Breeding With Chilled And Frozen Semen

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Artificial Insemination (AI) involves the introduction of sperm into the reproductive tract of the mare without natural mating. AI in the horse was first practiced long ago. Ancient Arabian texts describe how mares were successfully inseminated. In the late 18th Century, an Italian scientist took the idea up again. He performed a series of studies in which he placed stallion semen in the snow and found that he did not necessarily kill the spermatozoa (or "spermatic vermiculi" as he termed them), but merely made them inactive. Upon warming, their motility returned. The use of AI on a regular basis in horse breeding dates back to the beginning of the 20th Century.

The first organized AI programs were run on Russian stud farms. During the past two decades, AI has been accepted by many national and international breed authorities throughout Europe and America. In some countries, for example The Netherlands, several thousand mares are inseminated each year.

There are some breed restrictions to the use of AI, most noticeably the General Stud Book and The Jockey Club, which regulate the Thoroughbred. It is therefore important to know which breed registries permit AI and which do not.

For those of us currently involved in the equine breeding industry, it is obvious that AI is set to play an ever-increasing role in the future. It is equally apparent that there is a need for breeders and veterinarians alike to understand fully and to be familiar with the techniques of AI in the horse. This should ensure first of all the welfare of the horse and secondly the success of AI in the horse.

A successful AI program depends upon both the stallion and the mare, and it involves for the stallion the following components: a thorough examination for breeding soundness, confirmation that the stallion has semen of sufficient quality, and appropriate cooling and storage of the semen sample after collection.

It involves much more for the mare: a satisfactory breeding soundness examination; the induction of an ovulatory estrus; the accurate prediction of ovulation; correct timing of insemination relative to ovulation; appropriate storage, thawing, and handling of semen; correct insemination technique; post-insemination examination and treatments as required; and correct pregnancy diagnosis 14 to 16 days after insemination.
There are two key concepts—evaluation of the semen sample and a well-timed insemination. It is no use merely collecting a sample of semen; the sample must be thoroughly evaluated to make sure that it is of sufficient quality to be used in an AI program. The semen must be processed adequately to assure that it retains its fertilizing potential. Likewise, the sperm must be infused at the appropriate stage of the mare's reproductive cycle.

It is important to be aware that AI in the horse requires a high degree of veterinary input and is not a cheap alternative to natural breeding. It is vital that there is good communication at all times between the mare owner and stallion owner, and their respective veterinarians.

Advantages And Disadvantages Of Breeding With Chilled Or Frozen Semen

Although AI has many advantages, there are some drawbacks to consider.

Advantages of using AI include the following:

1) Eliminating the cost and stress of mare (and perhaps foal) transport.

2) Maximizing the efficiency of stallion usage as more mares can be bred from one ejaculate.

3) Increasing the availability of stallions both within and among countries.

4) Preventing the mare from returning home in poor condition and reducing the likelihood of mare injuries or of the foal's contracting disease.

5) Evaluating the semen on a regular basis, which means that any problems in the stallion's fertility will be identified more quickly.

6) Using extenders with proper antibiotics to preserve the longevity of sperm and minimize bacterial contamination.

7) Enhancing the safety of animals and animal handlers.

8) Reducing the risk of sexually transmittable and other diseases spreading through a breeding population or among farms.

Among the disadvantages of AI are these:

1) The higher costs due to the considerable technology and skill required to collect, evaluate, and ship semen properly.

2) The need for considerable amounts of paperwork and the need for tightly controlled regulations for the control of diseases to guarantee the export of
3) The requirement of adequate infrastructure for transporting the semen.

4) Semen from some stallions will not tolerate the cooling and/or the freezing and thawing process.

5) Mare owners in remote areas might not be able to have the stage of the mare's cycle determined.

For an AI program to be successful, strict attention should be paid to health precautions and hygiene. There should be strict adherence to Guidelines and National Codes of Conduct for Disease Control appropriate for your country to reduce the risk of disease transmission. Bacterial cultures from the stallion's semen, urethra, and prepuce submitted to an appropriate laboratory to identify any venereal pathogens should be performed. These samples need to be taken regularly if the stallion is involved in both a natural breeding and AI program.

The Stallion: Semen Collection And Handling

Most stallions easily can be collected artificially using one of several models of artificial vaginas (AVs; the Hannover model is illustrated in Figure 1). Although shape and size vary, AVs work with the same principle: a double rubber liner with a water jacket in between.

The stallion will ejaculate in response to appropriate temperature, pressure, and friction of a well-lubricated AV. As a rule, the inner liner temperature of the AV should be between 43° and 45° Centigrade. Selection of temperature and pressure will depend on stallion's preference. The AV should be well lubricated with a sterile, non-spermicidal lubricant. The container where the semen is collected should be dry, clean, and at body temperature in order to prevent damage due to "cold shock" of the sperm cells. Cold shock will result in premature death of the sperm and/or abnormal motility patterns. The changes resulting from cold shock in most cases will severely compromise the fertilization potential of spermat-ozoa.

Virtually all stallions of normal libido will ejaculate into an AV provided it has an appropriate temperature and pressure and is properly lubricated. The correct pressure can be achieved by adjusting the amount of water or air in the AV. It generally is preferable to overfill the AV since it is easier to remove than add water. The optimum temperature of the water immediately before collection is 48° C. This equates to a luminal temperature of 45° C. A marginal increase of water temperature (up to 52° C) might be beneficial if the stallion will not ejaculate. Depending on the stallion's preference, pressure and water temperature can be adjusted.

If the stallion does not mount quickly, it is worth considering the use of an AV model such as the Colorado, which contains a large amount of water and
thus retains heat better than a lighter model with less water (Figure 2). On the other hand, this model can be very heavy.

The Hannover AV has a partial partition at the distal end, and some stallions prefer to touch this with their glans penis for extra resistance. For very small ponies, a bull AV can be used.

A small amount of non-spermatotoxic lubricating gel must be applied before use. This might not be necessary if the plastic disposable liners are used. A roll of plastic film, together with a sterilizable bottle (such as a baby bottle), can be used as a disposable AV liner. These liners can be used with or without an in-line filter to prevent contamination of the ejaculate with dirt or gel.

It is important that the liner is non-spermicidal. If a rubber liner is used, it is important to clean, rinse in de-ionized water, and air dry the liner. Plastic liners are easier to use, but a few stallions seem to dislike their texture and will not readily use them.

The sterilized collection bottle should be kept in the incubator at 37°C until just before use, and maintained close to this temperature throughout the collection process. An insulated cover for the collection bottle is useful.

Stallions can be collected either mounting a mare in heat, a breeding phantom or dummy mare (Figures 3A and 3B), or on the ground with proper manual stimulation. The "teaser" or "jump" mare should be of adequate size, should be sturdy, should be placid, and should be free of contagious diseases and in standing estrus. To keep a mare in estrus, it is possible to ovariectomize her and treat with estradiol benzoate (50 mg per week). A tail bandage is applied and her perineal region and flanks cleaned. It is necessary that she stand still, so a twitch might be needed. To protect the stallion against being kicked by the mare, a breeding hobble (such as a rope around her hind legs and tied around her neck) can be used. To protect the mare against biting from the stallion, the mare's neck and shoulders can be covered with a cloth or shield (Figure 4).

Routine pre-collection hygiene measures for the stallion include cleansing of the penis with hand-warm water (40°C) and several disposable cloths. Cleansing begins at the glans penis, then the sheath and prepuce are washed. Special attention should be paid to the fossa around the urethral process with the urethral sinuses and the urethral diverticulum. These parts often are contaminated with smegma. Dry the penis thoroughly with a clean disposable towel, although some prefer not to dry the penis. Antibiotic or disinfectant solutions should not be used. If the stallion has not been used for collection or a long period (one to two weeks), the routine initially should be performed every time before collection. If the stallion is collected daily, it is suggested that the treatment be performed once or twice weekly. The majority of stallions accept the treatment well after they become accustomed.
Semen should be collected into a pre-warmed container, as mentioned earlier, and maintained at body temperature while in its raw state. Semen should be filtered to remove the gel fraction and other debris if an in-line filter was not used (Figure 5) and diluted with an appropriate pre-warmed extender. The physical characteristics of the ejaculate, including volume, concentration, color, and motility, should be evaluated. Motility is checked using a microscope with a warming table at 37° C. (Figure 6).

**Semen Preservation**

The temperature at which semen should be stored depends on the period of time from collection to insemination. Semen that is to be inseminated immediately can be used undiluted, but if the semen is to be used after 10 minutes, it is best diluted with pre-warmed extender. Semen to be used within six to eight hours after collection can be stored at room temperature (18-22° C) in a dark environment. Removing the seminal plasma by centrifugation might be beneficial for certain stallions. It is preferable that semen stored for such a period should not be subjected to fluctuations in temperature, and storage in a light-proof, air-tight container (such as a thermos flask) should be considered. If semen is intended to last longer than eight hours, it should be cooled down to 5-8° C over a two- to three-hour period to prevent too much energy loss of the spermatozoa.

The rate of cooling from room temperature is critical. Approximately 12 years ago, the Hamilton Thorne Company developed a container (The Equitainer) for transporting chilled equine semen (Figure 7). This consists of a strong container with a snap-lock that holds frozen canisters, insulation, thermal ballast bags, and an isothermolyzer. The container is special in that it has the ability to lower the temperature of the semen package approximately 0.3° C every minute until the temperature stabilizes at 4-6° C. The semen should remain constant at that temperature for 48 hours. This cooling rate was designed specifically after trials were conducted to determine sperm motility after cooling at various rates. If the total volume of the extended semen and thermal ballast lies between 120 and 170 ml, the semen will cool at the proper rate.

Special equipment, such as the Equitainer, is required for optimal survival of semen after transport. In my experience, the Equitainer is easy to use, durable, and works very well. Disposable containers for shipping chilled semen are available on the market, at less cost than the Equitainer, but sperm survival might not be optimal under certain conditions due to storage temperature fluctuation and reduced storage period.

In The Netherlands, a polystyrene box in which two 15 ml tubes with chilled semen can be placed is used to transport chilled semen (Figure 8). The polystyrene box is packed in a cardboard box, which can be placed in a refrigerated (at 5° C) transporter. Semen arrives at the insemination center to it.
later that afternoon, i.e., within eight hours of collection. This is an efficient and inexpensive system, but is only really possible in a small country such as The Netherlands, where the distances over which the semen needs to be shipped are smaller.

Some stallions have semen that transports well in the cooled state, while others do not. For that reason, it is advisable to test the storage ability of a stallion before his semen is shipped to a client. An ejaculate should be collected, diluted 1:2 with an appropriate extender, placed in an Equitainer, then examined at 12-hour intervals for sperm motility.

Because one usually packs at least one billion progressively motile sperm per insemination dose, final dilution rates will vary (1:1 to 1:6) according to the density of the semen collected. It is generally accepted that approximately 50% of spermatozoa are non-viable by the time of insemination due to the process of cooling and shipping the semen, and that is the reason one billion progressively motile spermatozoa should be shipped.

The ideal dilution rate for chilled semen is within the range of 25 to 50 million spermatozoa per ml. This means that in most cases, the volume of shipped extended semen can be kept in the range of 30 to 60 ml, which would seem optimal. Extended semen that is too dilute will result in large volumes being lost from the mare's reproductive tract via the cervix. The low spermatozoa concentrations might not achieve optimal pregnancy rates.

Each shipment of fresh or chilled semen has to be accompanied by documents with information on the stallion (identity and health status), the collection center, collection date, shipment date, and information about the semen quality and the number of sperm sent.

If life expectancy of the semen is longer than 72 hours, cryopreservation (freezing) seriously should be considered. There are several additional advantages of frozen semen:

1) Semen can be transported easily internationally (this also can be done with chilled semen in some cases).

2) Semen can be used from stallions which are away competing for long periods of time or recovering from injury.

3) Semen can be frozen from potentially valuable stallions and stored indefinitely.

There are several disadvantages:

1) Pregnancy rates using frozen semen often are disappointing. Indeed, pregnancy rates can be 0% if strict stallion selection and careful insemination routines are not practiced. There is considerable variation among breeds with
regard to the ability of semen to freeze satisfactorily.

2) Charges for freezing stallion spermatozoa are relatively high.

3) Frozen-thawed semen has a very short life span, which means that insemination must take place very close to ovulation. This means repeated and frequent veterinary examinations are necessary, which further increase costs.

Mare owners often pay large sums of money for frozen semen of unknown potential. Post-thaw motility results often are quoted as the basis for selling frozen semen. It would be better to select stallions on the basis of fertility (number of live foals produced from frozen semen inseminations) rather than post-thaw motility because the correlation between fertility and post-thaw motility of frozen semen could be poor. Stallion owners should be encouraged to sell semen only on the basis of confirmed pregnancies. When substantial fertility data have been accumulated on a particular stallion, his semen can be sold with an expected pregnancy rate in mind.

**The Mare**

Before a mare owner begins on a breeding program using chilled or frozen semen, the difficulties associated with the use of the technique, as well as an indication of the expected success rates, must be understood. Many mare owners seem to believe that once they have decide to breed a mare, she will become pregnant and have a live foal the following year. This is simply not true, and it is important that they are aware of the likelihood of success. For a typical set of circumstances, i.e., a reasonably fertile mare and good-quality semen, one can expect pregnancy rates of 55-70% per cycle with chilled semen and 35-50% per cycle with frozen semen. The overall pregnancy rates at the end of the season vary between 50% and 90%, with an average of about 75%. Of course, some mares lose the pregnancy and the resulting live foal rate ends up around 65%.

It also is important to realize the costs involved with using AI. Many mare owners expect costs to be decreased because of no transport of the mare. They do not realize that there can be considerable costs involved with the collection and transporting of semen and for monitoring the reproductive cycle of the mare and inseminating her at the appropriate time.

**Breeding Soundness Examination**

All mares should have a thorough pre-breeding assessment before breeding with chilled or frozen semen. This is to identify mares likely to conceive successfully and carry a foal to term. While unforeseen problems can occur, there are procedures that can help you and your veterinarian decide if a mare is suitable for breeding. The suitability for breeding does not refer to the quality of the horse or its temperament; rather it is an assessment of the genital health of that particular mare. Collectively, these procedures are
known as the breeding soundness examination.

First of all, a complete breeding history of the mare for the last five years should be taken. By answering a series of detailed questions, mare owners will help uncover information that otherwise would remain unknown. Some relevant questions are as follows:

**How old is the mare?** Fertility declines with age once a mare is more than 10 years old. This is not to say that old mares cannot become pregnant and have a foal, but in general, the older a mare is, the less likely she is to have a foal.

**Has the mare been to the stallion or inseminated previously?** If so, **has she become pregnant and delivered any foals?** Mares which have had foals have at least proven that they are capable of doing so. Maiden mares which have not had foals are more of an unknown quantity. In many cases, they are young mares and go in foal relatively quickly and easily. However, if the maiden mare is older, she will be less likely to get in foal. The breeding history should include a record of whether the mare has ever aborted, conceived twins, suffered dystocia, or failed to conceive.

The gynecological examination should consist of a thorough and systematic rectal examination (both manual palpation and ultrasound) of the cervix, uterus, and ovaries. The vagina and cervix also should be examined using a speculum (a narrow tube down which you can shine a light), manually by vaginal palpation, or both. In many cases, a swab of the lining of the uterus will be taken to see if there are any bacteria present or signs of an inflammation of the uterus (endometritis).

During estrus, your veterinarian should continue to monitor the cycle by daily (more frequently when frozen semen is being used) rectal palpation and ultrasound, and/or teasing, if available. Ultrasonographic examinations should be done prior to breeding. These exams provide information as to the number of follicles of ovulatory size, prevalence of uterine cysts, and any sign of inflammation and/or infection.

In many cases, mares which are intended for AI often are kept at the owner's home, where no teaser stallion is available for detection of estrus. This also could be true if, as is often the case (in England) for ease of examination, the mare is kept at the veterinarian's premises. Detection of estrous behavior in the absence of a teaser stallion usually is misleading and, therefore, the attending veterinarian must be prepared to induce and diagnose estrus in the absence of a stallion.

Prediction of ovulation is not easy and involves taking into consideration several findings and making a considered judgment. By a combination of daily rectal and vaginal palpations and ultrasound examination, an experienced veterinarian usually can make an accurate prediction of when ovulation will happen. Hormones frequently are used to induce ovulation. The
most commonly used method is the intravenous administration of 3,000 IU human chorionic gonadotrophin (hCG) once it has been established that the mare is in estrus with a soft follicle at least 35 mm in size and an edema pattern (Figure 9) visible in the uterus. Approximately 85% of mares will ovulate in the 24 to 48 hour period following hCG administration. Recently, use of a gonadotrophin releasing analog, deslorelin, has been found as effective in inducing ovulation as hCG.

**Timing Of Insemination**

Ideally, the stallion owner should be notified 48 hours prior to the desired breeding date and a clear communication channel opened between the mare owner's veterinarian and the stallion owner early in the cycle. Any application for import permits should have been made well in advance of the desired breeding date.

Accurate prediction of ovulation is important because the optimal time for AI with chilled semen is in the 24 hours leading up to ovulation. Pregnancy rates generally will decrease if insemination is outside this range. This time interval is shorter than if fresh semen or natural service is used. That is not to say that pregnancies will not occur when the interval exceeds 24 hours, but differences among individual stallions, different extenders, and different systems of cooling can cause a wide variation in the longevity of chilled semen not only in storage, but also in the mare’s reproductive tract.

With frozen semen, there is evidence that some semen might not remain viable as long as 24 hours following insemination, so insemination closer to ovulation is preferable. The regime most commonly used with frozen semen is to examine the mare every six hours as ovulation approaches. Insemination should be just before ovulation is anticipated. If semen supplies are limited, insemination can be withheld until ovulation is detected. This system ensures the use of a single insemination dose at a maximum of six hours after ovulation. In my experience, pregnancy rates do not begin to decline with frozen semen as long as insemination is within six hours following ovulation. Ideally, one would prefer to inseminate the mare in the eight hour period before ovulation. However, it is not possible to be 100% certain in predicting when ovulation will occur.

**Insemination Technique**

The mare should be identified from a passport or similar identity document and might need to be matched against an identity supplied with the semen. The documentation accompanying the semen should be checked and the paperwork should confirm that the stallion has passed all relevant health checks. Information on date and time of collection, motility, concentration, and type and ratio of extender used also should be included with each shipment. The veterinarian carrying out the insemination should certify that the semen has been received and that the identity of the mare has been checked and is the same as that described in the nomination agreement. It
should be further certified that only this mare has been inseminated and that any unused semen has been destroyed.

She should be prepared for insemination in a clean, well-lit environment; a crush (stock) for restraint is preferable. Her tail should be bandaged and tied out of the perineal region. The vulva and perineal area should be thoroughly cleansed with very dilute povidone-iodine solution or mild soap. This is then thoroughly rinsed off with fresh warm water and the perineal area dried with clean, soft, disposable (paper) towels.

If chilled semen is being used, the semen container should remain unopened until this stage. No attempt should be made to warm the semen prior to insemination of the mare. Since there might be a small delay between cleaning the mare and insemination, it might be helpful to empty the mare’s rectum of feces to prevent contamination of the area after cleansing. It is highly recommended that all the semen arriving should be inseminated as soon as the shipment arrives. Although many breeding farms ship semen for two inseminations, semen should **not** be stored for use 12 to 24 hours later. The oviduct (fallopian tube) of the mare is a far better incubator of spermatozoa than any transport system available. In addition, the first insemination might cause some degree of uterine inflammation, and any subsequent insemination will make this inflammation worse.

The semen should be gently mixed prior to loading into a sterile plastic syringe (without a rubber plunger). The syringe then should be attached to a sterile insemination pipette. The operator should use a "sterile" obstetric glove (i.e., a glove turned inside out). In certain circumstances, a sterile surgeon's glove should be placed over the clean rectal glove. It might be necessary to place a small amount of sterile, non-spermicidal lubricant on the top of the hand around the knuckles.

The catheter should be held with the tip behind the finger tip and the hand brought into the vulva (Figures 10 A and B). The external opening of the cervix should be located with the index finger, and a finger inserted into the cervical canal. The catheter is inserted alongside the finger and the catheter gently pushed forward. It is very important that the catheter reaches the mid or cranial uterine body and does not remain obstructed in the cervix. This passage through the cervix is not always easy. Deposition immediately cranial to the cervix should be avoided.

The syringe should be gently emptied, infusing the semen into the uterus. During the deposition of the semen, it is important that the tip of the catheter not be buried in the uterine mucosa or a uterine fold. Any resistance to the flow of semen should be corrected by a fractional withdrawal of the catheter.

A small amount of semen should be warmed to 37° C, after which it should be examined for progressive motility and gross abnormalities.

Once the semen has been thawed, the technique for insemination of frozen-
thawed semen is identical to that used for fresh or cooled transported semen, except that one should use warm instrumentation (catheter and syringe). No attempt need be made to increase the volume of semen frozen in small containers. Equine semen can be frozen in 0.5 ml straws like those used for cattle. In that case, between one and eight of them are required per insemination (Figure 11).

When several straws are required for one insemination, the thawed semen should be pooled, then inseminated. Plastic bags, aluminum sachets, pellets, 1 ml vials, 1ml straws, and 4.5 ml "Maxitubes" with ball seals at either end also are used for freezing semen. Each package has its own thawing method, and if thawing instructions are absent, the inseminator should obtain them before using the semen because one cannot assume that because semen is in a particular kind of package, that it should be thawed by a certain method. Obviously, an optimal packaging system has yet to be developed. Marking on semen packages also is variable.

Examination Following Insemination

The mare should be checked for ovulation by your veterinarian within 24 hours. It might be necessary to order a second delivery of semen if the time of ovulation has been miscalculated. It cannot be assumed that just because sufficient numbers of viable sperm have been inseminated at the optimum time relative to ovulation that pregnancy automatically will ensue in a mare. Breeding induces an acute inflammatory response, which is normal and beneficial. The reason many mares, particularly old maiden mares, fail to become pregnant is defective uterine clearance of this inflammatory "soup." It is the spermatozoa themselves that elicit the most acute inflammatory response. (A detailed discussion of the inflammatory response to breeding and the management of the susceptible mare will be covered in a future article and only brief details are given here.)

Ultrasonic examination of the uterus 12 to 24 hours after insemination often shows collections of fluid (Figure 12). These must be removed if optimum pregnancy rates are to be achieved. Oxytocin probably is the drug of choice. Subsequent intrauterine antibiotic treatments can be beneficial in certain cases.

Mares with defective uterine clearance are better treated in relation to insemination rather than waiting for ovulation. Large-volume lavage with warm saline solution in addition to oxytocin might be beneficial. The perineal conformation of the mare should be checked and a Caslick operation performed if necessary.

Examination of the mare for pregnancy should take place as early as possible using ultrasonography. This is best done 14 to 15 days after insemination. The 14 day pregnancy is 13 to 18 mm in size (Figure 13). The embryonic vesicle grows at a rate of approximately 3.5 mm/day at this stage of pregnancy and remains highly mobile, making thorough examination of the
In conclusion, the success of breeding with chilled or frozen semen depends on the fertility of the stallion, the fertility of the mare, and managerial practices. The end product (foal) is an interaction among all of these factors.

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About The Author

Jonathan F Pycock, BVetMed, PhD, DESM, MRCVS, RCVS Specialist in Equine Reproduction, is a 1983 graduate of the Royal Veterinary College, University of London, from where he obtained his PhD in 1988 for his thesis on breeding problems in the mare. He was in private equine practice until 1994, when he took a position as Associate Professor of Equine Reproduction at the University of Utrecht in The Netherlands. While there, he developed a special interest in artificial insemination with both chilled and frozen semen and worked extensively in those areas. He was awarded his Diploma in Equine Stud Medicine in 1994, and in 1995 became an RCVS recognized Specialist in Equine Reproduction. He returned to the UK in January 1997 to begin Equine Reproductive Services, a first opinion and referral private equine practice based in Yorkshire. He has published many papers and book chapters and recently completed editing the book Equine Reproduction and Stud Medicine. He is Chairman of the AI Committee of the British Equine Veterinary Association and is responsible for organizing the courses for technicians involved in equine AI.