Perspectives on Bovine Embryo Transfer

Reuben J. Mapletoft

WCVM, University of Saskatchewan, Saskatoon, Saskatchewan Canada S7N 5B4
Email: Reuben.mapletoft@usask.ca

■ Take Home Message

- Over a period of approximately 40 years, commercial bovine embryo transfer has become a large international business.

- On a worldwide basis, more than 500,000 embryos are produced annually from superovulated donors.

- The technology is well established, and superovulation and embryo collection are now done as frequently as every 30 days.

- Cryopreservation and direct transfer of frozen-thawed embryos is common-place with pregnancy rates near that of fresh embryos.

- Since the zona pellucida-intact bovine embryo can be made specified pathogen-free by washing procedures, thousands of frozen embryos are marketed internationally on an annual basis.

- In vitro embryo production is well established technology, but is generally too labor-intensive and costly for routine use in Canada.

- Polymerase chain reaction (PCR) technology is currently being used for sexing embryos, and this technology will be used for "embryo diagnostics" and "embryo genomics" in the future.

- Sex-sorted bovine semen is an established technology and is likely to be used increasingly in the future, especially for in vitro embryo production.

■ Introduction

The bovine embryo transfer industry arose in North America in the early 1970's (Betteridge, 2003). Continental breeds of cattle imported into Canada were very valuable and relatively scarce because of international health and trade restrictions. Embryo transfer offered a means by which their numbers could be multiplied rapidly. For several years, the most common use of embryo transfer in animal production programs was the proliferation of so-called desirable phenotypes. However, the University of Guelph introduced

the concept of MOET (multiple ovulation and embryo transfer) in 1987 (Smith, 1988). They showed that MOET programs could result in increased selection intensity and reduced generation intervals, resulting in improved genetic gains. The establishment of nucleus herds and "Juvenile MOET" in heifer offspring was shown to result in genetic gains that approached twice those achieved with traditional progeny test schemes. It is noteworthy that prior to the Guelph work, most embryo transfer done in Canada was in beef cattle, whereas approximately 75% of the embryo transfer work in Canada in 2010 involved dairy cattle (Table 1); approximately 65% of embryo transfer work in the USA continues to involve beef cattle (Stroud, 2009).

Embryo transfer is now commonly used to produce AI sires from proven cows and bulls (Teepker and Keller, 1989). In addition, new genomic techniques are being used increasingly to select embryo donors, especially for selection of dairy bull dams for superstimulation, where a genomic analysis is becoming essential (Seidel, 2010). Although economics would seem to preclude the use of embryo transfer techniques for anything but seed-stock production at this time, the commercial cattle industry can benefit by the use of bulls produced through well designed MOET programs (Christensen, 1991). The success of MOET programs has also led to the use of this technology to genetically test AI sires (Lohuis, 1995); bulls were proven by production records from siblings rather than offspring (Smith and Ruane, 1987). It was possible to genetically test a bull in 3.5 years as opposed to 5.5 years using traditional progeny testing schemes, which also resulted in shortened generation intervals. Results supported the theory, but physiology was a limiting factor in practice; superovulatory results made it difficult to produce the desired number of female offspring for genetic testing.

The first commercial embryo transfer programs relied on mid-ventral surgical exposure of the uterus and ovaries with the donor under general anesthesia. This necessitated surgical facilities and limited the use of the technology in the dairy industry because the udder of dairy cows hindered mid-ventral access to the reproductive tract. It was not until the mid-1970s that nonsurgical embryo recovery became sufficiently developed to be used in practice (Drost et al., 1976; Elsden et al., 1976; Rowe et al., 1976). In the early 1980s, nonsurgical embryo transfer techniques (Rowe et al., 1980) were adopted, allowing embryo transfer on the farm and the technology became attractive to dairy farmers.

Table 1. Summary of bovine embryo transfer activity in Canada in 2010
(CETA Statistics of 63 clinics across Canada; www.ceta.ca).

	DAIRY	BEEF	TOTAL
IN VIVO EMBRYOS			
Total number of donors	10,821	2,135	**12,956**
Total number of ova/embryos collected	126,351	29,898	**156,249**
Total number of transferable embryos	73,732	15,472	**89,204**
Total number of embryos frozen	47,784	12,494	**60,278**
Number of fresh embryos transferred	24,869	1,981	**26,850**
Number of fresh sexed embryos transferred	1,891	41	**1,932**
Number of frozen embryos transferred	23,533	4,753	**28,286**
Number of frozen sexed embryos transferred	1,818	47	**1,865**
Total number of Direct Transfer frozen/thawed embryos			**28,214**
PREGNANCY RATES			
Fresh Embryos *(based on 13,066 transfers)*			**58.3 %**
Frozen Embryos *(based on 14,758 transfers)*			**57.4 %**
Total number of embryos exported	8,178	4,894	**13,072**
Total number of embryos imported	152	147	**299**
OTHER TECHNOLOGIES (TOTAL NUMBERS)			
Total number of split embryos transferred			**1,605**
Total number of embryos biopsied for sexing			**4,880**
Total number of embryos biopsied for genetic testing			**80**

The embryo transfer industry grew rapidly in the late 1970s, both in terms of the number of practitioners and in the number of donors. Seidel (1981) reported that in 1979, more than 17,000 pregnancies resulting from the transfer of bovine embryos were recorded in North America. In 2009, Stroud reported that 539,683 in vivo-produced bovine embryos were transferred world-wide, of which 54% were transferred after on-farm freezing and thawing. In addition, 292,000 in vitro-produced bovine embryos were transferred, primarily in Brazil. North America has continued to be the centre of commercial embryo transfer activity with more than 53,000 donor cows superstimulated and more than 249,000 embryos transferred (46% of all embryo transfers).

Although the International Embryo transfer Society (IETS) was founded in 1974 by the practitioners of embryo transfer, a growing number of commercial embryo transfer practitioners have discontinued membership in favor of their

regional organizations. It is also clear that a growing majority of the IETS membership is composed of basic researchers representing government, industrial or academic institutions, including human medicine. However, the IETS has played a very important role in the dissemination of basic and applied information, allowing for the rapid growth of the embryo transfer industry. In particular, the Import/Export Committee of the IETS (now referred to as the Health and Safety Advisory Committee; HASAC) has been instrumental in gathering and disseminating scientific information on the potential for disease control with bovine embryo transfer. The Manual of the International Embryo Transfer Society *"A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures"* (Fourth Edition, 2010) has become the reference source for sanitary procedures used in export protocols.

In 1982, the American Embryo Transfer Association (AETA) was formed to unite and organize the commercial embryo transfer industry in the USA, and in 1984, the Canadian Embryo Transfer Association (CETA) was formed. Objectives included the establishment of standards of performance and conduct, and a liaison with Federal agencies for both domestic and international embryo transfer. These associations also interact directly with breed associations, producer groups and international groups such as the IETS. Their expressed purpose is to establish standards of practice to provide confidence within each country, and internationally, for the utilization of embryo transfer technology. In this regard, their Certification Programs are integral in ensuring that Embryo Transfer Practitioners are technically and ethically competent in the handling of embryos used in international trade.

Although there has been no appreciable increase in the number of embryos produced per superovulated donor over the past 20 years, the importance of follicle wave dynamics (Adams, 1994) and methods for the synchronization of follicular wave emergence (Bo et.al, 2002), have simplified the means by which superovulation might be achieved, resulting in increased embryo production per unit time. Donor cows are being superstimulated more frequently than in the past (often every 30 days without any reduction in ova/embryo production, and no effect on subsequent fertility), and more embryos are being produced per year with no change in the actual superstimulation protocol. The application of similar procedures to recipients has made estrus detection, and the need to wait for animals to "come into heat" unnecessary, facilitating management in commercial programs (Bo et al., 2002).

■ Disease Control

Several large studies have now shown that in vivo-produced bovine embryos do not transmit infectious diseases. In fact, the IETS has categorized disease agents based on the risk of transmission by a bovine embryo (see

Stringfellow and Givens, 2000, and The Manual of the International Embryo Transfer Society). Category 1 includes diseases or disease agents for which sufficient evidence has accrued to show that the risk of transmission is negligible, provided that embryos are properly handled between collection and transfer. Category 1 diseases include Enzootic bovine leukosis, Foot and mouth disease (cattle), Bluetongue (cattle), Brucella abortus (cattle), Infectious bovine rhinotracheitis, pseudorabies in swine and Bovine spongioform encephalopathy. Category 2, 3 and 4 diseases are those for which less research information has been generated.   However, it is noteworthy that none of the infectious diseases studied have been transmitted by in vivo-produced bovine embryos, provided embryo handling procedures were done correctly.   Consequently, it has been suggested that embryo transfer be used to salvage genetics in the face of a disease outbreak (Wrathall et al. 2004)).

Embryo Import-Export

The intercontinental transport of live animals costs several thousands of dollars, whereas an entire herd can be transported, in the form of frozen embryos, for less than the price of a single plane fare. Additional benefits of embryos include reduced risk of disease transmission, reduced quarantine costs, a wider genetic base from which to select, the retention of genetics within the exporting country, and adaptation. Over the last 10 years, embryo import regulations for many countries have been simplified. In the year 2002, approximately 30,000 embryos were frozen in North America for export purposes, and in 2010, more than 13,000 embryos were exported from Canada alone.

Although handling procedures recommended by the IETS make it possible to safely export in vivo-derived embryos originating from donors sero-positive to specified pathogens (Mapletoft and Hasler, 2005), it is a different story with embryos produced with in vitro techniques.   The structure of the zona pellucida of in vitro-produced (IVP) bovine embryos differs from that of in vivo-derived embryos (Stringfellow and Givens, 2000). It has been shown that a number of pathogens are more likely to remain associated with in vitro-derived embryos following washing than with in vivo-derived embryos (Stringfellow and Givens, 2000). This has potentially serious ramifications for the international movement of IVP embryos, and so protocols must be revised accordingly.

■ Cryopreservation: Direct Transfer and Vitrification

The development of effective methods of freezing embryos has made embryo transfer a much more efficient technology. For many years, embryos were frozen very successfully in glycerol, but because glycerol penetrated cell

membranes rather slowly, it also had to be removed slowly after thawing. This necessitated microscopic examination and time for dilutions in the lab. Recently, the use of highly permeating cryoprotectants such as ethylene glycol has allowed the direct transfer of bovine embryos. With this approach, the embryo straw is thawed in a water-bath, and its contents are deposited directly into the uterus of the recipient, much like AI. There is no need of a microscope or complicated dilution procedures. The cryoprotectant leaves the embryo in the uterus, without causing osmotic stress. In a study of the North American embryo transfer industry in 1998, pregnancy rates from direct transfer embryos were comparable to that achieved with glycerol (Leibo and Mapletoft, 1998). The freezing of bovine embryos is now common place and pregnancy rates are only slightly less than that achieved with fresh embryos (Table 1; Leibo and Mapletoft, 1998). During the year 2009, more than half the embryos collected in North America were frozen prior to transfer and more than 95% were frozen in ethylene glycol for direct transfer (Stroud, 2009). Although the skill-level required to transfer embryos frozen in ethylene glycol does not differ from transfer of embryos frozen in glycerol, no embryologist is needed at the time of thawing. Consequently, a growing number of direct transfer embryos are now being transferred by technicians with experience in AI. Pregnancy rates with frozen/thawed embryos are now only slightly less than that achieved with fresh embryos (Table 1; Leibo and Mapletoft, 1998).

Freezing and thawing procedures are time consuming and require the use of biological freezers and a microscope. Complicated embryo freezing procedures may soon be replaced by a relatively simple procedure called vitrification. With vitrification, high concentrations of cryoprotectants are used and the embryo in its cryoprotectant solution is placed directly into liquid nitrogen. Because of the high concentration of cryoprotectants and the ultra-rapid freezing rate used, ice crystals do not form; the frozen solution forms a "glass". Since ice crystal formation is one of the most damaging processes in freezing, vitrification has much to offer in the cryopreservation of oocytes, IVP embryos and biopsied embryos. However, its greatest advantage is its simplicity. In a rather large study conducted in Holland, pregnancy rates following direct transfer of bovine embryos vitrified in 0.25 ml straws did not differ from a control group frozen by traditional means in glycerol (Wrathall et al., 2004).

■ In Vitro Embryo Production (IVP)

Although each ovary contains hundreds of thousands of oocytes (eggs) at birth, many thousands undergo atresia and are lost, starting before birth. This tremendous loss of genetic material could be salvaged by harvesting oocytes from the ovary and using IVP techniques (Hasler et al., 1995). Bovine IVP is now a reasonably efficient procedure; transvaginal ultrasound-guided oocyte aspiration at frequent intervals, in combination with in-vitro fertilization (IVF) has proved its worth in improving the yield of embryos from designated

donors, salvaging irreplaceable genetics following slaughter in the face of infectious disease control or in culling for other reasons (see Hasler, 2003). IVF has also been used to produce thousands of embryos needed for scientific research, including efforts to produce embryonic stem cells.

A few laboratories have reported very modest successes in the production of pregnancies from IVP of embryos from calves (reviewed in Mapletoft and Hasler, 2005) which offers the potential for increased genetic gain by decreasing generation intervals even further (Smith, 1988). In addition, oocyte aspiration has proven to be safe and very successful in pregnant cows and is often used when high demand for offspring from a particular donor cow, or MOET programs necessitate the production of additional offspring. Oocytes with good viability have been collected once or twice weekly, or following superstimulation with gonadotropins as late as 90 to 150 days of gestation (Garcia and Salaheddine, 1998).

Several authors have directly addressed the question of using IVP as a substitute for in vivo production of embryos (Bousquet et al., 1998; Hasler et al., 1995). It is clear that pregnancies can be produced by IVP from donor females that were infertile (Hasler et al., 1995), but it is unclear whether IVP is a realistic alternative to conventional superovulation and embryo collection for production of embryos from reproductively healthy cattle. In 2009, more than 292,000 IVP embryos were transferred world-wide (Stroud, 2009), but this is accounted for almost entirely by the increase in activity in Brazil where IVP of embryos is done primarily in Bos indicus cattle.

One commercial embryo transfer unit in Canada has provided data comparing the efficacy of conventional embryo transfer to IVP (Bousquet et al., 1998). Success rates with IVP of bovine embryos in their hands greatly exceeded the published results of other commercial programs. The authors concluded that IVP would produce about 3.4 times more embryos and 3.2 more pregnancies in a 60 day period, assuming only one superovulation per donor. This was also higher than that reported by other commercial embryo transfer practitioners. In addition, as mentioned above, donor cows are often superstimulated and in vivo-produced embryos are collected every 30 days, now-a-days. Currently, under commercial conditions in North America, it would appear to be more expensive to produce pregnancies by IVP than with conventional superovulation and embryo transfer. For most breeders, this technology is an advantage only for extremely valuable cows that are infertile or fail to produce embryos following superstimulation.

It is common to produce IVP embryos from slaughterhouse-derived ovaries. Although this source of oocytes has little to offer from a genetic improvement perspective, and it presents biosecurity issues relating to international movement of embryos, it is a very import source of embryos for research. In addition, IVP embryos produced from slaughterhouse-derived oocytes have

been used to improve pregnancy rates in repeat breeding cows, and in those under heat stress. It has also been proposed as an inexpensive source of embryos to produce dairy-beef in dairy cattle or to induce twinning in beef cattle.

■ Prenatal Sex Determination

Determination of the sex of preimplantation bovine embryos with the use of the polymerase chain reaction (PCR) is a service offered by several embryo transfer practitioners. However, removal of the biopsy from the embryo requires a high level of operator skill, and embryo biopsy is an invasive technique that results in invasion of the integrity of the zona pellucida and some reduction in the viability of the embryo, especially following freezing. Although many livestock breeders request embryo sexing, it has not found widespread use in North America. It is labor-intensive and costly, and biopsied embryos do not survive freezing very well. During 2009, only 2500 sexed embryos were transferred in Canada (Stroud, 2009).

In the near future, PCR assays for use in the identification of other traits of economic importance may become available (Bishop et al., 1995). Marker-assisted selection (MAS), based on identifying genetic markers for unknown alleles of valuable traits, probably has a similar future (Georges and Massey, 1991). MAS can potentially be applied to embryo biopsies if sufficiently valuable markers can be identified. A PCR assay currently exists for simultaneous detection of the bovine leucocyte adhesion deficiency (BLAD) gene and the sex of embryo biopsies (see Hasler, 2003). It is probable that PCR techniques will be developed that permit the analysis of a large number of markers simultaneously from one biopsy (embryo diagnostics), and it is likely that genomic testing of embryos with single nucleotide polymorphism (SNP) technology will occur in the near future, again utilizing embryo biopsies and PCR technology (Seidel, 2010).

The flow cytometric technology used to separate X- and Y-bearing sperm into live fractions has been improved over the last 10 years (Johnson, 2000). With a purity of 90%, about 10 million live sperm of each sex can be sorted per hour (Seiderl, 2003). In AI field trials involving approximately 1000 heifers, pregnancy rates with 1.0×10^6 sexed, frozen sperm were 70 to 90% of unsexed controls inseminated with 20 to 40X as many sperm. A recent study involving 574 calves produced from sex-sorted sperm concluded that there were no differences in gestation length, neonatal deaths, calving ease, birth weight or survival rate to weaning (Tubman, 2003). The disadvantages of this technique are the slow speed of sorting, the decreased fertility of sexed sperm, especially in superovulated donor cows, the cost of the semen, and the availability of semen from specific bulls (Amann, 1999). For the embryo transfer industry, sex-sorted semen presently has its greatest use in IVP of bovine embryos.

■ Success Rates, Costs and Regulations

Success rates in terms of embryo production per superovulation attempt, and pregnancy rates following transfer have changed little over the last several years, except that we are now more able to control ovarian function and collect embryos more frequently. This has doubled embryo production in many donor cows. Pregnancy rates on a national basis are around 60 % with fresh embryos and 55 – 60 % with frozen/thawed embryos. Embryo transfer practitioners charge around $250 to superovulate and collect donor cows, and around $50 to freeze each embryo. It costs from $75 to $150 per embryo for transfer, depending on the embryo collection arrangement. The largest cost factor for embryo transfer still relates to recipients, and recipient management.

Breed association regulations vary so it is important to enquire before embarking on such a venture. Most embryo transfer practitioners are familiar with regulations and can provide guidance. The regulation of embryo transfer procedures fall under the veterinary act in all provinces, and only veterinarians can be certified by CETA and the CFIA for the exportation of embryos. However, certification is not necessary for embryo production for domestic purposes. Some provinces have exemptions from the veterinary act for the nonsurgical transfer of previously evaluated embryos e.g., Direct Transfer.   Thus, as indicated earlier, technicians are now transferring frozen/thawed embryos, much like artificial insemination.

■ Conclusions

In approximately 40 years, commercial embryo transfer in cattle has become a well established industry with more than 500,000 embryos being transferred on an annual basis throughout the world. Although this results in a very small number of offspring on an annual basis, its impact is large because of the quality of animals being produced. Embryo transfer is now being used for real genetic improvement, especially in the dairy industry, and most semen used today comes from bulls produced by embryo transfer.   However, the real benefit to embryo transfer is that in vivo-produced bovine embryos can be made specified pathogen-free by washing procedures, making this an ideal procedure for disease control programs or in the international movement of animal genetics. Techniques have improved over the past 40 years so that frozen-thawed embryos can be transferred to suitable recipients as easily and simply as artificial insemination. In vitro embryo production and embryo and semen sexing are also successful, but time and cost limit their widespread use. A combination of embryo transfer using proven cows inseminated with semen from proven bulls, followed by industry-wide artificial insemination appears to be the most common use of bovine embryo transfer in the near future.

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