

## Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production

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**Abstract.** Structural features of the dairy industry make it well situated to use embryo technologies as tools for enhancing the genetic merit of dairy cattle and improving fertility. Technologies dependent upon embryo transfer have the potential to increase the efficiency of quantitative genetic selection as well as marker-assisted selection, simplify cross-breeding and germplasm conservation procedures and allow incorporation of transgenes into dairy cattle. In addition, embryo technologies may prove useful in improving fertility in infertile populations of lactating cows. The realisation of the promise of embryo technologies has been constrained by suboptimal efficiency in the production of embryos, alterations in embryonic and fetal survival and development associated with *in vitro* embryo production and cloning, as well as other technical and societal concerns. Solutions to many of these constraints are possible and the use of embryo technologies in both nucleus and commercial herds is likely to increase. Eventually, embryo transfer may compete with artificial insemination as a dominant method for establishing pregnancies in dairy cattle.

### Introduction

The widespread use of artificial insemination has led the world dairy industry to achieve spectacular improvements in the genetic merit of dairy cattle for milk yield. The effectiveness of artificial insemination was made possible by structural features of the dairy industry that include the intensive nature of dairy production, the heritability of dairy traits, extensive collection of performance and pedigree records, involvement of government agencies and other organisations in genetic testing programmes and the embrace, by producers, of artificial insemination as a reproductive and genetic technology. Today, embryo transfer offers the opportunity for additional major improvements in the genetic improvement of dairy cattle. Technologies dependent upon embryo transfer have the potential to increase the efficiency of quantitative genetic selection as well as marker-assisted selection, simplify cross-breeding and germplasm conservation procedures and allow incorporation of transgenes into dairy cattle. In addition, embryo technologies may prove useful in overcoming some of the large decline in fertility of lactating dairy cattle that has occurred over the past 30–40 years (Royal *et al.* 2000; Stevenson 2001; Washburn *et al.* 2002; López-Gatius 2003).

The same features of the dairy industry that have allowed artificial insemination to become such an effective tool in genetic improvement can also contribute to the realisation of the possibilities that embryo technologies offer. Moreover, the institutional infrastructure that has developed to support the use of artificial insemination (bull studs, government and private breeding organisations, semen sales organisations), as well as new organisations devoted exclusively

to commercialisation of embryo technologies, are in place to provide the framework for facilitating the dissemination of embryo technologies in the dairy industry. One can envision a time when embryo transfer will compete with artificial insemination as a dominant method for reproduction in both registered and commercial dairy cattle. In such a scenario, it is likely that embryos would be produced predominately at large-scale, commercial embryo-production facilities. Compared with the typical embryo produced for transfer today, which is used primarily in elite herds, cost concerns would dictate that the bulk of embryos in such a production system would likely be derived by *in vitro* techniques for use in commercial herds. Many embryos could be made 'value added' by the use of one or more techniques that allow for gender selection, screening for inheritance of specific gene alleles and possible inclusion of a transgene.

The world dairy industry seems to be a long way from such a revolutionary change in breeding practices. As currently practised, embryo technologies are of limited importance in the dairy industry. Most calves are born as a result of artificial insemination or natural mating. Some breeding organisations use embryo transfer as a genetic selection tool, but most genetic selection is based primarily on sire progeny testing. The use of embryo transfer as a tool for enhancing reproductive efficiency is limited by the high cost of producing embryos and problems associated with the *in vitro* production of embryos that lead to poor embryo freezability, abnormal fetuses and calves and altered sex ratios (Hasler 2000; Thompson and Peterson 2000; van Wagendonk-de Leeuw *et al.* 2000; Peterson and Lee 2003). The opportunities of

nuclear cloning for expanding desirable genotypes and for improving the efficiency of the production of transgenic animals are limited by problems of abortion, congenital abnormalities and post-natal death attendant with this technique (Cibelli *et al.* 2002; Tsunoda and Kato 2002). Moreover, there are regulatory and societal problems attendant with the production of cloned animals and animals containing transgenes (Evans 1999; Galli *et al.* 2003).

Despite the poor penetration of embryo transfer into the dairy industry, technologies to allow for the increased use of embryos are either in place or should be soon. The purpose of the present review is to highlight some features of the current status of embryo technologies in the dairy industry and to relate the promise that these technologies offer to the dairy industry with the technological advances that will be required to realise that promise.

### **The scope of embryo transfer**

The efforts of the International Embryo Transfer Society (IETS) Data Retrieval Committee have been invaluable in characterising the degree to which embryo transfers are performed in livestock. Summaries of the Committee's activity are published annually in the IETS Newsletter (see Thibier 1999, 2001, 2002 for the data cited in this section). The accuracy of the data are limited somewhat because of problems inherent in collection of such data. Also, the published summaries do not allow ready distinctions to be made between dairy and beef recipients and this limits inferences that can be made regarding the scope of embryo transfer in the dairy industry. Nonetheless, it is possible to use the data to derive several conclusions regarding the impact of embryo technologies on cattle production in general and these conclusions are likely to be relevant to dairy cattle in particular.

Embryo transfer is still an uncommon event when placed in the context of the large number of cows in the world. For example, in 2001, the total number of embryo transfers performed for both dairy and beef recipients was approximately 500 000, whereas there were 125 000 000 milk cows worldwide in 2001 (USDA Foreign Agricultural Service 2003). For all types of embryos, the total number of transfers reached a peak in 2000: there were an estimated 570 301 transfers performed in that year compared with 472 622 transfers performed in 1998. The recent decline could be due to changes in economic conditions, as well as to the outbreaks of foot and mouth disease (Thibier 2002).

The embryo derived *in vivo* remains the predominant type of embryo used in embryo transfer. In 2000, 93% of recorded transfers were of embryos produced *in vivo* (528 540) compared with 7% for embryos produced *in vitro* (41 761). The same percentage of embryos was derived *in vivo* in 1998. However, the predominance of the *in vivo*-produced (IVP) embryo is less pronounced in Asia and Europe. In 2000, IVP embryos made up 17% of the reported embryos used for

transfer in Asia (predominantly Japan, Korea and Taiwan) compared with 12% for Europe and 0.8% for North America. Complete data are not available on the proportion of IVP embryos that are derived by transvaginal aspiration of oocytes from follicles (i.e. oocyte pickup (OPU)) compared with oocytes obtained from abattoir material. In Europe, the proportion of IVP embryos produced by OPU grew from 53% in 2000 to 76% in 2001. In contrast, all IVP blastocysts reported as being produced in Korea in 2000 were derived from ovaries collected from the abattoir. Currently (i.e. 2001), approximately half the transferred embryos recorded are cryopreserved: this is true for embryos produced *in vivo* (48.8% frozen) as well as for IVP embryos (49.2%).

North America and Europe are the predominant practitioners of embryo transfer in terms of total numbers of transfers, representing 49% and 22% of all transfers of *in vivo*-derived embryos in 2001. However, given the disparities in total dairy cattle numbers, data on the total number of transfers do not give an indication of the degree of penetration of embryo technologies in different countries. Japan, for example, although only having 971 000 milk cows in 2001 (Danish Milk Board; <http://www.mejeri.dk/view.asp?ID=547>), recorded 53 510 transfers of IVP embryos. In contrast, France, with 4 194 700 milk cows in 2001 (Weiler and Poschacher 2003), recorded 32 922 transfers. Thus, the ratio of embryos transferred to milk cows was 1 : 18 for Japan and 1 : 127 for France. To make accurate estimates of the impact of embryo technologies on a per milk cow basis, it will be necessary to breakdown numbers of total transfers into those for dairy and beef recipients.

### **Current and potential role of embryo technology in genetic improvement**

#### *Selection for quantitative traits*

Most production traits in dairy cattle are quantitative genetic traits in which phenotype is controlled by actions of numerous genes as well as by the changes in the activity of those genes caused by the animal's environment. Heritability estimates the proportion of the variation in a trait determined by additive genetic variation. The rate of genetic gain of a particular quantitative trait is the product of the intensity of selection, the amount of additive genetic variation and the accuracy of selection (the ability to determine accurately which animals are genetically superior) divided by the generation interval. The intensity of selection is determined by the proportion of animals retained for breeding. The accuracy of selection is determined by the heritability (i.e. the greater the proportion of variation due to genetics, the more accurate selection will be), as well as by the number of records for the animal and its relatives.

Artificial insemination has had such a beneficial impact on dairy cattle selection because it led to an increase in both the intensity of selection (because only a very small number of bulls are required to generate progeny) and the accuracy of

selection (because bulls are identified for genetic merit based on performance records of their daughters and other relatives). In addition, artificial insemination has allowed rapid dissemination of superior genetics from elite or nucleus herds to commercial herds. Embryo technologies can provide a further boost to the rate of genetic gain realised in dairy cattle for the same reasons as for artificial insemination. Embryo technologies can also be used to reduce generation interval through the collection of oocytes by OPU from immature heifers, although optimisation of this approach will depend upon overcoming problems of oocyte competence in prepubertal animals (Khatir *et al.* 1998; Salamone *et al.* 2001). The use of embryo technologies also makes possible factorial mating schemes, where females are bred to several males to increase the rate of genetic gain (Dekkers 1992; Leitch *et al.* 1994).

The impact of the most common embryo technologies in use today (superovulation and OPU–IVP schemes) on the rate of genetic selection will be less than for artificial insemination because the change in intensity and accuracy of selection is less than for artificial insemination. However, technological advances can change the situation. Recent advances in directing embryonic stem cells to differentiate into oocytes (Hübner *et al.* 2003) may make it possible at some point to produce female gametes in culture. In this way, the female could be made to produce gametes as abundantly as the male. Closer on the horizon is the use of nuclear cloning in breeding programmes. The use of nuclear cloning could, conceivably, allow for very intense selection of dams of sires and dams of dams (Dematawewa and Berger 1998) and could increase the accuracy of selection because performance can be recorded on multiple clones of an individual under different environments. Cloning could also allow rapid dissemination of superior genetics from nucleus herds to commercial herds. Although cloning technology is largely experimental, commercial companies producing bovine clones for agricultural purposes exist, including Cyagra in the US (Worcester, MA), the Laboratorio di Technologie della Riproduzione in Italy (Cremona) and RAB Australia/Clone International in Australia (Albury, NSW). Large-scale production of clones is being achieved in Japan and Korea. The IETS Data Retrieval Committee reported that Korea transferred 2526 cloned embryos in 2000 (Thibier 2001).

van Arendonk and Bijma (2003) have discussed aspects of breeding theory that describe limitations in the genetic change that can be achieved by the use of embryo technologies. Selection strategies, by their nature, reduce genetic variance and, because rate of genetic gain is dependent upon the amount of genetic variance, this reduction in genetic variance means that genetic gain decreases over time. Genetic variance can be partitioned into variance for each parent as well as Mendelian sampling variance. Because genetic selection reduces the genetic variance of the parents, but not the Mendelian sampling variance, data on siblings become

less important than the individual's record and progeny records. Meuwissen (1998) estimated that use of multiple ovulation–embryo transfer (MOET) in combination with efforts to reduce generation interval could increase the rate of genetic gain by 15%, but also the rate of inbreeding (by 80%). The degree of inbreeding in intensively selected dairy cattle breeds is accelerating (Thompson *et al.* 2000b, 2000c) and may eventually reach the point where inbreeding depression due to accumulation of homozygous recessive loci becomes severe and cross-breeding becomes necessary (Hansen 2000). Consideration of inbreeding in genetic strategies using embryo technologies limits the rate of genetic gain that can be achieved. For example, van Arendonk and Bijma (2003) have argued that required constraints on juvenile breeding schemes to reduce inbreeding will negate any possible increase in genetic gain caused by shortening of the generation interval.

Currently, the major use of embryo technologies in genetic selection is to increase the number of offspring from dams used to produce young bulls for progeny testing. As of May 2003, 66 of the Top 100 Total Production Index (TPI) International Holstein bulls were produced by embryo transfer. Reducing the number of females used to produce offspring can have significant effects on the rate of genetic gain. In one model, van Arendonk and Bijma (2003) estimated that increasing the number of female offspring from two to eight, and thereby reducing the number of females selected to produce offspring by a factor of 4, caused a 57% increase in the rate of genetic gain while also causing an increase in inbreeding. In another example, it was estimated that optimal economic returns under New Zealand conditions were obtained when reproductive technologies were implemented to decrease the number of cows required to produce one potential sire from the current 3.2 cows per bull calf to two cows per bull calf (Smeaton *et al.* 2003).

Smeaton and Vivanco (2001) estimated the economic return from using embryo technologies to increase genetic selection for heifer replacements under New Zealand conditions. Depending on the scenario, the rate of genetic gain was improved by 28%–33% after 10 years. However, genetic variation was reduced as well, suggesting that rates of genetic gain may be overestimated and that inbreeding would be a concern.

#### *Selection for specific alleles*

There are intensive efforts to identify specific gene loci that influence quantitative traits (i.e. quantitative trait loci (QTL)) so that marker-assisted selection could be practised to select animals inheriting alleles that confer increased production (for a review, see Kappes 1999). Markers can be for the actual gene influencing production or for regions of DNA in close proximity to the gene. Several markers have been identified in dairy cattle, including markers for milk production traits (Spelman *et al.* 2002; Freyer *et al.* 2003; Viitala *et al.* 2003) and resistance to mastitis (Klungland *et al.* 2001).

The eventual sequence of the bovine genome should lead to an increase in the number of QTL. Genetic markers have also been identified for several traits inherited in a Mendelian manner, such as coat colour (Joerg *et al.* 1996; Seitz *et al.* 1999), the polled condition (Schmutz *et al.* 1995) and several diseases caused by recessive alleles, including a deficiency of uridine monophosphate synthase (DUMPS; Schwenger *et al.* 1994) and bovine leucocyte adhesion deficiency syndrome (BLAD; Tammen *et al.* 1996).

Among the advantages of marker-assisted selection are an increase in the accuracy of selection (because of elimination of Mendelian sampling variance and variation in the phenotype caused by environmental factors) and a reduction in generation interval (because genotyping can be performed before the animal expresses the phenotype). Technological advances in techniques for pre-implantation genetic diagnosis by polymerase chain reaction or fluorescence *in situ* hybridization (Bredbacka 2001) mean that, as genetic markers are identified, it will be possible to select embryos for transfer based on a determination of the specific allelic inheritance of that embryo. Bovine embryos have been genotyped for DUMPS (Schwenger *et al.* 1994) and transgenes (Chen *et al.* 2002). Commercial testing of embryos for BLAD is now performed at Cyagra/Em Tran (Elizabethtown, PA, USA).

One genetically controlled trait of great interest to dairy producers is gender. In addition to allowing dairy producers to produce a greater proportion of female calves, sex selection to produce males or females can also improve the rate of genetic selection in genetic selection programmes by reducing the number of pregnancies required to produce potential sires and dams (Dematawewa and Berger 1998). Polymerase chain reaction-based sexing of bovine embryos is very accurate (Lopes *et al.* 2001) and a system for performing embryo sexing in the field is available commercially from AB Technology (Pullman, WA, USA). The development of sexed semen (Seidel 2003), which is now available commercially from companies such as Cogent (Chester, UK) and Goyaike (Escobar, Argentina), offers another alternative for regulating the sex ratio in embryos by use of such semen in superovulation or IVP protocols. The use of sexed semen in embryo production procedures will be a more cost-effective way to skew the sex ratio in cattle than methods based on embryo biopsy, particularly for embryos of lower economic value, such as may be used in commercial herds.

#### *Transgenics*

Integration of transgenes can be incorporated into mammalian cells, including those of cattle, by microinjection of embryos (Eyestone 1999; Behboodi *et al.* 2001), retroviral transmission (Chan *et al.* 1998), nuclear cloning using genetically modified cells as nuclei donors (Chen *et al.* 2002; Brophy *et al.* 2003) and by *in vitro* fertilisation using transgenic sperm prepared by restriction enzyme-mediated insertion (Shemesh *et al.* 2000). Transgenics has proven

successful as a method for producing pharmaceutically useful products in cow milk (van Berkel *et al.* 2002), but this use of transgenic technology will have little impact on the dairy industry. To use transgenesis to improve the efficiency of dairy production, it will be necessary to identify candidate genes that can influence production traits. Recently, it was demonstrated that the production of transgenic cows with additional copies of the genes for bovine  $\beta$ - and  $\kappa$ -casein resulted in increases in the amount of both caseins in milk (Brophy *et al.* 2003). Such an achievement illustrates the promise of transgenesis for increasing the yield of a valuable commodity, as well as changing the composition of milk to improve its use in specific food-manufacturing processes.

Few other specific genes have been identified as possible candidates for transgenesis into cattle used for milk production. It is likely that progress in the bovine genome sequencing project, identifying QTL and in understanding the molecular basis of animal function will lead to new genes of interest. Although it may be more efficient to select for QTL allelic variants using marker-assisted selection than to increase gene frequency using transgenesis, the latter technique allows genes identified by QTL mapping in another species to be incorporated into cattle. A possible example is the Booroola gene controlling multiple ovulation in sheep (Galloway *et al.* 2002), which may be useful to incorporate into cattle used for dairy–beef systems. Another example is that of genes encoding for antibacterial proteins to increase disease resistance (Kerr *et al.* 2001). Other possible genes that may be useful in dairy cattle transgenics and that have not yet been mapped are the *slick* gene, which controls hair growth in the Senepol and Carora breeds and that confers thermotolerance (Olson *et al.* 2003), and genes in *Bos indicus* that control thermotolerance at the cellular level (Paula-Lopes *et al.* 2003).

#### *Germplasm conservation*

Embryo technology can also be used as a method for germplasm conservation to maintain rare breeds or to reconstitute genetically valuable animals lost through injury or disease. Wells *et al.* (1998) used cloning technology to reproduce the last remaining cow of the Enderby Island cattle breed and Cyagra produced a clone of the Top 100 TPI Holstein bull Regancrest Emory Derry after his death. Another valuable bull that is listed in the Top 100 TPI International Index, Mtoto, has been cloned by the Laboratorio di Technologie della Riproduzione (Galli *et al.* 2003).

#### **Prospects for the use of embryo transfer as a reproductive management tool**

Pregnancy rates to first insemination in the UK declined from 56% in the period 1975–1982 to 40% during the period 1995–1998 (Royal *et al.* 2000). This historical decline in fertility of lactating dairy cows, which has been seen elsewhere around the world where intensive dairying has been the regular

practice (Lucy 2001; Stevenson 2001; Washburn *et al.* 2002; López-Gatius 2003), is an impetus for the development of new approaches to increase the calving rate in lactating dairy cows. The concept of using embryo transfer to achieve this purpose is based on the hypothesis that a significant proportion of pregnancy failures in inseminated lactating cows is the result of disruption in events occurring before the time when embryos are ordinarily transferred (Days 6–8 after oestrus). Embryo transfer involves a process of selection and only those embryos that achieve development to the morula or blastocyst stage are transferred. Thus, embryo transfer by-passes or reduces pregnancy failure due to anovulation, ovulation of oocytes with low developmental competence, compromised oviducal or uterine environment, insemination errors or damaged spermatozoa.

Whether embryo transfer can prove to be widely effective for increasing the pregnancy rate in lactating dairy cattle will depend upon: (1) the degree to which infertility in dairy cows is due to factors correctable by embryo transfer (i.e. events before the day of transfer); (2) the inherent developmental potential of the embryo being transferred; and (3) the level of fertility in the herd achieved by artificial insemination. Embryo transfer must also be an economical alternative to artificial insemination; the embryos must be inexpensive to produce and to transfer. So-called 'surplus' embryos that are produced by superovulation but not transferred into elite cows are one potential source of inexpensive embryos. However, the supply of these embryos will be insufficient if embryo transfer is practised on a wide scale in commercial herds. Rather, the most likely inexpensive source of embryos for large-scale embryo transfer programmes in commercial herds will be those produced from slaughterhouse oocytes by IVP. The costs of performing embryo transfer on commercial herds could be reduced by providing specialised training to dairy farm personnel so that they can become proficient in performing embryo transfer.

The causes of infertility in dairy cows are not completely understood and are multifactorial in origin (Lucy 2001). Poor oocyte competence is one source of infertility because the proportion of oocytes that cleaved and developed in culture following IVP was lower for cows of high genetic merit for milk yield than for cows of low genetic merit (Snijders *et al.* 2000). In addition, there was a reduction in embryo development score at Day 5 after ovulation for lactating cows compared with non-lactating cows (Sartori *et al.* 2002). There are other possible causes of infertility in lactating cows that exist after Days 6–8 of pregnancy that could, potentially, compromise embryonic survival after transfer. These include decreased circulating concentrations of progesterone (de la Sota *et al.* 1993; Chagas e Silva *et al.* 2002a; Sangsritavong *et al.* 2002) and insulin-like growth factor (IGF)-I (de la Sota *et al.* 1993). There was, however, no difference in late embryonic loss (between Days 28 and 84 of pregnancy) between lactating cows or heifers and no effect of milk yield or genetic

merit for milk yield on late embryonic loss (Silke *et al.* 2002). However, lactating cows that lost body condition did have higher late embryonic loss than other cows (Silke *et al.* 2002).

Few studies have been performed to determine pregnancy rates in lactating dairy cows used as embryo transfer recipients and results are inconsistent as to whether pregnancy rates following transfer are lower in lactating cows compared with heifers or dry cows (Table 1). Although Putney *et al.* (1988) detected little difference in pregnancy rate, Dochi *et al.* (1998) and Hasler (2001) found pregnancy rates in cows were lower than in heifers and Chagas e Silva *et al.* (2002a) found that pregnancy and calving rates were lower for lactating cows than heifers when frozen embryos were transferred, but not when fresh embryos were transferred. Taken together, these results suggest some inadequacy in the uterine environment of lactating cows that is more likely to compromise embryonic survival when embryos have lower developmental potential.

The usefulness of embryo transfer as a reproductive management tool will depend on the level of fertility in the herd. Thus, when pregnancy rates per artificial insemination are high, embryo transfer would not represent a viable alternative to artificial insemination as a reproductive technology. For example, in a study in Wisconsin (Sartori *et al.* 2003), the pregnancy rate at Days 25–32 for lactating cows with a single ovulation and receiving embryos produced by superovulation (mostly frozen–thawed embryos) was 40.3% compared with 35.6% for cows with a single ovulation and bred by artificial insemination. For cows with multiple ovulations, pregnancy rates were 51.7% and 50.0% for embryo transfer and insemination, respectively. In contrast, when pregnancy rates to artificial insemination are low, embryo transfer can be effective at increasing the pregnancy rate in lactating cows during periods of heat stress. Results of studies demonstrating the usefulness of embryo transfer in heat-stressed cows are shown in Fig. 1. In each of four studies (Putney *et al.* 1989; Ambrose *et al.* 1999; Drost *et al.* 1999; Al-Katanani *et al.* 2002), pregnancy rates for embryo transfer recipients were greater than for cows bred by artificial insemination. The only time embryo transfer was not effective was when IVP embryos were transferred after cryopreservation.

More widespread application of embryo transfer as a reproductive management tool will require further efforts to increase the pregnancy rates following transfer into lactating recipients. Pregnancy rates following transfer can be increased by: (1) improvements in culture systems so that embryos have a high potential for post-transfer development; (2) development of procedures to identify those embryos most suited for transfer; and (3) manipulation of the recipient to improve embryo survival after transfer. Recent research suggests that post-transfer embryonic survival can be improved by the addition of 9-*cis*-retinoic acid to maturation medium (Hidalgo *et al.* 2003) and vitamin E to embryo culture medium (Olson and Seidel 2000a). Pregnancy rates in

**Table 1. Pregnancy and calving rates achieved following embryo transfer in studies chosen to illustrate the effectiveness of embryo transfer in lactating recipients**

| Animal status                       | Embryo type                       | No. cows | Pregnancy rate<br>Days 40–60 (%) | Calving<br>rate (%) | Reference                          |
|-------------------------------------|-----------------------------------|----------|----------------------------------|---------------------|------------------------------------|
| Dairy cows                          |                                   |          |                                  |                     | Putney <i>et al.</i> 1988          |
| Lactating                           | SO-fresh                          | 199      | 53.7                             |                     |                                    |
| Dry                                 | SO-fresh                          | 444      | 59.7                             |                     |                                    |
| Dairy heifers                       | SO-fresh                          | 677      | 63.1                             |                     |                                    |
| British Friesian                    |                                   |          |                                  |                     | McEvoy <i>et al.</i> 1995          |
| Lactating                           | IVP-fresh <sup>a</sup>            | 97       |                                  | 49.5                |                                    |
| Lactating                           | IVP-fresh and frozen <sup>b</sup> | 97       |                                  | 34.6                |                                    |
| Cows, mostly dairy <sup>c</sup>     | SO-frozen                         | 482      | 28.9 <sup>d</sup>                |                     | Dochi <i>et al.</i> 1998           |
| Heifers, mostly dairy <sup>c</sup>  | SO-frozen                         | 791      | 46.2                             |                     |                                    |
| Dairy                               |                                   |          |                                  |                     | Hasler 2001                        |
| Cows                                | SO-fresh                          | 844      | 52.8                             |                     |                                    |
| Heifers                             | SO-fresh                          | 6612     | 70.5                             |                     |                                    |
| Cows                                | SO-frozen                         | 518      | 47.1                             |                     |                                    |
| Heifers                             | SO-frozen                         | 3477     | 60.9                             |                     |                                    |
| Holstein                            |                                   |          |                                  |                     | Chagas e Silva <i>et al.</i> 2002a |
| Lactating cows                      | SO-fresh                          | 82       | 50.0                             | 48.8                |                                    |
| Heifers                             | SO-fresh                          | 46       | 58.7                             | 52.2                |                                    |
| Lactating cows                      | SO-frozen                         | 83       | 34.9                             | 28.9                |                                    |
| Heifers                             | SO-frozen                         | 196      | 50.5                             | 48.5                |                                    |
| Holstein                            |                                   |          |                                  |                     | Moreira <i>et al.</i> 2002         |
| Lactating, cool season <sup>e</sup> | SO-frozen                         | 140      | 44.3                             |                     |                                    |
| Lactating, hot season <sup>e</sup>  | SO-frozen                         | 41       | 34.2                             |                     |                                    |

IVP, *in vitro*-produced; SO, superovulation.

<sup>a</sup>Two embryos transferred (36.4% twins born); year 1 of study.

<sup>b</sup>Two embryos transferred (29.1% twins born); year 2 of study.

<sup>c</sup>A total of 89% of recipients were Holstein, with the remainder various dairy and beef breeds; pregnancy diagnosis after Day 60.

<sup>d</sup>The arithmetic mean of least-squares means for cows in parities 1, 2 and 3 is presented.

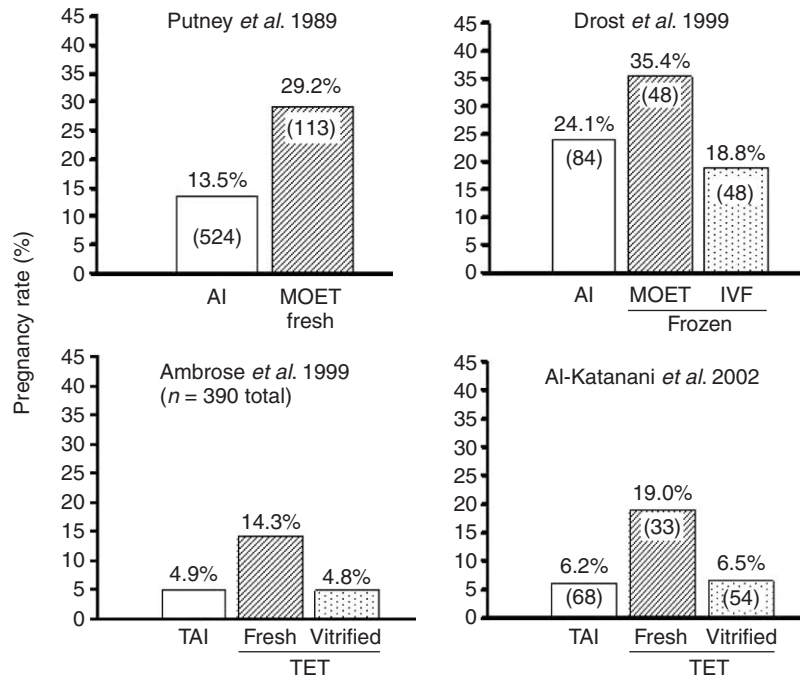
<sup>e</sup>Some donors and recipients received somatotropin, which increased pregnancy rate in a non-additive manner.

heat-stressed, lactating recipients were higher when receiving embryos cultured with IGF-I than when receiving control embryos (Block *et al.* 2003a). Such an effect could represent a general beneficial effect of IGF-I or indicate that IGF-I enhances embryonic resistance to heat stress. The ability to identify blastocysts that have a high probability for post-transfer survival is still based largely on morphological criteria (Van Soom *et al.* 2001) and there is a need to develop accurate, non-invasive procedures for determining embryo quality. Efforts to manipulate the recipient to increase pregnancy rate have been based largely on hormonal therapy. Treatment of lactating recipients with bovine somatotropin increased pregnancy rate in lactating cows (Moreira *et al.* 2002), but not in non-lactating animals (Block *et al.* 2003b; Hasler *et al.* 2003). Perhaps the beneficial effect of somatotropin relates to restoring the low concentrations of IGF-I in lactating cows (de la Sota *et al.* 1993). Treatment with gonadotrophin-releasing hormone (GnRH) at Day 11 after the putative day of ovulation tended to increase the pregnancy rate in lactating cows exposed to heat stress (Block *et al.* 2003a), but there was no beneficial effect of GnRH when administered at the time of transfer (Smith and Grimmer 2002).

Another potential constraint to the use of embryo transfer in lactating cows is the fact that oestrus is often of a short duration or low intensity (Dransfield *et al.* 1998). However, this problem can be overcome by the use of timed embryo transfer based on the ovulation synchronization protocols used for timed artificial insemination. Pregnancy rates following timed embryo transfer can be similar to those following transfer after a detected oestrus (Bó *et al.* 2002).

### The efficiency of embryo technologies

The growth of embryo technologies in the dairy industry has been constrained by the high cost of producing a live calf. For example, Smeaton and Vivanco (2001) estimated that a New Zealand farmer could reap a profit by using embryo transfer to increase the rate of genetic gain in the production of dairy heifer replacements only if the cost of the embryo technology was less than US\$16 more per cow per year. Similarly, Dematawewa and Berger (1998) estimated the breakeven cost for producing a clone for use in progeny testing schemes as US\$84 or less. Unfortunately, losses limit the efficiency of embryo production at each step in the process, beginning with recruitment of oocytes for embryo



**Fig. 1.** Effectiveness of embryo transfer for improving pregnancy rates of lactating dairy cows during heat stress. Experiments were conducted in Florida during the hot period of the year. Pregnancy was determined by rectal palpation at 40–60 days of gestation. AI, artificial insemination; MOET, multiple ovulation embryo transfer; IVF, embryo produced by *in vitro* fertilisation; TAI, timed artificial insemination; TET, timed embryo transfer. Note that all embryos in experiments by Ambrose *et al.* (1999) and Al-Katanani *et al.* (2002) were produced *in vitro*. Numbers above each bar represent the percentage pregnant and numbers in parentheses are the number of recipients.

production (either by superstimulation *in vivo* or retrieval from the animal for *in vitro* maturation and fertilisation) and including fertilisation failure, poor development of fertilised embryos to a stage suitable for transfer, failure of transferred embryos to establish a pregnancy, abortions and post-natal death losses. In this section, some factors that limit the efficiency of embryo production and increase its cost will be discussed.

#### Oocyte yield

The number of oocytes at birth in the newborn heifer is approximately 150 000 (Erickson 1966). From this bounty of gametes, the high-producing dairy cow usually produces only two to five calves in her lifetime. This lifetime yield of offspring can be substantially increased by MOET schemes and by IVP protocols, but inefficiencies remain and most oocytes remained unharvested.

Using superovulation, the average yield of transferrable embryos generated in dairy cattle is around four to five (Putney *et al.* 1988; Bousquet *et al.* 1999; Bó *et al.* 2002; Moreira *et al.* 2002; Merton *et al.* 2003). Embryo yield has been reported to be lower for lactating dairy cows than dairy heifers (Chagas e Silva *et al.* 2002b). Superovulation response is variable and unpredictable, although increased emphasis on

using purified preparations of follicle-stimulating hormone (FSH) and initiating superovulation protocols coincident with emergence of a follicular wave can reduce variability and increase yield (Kanitz *et al.* 2002; Mapletoft *et al.* 2002). One limitation with MOET schemes is the frequency at which animals can be subjected to superovulation (approximately every 30 days), so that the annual yield of embryos is usually no more than approximately 60 transferrable embryos. In addition, the procedure can only be used on animals that are either not pregnant or where it is not desired to achieve pregnancy. Thus, lactating cows can only be subjected to superovulation for a limited period after calving if one is attempting to maintain a 365-day calving interval.

Collection of oocytes from females by ultrasound-guided transvaginal collection of oocytes (i.e. OPU) offers the opportunity to produce embryos from cows not otherwise available for embryo collection, such as pregnant cows and prepubertal animals. Moreover, OPU can be conducted at intervals as frequently as twice a week, with a high yield of competent oocytes; the more frequent the collections, the lower the oocyte yield and the higher the oocyte quality (Merton *et al.* 2003). The reported number of transferrable embryos produced in a single round of OPU varies from 0.4 to 4.7 (Bousquet *et al.* 1999; Goodhand *et al.* 1999; Faber *et al.*

2003; Galli *et al.* 2003; Merton *et al.* 2003). Removal of the dominant follicle (Merton *et al.* 2003) and superstimulation with FSH can increase oocyte and embryo yield (Goodhand *et al.* 1999; Sirard *et al.* 1999). Moreover, delaying OPU until 36–48 h after the last FSH injection improved oocyte competence, as determined by blastocyst production following IVP (Sirard *et al.* 1999; Blondin *et al.* 2002). The technician is also an important source of variation in the success of OPU procedures. Compared with superovulation, the production of embryos using OPU requires more technical skill and sophisticated equipment and there is variation between teams of technicians in the success of OPU (Merton *et al.* 2003). However, the overall production of embryos using OPU can be high. Merton *et al.* (2003) estimated that approximately 50 freezable embryos can be produced per cow per year using superovulation compared with 150 embryos per year for OPU.

Ovaries collected at the abattoir are a ready source of large numbers of oocytes. Moreover, the proportion of oocytes that develop into blastocysts following *in vitro* maturation, fertilisation and culture is sometimes higher for oocytes collected from abattoir material than for oocytes retrieved by OPU (Merton *et al.* 2003); indeed, oocyte competence increases for approximately 4 h post-mortem (Blondin *et al.* 1997). The procedures for IVP are also simpler to perform when using oocytes recovered from abattoir ovaries because maturation, fertilisation and embryo culture can be performed in bulk. Should large-scale use of embryo transfer take place in the dairy industry, the abattoir-derived oocyte is a logical source for the production of such mass-produced embryos. Even though information is not available on the dam, the genetic merit of such embryos need not be inferior. Rutledge (1997) has shown that the genetic ability for the milk yield of cows sent to slaughter is only slightly less than that for the average cow in the herd. Moreover, bulls of high genetic merit can be used to produce embryos from these oocytes in a cost-efficient way because the cost of a straw of semen is amortized over several embryos. The use of the abattoir-derived oocyte in commercial beef embryo production is being practiced in Italy and Japan (Galli *et al.* 2003).

Although not practical today, new technologies under development have the potential to transform methods of oocyte production in the future. Research to understand the regulation of pre-antral follicular growth may lead to the development of procedures for culturing pre-antral follicles to a stage compatible with *in vitro* maturation and fertilisation. This has been achieved in the mouse (Eppig and O'Brien 1998) and live offspring have resulted (Liu *et al.* 2001). As mentioned earlier, it has been shown recently that mouse embryonic stem cells can give rise to oogonia that enter meiosis, recruit adjacent cells to organise into follicle-like structures and can undergo parthenogenetic activation and become blastocysts (Hübner *et al.* 2003). Despite problems in cattle in generating embryonic stem cells, such an accomplishment in mice offers the possibility that, at some point, stem cell lines

can be generated in cattle from genetically superior females to allow an unlimited number of oocytes to be derived.

#### *Inefficiencies associated with blastocyst production in vitro*

The proportion of bovine oocytes that become transferable embryos remains suboptimal, usually being no more than 50% and typically more like 15%–30%. The failure to achieve higher blastocyst yield is due to a combination of factors, including collection of oocytes with low competence for fertilisation and development (Hansen 2002), inadequate conditions during maturation and fertilisation (Rizos *et al.* 2002a), and suboptimal embryo culture systems (Thompson 2000). Efforts to improve oocyte maturation include developing protocols allowing the oocyte to undergo a period of arrest before maturation (Sirard *et al.* 1999; Blondin *et al.* 2002), performing OPU collections in superovulated recipients before and 24 h after the luteinizing hormone surge (Dieleman *et al.* 2002) and modifying the maturation medium to include epidermal growth factor (Lonergan *et al.* 1996), anti-oxidants (Mizushima and Fukui 2001) and 9-*cis*-retinoic acid (Hidalgo *et al.* 2003). Among the promising developments for improving conditions for embryo culture are supplementing culture medium with IGF-I (Matsui *et al.* 1995; Palma *et al.* 1997; Block *et al.* 2003a), vitamin E (Olson and Seidel 2000a) and hyaluronan (Stojkovic *et al.* 2002), reducing concentrations of oxygen (Iwata *et al.* 1998; Olson and Seidel 2000b; Thompson *et al.* 2000a) and glucose (Iwata *et al.* 1998; Gutierrez-Adan *et al.* 2001) and inhibiting oxidative phosphorylation (Thompson *et al.* 2000a). However, the effects of most of these treatments on embryo survival after transfer have not been determined.

The embryo produced *in vitro* is often different from the embryo derived *in vivo* in terms of morphology (Massip *et al.* 1995; Crosier *et al.* 2001; Rizos *et al.* 2002b), gene expression (Bertolini *et al.* 2002; Lazzari *et al.* 2002; Lonergan *et al.* 2003) and metabolism (Krisher *et al.* 1999; Khurana and Niemann 2000). Alterations caused by IVP can reduce embryo survival after transfer (Farin and Farin 1995; Farin *et al.* 1999; Hasler *et al.* 2003), freezability (Drost *et al.* 1999; Enright *et al.* 2000; Al-Katanani *et al.* 2002; Rizos *et al.* 2002a) and have consequences for conceptus development after transfer. Post-transfer problems associated with transfer of IVP embryos include alterations in allanosis development, increased rates of abortion, increased growth and aberrant gene expression of muscle, large calf size, increased neonatal mortality and a sex ratio skewed towards males (Farin and Farin 1995; van Wagtenonk-de Leeuw *et al.* 1998, 2000; Hasler 2000; Thompson and Peterson 2000; Behboodi *et al.* 2001; Crosier *et al.* 2002; Lazzari *et al.* 2002; Block *et al.* 2003a; Peterson and Lee 2003). An example of one IVP calf born with large calf syndrome is shown in Fig. 2. The birth of calves like this can compromise the health of the dam and be unsettling to producers.



**Fig. 2.** Calf born with large-calf syndrome. The calf, which was born on 20 December 1999, weighed 98 kg at birth. The photo was taken at 2 days of age. Also shown is Rocio Rivera, who produced the calf by *in vitro* production with an embryo culture medium consisting of CR1aa + 5% (v/v) fetal calf serum added at Day 5 after insemination. The calf died on 26 December 1999.

Reducing the problems of abnormal fetal development is critical to ensuring that technologies based on IVP gain widespread use in the dairy industry because farmers will be reluctant to transfer embryos that have a high potential for yielding a defective calf. Progress on these issues is underway. Problems associated with IVP can be reduced by removal of the serum in embryo culture medium (Wrenzycki *et al.* 1999; Rizos *et al.* 2003), although removal of the serum does not always prevent increases in calf birth weight due to IVP (van Wagendonk-de Leeuw *et al.* 2000; Lazzari *et al.* 2002). The increased proportion of male calves following embryo transfer with IVP embryos is likely due to the preferential toxicity of glucose for female embryos (Gutierrez-Adan *et al.* 2001; Larson *et al.* 2001; Peippo *et al.* 2001). There is some promise that freezability of IVP embryos can be improved by culture in hyaluronan (Stojkovic *et al.* 2002) or by improvements in vitrification protocols (Arav *et al.* 2002).

Somatic cell cloning is also associated with the same problems as IVP and in an often exaggerated manner, especially with respect to embryonic, fetal and neonatal losses (for reviews, see Cibelli *et al.* 2002; Tsunoda and Kato 2002; Edwards *et al.* 2003). There are also unresolved issues as to whether cloned animals that survive until adulthood have normal physiological function. There are reports that cloned heifers (Enright *et al.* 2002; Pace *et al.* 2002) have reproductive activity similar to that for controls. Repeated cloning to produce six generations of sequentially cloned mice resulted in apparently normal individuals with respect to learning, strength, coordination and ageing, although telomere length became longer with each generation (Wakayama *et al.* 2000). However, in another study, the average lifespan of cloned mice was less than controls (Ogonuki *et al.* 2002) and it is

possible that some clones will prove to have shorter lifespans either because of premature cellular senescence due to altered telomere lengths or epigenetic changes that persist into adulthood and make the animal more susceptible to specific diseases. The lifespan issue is one that has not yet been resolved by detailed experimentation and there is evidence that the impact of cloning on telomere length depends on species, cell line used for nuclear transfer and other factors (Kühholzer-Cabot and Brem 2002).

Another issue to be resolved is the degree to which epigenetic variation and differences in mitochondrial genetics (Steinborn *et al.* 2002) lead to between-clone variation in phenotype. In one experiment, the gene expression pattern in neonatal mouse clones was not identical to the founder animal (Humpherys *et al.* 2002) and, in another experiment, cloned mice were found to exhibit obesity not seen in control animals produced by IVP or in animals produced by natural mating of clones (Tamashiro *et al.* 2002). In cattle, there were no differences in the genome-wide methylation status between adult, lactating clones and age-matched lactating control cows (Cezar *et al.* 2003), although the possibility that a few genes did differ cannot be ruled out.

### Synopsis

The rate of progress in the development of embryo technologies has been phenomenal. The report of the birth of the first IVP calf occurred a little more than 20 years ago (Brackett *et al.* 1982) and the first mammal produced by nuclear cloning with adult cells was born in 1996, less than 8 years ago (Wilmut *et al.* 1997). Today, companies involved in the use of embryo technologies for beef and dairy production are located throughout much of the world. Given the difficulty in recreating and manipulating developmental events in the laboratory that usually occur in the closely regulated environment of the ovary, oviduct and uterus, it is not surprising that embryo technologies face technical issues that limit their usefulness in cattle-production systems. At the same time, the rapid nature of the progress in developing embryo technologies to date is an indication that many of the technical barriers existing today will be overcome. The key to greater implementation of embryo technologies in the dairy industry for improvement of genetic merit and for enhancing reproductive function will be the development of procedures that are cost-effective for the commercial producer as well as the producer of elite cattle. The potential power of these technologies means that the dairy cattle industry must come to terms with how the technologies can be implemented best to improve genetic merit without compromising genetic variance.

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