Forty years of embryo transfer in cattle: A review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminiscences
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Forty years of embryo transfer in cattle: A review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminiscences

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Abstract

After the first successful transfer of mammalian embryos in 1890, it was approximately 60 years before significant progress was reported in the basic technology of embryo transfer (ET) in cattle. Starting in the early 1970s, technology had progressed sufficiently to support the founding of commercial ET programs in several countries. Today, well-established and reliable techniques involving superovulation, embryo recovery and transfer, cryopreservation, and IVF are utilized worldwide in hundreds, if not thousands, of commercial businesses located in many countries. The mean number of embryos produced via superovulation has changed little in 40 years, but there have been improvements in synchrony and hormonal protocols. Cryopreservation of in vivo-derived embryos is a reliable procedure, but improvements are needed for biopsied and in vitro-derived embryos. High pregnancy rates are achieved when good quality embryos are transferred into suitable recipients and low pregnancy rates are often owing to problems in recipient management and not technology per se. In the future, unanticipated disease outbreaks and the ever-changing economics of cattle and milk prices will continue to influence the ET industry. The issue of abnormal pregnancies involving in vitro embryos has not been satisfactorily resolved and the involvement of abnormal epigenetics associate with this technology merits continued research. Last, genomic testing of bovine embryos is likely to be available in the foreseeable future. This may markedly decrease the number of embryos that are actually transferred and stimulate the evolution of more sophisticated ET businesses.

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1. Introduction

It is widely recognized that the first documented live birth resulting from the transfer of mammalian embryos was achieved by Walter Heape in 1890 [1]. In a subsequent paper, Heape described his technique for handling rabbit embryos, which involved spearing them on the tip of a needle and transferring them to a recipient without an intermediary step of placing them in holding medium [2]. Heape’s varied discoveries and successes in the field of reproduction were described in detail by John Biggers, who was awarded the Pioneer Award (PA) by the International Embryo Transfer Society (IETS) in 1990 [3]. Thirty years then passed before another success in producing mammalian offspring from embryo transfer (ET) was reported and it again involved rabbits [4]. The first birth of a calf resulting from ET is credited to E.L. Willet, et al., at the University of Wisconsin [5] and occurred 63 years ago. The research by Willet and the other early workers that led up to this first success in cattle is elegantly described by Keith Betteridge (2003 IETS-PA) in a paper published in Theriogenology in 2000 [6].

Protocols for the superovulation of cattle [7–9], appropriate media (for review see [10,11]), and the
surgical recovery and transfer of embryos [9,12] were developed primarily starting in the late 1940s and into the 1960s. These procedures, which led to the establishment of the modern ET industry in the early 1970s, are covered in a number of reviews, including some especially thorough contributions by Betteridge [13,14], Adams [15], Foote (2002 IETS-PA) and Onuma [10], and Seidel (2008 IETS-PA) [16].

In cattle, ET on a commercial basis has grown into a mature industry that is active today on a worldwide basis. Coincident with this 40th anniversary edition of *Theriogenology*, the commercial ET industry is today just a few years past 40 years of age. Also, the 40th anniversary of the IETS will be celebrated at the annual meeting in Reno, Nevada, in January 2014.

The modern livestock ET industry is a result of the pioneering efforts of two groups: The scientists who initially developed the procedures and techniques of ET, and the commercial ET practitioners who modified this technology, making it practical and available first to the cattle industry and then to other livestock species. When possible, the contribution of practitioners will be acknowledged. However, a fair amount of technology and ET improvements from the private sector have not been published and are available only in verbal, anecdotal form.

Seeking to provide comprehensive coverage of ET in cattle, whether on the basis of the historical development of the technology, the development and growth of the commercial industry, or the research applications that are utilized, is simply not possible in a short review paper. While preparing this manuscript, I sat in my home office surrounded by reprints of at least 1000 published papers dealing with some aspect of bovine ET, not including a number of books and published proceedings. A recent survey of PubMed showed a total of 2051 references on bovine ET, 609 on bovine superovulation, and 877 on bovine IVF.

The invitation to write this review for *Theriogenology* represents a daunting challenge; I was afforded a good deal of freedom and I have chosen to emphasize the following: to briefly cover the early history and development of bovine ET; outline the growth of the commercial ET industry, primarily in North America; and describe personal experiences in the growth and evolution of the commercial ET industry. However, my experiences in the ET industry are not particularly unique or unusual, but they span approximately 40 years, and I believe a personal view may sometimes be more interesting and informative than limiting coverage to published scientific papers, although I will emphasize key studies published in *Theriogenology*. Because of the breadth of the subject and space limitations, it is not possible to include all relevant references. Using IVF as an example, there are 170 pages of references in Ian Gordon’s (1998 IETS-PA) 1994 comprehensive review of cattle IVF [17]. Consequently, many fine studies published in journals other than *Theriogenology*, and many that have appeared in *Theriogenology* are not included in this review. I offer a sincere apology to any authors whose work I may have covered superficially or outright failed to include.

2. The early days of commercial ET

A large degree of recognition must be extended to Tim Rowson (1985 IETS-PA) and his colleagues at the Agricultural Research Council Unit of Animal Reproduction in Cambridge, Great Britain, for contributing much of the technology that got commercial bovine ET started in early 1970s. The Animal Research Council unit initiated surgical recovery and transfer of bovine embryos, which led to the establishment in the early 1970s of some of the first ET units in North America, including Alberta Livestock Transfer in Calgary Alberta; Modern Ova Trends in Norval, Ontario; Colorado State University (CSU) In Ft. Collins; Carnation Genetics in Hughson, California; and Cooding Embryological Science, Inc., in Foraker, Oklahoma. All of these units built and operated expensive surgical facilities for both the donors and recipients that underwent aseptic surgery under general anesthesia. These early commercial programs greatly extended and improved the work started by research programs such as the station in Cambridge. During this period of time there were also significant contributions being made from the growing ET industry in other areas of the world.

As technology rapidly improved and commercial ET units rapidly came on line, there were two noteworthy meetings of academicians and practitioners involved with both ET research and commercial services. In December 1975, a group of scientists and practitioners representing both North America and Europe met at Cambridge. A book edited by Rowson detailing the presentations at this meeting was published by the European Commission of the European Communities [18]. In July 1976, many of the participants in the Cambridge conference, plus a number of additional persons met to discuss ET during the 8th International Congress on Animal Reproduction in Krakow, Poland. The resulting monograph [19] detailing the papers and discussions from this meeting and the Rowson publication from the Cambridge meeting [18] were enormously helpful in advancing ET science into the commercial realm, and much of the science described remains accurate and relevant today. A few years later, in 1982, an excellent review edited by Cyril Adams of both the history and the current status of ET procedures was published [15].

In addition to development of technology, a second factor strongly influenced the early growth of the ET industry in North America. The legal importation of so-called exotic cattle from Europe was approved by the Canadian government [14,20]. These cattle, initially representing several breeds including Simmental, Limousin, and Charolais, could not be imported directly into the United States, but once they were in Canada, they could qualify for movement into the United States. Three-quarter and even half-blood females sold for very high prices and these cattle fueled the early ET industry in North America.

Starting in 1973 through 1975, the rapid increase in the number of Simmental calves born in the United States as a result of ET is shown in Table 1. Although more than 1500 Simmentals were reported born during this period, no Angus and only 21 Holsteins were recorded by their respective breed associations. Of the 33 cattle breeds...
The concept of chance in a happy or benevolent way means the occurrence and development of events by "good fortune" which can range from good to bad, but nonetheless, ET had a huge role in establishing the Simmental breed in North America. It is clear that by the late 1980s ET was being applied to many cattle breeds and that the initial surge applied mainly to Simmentals and other "exotic" breeds in North America had trailed off.

The definition of "serendipity" goes beyond just the concept of "luck," which can range from good to bad, but means the occurrence and development of events by chance in a happy or beneficial way. In 1973, I was in the final year of working on my PhD at the University of Illinois. My research involved a study of environmental influences on reproduction in a Canadian arctic rodent, specifically Dicrostonyx groenlandicus, known as the collared lemming. In October of that year, my wife, infant son, and I drove to Ft. Collins, Colorado, for a short vacation and visit with my brother and his family. While in Ft. Collins and owing to an entirely serendipitous series of events, I met George Seidel, who was just beginning his third year as an assistant professor at CSU and was busy setting up a bovine ET program with both commercial and third year as an assistant professor at CSU and was busy setting up a bovine ET program with both commercial and research applications. As a result of meeting George and telling him about my work with lemming embryos, he subsequently offered me a post-doctoral fellowship in his laboratory. I was to participate in the newly established bovine ET program at the university. George did not know that I had never taken a college course in the field of animal science, or that I really did not know the difference between Holstein and Hereford cattle. Phil Dziuk (2001 IETS-PA), who was on my PhD thesis committee at the University of Illinois, encouraged me to take the post-doc and make the jump from zoology to animal science, stating "there is a big future in embryo transfer."

The early months at CSU were a comprehensive learning experience for me and a physically demanding schedule for all the personnel in the ET unit, often involving long days. On mornings when a large number of embryos were recovered and suitable recipients were abundant, we assumed that the surgical transfers would continue well into the night, if not into the next morning. As word spread of these successes, a rapidly growing number of visitors showed up, were always made welcome, and in some cases remained for days or weeks. As the techniques in the ET program evolved and improved, they were readily shared with the visitors. Simmentals were the most common breed represented early in the CSU program and donor owners frequently visited and observed the procedures. Soon after joining the Colorado team, I dropped to the floor a glass Petri dish containing all 25 good and quite valuable embryos just collected from a donor. The owner watched in dismay as George Seidel tried in vain to recover the embryos off the floor. If not for a forgiving environment, my new ET career might have ended right then and there.

In 1975, Peter Eidsen arrived at the CSU ET unit, bringing with him valuable personal experience with ET in Australia. As Peter recently related to me, "My interest in ET was piqued with the Rowson-Cambridge publication in 1969 [22], where they described successful embryo recovery and embryo transfer and I thought this would be a method to discover some of the infertility problems which occur in general practice. . . . Following Rowson's instructions, we collected Charolais embryos in New Zealand and shipped them in an incubator to Australia, where we transferred them into recipients starting in 1972." Peter stated, "We quickly discovered that all too frequently donors do not always produce embryos, but unfortunately my client had read grossly exaggerated reports in American cattle magazines and, as a result, he assumed that I was not performing up to standard." Several things stated by Peter have not changed in the last 40 years!

Not long after his arrival at CSU, Peter shared with us his intention to develop a nonsurgical approach to embryo recovery. While in private practice he had met and worked with T.M. Sugie (1986 IETS-PA) and was convinced that surgical recoveries should be relegated to the past. In addition to the need for expensive surgical facilities, there was considerable concern regarding donors (approximately 10%) rendered either infertile or subfertile by the surgery [23]. Last, it may have been largely forgotten that the mid-ventral surgical approach made the collection of embryos from lactating dairy cattle impractical.

A relevant, serendipitous event recalled by Peter follows: "I was visiting a MD friend in Ft. Collins and on his desk was a box of Foley catheters—so I asked if I could have one and he said take the box. Later, we recovered an embryo using a Foley catheter in my first successful nonsurgical flush, and I knew that after 3 years and three countries visited this was the technique that would work without damaging the uterus if used correctly." Peter then extended the length of the Foley catheters with tubing and glue and further experimented with nonsurgically "flushing" donors. The flush medium was introduced into the uterus under pressure from gravity flow, and the outflow was collected in graduated cylinders. Many practitioners today use an almost identical protocol, with the exception that specifically designed, commercially available catheters and tubing are now available. I clearly remember the challenge of finding embryos among the blood and mucus in some of the initial flushes. However, the equipment and Peter's skill
evolved rapidly, leading to a widely cited publication on
the technique [24]. It should be pointed out that some-
what similar nonsurgical protocols were developed at the
Universities of Wisconsin [25] and Utrecht [26] were also
described in the same issue of Theriogenology as Elsden,
et al's paper.

Commensurate with the opening of several commer-
cial ET businesses starting in the early 1970s, there was
growing interest in the formation of an ET scientific so-
ciety. In May 1974, 25 individuals attended a meeting in
Denver, Colorado, to discuss the science and business of
ET and to explore the idea of forming a society [27]. As a
consequence, the IETS was officially formed on May 26,
1974, and the first meeting was held over 2 days in
Denver in January 1975. A total of 82 individuals, roughly
split in numbers between researchers and commercial
practitioners, became charter members of the IETS at this
meeting [28]. As an indication of the "state" of the sci-
cence at that time, I clearly remember seeing a number of
attendees busily hand drawing renditions of an eight-cell
bovine embryo photomicrograph shown on the screen
during a talk by George Seidel. The IETS grew somewhat
fitfully in the early years, with attendance at the early
meetings going from 82 in 1975 to 156 in 1979. Serious
money problems bedeviled the society in the early years
and both George and Sarah Seidel provided quite signif-
ificant personal time contributions in keeping the society
going. The IETS survived the early days and has held an
international meeting every year since 1975; membership
topped 1000 by the early 1990s. The formation of
numerous more commercially based associations in a
number of countries and regions began in the 1980s. This
has led to some retraction of membership in the IETS,
which currently stands at 748 members in 61 countries
(compliments of the IETS).

Another important link between the 40th anniver-
saries of Theriogenology and the IETS was the 1978 initi-
atation of annual publication of the IETS proceedings in
the January issue of the journal. This agreement continued
for 25 years, ending with the January 2003 issue. As a
consequence of this cooperation, the 1978 to 2003 'IETS'
editions of Theriogenology contain a wealth of informa-
tion related to ET. In addition, a number of issues have
carried the pre- and/or post-conference proceedings of
the IETS.

3. The ET industry after the adoption of nonsurgical
embryo recoveries

After the three papers on nonsurgical embryo
recovery cited above appeared in Theriogenology [24–26],
there was a rapid increase in the number of commercial
ET operations in North America, Great Britain, Australia,
Europe, and Japan. In early 1977, I left Colorado and
joined Robert Baker at a new ET company, Auld Croft
Farms, Ltd., in Mississauga, Ontario. Bob had recently
resigned a professorship at MacDonald College in Sainte-
Anne-de-Bellevue, Quebec, and we worked together at
Auld Croft in Ontario for nearly a year. Bob then left for
the United States, where he helped establish American
Embryos, Inc., in Middleville, Michigan, which went on to
figure in an important ET patent battle. With Bob's
departure, my family and I were anxious to return to the
United States and serendipity again influenced my career.
In January 1978, I was employed by Via Pax, Inc., another
Ontario ET entity, with the goal of establishing an ET
business in Pennsylvania. Shortly after arriving with my
family in Pennsylvania during a record-setting January
blizzard in 1978, I received instructions to fly to Milan,
Italy, and participate in a hectic, 2-week marathon
flushing Italian Holsteins. Upon returning to the United
States, my veterinarian colleague, Alan McCauley (former
faculty member at the Cornell University School of Vet-
erinary Medicine) and I proceeded rather quickly to
purchase the newly formed Via Pax business and rename
it Em Tran, Inc., a business we co-owned and operated
until 2001. With little competition and the demand for ET
services literally exploding, we hired and trained a
number of veterinarians, with up to five teams on the
road providing on-farm services many days. In time, a
number of these veterinarians went on to establish other
ET businesses in Pennsylvania and several other states.
While trying to provide on-farm ET services in more than
dozens states, including California, our business suffered
from growing too large, too fast, a not uncommon char-
acteristic of many early ET businesses. In time, we
concentrated more on in-house services (Fig. 1), by
building a spacious laboratory, cattle boarding facilities,
and a milking parlor. In addition, beef donors and
company-owned recipients were boarded on a number of
former nearby dairy farms. Although we continued to
provide on-farm services, a rapidly growing number of ET
practitioners also provided on-farm services. Conse-
quently, our program concentrated on in-house services
and reached a peak of approximately 300 donors boarded
and approximately 1000 recipient heifers under our
management. A number of similar large programs were
established in North America during the 1980s and
1990s. Many of them were supported by investment
programs, such as limited partnerships. Few of these
large programs remain in business, however.
In 1974, the US Patent office issued a patent entitled *Reproductive processes for cellular bodies* to L. Augspurger, a Michigan patent attorney with no personal experience in ET [29]. The patent contained approximately 100 specific processes covering every conceivable technique in the field of ET, plus many technical processes that had not yet been perfected, including some that were likely not technically possible. In 1981, the patent holder brought suits for patent infringement in US District Court in Chicago and injunctions forced three ET businesses to cease operation while legal proceedings took place. Substantial funds were raised by members of the American Embryo Transfer Association (AETA) and a vigorous defense was waged, which included an effective critique of the patent by George Seidel. When victory for the Augspurger lawsuit seemed questionable, an anonymous, generous ET practitioner purchased the patent, plus two others of Augspurger’s, with the stipulation that Augspurger dedicate the patents to public use. There have been several subsequent patents that might have had serious economic impact on the ET industry had they been upheld in court challenges.

3.1. Superovulation

Like most of the subheadings in this review on ET, superovulation is covered comprehensively in another paper in this issue of *Theriogenology* and, as a consequence, is only briefly covered herein.

The entire sequence of procedures involved in bovine ET usually starts with superovulation of the donor female. The earliest commercial work in North America utilized either commercially available FSH purified from porcine pituitaries (Armour-Baldwin Laboratories, Omaha, NB; 50-mg Armour standard FSH units) or eCG. Relevant to this early use of eCG, known in the past as pregnant mare’s serum gonadotropin, is another account by Peter Elsdon: “During our early efforts in Australia, FSH was not available so I made my own crude extract from equine pituitaries collected from a local horse slaughterhouse, and I prepared pregnant mare’s serum gonadotropin from mares whose blood I collected at 80 to 120 days pregnant. The resultant serum could only be used on a donor once because of the chance of anaphylactic shock. Nevertheless, we collected an average of 5.2 transferrable embryos in 1977 and 1978.” Later, after 5 years of commercial ET and research at CSU, Peter stated that the number of good embryos tended to be greater when FSH was used compared with eCG [30]. The advantages of using multiple injections of FSH versus a single injection of eCG were confirmed in a study by Boland, et al. in Dublin [31].

The first paper in *Theriogenology* involving bovine ET appeared in Volume 1, Issue 2 in February 1974 [32]. Not surprisingly, this study involved Keith Betteridge, a Canadian pioneer in the development of bovine and equine ET and, as discussed, the author of a number of review papers related to ET [6,13,14]. The study involved the superovulation of cows with eCG, with or without hCG. The authors made the observations that the superovulation protocol resulted in great variation in response, embryo recovery rates were adversely affected by excessive ovulation rates, and there was evidence of reduced responsiveness to repeat treatment.

The mean number of transferrable embryos from reproductively normal cows has remained relatively unchanged during the past 30 years for both beef [33] and dairy cows [34]. Looney, et al. [33] reported a mean of 6.2 “good” embryos from more than 2000 beef donors representing 14 breeds and we reported [34] a mean of 6.4 transferrable embryos from almost 700 individual Holstein donors, each superovulated for the first time. These means can be compared with those provided by the annual census of the AETA, in which the means were 7.0 for beef (>24,000 donors) and 6.3 for dairy (>15,000 donors) in 2011, the latest year for which results are available (compliments of the AETA). The lack of improvement in dairy cows is even more surprising when one considers that in the 1983 study [34], 45% of the donors were 7 years of age or older, whereas today there is a strong emphasis on flushing younger animals, including a significant percentage of virgin heifers as young as 8 months of age (personal communication). Also, with the widely recognized decrease in conception rates in dairy cattle after artificial insemination (AI) [35], it seems plausible that a drop in superovulation embryo numbers might have been anticipated. However, an assessment on mean numbers of embryos recovered in four different commercial programs showed no change over a 20-years period [36], supporting the data provided by the AETA.

For many years, FSH injections to initiate superovulation in cattle were started between Days 8 and 13 of the estrous cycle. This approach was quite reliable, and embryo numbers did not vary when donors were started on any of these days [34,37]. However, problems with this protocol included the necessity of knowing the date of a donor’s last estrus, plus the window of appropriate heat dates limited the ability to superovulate a group of cattle with scattered estrus dates. When progesterin ear implants (Synchro-Mate-B, CEVA Laboratories, Inc., Overland Park, KS) became available, it added another tool to methods of synchronizing donors for initiating superovulation. Although production of Synchro-Mate-B eventually ceased, the availability of silicone vaginal progesterone-releasing devices [38] represented a similar tool for controlling estrous cycles, especially when used in conjunction with various estrogens. A good deal of the early work in this area was conducted by Gabriel Bo in Reuben Mapletonf’s (2010 IETS-PA) laboratory at the University of Saskatoon [39,40], with a number of ensuing studies [41]. These studies have clearly shown that cattle can be effectively superovulated with the use of a progesterone-releasing device and estrogen.

For many years, the common dogma among ET practitioners was that donors should be allowed to undergo two estrous cycles between superovulation attempts. Even though it is common practice to inject prostaglandin after embryo collection, superovulated donors often do not return to estrus for 3 or more weeks. Consequently, this protocol leads to an interflush interval of 60 or more days. In the early 1990s, while managing
and flushing a large number of donor cows owned by Holland Genetics (CRV, Arnhem, The Netherlands), we learned that their ET staff had found that embryo production was not decreased when donors were superovulated at shorter intervals. As a consequence, by using progestin ear implants, we were able to superovulate the Dutch-owned donors at 40-days intervals without waiting for them exhibit two interflush estrous cycles. This change in superovulation protocols increased by 50% the number of embryos that were recovered and frozen per unit time for export to Holland Genetics. A group of [24] Red Angus primiparous heifers boarded at Em Tran, Inc., was superovulated on this accelerated basis. As with the Holland Genetics-owned Holsteins, these donors received a progestin implant and were given prostaglandin immediately after flushing and then watched for standing estrus. Ten days later, the implant was removed and superovulation started mid-cycle after the next estrus. If estrus was not observed within 3 days of implant removal, another implant was inserted and superovulation initiated five days later. As shown in Table 2, there was no decrease in the mean number (5.5) of freezable embryos produced over a sequence of 11 superovulations repeated at a mean interval of 39.7 days. In 47 cases of the total 264 attempted superovulations (17.8%), donors either did not come into estrus or produced zero freezable embryos but are included in the data totals. It is not clear why these Red Angus and, in fact, a large number of other donors in the same herd failed over a period of years to produce as many embryos as Black Angus under the same management conditions. Today, many ET practitioners routinely repeat superovulation of donors on a 30- to 40-days interval.

A new protocol that has increased the efficiency of superovulation without increasing embryo production involves the use of a slow-release formulation diluent for the FSH. The protocol, utilizing hyaluronan as diluent, was compared in a two-injection protocol versus the industry-wide standard eight-injection protocol using saline as diluent [42]. Embryo production for the slow-release formulation protocol was similar to the traditional protocol, on this accelerated basis. As with the Holland Genetics-owned Holsteins, these donors received a progestin implant and were given prostaglandin immediately after flushing and then watched for standing estrus. Ten days later, the implant was removed and superovulation started mid-cycle after the next estrus. If estrus was not observed within 3 days of implant removal, another implant was inserted and superovulation initiated five days later. As shown in Table 2, there was no decrease in the mean number (5.5) of freezable embryos produced over a sequence of 11 superovulations repeated at a mean interval of 39.7 days. In 47 cases of the total 264 attempted superovulations (17.8%), donors either did not come into estrus or produced zero freezable embryos but are included in the data totals. It is not clear why these Red Angus and, in fact, a large number of other donors in the same herd failed over a period of years to produce as many embryos as Black Angus under the same management conditions. Today, many ET practitioners routinely repeat superovulation of donors on a 30- to 40-days interval.

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Successful superovulation is influenced by many donor factors, such as breed, age, parity, and reproductive history. In addition, FSH preparation and superovulation protocol, climate, nutrition, and other management factors can influence superovulation outcome. Of no less importance, however, are semen quality and the timing of AI and skill of the inseminator. The low numbers of accessory sperm observed in embryos recovered from superovulated cattle indicate that sperm numbers at the site of fertilization are low [43]. Superovulated cattle are often inseminated twice, with one straw at 12 and 24 hours after the onset of estrus. There is not, however, any single insemination protocol for superovulated cattle that is widely accepted as superior. Evidence for a positive relationship between sperm quality and both fertilization rate and embryo quality is supported by data from a commercial ET program [44].

The availability of sex-selected semen has opened new problems and opportunities for ET. Although there remains a general reluctance among ET practitioners to recommend the use of sexed semen by clients, results in superovulated cattle [45,46] and in IVP systems have been encouraging [47].

### 3.2. Embryo recovery

For more than 35 years, most if not all embryo recoveries in cattle have been achieved by nonsurgical flushing methods. Silicone catheters in a variety of lengths and sizes are now manufactured and sold by several firms that specialize in ET equipment and supplies. These catheters are longer, with strategically placed holes, have longer cuffs than Foley catheters, and are autoclavable, making them far superior to traditional Foley catheters. A wide variety of flushing techniques are practiced with apparently comparable success rates. Using a stainless steel stylette to provide rigidity, some practitioners thread the catheter through the cervix and inflate the cuff in the internal cervical os. Others thread the catheter half way up one horn, or in some cases, even further, and flush each horn individually. Some practitioners introduce the flushing medium into the uterus with the aid of gravity flow, whereas others use a large syringe and sequentially add smaller volumes of medium. Also, there now is a wide selection of embryo filters available that allow the flush medium to flow through and be discarded, while trapping ova and embryos within the filter (Fig. 2). The earliest models of embryo filters appeared in 1986 and up until then the flush medium was collected in a 1-L plastic, autoclavable graduated cylinder, or Erlenmeyer flask (Fig. 2). The fluid was then allowed to settle for approximately 45 minutes and then all but the last 100 mL was syphoned off and discarded. The greatest disadvantages of this system were the delay in proceeding with searching the flush for embryos and the challenge of handling large, awkward cylinders during the flushing procedure.

#### 3.3. Media: Flushing and holding

As described in several reviews [6,11], homologous serum was frequently used for both recovering and

### Table 2

Mean number of freezeable embryos produced at 40-days intervals in sequential superovulations of 24 Red Angus primiparous heifers.

<table>
<thead>
<tr>
<th>Sequence</th>
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<tr>
<td>No. donors</td>
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<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>No. embryos</td>
<td>5.5</td>
<td>5.7</td>
<td>3.7</td>
<td>5.8</td>
<td>4.5</td>
<td>6.2</td>
<td>4.6</td>
<td>4.9</td>
<td>5.8</td>
<td>4.3</td>
<td>8.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Unpublished data from Em Tran, Inc.
holding bovine embryos during the early days of ET research. The early development of ET media was thoroughly described in reviews by Foote and Onuma [10] and Seidel [11].

Most ET practitioners made their own media before the availability of commercially manufactured media specifically intended for ET. Utilizing reverse osmosis purified water, modified Dulbecco’s phosphate-buffered saline (DPBS) with 1% heat-treated new-born calf serum for flushing and 10% new-born calf serum for holding embryos was made in the laboratory at Em Tran, Inc., for many years. Holding medium was produced in multi-liter quantities, frozen in small aliquots, and thawed daily for use. The medium was then stored through the working day in a 35-mL disposable syringe (Monoject, Sherwood Medical, St. Louis, MO), and expressed through a 0.25-μm filter into Petri dishes as needed.

Starting in late 1983 and early 1984, a number of ET businesses in the United States reported a decrease in recipient pregnancy rates (personal communication). We later reported [48] that overall pregnancy rate in Em Tran, Inc.-owned Holstein heifers dropped from 71% in 1983 to 53% in 1984. Most notable were numerous total failures in which no pregnancies were produced after transfers both on-farm and in-house. Researchers at Granada Genetics, Inc., Marquez, Texas, shared their concern (direct communication) that maintaining holding medium in Monoject (Sherwood Medical Co.) syringes was responsible for the sharp conception rate decline observed in their business. Experimental results demonstrating the syringe toxicity was later reported at the 1986 IETS conference [49,50]. In the study conducted at Em Tran, Inc., involving the effect of different volumes of DPBS stored in Monoject syringes for 3 hours, survival of mouse embryos exposed to the medium from full syringes was 67%, but dropped to 0% when only 10 mL of disinfection by-products was held in the syringes (Table 3). By comparison, survival of control embryos was 84% in medium not exposed to any type of syringe. Similar results were achieved in a mouse embryo study conducted in the laboratory of Robert Foote at Cornell University (unpublished data).

Because of the substantial financial losses incurred by Em Tran, Inc., a suit claiming product liability was filed in Federal Middle District Court, Civil Action No. 86-0268, in Harrisburg, Pennsylvania, and a 7-days jury trial was conducted in August 1986. The jury found in favor of the plaintiff and ultimately damages were paid. The importance of this relative to the ET industry includes the following: The syringes had previously been nontoxic as used by the ET industry. Toxicity became evident only after Sherwood Medical changed from ethylene oxide to gamma radiation to sterilize the syringes. Evidence was introduced that gamma radiation affected the rubber gaskets, facilitating the release of a toxic substance into the syringe contents; the US Department of Health and Human Services had warned approximately 200 human genetic laboratories using similar syringes for collecting and storing amniotic samples owing to evidence of syringe toxicity. Neither the U.S. Food and Drug Administration (FDA) nor Sherwood Medical issued any warnings to veterinary consumers. The only testing of plastic syringes mandated by the FDA involved a rabbit skin test, which is markedly less sensitive to toxicity compared with pre-implantation embryos. Today, a number of specialized plastic and silicone products are available to the ET industry, and as a result of the lawsuit the rubber composition of the Monoject syringes was changed. However, there continue to be cases where the composition of plastics and other synthetics change without notice.

In a comprehensive 1990 study of materials toxicity using mouse embryo development as an endpoint, the Monoject syringes performed quite well with 1 hour of medium exposure [51]. However, mouse embryo development was extremely poor when embryos were cultured in media exposed to four brands of syringes for 16 hours, including Monoject. As a result of these syringe problems, most ET practitioners currently only use
syringes for media handling that are entirely composed of plastic, without a rubber gasket on the plunger.

Presently, there are a number of companies that manufacture media specifically designed for bovine flushing, holding, and cryopreservation. Serum is no longer a component of most ET media and has largely been replaced by either BSA or a synthetic surfactant such as polyvinyl alcohol. Also, today there are some efficacious, specialized ET media available for bovine ET that are entirely synthetic and do not require refrigeration [52].

3.4. Embryos

Because of the limitation of numbers, traditionally it has been difficult to accumulate data in academic research programs on evaluations of the relationship between embryo age, stage, and quality on pregnancy rates. As commercial programs grew in number and size, the effects of these embryo variables were addressed in retrospective analyses of pregnancy data [53–55].

In 1983, embryo photomicrographs and detailed descriptions of four different quality ratings of bovine embryos were published along with conception data from a commercial ET program in California [56]. The embryo rating descriptions in this paper have been widely adopted by both ET researchers and practitioners, are recognized by the IETS [57], and are used for international labeling of embryos. However, there were not clear differences in conception rates relative to embryo stage and quality in this study [56], probably because the sample sizes were relatively small and the conception rate with the best embryos was only 45%. A thorough review describing the evaluation of embryo quality was covered by Merton [58].

In 1987, we published two retrospective analyses of Em Tran, Inc., data involving approximately 7000 [48] and later, an analysis of an additional approximately 12,000 [59] transfers of fresh embryos. There were highly significant differences in conception rate among quality 1, 2, and 3 embryos and although quality 4 was numerically lower, the sample size was too small to achieve significance. The scale used for grading embryos in this study was different than that recognized by the IETS in that we broke down IETS quality 1 (excellent and good) into quality 1 (excellent and quality) and 2 (good quality). Stages 4 (late morula), 7 (expanded blastocyst), and 8 (hatched blastocyst) resulted in lower pregnancy rates than stages 6 and 7 (early and mid blastocysts) when the recipients were all Holstein heifers owned and managed by Em Tran, Inc. [48]. However, there were no differences in pregnancy rates among embryos, all stages combined, recovered on Days 5 through 8 after estrus, whereas embryos recovered from donors on Day 9 resulted in a lower pregnancy rate. In the second study [59] involving a variety of business- and client-owned recipients composed of both cows and heifers of both beef and dairy breeds, qualities 1, 2, and 3 again resulted in different pregnancy rates, but neither embryo stage nor age was related to pregnancy rate. A relatively typical group of Day 7 embryos and ova from one donor is shown in Figure 3. This photomicrograph illustrates the variation in stages (4 and 5) and qualities (1 and 2) often seen in embryos from a given donor.

The take-home message regarding bovine embryo factors is that commercially acceptable pregnancy rates can be achieved after transfer of embryos representing a fairly wide range of donor breeds and ages, as well as embryo stages and quality.

3.5. Embryo transfer and recipients

Bovine embryos were almost exclusively transferred surgically via midline incision in the pioneering ET businesses in the early 1970s. Unfortunately, this approach eliminated the possibility of transfers being conducted on-farm or anywhere outside a well-equipped surgical facility. Also, as mentioned, it prevented transfer into lactating dairy cattle. Despite the fact that nonsurgical flushing virtually universally replaced commercial surgical embryo recoveries starting in 1975 and 1976, surgical transfer via flank incision was widely utilized into the early 1980s. While we were working together in Canada, Bob Baker introduced me to a transfer approach via flank incision utilizing a local anesthetic block. This protocol was much faster than a midline transfer, could
be performed on-farm, and required a minimum of equipment. The flank transfers proved to work quite well and resulted in high pregnancy rates. The paralumbar area of the recipient flank ipsilateral to the CL was clipped, locally anesthetized, and surgically disinfected (Fig. 4). The ET practitioner exteriorized a loop of the uterine horn through a surgical incision and punctured the wall of the horn with a bone pin. A technician then transferred the embryo into the uterine lumen using a 20-ga catheter attached to a 1-mL syringe (Fig. 4). Another characteristic of this transfer method is that most veterinarians learned it quickly and achieved high conception rates without having any previous experience. Successful nonsurgical transfer actually requires a great deal more experience, specifically extensive experience in rectal palpation (Al experience is also advantageous), than did the now discontinued surgical approach.

One of the recipient factors of concern to early ET practitioners was the side of transfer relative to the side of the CL. In an early issue of Theriogenology (1977), Tervit et al. [60] looked at this question, but failed to answer it conclusively. Although transfers to the horn ipsilateral to the CL had a numerically greater conception rate (54%) than transfers to the contralateral side (39%), the sample sizes were too small to attain significance.

With statistical help from Bob Foote (2002 IETS-PA), we performed a retrospective analysis of more than 7600 flank transfers performed over a 6.5-years period into Holstein recipients owned and managed by Em Tran, Inc. The results [48] included a pregnancy rate of 71% at 60 days of gestation and involved an analysis of 14 factors related to donors, embryos, and recipients. Among recipient factors that did not influence conception rate were the size of the CL, the side of the transfer, and the use of a recipient once versus twice (after an unsuccessful first transfer). Surprisingly, pregnancy rates were greater among recipients induced into estrus with prostaglandin compared with those exhibiting natural estrus. As expected, estrus synchrony between recipients and donors influenced pregnancy rate. In a study using Angus beef recipients, there also was no influence of CL size on pregnancy rate, nor did luteal volume or progesterone concentration among recipients differ among recipients that did or did not become pregnant [61]. In a different approach to the question of progesterone and recipients, Lonergan et al. [62] manipulated the level of progesterone in recipients receiving multiple embryos. Although this study did not address actual pregnancy rates, it showed that embryos transferred into recipients developed faster in a high progesterone environment. Although there obviously is a minimal level of progesterone necessary to establish and maintain pregnancy, the presence of a palpable CL in the recipients in these two studies obviously was sufficient to provide an adequate concentration of progesterone.

Early attempts at nonsurgical transfer were recited in some of the reviews cited. Also, in the first issue of Theriogenology that carried the annual proceedings of the IETS (1978), Sreenan [63] reviewed the subject of nonsurgical transfers. Although there were some encouraging results cited in the review, surgical transfers predominantly resulted in somewhat higher pregnancy rates than nonsurgical transfers. One problem during the 1970s was the lack of a specific instrument designed for transfer of embryos. Open-end AI guns were successfully utilized by some practitioners [55], but it was not until after IMV introduced a specific ET instrument (1984–85 in North America, compliments of IMV Technologies) that nonsurgicals completely replaced flank surgical transfers. We were reluctant to make the transition to nonsurgical transfers because we had achieved a consistently greater pregnancy rate using flank surgery. We made the transition at both Em Tran, Inc., and Em Tran-West, Inc., over a period of about 18 months, slowly phasing in the nonsurgicals starting in 1986. The results at Em Tran-West, Inc. (Table 4) clearly showed with fresh embryos that the transition from surgical to nonsurgical transfers did not affect pregnancy rates in either recipient heifers or cows [59]. However, pregnancy rates for cows were numerically lower and, in fact, significantly lower when frozen embryos were transferred nonsurgically into cows.

Although ET conception rates were greater in dairy heifers compared with cows in many studies, rates were similar among dairy heifers, beef cows, and beef heifers in one study [59]. Also, several other factors have great latitude in North America, including season, year, and synchrony between the embryo age and recipient estrus.

Fig. 4. (A) The clipped, surgically scrubbed, and anesthetically locally blocked flank of a Holstein heifer. (B) The transfer of an embryo with a plastic pipette by a technician into the lumen of the uterine horn while the horn is exteriorized by a gloved surgeon.
Perhaps the most important factor influencing the success of transferring embryos, assuming good quality embryos and practitioner competence, is recipient management. Management can encompass many factors, including disease status, nutrition, adequate cattle handling facilities, and reproductive health. Stroud and Hasler [44] provided a number of examples of how various management problems negatively impact pregnancy rates in ET recipients.

3.6. Cryopreservation

The announcement in 1972 of the survival of mouse embryos after cryopreservation at −196 °C and thawing was groundbreaking [64]. Success with bovine embryos followed shortly thereafter and in 1973 the birth of the first calf resulting from transfer of a cryopreserved embryo was announced by Wilmut (2011 IETS-PA) and Rowson [65]. A comprehensive review of the early reports involving cryopreservation of bovine embryos was covered in detail by Ralph Maurer [66] in the first proceedings (1978) of the IETS printed in Theriogenology.

Successful incorporation of cryopreservation into commercial ET lagged behind the basic procedures of superovulation, flushing, and transfer. In September 1977, I was able to visit Steen Willadsen (2005 IETS-PA) in his laboratory in Cambridge. He was generous with his time and advice on freezing cattle embryos, and I returned home full of optimism, but it was about 5 more years before I was successful in establishing a reliable cryopreservation program. In the years between my visit to Cambridge and a consistently successful protocol, the technology evolved from glass ampules, serial additions of diluted DMSO, and a two-stage 3-hours cooling program involving cooling rates of both 0.1 °C and 0.3 °C per minute. The successful programs we and most practitioners used in the early 1980s involved 0.5-mL straws, embryos equilibrated directly in 10% glycerol and a one-stage cooling program at about 0.4 °C per minute that lasted only about 1.5 hours.

The importance today of reliable cryopreservation to the ET industry is shown in Table 5. In the most recent datasets available (2011) from the AETA and Canadian Embryo Transfer Association (CETA), approximately 70% of all bovine embryos collected were frozen and not transferred fresh. The percentage frozen has risen steadily over the years and in the United States is higher for beef versus dairy embryos (79% and 59%, respectively) and is similar for Canada (beef, 81%; dairy, 65%; compliments of the AETA and CETA). Early in the ET industry, before reliable cryopreservation protocols, there was an emphasis on achieving as many embryos as possible transferred into suitable recipients. Cattle owners often put a high priority on transferring all usable embryos. In 1980 and 1981 at Em Tran, Inc., we discarded more than 500 embryos of 4772 collected because of a lack of recipients. Figure 5 is an extremely productive donor cow with 10 calves produced from 16 embryos transferred. The 16 embryos were only half of the total number (32) of transferrable embryos produced from the flush, but only 16 recipients were available. The additional 16 embryos were flown overnight to another ET facility where they were frozen in a somewhat experimental fashion, with none surviving when they were subsequently thawed.

Commercially, embryos selected for cryopreservation include most good quality embryos ranging from stages 4 to 7. This is necessary because embryo stages can vary somewhat within a flush and donors that came into estrus over a 2 or even 3-days range may need to be flushed as a group on the same day.

Approximately 30 years ago, Joe Wright [67] showed no differences in survival rate of frozen/thawed embryos ranging from stages 4 to 7; however, the sample sizes were rather small. Likewise, we detected no

### Table 4

Comparison of 60-days conception rates after surgical or nonsurgical transfer of fresh or frozen-thawed embryos to cows and heifers at Em Tran-West, Inc.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Fresh embryos</th>
<th>Frozen embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. transfers</td>
<td>% Pregnant</td>
</tr>
<tr>
<td>Surgical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heifers</td>
<td>1485</td>
<td>80a</td>
</tr>
<tr>
<td>Cows</td>
<td>491</td>
<td>70a</td>
</tr>
<tr>
<td>Nonsurgical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heifers</td>
<td>590</td>
<td>79a</td>
</tr>
<tr>
<td>Cows</td>
<td>84</td>
<td>61b</td>
</tr>
</tbody>
</table>

a,b Values in columns without common superscripts differ significantly
\( (a \text{ vs. } b, P < 0.001; c \text{ vs. } d, P < 0.005) \) [59].

### Table 5

Numbers of reported donors collected, embryos recovered and embryos frozen before transfer in the US and Canada in 2011.

<table>
<thead>
<tr>
<th>Factors</th>
<th>USA</th>
<th>Canada</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. donors collected</td>
<td>41,151</td>
</tr>
<tr>
<td></td>
<td>No. embryos recovered</td>
<td>274,887</td>
</tr>
<tr>
<td></td>
<td>No. embryos frozen (% of recovered)</td>
<td>197,060 (72)</td>
</tr>
</tbody>
</table>

Compliments of the American Embryo Transfer Association and Canadian Embryo Transfer Association.

**Fig. 5.** Windy Mont Matt Kathy, Holstein cow with 10 calves born after transfer of 16 embryos in 1978, before commercial cryopreservation of embryos. Superovulated and flushed starting at age 5, Kathy went on to produce more than 100 embryo-transferred calves in the next 5 years while continuing to carry three of her own calves.
differences in survival of stages 4 to 7, but the number of stage 7 embryos was small [59]. For years, increasing numbers of ET practitioners had declared a reluctance to freeze stage 7 embryos. In 2011, I acquired a large dataset (≈72,000 transfers) from four ET North American businesses of pregnancy rates versus frozen embryo stages [68]. Stage 7 embryos had a lower pregnancy rate (43.8%) than stages 4 and 5 (53.7% and 55.3%, respectively), whereas stage 6 was also slightly but significantly lower (52.1%). These reductions in pregnancy rate do not entirely eliminate the commercial value of freezing stage 6 and 7 embryos. However, the evidence now clearly supports avoiding freezing stage 7 embryos whenever possible. Further research on developing a protocol for these expanded blastocysts is needed.

For more than 10 years, glycerol was the favored cryoprotectant for bovine embryo cryopreservation. The protocols became greatly shortened and simplified by loading 0.25-mL straws with embryos that had been exposed to 10% glycerol for approximately 10 minutes, placing them in a programmable freezer holding at −6.0 °C, and after seeding, lowering the temperature at 0.4 °C to 0.6 °C per minute to −32 °C followed by plunging into liquid nitrogen. The downside of this system is that upon thawing the embryos had to be removed from straws and rehydrated through a series of sucrose dilutions and then reloaded into straws for transfer to recipients. The announcement in 1992 of a direct transfer (DT) cryopreservation system using ethylene glycol (EG) as a cryoprotectant had a rapid and positive effect on the world-wide ET industry [69]. Although they are frozen in a manner very similar to glycerol in straws, DT straws are loaded directly into a transfer gun after thawing, thus saving time and the need for a microscope and experienced embryologist. In the last survey on the subject in 2009, more than 99% of beef and 94% of dairy embryos in the United States were frozen in EG for DT (AETA). Cryopreservation by vitrification, which involves the use of highly concentrated aqueous solutions of cryoprotective agents that prevent ice crystal formation during cooling, is also characterized by exceedingly fast cooling. Vitrification is sometimes touted as a superior method of cryopreserving in vivo-derived bovine embryos compared with traditional ‘slow’ freezing. However, the pregnancy rate from frozen-thawed bovine in vivo-derived embryos is only about 10 percentage points lower than fresh embryos of the same quality rating [59]. This is true whether the embryos are frozen in glycerol or EG (unpublished). Consequently, there is minimal room for improvement of freezing technology, assuming that frozen embryo pregnancy rates are unlikely to exceed those of fresh embryos.

Most vitrification systems utilize ‘carrier tools’ other than straws to achieve rapid chilling rates [70]. Studies involving vitrification of in vivo-derived embryos in 0.25-mL straws, with a column of diluent included in the straw, to facilitate DT, have achieved satisfactory pregnancy rates [71,72]. However, the pregnancy rates were no larger than those achieved with standard slow frozen DT/EG straws.

Another advantage often attributed to vitrification is the lack of the need for a programmable freezer, plus the speed of the actual chilling step in the protocol. However, it is not uncommon in commercial ET to freeze 10 to as many as 100 or more embryos in a day’s work that is often conducted on-farm. Vitrification of one or a few embryos is quite convenient and considerably faster than slow freezing the same number of embryos. Once the number of embryos exceeds a small number, however, slow freezing is faster than vitrification when calculated on a per embryo basis. In addition, once straws are loaded and placed in a programmable freezer, the ET practitioner or technician is free to handle other tasks.

3.7. Import/export of embryos

It was first demonstrated by the Cambridge group in 1972 that bovine embryos could be transferred to the oviducts of rabbits, removed after 4 days, transferred into recipients, and result in the birth of live calves [73]. This technique was used on a small scale for export of embryos into the early 1970s [74], although in vitro storage in liquid medium was used more commonly.

One afternoon in September 1977, I came down the stairs of a Malev Airlines Tupolev jet with a veterinary colleague to be greeted on the Budapest tarmac by two Russian soldiers armed with submachine guns and accompanied by a leashed, but intimidating-looking Alsatian. In my pocket were five glass vials containing 30 embryos from separate Holstein donors that had been flushed the previous morning on farms near Toronto, Ontario. The World Bank financed the program for Hungary, but it was up to us to organize and make it successful. After nearly 3 hours in customs and immigration, we squeezed into a Russian Lada and headed for a state collective farm in the country. Incredibly, despite my forceful protestations and before starting transfer of the embryos, we had to attend a ‘celebratory’ dinner in the village café with a dozen government veterinarians eating and toasting the success of our program until late in the evening.

A long night of frustrating work at the state farm followed that dinner. Instead of the detailed list of equipment and supplies that we had been promised, we had to make due with a traditional straight razor, lidocaine in 5-mL glass ampules along with a glass syringe and a single dull needle, a single scalpel that was sharpened on a whet stone, and penicillin in 10-mL ampules. After each frustrating attempted transfer of an embryo, a group of government veterinarians in business suits toasted the event with liquor that was offered off a tray held all night by a young woman from the farm. Some weeks later we were not too surprised to hear that only one recipient had died of peritonitis and there was one confirmed pregnancy! The transfer conditions had been such that we would not have been surprised with more deaths and no pregnancies. This account is probably not surprising to those readers who participated in some of the early fresh embryo export adventures to import locations with no experience in ET. The development of reliable bovine embryo cryopreservation protocols
allowed a rapid growth and, usually, a great degree of success in the export/import of embryos.

Another serendipitous event involving my ET career occurred in 1985 when Jake Chardon, the CEO of Holland Genetics, paid an unexpected visit to Em Tran, Inc. Unhappy with the management and embryo production of some Holland Genetics-owned Holstein cows housed in another state, he asked us if we would be willing to provide management, embryo freezing, and export to The Netherlands. Three skinny cows soon arrived with hooves in such serious need of trimming that they basically skied down the truck ramp. Within a month of arrival, embryos from all three cows were stored in liquid nitrogen. What followed was a mutually beneficial, long-term relationship during which we boarded more than 200 Holland Genetics-purchased and -owned cows that produced some 10,000 frozen embryos exported to The Netherlands. Undoubtedly, we were not the only ET business that could have provided the needed services to Holland Genetics, but we just happened to be the first business that Jake Chardon visited on his tour of the United States ET industry.

Early in the embryo export/import programs, there were few established health regulations relating specifically to embryos. The frozen embryos in the first few shipments from Em Tran, Inc., to The Netherlands were thawed and transferred into quarantined recipients that then calved while still in quarantine. Rather quickly, however, infectious disease transmission via embryos was addressed by various agencies within a number of countries. The IETS took a lead role in listing specific diseases into risk categories based on published studies. Many countries no longer require specific testing of donors for diseases that are listed by the IETS as Category 1 (“those for which sufficient evidence has accrued to show that the risk of transmission is negligible”) [55]. A specific protocol for the handling and washing of embryos destined for export is also prescribed by the IETS and widely accepted internationally. The history of this subject was thoroughly covered in a fine review [75].

3.8. Embryo micromanipulation

In the early 1980s, a new technology became available to the ET industry. Termed “splitting,” it involved producing identical half, known as “demi,” embryos by splitting the embryonic mass with a micro-blade. Like so many of the techniques and protocols involved in ET, some of the pioneering efforts leading to commercial splitting of bovine embryos were conducted at the Animal Research Station in Cambridge [76,77]. The first commercial applications of embryo splitting involved the use of two micromanipulators and the transfer of both demi embryos clad in a zona [78,79]. This protocol necessitated the use of two micromanipulators and the availability of a second zona, usually obtained by removing the vitellus from unfertilized ova. Tim Williams and Techan Takeda, both former students at CSU, provided significant on-site help in developing a successful embryo splitting program at Em Tran, Inc. [80], which was characterized by high conception rates for demi embryos transferred either surgically or nonsurgically. When it became clear that conception rates with zona-free demi embryos were comparable to those with zona-clad demis, the ET industry responded accordingly [81,82]. Production of zona-free embryos is both quicker and requires less equipment. In this protocol, the embryo adheres to the bottom of a Petri dish in surfactant-free medium and the embryo is split from above with a micro-blade attached to a single micromanipulator or handheld. The resulting demi embryos are then transferred zona-free.

Initial enthusiasm for embryo splitting technology among dairy cattle breeders rather quickly diminished when it became difficult to justify the costs associated with increasing the number of bull calves. A peak in commercial embryo splitting occurred about 1986 when the Holstein Association reported the registration of 158 female calves. This would have represented approximately 300 embryos, with half of them males and a nearly 50% conception rate for the female demis. In 2011, a total of only 36 dairy embryos of all breeds were reported split in the AETA survey. In addition to the problem of increasing the number of dairy bull calves, the decreased demand for splitting is also likely owing to the inconvenience of on-farm splitting as well as the increased emphasis on freezing a majority of embryos from most donors.

In addition to splitting, sex determination of embryo biopsies by polymerase chain reaction is another technology utilized by the ET industry. This technology was adapted to relatively simple systems for sexing bovine embryos in the late 1980s [83]. A plethora of papers dealing with sexing embryos utilizing polymerase chain reaction and electrophoresis was published throughout the 1990s. Few commercial ET practitioners adopted the technology, however. We basically avoided it owing to some of the horror stories circulating about the time involved in sexing a group of embryos plus the issue of DNA contamination that led to false male identifications. In 1995, I read a paper and was impressed with the description of a new approach to sex determination by polymerase chain reaction developed in Finland [84]. This technique required no electrophoresis and thus was faster than conventional protocols and, importantly, avoided the risk of DNA contamination of the work/laboratory site. Remarkably, within 2 weeks of inquiring about the technology to Peter Bredbacka, the senior author of the paper, he was in my laboratory teaching us the technique. Years of collaboration and continuing friendship followed and we utilized Peter’s system (Finnzymes Oy, Espoo, Finland) quite successfully [85]. We avoided trying to use sexing on-farm, and used a permanent micromanipulation set up in our home laboratory; by limiting biopsy procedures to the morning, we were able to either freeze or transfer the desired sex embryos in the afternoon. This avoided the long days that are so typical when additional ET procedures, other than flushing, transfer and/or freezing are conducted on-farm.

There are undoubtedly more published papers dealing with sexing of bovine embryos (97 listed by PubMed) than there are practitioners actually offering the service
in the North America and probably internationally as well. The IETS does not collect data on embryo biopsy/sexing, but in the United States only 0.3% of the embryos collected in 2011 were biopsied for sex (AETA). In Canada, sexing has been utilized on a wider basis; 3.7% of the embryos were sexed in 2012 (CETA). It is not clear why the procedure has been used more extensively in Canada compared with the United States, but a small number of very active Canadian practitioners may account for this 10-fold difference.

3.9. In Vitro Fertilization

*In vitro* fertilization has been adopted as a generic phrase that often includes the procedures of IVM, IVF, and IVC. All three procedures usually are conducted in sequence to produce embryos exclusively in *vitro* (IVP).

In 1959, the rabbit was the first mammalian species in which live offspring were known to have been produced by IVF (M.C. Chang, 1983 IETS-PA) [86]. The next reported success was with laboratory mice in 1968 [87]. Subsequent progress with *in vitro* technology in mice, however, often did not extend to livestock species, because the mouse did not prove to be a good procedural model. In 1977, IVF in cattle was first accomplished with semen capacitated in the oviduct or uterus of cows in estrus or the uterus of a rabbit [88]. The first live calf resulting from IVF was born in 1981 as the result of the transfer of a four-cell embryo into the oviduct of a recipient cow [89]. Pregnancies also were achieved after transfer of IVM–IVF bovine embryos cultured in the oviduct of a sheep for 5 days [90]. Two calves were born after transfer of embryos resulting from IVF with sperm capacitated using calcium ionophore and the resulting zygotes cultured in rabbit oviduct [91]. The first calves produced entirely from IVM, IVF, and IVC were born in 1987 [92]. However, throughout the 1980s bovine *in vitro* techniques remained primarily research technology with little utilization by the ET industry.

The first repeatable, efficient technique involving transvaginal ultrasound-guided aspiration was developed in 1988 [93] and has become the predominant technique for oocyte collection from living cattle. This technique has become widely known as ovum pick-up (OPU). More recently, comprehensive studies on the schedule and frequency of OPU with and without FSH stimulation have been conducted [94,95].

In addition to OPU, several breakthroughs in the 1980s involving all steps in the IVP process set the stage for adoption of the technology by the commercial ET industry. Perhaps two of the most noteworthy findings were the discovery that IVF of bovine oocytes proceeds most efficiently at 39 °C [96] and the development of *in vitro* capacitation techniques utilizing heparin [97].

We made a decision to explore the use of IVP in our commercial program in 1991 and I received a good deal of help from Jerry Yang, who at that time was completing his PhD at Cornell. However, progress was slow, fertilization rates were low, and few embryos developed in our experimental IVC system. When the program was close to abandonment, serendipity struck again. The announcement that Granada Genetics had entered bankruptcy was an unexpected shock to both the ET industry and to the suddenly unemployed, talented staff members of the company. However, this unfortunate business failure turned out to be hugely beneficial to my business because I was able to quickly bring Charles Looney into our laboratory and he effected an immediate and great improvement in all aspects of our IVF program. The rest is history, and the results of the first few years of our IVF program were published in 1995 and presented at the annual IETS conference [98]. This program almost exclusively involved OPU of oocytes from donors with fertility problems. Several hundred donors that had performed unsatisfactorily in conventional superovulation/flush programs entered the program and almost all of them produced at least one pregnancy. However, the mean number of oocytes collected via OPU was only 4.1 [98] and 5.3 in a similar program as reported by Charles Looney at Granada Genetics [99].

Because Day 0 in relation to heat is the day of estrus, whereas in most IVP programs Day 0 relates to the day of IVF, IVP embryos are in fact actually 1 day older than *in vivo*-derived embryos usually considered to be the same age. For example, a – 1 recipient (Day 6) is not just 1 day younger than a Day 7 recipient, but is in fact actually –2 days in asynchrony. A retrospective analysis of the pregnancy rate of more than 4500 IVP ETs showed that a significantly higher pregnancy rate of 5 percentage points was achieved by transfer of Day 7 IVP embryos into Day 7 or 8 recipients compared with Day 6 recipients [100]. This observation also is supported by data recently provided by Trans Ova Genetics, Inc. (unpublished data), showing a loss of 8 percentage points when IVP embryos were transferred into Day 6 versus Day 7 or 8 recipients. A percentage point loss of 5 or 8 points, when the conception rate is around 50% translates in an actual decrease of 10% to 16% fewer pregnancies. Data such as these generated by the ET industry clearly show why it is so difficult to detect small but important effects in studies which are limited by animal numbers.

During the period when the emphasis in North America was still on using IVP from problem donors, a sizable IVP industry developed in Japan and Brazil and, to some degree, in Italy and The Netherlands. The IETS did not start tabulating statistics on IVP embryos until the annual report in 1996 (compliments of the IETS). In that year, Japan reported that more than 4600 IVP embryos were produced, which was the greatest number among any of the countries covered in the report. No statistics on IVP embryos were available from North or South America. By 2002, the numbers had changed dramatically and more than 48,000 IVP embryos were reported as having been transferred in South America, 22,000 in Asia, and 11,000 in Europe.

In addition to breed differences in success of OPU/IVP, there are significant differences related to donor age. Although cleavage percentage did not differ, the percentage of good embryos relative to the percentage cleaved varied significantly relative to age in Holsteins, with mature cows the greatest, followed by heifers over 9...
months of age, heifers age 6 to 8 months and calves age 2 to 3 months, which were the lowest [101].

The replacement of traditional ET in Brazil by OPU/IVP started in 1998 and 1999 [102] and grew quite rapidly, with the remarkable total of more than 318,000 IVP embryos transferred in 2011 (IETS) compared with 35,563 in vivo-derived embryos transferred. Worldwide, Brazil accounted for approximately 86% of all the IVP transfers in 2011. The leveling off of conventional ET and the rapid rise in production of IVP embryos is shown in Figure 6. The change in the Brazilian ET industry has been attributed to a number of factors, including the lower number of embryos produced by superovulation of Bos indicus compared with B taurus breeds, the relatively high cost of FSH, the large oocyte production via OPU of B indicus cattle, and modest labor costs. Much of the early growth of the Brazilian ET market was fueled by Nelore beef donors, which in one study produced a mean of 23 usable oocytes, with a maximum of 128 per nonstimulated OPU session [103]. In addition, Gir cows, a B indicus dairy breed, produced more oocytes than Holstein dairy cows [104]. The Brazilian IVP industry has continued to expand and is now operating in several other South American countries and South Africa, and one company has recently initiated a large program in Russia (In Vitro Brazil, personal communication).

L’Alliance Boviteq, Inc., is probably the current leader in production of IVP embryos from OPU in Canada; total production of IVP embryos for the country went from just 287 in 2004 to 4453 in 2012 (CETA). In the United States, with three major companies offering services (OvaTech LLC, Gainesville, FL; Sexing Technologies, Navasota, TX; and Trans Ova Genetics, Sioux Center, IA), IVP production from OPU totaled 2078 in 2004 and reached 40,602 from 8689 OPUs in 2011 (AETA). Trans Ova Genetics is currently the largest producer of IVP embryos in the United States, with laboratories in Iowa, Texas, and Maryland. The emphasis in this program is on reproductive healthy cows and heifers, including OPU on cows in early pregnancy. Trans Ova operates under a license (XY, Inc., Navasota, TX) to sex semen, and oocytes from many donors are fertilized with “resorted” semen (previously unsexed, frozen semen which is thawed, sorted, and then used for IVF immediately). The head of the laboratory, Hong Wei, reported that cleavage and blastocyst development rates are larger with resorted semen compared with sexed semen available from commercial bull studs. Projected production for 2013 includes approximately 15,000 OPUs resulting in more than 70000 IVP embryos (unpublished data, personal communication). All of the North American IVF programs include stimulation of most donors with FSH and OPU performed once every 14 days. At Trans Ova, this has resulted in the collection of 18 to 20 oocytes per OPU, with a 25% blastocyst development rate. This is in contrast with Brazil, where most B indicus donors, characterized by large ovarian follicle counts, do not receive FSH, and OPU is performed weekly.

Proposals have been made for dealing with some of the problems associated with IVP embryos [105]. One issue that has not been entirely resolved among the problems that are associated with production and commercial utilization of IVP embryos is the question of the normalcy of IVP embryos and ensuing pregnancies after transfer. As first shown in sheep [106,107] and then in cattle [98,108–111], problems associated with pregnancies and neonatal issues are often included in what has become known as the “large offspring syndrome” (LOS). Larger fetuses and heavier birth weights are only one, and not necessarily the most serious, of the problems associated with LOS. Increased abortions throughout gestation, abnormal placentas, delayed and poor labor in recipients, and an extremely large increase in the incidence of hydroallantois are among the conditions recorded. The LOS syndrome largely has been attributed to IVC systems containing serum, with and without co-culture. Various co-culture IVC systems containing serum have been shown to support a high rate of blastocyst development and thus there was some reluctance by the ET industry to discontinue the use of serum and/or co-culture. Semidened systems in which BSA replaced serum have been shown to perform nearly equally to co-culture [112]. Some of the problems greatly decreased when serum was replaced by BSA [113], and many thousands of IVP-derived pregnancies and calves have been produced in the past few years, primarily in Brazil, North America, Europe, and Japan. Unfortunately, there is a lack of peer-reviewed published data describing the current status of LOS problems in the commercial ET-IVP industry.

3.10. Cloning

The announcement of the birth in 1996 of “Dolly” the lamb, representing the first mammal successfully cloned from a somatic cell [114], created worldwide coverage in the lay press. Optimism regarding commercial applications of cloning by somatic cell nuclear transfer (SCNT) soon followed [115,116]. Starting in the late 1990s, several businesses were established in the United States to offer commercial cloning services for a number of species, including cattle. Cyagra, Inc., which ceased operation in the United States in 2010, was established in 1999 as a division of Advanced Cell Technology in Massachusetts. In 2002, Cyagra was purchased by an Argentinian businessman and moved into the former facilities of Em Tran, Inc., in Pennsylvania. Several hundred cattle clones were

Fig. 6. Comparison of the annual number of in vivo-derived and in vitro embryo produced embryos transferred internationally since 2000 through 2011. (Compliments of the International Embryo Transfer Society.)
produced at Cyagra, and the cloning data base contributed to the risk assessment by the Center for Veterinary Medicine division of the FDA.

The only other large, North American cloning company, ViaGen, was founded in Austin, Texas, in 2002 to provide commercial bovine, equine, and porcine gene banking, cloning, and genomics services. In 2003, ViaGen acquired ProLinia of Athens, Georgia, and then was sold, in turn, in 2012 to Trans Ova Genetics in Iowa.

In January, 2008 the FDA released a 968-page Final Risk Assessment (no. 179) entitled “Use of Animal Clones and Clone Progeny for Human Food and Animal Feed.” The report concluded that meat and milk from healthy cloned animals or their offspring are as safe as those from ordinary animals, effectively removing the last U.S. regulatory barrier to the marketing of meat and milk from cloned cattle, pigs, and goats. Before this, milk, meat and, in some cases, offspring from cloned cows were not marketed and semen from bulls was not frozen and sold for AI. Currently, there remains a voluntary agreement on the part of the main producers of bovine clones in the United States not to market meat or milk from clones because consumers remain wary of such food.

Although numerous anatomic, physiologic, and epigenetic problems have been documented for SCNT-derived pregnancies and calves [117–119], there have been optimistic proposals for improving the technology [120]. However, the cost of commercially producing cloned cattle is variously estimated at $15,000 to $20,000 apiece, and this seems to have greatly limited the utilization of the technology by cattle breeders. The author’s only experience with cloning was the sale of Em Tran, Inc., to Cyagra, Inc., and it is unlikely that the majority of commercial ET practitioners either have had or will have any involvement with transferring cloned embryos in the foreseeable future.

4. The future

Several papers have addressed the future of ET [121–124]. In addition, many other papers have dealt with single, specific technologies that may eventually become part of the ET industry. Several technologies, such as cloning and transgenics, have been utilized commercially only on a limited basis. Significant improvements in these technologies will be necessary for any substantial increase in utilization to occur. In his 1991 paper entitled “Embryo transfer: the next 100 years” [121], George Seidel listed 10 recent technologies of “pertinence” to embryo technology. Several of his choices have, without question, had significant impact on commercial ET, including estrus and ovulation synchronization, sperm sexing, cryopreservation technology, and in vitro technology.

In his 2006 paper dealing with ET “achievements and perspectives” [124], Keith Betteridge discussed, in addition to a number of other relevant subjects, the growth of commercial IVP and the potential problems with epigenetic effects on in vitro embryos. Pertinent to this subject, abnormalities in epigenetic control of gene expression recently were reviewed [125,126] in the context of both IVP and SCNT bovine embryos and other mammalian species.

Growth in the number of IVP embryos produced yearly in the commercial ET industry has increased the demand for improving all aspects of IVF technology. It is widely agreed that IVC, whether based on single or sequential media, does not mimic the changing environment encountered by embryos descending the oviduct and entering the uterus. In addition, embryos cultured in conventional Petri dishes, whether in large volumes or microdrops, may not be exposed to an ideal ratio of media volume to embryo mass. Microfluidic culture systems have been proposed as providing solutions to some of these problems [127,128]. However, conventional ET procedures are unlikely to be affected by the development of microfluidics technology.

In addition to changes in technology, unpredictable events affect commercial ET. For example, in May 2003 Canada confirmed its first native case of bovine spongiform encephalopathy disease, which happened to be in an Alberta cow. The discovery prompted the United States and other nations to ban imports of Canadian cattle and beef. From 2000 to 2002, the CETA reported a mean of approximately 3260 beef donors flushed annually. After the import ban, the number of Canadian beef donors flushed dropped to approximately 2600 in 2003 and only approximately 2000 in 2004. Although there has been some recovery, the beef ET business has never fully recovered in Canada. Much as the periodic drop in milk prices has negatively impacted the dairy ET business in the United States, unpredictable disease outbreaks can have profound, negative effects on the ET industry.

Unexpected disease outbreaks have the potential to have a direct and negative impact on ET, but also to energize and strengthen the resistance of animal rights groups to ET and other reproductive technology. Among the problems raised in my 2003 paper dealing with the future of ET [122], I pointed out three European countries outright that prohibited collection of oocytes via OPU, and slaughterhouse collection of oocytes was permitted only for research purposes in five European countries. The well-publicized and publically supported closing of all horse slaughterhouses in the United States has made in vitro research in horses frustratingly difficult.

The costs associated with performing ET services have risen somewhat dramatically over the years. In 1978, the cost per dose of the original Armour FSH-P was $6.50 and the ET practitioner charge per donor superovulated and flushed was $500. Today, the cost of the available FSH preparations exceeds $100 per dose and the ET practitioner fees are half or less than what they were 35 years ago. The ET business has become quite competitive and, depending on the country or state, non-veterinarians are providing an increasing share of the procedures, especially the transfer of frozen embryos. It is likely that an increasing proportion of ET procedures will be performed by noncredentialed technicians in the future.

Genomic evaluation of cattle recently has been reviewed [129,130], and the growing importance of AI sire genomic selection is undisputed. Using genomics, more bull calves are being screened and fewer calves are
actually being purchased by bull studs and undergoing pregnancy testing [131]. In fact, a satisfactory genomic profile is almost a necessity today to secure a sales contract on a dairy bull calf from a bull stud. The power of genomic selection is greatest for the Holstein breed owing to the enormous amount of production data and sire information available for cows and bulls of this breed. In addition, genomic information is also being used for selection of Jersey and Brown Swiss bull calves (Select Sires, Inc., Plain City, OH). Among beef breeds, genomic information is strongest for Angus cattle, followed by Herefords and Simmental.

The advantages of applying genomic selection to embryos were recently covered by Seidel [132]. With the use of single nucleotide polymorphism (SNP) chips, embryo genomic information will appeal to cattle breeders because they will save money on unwanted ET service costs, recipients, and unwanted calves. In addition, genomic evaluation of embryos will decrease generation intervals. Identification of SNPs in embryos requires whole genome amplification of the embryo biopsy, followed by SNP analysis, which is now available using several different, commercially available chips that respond to as many as 800,000 different SNPs. Several ET/reproductive technology businesses are currently working toward introducing genomic analysis of embryos to cattle breeders in the foreseeable future (unpublished data). Undoubtedly, this technology will appeal to many cattle breeders, but it will also force a rather drastic reorganization of ET businesses who want to offer this service.

Vitrification has become the system of choice for cryopreservation of human embryos [133]. In addition, vitrification is often proposed for cryopreservation of bovine IVP, SCNT, and biopsied in vivo-derived embryos. However, currently available vitrification protocols using 0.25-ml straws as the container have not resulted in satisfactory pregnancy rates (unpublished data). If and when there is widespread genomic analysis of bovine embryos, an efficacious and practical DT vitrification system for biopsied bovine embryos will be valuable.

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