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Freezing Embryos

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With breed registries admitting more than one foal per mare per year, the use of frozen embryos is becoming more mainstream.

The freezing of equine embryos is getting increased attention from researchers. For years embryo freezing for horses has lagged well behind the same procedure in the bovine industry. The reasons have been quite basic. First, equine embryos are more difficult to harvest in suitable numbers and more complicated to freeze than their bovine counterparts.

Second, there hasn't been that much demand. One reason for the lack of demand was the fact that many registries imposed restrictions on embryo transfer of frozen embryos. In a number of cases, those restrictions recently have been lifted.

It now appears, says E.L. Squires, MS, PhD, professor in the Department of Biomedical Sciences at Colorado State University's (CSU) veterinary school, that embryo freezing is heading toward its own niche in the horse industry.

The reasons are in direct contrast to the reasons for slow progress in the past. Now, science is at work to superovulate mares--so they release greater numbers of eggs and, thus, produce multiple embryos--and new and improved methods have been found to successfully freeze equine embryos.

Squires says CSU researchers are in the midst of a project examining an improved procedure for superovulation of mares. Without superovulation, he points out, there would be less demand for embryo freezing. If only one embryo is recovered, it very often goes immediately into a recipient mare and doesn't require storage. Preliminary results of the CSU study (which were not available at press time, Oct. 4) will give researchers a strong indication of the new procedure's success rate, Squires says.

The scientific study of equine embryo freezing and embryo transfer is relatively new. The study of equine embryo collection and transfer began in 1970, but it wasn't until 1982 that the first foal was born as the result of transferring an embryo that had been frozen. That occurred in Japan.

The leading countries involved in embryo transfer, according to Squires, are the United States, Argentina, and Brazil. Other countries using embryo transfer to a lesser extent, he says, are Australia, Canada, Italy, Germany, and France.

Progress was slowed in the early years of research because many registries approved the registering of only one foal per mare per year. A big breakthrough in that area came in 2002 when the American Quarter Horse Association (AQHA) approved the unlimited registration of foals from a given mare during a year by using embryo transfer.

"The major candidates for embryo transfer," Squires says, "include older mares with poor reproduction histories that are unable to produce a foal by conventional natural mating or artificial insemination and show mares that are competing in racing, polo, or other performance events."

The success or failure of embryo transfer, even with nonfrozen embryos, depends on a number of factors, according to Squires. Here is what he had to say in a recent paper he presented on the state of embryo technologies in the horse:

"Factors that affect embryo recovery include day of recovery (age of embryo), number of ovulations, age of the donor mare, and quality of the semen. Mean embryo recovery (rate) per cycle from single-ovulating mares in commercial embryo transfer programs is approximately 50%. Mares that spontaneously double- or triple-ovulate during a given cycle have higher embryo recovery rates than single-ovulating mares. Certain breeds--Thoroughbreds, draft horses, and Warmbloods--are more prone to having multiple ovulations during a given cycle.

"The major factor affecting embryo recovery is the mare's reproductive history," Squires says. "Older mares with poor reproductive histories produce fewer embryos. Causes of reduced embryo recovery from these older mares include uterine and oviductal pathology (the structural and functional manifestations of disease) and increased early embryonic death.

"The quality of semen used to inseminate mares also affects embryo recovery," he continues. "Generally mares inseminated with fresh semen are more likely to produce an embryo than those inseminated with either cooled or frozen-thawed semen."

The size of the embryo being recovered and transferred is highly significant in success rates, especially when freezing is involved. Squires tells us that embryos that are beyond six to 6½ days of development and more than 0.3 millimeters in diameter are not good candidates for freezing and subsequent transfer. The larger the embryo, the lower the pregnancy rate when transferred.

Yet, with all that being said, it appears obvious that freezing of embryos, especially when superovulation is involved, can have some distinct advantages.

With multiple embryos, for example, some could be frozen for later use if the mare becomes infertile or dies. Freezing embryos also reduces the need for synchronizing recipient mares. When fresh or cooled embryos are transferred to a recipient mare, she must be in the same stage of the estrous cycle as the donor. With frozen embryos, the thaw and transfer can take place whenever the recipient is in a state where her uterus will accept and nurture the embryo.

Another advantage of freezing equine embryos is the ability to import and export embryos from matings around the world.

Squires says that among the prime candidates for embryo flushing and freezing are mares late in the breeding season. Under such circumstances, he points out, the embryo or embryos could be flushed, frozen, and stored until early the following season.

Freezing Problems

One of the prime problems encountered in freezing equine embryos has been the formation of ice crystals during the freezing process that can damage the embryos. A variety of substances, with an emphasis on glycerol (a colorless, odorless viscous liquid chemical compound commonly used in pharmaceuticals), have served as antifreeze to reduce ice formation and lower the freezing point for the embryos. Technically, these substances are called cryoprotectants.

The standard approach to freezing embryos in the past involved a slow cooling process after collection and storage of the embryo in liquid nitrogen. The process had some drawbacks. One, it wasn't a speedy approach, and two, it required some specialized equipment for the freezing.

Not totally satisfied with the slow-cool freezing approach, researchers, including a group at Colorado State, began examining other approaches. Enter vitrification.

Vitrification

Vitrify comes from the Latin root *vitreous*, which means "glassy." In the case of vitrification of embryos, the process of vitrification rapidly converts a liquid into a glasslike solid that can be preserved in a frozen state, but minus the destructive ice crystals that often are a part of the slow-cool freezing process. Squires explains: "Biologically, the equine embryo is unique in that it forms an acellular protein membrane called the capsule, which may impede penetration of the cryoprotectant, making freezing of blastocysts and expanded blastocysts quite difficult."

With vitrification, the formation of ice crystals is, generally speaking, eliminated.

There are other advantages to vitrification, according to Squires. For one, the process is faster than the slow-cool freezing approach, and it is much simpler and cheaper. A specialized machine for the freezing of embryos costs in the neighborhood of $5,000, Squires says. With vitrification, a veterinarian can buy a kit that contains all of the materials necessary and carry out the procedure in any locale.

Is the vitrification approach successful? Based on research conducted by Squires and his CSU colleagues, the answer is yes.

Jason Hudson, DVM, reported the results of one of the most recent CSU studies in a scientific paper. There were several goals for the study, according to Hudson. One goal was to study the effects of vitrification on embryos that had already been cooled and stored 19 hours to find out if there would be a difference in pregnancy rates between embryos that were vitrified after cooling and embryos that were vitrified immediately after collection. Another aspect of the study involved using embryos from superovulated mares to determine if the production of multiple embryos had a deleterious effect on vitrification and subsequent pregnancy.

As a precursor to this study, CSU scientists (including Elaine Carnevale, DVM, PhD) had researched the vitrification process. In that study, 48 embryos less than 0.3 millimeters in diameter had been collected and vitrified shortly after collection. An ethylene glycol-glycerol solution was used in the vitrification process. The 48 embryos were transferred to synchronized mares, and 26 of them (54%) resulted in pregnancies at 14 to 16 days. All of the research mares were single-ovulating mares.

Here is what Hudson says in his introduction to a report on the more recent research project: "The vitrification process is quicker than slow-cool cryopreservation and does not require specialized equipment. In addition, dilution of the cryoprotectants can occur in the straw (where the vitrified embryos are stored) and the embryo can be transferred directly into the mare without removal from the straw.

"Cooling and storage of equine embryos for 12 to 24 hours at 5°C (41°F) before transfer is standard practice in the equine industry," he continues. "Pregnancy rates are similar for embryos cooled and stored for 24 hours compared with embryos transferred immediately upon collection. Perhaps embryos can be collected on the farm, cooled and stored, then sent to a special facility for vitrification."

Thirty-eight mares were involved in the study. They were treated with a hormone to stimulate superovulation. The vitrification process for all embryos collected worked like this: First, the embryos were harvested and placed in a holding medium. Then they were immersed in the first vitrification solution comprised of glycerol (VS1) for five minutes. Then the embryos were transferred to VS2, comprised of glycerol and ethylene glycol for five minutes. In the third step, the embryos were placed in VS3, again involving higher concentrations of glycerol and ethylene glycol,

and they were loaded into a straw that was heat-sealed at both ends.

The third procedure is the tricky one because it must be completed very quickly. As part of the process, the straws were placed in a cooled plastic goblet that was held vertically in liquid nitrogen vapor within an insulated container.

According to Hudson's report, this allowed liquid nitrogen to surround the goblet and produce a mixture of cooled air and nitrogen vapor around the straw at a temperature of -196°C (-320°F). The straw remained in the vapor for one minute before the goblet and the straw were plunged into liquid nitrogen. Straws were then placed in a liquid nitrogen storage tank until they were warmed for transfer into recipient mares.

When it was time to transfer the embryo, the straw was removed from the liquid nitrogen tank, held in the air at room temperature for 10 seconds, then plunged into a bath of water at 20-22°C (68-71.6°F) for an additional 10 seconds. Then the straw was removed from the water bath "and flicked like a clinical thermometer four to five times to assure mixing of solutions," says Hudson.

Following the thawing process, the embryos were transferred to recipient mares.

All of the research results were positive. Superovulation did not appear to affect the embryos in any negative way. It was the same with embryos that were cooled and vitrified. There was no significant difference in pregnancy rates between those embryos and embryos that were vitrified immediately after collection. The overall pregnancy rate for vitrified embryos involved in the study was 70%, similar to rates obtained for nonfrozen embryos and slow-cooled frozen embryos.

Hudson says in the conclusion of the report: "In summary, cooling of embryos for 12 to 19 hours before vitrification did not reduce pregnancy rates after warming and transfer. Embryos less than 300 microns in size can be cooled, vitrified, thawed, and directly transferred into recipient mares with high pregnancy rates. Further studies are needed to determine whether other slow-cooling methods for freezing or other vitrification methods can be used to freeze embryos greater than 300 microns in size."

Take-Home Message

It would appear the frozen embryo is here to stay in equine breeding. Further research likely will help establish where it fits in the industry of assisted reproduction.

Seek the advice of a qualified veterinarian before proceeding with any diagnosis, treatment, or therapy.