed equine spermatozoa (Barbacini *et al.* 1999; Barbacini 2000; Vidament *et al.* 2000; Samper 2001; Sieme *et al.* 2003). The per-cycle pregnancy rate in a survey conducted by Samper (2001) was 56.7% for 578 mares inseminated in a clinical setting with frozen semen from a variety of sources and 53.6% and 52.3% for mares inseminated in Italy during the 1999 and 2000 seasons respectively (Barbacini 2000).

### ***Receiving and Inseminating Cooled Transported Semen***

Before semen is ordered, the mare should be ultrasonographically monitored to confirm normal cycling and ovulation. The stallion management should then be notified the first day of oestrus. Afterwards, the mare should be examined frequently during oestrus so that the stallion management can be informed at least 24 hours in advance of the desired insemination time. Mares should be inseminated based on impending ovulation rather than daily from the first day of oestrus. Most normally cycling mares can be inseminated with one or two shipments per cycle if frequent genital examinations are performed.

Receiving and inseminating cooled, transported semen is very simple and does not require expensive equipment related to semen handling. After the shipping container arrives and before it is opened, prepare the mare for insemination as for fresh semen AI. Open the container and gently mix the cooled semen inside the whirl-pack bag or the plastic vial. Open the specimen bag or vial and draw up the entire insemination dose (provided the volume does not exceed 50-60 ml) into an all-plastic non spermicidal syringe. Place a small aliquot into a clean vial or test tube and warm to 37° C (5-10 min) in a controlled temperature water bath for evaluation of spermatozoal motility. The syringe is connected to an insemination catheter which is then introduced through the mare's vagina and cervix into the uterine body with the instrument guarded by a gloved hand previously lubricated with a sterile, water soluble, non-spermicidal gel. The semen is then expressed into the lumen of the mare's uterine body. Note that it is not recommended that the semen be warmed prior to insemination. All semen handling may be performed at room temperature.

### ***Shipping and Storage of Frozen Semen***

Frozen spermatozoa lose fertilising 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 minimising exposure to increased temperatures. Frozen semen is typically transported in a nitrogen vapour container. These cryogenic containers maintain near liquid nitrogen temperatures (around -190° C) for days or weeks without the use of hazardous liquid nitrogen. Vapour phase containers work by absorbing nitrogen into a thick layer of absorbent material that surround the inner cavity of the container where the semen is stored. These type of 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 in order to provide maximum holding time.

One of the main benefits of using frozen semen is the fact that it can be shipped to the veterinarian or the mare owner in advance, thus eliminating the anxiety and logistical headaches associated with cooled semen breeding. Ideally, the semen can be ordered in advance of the mare's oestrus 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 oestrus 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. The canister should be slightly lifted into the neck of the shipper then the individual straws or the plastic goblet should be gently grasp using a pre-cooled haemostats or tweezers and the canister lowered back into the container. The canister or straws should never be lifted above the frost line (visible about 5cm from the top of the container neck) until it is the time to transfer the semen. While holding the semen well inside the shipper, a canister has to be lifted from the storage container slightly up into the neck, again never above the frost line. As quick as possible transfer the straws from the shipper into the liquid storage canister and lower back down into the liquid nitrogen.

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 aluminium containers directly on concrete floors as this will erode the aluminium.
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  $\frac{1}{2}$  capacity.
6. Inspect the neck cork regularly for damage and replace if necessary to maintain maximum holding time.

### ***Thawing and Insemination***

During cooling and freezing sperm undergo a series of chemical and physical changes that include partial dehydration, cryoprotectant penetration of cells, reorganisation of membrane lipids and proteins, exposure to high salt concentrations and exposure to inter and intracellular ice crystals. Cryopreservation protocols are designed to minimise the negative effects of these stresses. Thawing sperm 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 reorganise and cryoprotectants diffuse out of the cells. Thawing semen improperly, i.e. too fast or too slow for the freezing protocol employed will reduce the viability of the sperm and decrease 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 30 seconds 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 sperm will die rapidly kept at 39° C or 40° C whereas they will survive quite well at 34° C or 35° C. Some freezing laboratories recommend thawing semen frozen in 0.5 ml straws at 75° C for 7 seconds. While this technique works well for recovering sperm motility when performed correctly, the risk of damaging the sperm from exposure to 75° C temperature for more than exactly 7 seconds 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 seconds. 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 up into a standard insemination pipette to inseminate the mare. Although most commercial laboratories now freeze semen in 0.5 ml straws, some semen is still frozen in 4 or 5 ml macrotubes. These straws are generally thawed at 50° C for 45 seconds. With these straws it is critical that they do not remain in the 50° C water bath for more than 45 seconds to prevent damage from exposure to elevated temperatures.

### ***Strategies for AI with Frozen Semen***

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 hours prior to or within 6 hours 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 3 to 4 times per day examinations of mares inseminated with frozen semen. In a retrospective study performed at Cristella (Barbacini *et al* 1999) on 559 warmblood mares inseminated only once per oestrus a 76.7% and 41.25% seasonal and per cycle pregnancy rates were obtained. The following protocol was used in this study as soon as a cyclic mare was detected in oestrus:

- ✓ Daily ultrasonographic monitoring of ovarian activity until one or more follicles with a diameter of at least 35-40mm was detected.
- ✓ Induction of ovulation by intravenous administration of 2.000 IU human chorionic gonadotrophin (hCG).
- ✓ Ultrasound scan examination 12 hours after hCG treatment, then every 4-6 hours until ovulation occurred.
- ✓ Insemination.

By using this protocol it was possible to inseminate all the mares in a period of 6 hours pre- and 6 hours post-ovulation.

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 monitoring of ovarian activity during oestrus until one or more follicles with a diameter of at least 35-40mm is detected.
- ✓ Induction of ovulation by intravenous administration of 2.000 IU human chorionic gonadotrophin (hCG).
- ✓ Two inseminations performed at 24 and 40 hours post-injection.

Using this insemination schedule, mares that ovulate 18 to 52 hours after administration of the ovulatory agent will have had sperm deposited in the reproductive tract within 12 hours prior to ovulation or within 6 hours after ovulation or both. In a clinical trial conducted at Cristella (Reger *et al.* 2003) 26 of 34 mares conceived (76%) after two timed inseminations vs. 15 of 21 (71%) conceiving following a single insemination within 6 hours post-ovulation. Moreover, in a controlled study performed at Colorado State University (Reger *et al.* 2003) no difference in embryo recovery rates was reported for mares inseminated once within 6 hours post-ovulation with  $800 \times 10^6$  total frozen-thawed sperm (60%) vs. mares inseminated twice at 24 and 40 post-deslorelin with  $400 \times 10^6$  total sperm per insemination (55%).

During the 2002 and 2003 breeding seasons, data was collected from Select Breeders Service affiliated laboratories from 408 mares (Squires *et al.*, submitted for publication). These mares were inseminated with semen from numerous different stallions whose semen was frozen by various laboratories in Europe and in the USA. The overall first cycle and seasonal pregnancy rates were 46% and 75% respectively. No difference was observed in first cycle pregnancy rates between mares inseminated once (50%) or multiple times (47%) per cycle.

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 to 8 hours after ovulation. This usually requires that mares are boarded at a clinic or late night farm calls must be made by the practitioner to insure that the post-ovulation insemination is performed within the critical 4 to 6 hours window after ovulation. The cost in veterinary care to the mare owner is substantial and often discourages them from utilising frozen semen. Stallion owners who sell semen by the dose for hundreds or even thousands of euros are forcing the mare owners to utilise 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.* (2003). In this study, mares inseminated twice per cycle at 24 hours intervals had a 50% per cycle pregnancy rates and mares inseminated once averaged 42% per cycle pregnancy rate. Pregnancy rates for mares inseminated once within 12 hours prior to ovulation or once within 12 hours post-ovulation were 41% and 50%, respectively. Samper (2001) and Vidament (1997) also reported that pregnancy rates with frozen semen were higher when mares were inseminated more than once per cycle.

A two-dose timed insemination protocol allows a practitioner to examine mares once daily during normal hours without compromising fertility. 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 minimise invasion of the susceptible uterus. Older mares may also be less responsive to ovulatory agents such as hCG (Barbacini *et al.* 2000). Although not examined directly in this study, the lower conception rate for older mares bred with multiple vs. single inseminations may be due to these factors.

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