

Compendium

Artificial Insemination and Embryo Transfer in Mares

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Abstract: Mares can be artificially inseminated with chilled or frozen semen to increase the revenue from their offspring. Embryo transfer can be used to produce more than one foal from a single mare per season. Recent advances in using equine follicle-stimulating hormone to induce superovulation in mares have stimulated research on preserving equine embryos. Equine embryos are usually collected on day 7 or 8 after ovulation, and younger (day 6.5) embryos are typically cryopreserved. Cryopreservation improves the ability of veterinary clinicians to preserve embryos for implantation in recipient mares and facilitates international exchange of embryos.

Anecdotal evidence suggests that, in the 1300s, horses were the first domestic animals to undergo artificial insemination (AI). To mate mares with outstanding stallions owned by enemies, Arabs are said to have collected semen from the vaginas of their enemies' mares and introduced it into their own mares. However, more reliable evidence suggests that AI was first performed in dogs and that the development of AI in horses paralleled that of several other domestic species at the end of the 18th century.¹ In contrast, the development of embryo transfer in horses has lagged behind that of other species, most notably cattle. The difference is a result of interest in the procedure within the respective industries and in the technical challenges of working with diverse species. Initially, difficulties in transporting equine semen restricted AI to the premises where semen was collected. However, improvements in semen extenders and cryoprotectants have allowed AI using cooled or frozen semen to become a major breeding tool. The technical reasons why equine embryo transfer has lagged behind its bovine counterpart are steadily being addressed. Specifically, stimulation of multiple ovulations through exogenous gonadotropin (equine follicle-stimulating hormone [FSH]) and the development of efficient methods of nonsurgical embryo transfer have contributed to a resurgence of interest in embryo transfer technology in horses. However, embryo cryopreservation, which allows long-term storage of embryos and has successfully been used to improve cattle production, remains problematic in horses because of the size and morphology of the embryo at the time of collection. If the problems are overcome, equine embryos could be frozen to preserve valuable genetic lines and sustain embryo viability when a suitable recipient mare is not readily available. Therefore, current research is focused on establishing optimal freezing techniques for equine embryos when they are most successfully collected (i.e., 7 or 8 days after ovulation).

Artificial Insemination

Factors that influence the success of AI in mares include the following:

- Whether fresh, chilled, or frozen semen is used
- The quality of the semen
- The number of spermatozoa used per insemination
- The preparation (extending, chilling, or freezing) of the semen
- The number of inseminations
- The timing of insemination
- The anatomic placement of semen

When natural breeding was compared with AI, no difference in pregnancy rates was found in fertile mares: 23 of 31 mares (74.2%) became pregnant after natural breeding with one of three stallions, while 51 of 68 mares (75%) conceived when artificially inseminated with fresh or chilled semen from the same stallions.² However, it is very important for mares to be inseminated as close to the time of ovulation as possible. The optimal time for AI using chilled semen is within 24 hours before expected ovulation. The insemination should be repeated after 48 hours if the mare has not ovulated. When frozen semen is used, every attempt should be made to inseminate the mare within 12 hours before ovulation or within 6 hours after ovulation. If only one dose of frozen semen is available, the mare must be examined by ultrasonography at 6-hour intervals and inseminated as soon as ovulation is detected.

Because the timing of insemination in relation to the occurrence of ovulation is crucial, the prediction of ovulation is very important. To predict when ovulation will occur requires repeated manual and ultrasonographic reproductive examinations to identify changes in (1) the appearance of the uterus, (2) follicular size and turgidity, and (3) the echogenicity of the follicular rim. During estrus, the endometrial folds become markedly edematous

and display a typical “cartwheel” pattern. Clinical observation can detect the rate at which edema increases in the endometrium during the first few days of estrus concomitant with increases in follicular size until the endometrium becomes maximally edematous. As ovulation approaches, endometrial edema normally decreases. Just before ovulation, the follicle becomes softer and painful and may become triangular or irregularly shaped. At the same time, the echogenicity of the follicular wall increases and an anechoic line appears around the follicle. These changes are related to increased vascularity of the follicular wall and lead to edema, hemorrhage, and hyperemia in the follicular layers. Color Doppler ultrasonography can be used to quantify these changes in the follicle before ovulation.³⁻⁵

To improve the chance of detecting ovulation or to time ovulation with the availability of semen, ovulation can be induced with deslorelin (1.5 mg) or human chorionic gonadotropin (2500 IU). If chilled semen is used, a single insemination should be performed approximately 24 hours after induction of ovulation, and ovulation should be confirmed the following day. When frozen semen is used, ultrasonographic examinations should start 12 to 24 hours after ovulation has been induced; these examinations should be performed every 6 hours until the mare has ovulated. Because of postovulatory aging of the oocyte, insemination more than 6 to 12 hours after ovulation increases the rate of embryonic loss no matter what semen preparation is used.⁶ When frozen semen is used, pregnancy rates can be as high as 70% when insemination occurs within 6 hours after ovulation and 50% when the postovulatory insemination interval is extended to 12 hours.^{6,7} When more than one dose of frozen semen is available during a given heat cycle, the mare should be inseminated at 24 hours after induction of ovulation and again 16 hours later (40 hours after induction of ovulation), even if the mare has ovulated by the second time point. This ensures that viable sperm are in the oviduct for 18 to 52 hours after induction of ovulation.

There is no consensus on the minimum number of progressively motile spermatozoa required for AI, and data vary between laboratories. However, when mares are inseminated with fresh or chilled semen, a dose of 500 million progressively motile spermatozoa at the time of AI will ensure an acceptable pregnancy rate.⁸ Internationally, a minimum of 300 million progressively motile spermatozoa per dose is required for thawed frozen semen. However, if semen from a highly fertile stallion is used, the insemination dose can be reduced to 100 million progressively motile spermatozoa without reducing fertility.⁸

The standard insemination technique is to place the semen in the uterine body. However, when frozen semen is used, insemination deep into the uterine horn, ipsilateral to the dominant follicle, is advisable. This technique increases the number of sperm recovered from the oviduct, increasing the pregnancy rate in mares, especially if <50 million progressively motile spermatozoa are inseminated.⁹ Deep intrauterine AI may help achieve acceptable pregnancy rates when sex-sorted semen is used (a dose typically contains only 5 to 25 million progressively motile spermatozoa); however, the interest in sex determination of foals is limited.

The volume of extender used to suspend sperm cells in an insemination dose appears to have little effect on pregnancy rates. Doses of extender can vary from 0.5 to 120 mL, with no significant effect on fertility.⁸

Chilled semen shipments usually contain two insemination doses. Practitioners often debate whether to inseminate mares once with a double dose or twice, 24 hours apart. Both the number of viable spermatozoa and the timing of insemination are important in achieving pregnancy. In two independent studies, insemination of mares using one dose of semen on 2 consecutive days slightly improved pregnancy rates in one study¹⁰ but had no effect in the other study.¹¹ This suggests that if the quality of semen is suboptimal, the timing of insemination in relation to ovulation is more important

than the absolute dose of semen, but this needs to be definitively determined. However, in one of the studies,¹⁰ there were notable differences between stallions relative to the timing of AI using chilled semen. One of the three stallions used in the study impregnated more mares (six of eight [75%]) when the mares were inseminated twice than when they were inseminated once with a double dose (two of 10 [20%]).¹⁰ This suggests that interstallion variation is another factor that can influence the success of insemination using fresh, chilled, or frozen equine semen.

Frozen semen is thawed in a water bath; however, bath temperature and immersion time may differ between semen processors. Therefore, directions for thawing should be followed as designated. Semen frozen in 0.5-mL straws is most commonly thawed at 37°C (98.6°F) for 30 seconds, while 5-mL semen containers (macrotubes) are thawed at 50°C (122°F) for 42 seconds. The straw(s) should be dried thoroughly after removal from the water bath. It is practical to cut the straw(s) that constitute an insemination dose at one end and pour the entire volume of semen into a warm tube or directly into a warm syringe case. To prevent osmotic shock of the sperm cells, semen should not be mixed with extender after thawing.¹²

Progressive sperm motility is most commonly evaluated before AI. However, because sperm motility alone is a poor predictor of

Key Points

- When frozen semen is used, inseminating mares twice (i.e., 24 and 40 hours after induction of ovulation) is much less time-consuming than other methods and provides good results. It is recommended to insert both doses of chilled semen into the mare when the shipment arrives.
- The best predictor of fertility is the stallion's past conception rate per cycle.
- Giving oxytocin before the last uterine flush has been found to increase embryo recovery rates.
- Small equine embryos can be successfully vitrified, even after 12 to 24 hours of storage.
- Treatment of recipients with prostaglandin synthesis inhibitors can increase pregnancy rates.
- Embryos should not be collected from mares that are under heat stress, which decreases embryo recovery rates.

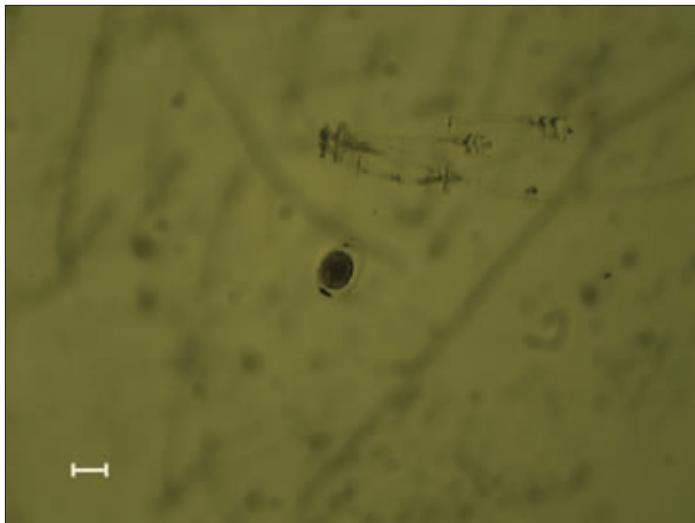


Figure 1. An equine embryo 6.5 days after ovulation. (bar = 100 µm)

fertility, sperm morphology should also be assessed, particularly when repeated use of semen from a specific stallion results in poor conception rates. Sperm cells can be stained with eosin-nigrosin and evaluated under oil immersion using a light microscope at $\times 1000$ magnification to determine the total number of morphologically normal cells. In an average stallion, approximately 50% of spermatozoa are morphologically normal.¹³ Sperm defects that affect sperm penetration and attachment, such as damage to the acrosome, require special staining techniques or electron microscopy to detect. In general, it is best to request the stallion's past conception rate per cycle, as this is the best predictor of stallion fertility.

Embryo Transfer

Embryo transfer makes it possible to obtain more than one offspring per year from valuable animals. Superovulation and embryo cryopreservation by slow freezing or vitrification have both had limited success in horses. The purpose of superovulation is to obtain more embryos at one time to make embryo transfer more efficient. Superovulation in horses is much less successful than in cattle. The standard equine FSH therapy protocol starts 5 to 6 days after ovulation is detected in the donor mare, when the diameter of the largest follicle is 20 to 25 mm. (Currently, no equine FSH products are commercially available in the United States). Starting on day 5 to 6 after ovulation, equine FSH is injected at a dose of 12.5 mg twice daily, and luteolysis is initiated using prostaglandin F_{2α} on the second day of FSH therapy. When the diameter of the largest follicle is >35 mm, FSH therapy is discontinued. After a 36-hour "coast" period, ovulation is induced using human chorionic gonadotropin or deslorelin, and the mare is inseminated twice. An average of 1.9 embryos have been recovered after superstimulation using equine FSH compared with an average of 0.5 embryo in control groups.¹⁴

If ovulation occurs from more than three follicles at once, the embryo recovery rate per ovulation decreases. One reason for

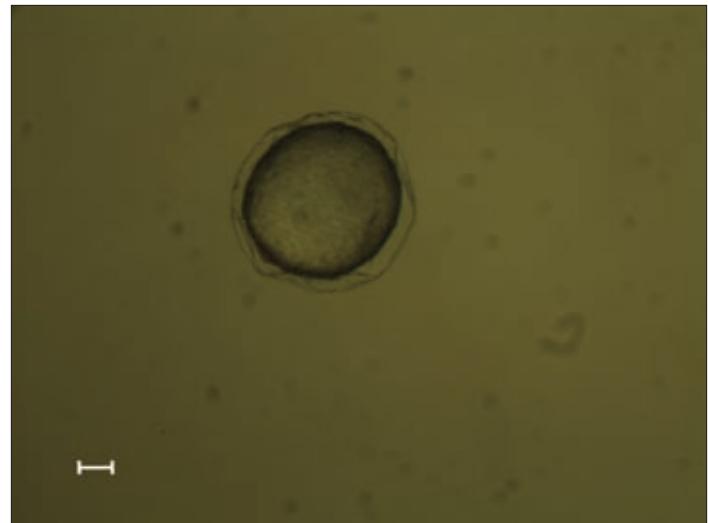


Figure 2. An equine embryo 7.5 days after ovulation. (bar = 100 µm)

this is that ovulation in mares occurs only through the ovulation fossa, and it is thought that the competing follicles cause a physical blockage, preventing the ova from reaching the ovulation fossa.¹⁵ It has also been proposed that the decrease in the embryo recovery rate might be caused by disruption of oocyte transport through excessive accumulation of coagulated blood in the ovulation fossa after superovulation.¹⁶

The flushing procedure for embryo recovery in horses is straightforward and easy to perform. To flush out the embryo(s), the uterus is repeatedly irrigated with 1 to 2 L of medium, which is passed through an embryo filter. Up to 8 L of flushing medium is required. The combination of administering oxytocin before the last flush, allowing the medium to remain in the mare's uterus for approximately 3 minutes, and massaging the uterus per rectum increases embryo recovery rates by approximately 10%.¹⁷ The embryo arrives in the mare's uterus as late as day 6 to 6.5.¹⁸ Embryos can first be recovered on day 6 to 6.5 with diameters <300 µm, but average diameters are 400 to 1200 µm on days 7 to 8.¹⁹

The difficulty associated with embryo cryopreservation in horses is related to the size and morphology of equine embryos when they are most readily collected. The timing of embryo collection significantly affects the success of embryo recovery and cryopreservation in horses. When the embryo enters the uterus on day 6 to 6.5 after ovulation, it is surrounded only by the zona pellucida¹⁸ (FIGURE 1). During the next 24 hours, a glycoprotein capsule forms and expands between the embryo and the zona pellucida, leading to rupture and removal of the zona pellucida²⁰ (FIGURE 2). The presence of the glycoprotein capsule complicates the conventional freezing process because the thick embryonic capsule of large equine embryos prevents penetration of cryoprotective agents.^{21,22} Therefore, although pregnancy rates after conventional cryopreservation of late morula and early blastocyst embryos (<300 µm; collected on day 6.5 after detection of ovulation) are comparable to transfer of fresh embryos (64% versus 70%, respectively),²³ the rates are poor after transfer of slow-frozen

expanded blastocysts (400 to 1200 µm; collected on day 7 or 8).²⁴ Embryo recovery rates are low at day 6.5 but increase to acceptable levels of 60% to 65% at day 7 or 8.¹⁹ Consequently, the collection rate is (1) poor when embryos respond best to conventional freezing and (2) optimal when embryos respond poorly to conventional freezing.

To resolve this problem, researchers have explored embryo vitrification. In contrast to conventional freezing methods, which use slow cooling rates of 0.3°C to 0.5°C (32.5°F to 32.9°F) per minute and low concentrations of cryoprotectants, vitrification involves incubation for a short time in more concentrated cryoprotectants, followed by plunging into liquid nitrogen. Consequently, during vitrification, the cooling rate in 0.25-mL straws is much faster (up to 20,000°C [36,032°F] per minute), and the solution goes directly into a glass or a vitreous state, avoiding ultrastructural damage caused by ice-crystal formation.²⁴ In one study,²⁵ success after transfer (pregnancy rate: 65%) was not affected by storage of <300 µm-diameter embryos for 12 to 24 hours between cooling to 5°C (41°F) and vitrification. Therefore, small equine embryos can be cooled slowly in a passive cooling device and shipped to a laboratory for vitrification. However, success of vitrification has remained poor for equine embryos after capsule formation. Current research at The University of Georgia is evaluating whether vitrification could result in more successful pregnancies after transfer of large (expanded) equine blastocysts.²⁶

Embryo recovery rates in performance mares are approximately 40%. In one study,²⁷ 30 minutes of exercise per day (starting 2 weeks before breeding and continuing until embryo collection on day 7 after ovulation) reduced the embryo recovery rate (34% in the treatment group compared with 63% in the control group) and embryo quality. At the end of the daily exercise period, rectal temperatures increased by a minimum of 1.2°C (2.2°F) to a maximum of 2.4°C (4.3°F). It was concluded that the reduction in embryo recovery and morphology may be related to thermal stress.²⁷

Equine embryos migrate throughout the uterus until day 15 or 16. Therefore, the area to which the embryo is transferred is relatively unimportant, and it is deposited into the uterine body or the base of one horn. A regular AI pipette is used for embryo transfer of large (day 8) embryos instead of a special embryo transfer gun because of the pipette's larger inner diameter. Manipulation of the uterus during embryo transfer may cause the release of prostaglandin F_{2α} from the endometrium. In cattle, administration of the prostaglandin synthesis inhibitor flunixin meglumine before transfer increased the pregnancy rate; this may also occur in equine recipients.²⁸ Ovulation of the donor and recipient mares should be synchronized to +1 to -3 days (i.e., the recipient mare can ovulate 1 day before to 3 days after the donor mare).

Conclusion

The development of an effective embryo cryopreservation technique would greatly (1) increase the flexibility to implant embryos as recipients become available and (2) facilitate exchange of embryos, particularly internationally. The need to successfully cryopreserve

equine embryos is becoming even more evident with current advances in applying superovulatory regimens to mares receiving FSH. Adequate recipient management and selection in embryo transfer programs are also important factors and optimize the desired outcome—a healthy foal.

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- 1. The optimal time for AI using chilled semen in a mare is within**
 - a. 48 hours before ovulation.
 - b. 24 hours before ovulation.
 - c. 12 hours after ovulation.
 - d. 24 hours after ovulation.
- 2. Ovulation in a mare in estrus can be predicted by**
 - a. follicular size and shape.
 - b. the softness of the follicle.
 - c. the echogenicity of the follicular wall.
 - d. all of the above
- 3. The minimum number of progressively motile spermatozoa required for successfully breeding a mare**
 - a. depends on the stallion's fertility and varies between laboratories.
 - b. is 100 million.
 - c. is 300 million.
 - d. is 500 million.
- 4. Which of the following is the best predictor of a stallion's fertility?**
 - a. progressive motility of spermatozoa
 - b. sperm-cell morphology
 - c. the stallion's conception rate per cycle
 - d. semen concentration
- 5. To induce superovulation in a mare, twice-daily treatment using equine FSH is started**
 - a. after induction of ovulation and detection of a dominant follicle of >35 mm in diameter on one of the ovaries.
 - b. when the largest follicle on the ovaries is 20 to 25 mm in diameter.
 - c. 5 to 6 days after detection of ovulation in the donor mare.
 - d. b and c
- 6. The results of embryo collection in mares can be optimized by**
 - a. injecting oxytocin before the last flush.
 - b. waiting 3 minutes after infusion of the last portion of medium.
 - c. uterine massage per rectum.
 - d. all of the above
- 7. Equine embryos are most commonly collected on day 7 or 8 after ovulation, when they**
 - a. are <300 µm in diameter.
 - b. have an average diameter of 400 to 1200 µm.
 - c. are surrounded by a glycoprotein capsule.
 - d. b and c
- 8. Cryopreservation of embryos by vitrification involves**
 - a. incubation in higher concentrations of cryoprotectants.
 - b. a longer incubation time than during a conventional slow freezing procedure.
 - c. a higher cooling rate.
 - d. a and c
- 9. During transfer, equine embryos can be deposited in**
 - a. the uterine horn ipsilateral to the corpus luteum.
 - b. the uterine body.
 - c. the uterine horn contralateral to the corpus luteum.
 - d. all of the above
- 10. An embryo collected on day 8 after ovulation of a donor mare can be transferred on day ____ after ovulation of a recipient mare.**
 - a. 9
 - b. 5
 - c. 3
 - d. a and b