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History and perspectives on bovine embryo transfer

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Abstract

On a worldwide basis, more than 750,000 embryos are produced annually from superovulated donors and more than 450,000 embryos are produced using *in vitro* techniques. Superovulation and embryo collection are done as frequently as every 30 days. Cryopreservation and direct transfer of frozen-thawed embryos results in pregnancy rates near that of fresh embryos. Since the zona pellucida-intact *in vivo*-produced 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 used widely in countries like Brazil and Japan. Polymerase chain reaction (PCR) technology is currently being used for sexing embryos, and this technology is beginning to be used for "embryo diagnostics" and "embryo genomics". Sex-sorted bovine semen is also readily available and is being used increasingly, especially for *in vitro* embryo production.

Keywords: export, genetic improvement, import, IVF, sexing.

Introduction

For an historical perspective on assisted reproduction, the reader is referred to a comprehensive review of farm animal embryo transfer and its associated technologies (Betteridge, 2003). In brief, the first successful transfer of mammalian embryos was performed by Walter Heape in 1890. Heape transferred two four-cell Angora rabbit embryos into an inseminated Belgian doe, which subsequently gave birth to four Belgian and two Angora young (Betteridge, 2003). There were no reports of further success in mammalian embryo transfer until the 1920s, when several investigators again described embryo transfer in rabbits. Warwick and colleagues did considerable work on embryo transfer in sheep and goats in the 1930s and 1940s (Referenced in Betteridge, 1981, 2003), but it was Umbaugh (1949) who reported on the first successful embryo transfers in cattle. He produced four pregnancies from the transfer of cattle embryos, but all the pregnancies were terminated before full term. In 1951, the first embryo transfer calf was born in Wisconsin following the surgical transfer of an abattoir-derived day-5 embryo (Willett *et al*., 1951; Referenced in Betteridge, 1981).

It was Rowson and colleagues who developed much of the technology that later found commercial use. Indeed, Betteridge (2003) has referred to Rowson as a founding father of embryo transfer in farm animals, and the International Embryo Transfer Society recognized his stature with the title of Founding Honorary President. In 1972, Rowson organized the first international course on bovine embryo transfer in Cambridge that brought together 13 veterinarians from around the world. Several of these registrants became the founding members of the International Embryo Transfer Society (IETS) and practitioners of commercial embryo transfer (Referenced in Betteridge, 2003).

The bovine embryo transfer industry as we know it today arose in North America in the early 1970's (Betteridge, 1981, 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. However, it was private veterinary practitioners and small commercial companies who developed the technology for commercial use; they took techniques from the laboratory to the field. These pioneers encountered countless practical difficulties and founded the IETS to facilitate open discussion which they considered necessary if progress was to be made.

Embryo transfer organizations

The IETS was founded in 1974, with 82 Charter Members, representing researchers, academics and veterinary practitioners from around the world (Carmichael, 1980; Schultz, 1980). The IETS became the main forum for scientific and regulatory exchange and discussion in the field of embryo transfer and associated technologies. The Proceedings of the Annual Meeting of the IETS, which were published as the first issue of Theriogenology each year, served as a yard-stick with which to measure changes in emphasis and intensity of activity in embryo transfer. More recently, the IETS Proceedings have been published in the first issue of Reproduction, Fertility and Development. It is noteworthy, that since 1978, the proceedings of the Annual Meeting of the IETS have been published and available to registrants at the time of the meeting.

With the founding of regional embryo transfer organizations, a growing number of commercial embryo transfer practitioners have discontinued membership in

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the IETS in favor of their regional organizations. A growing number of the IETS members have been basic researchers representing government, industrial or academic institutions, including human medicine (Hasler, 2003). However, the IETS has played an 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"* has become the reference source for sanitary procedures used in embryo export protocols (Stringfellow and Givens, 2010).

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. They established 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 (Mapletoft and Hasler, 2005).

The Brazilian Embryo Technology Society (SBTE) was founded in Brasilia in 1985 (Rubin, 2005) as a private, not-for-profit organization of professionals dedicated to embryo transfer technology. Member's interests related primarily to cattle, horses and small ruminants, but also included swine, companion animals, laboratory species and wild and endangered species. Its stated goal is to serve as a forum for the exchange of information among practitioners, scientists, educators, livestock breeders and students as well as suppliers in the area of reproductive biotechnology. SBTE aimed to promote the science of animal embryo technology by encouraging effective research, disseminating scientific and educational information, maintaining high standards of ethics and cooperating with other organizations with similar objectives.

The application of commercial embryo transfer in cattle

Genetic improvement

With the development of commercial embryo transfer in the 1970s, its most common use 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 increased 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 84% of the embryo transfer work done in Canada in 2011 involved dairy cattle. On the other hand, approximately 61% of embryo transfer work in the USA continues to involve beef cattle (Stroud, 2012).

Embryo transfer is now commonly used to produce AI sires from the top producing cows and proven bulls (Teepker and Keller, 1989). In addition, new genomic techniques are being used increasingly to select embryo donors; genomic analysis has become essential for the selection of bull dams to be used in embryo transfer (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 has benefited from the use of commercial 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.

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 (Stringfellow and Givens, 2000; Mapletoft and Hasler, 2005). Category 1 diseases include 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. This includes inspection of the zona pellucida at >50X magnification and washing/trypsin treatment procedures. 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. 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 ability to utilize embryos in preventing the transmission of infectious disease makes them ideal for the international movement of animal germ plasm. The intercontinental transport of live animals also costs 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 for the international movement of animal genetics include reduced risk of disease transmission, reduced quarantine costs, a wider genetic base from which to select, the retention of the original genetics within the exporting country, and adaptation. Over the last 10 years, embryo import regulations for many countries have been simplified. In 2011, approximately 30,000 embryos were frozen in North America for export purposes, and 13,737 embryos were exported from Canada alone (Stroud, 2012).

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

Embryo transfer technology

Although the applications and techniques associated with bovine embryo transfer have been reviewed extensively (Mapletoft, 1985, 1987), a brief historical perspective may be useful. Early investigators described non-surgical embryo recovery techniques (Rowson and Dowling, 1949), but these were not successful, and so all embryo recoveries and transfers were performed surgically in the early 1970s. These 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 1976 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 also developed, allowing for on farm embryo transfer.

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 more than 17,000 pregnancies resulted from the transfer of bovine embryos in North America in 1979. More recently, Stroud (2012) reported that 572,432 *in vivo*-derived bovine embryos were transferred world-wide in 2011, of which 54% were transferred after freezing and thawing. In addition, 373,836 *in vitro*-produced bovine embryos were transferred, 85% of which were in Brazil. In 2011, North America continued to lead in commercial embryo transfer activity with collection of 54,837 donor cows and the transfer of more than 248,615 embryos (43% of all embryo transfers).

Although there has been no appreciable increase in the number of embryos produced per superovulated donor cow over the past 20 years, the importance of follicle wave dynamics (Adams, 1994) and methods for the synchronization of follicular wave emergence (Bó *et al*., 1995, 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), and more embryos are being produced per year with no change in the actual superstimulation protocol. The application of similar procedures in recipients has made estrus detection, and the need to wait for animals to "come into heat" unnecessary, facilitating fixed-time embryo transfer (Bó *et al*., 2002).

Cryopreservation, direct transfer, and vitrification

The development of effective methods of cryopreserving bovine embryos (Wilmut and Rowson, 1973; Leibo and Mazur, 1978) made embryo transfer a much more efficient technology, no longer depending on the immediate availability of suitable recipients. Pregnancy rates are only slightly less than those achieved with fresh embryos (Leibo and Mapletoft, 1998). Recently, the use of highly permeating cryoprotectants, such as ethylene glycol, has allowed the direct transfer of bovine embryos (Voelkel and Hu, 1992; Hasler *et al*., 1997). In a study of the North American embryo transfer industry, pregnancy rates from direct-transfer embryos were comparable to those achieved with glycerol (Leibo and Mapletoft, 1998), and in 2011, more than 95% of frozen-thawed embryos were transferred by Direct Transfer (Stroud, 2012). In addition, a growing number of direct-transfer embryos are being transferred by technicians with experience in AI.

Freezing and thawing procedures are time-consuming and require the use of biological freezers.

Complicated embryo freezing procedures may soon be replaced by a relatively simple procedure called vitrification (Rall and Fehy, 1985). With vitrification, the embryo in high concentrations of cryoprotectants is placed directly into liquid nitrogen. As a result of the high concentration of cryoprotectants and the ultra-rapid rate of freezing, ice crystals do not form; instead 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 and *in vitro*-produced embryos. However, its greatest advantage is its simplicity. Vitrification is now widely used experimentally and in vivo-derived bovine embryos have been vitrified successfully in 0.25 ml straws for direct transfer (van Wagtendonk-de Leeuw *et al*., 1997).

In vitro embryo production

Bovine *in vitro* embryo production (IVP) is now a well-established and efficient procedure (Brackett and Zuelke, 1993). Moreover, ovum pick-up (OPU) at frequent intervals, in combination with *in vitro* fertilization, has improved and increased the yield of embryos from designated donors (Garcia and Salaheddine, 1998). *In vitro* fertilization has also been used to produce the thousands of embryos needed for scientific research, including efforts to produce embryonic stem cells; the constituent oocyte maturation and embryo culture techniques are integral parts of the procedures for cloning and transgenesis (Campbell *et al*., 1996; Niemann and Kues, 2003). A few laboratories have also reported very modest successes in producing pregnancies with IVP embryos from calves (Duby *et al*., 1996; Fry *et al*., 1998; Taneja *et al*., 2000), which offers the potential for decreasing generation intervals (Betteridge *et al*., 1989). In addition, OPU has proven to be safe and very successful in pregnant cattle.

Several authors have directly addressed the question of using IVP as a substitute for *in vivo* embryo production (Sinclair *et al*., 1995; Hasler, 1998; Bousquet *et al*., 1999). At present, under commercial conditions in North America, IVP appears to be more expensive than conventional in vivo embryo production. For most breeders, this technology is an advantage only for extremely valuable cows which are infertile or fail to produce embryos after superstimulation. Indeed, the number of IVP embryos produced globally in 2011 as compared to 2010 was up less than 1%. However, IVP in Brazil in 2011 increased by 20% over 2010 resulting in 318,116 transferrable embryos. Brazil accounts for 86% of the world's total IVP. In 2011, 53,019 OPU sessions were performed in Brazil, yielding an average of 15 oocytes and 6 embryos per session. As a result, IVP numbers have surpassed that of *in vivo* embryo production in Brazil; it will be interesting to see if the trend continues for other countries in the world.

The efficiency of frozen IVP embryos will likely determine the acceptance of IVP technology by other countries (Hasler *et al*., 1995). So far, the majority of the IVP embryos have been transferred fresh, not frozen. However, data vary according to regions of the world. Worldwide 8% of the IVP embryos transferred in 2011 were frozen-thawed, while only 5% of IVP embryos were frozen in Brazil (Stroud, 2012). However, Brazil reports transferring more frozen-thawed IVP embryos each year, and results are improving.

Adoption of new technologies

Prenatal determination of sex potentially has great economic impact (Seidel, 2003) and the polymerase chain reaction (PCR) to determine the sex of bovine embryos is a service offered by many embryo transfer practitioners (Thibier and Nibart, 1995). However, embryo biopsy requires a high level of operator skill, and is an invasive technique resulting in disruption of the integrity of the *zona pellucida* and some reduction in the viability of the embryo, especially after cryopreservation. In the near future, PCR assays to identify other traits of economic importance will no doubt 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). Like genotyping of specific alleles, 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 gene and the sex of embryo biopsies (Hasler, 2003). It is probable that PCR techniques will be developed that permit the analysis of a large number of markers from one biopsy leading to the concept of "embryo diagnostics". It is also 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 15 years (Johnson *et al*., 1994; Johnson, 2000). Approximately 10 million live sperm of each sex can be sorted per hour (Seidel, 2003), with a resulting purity rate of >90%. In AI field trials, pregnancy rates following insemination with 1 million sexed, frozen sperm were reported to be 70 to 90% that of unsexed controls inseminated with 20 to 40 million sperm (Seidel *et al*., 1999). A recent study which compared 574 calves produced from sex-sorted sperm with 385 control calves concluded that there were no differences in gestation, neonatal deaths, ease of calving, birth weight or survival rate to weaning (Tubman *et al*., 2003). The disadvantages of flow cytometry are the slow speed of sorting, the decreased sperm viability (pregnancy rates), especially in superovulated donor cows, the cost of the semen, and the availability of

semen from specific bulls (Amann, 1999). It is likely that sexed semen will have the greatest use in IVP of bovine embryos in the near future.

Summary and conclusions

Commercial embryo transfer in cattle has become a well established industry. Although a very small number of offspring are produced 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 gain, especially in the dairy industry, and most semen used today comes from bulls that have been produced by embryo transfer. An even greater benefit of bovine embryo transfer may be that *in vivo*-derived embryos can be made specified pathogen-free by washing procedures, making this an ideal process 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 is normally done. *In vitro* embryo production and embryo and semen sexing are also successful. A combination of embryo transfer using proven cows inseminated with semen from proven bulls, appears to be the most common use of bovine embryo transfer.

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