

New Assisted Reproductive Techniques for Horses

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Introduction

Although embryo transfer provides a means of obtaining pregnancies from some mares that might not otherwise be capable of producing offspring, some mares cannot provide embryos for transfer. Mares in which embryo transfer may not be successful include those with: 1) failure of ovulation, 2) chronic endometritis (uterine inflammation), or 3) anatomical problems (e.g., cervical adhesions, etc.). However, these mares could be used as oocyte (egg) donors and continue to produce foals through other assisted reproductive techniques such as oocyte transfer, in vitro (test tube) fertilization or intracytoplasmic sperm injection. In addition, stallions with low sperm numbers and/or poor semen quality could also benefit from some of these newer technologies. Other reproductive technologies that are being developed include sexed semen, low-dose insemination procedures and cloning.

The successful application of new assisted reproductive procedures requires that equine gametes (oocytes and sperm) are collected and handled appropriately. For successful fertilization, the oocyte must be viable and at the correct stage of maturity. The ovaries contain many thousands of “resting” oocytes, that are in an inactive state; during each estrous cycle, groups of resting oocytes are “selected” and induced to start a growth phase that involves changes in the oocyte itself and the cells of the follicle surrounding it. Although many (> 20) oocytes start growing during each cycle, only one or sometimes two oocytes and their associated follicles develop to full maturity and ovulate; the remainder undergo a degenerative process called atresia. At the time ovulation, the oocyte has reached full maturity and is ready to be fertilized.

For commercial application of new assisted technologies, oocytes are primarily collected by transvaginal ultrasound-guided follicle aspiration (TVA). Oocytes can be collected from mature follicles that are ready to ovulate or from immature follicles. Oocytes collected from small, immature follicles must undergo maturation in vitro. The development of successful culture systems for routine in vitro maturation of equine oocytes is currently an area of considerable research interest.

Oocyte Transfer

Oocyte transfer involves collection and surgical transfer of a donor mare’s mature oocyte into a recipient mare’s oviduct, so that fertilization and subsequent embryonic development

occur within the reproductive tract of the recipient. This procedure has been referred to as gamete intrafallopian transfer (GIFT), which implies that both gametes (oocyte and sperm) are transferred into the oviduct; however, as it is currently being performed in the mare, this procedure involves only the transfer of an oocyte, since the recipient mare is inseminated with semen from the desired stallion using standard insemination procedures. Because the recipient mare is inseminated, her oocyte must be removed to prevent it from being fertilized.

Oocyte transfer does not require ovulation to occur in the donor and completely bypasses all of the tubular genitalia (oviduct, uterus, etc.) of the donor mare; therefore, it may be especially well suited for donor mares with failure of ovulation and/or those with chronic problems associated with the reproductive tract that interfere with fertility. In addition, oocyte transfer has been proposed as an alternative method of obtaining pregnancies from any mare in which embryo transfer has been unsuccessful regardless of the underlying reason.

Although the first equine oocyte transfer resulting in the birth of a foal was reported in 1988, it has only been recently the procedure has been performed as a clinical procedure. In a recent report, Carnevale et al. at Colorado State University (see suggested reading list) described their results using oocyte transfer in a commercial breeding program involving 38 mares (mean age 21 years) with reproductive problems. Donor mares consisted of those with a wide range of reproductive abnormalities including persistent endometritis, pyometra, cervical fibrosis, repeated development of anovulatory hemorrhagic follicles, and idiopathic reproductive failure. Oocytes were collected from donor mares using transvaginal ultrasound-guided follicle aspiration, which was well tolerated by the donor mares without any complications. Oocyte collection rates were high with oocytes recovered during 80% of cycles. Of a total of 90 oocytes recovered from these donor mares during 99 cycles, 75 were transferred into 64 recipient mares, which resulted in 20 mares becoming pregnant (31%).

Carnevale et al. noted the success of oocyte transfer in their study was affected by oocyte and semen quality. The most important factor affecting oocyte quality was mare age. Reduced fertility in mares ≥ 20 years old has been associated with poor oocyte quality, since embryo development rates were significantly reduced when oocytes from aged compared to young mares were transferred into young recipient mares. In addition, morphological evaluation of oocytes from aged and young mares using light and electron microscopy demonstrated that although some oocytes from aged mares are morphologically similar to oocytes from young mares, more oocytes from aged mares had morphological anomalies. Because semen quality also affected the success of oocyte transfer, the authors recommend the use of stallions with high-quality semen to maximize the success of the procedure.

This work clearly demonstrates that oocyte transfer can be used to obtain pregnancies from mares with a wide range of reproductive problems that otherwise would probably not be able to produce offspring; however, because it is typically older mares that suffer chronic fertility problems and oocyte quality is lower in aged mares, the efficiency of oocyte transfer will be lower in aged mares compared to younger mares. More recently, Carnevale et al. described the successful establishment of pregnancies and birth of one foal from oocyte transfer using oocytes that had been collected from mares after euthanasia. This has relevance to circumstances

involving the untimely death of mares; oocyte transfer can potentially be used to obtain pregnancies from mares post-mortem.

In Vitro Fertilization (IVF)

IVF has been used successfully for many years in the treatment of human infertility, and techniques are now available for producing large numbers of IVF embryos from several domestic species. Although the first foal produced by IVF was reported in 1991, a repeatable method for successfully performing IVF in horses has not been developed. The primary biological barrier to IVF appears to be an inability to effectively capacitate equine sperm in vitro, a process that is necessary in order for sperm to be capable of fertilizing an oocyte. Because of the lack of progress in this area, recent research has focused on alternatives to traditional methods of IVF.

Intracytoplasmic Sperm Injection (ICSI)

ICSI is one of the modified forms of IVF being developed for use in horses that involves using a micro-manipulator to inject a single sperm cell into the cytoplasm of a mature oocyte. By mechanically fertilizing an oocyte (i.e., placing the sperm cell into the oocyte), ICSI eliminates the need for the sperm cell to bind and penetrate the oocyte, which appears to be the aspect of sperm-oocyte interaction that fails to occur appropriately during standard IVF procedures.

The first foal produced using ICSI was reported in 1996, and since that time there have been further reports of the success of the ICSI procedure. However, factors affecting the success of ICSI are poorly understood and commercial application of the procedure in horses has not been reported. Although it is still an experimental procedure, ICSI holds great potential for assisted reproduction in the mare. In addition, because ICSI requires only a single sperm cell, it has tremendous potential application for stallion management (e.g., subfertile stallions, frozen semen, etc.).

Sexed Semen

There is considerable interest in developing methods of separating X- and Y-chromosome bearing sperm, to produce “sexed” semen, that when inseminated allows gender selection of the resulting offspring; insemination of X-bearing sperm cells will result in female offspring, and insemination of Y-bearing sperm cells will result in male offspring. The first foal (a filly) conceived with “sexed” semen was born during the summer of 1998. Since then, many more foals of pre-determined gender have been born using sexed semen. Currently, semen sexing is commercially available through XY Inc. in Fort Collins, Colorado, USA.^A

Semen is “sexed” using a flow cytometer, an instrument that physically separates X- and Y-bearing sperm cells; however, using current instrumentation, only 1,000 to 1,500 viable, sexed sperm cells can be sorted per second. Although that appears to be an extremely fast rate of sorting, at that rate, it would require 4 to 5 days of continuous operation to produce enough “sexed” sperm cells for one insemination dose using the standard number of sperm required for conventional insemination of fresh semen (i.e., 500 million progressively motile sperm). That limitation has been responsible for stimulating research to develop new insemination methods that are successful when a limited number of sperm cells are used. In addition to use with sexed

semen, low-dose insemination techniques may be beneficial when inseminating frozen-thawed semen of low quality or limited quantity, or when inseminating semen from subfertile stallions.

Low-Dose Insemination

As the name implies, low-dose insemination involves placement of a substantially smaller number of sperm in the female reproductive tract compared to the number used for conventional artificial insemination. The primary impetus for the development of low-dose insemination techniques has been for use with sexed semen as described above. However, the application of low-dose insemination techniques has relevance to other situations when sperm numbers are limited (e.g., frozen-thawed semen) and/or of marginal quality.

Three methods of “low-dose” insemination have been investigated: 1) surgical insemination into the oviduct, 2) transcervical endoscopic insemination at the utero-tubal junction and/or into the oviduct, and 3) deep intra-uterine insemination near the utero-tubal junction. Although surgical oviductal insemination can achieve pregnancies with extremely low numbers of spermatozoa (50,000 to 150,000), it has limited practical application at this time. Compared to oviductal insemination, endoscopic insemination is more practical and can achieve pregnancies with a slightly higher number of spermatozoa (~ 1 million), but requires expensive equipment that is not well suited to field use. Currently, deep intra-uterine insemination is the most practical method of low-dose insemination because it can be performed in the field with readily available equipment, but it requires more spermatozoa (5 to 25 million) than oviductal or endoscopic insemination. Continued research is needed to further refine low-dose insemination techniques.

Nuclear Transfer (Cloning)

Nuclear transfer is a method of generating a genetic clone of an animal, which gained widespread recognition when it was first used to produce “Dolly” from a cultured mammary gland cell that had been isolated from an adult ewe. Since then, cattle, goats, pigs, mice, rabbits, cats, mules, a horse and rats have been cloned using nuclear transfer. Although the term nuclear transfer implies only the nucleus of the donor cell is transferred during the procedure, as it is currently performed, an entire donor cell is fused with a suitable host cell, which in most cases is a mature oocyte from which the nuclear genetic material has been removed. By removing the genetic material from the host oocyte and fusing the enucleated oocyte with the donor cell, the donor cell DNA serves as the template for subsequent gene expression, which leads to the development of a genetic clone of the donor animal. The host oocyte provides appropriate cellular characteristics (organelles, enzymes, etc.) that are necessary for the initiation and coordination of embryonic development.

In May, 2003 we reported the birth of a cloned mule, which was the first successful cloning of an equine species. Since then, two more genetically identical cloned mules have been born. Soon after the birth of our first cloned mule, a team of scientists in Italy reported the birth of a cloned horse. Production of offspring using nuclear transfer offers tremendous potential for preserving valuable genetic material of endangered and/or exotic equine species such as the Mongolian Wild Horse (Przewalski’s horse). Similarly, it will allow the preservation of genetics from individual animals that would otherwise not be able to reproduce (e.g., geldings). In

addition, because of the companion animal role that horses fill for some individuals, it is likely that some horse owners will utilize nuclear transfer as a means of cloning individual animals for emotional fulfillment. Although some breed associations do not allow cloning (e.g., Jockey Club, American Quarter Horse Association), for some equine sporting activities (e.g., Dressage) breed registry status is irrelevant, which eliminates that impediment to the use of cloning.

Horse owners who might be interested in having a horse cloned can have their veterinarian assist them by “banking” tissue from the animal(s). There are currently several commercial companies^B that will isolate and store cells from tissue samples collected from animals for subsequent cloning purposes. These companies typically provide the veterinarian with a tissue collection and transport kit; the procedure involves collecting a small skin biopsy, which is then placed in tissue culture medium and returned to the company where cells are grown in tissue culture. Ideally, tissue should be collected from a live animal; however, in an emergency, it may be possible to collect a suitable sample soon after death. Once viable cells are growing in culture, they can be used immediately for cloning, or they can be harvested and stored frozen in liquid nitrogen for use sometime in the future.

Summary

For mares in which embryo transfer cannot be performed successfully, newer assisted reproductive techniques such as oocyte transfer, IVF or ICSI may provide a means through which offspring can be obtained. In addition, sexed semen, low-dose insemination and nuclear transfer cloning procedures will provide horse owners with new options for the reproductive management of their animals. Although these new technologies are just beginning to have clinical application at this time, with continued development, they will offer new alternatives to our approach of managing subfertile/infertile mares and/or stallions.

Suggested Reading

1. Carnevale EM, Squires EL, Maclellan LJ et al: Use of oocyte transfer in a commercial breeding program for mares with reproductive abnormalities. *J Am Vet Med Assoc* 218:87-91, 2001.
2. Carnevale EM, Maclellan, LJ, Coutinho da Silva, MA et al: Pregnancies attained after collection and transfer of oocytes from ovaries of five euthanatized mares. *J Am Vet Med Assoc* 222:60-62, 2003.

Footnotes

- A. XY Inc., 1108 North Lemay Avenue, Fort Collins, Colorado, 80524 USA, tel 970-493-3113, fax 970-493-3114, e-mail: info@xyinc.com, internet: www.xyinc.com.

- B. Tissue banking companies:
 - Cyagra, Inc., Worcester, Massachusetts, USA
 - Geneticas, Los Angeles, California, USA
 - Genetic Savings & Clone, Sausalito, California, USA
 - Lazaron BioTechnologies LLC, Baton Rouge, Louisiana, USA
 - PerPETuate, Inc., Farmington, Connecticut, USA