SYMPOSIUM: REPRODUCTIVE TECHNOLOGY
AND GENETIC IMPROVEMENT

Current Status and Potential of Embryo Transfer
and Reproductive Technology in Dairy Cattle

JOHN F. HASLER
Em Tran, Inc.
Elizabethtown, PA 17022

ABSTRACT

Significant use of embryo transfer in dairy cattle commenced with the introduction of nonsurgical embryo recovery in the mid-1970s and developed with the use of nonsurgical transfers in the late 1970s. Numbers of registered Holstein calves from embryo transfer doubled yearly through 1980, after which the rate of increase slowed; the total reached nearly 19,000 calves in 1990. However, the efficacy of superovulation procedures and commercial success rates of transferred fresh embryos have not improved the past 10 to 15 yr. Fertilization rates in superovulated donors remain low. Although embryo-splitting techniques were perfected in the early 1980s, they are not used widely. A practical, commercial embryo-sexing procedure remains unavailable. Recent significant improvement is apparent in the technology of ultrasound-guided oocyte collection and in vitro oocyte maturation, fertilization, and embryo culture. In the future, this technology may be used in conjunction with sperm separated by sex with a flow cytometer. Modest numbers of embryo clones have been produced in several commercial programs via nuclear transfer techniques. However, the efficiency of gene transfer experiments involving ova of cattle and other domestic species has been low. Recently, DNA probe technology has begun to provide genotype information for cattle and will ultimately be applied to embryos.

INTRODUCTION

Commercial embryo transfer (ET) in cattle began in North America during the early 1970s largely because of the availability of PGF₂α and the high prices and demand for several breeds of "exotic" beef cattle. During the early years of the ET industry, embryo recoveries were performed via a midventral surgical technique; the donor was under general anesthesia in a surgical facility. During this period, ET was not widely utilized in the dairy industry because the udder of dairy cows hindered midventral access to the reproductive tract. In addition, in some donors, the formation of adhesions following surgery sometimes led to loss or impairment of fertility (49). Consequently, few reproductively healthy dairy cows were transported to surgical ET facilities.

Early attempts at nonsurgical recovery of bovine embryos did not prove successful for various reasons (43, 45, 184, 208). However, in 1976, several groups (44, 50, 183) reported an efficient, nontraumatic, nonsurgical technique utilizing Foley catheters (C. R. Bard Inc., Covington, GA). Following the introduction of the nonsurgical "flushing" technique, the application of ET in the dairy industry grew rapidly. As shown in Table I, the first Holstein calves produced by ET were registered in 1974. Nonsurgical transfer techniques were developed and refined by numer-
TABLE 1. Holstein registrations from embryo transfer (ET) for 1974 to 1990.

<table>
<thead>
<tr>
<th>Birth year</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1974</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1975</td>
<td>5</td>
<td>10</td>
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<td>1976</td>
<td>41</td>
<td>93</td>
<td>134</td>
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<td>101</td>
<td>164</td>
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<tr>
<td>1978</td>
<td>293</td>
<td>332</td>
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<tr>
<td>1979</td>
<td>705</td>
<td>782</td>
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</tr>
<tr>
<td>1980</td>
<td>1598</td>
<td>1968</td>
<td>3566</td>
</tr>
<tr>
<td>1981</td>
<td>2419</td>
<td>3403</td>
<td>5822</td>
</tr>
<tr>
<td>1982</td>
<td>3166</td>
<td>5132</td>
<td>8298</td>
</tr>
<tr>
<td>1983</td>
<td>3807</td>
<td>6399</td>
<td>10,206</td>
</tr>
<tr>
<td>1984</td>
<td>4359</td>
<td>8452</td>
<td>12,811</td>
</tr>
<tr>
<td>1985</td>
<td>4616</td>
<td>9952</td>
<td>14,568</td>
</tr>
<tr>
<td>1986</td>
<td>4957</td>
<td>10,393</td>
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<td>1987</td>
<td>5187</td>
<td>9975</td>
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<tr>
<td>1988</td>
<td>6252</td>
<td>11,387</td>
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</tr>
<tr>
<td>1989</td>
<td>6783</td>
<td>11,138</td>
<td>17,921</td>
</tr>
<tr>
<td>1990</td>
<td>7188</td>
<td>11,539</td>
<td>18,727</td>
</tr>
<tr>
<td>Total</td>
<td>51,478</td>
<td>91,120</td>
<td>142,598</td>
</tr>
<tr>
<td>1990 Non-ET</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>-30,000</td>
<td>-365,000</td>
<td>-395,000</td>
</tr>
</tbody>
</table>

1Not completed.


<table>
<thead>
<tr>
<th>Breed</th>
<th>Evaluation</th>
<th>Number</th>
<th>Percentage by ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holstein</td>
<td>TPI</td>
<td>100</td>
<td>44</td>
</tr>
<tr>
<td>Jersey</td>
<td>PTA-Protein</td>
<td>50</td>
<td>22</td>
</tr>
<tr>
<td>Brown Swiss</td>
<td>PTI</td>
<td>33</td>
<td>27</td>
</tr>
</tbody>
</table>

1ET = Embryo transfers; TPI = Type-Production Index; PTA-protein = PTA for percentage and pounds of protein; PTI = Production-Type Index.
TABLE 3. Variation in superovulation among 5 Holstein cows.

<table>
<thead>
<tr>
<th>Cow</th>
<th>Super-ovulations (no.)</th>
<th>Mean total ova (%</th>
<th>Fertilized Degenerate (no.)</th>
<th>Freezable (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13</td>
<td>12.2</td>
<td>74</td>
<td>3.8</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>5.4</td>
<td>64</td>
<td>.8</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>18.6</td>
<td>55</td>
<td>1.3</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>10.3</td>
<td>93</td>
<td>.1</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
<td>8.7</td>
<td>90</td>
<td>1.2</td>
</tr>
</tbody>
</table>

high LH concentration (28, 41, 88). In studies utilizing commercial FSH preparations in which the LH content was assumed to be higher in one product than another, there were no differences among superovulatory responses because of the use of different products or different batch lot numbers of the same product (15, 33, 242).

In 1983, the first superovulation of 984 Holsteins at Em Tran (Elizabethtown, PA) yielded an average of 5.1 embryos (81), and this average has not improved during the intervening years. With a range of 0 to 101 ova and 0 to 50 embryos, the average, during a 13-yr period at Em Tran, with over 6000 different Holstein females superovulated one or more times, was approximately 4.5 to 5.0 usable embryos per superovulated Holstein donor. Enormous variation is evident in the responses of individual cattle to superovulation. Table 3 shows very large differences (P < .001, by ANOVA) in fertilization rates and embryo numbers among 5 Holstein cows superovulated 9 to 15 times each. On the low end, cow B averaged only 5.4 ova per superovulation compared with 18.6 for cow C. Simply increasing the amount of FSH administered to a cow has not resulted in the production of more embryos (36, 153).

Some other variables related to superovulation are relatively well understood. For example, initiating superovulation early in the estrous cycle resulted in reduced responses (70, 125, 205). Age, to at least 10 yr (37, 81, 124), and repeated superovulations (40, 42, 81) had, at best, only a small effect on the number of embryos produced. However, older cows selected for superovulation and females undergoing repeated superovulations represent highly selected individuals. Neither the use of GnRH or analogs (61, 163, 164) nor the use of human chorionic gonadotropin (108) in conjunction with superovulation regimens has increased the number of embryos.

The number of small follicles observed via ultrasound prior to superovulation was positively correlated with the size of the response and the number of embryos collected (181). There have been a number of attempts to increase the number of follicles likely to respond to superovulation by injecting one or two small doses of FSH or PMSG early in the estrous cycle. This treatment resulted in increased embryo production in some studies (155, 215, 224) but not in others (71, 73, 171, 176). Ultrasound also was used to increase the understanding of follicular wave cycles during the bovine estrous cycle (62, 106). This work indicated that a dominant follicle develops in each follicular wave and inhibits the maturation of other follicles in that wave. Some recent work (72, 77) suggests that initiating superovulation in the presence of a dominant follicle reduces ovarian responses. However, several studies failed to demonstrate a correlation between the presence of a dominant follicle and the size of the superovulatory response (71, 238). The mere presence of a dominant follicle may be less important than its suppressive capability (189). More research in methods of assessing the functional characteristics of dominant follicles may lead to improvements in superovulation of cattle.

Recently, bovine FSH has been produced by recombinant DNA technology (27). Initial tests of this product for superovulation of cattle indicated a remarkably high production of embryos because of both a high level of superovulation and a high fertilization rate (239). In a subsequent study, the degree of superovu-
lation was moderately large in Hereford heifers and very high in Brahman heifers with high fertilization rates for both (12). This recombinant FSH has not yet been approved for use by the FDA.

**FERTILIZATION**

Fertilization of the ova produced by superovulation is a component of ET with significant room for improvement. In a 1984 study of 984 Holstein cows and heifers superovulated one time each, 61% of the 8771 recovered ova were fertilized (81). In a current study of 1337 repeat superovulations of approximately 150 Holstein cows at Em Tran, 64% of the 11,537 ova were fertilized. Another large commercial ET company reported a fertilization rate of 63% in 476 superovulated Holsteins (124). Cows in these studies were inseminated with 2 to 4 straws of frozen semen from a variety of commercially popular bulls. Fertilization was higher (70 to 80%) for superovulated beef cattle in some commercial ET programs (23, 127, 191). In single-ovulating cows that were not superovulated, fertilization rates often range from 85 to nearly 100% (84, 180). In direct comparisons between superovulated and nonsuperovulated cattle, fertilization rates were inevitably higher in the single-ovulating females (84, 187, 196). It has not been proven that sperm transport is inhibited in superovulated versus single-ovulating cattle. However, accessory sperm counts have averaged fewer than 1 per zona from superovulated cows versus over 40 per zona of ova from single-ovulating cows in some studies (84, 187). A histological study demonstrated bilateral sperm reservoirs in the uterotubal junctions and isthmus of single-ovulating cattle, whereas superovulated cattle exhibited only unilateral or a lack of reservoirs (91). In some studies (129, 198), but not in others (84, 139), fertilization rates in single-ovulating cattle were higher following semen placement in the uterine horn versus insemination in the body of the uterus. Fertilization rates in superovulated cattle were not improved by inseminating more than twice (12 and 24 h postestrus) in one study (225) or once (at 12, 15, or 24 h) versus three or four times in other studies (130, 190).

The cost of semen in a commercial ET program is often an important consideration. It is tempting to use more semen from bulls suspected or known to be less than average in fertility. However, fertilization rates in Holstein cows at Em Tran did not increase when 4 or 5 versus 3 straws of semen were used. Hawk et al. (83) increased the fertilization rate from 53% in superovulated cows inseminated with 70 million frozen sperm to 93% when 4.4 billion fresh sperm were used. Even with 93% fertilization, however, only 32% of the fertilized ova exhibited accessory sperm.

Fresh and frozen semen from bulls selected for high and low nonreturn rates was used to inseminate 160 superovulated beef heifers, and ovulated ova were recovered from the heifers at slaughter (25). The 89 and 70% fertilization rates of superovulated ova for the two treatment groups correlated highly with the nonreturn rates of the sires. Analysis of some currently popular Holstein sires used on superovulated cows at Em Tran indicated significant differences ($P < .001$, by ANOVA) in fertilization rate and in the number of embryos per flush (Table 4). The differences in fertility of the 9 bulls listed in Table 4 are representative of the variation seen in the total data set of inseminations using 96 bulls, resulting in 1210 embryo recoveries. The differences in fertilization rate among bulls were demonstrated within in vitro studies (201) in which significant differences existed among bulls in the proportion of fertilized ova that continued to develop to the blastocyst stage. In another in vitro study, fertilization and first cleavage rates were similar among bulls, but neither was correlated with the percentage of development to blastocysts (55). Similarly, in Table 4, which represents an in vivo comparison, differences were significant among bulls in the percentage of degenerate embryos. These data demonstrate differences among bulls not only in fertility, but also in the viability of preimplantation embryos. This concept is further supported by the report from a commercial ET program that the pregnancy rate of transferred embryos was significantly affected by the Holstein sire used to inseminate the donor (29). Recent data demonstrated a positive relationship in the number of accessory sperm and the quality of embryos in single-ovulating Holstein cows (32). These data appear to indicate that increasing the number of sperm contacting superovulated ova might improve not only fertilization rate, but also embryo quality.
TABLE 4. Fertility of AI sires used on embryo donors.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Flashes</th>
<th>UPO(^1)</th>
<th>Degenerate ovum</th>
<th>Frozen ovum</th>
<th>Mean frozen ovum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no.)</td>
<td>(%)</td>
<td>(no.)</td>
<td>(no.)</td>
<td></td>
</tr>
<tr>
<td>Ambrose</td>
<td>46</td>
<td>40</td>
<td>10</td>
<td>49</td>
<td>4.9</td>
</tr>
<tr>
<td>Blackstar</td>
<td>206</td>
<td>38</td>
<td>13</td>
<td>48</td>
<td>4.8</td>
</tr>
<tr>
<td>Cubby</td>
<td>39</td>
<td>35</td>
<td>5</td>
<td>60</td>
<td>4.1</td>
</tr>
<tr>
<td>Leadman</td>
<td>178</td>
<td>32</td>
<td>16</td>
<td>49</td>
<td>4.6</td>
</tr>
<tr>
<td>Michael</td>
<td>74</td>
<td>20</td>
<td>18</td>
<td>61</td>
<td>4.7</td>
</tr>
<tr>
<td>Southwind</td>
<td>61</td>
<td>31</td>
<td>15</td>
<td>62</td>
<td>4.3</td>
</tr>
<tr>
<td>Tesk</td>
<td>51</td>
<td>39</td>
<td>8</td>
<td>51</td>
<td>3.8</td>
</tr>
<tr>
<td>Wister</td>
<td>37</td>
<td>55</td>
<td>10</td>
<td>35</td>
<td>2.5</td>
</tr>
<tr>
<td>Laban</td>
<td>20</td>
<td>53</td>
<td>19</td>
<td>27</td>
<td>2.2</td>
</tr>
<tr>
<td>Total(^2)</td>
<td>1210</td>
<td>35</td>
<td>13</td>
<td>51</td>
<td>4.4</td>
</tr>
</tbody>
</table>

\(^1\)UFO = Unfertilized ovum; mean frozen = mean number of embryos frozen per flush.
\(^2\)Total values for all 96 bulls used in 1210 flushes.

RECIPIENTS

Any ET procedure ultimately depends on the availability of a suitable recipient. Numerous factors related to bovine females have been studied in order to maximize the rate of pregnancy. A classic concern is the importance of the synchrony of estrus between the recipient and the donor of the embryo. In 1969, Rowson et al. (186) showed that a deviation of ± 2 d could result in pregnancy, but a later study (185) indicated a large drop in pregnancy rate with a deviation of only 1 d. However, the data from the second study (185), which form an almost perfect bell-shaped curve, do not apply in all situations. The effect of asynchrony on pregnancy rate in that study (185) may have been magnified by the young age of the embryos. The graphs in Figure 1 represent pregnancy rates as they relate to donor versus recipient estrous synchrony from five large commercial ET programs. The bottom curve represents over 13,000 transfers, primarily into beef cows that came into estrus naturally. Similarly, the two curves in the middle represent transfers primarily into beef cows. The top two, very similar curves represent a total of more than 8000 embryos transferred into Holstein heifers, most of which had been synchronized with PGF\(_{2α}\). It is not clear why there are such differences among some of the commercial programs represented. The curve from Putney et al. (168) is based on more than 13,000 transfers, of which 1320 were dairy cattle. A higher percentage of dairy heifers (63%) in this study became pregnant following ET than either beef cows (57%) or heifers (49%). Synchrony may become less important with a higher average pregnancy rate. In four of the five programs, it was advantageous that the recipient was in estrus before the donor.

Heifers synchronized with PGF\(_{2α}\) exhibited a higher pregnancy rate following ET than recipient heifers in natural estrus in one study (80), but there was no difference in another.

Figure 1. Effect of donor-recipient synchrony of estrus on the pregnancy rate of recipients. Recipient in estrus before donor (+) and recipient in estrus after donor (–). Key to references and sample sizes: (O) (29) (n = 1202); (♦) (80) (n = 6996); (●) (168) (n = 13,205); (Δ) (38) (n = 13,164); and (○) (199) (n = 2016).

The quality of the corpus luteum of recipients was not correlated with pregnancy rate (29, 39, 80, 207). Determination of serum progesterone concentrations in recipients on the day of ET has been investigated by numerous workers as a possible means of predicting the success of ET. In most studies, there was no difference in average blood progesterone on the day of ET in recipients that became pregnant after ET and those that did not (79, 147, 148, 174, 207). However, in some reports (3, 147, 148, 174, 207), pregnancy rates were lower among recipients for which progesterone concentrations were below 1 to 2 ng/ml of serum. Pregnancy rate in recipients injected with progesterone at the time of ET was not higher than that in control females (223). However, in another study (188), recipients that received progesterone either by injection or by intravaginal sponge and that were in estrus 12 to 48 h after the donor sustained a pregnancy rate of 53 versus 37% in untreated controls. Injection of progesterone on d 1 to 5 in recipients reduced the effect of a 3-d asynchrony between d-8 embryos and d-5 recipients (64). The pregnancy rate in progesterone-treated females was 47 versus 5% in untreated controls. In attempts to increase progesterone secretion from the corpus luteum, human chorionic gonadotropin was injected into ET recipients without improving embryo survival (76, 128, 188). Similarly, treatment of recipients with a GnRH analog, either at the time of ET or 4 to 7 d later, did not increase pregnancy rate (47). The β-mimetic agent clenbuterol, utilized in order to maintain uterine quiescence, did not increase pregnancy rate when it was injected prior to nonsurgical ET (1, 9, 132, 223) or surgical ET in one study (9), but pregnancy rate increased when clenbuterol was used prior to surgical ET in other studies (30, 132).

**EMBRYO FREEZING**

During the 1970s, commercially recovered bovine embryos in most cases were either transferred to recipients within hours or discarded. The unpredictability of embryo production from a given donor and the necessity for donor-recipient synchronization of estrus frequently created discrepancies between the number of embryos and the number of suitable recipients available. Although it is possible to culture bovine embryos with excellent survival at ambient temperature (80) or 0°C (121) for at least 24 h, suitably synchronized recipients still must be available. Only by long-term storage, necessitating freezing, can surplus embryos be preserved for transfer at a later date to suitably synchronized recipients.

In 1972, the survival of cryopreserved mammalian embryos was proven for the first time with the birth of live mice that had been frozen as 2- to 8-cell embryos, stored at −196°C, and transferred to recipients (227). Amazingly, this study reported a 65% post-thaw survival rate for almost 1000 mouse embryos. Although the birth of the first calf resulting from ET of a frozen embryo was announced in 1973 (237), it was some years before the reliability of freezing cow embryos approached that of mice. Improved techniques for the freezing of bovine embryos resulted from the investigation of a number of factors, including comparisons of types and concentrations of cryoprotectants, optimal rates of freezing and plunge temperature, influence of the stage and quality of embryos, methods of cryoprotectant removal, thaw temperature, and cryoscopic examination of cytoplasmic responses during freezing and thawing (18, 52, 102, 112, 113, 145, 146, 231). By the early 1980s, some commercial ET companies reported pregnancy rates from frozen bovine embryos exceeding 50% (157, 200, 241). Reliable freezing technology led to the rapid development of an international trade in frozen embryos; pregnancy rates exceeded 60% in some cases (143, 214, 219).

Pathogens have not been demonstrated inside the zona of cattle. It is now generally accepted that the intact zona pellucida is an effective barrier to the passage of microbes, including viruses (202). A number of both viral and bacterial pathogens are effectively removed from the outside of the bovine zona pellucida either by rinsing the embryos 10 times in culture medium or, for some pathogens, by exposing the embryos briefly to trypsin (202). The pregnancy rate of embryos exposed to trypsin was not compromised (82). These findings have led to the adoption by many countries of specific import regulations that recognize that it is safer to import properly handled preimplantation embryos than live
animals. For example, between 1983 and 1986, France imported more than 1000 Holstein embryos from donors tested only for brucellosis and tuberculosis (214). The embryos were rinsed 10 times prior to freezing, and none of the resulting calves was clinically positive for any diseases. Although a significant number of the embryo donors were positive for bovine leukemia virus and infectious bovine rhinotracheitis virus, all of the recipients remained serologically negative. The International Embryo Transfer Society (Champaign, IL) recognizes a yearly updated list of pathogens ranked according to scientifically documented risk of transmission by ET. Table 5 lists the pathogens for which the risk was considered to be the lowest as of 1992. These findings facilitated the export from North America of frozen embryos from Holstein donors. The Holstein Association (Brattleboro, VT) reported that 3200 export certificates for frozen embryos were processed in 1990, which may represent 10,000 to 12,000 embryos.

Several additional approaches to embryo freezing may have the potential to impact the ET industry in the future. In 1982, Leibo et al. (120) first described a system in which an embryo is frozen in a straw along with a separate volume of liquid necessary to rehydrate the embryo. After thawing a one-step straw, the freeze solution, containing glycerol and the embryo, and the rehydration solution, containing sucrose, are mixed by shaking the straw. Within a few minutes, the embryo can be transferred nonsurgically directly from the straw. The patented one-step system (115) eliminates the need for using a microscope, unloading thawed embryos, rehydrating them, and then reloading them again into straws. Consequently, it eliminates the need for specialized technical expertise, reduces equipment needs, and saves time. This technique has not been widely adopted by the ET industry, probably because the pregnancy rate was between 26 and 42% (116, 117), which is lower than that rate achieved with traditional technology in the ET industry. However, this may be a reflection of the conditions under which the field trials were conducted rather than of the potential of the freezing system. Pregnancy rates approximated 50% for other, similar, single-step systems (134, 175). Fahy et al. (58) first described different approach to preservation of embryos during freezing: vitrification, a phenomenon in which highly concentrated solutions of cryoprotectants fail to crystallize when rapidly cooled, passing instead from the liquid state to an unstructured glassy state. Vitrification involves exposing embryos to a mixture of different permeating cryoprotectants and abruptly plunging them into liquid nitrogen (118). Although relatively toxic cryoprotectants are used in the vitrification procedure, 80% of frozen thawed mouse embryos developed in in vitro culture (IVC), and a 17% pregnancy rate was produced by ET (173). There are a few reports (35) of ET pregnancies produced from vitrified embryos; one study (134) cited 9 pregnancies from 23 ET. Other than the ease of the verification freezing procedure, advantages over conventional freezing procedures are not clear.

CLONING

The word "clone", derived from the Greek word for twig, suggests the asexual production of new plants by breaking off twigs (193). In the context of animal breeding and ET, cloning implies the production of a number of identical copies of an individual animal. Unfortunately, no one has been successful in making clones from the somatic cells of adult mammals. Clones in mammals have been produced by several techniques only from preimplantation embryos. Consequently, in the case of dairy cattle, if predictable phenotypes and milk production are desired, cloned female embryos must be frozen and stored for approximately 3 yr while one clone reaches maturity after ET and goes through progeny testing. The im-

TABLE 5. Category 1 diseases recognized by the International Embryo Transfer Society in 1992.1

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Trypsin required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukosis</td>
<td>No</td>
</tr>
<tr>
<td>Foot and mouth disease</td>
<td>No</td>
</tr>
<tr>
<td>Brucellosis</td>
<td>No</td>
</tr>
<tr>
<td>Bluetongue</td>
<td>No</td>
</tr>
<tr>
<td>Infectious bovine rhinotracheitis</td>
<td>Yes</td>
</tr>
<tr>
<td>Pseudorabies (swine)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

1Category I diseases are those for which sufficient evidence has accrued to show that the risk of transmission is negligible, provided that the embryos are properly handled between collection and transfer.
mediate advantage of cloning to cattle breeders is as a tool for increasing the number of embryos produced through superovulation. For research purposes, identical animals provide a large advantage over randomly bred animals (14). Nicholas and Smith (144) described the predicted rapid genetic advances of breeding programs based on genetic selection of clones.

**Embryo-Splitting**

Embryo-splitting on a commercial basis owes its roots to experimental studies of separating the early embryonic blastomeres of species such as the mouse (211), rabbit (141), and pig (142). Willadsen and Polge (230) first described a technique in 1981 by which bovine monozygotic twins were produced from the bisection or splitting of one embryo. Following this, the technology was refined, and successes were achieved with different kinds of splitting instruments and embryos of varying ages (149, 232). Application of embryo-splitting within the ET industry began in the mid-1980s, and a number of groups reported high success rates after both surgical and nonsurgical transfer of both zona-clad and zona-free demi-embryos (Table 6). The pregnancy rates in this table, which range from 50 to 76%, translate into 1.0 to 1.52 pregnancies per original embryo, an obvious advantage over the transfer of intact embryos. To date, cloning by embryo-splitting in commercial ET has been limited in the bovine to production to two demi-embryos per parent embryo. Early research in the mouse suggested that the embryological transition from morula to blastocyst, occurred after a genetically determined number of cleavage divisions, not after a certain number of cells were present (213). As a consequence, the number of segments into which an embryo could be divided, with survival, is limited by the minimum number of cells necessary to form a competent blastocyst. It was recently shown that a high percentage of embryos derived from single blastomeres, isolated from 4-cell bovine embryos developed in culture (131). These embryos contained approximately one-third the number of cells found in normal control blastocysts. Although Willadsen and Polge (230) reported a high initial survival rate of bovine “quarter” derived from separating 8-cell embryos into four 2-cell embryos, the pregnancy rate after ET was low. In addition to low success, blastomere separation of 4- or 8-cell embryos is not commercially feasible because it necessitates the surgical recovery of the embryos from the oviducts and the use of an intermediate recipient, such as a sheep, until the embryos are old enough to be transferred to the uterus of a bovine. Splitting of bovine morulae would theoretically be even less successful than blastomere separation because of the inevitable loss of some cells by the splitting process (203).

At least one bull stud embarked on a program of purchasing pairs of twin bulls and evaluating them in a progeny test program (19). The Holstein Association recently initiated a study of performance and type differences between female twins produced by embryo-splitting (110). An initial finding, based on 40 pairs of twins maintained within the same herds, was that 53% of the females produced at the same level, within 909 kg (2000 lb) of milk per lactation, as their twins. The currently used mathematical model predicted that only 37% of the pairs would be within 909 kg. Continued studies of this type may increase the understanding of heritability versus environmental components of milk production and other traits. In spite of the usefulness of such studies, embryo-splitting has not become widely adopted by the dairy breeders utilizing ET. Table 7 shows the annual registration of Holstein calves that were

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**TABLE 6. A comparison of percentage of pregnancies obtained with split bovine embryos by different researchers.**

<table>
<thead>
<tr>
<th>Transfer</th>
<th>Zona</th>
<th>(105)</th>
<th>(74)</th>
<th>(8)</th>
<th>(210)</th>
<th>(3)</th>
<th>(197)</th>
<th>(119)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical</td>
<td>With</td>
<td>60</td>
<td></td>
<td>64</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical</td>
<td>Without</td>
<td>57</td>
<td>72</td>
<td></td>
<td>60</td>
<td>57</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Nonsurgical</td>
<td>With</td>
<td>57</td>
<td>50</td>
<td></td>
<td>60</td>
<td>57</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Nonsurgical</td>
<td>Without</td>
<td>57</td>
<td>72</td>
<td></td>
<td>60</td>
<td>57</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

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TABLE 7. Registration of Holsteins resulting from split embryos for 1982 to 1990.

<table>
<thead>
<tr>
<th>Birth year</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>1983</td>
<td>19</td>
<td>25</td>
<td>44</td>
</tr>
<tr>
<td>1984</td>
<td>39</td>
<td>51</td>
<td>90</td>
</tr>
<tr>
<td>1985</td>
<td>100</td>
<td>149</td>
<td>249</td>
</tr>
<tr>
<td>1986</td>
<td>64</td>
<td>158</td>
<td>222</td>
</tr>
<tr>
<td>1987</td>
<td>34</td>
<td>73</td>
<td>107</td>
</tr>
<tr>
<td>1988</td>
<td>35</td>
<td>61</td>
<td>96</td>
</tr>
<tr>
<td>1989</td>
<td>39</td>
<td>73</td>
<td>112</td>
</tr>
<tr>
<td>1990</td>
<td>36</td>
<td>81</td>
<td>117</td>
</tr>
<tr>
<td>Total</td>
<td>370</td>
<td>671</td>
<td>1041</td>
</tr>
</tbody>
</table>

produced by embryo-splitting. Clearly, after a peak in 1985 to 1986, the number produced yearly declined and reached a plateau at a modest number. There are several possible reasons for this. The splitting equipment employed by most ET practitioners is not easily utilized on the farm. Simplified splitting techniques (182, 233) that do not require the use of a micromanipulator have not been widely adopted by the ET industry. Perhaps more importantly, the inability to know the sex of embryos to be split may discourage breeders from increasing the number of calves produced by this technology. As seen in Table 7, only approximately one-half of the bull calves resulting from splitting have been registered since 1986.

Nuclear Transfer

Mammalian cloning evidenced by the birth of three live mice was announced first in 1981 (92). This achievement resulted from the transfer of nuclei from the inner cell mass of blastocysts into fertilized ova from which the pronuclei were removed. These results have not been repeated (136, 179). In 1983, the success rate of cloning in mice was high, resulting from an improved technique of removing the pronucleus from the recipient egg and from the use of Sendai virus to fuse the donor pronucleus and recipient egg (135). However, because single-cell pronuclear donor embryos were used, this technique only had the potential to produce one copy of each donor embryo. Further attempts to produce clones from multicellular mouse embryos using pronuclear zygotes as the recipient were unsuccessful (136, 179). Additional research led to a clearer understanding of the importance of synchrony between the donor and recipient eggs, involving various nuclear and cytoplasmic factors. Tsunoda et al. (216) were able to produce mouse clones by fusing 8-cell nuclei to enucleated 2-cell blastomeres. Cloning technology was significantly improved when Willadsen (228) showed that single blastomeres from 8- and 16-cell sheep embryos that were fused to enucleated, unfertilized sheep ova supported development to the blastocyst stage. Lambs were born after ET of clones from the 8-cell donors. The percentages of cloned embryos that reached the blastocyst stage were similar whether Sendai virus or an electrofusion apparatus was used to achieve fusion between the donor and recipient membranes. The success in using unfertilized oocytes as recipients led to the concept that the oocyte cytoplasm had the capacity to "reprogram" the mitotic clock of the donor nucleus, thereby turning it back to start the first cell cycle [for discussion, see (204)].

The first cloned calves resulted from the electrofusion of blastomeres from 9 to 15-cell morulae to enucleated oocytes (165). The first report from a commercial bovine cloning program indicated a 23% pregnancy rate in recipients receiving embryos cloned from 5- to 6-d-old embryos composed of between 16 and 64 cells. (17). Pregnancies were obtained from clones derived from both fresh and frozen donor embryos. In addition, pregnancies were obtained from second generation clones, i.e., clones derived from clones. The efficiency from the start of the procedure to the end was low. For example, using blastomeres obtained from 5-d-old donor embryos, electrofusions between donor blastomeres and recipient oocytes were attempted 882 times, resulting in 636 fusions (72%), of which 196 (22%) developed to at least the morula stage after 6 d of culture in sheep oviducts. If all 196 clones had been transferred, the quoted pregnancy rate of 23% would have produced 45 pregnancies, or 5.1% of the original 882 donor blastomeres. This involved the use of recipient oocytes surgically recovered just prior to ovulation from superovulated cattle. A subsequent, much less expensive approach utilized in vitro matured oocytes from ovaries obtained from
2866 HASLER

the slaughterhouse (10). The rate of cloning and the pregnancy rate after ET of clones were similar between recipient oocytes matured in vitro and in vivo. The birth of the first 100 calves resulting from ET of cloned embryos was recently announced by another commercial organization (229). The pregnancy rate in that study (229) was 38% at 90 d of gestation. No technology related to production of the clones was presented, but it was noted that some calves seemed exceptionally large at the time of birth. Although over 30 clonal pregnancies have been produced from one original donor embryo, a large degree of variation exists in the ease with which individual embryos can be cloned (K. Bondioli, personal communication). As of April 1991, the Holstein Association and registered 64 calves resulting from ET of cloned embryos.

CHIMERICAS

According to Greek mythology, chimeras were fire-breathing monsters, with a lion's head, a goat's body, and a serpent's tail. Embryologically, chimeras represent an organism composed of genetically different cell populations. Tarkowski (212) and Mintz (138) independently produced the first mouse chimeras by aggregating two early cleavage stage embryos that combined into a single embryo. The resulting offspring had tissues derived from both embryos. Subsequently, mice with 6 or 8 parents have been produced (156). Mouse chimeras have been extremely valuable in studying germ cell distribution, sexual differentiation, and immunological tolerance. Manipulations to produce chimeras with trophoblast and inner cell mass originating from embryos of different species may provide a means to overcome placental incompatibilities between donors and recipients. For example, neither domestic goats nor sheep carry the embryo of the other species to term. However, chimeras were constructed so that each recipient carried young of the opposite species, whereas the placental tissue was derived from the same species as the recipient (59). Chimeras were produced in cattle by aggregating morulae (22) and by injecting the cells of one embryo into the blastocoelic cavity of another (209). Because of the unpredictable composition of chimeric offspring, it is not likely that this technology will be applied to cattle commercially. However, it may be possible to produce transgenic cattle by inserting the desired gene into embryonic stem (ES) cells, which then would be injected into the blastocoelic cavity of the host embryo, as has been done in mice (for review, see (21)). The resulting chimera may or may not express the transferred gene in its germ line. This would be a slow and expensive approach, requiring two generations to produce the desired genotype, and then only if the gene is in the germ line.

SEXING OF EMBRYOS AND SEMEN

Embryos

Sex determination of preimplantation cattle embryos utilized in ET has economic importance. The sex ratio at birth in dairy cattle produced by artificial insemination was reported to be 50.8% males (60), which compares closely with the 51.1% recorded in ET calves of mixed breeds (103). Male embryos develop, on the average, faster than female embryos, both in vivo (5) and in vitro (6). However, this phenomenon cannot be utilized accurately for sexing embryos on an individual basis (5). Karyotyping is an invasive approach to embryo-sexing that involves obtaining cells from an embryo, preparing metaphase spreads, and identifying the sex chromosomes. Definitive karyotypes were obtained in only 58% of hatched blastocysts (78) and in 62% of d-7 embryos (172). In addition to its low efficiency, this invasive technique is not suitable for embryos requiring an intact zona in order to be frozen or exported.

Two noninvasive approaches to embryo-sexing have been investigated: 1) colorimetric monitoring of X-linked enzyme activity prior to X chromosome inactivation and 2) immunodetection of H-Y antigen with antibodies. Both of these techniques were only moderately accurate in sexing mouse embryos (226) but has not been adopted by the ET industry. Seidel (194) reviewed the economics, advantages, and disadvantages of producing ET calves from sexed embryos based on a hypothetical 80% rate of accuracy.

Development of probes specific for Y chromosomes has led to an evolution of increasingly accurate and rapid sexing techniques. Embryo sex determination with probes specific for Y chromosomes required about 30 h using an immunocytochemical technique (123) and 8 d with a radioactive isotope technique (16). Utilization of the polymerase chain reaction for amplification of DNA sequences specific for Y chromosomes shortened the assay time to a few hours (86, 159). In spite of the speed and accuracy of this technique, it still requires invasion of the zona and the use of a micromanipulator. In addition, current polymerase chain reaction technology is not likely to be usable in on-farm ET situations because of risk of contamination (87). Consequently, the primary use of this technology in the future may be in conjunction with cloning.

Semen

In many cases, even if a group of bovine embryos could be accurately and rapidly sexed, some of them would be discarded. Use of sex-selected semen on superovulated donors would increase, if not double, the number of embryos of the desired sex. Theoretically, semen can be sex selected either by physical separation of the X- and Y-bearing sperm populations or by selectively killing or inactivating one or the other population. Over the years, success has been claimed for many different bovine semen-sexing techniques [reviewed by Amann, (2)]. None of these techniques has been credited with significantly skewing fetal sex ratios in field trials.

Dead sperm from mice (Microtus) were separated by flow cytometry into sex-determining populations with an accuracy of about 80% (162). Improvements in the flow cytometric cell sorting equipment and in staining of the sperm led to 90% accuracy in separation of bull sperm that retained the ability to form pronuclei when injected into hamster oocytes (98). Furthermore, the technology was used to demonstrate that bovine semen samples purportedly enriched in X- or Y-bearing populations by a number of different techniques, were, in fact, unaltered in sex ratio (161). Rabbits inseminated with sperm sorted by flow cytometry produced 81% males and 94% females (99). Because only approximately 350,000 sperm/h can be sorted by flow cytometry, this technology is not currently useful in cattle insemination programs. However, this technique can possibly be used to provide adequate numbers of sex-selected sperm for in vitro fertilization (IVF) procedures.

GENE TRANSFER

Mammalian gene transfer was first described in mice in 1980 (68). Shortly thereafter, Palmiter et al. received a great deal of attention for the production of transgenic mice carrying the structural gene for rat (150) and human (151) growth hormone. Some of the transgenic mice grew twice as large as controls, and growth hormone concentrations were as much as 800 times higher. Half of the transgenics (151) transmitted the growth hormone gene to their offspring, which also grew faster than controls. These early successes in mice catalyzed numerous attempts to produce transgenic livestock; a major effort was directed to pigs. In the previously mentioned first success with growth hormone (150), 7 transgenic mice resulted from 170 injected ova (4%). In contrast, Pursel et al. (167) reported in a review paper that only 73 transgenic pigs were produced out of a total of almost 11,000 (0.7%) ova injected. In addition to the very low efficiencies, there has been no expression to date of a commercially useful transgene in pigs. Although some pigs that were transgenic for growth hormone exhibited enhanced growth rates and feed efficiencies, they exhibited a variety of problems, including lameness, gastric ulcers, and lack of libido.

Progress in producing transgenic livestock has lagged behind that in mice for several reasons. Mouse ova and embryos are readily available and inexpensive. The pronuclei of mice are clearly visible within the cytoplasm, whereas the ova of cattle (221) and pigs (222) must be centrifuged to displace cytoplasmic lipid granules, which permits visualization and micropuncture of the pronuclei. Also, a much greater research effort has been directed to mice and has resulted in production of hundreds of different transgenics, including some very valuable models for the study of human disease [for review, see (90)]. Consequently, as pointed out by Pursel et al. (166), much more is known regarding the conditions favoring gene transfer in mice than in any other species.
There are only a few reports of transgenic cattle. Four transgenic 60-d fetuses were produced from 1325 centrifuged, fertilized ova, of which 819 had been injected with a construct of chloramphenicol acetyltransferase structural gene (13). The same research group (13) later reported the birth of one calf known to be transgenic with a human estrogen receptor gene linked to a skeletal actin promoter (133). Still other pregnancies had not reached term at the time of the report (133); however, only 79 pregnancies were produced from a starting total of 1704 (4.6%) injected ova. One of the preceding studies (13) utilized the sheep oviduct as an intermediate host for 6 d between the time of gene insertion into ova and the nonsurgical transfer of embryos into bovine recipients. Taking into account the failure to recover 16% of the ova from sheep oviducts, 17.6% of injected ova developed into embryos that developed normally. When a much less expensive system, IVF, was utilized for gene-injected ova, a similar percentage (15%) developed into embryos (133). Another laboratory (85) reported a higher proportion of development (17 to 47%) when DNA-injected bovine ova were cultured in ligated rabbit oviducts for 7, 8, or 9 d.

Another approach to gene transfer, mentioned in the section on chimeras, involves the use of ES cells. First described in 1981 (for review, see (177)), they represent pluripotent cells isolated from the inner cell mass of blastocysts. Mouse ES cells can be maintained indefinitely in vitro in an undifferentiated state. They can be injected into the blastocoelic cavity of mouse blastocysts, whereby they contribute to the formation of chimeras. Consequently, DNA can be inserted into ES cells, and chimeric, transgenic mice can be produced (178). Unfortunately, although ES-like cells have been isolated from bovine blastocysts (54, 206), their ability to contribute to the germ layer after transfer to a blastocyst has not been demonstrated. Wilmut et al. (235) suggested a future strategy for more efficient production of bovine clones if bovine ES were to become available. By inserting DNA into ES cells rather than into the pronuclei of early embryos, only those ES cell lines in which the DNA is incorporated would be selected for use. The next unique step in the procedure would be to produce clones by electrofusing single, transgenic ES cells to enucleated oocytes. This would avoid the lengthy and expensive process of producing two generations of cattle, which would be necessary if the ES cells were transferred into blastocysts that would then develop into transgenic chimeras. This system cannot be attempted at present in either cattle, because of the lack of ES cells, or in mice, because it has not proven possible to produce clones with mouse inner cell-mass cells.

Notwithstanding the low rates of success in producing transgenic cattle, there is interest in getting growth hormone gene constructs into dairy cattle with the goal of increased milk production. Because the metallothionein promoter used in previous sheep and pig projects causes continuous transcription of the growth hormone gene, a more suitable promoter must be found (220). At present, few genes controlling traits of economic importance are known in cattle. However, a number of applications for gene transfer in dairy cattle directed at changes in milk composition have been suggested (235). Suggested applications are related to the protein composition of milk because it "is controlled very directly by gene expression" (235). Gene transfer related to milk proteins falls into two general categories: 1) changes in concentrations of particular proteins, which, depending on the protein, could result in improvements in the response of milk to processing, improvements in cheese production, increases in the digestibility of milk by humans, and increased resistance of the transgenic cow to mastitis and 2) production of pharmaceutical proteins. The results of research in mice and sheep that are relevant to these potential applications in dairy cows were reviewed by Wilmot et al. (235, 236). The birth of a transgenic dairy bull calf carrying a gene for the milk-specific production of human lactoferrin was recently announced (107). This calf was one of 2 transgenics (the other of which was a mosaic) out of 21 calves that resulted from 129 embryos transferred to recipients. The project started with the follicular aspiration and in vitro maturation (IVM) of 2297 oocytes, successful IVF of 1358, gene injection of 1154, and development to the blastocyst stage in culture with subsequent ET of 129. Two reports simultaneously published detailed the production of several transgenic...
goats with human-type plasminogen activator in their milk (46) and three transgenic ewes with human antitrypsin in their milk (240). The efficiency of producing the transgenic goats and sheep in terms of the number of embryos that were gene injected was somewhat higher than that described for the in vitro system utilized to produce the transgenic bull. Expensive projects such as these often have strong financial inducements to seek patents for transgenics. The status of patenting transgenics was reviewed by Raines in 1990 (170). On June 13, 1991, US senator Mark Hatfield introduced a bill to impose a 5-yr moratorium on the granting of patents for genetically engineered vertebrate or invertebrate animals (US Senate bill number 1291).

**IVF AND IVC**

The advantages of mice versus cattle in reproductive research have perhaps been most evident in the development of IVM, IVF, and IVC systems. Mouse ova and embryos can be inexpensively obtained in large numbers at any stage of development. Until the recent development of efficient bovine IVF systems, embryo culture experiments were dependent on surgical collection of early embryos or nonsurgical collection of older embryos from superovulated cattle. Seidel's literature review (192) of bovine embryo IVC between 1970 and 1977 documented the widespread use of small sample sizes. Of 47 experimental groups in 15 papers, the embryo sample size was 20 or smaller in 22 groups. Much greater progress was made during this period than was possible with cattle or other livestock by the use of mouse ovum culture systems, which utilized large numbers of ova in multifactorial experiments. However, mouse IVC systems do not necessarily work well for other species, including cattle. For example, the developmental IVC block that occurs at 2 cells in mice occurs at 8 to 16 cells in cattle, and the culture conditions to overcome it differ for the two species (11). The temperature of culture systems for mice is routinely 37°C, and most early work in cattle was performed at 37°C in spite of the fact that the body temperature of bovines averages approximately 39°C. Lenz et al. (122) showed that a significantly higher percentage of ova were fertilized at 39 versus 37°C. Only after large numbers of fertilized bovine ova became available were IVC systems rapidly improved.

Bovine IVF was first achieved in 1977 with semen that was capacitated in the oviduct or uterus of estrual cow or the uterus of a rabbit (93). The first live calf resulting from IVF was born in 1981 (20). During the evolution of IVF, fertilization rates remained modest when media of high ionic strength were used for capacitation of sperm (75, 101). The IVF protocols changed significantly when the effectiveness of capacitation with calcium ionophore (24) and heparin (152) was reported. The first studies of IVF in cattle depended on surgically recovering, via laparotomy or laparoscope, the activated ova from superovulated females (109). Improvements in culture systems led to IVF of IVM ova in numerous laboratories (24). Consequently, IVF and IVC experiments were able to use ova obtained from slaughterhouse ovaries. At present, high rates of IVF are possible with rather different systems. An IVF rate of 88% was achieved with 24-h incubation of ova and sperm using heparin capacitation a medium containing HEPES and modified Tyrode's salt solution, but only 35% fertilization resulted from 6-h incubation (243). In a defined medium using calcium ionophore capacitation, 6-h incubation resulted in 87% fertilization (97).

To be commercially useful, IVF embryos must develop and remain viable for approximately 7 days. In the early 1970s, Lawson et al. (111) showed that 1- to 8-cell bovine embryo cleaved when cultured in the rabbit oviduct, and pregnancy rate was high following ET. Similarly, sheep, rabbit, and cow oviducts were used to culture IVF embryos (114); however, various coculture IVC systems have largely replaced in vivo systems. Bovine trophoblastic vesicles significantly enhanced the development of bovine embryos from the 1-cell to the morula stage (26). More recently, bovine oviductal cells substantially improved the IVC development of bovine embryos (56). Improvement was similar with conditioned medium, from which oviductal cells were removed prior to IVC, and with frozen-thawed conditioned medium (57).

Reported pregnancy rates from ET of IVF and IVC embryos are based on small numbers but are apparently in the 50 to 70% range (56,
Despite the report that blastocysts produced by IVF and IVC contained only one-half the number of cells found in oviduct-cultured embryos (94), also use of a double staining technique demonstrated that the IVC embryos contained only one-half the number of inner cell-mass cells (95). However, d-5 IVF morulae were reported to have similar numbers of cells whether they were produced by IVC or cultured in the rabbit oviduct (48).

It was recently demonstrated that 31% (63) or 47% (140) of IVM and IVF embryos reached the late morula or blastocyst stage in vitro without coculture. Thus, in just a few years bovine culture systems have evolved from the necessity of oviductal culture in an intermediate host, to coculture systems, to very promising IYC successes without coculture.

With the goal of producing twin pregnancies in beef cows, a large commercial project was developed to provide NF embryos for ET into previously inseminated cows (67). Because it is less critical for the IYF embryos in this program to have genetic value than in some other situations, the ova originate from slaughterhouse ovaries, and IVF is by appropriate beef sires. In contrast, application of IVF and IVC in dairy cattle necessitates that the ova come from genetically superior donors. Laparoscopic collection was difficult (109) and has not been widely adopted for use. In addition, timing of collection relative to onset of ovulation is critical. Ultrasound-guided aspiration of antral follicles appears to be a promising technique. Initial studies demonstrated that an average of 5 (160) to 10 (217) ova per attempt could be aspirated on a once or twice weekly basis. There was no detectable, detrimental damage to the ovaries after 17 to 27 aspiration attempts (217). Widespread use of IVF embryos requires that they be frozen. However, few data are available on the survival of frozen-thawed embryos resulting from IVF.

Zona drilling, a technique that involves chemical dissolution of a hole in the zona, may have an application when sperm for IVF are in low concentration. There may be problems with polyspermy associated with zona drilling in human IVF [for review, see (100)]. However, a recent study showed enhanced IVF and IVC development with no increased polyspermy in bovine ova that had been zona drilled (104). Sperm injection involves inserting single sperm into the ooplasm of activated oocytes. In the bovine, exposure of sperm-injected ova to a calcium ionophore greatly enhanced activation of the ova (244). Furthermore, it was recently shown that sperm killed by repeated freezing and thawing could fertilize ova when injected into the ooplasm (69). Four pregnancies resulted from ET of embryos fertilized by injection of dead sperm. Another variation of sperm injection, involving placement of single sperm into the perivitelline space, resulted in low (9 to 11%) fertilization rates (89). Zona drilling and sperm injection are both techniques that might benefit from the availability of semen that was sex selected by flow cytometry (98). Finally, it was recently shown that primary ovarian follicles from 12- to 16-d-old mice could be grown in vitro to the preovulatory stage (169). Also, mice were born after ET of embryos produced from an IVF and IVC system that utilized oocyte-granulosa cell complexes taken from 12-d-old mice and cultured in vitro for 10 d (53).

**GENE PROBES**

Complementary to specific chromosomal sites, DNA probes represent one of many tools developed by molecular geneticists. Applications to livestock have been a very small offshoot of the numerous and, in some cases, very large genetic engineering projects involved primarily with human medicine and plant genetics. As mentioned in the section on sexing of semen, specific DNA probes have been developed that will hybridize with repetitive DNA sequences found exclusively on the bovine Y chromosome (16). In addition, several commercial companies have developed probes for the prolactin (31), κ-casein (137), and β-lactoglobulin (65) loci, all of which are linked to increased milk yield, protein yield, or both. Recently, probes for the weaver trait in Brown Swiss and for bovine leucocyte adhesion deficiency were made commercially available (J. M. Massey, personal communication). Markers closely linked to the bovine polled trait and to red color in Holsteins are being sought. The development of DNA probes such as these is based on the genetic mapping of DNA markers. Markers, which represent restriction fragment length polymorphisms, are mapped by
the study of inheritance patterns in bovine families, combined with DNA hybridization techniques and the use of polymerase chain reaction amplification. Several laboratories are engaged in the development of bovine genomic maps, and at least one commercial laboratory reported that over 200 markers have been identified (66).

Specific DNA probes available for cattle owners are being used on blood samples from cattle of any age and, in the case of the Y-specific probe, on 4- to 10-cell embryo biopsies (86). Continued improvements in IVF and IVC techniques and increasing availability of probes make it highly likely that single blastomeres of embryos will be monitored in various ET projects. The substitution of traditional genetic selection based on phenotype for DNA markers with probe technology has become known as marker-assisted selection (66). Georges (66) suggested the use of the term "velogenetics" for the selective utilization by ET of embryos identified through gene probes. Technology permitting, fetal oocytes would be obtained and matured through IVF and IVC, and superior embryos would again be selected by gene probes and then transferred. This approach significantly decreases the number of recipients needed to produce the best from a potentially large group of IVC embryos. It also decreases generation time from the use of fetal oocytes. Obviously, this technology also synergizes very well with cloning and gene transfer techniques.

CONCLUSIONS

Within the current boundaries of the state of the art, commercial bovine ET routinely results in the production of 5 to 7 usable embryos per superovulated donor and a pregnancy rate of 65 to 80% with fresh embryos and a 55 to 70% pregnancy rate with frozen embryos transferred into suitable recipients. Marked improvements in these pregnancy rates are unlikely. Probably 5 to 10% of the females in any group of recipients are unsuitable for use because of undetected fertility problems. The system may be close to its biological limits of success in the ET of fresh embryos. Field trials involving attempts to enhance pregnancy rate with various hormones and drugs showed no improvement over controls when the pregnancy rate in the untreated, control recipients was at the industry average. There is apparently no substitute for good management of recipients.

There would appear to be more possibility for improved pregnancy rates with frozen-thawed embryos, which are currently 10 to 15 percentage points below those produced from fresh embryos. Survival of embryos may be enhanced by new procedures such as vitrification. This procedure may have a genuine advantage over traditional procedures if the problem of toxic cryoprotectants can be reduced. There are some unpublished indications that vitrification may be the method of choice for IVC embryos produced by IVF and cloning, which are usually considered to be more fragile than in vivo embryos. Also, the delivery system for frozen embryos is currently a limiting factor in the efficient use of frozen embryos in the field. Traditional thawing techniques require the use of a microscope and the movement of thawed embryos through two or three rehydration solutions, after which the embryos are reloaded into straws for transfer. Single-step systems eliminate these procedures and make it practical for a technician to transfer a single frozen embryo on-farm, much as a single insemination is currently provided.

If only small increases are possible in pregnancy rates from transferred embryos, there may be more opportunity to achieve greater numbers of pregnancies per donor by increasing the number of usable embryos per flush. One approach is to learn how to know more exactly when to initiate each superovulation attempt for individual donors. Perhaps a combination of endocrine and ultrasound monitoring will permit the start of superovulation on the optimal day for a given estrous cycle in a donor. However, given the commercial necessity of using sires with quite variable fertility and the variation among donor females, it may not be possible to push fertilization rates of superovulated ova much higher.

The rate of progress within different areas of the ET field is extremely variable. Present knowledge of how to increase the chances of making an ET heifer pregnant has not increased in the past 10 yr, and the same number of embryos are obtained now from superovulated donors as 15 yr ago. As originally practiced, ET only provided a means to increase the number of calves per unit of time from a
donor female. In the early 1980s, specific genetic selection schemes, such as multiple ovulation embryo transfer (MOET) programs, were developed to use ET as a means of decreasing the time necessary to provide a genetic evaluation on bulls. Now, as pointed out by Seidel (198), we have recently started using a number of new technologies, some of which probably were not even thought of 20 yr ago. The polymerase chain reaction, for example, was perfected less than 10 yr ago and is now being utilized in several areas of cattle breeding and ET. Other less exotic but potentially powerful techniques, such as separation of X- and Y-bearing sperm, IVF and IVC, and cloning are starting to make an impact within traditional ET programs. Based on current technology, it seems reasonable that, in the near future, primary follicles from bovine fetuses will be cultured in vitro over a period of many weeks to maturity. As proposed in a volution genetics scheme, four generations of selection could be achieved in 28 mo by utilizing ova from 7-mo-old fetuses, IVM, IVF and IVC, gene probes, and ET. Thus, ET could become part of a genetic selection program in which there is no need for calves to be born. However, there will continue to be a need for efficient ET techniques to produce on-farm pregnancies from the results of this futuristic selection and production technology.

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