Bovine Embryo Transfer– Present and Future

Kevin G. Hubbert, BS, DVM*
Steven M. Hopkins, DVM**

Introduction

Bovine embryo transfer (ET) has evolved in the past few decades from basic research, to applied research, to its presence as a multimillion-dollar industry. ET is a technique for genetic manipulation which allows the producer to improve the production capacity of his livestock. This article describes some of the current techniques of ET being used and some of the techniques of genetic manipulation being researched which may find future commercial application in the field of ET.

Currently used techniques include superovulation, nonsurgical recovery of embryos, splitting of embryos and cryopreservation of embryos.

Superovulation

Superovulation involves the use of gonadotrophic hormones to rapidly increase the number of mature follicles and their oocytes. This surge of mature follicles leads to ovulation of more oocytes during one estrus cycle (up to 64 oocytes)[1] than would normally occur. Currently used gonadotrophins include equine chorionic gonadotrophin (ECG) and follicle stimulating hormone (FSH). Work by Elsden *et al.*[2] indicates that superovulation with FSH resulted in more corpora lutea, recovered embryos, and pregnancies than with the use of ECG. Both FSH and ECG are routinely used in industry with good results. Programs using FSH require that the hormone be given twice a day for several days to induce superovulation, whereas ECG programs require a single administration. This is due to the short half-life of FSH ($T\frac{1}{2} = 2-5$ hrs.) and the relaltively long half-life of ECG ($T\frac{1}{2} = 12$ days)[3] in the bovine system. Administered along with the gonadotrophins is prostaglandin $F_2\alpha$ ($PGF_2\alpha$).[4] This is given in combination with the gonadotrophin treatment to ensure a predictable interval between termination of gonadotrophin injections and estrus. When $PGF_2\alpha$ is given to superovulated donors, estrus follows in 40 to 72 hours.

Non-surgical Recovery

The techniques of non-surgical recovery of bovine embryos have improved greatly in the past decade.[4,5,6] The constant improvement of techniques and manipulative skills of the veterinarians and technicians performing the flushes has led to recovery rates of 71% and 92%[5] from non-superovulated and superovulated donors, respectively. Along with the good recovery rates seen with non-surgical recovery, the problems associated with surgical recovery[4] are avoided. These problems include adhesions of the reproductive tract and subsequent infertility and/or sterility of the donor. The use of non-surgical recovery has led to on-the-farm recoveries since elaborate operating facilities are not needed. This enables the producer to continue to milk lactating dairy cows, avoid damage to the reproductive tracts of valuable donors, and repeatedly collect embryos from his top-producing cattle.

Embryo Splitting

Embryo splitting, a tedious procedure for the production of identical calves, is currently being used commercially.[7] This is a relatively simple procedure with the limiting factor for its routine use being the expense of the required equipment.[8] The embryos used for splitting are collected 6 to 7 days after estrus.[7] These embryos are at the morula and early blastocyst stages of development; each is sur-

*Dr. Hubbert is a 1984 graduate of the College of Veterinary Medicine at Iowa State University.
**Dr. Hopkins is an assitant professor in Veterinary Clinical Sciences at Iowa State University

rounded by a zona pellicuda. At this stage of development, the embryo is organized in a tight ball of cells, each of which is totipotential and therefore capable of forming a completely normal fetus after splitting. The procedure, as described by Williams *et al.*,[7] involves washing the embryo to be split in modified Dulbecco's phosphate buffered saline solution with 10% heat-inactivated steer serum, then placing it in a microdrop with an unfertilized ovum. The microdrop is then overlayed with paraffin oil. The micromanipulation apparatus[7,8] consists of a pair of Leitz micromanipulators which control the microtools (consisting of a holding pipet, microsurgical blade, suction pipet and injection pipet) and a compound microscope with 125× magnification. The zona pellucida of the unfertilized ovum is split and the contents are removed, leaving a surrogate zona pellucida. The zona pellucida of the embryo is then incised while holding the embryo with the suction pipet. Next, the microsurgical blade is used to split the embryo into 2 demi-embryos. One of the demi-embryos is removed with the injection pipet and placed into the surrogate zona pellucida, leaving the other demi-embryo in the original zona pellucida. This results in two genetically identical embryos. This doubling of the number of transferable embryos collected from one flush will increase the number of calves from the donor.

Cryopreservation

Cryopreservation of bovine embryos is being used at an ever-increasing pace in the embryo transfer industry. Cryopreservation, while still in its developmental stages, is a viable economic alternative under special circumstances. The advantages to freezing embryos include[1]: embryos can be stored indefinitely;[2] surplus embryos can be stored until recipients can be found;[3] in beef herds, embryos can be collected during the nonbreeding season and transferred during the breeding season;[4] a demi-embryo can be frozen while the corresponding demi-embryo is karyotyped or transferred.

There are disadvantages to freezing embryos, such as the expense of a freezing machine, and the embryo mortality associated with freezing and thawing. Shea *et. al.*[9] found that freezing and thawing of good quality embryos (as judged by morphology) resulted in 40% pregnancy rates after transfer to recipients. Other studies with frozen-thawed embryos have obtained from 5%[11] to 60%[12] pregnancy rates.

Countless articles on cryopreservation have been written in the past decade; most have dealt with improvement of previous techniques, protocols and cryoprotectants used for freezing embryos. The basic process and theory behind these techniques, as described by Seidel *et. al.*,[11] will be discused here. The main objective of freezing embryos is to remove all unbound, intracellular water by slowly dehydrating the embryo, then plunging it into liquid nitrogen. The embryos are transferred from flushing media to modified Dulbecco's phosphate-buffered saline (PBS)[10,11,12,13] with a cryoprotectant. The two cryoprotcctants used most often are dimethyl sulfoxide (DMSO)[11,12,13] and glycerol.[10,11] The purpose of the cryoprotectant is to protect the cells of the embryo (during cooling to temperatures below 0°C) from damage by intracellular crystals and increasing solution effects. The embryos are first cooled rapidly to either −6C[10] or −7°C,[11,12,13] at which point ice crystal formation is induced mechanically (seeding). The intracellular water of the embryo does not freeze at these temperatures, nor does all of the extracellular water. The solutes (primarily NaCl and cryoprotectant) of the extracellular water lower the freezing point of the water; therefore, the ice crystals formed first are composed of hypotonic water. The remaining hypertonic extracellular water osmotically dehydrates the cells of the embryo. As the entire system is slowly cooled, the extracellular ice crystals continue to grow and dehydrate the cells. The aim of this stage of the procedure is to remove most of the unbound intracellular water. The amount of water removed is limited by the speed at which water crosses the cell membrane of the embryo. If the cooling is too rapid, extracellular water is frozen in large and small crystals. After slow thawing, the small crystals melt and recrystalize in the large ice crystals, promoting ice growth. Once most of the intracellular water is removed by slow cooling, the embryos are plunged into liquid nitrogen. Thus, the goal is to manipulate the cooling rate of the embryo in solution to prevent formation of large, damaging intracellular ice crystals and to rehydrate the embryonal cells. Once a dependable, inexpensive and relatively simple method of freezing and thawing embryos and

ending up with good viable embryos is developed, cryopreservation will find a greater use in the embryo transfer industry.

Other methods of genetic manipulation being considered include nuclear transplantation, separation of blastomeres, androgenesis (crossing a bull with a bull) or gynogenesis (crossing a cow with a cow), cloning, and chimeria production.[13] These techniques may find some use in commercial raising of cattle. Three techniques which will be of more commercial use are sexing of embryos, in vitro fertilization and sperm capacitation.

Embryo Sexing

The ability to sex embryos could mean a great deal to both the beef and dairy producer alike. Being able to select female embryos for transfer in a dairy herd could mean the addition of potentially high-producing cows to the milk line as opposed to calving an ET bull calf which may be of limited value. This holds true also for the beef producer who can develop a high producing herd faster with artificial insemination and ET heifer calves than with ET bull calves.

Sexing of embryos will be a needed technique until reliable separation of X- and Y-chromosome-bearing spermatozoa becomes commercially feasible. This procedure is currently done only in research. Biopsying morulae (i.e. splitting early blastocytes and karyotyping the removed cells[14]) is one method of sexing embryos. Most embryos continue to grow normally after biopsy, and this can be highly accurate if suitable metaphase chromosomes are available. Unfortunately, these procedures are tedious and time-consuming, and usable preparations are obtained from only about two-thirds of the embryos.[14] A fringe benefit of karyotyping is detection of chromosomal abnormalities.

An alternative to karyotyping is the detection of H-Y antigen, a gene product of the Y-chromosome. White *et al.*[15] conducted a study in which two procedures were used to detect the H-Y antigen. One method used cytolysis of H-Y antigen-positive embryos to detect the H-Y antigen. This procedure (performed on mice) was 81.1% accurate at predicitng female embryos; however, it also had 18.9% false positives (males). Another method used is binding of a fluorescein isothiocyanate (FITC)-labeled antibody to the H-Y antigen. This procedure was 78.3% accurate in determining males, but 21.7% false positives (females) were seen. This FITC procedure was also conducted with mice.

In Vitro Sperm Capacitation and Fertilization

In vitro sperm capacitation and fertilization are not well understood in any of the mammalian species; however, a great deal of work has been done and is being continued in this particular field.[17,18,19,20] The physiological changes which the spermatozoon must undergo before it is able to penetrate and fertilize the oocyte are not well understood. These changes are termed capacitation. Capacitation, is a change at the molecular level within the sperm membranes. This change affects the metabolism of the sperm and leads to an increase in the flagellation of the sperm, and therefore a change in the character of the sperm movement. Also involved in the capacitation process is the removal of decapacitation factor, normally produced in the epididymis or accessory sex glands. In the female reproductive tract, capacitation occurs as the sperm move through the uterus and oviducts. Another process the spermatozoon must undergo before it can fertilize the oocyte is the acrosomal reaction. This reaction occurs as the sperm penetrates the cumulus oophorus and comes in contact with the zona pellucida. This reaction takes place at the acrosomal membrane and involves the release of several enzymes. Two of these enzymes are hyaluronidase and acrosin. The release of these enzymes when the inner acrosomal membrane is in close association with the zona pellucida facilitates the penetration of the sperm into the zona pellucida.

Under in vivo conditions fertilization takes place in the ampulla. After capacitation and the acrosomal reaction have occurred and the sperm has entered the vitellus, the process of embryonic development beings. The oocyte, which has stopped dividing at the second metaphase of meiosis, begins to divide and complete meiosis. The emission of the 2nd polar body (Barr body) and the formation of the male and female pronuclei occur next, followed by breakdown of the nuclear membranes of the pronuclei and the alignment of the chromosomes on the mitotic spindle. The diploid complement of the chromosomes is thus restored, and cleavage of the embryo begins.

Methods of sperm capacitation in vitro[19,20] and in the reproductive tracts of cattle and sheep[18,20] indicate that capacitation of sperm and the subsequent penetration of the sperm into the oocyte and initiation of cleavage of the embryo can be accomplished outside of the donor cow's oviduct. Brackett *et al.*,[17] using a high-ionic-strengh defined medium (380 m0sm/kg) for culture and capacitation of sperm, reported 34 (43.6%) of 78 ova and 14 (19.7%) of 66 follicular oocytes were fertilized in vitro. The fertilized oocytes divided to the 8-cell stage in culture media. Sirard *et al.*,[19] using a high-ionic-strength defined medium similar to Brackett's (modified by the addition of bovine serum albumin fatty-acid-free), found that fertilization and cleavage could be achieved. The rate of capacitation ranged from 14% to 46%, and that of cleavage from 0% to 40%, indicating that continued study needs to be done to find a repeatable and reliable medium and method for in vitro fertilization.

One documented case of in vitro capacitation and fertilization resulting in the birth of a normal calf has been confirmed by blood group compatibility testing.[17] This gives encouragement that a reliable method for in vitro fertilization is going to be a reality in the not-so-distant future.

Possible applications[4] of in vitro fertilization are: (1) to circumvent infertility of a female due to disease of the reproductive tract; (2) to provide large numbers of oocytes from cows terminally ill or from cows on the slaughter house floor, and (3) to provide the extended use of rare semen on a greater number of oocytes than would normally be possible by in vivo fertilization.

Summary

As stated in the introduction, the purpose of this paper is to introduce the reader to some of the techniques currently in routine use in ET. These techniques include: (1) superovulation with gonadotrophins, which provides a surge of mature oocytes for fertilization and collection; (2) the use of non-surgical flushing, which enables the veterinarian to repeatedly collect embryos without damaging the donor's reproductive future; and (3) the splitting of embryos, enabling the veterinarian to increase the number of embryos produced from a single flushing. Also, the use of cryopreservation would enable the producer to preserve an embryo for an indefinte period of time.

The author has also introduced two methods derived from the research involved with ET which he feels will be profitable to the cattle industry in the future. These are the sexing of embryos and the in vitro capacitation and fertilization of spermatozoa and oocytes, respectively.

The combination of some or all of these techniques permits the cattleman or dairyman to manipulate the genetics of his/her livestock to produce faster growing, better feed converting and better milking animals, which will bring him/her a profit at the market place.

REFERENCES

1. Boland, MP, Crosby, TF and Gordon, I: Morphological Normality of Cattle Embryos Following Superovulation Using PMSG. *Theriogenology* 10: 175–180.
2. Elsden, RP, Nelson, LD and Seidel, GE, Jr.: Superovulating Cows with Follicle Stimulating Hormone and Pregnant Mare's Serum Gonadoptropin. *Theriogeniology* 9, 17–26.
3. Elsden, RP and Seidel, GE, Jr.: Embryo Transfer Procedures For Cattle. Published by Colorado State University, 1982 Short Course.
4. Seidel, GE, Jr.: Superovulation and Embryo Transfer in Cattle. *Science* 211: 351–358.
5. Elsden, RP, Hasler, JF and Seidel, GE, Jr.: Non-Surgical Recovery of Bovine Eggs. *Theriogenology* 6: 523–532.
6. Tervit, HR, Cooper, MW, Goold, PG and Haszard, GM: Non-Surgical Embryo Transfer in Cattle. *Theriogenology* 13: 63–71.
7. Williams, TJ, Elsden, RP and Seidel, GE, Jr.: Bisecting Bovine Embryos: Methods, Applications, and Success Rates. *Postdoctoral Thesis.* 1982.
8. Seidel, GE, Jr.: Proccedings of the Workshop: *IXth Annual Meeting of the International Embryo Transfer Society.* 1983.
9. Shea, BF, Jazen, RE, McAlister, RJ and McDermand, DP: Freezing of Bovine Embryos: Effects of Embryo Quality, Time from Thawing to Transfer and Number Frozen Per Vial. *Theriogenology* 20: 205–211.
10. Seidel, GE, Jr., Elsden, RP, Takeda, T and Farrand, GD: Field Trials with Cryopreserved Bovine Embryos. Published by Colorado State University, 1982 Short Course.
11. Schneider, U and Hahn, J: Recent Results of Frozen and Thawed Bovine Embryos. *Theriogenology* 11: 108.
12. Tervit, HR and Elsden, RP: Development and Viability of Frozen-Thawed Cattle Embryos. *Theriogenology* 15, 395–403.
13. Seidel, GE, Jr.: Applications of Microsurgery to Mammalian Embryos. *Theriogenology* 17: 23–34.
14. Hare, WCD and Betteridge, KJ: Relationship of Embryo Sexing to Other Methods of Prenatal Sex Determination in Farm Animals: A Review. *Theriogenology* 9: 27–43.

15. White, KL, Linder, GM, Anderson, GB and Bon-Durant, RH: Cytolytic and Fluorescent Detection of H-Y Antigen on Preimplantation Mouse Embryos. *Theriogenology* 19: 701-705.
17. Brackett, BG, Bousquet, D, Boice, ML, Donawick, WJ, Evans, JF and Dressel, MA: Normal Development Following In Vitro Fertilization in the Cow. *Biol. Reprod.* 27: 147-158.
18. Iritani, A and Niwa, K: Capacitation of Bull Spermatozoa and Fertilization In Vitro of Cattle Follicular Oocytes Matured in Culture. *J. Reprod. Fest.* 50: 119-121.
19. Sirard, MA, Lambert, R and Beland, R: Evaluation of Bovine Capacitation by In Vitro Fertilization. Theriogenology *21* 261.
20. Wright, RW, Jr. and Bondioli, KR: Aspects of In Vitro Fertilization and Embryo Culture in Domestic Animals. *J. Anim. Sci.* 53: 702-729.

Primate Conference Scheduled

Leading primatologists from around the world will be among the speakers at "Primates—The Road to Self-sustaining Populations," a week-long conference on endangered primates. Countries represented include England, The Netherlands, Australia, Indonesia, Kenya, Brazil, and the United States.

The meeting, June 24-28, 1985, in San Diego, is cosponsored by the Zoological Society of San Diego and Morris Animal Foundation, Englewood, Colorado.

Speakers will focus on preserving vanishing primate species, both in the wild and through zoo breeding programs. Experts on reproductive physiology, management of captive populations, zoo furnishings, diseases of primates, genetics, and populations in the wild will present papers and lead round-table discussions.

Registration fee for the meeting is $150 ($125 before April 1, 1985), or $35 for students. For information, contact:

Morris Animal Foundation
45 Inverness Drive East
Englewood, CO 80112
or
Zoological Society of San Diego
P.O.Box 551
San Diego, CA 92112-0551