Chapter 1
Historic frame of reference

The first transfer of a bovine embryo was reported in 1949 (Umbaugh, 1949), and the first calf from embryo transfer in 1951 (Willett ***et al.***, 1951). Milestones in the development of this technology have been evaluated from the point of view of their significance to our current knowledge of reproduction and to the improvement of animal agriculture (Betteridge, 1981; Adams, 1982; Betteridge, 1986). Application of embryo transfer to the cattle industry began in the early 1970s when European dual-purpose breeds of cattle became popular in North America, Australia and New Zealand. Breeders and speculators sought means to circumvent the high costs and lengthy quarantine periods linked to the importation of European breeding stock and to capitalize on premium prices that progeny from these rare dams and sires could command.

Thus, demand for embryo transfer services existed in advance of the ability of veterinarians and reproductive physiologists to supply them. This considerable economic incentive, however, inspired rapid development of practical techniques for superovulation and surgical recovery and transfer of bovine embryos, and the establishment of clinics to sell the technology to the public. Because the value of embryo transfer offspring was based on scarcity, however, the exercise was self-defeating. The inflated market for European dual-purpose breeds in North America collapsed abruptly in 1977 because the numbers of these animals increased markedly as a result of embryo transfer. During this short-lived boom, nevertheless, techniques had been improved and costs reduced. Notable was the development of procedures for non-surgical recovery and transfer and cryopreservation of embryos. With these improvements and a more realistic economic motivation, the industry now plays a useful role in the cattle industries of many countries (Seidel and Seidel, 1981).

Chapter 2
Applications of embryo transfer

INCREASE REPRODUCTION OF FEMALES

The main use of embryo transfer in cattle has been to amplify reproductive rates of valuable females. Because of low reproductive rates and long generation intervals, embryo transfer is especially useful in this species. Cattle may be valuable for many reasons, including scarcity, proven genetic value, or having unique characteristics such as disease resistance. Ideally, embryo transfer is used to satisfy both genetic and financial objectives simultaneously, i.e. milk or meat production increase or greater efficiency, and the investment returns financial benefits as well. It is possible to increase reproductive rates of valuable cows by an average of tenfold or more in a given year and fivefold or more per lifetime with current embryo transfer techniques. This amplification will increase substantially as new technologies, such as maturing oocytes ***in vitro***, are perfected.

Increased reproductive rates of donors with routine embryo transfer procedures are nearly always at the expense of reduced reproductive rates of recipients. This means that fewer calves will be produced from donors and recipients combined that if both reproduced conventionally. This is because potential recipients must be on hand to await embryos, which means recipients do not become pregnant as soon as they would conventionally. This waiting can be minimized with good management, and is often justified by the increased reproductive rates of donors. An exception to decreased reproduction with embryo transfer can occur when a substantial proportion of recipients receive two embryos (or two demi-embryos). Such twinning programmes, however, are currently a very minor use of embryo transfer.

The degree of amplification of reproduction of donors by embryo transfer will be considered in detail and in the context of other factors in Chapter 12. A caveat is that people tend to advertise their spectacular successes and minimize their failures. There is even a bias in scientific reports because experiments with poor results tend not to be published. Thus one must be careful to analyse the complete picture. Success rates can be especially poor in new programmes and in hostile environments; with good management, they can also be excellent.

CIRCUMVENT INFERTILITY

It is possible to obtain offspring from genetically valuable cows that have become infertile due to injury, disease, or age by means of superovulation and embryo transfer (Bowen ***et al.***, 1978; Elsden ***et al.***, 1979), although success rates are only about one-third of those achieved with healthy, fertile donors. Infertile heifers and cows with genetically caused subfertility should not be propagated. Although success rates are low, it is possible to recover oocytes from genetically valuable, moribund cows, fertilize them ***in vitro***, transfer them, and obtain offspring (Shea, 1978); techniques require only systematic optimization before they are applied in the field.

Choice of superovulatory and recovery procedures varies with the type of infertility (Table 1). For example, cows with cystic ovaries might be most effectively superovulated according to a regimen based on the insertion and removal of a progestin implant or intravaginal device rather than on injection of prostaglandin F_2 alpha.

IMPORT/EXPORT

The desire to improve herds of cattle, to increase variation in the gene pool, and to introduce new breeds, has motivated the importation/exportation of breeding stock. In the past, trade has been primarily either in young animals with outstanding pedigrees or semen. Animals have the advantage of being 100 percent of the desired new genotype and are usually of breeding age so that impact on the herd is immediate. The disadvantages are that costs, especially for transportation, are very high, and that there is a high morbidity rate if the new environment is markedly different in management, climate, or endemic pathogens. Moreover, if cows are imported, the genetic influence on the general population is limited until their bull calves reach breeding age. While the genetic influence of imported semen can be distributed over a larger portion of the herd, offspring have only 50 percent of the new genes and will not become producing members of the herd for two to three years. With imported embryos, the resulting offspring have 100 percent of the desired genes, but as with artificial insemination, it will be several years until the resulting animals become producers. The relative advantages and disadvantages of importing animals, semen and embryos are summarized in Table 2.

TABLE 1
Therapy for various types of infertility based on embryo transfer procedures

Cause of infertility	Procedure
Uterine infection	In cases of persistent pyometra in which volumes of fluid and debris build up in the uterus, it is often efficacious to flush the uterus with 0.9 percent NaCl until the recovered fluid is clear, and then to administer prostaglandin F_2 alpha. Penicillin or oxytetracycline may be infused for three to four days but, if not done carefully, can do more harm than good. Normal embryo transfer procedures can be followed after the next oestrus. In cases of subclinical or recurrent endometritis, repeated superovulation and embryo transfer may result in the “rescue” of viable embryos from the toxic environment to develop in the healthy uterus of the recipient.
Repeat breeder	Heifers in this category should not be propagated. With normally cycling, parous cows one should try at least three times to recover a single ovum to diagnose if there is ovulation, and if the ovum has been fertilized. It may be helpful to use raw semen. If a morphologically normal embryo at the expected stage of development is recovered, consider progesterone therapy during gestation.
Aged repeat breeder	Problem likely due to “worn-out” uterus; normal superovulatory treatment and transfer of embryos to uterus of younger recipient is often effective.
Chronic abortion	Recovery of the embryo before the abortion-causing condition is active can often circumvent the resulting infertility.
Cystic ovarian disease	Often superovulatory treatments based on the insertion and removal of a progestin implant rather than on prostaglandin F_2 alpha injection are effective (see Mapletoft ***et al.,*** 1980, for details). If the cystic ovarian condition appears to be hereditary, no propagation should be attempted.
Adhesions of ovaries or	Superovulation increases the chances that at least a few ova will be picked up if there are adhesions. A combination of superovulation with

blocked oviducts	surgical recovery or laparoscopy helps to diagnose the cause of the infertility and can result in recovery of viable embryos for transfer. Some conditions can be corrected surgically (e.g., flushing plugs of debris from the oviduct), although relieving adhesions is rarely of lasting benefit. In the future, it may be efficacious to recover oocytes from the ovaries of such cows laparoscopically for fertilization ***in vitro.***

Valid and serious sanitary and economic concerns have resulted in strict regulation of trade in breeding stock and semen (International Zoosanitary Code, 1986). For example, in order to prevent the introduction of infectious diseases, calves and adult cattle must often undergo a lengthy quarantine and testing programme before they may be imported; the collection and processing of semen is similarly regulated. Conditions of importation vary widely and frequently require months to years to carry out, many of them in advance of a proposed sales agreement. Thus, logistics are quite complicated and costly.

TABLE 2
Comparison of importing germplasm as postparturient animals, as semen or as embryos

Advantages	Disadvantages
Postparturient animals	
Animals productive quickly	Expensive
	Animals often succumb to disease
	Chance of introducing exotic disease
	Complex transportation logistics
	Limited immediate genetic influence if females are imported
Semen	
Inexpensive	Need to grade up to get pure-bred animals*
Low risk of disease transmission	Need for AI technology
Hybrid vigour, F_1 and F_2*	Long wait until animals productive
Simple transportation logistics	
Passive immunity from native dam	
Embryos	
Very low risk of disease transmission	Need for ET technology
Costs may be lower than animals	Long wait until animals productive
Simple transportation logistics	
Passive immunity from native dam	

* If changing from one breed to another

Few infectious organisms are spread by routine embryo transfer procedures (Hare, 1986), and such procedures do not result in rates of abortion or incidence of abnormalities among offspring that differ from those of the normal population of cattle (King ***et al.***, 1985). Such characteristics of embryos as protection by the zona pellucida, minute size, exposure only to a very circumscribed environment, and lack of body systems to host pathogens (e.g. respiratory, digestive, circulatory systems) result in significant barriers to infection. In addition, it is possible to wash, treat, and physically examine the individual embryo, which provides additional, very effective safeguards. Thus, importation of genetic material in the form of embryos is innately safer than importation of post-natal animals or semen (Stringfellow, 1985; Hare and Seidel, 1987). Regulatory officials recognize this fact and are drafting realistic conditions for importation that are less time-consuming than those required

for post-natal animals. Health regulations pertaining to the collection and processing of the semen used to produce embryos intended for export, however, may still apply.

The decreased risk of compromising the health of national herds in itself makes embryo transfer the method of choice for importing breeding stock in many cases. Other advantages are that the offspring will be 100 percent of the desired genotype and will adapt more readily to the new environment because of passive immunity acquired from the recipient. There is still a potential for problems of unthriftiness and disappointing production if the type of cow is inappropriate for the new environment, as for example, a high-producing North American dairy cow would be for an extensive management system based on range foraging.

Costs of importing embryos are often lower than importing post-natal animals, and it is possible to change the breed of a herd within a single generation. Nevertheless, costs are still a great deal higher than importing semen, and conventional embryo transfer remains a less potent tool for genetic progress than artificial insemination programmes based on intensive selection.

MOET PROGRAMMES

The term MOET, multiple ovulation and embryo transfer was coined by Nicholas and Smith (1983) to consider embryo transfer and related technology in the context of optimizing genetic improvement of cattle. Most MOET schemes require one or a few large nucleus herds. The resulting genetic improvement would be disseminated to the general population by embryo transfer, artificial insemination, or more practically by young bulls to be used in natural breeding. MOET procedures rely on advanced technology, which at first seems inappropriate for less developed countries. However, nearly all of the advanced technical procedures would be carried out at one or a few central sites, which may be especially appropriate for some applications in many less developed countries. There are both practical and theoretical advantages to MOET, which will be discussed after a description of MOET procedures.

To appreciate why MOET procedures are effective, it is necessary to consider briefly conventional animal breeding procedures. Improved animals result from the following practices.

Identify genetically valuable animals accurately so that the best can be used as parents of the next generation. This can be done by performance testing, progeny testing and pedigree analysis. Performance testing measures the animal itself, e.g. rate and efficiency of growth, milk production or the degree of calving difficulty (as a trait of the calf or the mother). Because there is some genetic component to such performance, a partial measure of genetic value is obtained. Advantages of performance testing include low cost, rapid availability of data and ability to test many or all of the animals in the population. Disadvantages are low accuracy (in many cases one measurement per animal), confusion by environmental factors (in some cases deliberate manipulation in order to make certain animals look good) and sex limitations, e.g. one cannot performance test a bull for milk production.

Progeny testing measures traits in offspring of animals and in many respects is the converse of performance testing. It is not sex limited and can be done over a variety of environments in ways that are not likely to be misleading. However, it is expensive, data are not available until the next generation and only limited numbers of animals can be progeny tested. Accurate progeny testing is difficult with cows because of limited numbers of offspring. In many cases a performance test is used to pre-select animals for progeny testing.

Pedigree analysis simply uses information available on relatives, for example, the genetic value of parents or siblings.

Use high selection intensity so that only the best animals genetically are selected as parents. Genetically superior cattle are propagated selectively by artificial insemination and embryo transfer, by keeping offspring from only the best cows and by using only the few best bulls in natural breeding systems. Because of low reproductive rates of cows, most genetic progress is made by selecting bulls and obtaining many progeny per bull.

Minimize the generation interval. If selection steps can be made every three years, genetic progress will be nearly twice what it would be with selection every six years. Progeny testing lengthens the generation interval because data are not available until the next generation, which often dissipates the advantages of the increased accuracy.

The main objective of MOET is to select on the basis of performance tests and pedigree analysis in order to reduce the generation interval, in comparison to progeny testing procedures used currently. Selection intensity is increased on the female side with superovulation and embryo transfer. In MOET schemes, genetic progress increases slightly if embryos are split so that more accurate assessments of genetic value are obtained. This occurs because two phenotypic measurements are made on the same genotype. Furthermore, reliability of measurements is increased because all animals are kept in one or a few herds under controlled conditions, and thus can be compared to each other accurately without bias.

MOET procedures should be especially useful for improving milk production (Nicholas and Smith, 1983) therefore this discussion is based on dairy cattle. However, MOET can also be used for beef cattle. Populations of the order of 1 000 animals (donors, calves, recipients) are required to make MOET procedures work optimally without increasing inbreeding more than 0.5 percent per generation.

To begin such a herd, the best females available are gathered, superovulated and bred to the best bulls available. The embryos are then collected and bisected to maximize production (or, in the future, cloned by nuclear transplantation so that many identical females result per embryo). Sexing of sperm or embryos would further improve this system. The progeny are then compared for such traits as milk production and milk composition, and the best sets are used to become parents of the next generation. Embryos may also be collected from heifers and then frozen, so that embryos are available as soon as selection has occurred in the fourth or fifth month of the first lactation. These procedures are repeated continually with each generation.

After several generations, the average genetic value of selected animals in these nucleus herds will exceed the average genetic value of selected animals from outside the herd, even most progeny-tested bulls. Thus, bulls that are siblings of the best females in the nucleus herd are selected as sires for the next MOET generation and for the general population because they are genetically superior (on the average) to bulls available elsewhere, even though they are not progeny tested.

A variation on MOET that may be an option in the future is to use cloning in another context. Some embryos of a clone would be frozen so that if a particular clone proved to be valuable, many cloned embryos would be made by serial nuclear transplantation. These embryos could then be disseminated to the population, thus greatly increasing the genetic value of the population. For genetic progress to continue, females of the best clones would be mated to the best bulls to obtain even better clones in the long run. It is likely to be some years before such a scheme becomes feasible, even in developed countries. However, MOET schemes are potentially very useful without cloning at all, and may be especially valuable in the absence of contemporaneous genetic improvement schemes, which require sophisticated data gathering systems. Such systems for cattle populations are frequently unavailable in less-developed countries.

TWINNING

Most of the world's cattle are of beef breeds, and most of the calves born to dairy cows are used primarily for meat. Beef production is inherently inefficient biologically. Since about 70 percent of nutrients consumed by dams are for body maintenance and the other 30 percent go to producing the foetus and milk to feed the calf (Seidel, 1981b), it should theoretically be possible to produce twice as many calves with only 30 percent more nutrients if cattle had twins. Probably a 60 percent increase in feed costs is more realistic due to higher morbidity and mortality and slower growth rates with twins. In practice, one would probably decrease cow numbers and increase calf numbers (due to twins) so that the amount of nutrients used per farm would remain constant. There is a great advantage to twinning if nutrients are limited and management capabilities are high.

There are dozens of studies (for example, Anderson, 1978) demonstrating that twinning can be successful, both in terms of calf survival and high fertility with acceptable intervals between parturition and conception while cows are suckling twins. However, most of these studies were conducted by highly motivated researchers with considerable resources. In routine cattle management programmes, farmers universally show an aversion to twinning, because the calves often die or do poorly, and there is a higher incidence among cows of death, retained placenta, decreased milk production and lower fertility after parturition. The majority of these problems are attributable to the fact that the farmers were not expecting twins to be born, and therefore, did not adjust management procedures accordingly.

Many studies have involved the production of twins by embryo transfer (Anderson, 1978). To date, other methods, for example administering low doses of gonadotrophins to cause twin ovulations, have not been efficacious, while twinning by embryo transfer has proved too complex and expensive to be profitable. The schemes that have worked at all for farmers have been heavily subsidized. However, in some situations, it may be profitable to obtain oocytes from slaughterhouse ovaries, mature and fertilize them ***in vitro***, culture them to the early blastocyst stage and freeze them for twinning purposes (Lu ***et al.***, 1987). Such an enterprise would have to rely on huge volumes of embryo production, and delivery of embryo transfer services very inexpensively, for example by systems similar to artificial insemination programmes.

At the time of writing, a huge effort is under way in several countries in the European Economic Community to exploit twinning by embryo transfer because of a marked shortage of calves to grow for beef purposes. The shortage and consequent high value of calves was caused by surplus numbers of dairy cows being used for milk production, whereas traditionally they produced most of the calves for beef. This shortage of calves is likely to moderate as more farmers switch to production of beef calves, but mean while considerable progress in technology of oocyte maturation and ***in vitro*** fertilization for commercial purposes is likely. Undoubtedly, there will be other situations in various countries in which twinning cattle will be profitable. However, due to the complex management requirements, such programmes will be appropriate only in very special situations, at least for the rest of this century.

EMBRYO TRANSFER AS PART OF OTHER BIOTECHNOLOGY

Many other potential applications of embryo transfer could be cited, but only three will be considered.

Detection of carriers of undesirable Mendelian recessive traits via embryo transfer is very effective for both cows and bulls. For certain traits like syndactyly and dwarfism, there is a shortage of homozygous, fertile females to use as mates for suspected carrier bulls. Embryo transfer is an obvious means of amplifying gamete (and embryo) production of such females so that bulls can be tested for carrier status. Embryo transfer also provides a method of testing daughters of carrier bulls to determine which half does not have the deleterious allele. Since at least seven defect-free calves are required to be 99 percent certain that a given animal is not a carrier, it would normally take longer than the average reproductive lifespan of a cow to test this; furthermore, all the calves produced during the

test would be carriers because of using semen from a double recessive bull. With superovulation and embryo transfer, one or two courses of superovulation will provide enough embryos to test most cows; moreover, recipients can be twinned and the foetuses examined at about two months of gestation to diagnose many of these defects. Thus, with embryo transfer a quick answer is possible to a problem that is otherwise intractable.

Exploitation of other technologies that require manipulating the oocyte or embryo ***in vitro*** depends on good embryo transfer techniques for success. Such technologies include ***in vitro*** fertilization, sexing, production of transgenic animals, bisection of embryos and cloning by nuclear transplantation.

From the standpoint of research, embryo transfer is a powerful tool for separating foetal and maternal effects. For example, is declining reproductive efficiency with age due to an aged ovum or an aged reproductive tract? Applications in research are considered in detail by Kuzan and Seidel (1986). As is described in Chapter 10, production of identical twin animals by transfer of bisected embryos for use as experimental animals greatly reduces research costs since much smaller treatment groups are needed to obtain statistically significant results.

Chapter 3
Managing donor and recipient herds

DONOR SELECTION

There are two broad criteria for selecting donors for most embryo transfer programmes: (1) genetic superiority, that is animals that contribute to the genetic objectives of the programme, and (2) likelihood of producing large numbers of usable embryos. In the majority of embryo transfer programmes, in both developed and less-developed countries, superiority is determined in practice by market forces. For example, it makes good sense to select donors whose offspring can be sold at a profit above embryo transfer expenses. Obviously, it is inappropriate to produce animals that will not be accepted by farmers. Educational programmes and demonstration projects may be required before new types of cattle can be introduced into an area.

In some cases, the sole criterion for selection is scarcity, and embryo transfer is used to increase numbers of animals available. This may be required to determine whether a new type of animal fits the environment or to get enough animals to develop appropriate management systems. If the objective is to conserve germplasm of indigenous breeds by cryopreservation of embryos, one may wish to select a random sample of donors (and sires) or insure that a range of phenotypes within the breed is used.

In many cases, objectives measures of genetic superiority can be used, for example milk production, milk composition, growth rates, calving ease and disease resistance. Because phenotypic superiority may not indicate genetic superiority, it is usually desirable to consult someone trained in animal breeding so that the best donors are selected to meet objectives.

Selection of donors for embryo production is frequently overlooked; indeed, in using embryo transfer to circumvent infertility one often selects against this trait. Although embryo production should be secondary to genetic superiority, it should be considered seriously. Healthy, cycling cattle with a history of high fertility make the most successful donors. When there is a choice, animals without calving problems, such as retained placenta, should be used. Donors at least two months post-partum produce more embryos than those closer to calving. Young cows seem to yield slightly more usable embryos than heifers under some conditions (Hasler ***et al.***, 1987). Lactation in either beef or dairy cows does not decrease response to superovulation provided that cows are cycling and not losing weight. Extremely fat cows make poor donors, both because they do not respond well to superovulation and because their reproductive tracts are more difficult to manipulate. Sick animals usually do not produce many good embryos.

Frequently, the factors described above are beyond the control of personnel working in an embryo transfer programme, but the following steps can sometimes be taken. First, when there is a choice, use animals for donors that are intrinsically fertile, are at least two months post-partum and otherwise in good reproductive health. Second, encourage management practices that minimize or circumvent potential problems such as having animals gaining

weight at the time of embryo transfer. Third, develop strategies to deal with problems caused by the embryo transfer programme itself. For example, repeated superovulation of the same donor means that it will not be going through an annual reproductive cycle; cows tend to get fat under such circumstances.

SELECTION OF SIRES

Since half of the genes come from the male, it is extremely important to use genetically superior bulls. In fact, selecting the male is usually more important than selecting the donor female because males will normally be bred to many females and can be selected more accurately than females. Likewise, it is necessary to select fertile bulls and fertile semen. Sperm transport is inhibited in superovulated cows (Hawk, 1988), which makes it especially important to use high quality semen.

MANAGEMENT OF DONORS

Donors are located either on the farm under production conditions or at an embryo transfer centre, frequently under intensive management. Both situations have advantages and disadvantages. Keeping donors on the farm is usually the less expensive alternative. Also, less input for labour and management is required on the part of embryo transfer personnel, especially if donors are lactating. However, it is extremely important to have good communication with the personnel who manage donors on the farm. Simple, written protocols are essential. Planning is very complex, since a series of steps occurs over a period of weeks. In most cases, personnel will visit the farm to collect the embryos. However, donors can be trucked to the embryo transfer centre without lowering success rates if they are not stressed unduly. Three to four hours of travel in trucks or trailers does not seem to be a problem.

It is important that suitable facilities are available for on-the-farm programmes. This includes equipment for handling cattle, such as chutes and head catches, a refrigerator for keeping drugs, and an appropriate site to work with the embryos. Personnel at the farm must have certain skills and, above all, be extremely conscientious. For best results, palpation skills are required to determine if a corpus luteum is present when donors are superovulated; in most cases artificial insemination and semen handling skills are also needed.

If it is necessary for embryo transfer personnel to go to the farm frequently to perform most of the steps, the financial advantage of having donors on the farm will be lost. On the average, success rates with donors on the farm are lower than if donors are at an embryo transfer facility, primarily because they are monitored more closely at embryo transfer centres. Nevertheless, success rates on some farms are as good as or better than those at embryo transfer centres. Obviously, success is highly correlated with the management skills of the farmer. In most cases of embryo collection on the farm, the facilities and management capabilities are also needed for recipients (see below).

If reasonable numbers of donors (e.g. 25–100) are assembled at a central facility, embryo collection, processing and transfer can be done efficiently. Clearly, under these circumstances, facilities for large numbers of donors (and recipients) are required. Concentrating cattle in this way invites disease. This is exacerbated by assembling animals from various sources which may introduce diseases from each source. The net result is that great attention must be directed to herd health management for successful programmes, particularly when valuable cattle are involved. This includes quarantine of incoming animals, and vaccination and testing programmes. If done systematically and conscientiously, herd health programmes tailored to local conditions are usually very effective.

Another problem encountered when concentrating cattle is their feed. In many climates pasture is not an option for a central embryo transfer facility because cattle would be too scattered to manage efficiently. Local conditions, such as availability of different kinds of

feed, amount of rainfall, etc., dictate how this problem will be dealt with. Proper nutrition is extremely important, but beyond the scope of this manual. Donors definitely should not be losing weight at the time of superovulation.

Other aspects of donor management, such as oestrus detection, are covered below. Conscientious and gentle handling of donors is a very important component of successful donor management.

SELECTION OF RECIPIENTS

As with donors, management of recipients is fundamentally different if they are located at an embryo transfer centre rather than on the farm; therefore, these situations will be considered separately. Frequently, using the farmer's own recipients simply is not feasible because of insufficient suitable animals. Another consideration is management capabilities of the farmer. In North America, one simple criterion has been useful to assess management capabilities; if successful artificial insemination programmes have been carried out in previous years, there is a reasonable chance that an embryo transfer programme using the farmer's recipients will be successful. If artificial insemination programmes have failed, embryo transfer is also likely to fail.

A common question is whether to use cows or heifers as recipients. The big advantage in using cows is there is less difficulty with calving (King ***et al.***, 1985). Conversely, heifers are easier to manage than cows because they are not lactating, which requires milking or calf management. One must be cautious if candidates for recipients are not lactating (and not pregnant), because this frequently means that they were culled for reasons that will make them subfertile. Heifers generally have higher fertility than cows, especially dairy cows. On the other hand, it is more difficult to transfer embryos non-surgically in heifers than in cows. Clearly, cows and heifers both have disadvantages and advantages, and the choice depends on careful analysis of the factors just described for each particular situation.

MANAGEMENT OF RECIPIENTS

On-the-farm recipient programmes must be tailored to the resources available. The following are essential:

- Cycling animals, not exposed to bulls;
- Animals in a good state of nutrition, preferably gaining weight;
- Herd health programmes, particularly for brucellosis, trichomoniasis, and other abortifacients. Note that severe problems can occur if a herd becomes free of a particular pathogenic organism and younger animals do not develop protective antibodies. This can be prevented by vaccination against diseases likely to recur. This also provides antibodies in colostrum;
- A simple, permanent means of easily identifying each animal;
- Facilities to keep many animals in close proximity to the treatment area for synchronization treatments, oestrus detection and embryo transfer;
- Appropriate chutes, head catches and pens for sorting;
- An excellent oestrus detection programme;
- Conscientious personnel.

On-farm recipient programmes can often only be efficient during a two-to three-week period of the year two to three months after the peak calving season. At other times, there are often too few animals available as suitable recipients without keeping cows non-pregnant for long periods. Also, with such short programmes, potential recipients which are not used or are not becoming pregnant to embryo transfer, can become pregnant at the next oestrous cycle and thus do not need to be culled because of not fitting into the annual breeding/calving programme. Sometimes such considerations are minor; for example, dairy heifers may be more available for extended times than beef heifers. Very large herds

without a pronounced breeding season have more flexibility.

Management of recipient herds at embryo transfer centres can be a huge logistical undertaking. Effective herds usually number at least several hundred head if pregnant recipients are included. These herds are the most expensive aspect of embryo transfer programmes, primarily because normal, healthy, fertile females are deliberately kept non-pregnant (essentially out of production) waiting for embryos. The waiting time can be minimized by using frozen embryos as a buffer and having a smaller recipient herd. Excess embryos are frozen when insufficient recipients are available; conversely, when excess recipients are available, frozen embryos can be thawed and transferred. Despite the advantages of frozen embryos, recipients often remain in the recipient herd a fairly long time. Many fail to get pregnant, and usually a definitive pregnancy diagnosis can be made only four to six weeks after embryo transfer (five to seven weeks after oestrus), at which time non-pregnant animals can be recycled. Blood or milk can be tested for progesterone 22–24 days after oestrus or returns to oestrus can be monitored to identify some of the non-pregnant recipients sooner so that they can be reused.

How many opportunities should a recipient have to become pregnant before culling her? The consensus is that in nearly all circumstances a recipient should be given a second chance and, in most circumstances, a third chance. However, after three failures following transfer of an embryo of good to excellent quality with no evidence of technical problems, the recipient should be culled as a poor risk. The exception is if pregnancy rates are rather poor on the average, e.g. below 45 percent per transfer, in which case recipients should possibly be given a fourth chance.

The next logistical question is to what extent oestrus synchronization should be used (see Chapter 8 for details on methods). About 5 percent of recipients should be in oestrus spontaneously on any given day. If embryo transfer work is done on an essentially daily basis, with an average of two or more donors per day, most of the recipients coming into oestrus will be required as recipients, and oestrus synchronization will not be advantageous on most days. On the other hand, if embryo transfer is scheduled less than three or four times per week, oestrus synchronization will be very useful. There is some evidence that oestrus synchronization with prostaglandins may result in higher pregnancy rates than natural oestrus (Hasler ***et al.***, 1987).

The following summarizes essential components of successful recipient management at an embryo transfer centre:

- A steady source of high quality animals;
- A sound herd health programme including quarantine, vaccination, blood testing, parasite treatment, etc.;
- Adequate physical facilities, including feed storage;
- A good, planned nutrition programme;
- An easy-to-use but complete record-keeping system;
- Permanent, easily visible, positive identification;
- A sound strategy to minimize inventory of animals while maintaining an adequate supply along with a plan to market culls profitably;
- A good oestrus-detection programme;
- Conscientious, patient personnel; in many cultures, on the average, women are superior to men in this respect;
- A financial policy requiring timely payment of bills, not releasing animals until bills are paid, etc.

The specific details of facilities, health tests, types of animals used, nutrition and so on vary so much from country to country that they are not presented here. However, the general principles discussed apply universally; failure to consider these factors will result in a propensity to failure of the entire programme.

OESTRUS DETECTION

Nearly all steps in embryo recovery and transfer are timed in relation to the onset of behavioural oestrus; clearly, accurate oestrus detection is essential. Physiological characteristics of the reproductive tract change greatly throughout the stages of the oestrous cycle. On day 1 after oestrus, for example, the oviduct provides an ideal milieu for the recently fertilized ovum, but the uterine environment on this same day is lethal.

Oestrus detection must be done carefully and conscientiously; accuracy is as important for recipients as it is for donors since embryo transfer success depends on the oestrous synchrony of both. Since considerable behavioural oestrus occurs at night, oestrus detected in the morning may have begun up to half a day earlier. Thus, recipients observed to be in oestrus one-half day out of synchrony with the donor may, in fact, be a full day off.

Oestrus detection is both art and science. The method that generally works best is to move around in each pen for 10–15 minutes or more while gently moving the cattle around and chasing up animals that are lying down. Detection cannot be done properly while sitting on the fence, although this may be an initial step. Some cows are more active in mounting other cows or stand to be mounted for a longer period than is normal (up to 30 hours); others show very few signs of oestrus and may not be observed to stand to be mounted ("silent" oestrus). It may help in the case of such cows to place them with a different group of cows to check oestrus. Display of behavioural oestrus among a group may be modified by treatment with synchronizing drugs such as progestagen or prostaglandin F_2 alpha because so many are in oestrus at once. Removing cows already found in oestrus in this situation often improves the chances of detecting others.

Every donor and recipient should be checked visually for oestrus at least twice each day—early in the morning and late in the afternoon and, ideally, more often, especially in the case of donors. Each animal will be in one of three categories each time oestrus is checked: (1) not in oestrus, (2) suspicious, or (3) in standing oestrus. The latter two categories should be recorded, together with the date, time and the animal's identification number. Cows in oestrus stand when mounted by others. Suspicious signs include ruffled rump hair, restlessness, bawling, walking the fence, nudging, mounting, sniffing, tail raising, discharge of clear mucus from the vulva, and swelling and inflammation of the vulva. Not every cow showing one of these characteristics should be recorded as suspicious, but a cow should be watched closely for standing oestrus and recorded as suspicious if it displays most of these characteristics.

Metoestrous bleeding—blood from the vulva, which is also seen frequently on the tail or hind-quarters of the animal—often occurs one to three days after oestrus. This bleeding is a good sign that a cow is cycling normally and should always be recorded for donor and recipient cows, especially if standing oestrus was not detected one to three days earlier.

As a further aid to accurate oestrus detection, a calendar should be kept for donors and, in some cases, for recipients too. When oestrus is detected, the donor's identification should be recorded on the calendar 18 days later. Thus, donors that were in oestrus 18–24 days earlier can be observed closely for oestrus. As soon as practical after recording oestrous behaviour on the form that the technician carries with him while observing the cows (see Chapter 16, example 4), the information should be transferred to a notebook in which daily tabulations are kept, and to individual data cards for each animal. Another option is to use a microcomputer system. Whenever data are transcribed, they should be checked for accuracy by a second person.

Aids to oestrus detection, such as chalk on the tailhead, are useful as long as they do not become a crutch for visual detection. Such aids are recommended for donors for the oestrus resulting from superovulation. Foote (1975) reviews aids and management schemes for oestrus detection. For an embryo transfer programme, the use of detector

bulls, vasectomized or with a blocked or deviated penis, is not advisable because bulls may spread venereal disease. Moreover, these procedures may not ensure total sterility. If a teaser animal is used, it should be an androgenized female.

PREGNANCY DIAGNOSIS

A detailed discussion of pregnancy diagnosis is beyond the scope of this manual. Nevertheless, some comments with regard to pregnancy diagnosis within embryo transfer programmes seem desirable. The first good indicator of pregnancy is failure of the recipients to show oestrus 18–24 days after the pre-transfer oestrus; obviously, the converse, showing oestrus, indicates non-pregnancy, although a small percentage of pregnant animals are in behavioural oestrus about three weeks after the previous oestrus. Progesterone assay of milk or blood samples 22–24 days after the pre-transfer oestrus is = 95 percent accurate in diagnosing non-pregnancy and about 80 percent accurate for pregnancy. However, with good oestrus detection, one gets about the same information as with progesterone tests. In other words, if the accuracy of oestrus detection is so poor that progesterone tests provide much additional information, the embryo transfer programme is likely to fail because of poor oestrus detection. Another point is that neither oestrus detection nor progesterone assay gives sufficiently accurate information for definitive pregnancy diagnosis on individual recipients. The information from returns to oestrus is very useful on a population basis, however, because it gives early information concerning success or problems.

At about day 26 of pregnancy in heifers and day 28 in cows, pregnancy can be diagnosed accurately under field conditions by ultrasonography or even earlier in very skilled hands (Kastelic ***et al.***, 1988). In most embryo transfer programmes, ultrasonography equipment is not justified, although it is very useful for a variety of purposes. When costs of ultrasonography equipment decline to half current prices, such equipment will probably be considered indispensable and will come into general use. A serious problem with early pregnancy diagnoses is that about 10 percent of 28-day pregnancies will not go to term. It has been found that 95 percent of two-month and 97 percent of three-month embryo transfer pregnancies go to term (King ***et al.***, 1985).

Pregnancy diagnosis can usually be diagnosed definitively by palpation per rectum after day 35 of pregnancy. Of course, ultrasound can also be used at these later stages. We do not recommend palpation prior to day 45, both because the conceptus is more fragile at early stages and because the information is not definitive anyway due to occurrence of spontaneous abortion even in the absence of palpation. Thus, our recommendation is to palpate per rectum at 45–60 days of gestation and confirm this with another palpation one month later.

Cows that show oestrus may be checked earlier than 45 days by palpation or ultrasonography. If they are, in fact, non-pregnant they can be recycled for use as recipients.

It is often useful to distinguish between an embryo transfer pregnancy and one from artificial insemination or natural breeding during the next cycle. The following steps will make it possible for most non-pregnant recipients to be bred or reused as soon as possible. First, it is imperative not to breed recipients that show oestrus earlier than normal, that is artificial insemination or exposure to bulls should be delayed 17–18 days from pre-embryo transfer oestrus. This makes the 90–95 percent of non-pregnant recipients without short cycles eligible for breeding. Pregnancy diagnosis is then done in a window of time that permits unequivocal distinction between the embryo transfer pregnancy and a possible subsequent one, which will be at least 17 days younger. With rectal palpation, this window may extend to about 65 days after pre-transfer oestrus for skilled palpators, and perhaps to 60 days for less skilled ones. Note that a good portion of this window is needed if embryo transfer was done over a seven-to ten-day period, if all recipients are to be examined at once, and recipients are not palpated prior to a 45-day pregnancy.

One other point is that it is common to diagnose pregnancy per rectum by “slipping membranes”. This method should not be used, at least not prior to day 50, because it leads to abortion in a significant, though small, percentage of cases (Abbitt ***et al.***, 1978). Palpation should be done on the basis of fluids, tone and size of the uterine horn. The small percentage of false positives due to undiagnosed uterine pathology is lower than the abortion rate caused by slipping membranes.

MANAGING PREGNANT RECIPIENTS

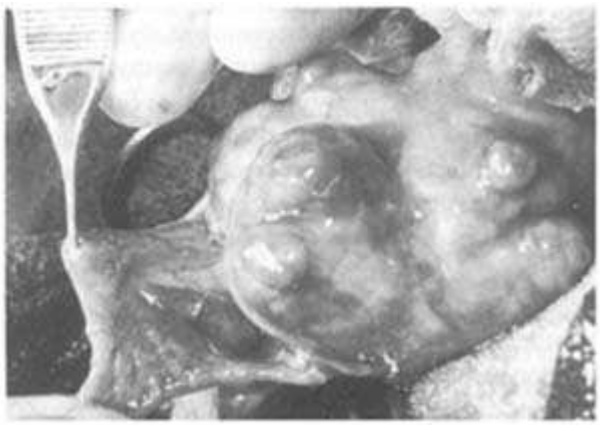
Since pregnant recipients are carrying valuable calves, they should receive better than average care. Nutrition is clearly important as well as prevention of abortion. The most critical time is at parturition. It is easy to lose 10 percent of calves at and within a few days of birth (King ***et al.***, 1985); most of these losses are due to poor management. It is especially costly to lose the calf after the huge investment up to that point. Of course, recipients often calve on individual farms not under direct supervision of embryo transfer personnel. Even so, it is wise to provide information on management at calving (e.g. ensuring that calves receive colostrum) to the personnel responsible so that embryo transfer programmes do not get a bad reputation.

Chapter 4
Superovulation

SUPEROVULATORY TREATMENTS

Superovulation is a very inefficient method of obtaining oocytes from bovine ovaries and is likely to be replaced by other approaches within the next decade. However, superovulation results in about ten times more embryos than single ovum recovery. Without superovulation, a usable embryo can be recovered about 60 percent of the time from normal donors by skilled technicians. Under similar conditions, superovulation usually yields an average of six usable embryos, although the variation is astounding (Figure 1). Normally, no embryos are recovered from 20–30 percent of superovulated donors and only one to three embryos are obtained from another 20–30 percent (see Table 11). An ideal response of five to 12 embryos is obtained from about one-third of the donors. However, a small percentage of donors yield more than 20 good embryos and, very rarely, more than 50.

FIGURE 1
Superovulated bovine ovary photographed during a surgical recovery procedure. Also note opening to fimbria



The two generally accepted methods of superovulating cattle are based on two different gonadotrophins, although there are many minor variations of these methods. The simplest is to give an intramuscular (i.m.) injection of 1 800–3 000 IU (usually 2 000–2 500 IU) of pregnant mare's serum gonadotrophin (PMSG), more correctly designated equine chorionic gonadotrophin (eCG), followed by a luteolytic dose of prostaglandin F_2 alpha or an analogue i.m. two to three days later. A second prostaglandin injection is often given 12–24 hours after the first, and seems to improve embryo production.

The second method of superovulation is to give eight to ten injections of follicle stimulating hormone (FSH) subcutaneously (s.c.) or i.m. at half-day intervals. Intramuscular injection is more reliable under field conditions. As with PMSG, prostaglandin F_2 alpha is given 48–72 hours after initiation of treatment with the fifth, sixth, or seventh FSH injection. The most common FSH regimen is 6,6,4,4,2,2,2, and 2 mg at half-day intervals with prostaglandin F_2 alpha given with the sixth or seventh FSH injection. About 20 percent more gonadotrophin

should be given to cows weighing over 800 kg. Sometimes, higher doses are used for the first two days; others give 5 mg for each injection. There are few studies with adequate numbers of donors per treatment group in which constant and decreasing doses have been compared, so reliable conclusions cannot be drawn regarding efficacy of such regimens.

A special problem with most commercially available FSH products is that they are quite impure, frequently with less than 5 percent biologically active FSH. The most commonly used product is currently marketed by Schering (see Chapter 17); over the last 25 years, essentially the same product has been marketed under various brand names, including Burns-Biotech, Reheis and Armour. It is obtained from swine pituitaries. Potency is determined relative to an “in-house” Armour standard, so the actual weight of the material in the bottle has little to do with the weight equivalent on the label. Recently, other FSH products of various purities have become available. Most feature low contamination with luteinizing hormone (LH). Although addition of large amounts of LH to FSH reduces its efficacy for superovulation of cattle, there are few convincing studies that the small amount of LH found in commercial batches of FSH decreases efficacy greatly. In fact, some LH may be needed for optimal superovulatory responses. The other impurities in FSH seem to be of little consequence, other than making it difficult to compare dosages among products, which may range from 1 to 50 percent pure. This results in widely different weights of product per dose. Production of bovine FSH either by cells in tissue culture or in milk of transgenic animals from genes cloned by recombinant DNA techniques will result in a more standardized preparation and eliminate much of the confusion in both commercial and experimental applications.

An annoying feature of most commercial FSH products is that insufficient sterile diluent is supplied for practical use. Usually FSH should be diluted at 1 or 2 mg/ml in saline so that a reasonable volume can be injected under field conditions.

In recent years, FSH has surpassed PMSG as the method of choice for superovulating cattle. In most studies comparing the two procedures, the FSH treatment has resulted in slightly higher numbers of usable embryos. However, PMSG works nearly as well. Everyone agrees that PMSG results in a much larger ovary, generally double the volume of one treated with FSH. This is probably related to its very long half-life (five days) in cattle (that of FSH is several hours) which results in continued recruitment of follicles after ovulation, very high progesterone levels and, probably, abnormalities in ovum transport. These problems have been ameliorated by injection of a commercially available antibody to PMSG given at the conclusion of the superovulatory treatment when the donor comes into oestrus. This results in a response more similar to that of FSH, including a small ovary at the time of embryo collection.

Other products that have been used for superovulating cows include equine anterior pituitary extract and human menopausal gonadotrophin (which also contains considerable LH). The former generally is not available commercially and the latter is too expensive for routine use.

It is possible to superovulate without giving prostaglandin F_2 alpha by starting gonadotrophin treatment on day 15 or 16 of the cycle of heifers and on day 16 or 17 of cows (oestrus = day 0). This method, which depends on natural luteolysis, is not recommended because the mean number of embryos recovered is reduced, the response is more variable and timing of the onset of oestrus is less predictable. Furthermore, most flexibility in scheduling donors is lost.

An alternative to using prostaglandin F_2 alpha is based on progestin withdrawal rather than luteolysis. The progestin can be injected, implanted subcutaneously, given via a vaginal coil or fed orally. Implants or vaginal coils are preferred because systemic concentrations of progestin drop rapidly when these devices are removed. Probably the most widely used progestin withdrawal system for superovulating cows is Syncromate-B (norgestomet) (see Chapter 8). However, for superovulation, the implant is often used without the injection of

progestin and oestrogen. The implant (two implants in large cows) is generally given on day 10–12 of the oestrous cycle and removed on day 18–20. Sometimes prostaglandin is given as well. FSH or PMSG injections are initiated two and a half to three days before implant removal. This system is generally not used for normal donors because of the time and expense. However, it is particularly suited to superovulating cows with cystic ovaries or for pre-puberal heifers.

When prostaglandin F_2 alpha-induced luteolysis is used for superovulation, it is best to initiate gonadotrophin treatment between days 9 and 14 of the oestrous cycle. Mean response is usually lower if treatment begins later or earlier, especially prior to day 5.

Another factor to consider is the optimal interval between superovulations when donors are treated repeatedly. Repeating superovulation at 15–20-day intervals works poorly. The most common recommendation is 45–60-day intervals, although one study gives reasonably convincing data from large numbers of animals that shorter intervals work well (Looney, 1986).

The treatments discussed so far should not strictly speaking be termed “superovulatory treatments” since their effect is production of additional mature follicles. These extra follicles actually ovulate in response to an endogenous LH surge, which in turn is triggered by secretion of gonadotrophin-releasing hormone (GnRH) in the presence of the high concentrations of oestradiol-17-beta resulting from follicular growth. Some workers inject GnRH or human chorionic gonadotrophin (hCG) 48 hours after prostaglandin or at the beginning of oestrus to augment endogenous LH. This is necessary for good superovulation for some species, but not for cattle. Exogenous LH or GnRH is useful to induce ovulation under special circumstances, e.g. superovulation of pre-puberal calves or timing the LH surge for experiments. However, most studies using FSH for routine superovulation indicate that this treatment does not result in more transferable embryos (Prado-Delgado ***et al.***, 1989), probably because the LH surge is frequently induced too early or too late for individual cows. When PMSG is used for superovulation, GnRH injection at the time of oestrus may yield slightly more embryos, but this requires further replication.

INSEMINATION

Following superovulatory treatment, the donor should be observed closely for signs of oestrus. Superovulated cows sometimes do not display oestrous behaviour as clearly as untreated cows, therefore such oestrous detection aids as KaMaR indicators (see Chapter 17) are helpful. About 10 percent of donors never show behavioural oestrus. These animals should not be bred.

The time when the donor is first observed in standing oestrus is the reference point for insemination treatment. Because the multiple follicles ovulate over a period of time and transport of sperm and ova is altered by superovulatory treatment, it is wise to breed more often and use more semen than normal. Freshly collected liquid semen is slightly superior to high quality frozen semen since unfrozen spermatozoa probably remain viable in the female reproductive tract longer.

If liquid semen is available, between 10 and 50 × 10^6 motile spermatozoa are inseminated 12 hours after the donor is observed in oestrus, and a similar quantity 12 hours later. If frozen semen is used, one ampoule or straw each time is inseminated 12 and 24 hours after the donor was first noticed in oestrus. Some people recommend two doses per insemination, particularly at the second insemination. Semen is thawed in a water bath at 35 °C (95 °F) and inseminated immediately. Some workers recommend more frequent inseminations beginning, for example, immediately after first observation of standing oestrus. We are not aware of scientific data indicating that such early insemination is appropriate. If only one insemination is to be done, it should be at 24 hours after oestrus was first detected.

Altered hormonal patterns make the reproductive tract of a superovulated donor a more hostile environment than that of an untreated cow; therefore, semen must be of the highest quality. Use of poor or mediocre semen often results in collection of unfertilized ova or degenerate embryos. Proper semen handling techniques are beyond the scope of this manual, but they cannot be overemphasized (see, for example, Pickett and Olar, 1980).

The inseminator must be gentle and must use hygienic techniques because the stress of superovulatory treatment makes a cow's upper reproductive tract extremely sensitive. Excessive manipulation of the tract could result in adhesions and the failure of the fimbriae to pick up all ova. In addition, with high numbers of ovulations and enlarged ovaries, more haemorrhaging than normal occurs and the relative size of the organs changes. This also increase the likelihood that adhesions will form. Infection introduced at the time of insemination could reduce rates of fertilization and recovery. Moreover, many follicles may not have ovulated by the time of the first insemination; therefore, the reproductive tract should be manipulated as little as possible to reduce risk of follicular rupture. For these reasons, ovaries definitely should not be palpated at the time of insemination.

Chapter 5
Recovery of embryos

Prior to 1976, most bovine embryos were collected via mid-line laparotomy or, less commonly, via a flank incision. In that year, several groups published efficacious methods for non-surgical (transcervical) recovery of embryos, and the industry changed to these procedures rather abruptly (see Betteridge, 1977, for review).

In most cases, embryos are recovered six to eight days after the beginning of oestrus (day 0). Embryos can be recovered non-surgically as early as four days after oestrus from some cows, but prior to day 6 recovery rates are lower than on days 6 to 8. Embryos can also be recovered on days 9 to 14 after oestrus; however, they hatch from the zona pellucida on day 9 or 10, making them more difficult to identify and isolate and more susceptible to infection. After day 13, embryos elongate dramatically and are sometimes damaged during recovery or become entangled with each other. Procedures for cryopreservation and bisection have been optimized for day 6–8 embryos, which is another reason for choosing this time. A small percentage of embryos remain in the oviduct after day 7. Unfortunately these are not recoverable with current non-surgical procedures.

NON-SURGICAL RECOVERY OF EMBRYOS

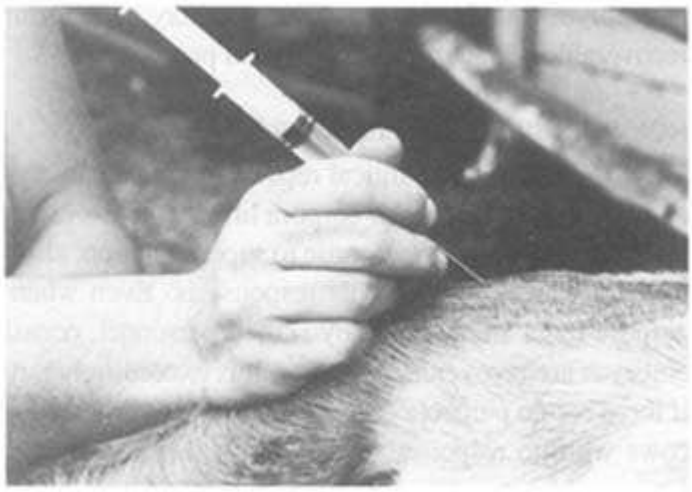
The first step in non-surgical recovery is to palpate the ovaries per rectum to estimate the number of corpora lutea. This is very difficult to do accurately if there is a large response to superovulation, although it is not critical to determine how large this response is. Even when only two or three corpora lutea are palpated by skilled personnel, occasionally four or five embryos are recovered. However, it is exceedingly rare to obtain embryos if there are no palpable corpora lutea by day 7. Under most circumstances, cows with no response are not worth flushing, although occasionally an embryo is recovered. It is rare to recover more than one embryo from cows with one palpable corpus luteum. In many situations, donors are palpated the day before recovery or the morning of recovery so that logistical plans can be made, for example, to flush those donors with poor responses first (or last) and cancel those with no response. Ultrasonography (Pierson and Ginther, 1988) provides more accurate information about responses than palpation, but currently this expensive equipment can only be justified in research contexts or in large embryo transfer programmes.

Epidural anaesthesia is recommended for non-surgical recovery procedures. The tailhead should be clipped, then scrubbed with iodine soap and swabbed with 70 percent alcohol to prevent infection of the spinal column. The site of the epidural injection is illustrated in Figure 2. A frequent error is to inject too much anaesthetic too far forward, which can cause the cow to lose control of the rear legs and fall down in the chute. We recommend injecting 5 ml of a sterile 2 percent solution of procaine in water using a new 18-gauge needle each time. Good epidural anaesthesia can be monitored by flacidity of the tail.

While the epidural anaesthesia is taking effect, the tail should be secured to one side out of

the way, for example, by tying it to a cord looped loosely around the cow's neck. It should not be tied too securely to something stationary, like the chute, for fear of the tail breaking if the cow falls or if personnel forget to loosen the cord before releasing the cow. The rear end of the cow should be cleaned of mud, manure, loose hair, etc., and then the vulvar area scrubbed thoroughly with iodine soap and rinsed carefully with swabs of 70 percent alcohol (Figure 3A). Sufficient time should be allowed for the lips of vulva to dry before inserting the recovery instrument to avoid carrying any alcohol into the uterus; disinfectants are extremely toxic to embryos. For the same reason, an assistant should open the labia gently when the cervical dilator or the recovery device is inserted (Figure 3B).

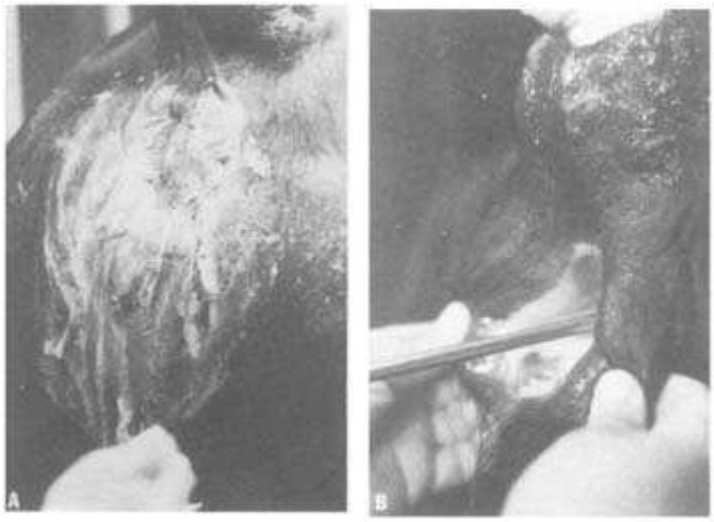
FIGURE 2
Site of epidural anaesthesia



Recovery procedures are carried out by manipulation per rectum. Because of the epidural anaesthesia, the rectum can balloon easily due to entry of air during removal and re-insertion of the hand. Once air has entered, it is extremely difficult to work effectively. A simple air pump attached to a length of tubing (Figure 4) to evacuate air from the rectum is an excellent investment, because ballooning of the rectum occurs occasionally, even with skilled personnel. Even so, the best strategy is to prevent entry of air as much as possible.

The basic instrument for non-surgical recovery is the Foley catheter (Figure 5). Generally 18- to 24-gauge sizes are used. It is best to use as large a catheter as can be introduced easily to achieve good rates of flow. Most people prefer two-way catheters, one passage for air and one for fluid, because rates of flow are higher than with three-way catheters, which have two smaller passageways for fluid. The disadvantage of the two-way catheter is the dead space or column of fluid that remains in the catheter during filling and never reaches the uterus. Teflon-coated Foley catheters are recommended to reduce the possibility of embryos sticking to the catheter.

FIGURE 3
(A) Scrubbing vulvar area with tamed iodine soap; (B) Assistant gently opening vulvar labia to avoid contamination of cervical expander or collection catheter during insertion



It is possible to purchase custom-made embryo collection devices. Usually these are very expensive, and cannot be justified. They generally have two advantages over standard Foley catheters in that they are slightly thinner and somewhat longer. For large, older cows, the standard Foley catheter can be lengthened by combining it with a second catheter using glass connecting pieces. Usually this works as well as the expensive, custom-designed catheters.

FIGURE 4
Length of tubing attached to a vaccum pump to evacuate air from the rectum

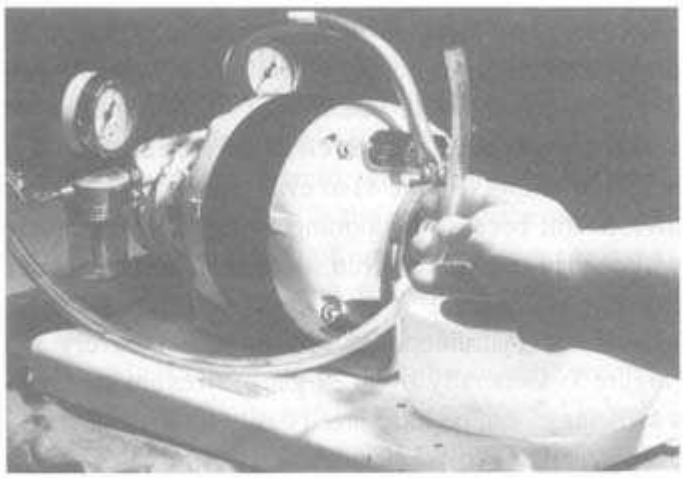
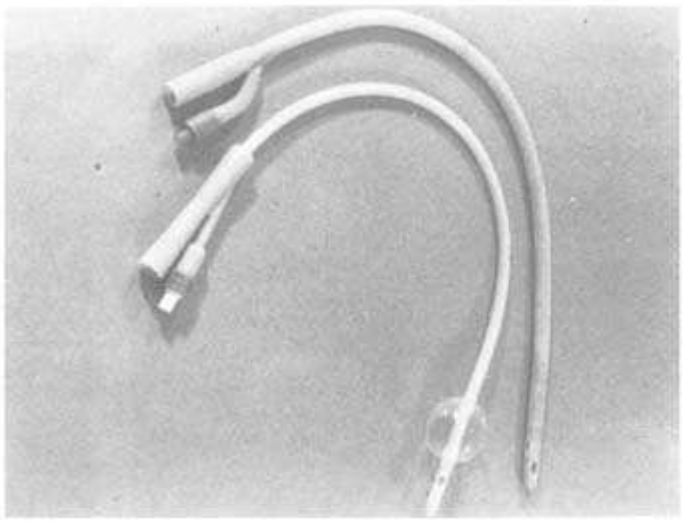


FIGURE 5
Two-way Foley catheters with and without balloon inflated (first appeared in Kuzan and Seidel, 1986)



Before removal of the Foley catheter from the paper envelope used for sterilization, the air system to the balloon is checked to see that it and the balloon will hold air. The Foley catheter is rinsed with sterile saline, and finally a sterile metal stylet such as the plunger of a Cassou insemination gun (Figure 6) is inserted into the lumen. The system is then ready for insertion into the cow. An assistant parts the labia of the vagina and the device is manipulated through the cervix.

Sometimes great difficulty is encountered in manoeuvring the Foley catheter through the cervix, particularly with heifers of some breeds. As soon as there is a hint of difficulty, the apparatus should be withdrawn and a stainless steel cervical expander (Figure 7) should be inserted first. ***A gentle, patient technique is essential.***

FIGURE 6
Insertion of stylet to make Foley catheter rigid for inserting through the cervix and positioning in the uterus

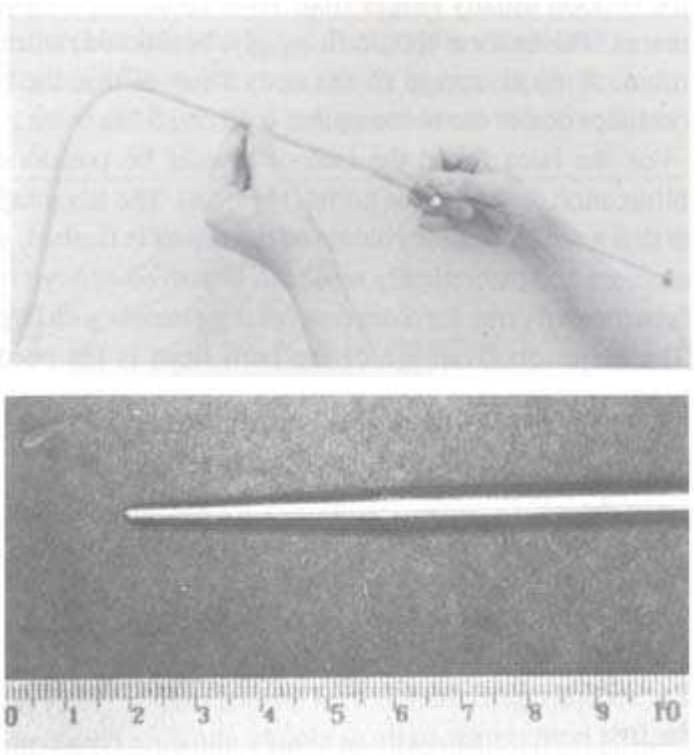


FIGURE 7
Tip of cervical expander; expander is a 48-cm stainless steel rod, 6.3 mm in diameter, that tapers in the last 4 cm to a 3-mm rounded tip

There are two fundamentally different approaches to positioning the balloon of the Foley catheter for non-surgical recovery procedures. These are commonly referred to as “body” and “horn” flushes. For the body flush, the catheter is inserted into the uterine body and the balloon is inflated just past the cervix. Some technicians prefer air, others, 0.9 percent sterile NaCl solution to fill the balloon; we recommend air. The single most common error in non-surgical recovery, especially with horn flushes, is overinflation of the balloon. This leads to rupture of the endometrium and loss of flushing fluid (and embryos) into the uterine tissue, from which recovery is impossible. Once this occurs, the only recourse is to reposition the balloon more anteriorly, precluding a body flush. The amount of air used to inflate the balloon usually ranges from 10 to 20 cc, depending on the size of the uterus. The balloon should fit snugly, but should not rupture the endometrium. A disadvantage of the body flush is that the balloon sometimes occludes one of the horns so that only one fills.

For the horn flush, the balloon should be positioned at the palpable bifurcation of the uterine horns (Figure 8). The advantage of the horn flush is that a much smaller volume of the uterus is flushed, which requires less medium and theoretically results in improved embryo recovery rates. This is particularly true for older cows of large breeds with large, pendulous uteri. The major disadvantage of the horn flush is the need to reposition the catheter in the second horn after flushing the first, which requires detaching the inflow-outflow tubing, deflating the balloon and reinserting the stylet. This prolongs the flushing procedure considerably.

In our laboratory, we frequently use a hybrid technique. We start with a horn flush on the side with the largest response and then gently retract the Foley catheter so that the balloon lodges in the uterine body. This sometimes occurs in the course of flushing without a deliberate attempt and illustrates the subtle situation of having enough air in the balloon, but not too much. The second uterine horn is then flushed from the body position by occluding the first horn transrectally or simply allowing both horns to fill and empty.

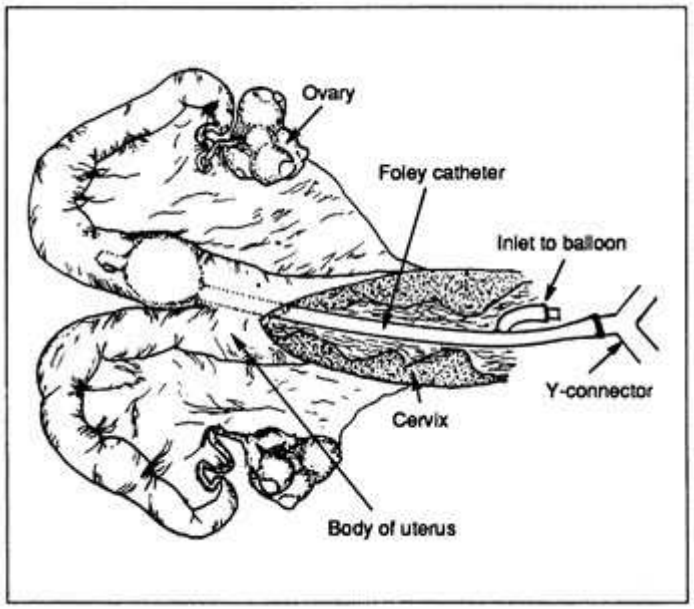
The uterus can be filled and emptied with either a continuous-flow system or in aliquots, for example, by repeatedly inserting and recovering 50 ml of fluid from a syringe. The same 50 ml may be reflushed two or three times before it is examined for embryos, or each aliquot may be used only once. With the continuous-flow system, the volume of fluid in the uterus is controlled by clamps on the inflow and outflow tubes. The aliquot method is best suited to

horn flushes. Both continuous-flow and aliquot methods have staunch advocates.

We recommend the continuous-flow system, which we find less cumbersome (Figures 9 and 10). We place 2 litres of medium in a disposable plastic intravenous infusion bag or Ehrlenmeyer flask held about 1 metre above the cow. This provides the proper pressure from the force of gravity for filling the uterus at the optimum rate. We use 1/4 -inch (inside diameter) Tygon tubing to connect the infusion bag and outflow tube to a Y-connector on the Foley catheter (Figure 10 and Chapter 17).

With either system, the principle is to fill and empty the uterus four to six times. With each successive filling, the uterus tends to expand, particularly in older cows, so that more and more fluid is required for an effective flush.

FIGURE 8
Position of Foley catheter for uterine horn flush

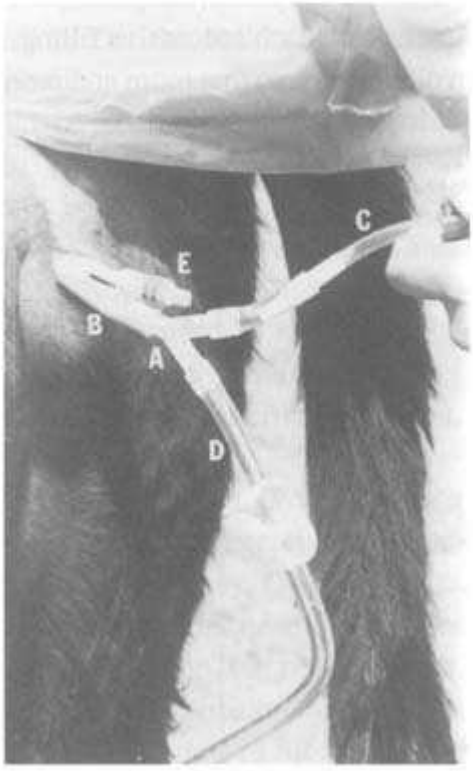


A good initial reference point is to fill the uterus until the degree of distension is equivalent to a 45-day pregnancy, which takes more fluid with body than with horn flushes and, similarly, more with cows than heifers. With body flushes, horns may be filled alternately or both horns filled and emptied together. Some technicians emphasize massaging the uterine horns to loosen embryos from endometrial folds, while others emphasize expanding the uterus to dislodge the embryos from the folds. Good rates of flow upon emptying are essential for good rates of recovery.

There is also debate about the need to occlude the utero-tubal junction to prevent retrograde flow into the oviduct. This may be a problem if the uterus is fully distended, but otherwise is probably an infrequent occurrence. Nevertheless, we recommend occluding the utero-tubal junction with the thumb and index finger when the filling cycle is at its peak, and gently massaging the uterus with the other three fingers.

FIGURE 9
Continuous-flow system for recovery of embryos with medium flowing through the Foley catheter into a graduated cylinder. Technician has clamped with a haemostat the inflow tubing from the Ehrlenmeyer flask held above assistant's head

FIGURE 10
Y-connector
(A) attached to fluid canal
(B) of Foley catheter. Note inflow
(C) and outflow
(D) tubing and air canal
(E) to inflate balloon of Foley catheter



ISOLATION OF EMBRYOS

Fluid from the uterus is usually collected in either 2-litre graduated cylinders or through a 75-µ mesh filter (Figure 11). When cylinders are used, the flush fluid is allowed to sediment for 25 minutes. Most normal embryos will settle to the bottom of the cylinder within this time. All but 150 ml is siphoned off with narrow-bore flexible tubing from each cylinder into another cylinder, which is set aside for resiphoning. The bottom 150 ml of fluid is swirled and poured into flat-bottomed dishes scored to divide the bottom into squares (see Figure 12). Each cylinder should then be rinsed at least twice with 20 ml of medium to dislodge any retained embryos.

With the filtration method of isolating embryos, fluid passes through the filter unit and is allowed to escape through a short length of tubing (see Figure 11A); outflow is controlled by means of a clamp. To prevent dehydration, at least 1 cm of medium should be retained in the filter to cover the filter grid on which the embryos rest. To recover the embryos from the filter, one swirls the filter container and pours the contents into a searching dish, and then quickly rinses the filter (Figure 11C) in concentric circles while holding it partly inverted, moving from the outer rim of the grid to the centre, using a 22-guage needle mounted on a 30-cc syringe containing flushing medium ***without serum or BSA***. The omission of protein from the medium for this step is important to prevent foaming when it is ejected from the needle, since embryos are easily lost among the bubbles, which persist for hours. The sides of the filter and the grid should be rinsed several times until all vestiges of mucus and cellular debris are gone. This takes considerable rinsing at high pressure. Medium containing 0.4 percent BSA or 10 percent heat-inactivated serum should be added to the searching dish after the filter has been thoroughly rinsed to keep embryos from floating and sticking to the dish or pipette.

The filter method is considerably faster than the cylinder method, although in the case of about 5 percent of donors, the filter becomes clogged with mucus, so a second filter must be used, and both must be rinsed to recover embryos. With conscientious effort, the cylinder method is just as efficacious as the filter method, and considerably less expensive with respect to materials; however, it is more labour intensive. Thus, it seems to us that the filter method should be used when labour is scarce and expensive and the cylinder method

should be used when labour is available and capital is not.

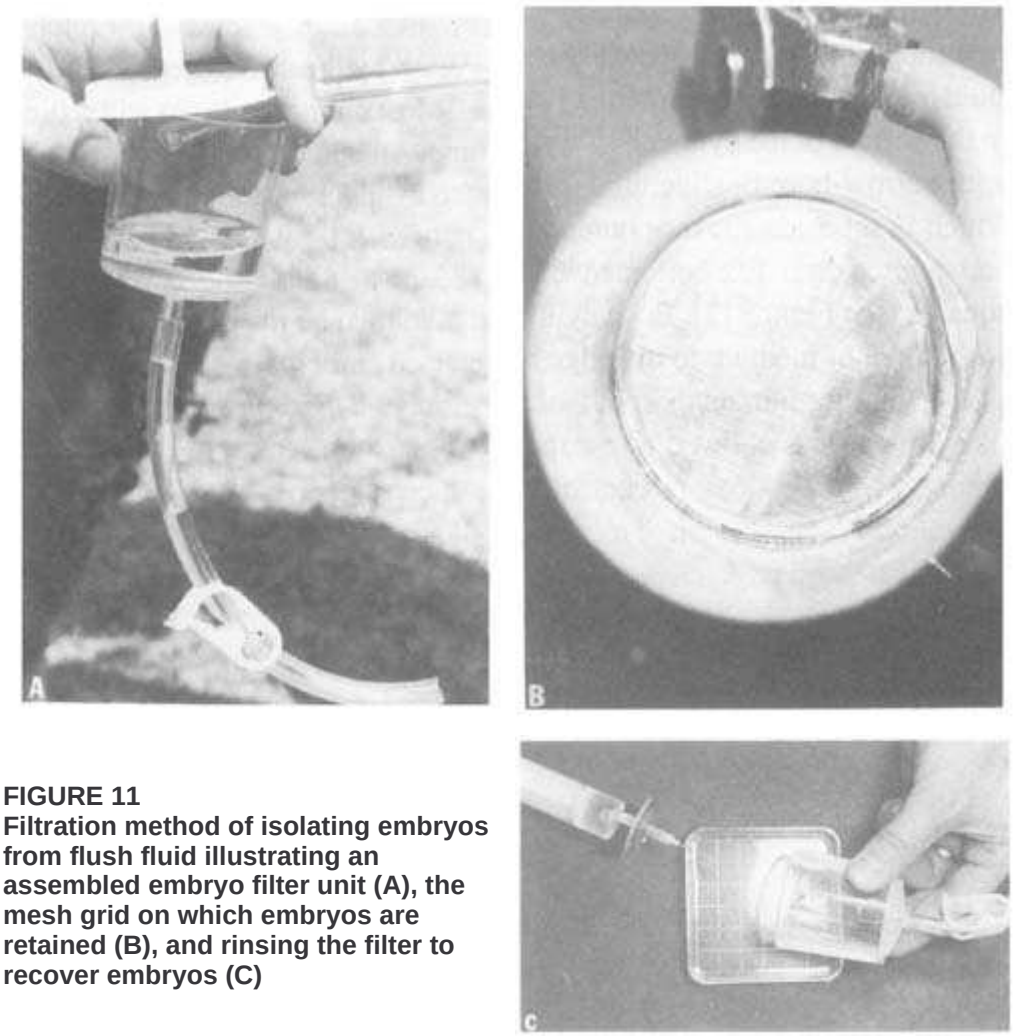


FIGURE 11
Filtration method of isolating embryos from flush fluid illustrating an assembled embryo filter unit (A), the mesh grid on which embryos are retained (B), and rinsing the filter to recover embryos (C)

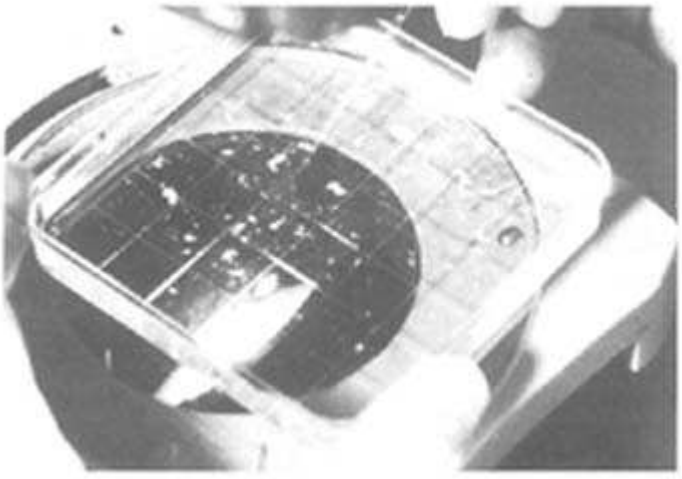
The flush fluid should be examined systematically at about 10–14X magnification to locate embryos (see Figure 12). They should be transferred to fresh medium as soon as they are found, and washed through at least three changes of medium (ten changes if there is a chance of export or if infection is suspected; see Chapter 14) as soon as possible. It is a good idea for two people to examine each dish twice. The fluid siphoned from the cylinders should also be resiphoned if that method is used. It pays to be painstaking in searching for embryos. Dishes that are not being examined should be covered and stored where they will not be exposed to excessive light. As soon as all embryos have been located and washed, they should be evaluated and prepared for either immediate transfer or cryopreservation. It is important to record pertinent data promptly to avoid embarrassing errors and loss of information (see Chapter 16).

REFLUSHING AND PREVENTION OF MULTIPLE PREGNANCIES IN DONORS

In some cases reflushing the uterus is appropriate. A second epidural injection is essential. This should be done at least two to three hours after the first flush so that the first epidural block wears off to avoid ballooning of the uterus when equipment is positioned. We have reflushed several hundred donors. Unless clear technical problems have occurred during the first flush, we usually do not recover additional embryos if none was recovered in the first flush. However, we frequently recover a few additional embryos on reflushing if there was a good ovarian response and a good rate of recovery of transferable embryos with the first flush. Reflushing results in about 10 percent more embryos, averaged over many

donors.

FIGURE 12
Examination of flush fluid square by square at 10X magnification to locate embryos. Container is 100×15 mm with 13-mm grid



Despite good flushing procedures by experienced technicians, not all embryos are recovered. A few are inaccessible in the oviduct, but some are simply missed. The variability in this step is in need of further research. It is particularly frustrating to recover no ova when there is a good ovarian response and no indication of problems with recovery procedures. However, as there were similar situations with surgical recovery of embryos, it is unlikely that the method of recovery is at fault. In our experience, it is best to give donors a luteolytic dose of prostaglandin F_2 alpha, or an analogue, after the flushing procedure. Of course, this may not be appropriate for certain infertility cases.

If prostaglandin is not given, donors should be examined ultrasonographically or palpated at 40–50 days after breeding to diagnose potential multiple pregnancy. Even with a luteolytic dose of prostaglandin, an occasional donor remains pregnant. Some recommend two prostaglandin injections at one-week intervals. Note also that most donors return to oestrus later than two to five days after prostaglandin, in contrast to non-superovulated cows.

Chapter 6
Maintaining embryos in vitro

Embryos derive nutrients from the fluid in which they are bathed; moreover, of perhaps more importance, embryos depend on the ambient fluid to maintain their physiological integrity. To date, no chemically defined medium has been formulated to support normal development of bovine embryos satisfactorily for longer than about 24 hours. However, several media are commercially available (or can be prepared easily) that adequately maintain embryos for the usual interval between collection and transfer. For longer periods, cryopreservation is recommended.

STORAGE CONDITIONS

For short periods of culture, the nutrient properties of the medium are far less critical than ***pH, osmolality, temperature, sterility, and lack of toxicity***. An embryo's range of tolerance for these properties is narrow. Inadequate control of these aspects of culture accounts for many failed transfers. In addition, embryos are damaged if exposed to excess light (e.g. microscope lights) for prolonged periods, but normal lighting or daylight in the work area is not harmful if embryos are not exposed for more than half an hour.

Body temperature of cattle is 39°C, and for certain purposes this temperature is appropriate. However, for routine commercial embryo transfer we do not recommend storage above 37° C because thermometers, even those on incubators, are frequently incorrect by 1° or 2°C. Culturing embryos at 41°C is extremely damaging, whereas storing them for up to 12 hours between 18° and 37°C is not.

MEDIA

The simplest medium is sterile saline (9 gm NaCl/litre of sterilized, deionized or distilled water). Saline will not support embryonic development, but will keep embryos alive for a few hours; it is better to use saline and transfer embryos quickly than to use a more complex medium without a proper buffering system. Moreover, saline can be steam-sterilized (at least 30 minutes at 121°C under a pressure of 104 kilopascals) if a 0.22-µm biological filtration system is not available. A serious problem with saline, however, is that embryos will float or stick to plastic or glass unless a macromolecule such as bovine serum albumin (BSA) or serum is added, both of which require membrane filtration for sterilization. BSA and serum are sterile when purchased.

TABLE 3
Recommended culture conditions

pH	7.2–7.6
Osmolality	270–310 mOsM/kg

Humidity	100 percent
Temperature	Room temperature (15–25°C) or 37°C in incubator
Buffer	Phosphate or bicarbonate ion (latter must be maintained under 5 percent CO_2 atmosphere)*
Sterilization	Filtration of medium through 0.22-µm-pore membranes, aseptic techniques; sterile equipment; addition of 100 IU penicillin G, and 50µg streptomycin sulphate per ml, or 25 µg/ml gentomycin sulfate; addition of antimycotics sometimes indicated
Macromolecule	Sterilized, heat-inactivated serum or serum albumin (e.g. Fraction V, bovine serum albumin)

* There is anecdotal evidence that HEPES buffer is detrimental to bovine embryos.

For most applications, we recommend a modified Dulbecco's phosphatebuffered saline (PBS). It is easy to use because it does not have to be equilibrated and maintained in an atmosphere of 5 percent CO_2 in air. It can be prepared from stock reagents (Table 4) or purchased in either ready-to-use or concentrated form, which must be diluted with sterile, deionized or distilled water. A frequent error is failure to dilute concentrated medium; embryos contract markedly when exposed to the high osmolality. Commercially available Dulbecco's PBS does not usually contain antibiotics, sodium pyruvate, glucose or macromolecules.

The least important ingredient is the Na pyruvate; neither Na pyruvate nor glucose is needed for most applications. The $CaCl_2$ and $MgSO_4$ can also be eliminated if embryos are to be kept ***in vitro*** only a short time. If these are omitted, osmolality should be adjusted by adding extra NaCl.

TABLE 4
Modified Dulbecco's phosphate-buffered saline (to make 10 litres)

Mixture One	**Amount**	**Function**
$CaCl_2.2H_2O$	1.32 g	Membrane/enzyme function
$MgSO_4.7H_2$	1.21 g	Membrane/enzyme function
The above may be weighted in advance and stored indefinitely in a sterile bottle under refrigeration		
Mixture Two	**Amount**	**Function**
NaCl	80.0 g	Osmotic balance; neutralize charge cell membrane
KCl	2.0 g	
Na_2HPO_4	11.5 g	Buffer to maintain pH
KH_2PO4_4	2.0 g	Buffer to maintain pH
Glucose	10.0 g	Energy source
Na pyruvate	0.36 g	Energy source
Streptomycin sulfate	0.5 g	Prevent growth of microorganisms
Na penicillin G	1 000 000 units	Prevent growth of micro-organisms
Mixture Two may be weighed in advance and stored dry in a sterile bottle under refrigeration for six months		
Combination of mixtures One and Two		
Dissolve the reagents in mixture Two in 8 litres of deionized or distilled water. Dissolve mixture One in 2 litres of deionized or distilled water. Add these 2 litres to the 8 litres		

stirring constantly. Other methods of dissolving these ingredients often result in the formation of a precipitate. Sterilize medium by passage through a 0.22-µm bacteriological filter.

Other media used to culture bovine embryos include Tissue Culture Medium-199 (TCM-199), Ham's F-10 medium, and Brinster's Mouse Ova Culture Medium-3 (BMOC-3). All of these are commercially available. TCM-199 with Hank's salts does not depend on CO_2 for buffering, but TCM-199 with Earle's salts as well as Ham's F-10 and BMOC-3 must be maintained under an atmosphere of 5 percent CO_2 in air.

MACROMOLECULAR SUPPLEMENTS

Supplementation of media with a large protein molecule decreases surface tension, which reduces the tendency of the embryo to float or adhere to plastic or glass, and helps to inactivate heavy metals and other toxins. However, macromolecules derived from serum are also a possible vector for viral infection of embryos. BSA and bovine serum are currently recommended sources of macromolecules. Polyvinyl alcohol and polyvinyl pyrrolidone are being studied as possible non-biological macromolecules to circumvent the danger of infection. These macromolecules do not function as well as BSA in reducing surface tension or chelating toxins.

BSA (Fraction V) should be added to medium just before use at a concentration of 0.05–0.1 percent for flushing and 0.4 percent for culture. The powder should be poured ***very gently*** on the surface of the medium and be allowed to dissolve for about 20 minutes without stirring or shaking (otherwise it turns into a glutinous blob or makes the medium unusably frothy). After the BSA has dissolved, the container should be inverted gently five or six times to mix in the BSA completely. The medium should then be sterilized by passage through a 0.22-µ bacteriological filter. Purer types of BSA are also acceptable. BSA can also be purchased as an aqueous solution, e.g. 7 percent BSA in water.

If serum is used, steer or calf blood should be harvested into sterile, sealable containers directly from the vein (to avoid contaminants from skin and hair). After coagulation, clots are cut every 2–3 cm with a sterile knife, and the blood stored overnight at 5°C. Clots are then filtered out and the serum centrifuged at 2000 X g for 12–15 minutes. The sediment is discarded and the supernatant recentrifuged. This step is repeated once again. The final supernatant is sterilized by passage through a 0.22-µ bacteriological filter into sterile containers of convenient size. In order to inactivate the protein complement, which can be toxic to embryos, serum must be treated in a 56°C water bath for ***exactly*** 30 minutes after the serum has reached 56°C. Serum can be frozen and stored for up to eight months provided that the containers are tightly sealed. For quality control, one aliquot from each batch of serum should be incubated overnight in a sterile, sealed container at 37°C and examined the next day for bacterial contamination. For some situations, such as international movement of embryos or maintenance of specific-pathogen-free herds, additional sterilization of serum by gamma irradiation is recommended (Manual of the International Embryo Transfer Society, 1987). Heat-inactivated, sterilized serum is added to medium instead of BSA at a concentration of 0.5–1 percent for flushing and 10 percent for storage.

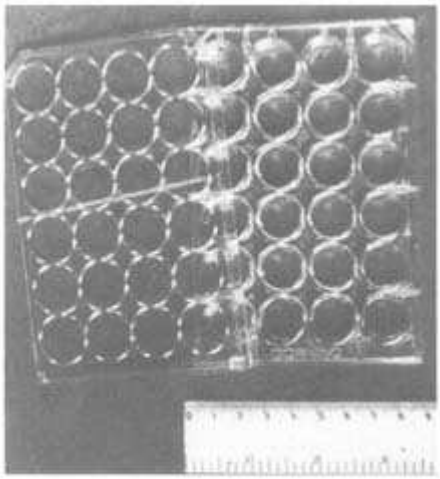
CONTAINERS

Embryos should be stored in small (<5 ml), sterile, transparent, sealable, inert containers. Small test tubes, Petri dishes or multi-well plates are convenient for routine use (Figure 13), but small test tubes are recommended if embryos must be moved any distance before loading into straws. If the embryos are to be stored in uncovered containers for more than 20 minutes, however, the medium should be covered with a thin layer of non-toxic paraffin oil to prevent evaporation and contamination and to regulate the rate of gas exchange between the medium and the surrounding atmosphere. Paraffin oil can also be used with

other types of containers and is recommended if sealable containers are not used. Except while they are being manipulated, embryos should be stored in a dark, dust-free incubator or cabinet (e.g. a refrigerator or an ice chest with a tightly-fitting lid set at room temperature or 37°C).

FIGURE 13

Left: Plastic multi-well plate with 16 × 17-mm wells (lid to left);
Right: 60 × 15-mm and 35 × 10-mm culture dishes, and a 12 × 75-mm test tube



PIPETTES

Many devices have been used to manipulate embryos. Standard Pasteur pipettes are much too large and the tips require fire polishing to prevent damage to embryos. We prefer to make our own pipettes from Pyrex glass tubing with a 4-mm outside diameter. Glass, in 15-cm lengths, is heated in the centre with a Bunsen burner and pulled to make an outside diameter of less than 1 mm. This is then scored with a diamond pencil and broken to make two pipettes. All ends are fire polished. After pipettes have been washed and rinsed thoroughly, they are placed in clean glass test tubes with screw tops and sterilized (and dried) by dry heat.

For use, pipettes are connected to a 0.5- or 1-cc syringe or plastic mouthpiece with rubber tubing (see Figure 32). Pipettes are made in batches of several hundred. They can be reused if debris does not adhere and they are placed in a soapy water bath immediately after use. In practice, we discard most of them after one use. Alternatives to making pipettes are to use tomcat catheters, 0.25-cc French straws (which, however, are too large for good washing procedures), disposable micropipettes, or pipette tips of various sorts. Care must be taken to wash, sterilize, and rinse these devices with sterile medium prior to use (see Chapters 15 and 18).

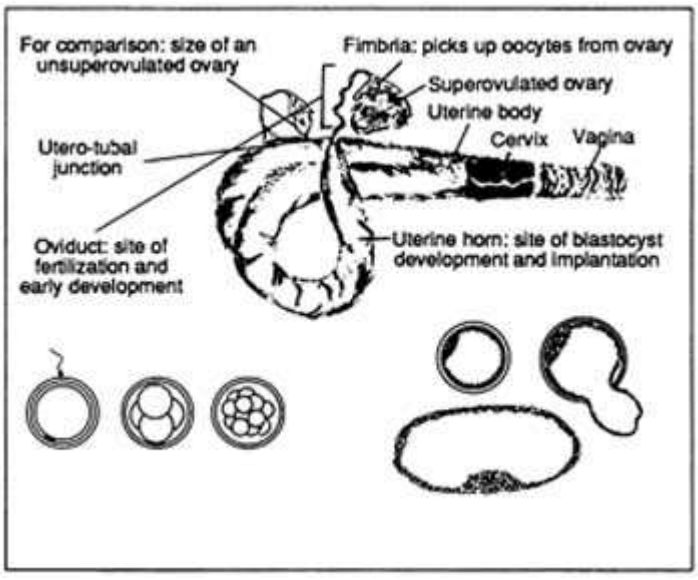
Chapter 7
Evaluation of embryos

EMBRYOLOGICAL TERMINOLOGY

In mammals, the female gamete is called an egg or ovum; the correct technical term for the newly ovulated female gamete is an oocyte. Upon fertilization, the oocyte becomes a one-cell embryo, sometimes referred to as a zygote. The embryo then divides into two-cell, four-cell, etc. stages. At the 16-cell stage, the embryo becomes a morula (Latin for mulberry). When a cavity (blastocoele) forms between the cells of the embryo, it is termed a blastocyst. To add further confusion, all of these stages of embryos are frequently called eggs or ova. Embryos of various stages are illustrated in Figures 14 to 25.

The first three divisions of the embryo are called cleavage divisions; thus, one-to eight-cell embryos are defined as cleavage stages. During this time the embryo actually decreases in weight. Only at the morula stage does the embryo begin to weigh more than at the one-cell stage.

FIGURE 14
Location of different developmental stages of bovine embryos in reproductive tract (first appeared in Hoard, *Dairyman*, 10 March 1988, p. 246)

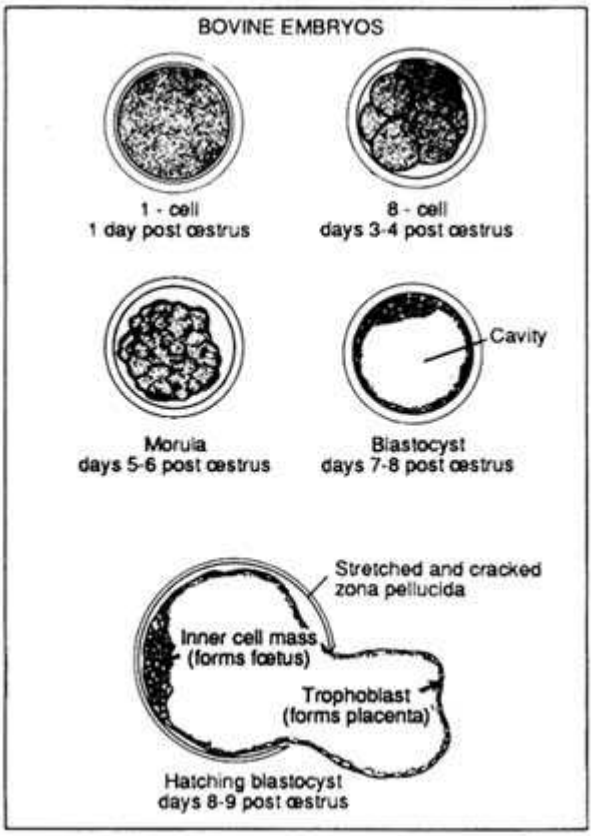


During the morula stage, cells of embryos change from spherical to polygonal in shape. This phenomenon is termed compaction. During compaction, specialized junctions from between cells, so the cells can communicate with each other. Frequently, compacted morulae are termed tight morulae. Compacted morulae are smaller than pre-compacted embryos. Compaction is an excellent sign that the embryo is developing normally; lack of compaction by six days after oestrus in cattle indicates retarded development.

As the morula develops into a blastocyst, it forms a cavity, the blastocoele, by expending

energy to pump fluid between the cells. Thus blastocyst formation also is indicative of continued normal embryonic development. Conversely, lack of blastocoele formation by seven to eight days after oestrus in cattle signifies retarded development.

FIGURE 15
Diagram of normal bovine embryos



The zona pellucida is a gelatin-like capsule that surrounds the oocyte and early embryo. It has receptors for sperm that are inactivated after fertilization, it keeps the cells of the pre-compaction embryo together, and protects these young cells from the immune system and from pathogens. If the zona pellucida is removed from pre-compaction embryos, the cells come apart upon embryo transfer and then degenerate. When the blastocoele becomes very large, the embryo expands (normally eight to nine days after oestrus), which thins the zona pellucida. This is the expanded blastocyst stage. After one to one days more, the expansion is so great that the embryo hatches out of the zona pellucida, perhaps aided by enzymes. Hatched blastocysts become elipsoid in shape 11–13 days after oestrus, and then elongate markedly by 14–16 days post-oestrus. By day 17–19 the embryo elongates sufficiently to reach the tip of both uterine horns.

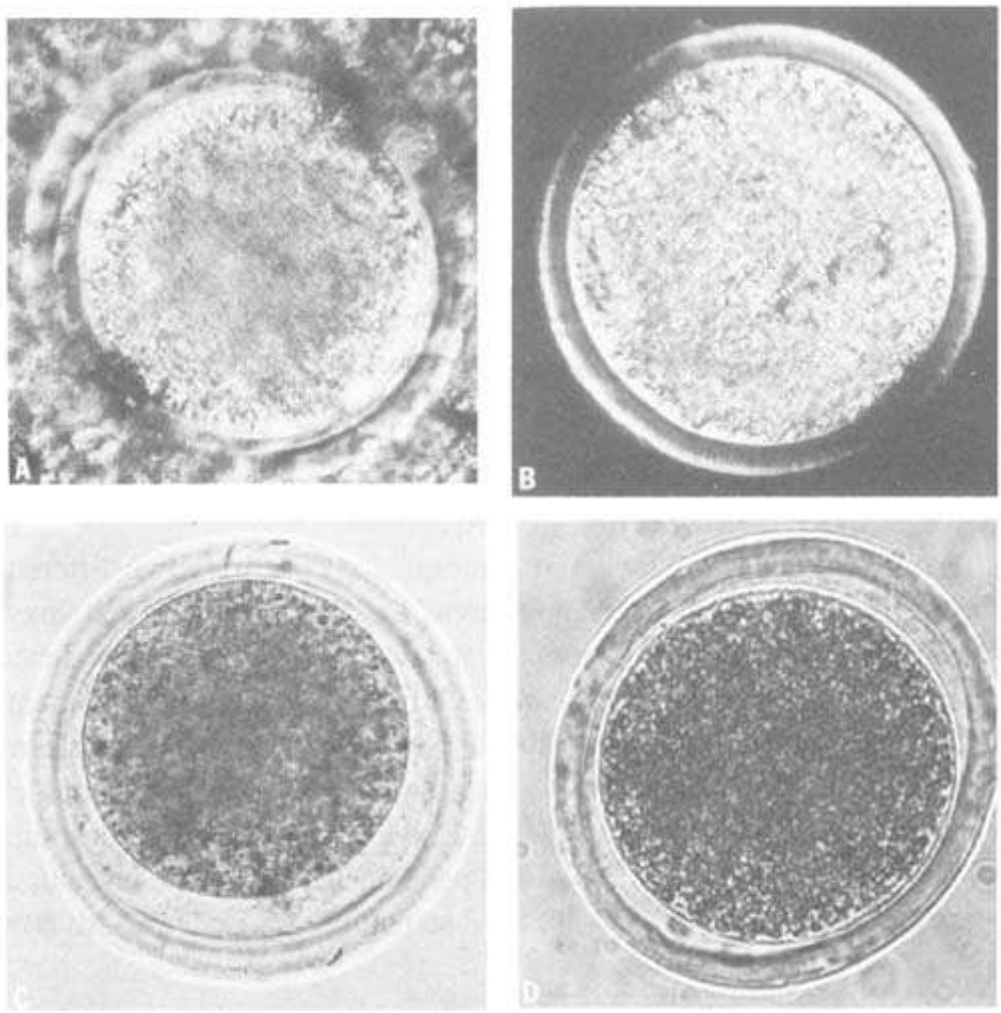
EVALUATION

For many beginners, the most intimidating aspect of the embryo transfer process is morphological evaluation of embryos. Obviously, there is no profit in transferring unfertilized ova or degenerate embryos, nor in discarding perfectly normal ones. Both errors are common when people are first gaining experience, and not infrequent when more seasoned personnel make hasty decisions. There are three elements to successful evaluation of embryos: training, experience and proper equipment.

Training includes learning the correct morphology of embryos at different times post-oestrus and the meaning of deviations from normal morphology. One must also learn how to manipulate and examine embryos. Experience is gained by examining many embryos at different stages of development. Ideally hundreds of embryos should be studied under the guidance of someone experienced in this area. Photographs, drawings or slides of various kinds of embryos are very useful. However, they can only substitute partially for real

embryos. Experienced personnel can evaluate more than 95 percent of ova accurately with a good stereomicroscope at 30X to 40X magnification or less. However, a small percentage of embryos require a compound microscope (at least a 10X objective with 8X to 20X eyepieces) for accurate evaluation. For learning purposes, a compound microscope is especially useful. Most compound microscopes are poorly designed to examine embryos, and working distance (distance from the embryo to the objective lens) is frequently short. These limitations make it easy to spill the dish containing embryos and to contaminate the fluid containing the embryos with the objective lens.

FIGURE 16
(A) Follicular oocyte with adherent follicle cells. Nomarski optics. (B) Follicular oocyte after removing follicle cells. Nomarski optics. (C) Normal appearing 1-cell ovum recovered five days after oestrus. Note spermatozoa in the zona pellucida. Bright-field optics. (D) Normal, unfertilized, ovulated oocyte recovered three days after oestrus. Bright-field optics. (Figures 16B, 19C, 20B, 20C and 23B first appeared in *Science*, 211: 351–358, copyright, AAAS, 1981)



Embryos collected six days post-oestrus should be post-compaction or so-called tight morulae. They should have 50–80 cells. Although it is impossible to count cells accurately in post-compaction embryos without resorting to procedures that damage embryos, it is useful to make estimates of cell numbers. Embryos should be generally spherical or ovoid, not too light nor too dark in colour (Figure 21B and C illustrates unacceptable extremes), and have uniform cell size. Deviations from normal include irregular cell sizes, large vacuoles in cells, areas of degeneration in the embryos, some cells not compacted with the main cell mass (termed extruded or excluded blastomeres), and a damaged zona pellucida. Nearly 20–30 percent of good embryos have some detectable morphological abnormality such as a few

excluded blastomeres. Most of these abnormalities are a matter of degree. If part of the embryo appears degenerate, but the bulk of the embryo appears normal, it has an excellent chance of developing into a normal calf (e.g. Figure 22B); morphologically abnormal embryos do not result in abnormal calves. Note that pregnancy rates with bisected embryos (see Chapter 10) are really quite good, which means that half of the cells can be degenerate without markedly lowering pregnancy rates.

FIGURE 17
(A) Unfertilized oocyte recovered five days after oestrus. Nomarski optics. (B) Same ovum as in (A) with bright-field optics. (C) Cracked, empty zona pellucida recovered five days after oestrus. Nomarski optics. (D) Unfertilized oocyte recovered six days after oestrus. Note blisters of clear cytoplasm. Nomarski optics

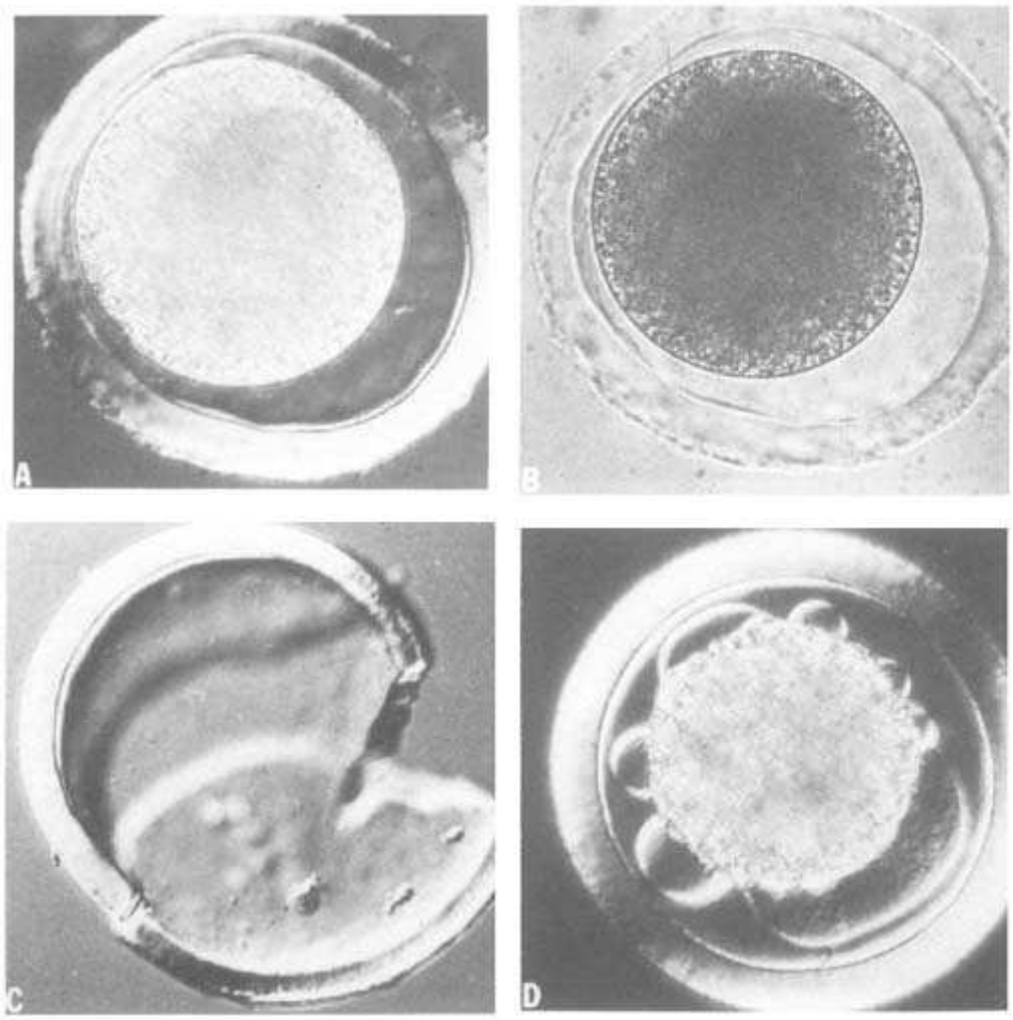


FIGURE 18
(A) Degenerate, unfertilized ovum recovered five days after oestrus. Nomarski optics. (B) Unfertilized ovum with two fragments of cytoplasm. Note large vesicles within cytoplasm. Bright-field optics. (C) Fragmented ovum, likely unfertilized recovered five days after oestrus. Bright-field optics. (D) Disintegrated ovum, probably unfertilized. Bright-field optics

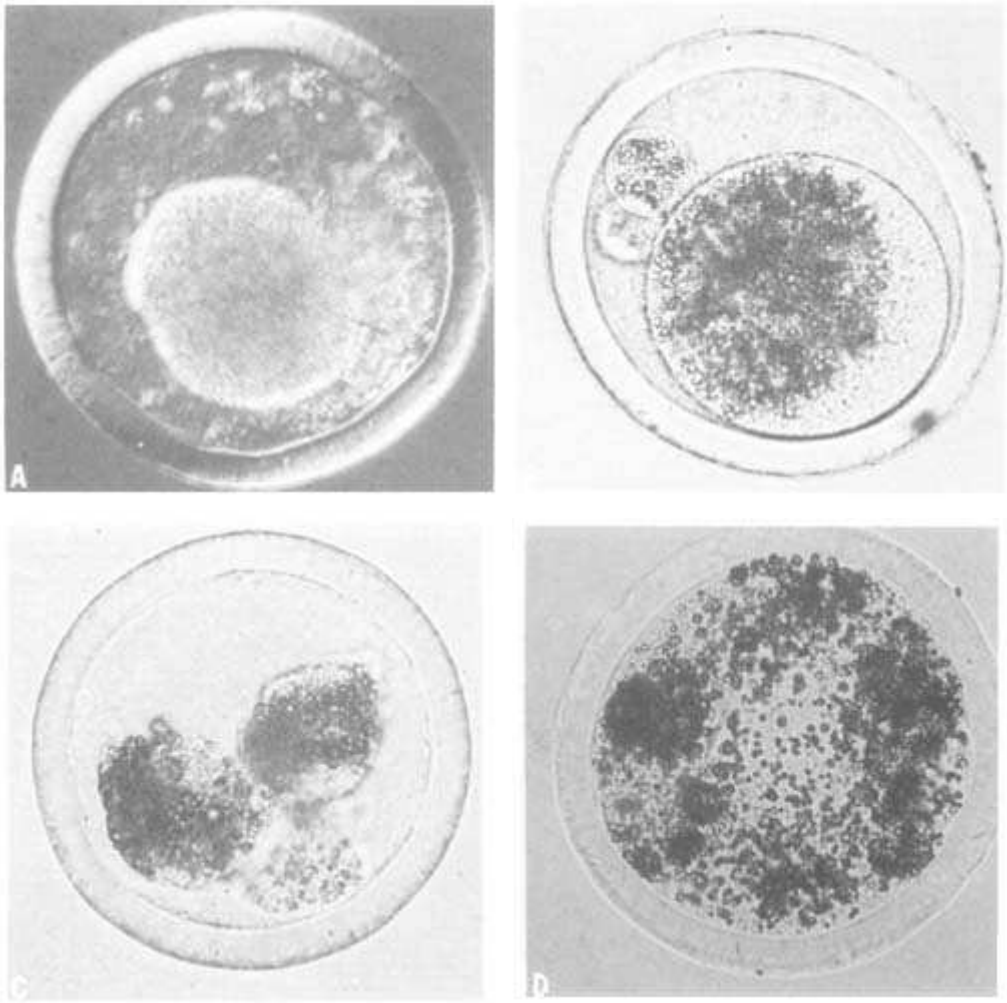
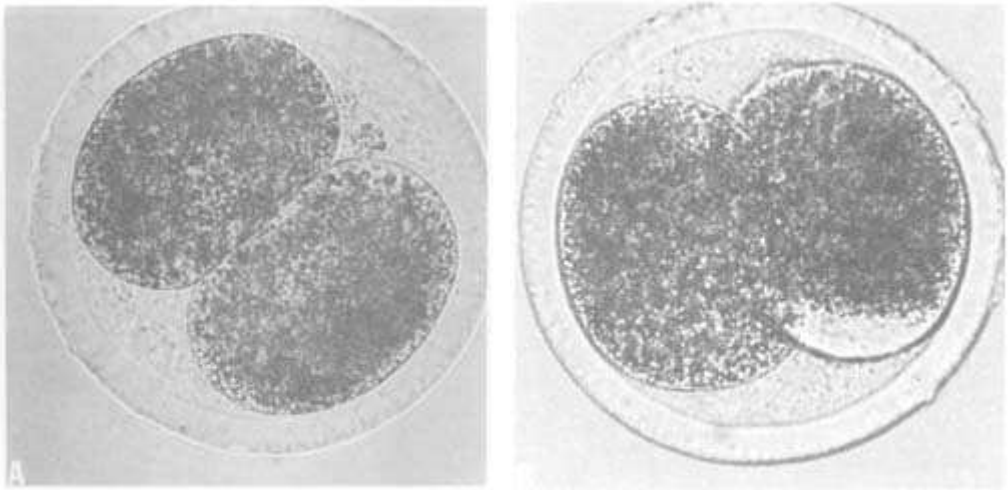
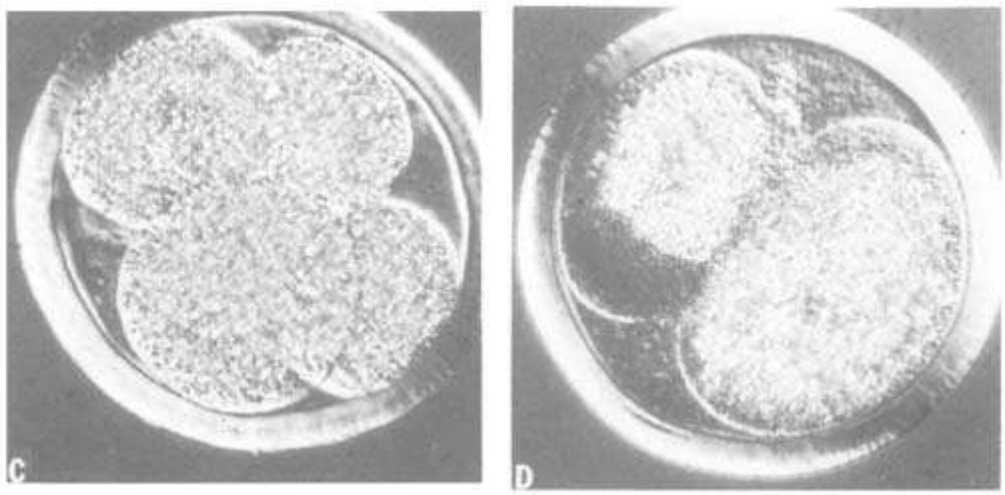


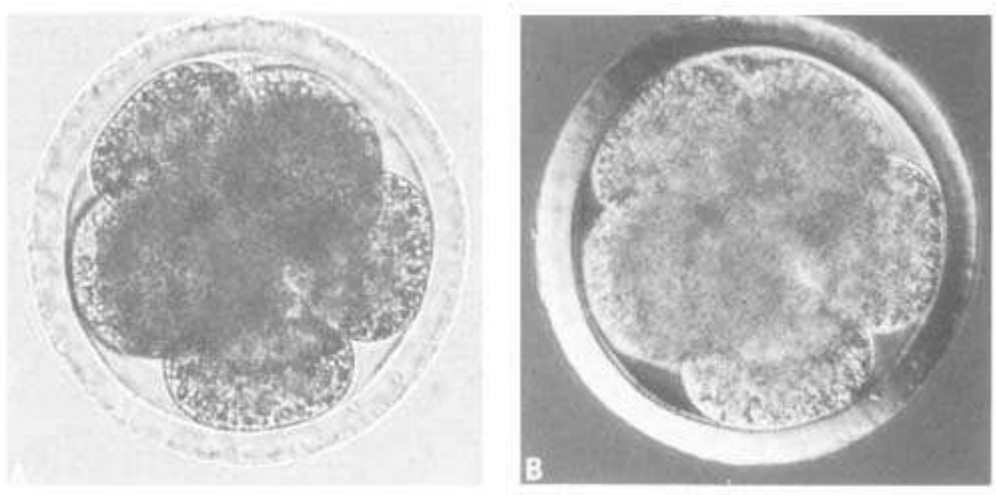
FIGURE 19
(A) Normal appearing 2-cell embryo recovered four and a half days after oestrus, bright-field optics. (B) Degenerating 2-cell embryo recovered five days after oestrus. Note clear cytoplasm in one blastomere. (C) Normal 4-cell embryo recovered two and a half days after oestrus. Nomarski optics. (D) A 2-cell embryo recovered five days after oestrus. Note clear cytoplasm. Nomarski optics





Day-7 embryos should be early blastocysts. As mentioned earlier, presence of a blastocoelic cavity is a good sign. Day-8 embryos should have a large blastocoele and some should be expanding, i.e. the diameter should be increasing so that the zona pellucida is thinned. A distinct, inner cell mass should be present. Other aspects of morphology should be as described earlier in this section. As with day-6 embryos, various imperfections are not uncommon in perfectly acceptable embryos.

FIGURE 20
(A) Normal 8-cell embryo recovered three days after oestrus. Bright-field optics. (B) Same embryo as in (A) but Nomarski optics. (C) Normal 12- to 14-cell embryo recovered four days after oestrus. Nomarski optics. (D) Severely retarded 12- to 14-cell embryo recovered six days after oestrus. Bright-field optics.



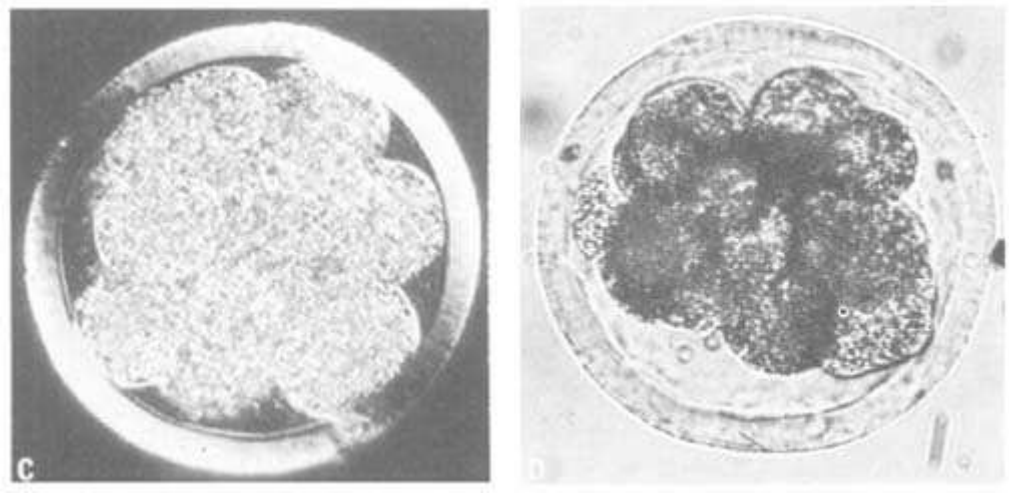
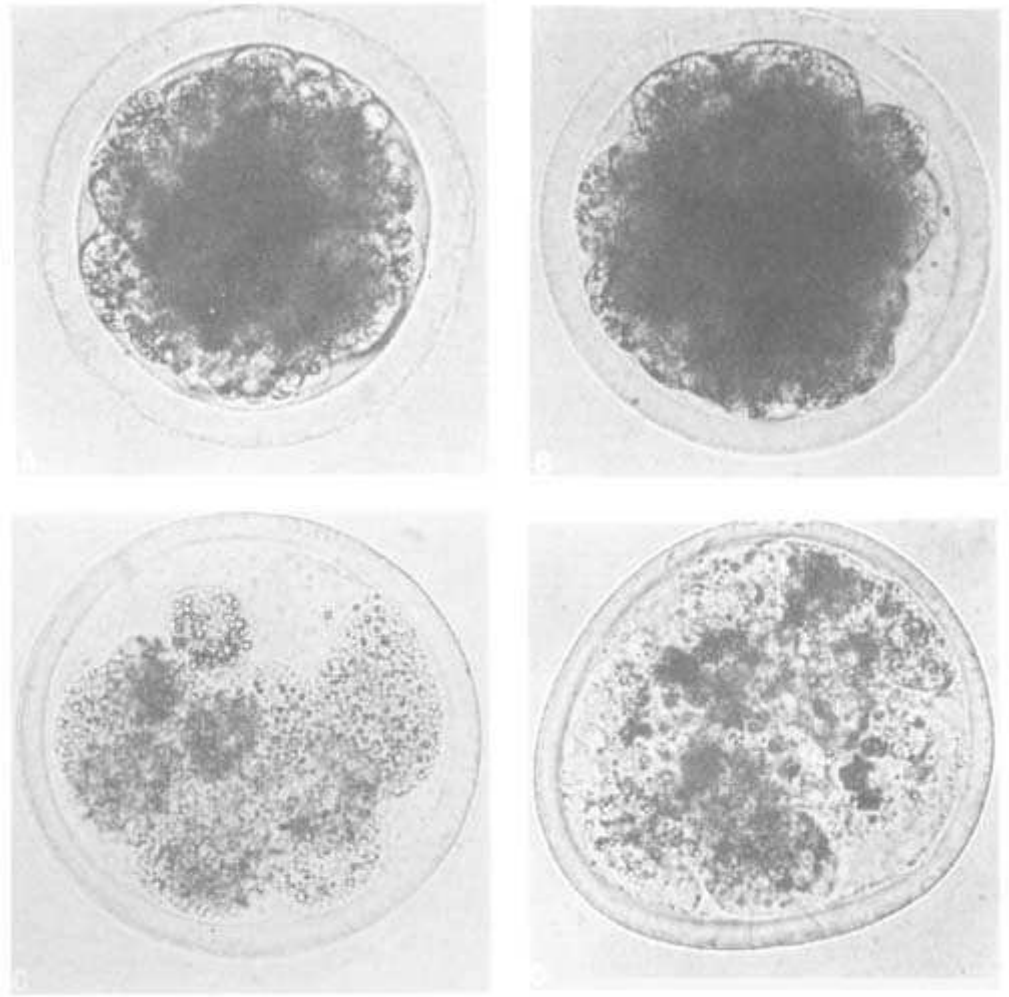


FIGURE 21
(A) Uncompacted morula recovered three days after oestrus, probably degenerating. (B) Uncompacted morula recovered three days after oestrus; dark cytoplasm. (C) Severely retarded and degenerating embryo recovered six days after oestrus. (D) Severely degenerate embryo recovered seven days after oestrus.
All are bright-field optics.



In our laboratory at Colorado State University, we have evaluated nearly 15 000 bovine ova over the years. About one-third of these have been unfertilized or severely degenerate; perhaps the most important task in evaluating ova is to identify these and fail to transfer

them so that pregnancy rates are not lowered. The single most difficult task for people learning to classify embryos is to distinguish between tight morulae and unfertilized oocytes (note that unfertilized embryo is improper terminology and internally contradictory), which can look very similar in size and texture. The unfertilized ovum has a perfectly smooth cell membrane, at least over a part of the cell, while the tight morula will have a slightly scalloped appearance.

FIGURE 22
(A) Newly compacted morula recovered seven days after oestrus. Bright-field optics. (B) Compacted morula recovered seven days after oestrus with several excluded cells; good morphological quality. Nomarski optics. (C) Compacted morula recovered seven and a half days after oestrus with many large, excluded cells, fair morphological quality. Bright-field optics. (D) Poor quality morula with many degenerate cells. However, the small, compacted mass to the lower left has a small chance of developing into a calf. Bright-field optics

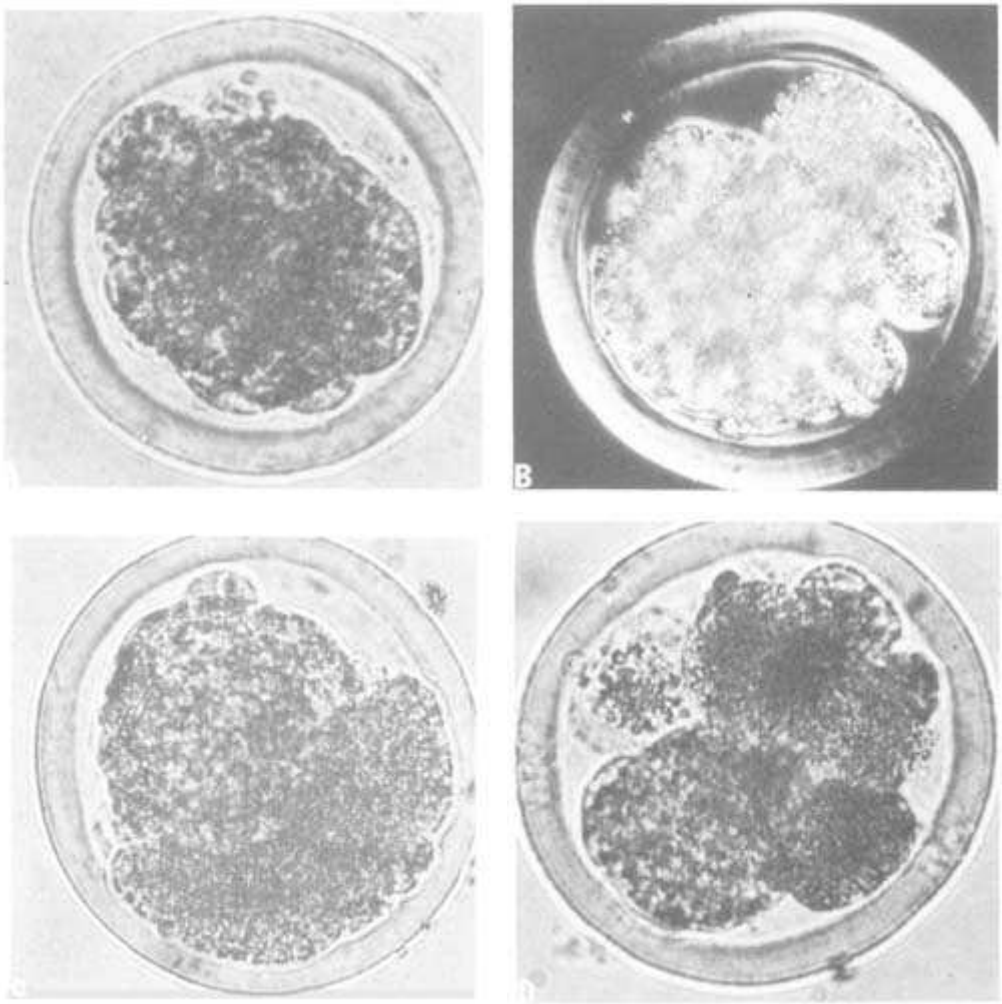
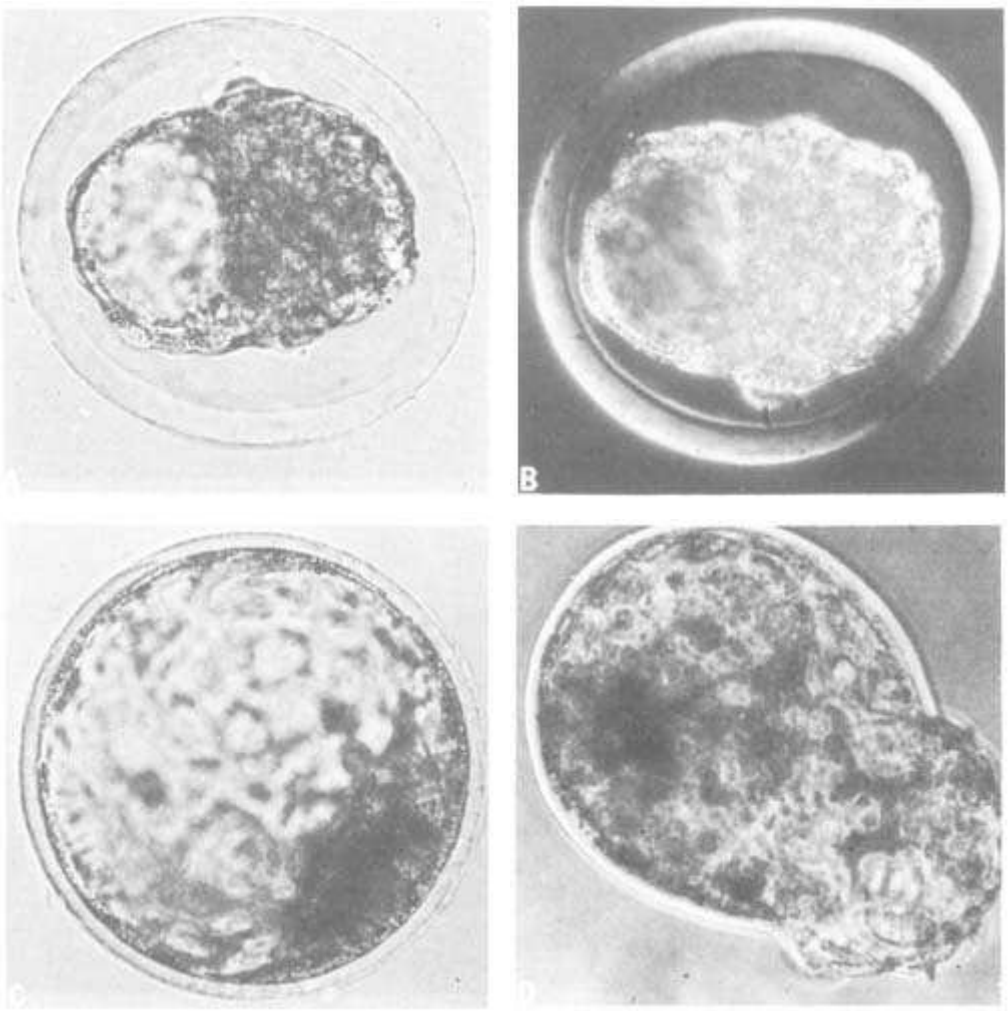


FIGURE 23
(A) Normal, early, expanded blastocyst recovered seven days after oestrus. Bright-field optics. (B) Same embryo as in (A) but Nomarski optics. (C) Normal, expanded blastocyst recovered seven and a half days after oestrus. Note the thinned zona pellucida. Bright-field optics. (D) Hatching blastocyst typically found nine days after oestrus. Bright-field optics



With experience, these two types of ova can be distinguished easily, especially with a compound microscope (Figures 24 and 25). Occasionally they are classified incorrectly, even by experts who do not take sufficient time (really only 5–10 seconds) to evaluate the embryos correctly. A second, much more rare misclassification occurs when unfertilized ova degenerate in the centre and become quite clear, resembling a blastocyst at first glance (Figure 25D). Most other misclassifications are a matter of degree in distinguishing among good, fair and poor embryos (see below). An excellent treatise on ovine embryo morphology is authored by Wintenberger Torres and Sevellec, 1987. Bovine and ovine embryos are nearly identical morphologically.

FIGURE 24
(A) Good quality compacted morula with a few degenerate cells recovered six and a half days after oestrus. Bright-field optics. (B) Unfertilized ovum recovered seven days after oestrus, easily mistaken for a morula with a dissecting microscope. Bright-field optics. (C) Degenerate, probably unfertilized ovum, can be mistaken for morula at lower magnification. (D) Degenerate, unfertilized ovum, easily mistaken for morula at lower magnification

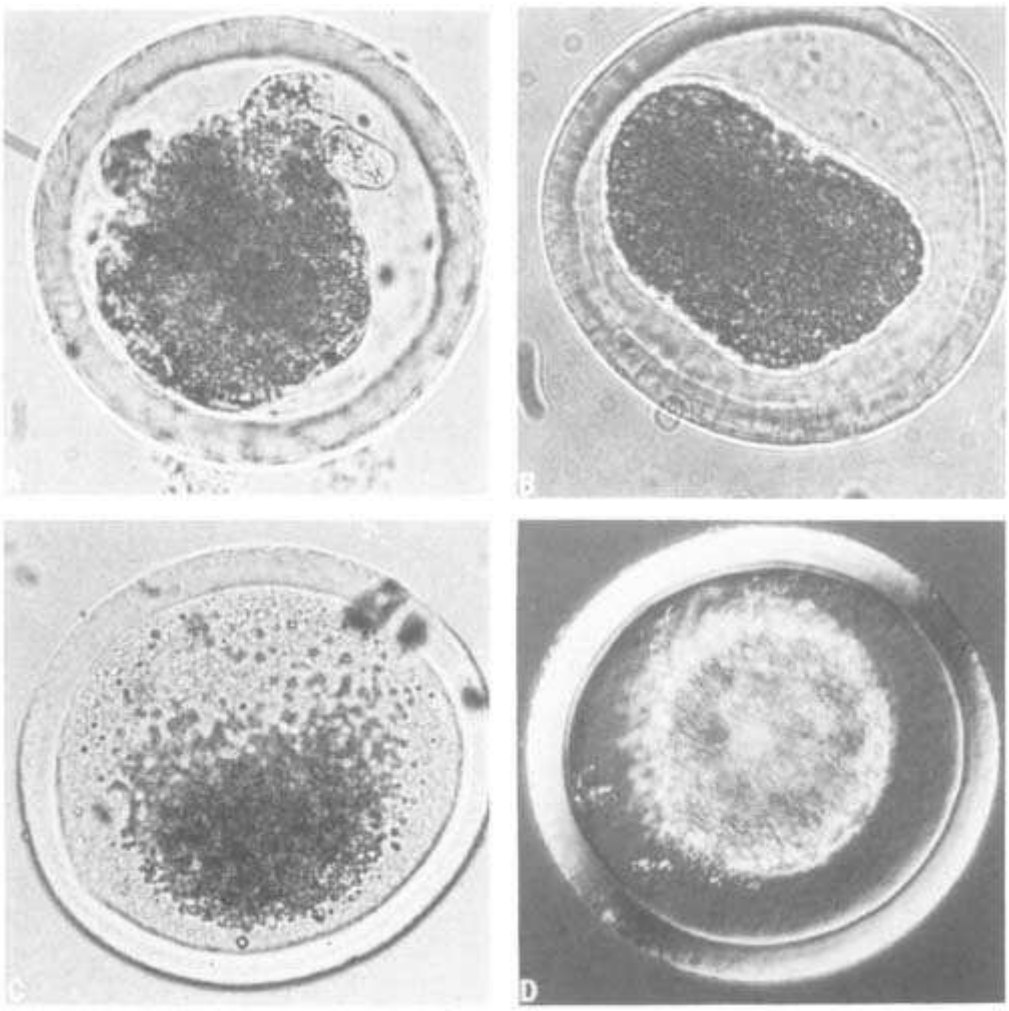
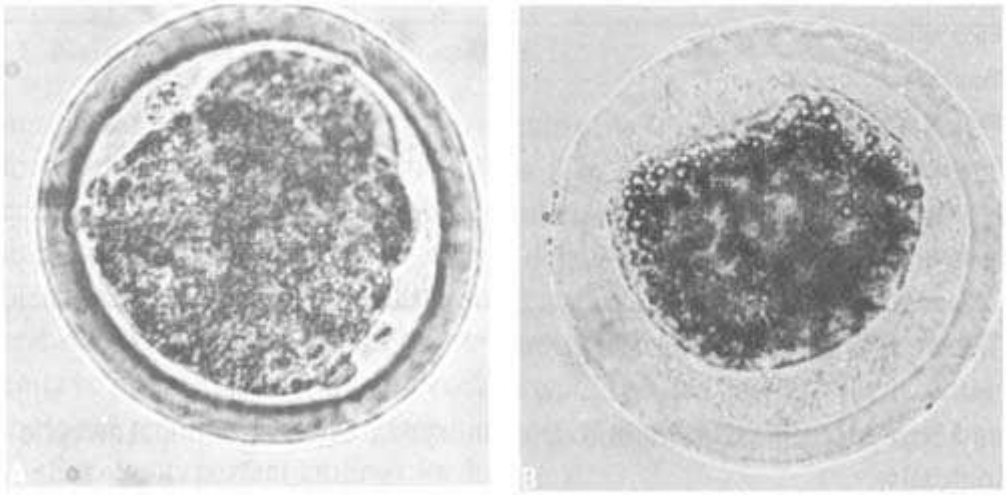


FIGURE 25
(A) Newly compacted morula recovered six days after oestrus (good quality) but with one large and probably abnormal cell to the upper right. (B) Unfertilized ovum easily mistaken for a morula. (C) Normal blastocyst recovered seven and a half days after oestrus. (D) Unfertilized ovum with large vesicle recovered five days after oestrus, easily mistaken for a blastocyst at lower magnification.
All are bright-field optics



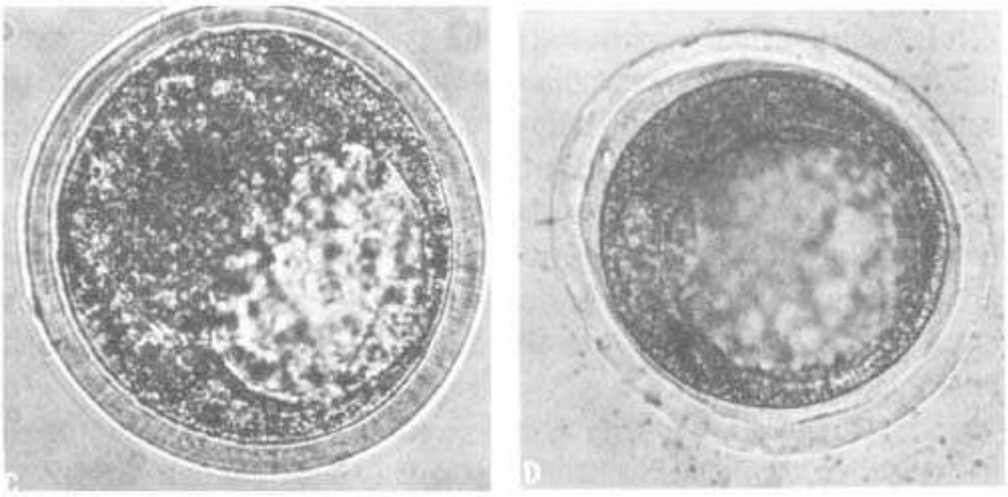


TABLE 5
Stage of normal embryonic development as a function of days after donor's oestrus

Stage of development	Days after onset of oestrus
1-cell	0–2
2-cell	1–3
4-cell	2–3
8-cell	3–5*
16-cell	4–5*
Early morula	5–6
Tight morula	5–7
Early blastocyst	7–8
Blastocyst	7–9
Expanded blastocyst	8–10
Hatching blastocyst	9–11

* Embryos usually move from the oviduct to the uterus at the 8- to 16-cell stage.

The proper procedure for classifying embryos is to isolate them, remove debris (which occurs automatically in the process of washing them three times), and then separate them into groups of transferable (or freezable or splittable) and non-transferable (unfertilized or severely degenerate) groups. Each ovum should then be carefully examined individually by focusing up and down and in certain cases rolling the embryo with a pipette or by shaking the dish. Those classified incorrectly should be placed in the proper groups and the non-transferable ova set aside. In cases in which classification is uncertain, ova should be examined with a compound microscope.

In our laboratory, we classify all ova into six categories. For those frozen or transferred, the final classification is generally made just before freezing or transfer. We fill out a form before making the final classification, which forces the evaluator to record (and thus take into account) the following criteria: age (days post-oestrus), cell number, compaction status, variability in cell size, colour of cytoplasm, areas of degeneration, numbers of excluded blastomeres, size of peri-vitelline space, stage of embryonic development, and number of days the embryo is retarded from normal (e.g. a four-cell embryo recovered five days after oestrus would be two days retarded). After these are recorded, ova are placed into one of

the following six categories:

TABLE 6
Pregnancy rates of embryos classified into quality groups based on gross morphology

Classification	No.	Percentage of embryos	Pregnancy rate
Excellent	275	54	63[a]
Good	152	30	58[a]
Fair	42	8	31[b]
Poor	42	8	12[c]

[a, b, c] Pregnancy rates with different superscripts differ, P<05.

- Excellent: perfect embryo for its age
- Good: trivial imperfections such as oval zona pellucida, a few, small excluded cells, or slightly asymmetrical shape
- Fair: definite but not severe abnormalities such as moderate numbers of excluded cells, small size, small amounts of degeneration, or retarded in development by up to one day
- Poor: considerable degeneration, vesiculated cells, greatly varying cell size, failure of compaction, very small and/or retarded by two days in development
- Degenerate: severely degenerate and not worth transferring
- Unfertilized or two- or three-cell.

It is often impossible to determine if an ovum is a severely degenerate embryo or is unfertilized. Even two-or three-cell embryos may in fact be fragmented, unfertilized ova. Table 6 (from Elsden ***et al.***, 1978) provides a distribution of embryos into the four categories considered transferable as well as pregnancy rates for each group. Clearly, this classification system ranks embryos reasonably well on a statistical basis. Of course, it is far from ideal from the standpoint of sorting embryos into the group that will result in calves and the group that will not. As a rule of thumb, only good and excellent embryos are suitable for splitting, and only fair, good, and excellent ones are suitable for freezing. Results of freezing fair quality embryos are marginal.

Chapter 8
Transfer of embryos

In cattle, embryos are routinely transferred to the uterine horn. This is because nearly all embryos are recovered non-surgically from the uterus, and therefore should be returned to this site. Furthermore, it is much easier to transfer embryos to the uterus than to the oviduct.

Both surgical and non-surgical methods of embryo transfer can be made to work well. Under most circumstances, non-surgical transfer is greatly preferred, although surgical transfer can be done quite rapidly, even in rather primitive circumstances.

SURGICAL TRANSFER

Although thousands of embryos have been transferred via mid-line abdominal incision to cows under general anaesthesia, in most circumstances a flank incision is far more practical. Recipients are placed in squeeze chutes that give access to either flank. The CL is located by rectal palpation and the flank ipsilateral to the CL is clipped, washed with soap and water, and sterilized with iodine and alcohol. About 60 ml of 2 percent procaine is given along the line of the planned incision. In everyday practice this seems more reliable than using a paravertebral block. Having scrubbed, the surgeon makes a skin incision about 15 cm long, high on the flank, just anterior to the hip. Muscle layers are separated, and the peritoneum is cut. The surgeon inserts a hand and forearm into the incision, locates the ovary, usually about 25 cm posterior to the incision, and visualizes or palpates the CL. The uterine horn is exteriorized by grasping and stretching with the thumb and forefinger the broad ligament of the uterus, which is located medial to the uterine horn. The uterine horn itself is very fragile. A puncture wound is made with a blunted needle through the wall of the cranial one-third of the exposed uterine horn. Using about 0.1 ml of medium in a small glass pipette (<1.5 mm outside diameter), an assistant draws up the embryo from the storage container. The pipette is then inserted into the lumen of the uterus, and the embryo is expelled. It takes some experience to be confident that the embryo has been deposited in the lumen. The incision is then closed, using two layers of sutures. With practice, the surgery takes about 15 minutes.

NON-SURGICAL TRANSFER

The big problem with non-surgical transfer is the difficulty in becoming proficient in this technique. First, it is necessary to be able to palpate ovaries accurately in order to select the side of ovulation. Pregnancy rates are markedly lowered if embryos are transferred to the uterine horn contralateral to the corpus luteum (Seidel, 1981a). Also, recipients should be rejected if no corpus luteum is present or pathology of the reproductive tract is noted. Even very experienced palpators make some errors in palpating corpora lutea.

The next step is to pass the embryo transfer device through the cervix. This is more challenging during the luteal phase, which is when embryos are transferred, than during oestrus, when artificial insemination is done and the cervix is more open. Heifers present a special challenge because of the small cervix; some breeds of cattle are more difficult than

others, e.g. certain ***Bos indicus*** breeds require greater skill. The best training prior to undertaking non-surgical embryo transfer is experience in artificial insemination. Ideally, the trainee will have inseminated hundreds of cattle artificially, including a large number of heifers.

The third step with non-surgical transfer is to be able to insert the tip of the instrument into the desired uterine horn quickly, smoothly and atraumatically. Some people never master this technique, and others require hundreds of transfers to become proficient. This is not surprising since pregnancy rates from artificial insemination are usually markedly lower for the first 50–100 cows bred by a newly trained inseminator than after he or she has become proficient. Well-trained inseminators generally require 100–200 non-surgical transfers until their pregnancy rates plateau; others usually require more. Most technicians who are successful with non-surgical transfer had low pregnancy rates for their first 100 non-surgical transfers.

One approach for people starting an embryo transfer programme is to begin with surgical procedures until acceptable pregnancy rates are achieved. If one begins with non-surgical transfer and pregnancy rates are low, it is difficult to distinguish among problems such as identifying usable embryos, problems with media, problems in storing embryos from collection to transfer, poor non-surgical embryo transfer technique, recipient problems, etc. Pregnancy rates are frequently low with surgical embryo transfer also, but one of the problems just mentioned is usually the cause, not the surgical transfer. Once the entire sequence of superovulation, embryo recovery, surgical embryo transfer, etc., is working well, it is advisable to switch to non-surgical transfer until proficiency is achieved. A frequent error is to have a number of embryo transfer teams work in a given province or country, none of which become proficient because of insufficient opportunities to gain experience. The result is that proficiency is attained slowly or not at all, and the programme is abandoned because of poor results.

Some people believe that there is a 5–10 percent advantage in pregnancy rates with surgical transfer, even when very proficient technicians are doing the non-surgical transfer. Even if this is true, in most circumstances nonsurgical transfer is still preferred because it is less expensive, it is quicker and does not involve surgical procedures. This may also obviate the need for veterinary supervision, which is required for surgery in many countries.

LOADING STRAWS

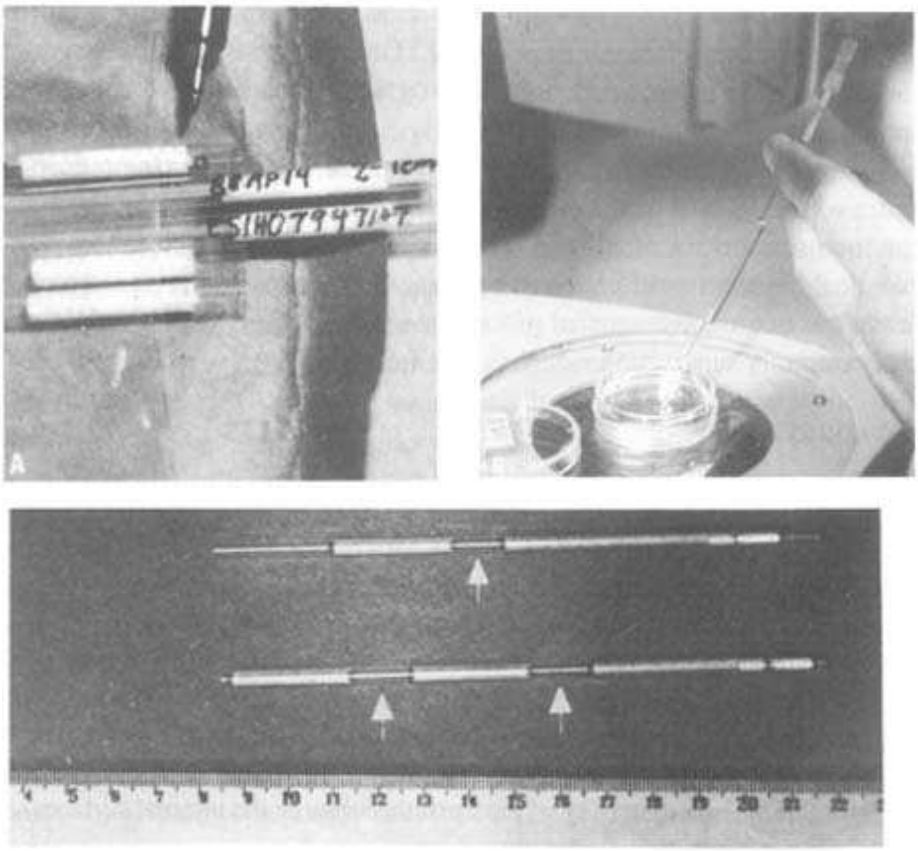
We recommend loading straws for embryo transfer as illustrated in Figure 6. The first step is to take a sterile 0.25-cc straw, shortened by 1 cm before sterilization, label it and rinse it twice with medium to removed any toxic contaminants, taking care not to wet the cotton plug and to discard the rinses. A plastic 1-cc tuberculin syringe fits snugly over the straw for aspirating and expelling fluid. The straw is filled nearly one-third full of fluid, then with a 5-mm column of air, then another column of fluid containing the embryo, one-third the length of the straw, then another short column of air, and finally more fluid to fill the straw and wet the cotton plug. Care must be taken not to compromise the sterility of the tip of the straw or the internal surfaces.

NON-SURGICAL TRANSFER EQUIPMENT

The most commonly used instrument for non-surgical transfer is the standard Cassou inseminating gun for French straws (Figure 27). There are dozens of other basically similar devices. Many have been tried in the Embryo Transfer Laboratory at Colorado State University. None of these more expensive devices gave improved pregnancy rates with ***Bos taurus*** cattle, and many were difficult to load without compromising sterility. Thus, our recommendation remains to use the standard French straw gun with a 0.25-cc French straw because it is inexpensive and easy to use correctly. We advise cutting about 1 cm off the standard 0.25-cc French straw to give better control of the tip after covering the straw and gun with the sterile sheath. We also recommend placing a sterile plastic bag over the

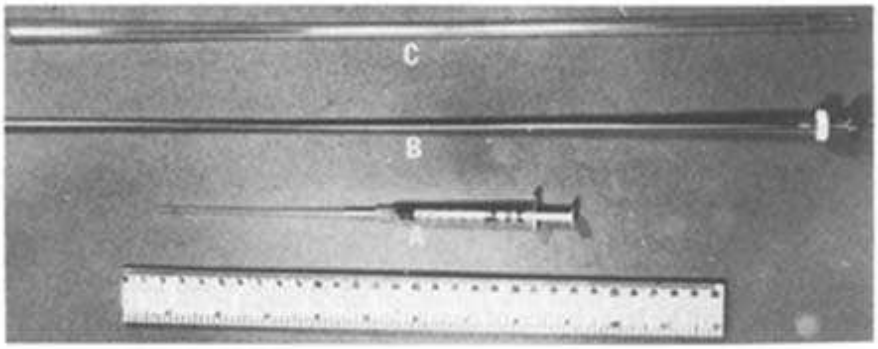
instrument as it is placed in the vagina, and piercing the plastic bag as the instrument enters the cervix.

FIGURE 26
Steps in loading a 0.25-cc plastic straw in preparation for transfer (or freezing) an embryo: labelling (A), aspirating embryo in second column of fluid (B), and the loaded straws (C). Note the air bubbles (arrows) to compartmentalize the straw. The top straw is loaded for freezing and the bottom straw is loaded for transfer, with a third column of medium as an added measure of safety



There are three situations in which an instrument other than the standard Cassou insemination gun may be advisable. Most instruments designed specifically for non-surgical transfer are longer, thinner and have a smoother tip than the sheath on the French straw gun for artificial insemination. This makes them somewhat easier to pass through the cervix. This can be especially helpful for beginners, although it is usually of little value to experienced technicians except for some heifers and certain breeds with difficult cervixes. This constitutes the second situation for the special instruments, i.e. when mostly heifers are used as recipients or difficult cervixes are experienced. The third situation in which a different instrument can be helpful is in large breeds of cows with long uteri, such as older Holstein-Friesian cows. Many of the instruments for non-surgical transfer are longer than the standard French straw gun, and these are somewhat easier to use for the long uterus. Perhaps the most used of these special instruments is the miniaturized embryo transfer syringe made by Cassou, which uses blue sheaths (see Chapter 17). Straws should be shortened by 0.5 cm for this instrument.

FIGURE 27
Non-surgical transfer equipment illustrating a 0.25-cc plastic straw attached to a syringe (A), a Cassou inseminating gun (B), and the sheath (C)



We reiterate, however, that while it may be desirable to invest in several of the expensive non-surgical transfer devices (which also use expensive sheaths), under most circumstances large numbers of these devices cannot be justified, and the standard Cassou gun should be used.

ANAESTHESIA

Epidural anaesthesia is recommended for routine non-surgical transfer (see Figure 2). This relaxes rectal musculature, making it easier to manipulate the reproductive tract gently as is required for high pregnancy rates. Very experienced technicians sometimes do not use epidural anaesthesia. However, under most conditions this is probably unwise because of the occasional difficult animal. Epidural anaesthesia clearly costs some minutes in time, and occasionally effective anaesthesia is not attained. The problem of waiting several minutes until the rectal muscles relax can be circumvented by having an assistant give the epidural injection about five minutes before embryo transfer while the technician is transferring the embryo to the previous recipient. The procedure for epidural anaesthesia is the same as for non-surgical embryo recovery.

TRANSFER PROCEDURE

The actual embryo transfer process is similar to the method used for artificial insemination, except that the transfer gun is passed well up the uterine horn ipsilateral to the corpus luteum. A good site to aim at is the palpable bifurcation of the uterine horns. Some technicians go a bit further by straightening the uterine horn progressively just before the gun is passed. The key is to pass the gun without damaging the endometrium. Therefore, it is better to insert the instrument less deeply and not cause damage. Speed is quite important once the cervix is passed but, at the same time, techniques must be gentle. Because cattle tend to move around when confined in a chute, there will be less chance of damaging tissues if the procedure is done quickly. As with artificial insemination, the plunger should be depressed firmly, but not too rapidly.

SYNCHRONY OF REPRODUCTIVE CYCLES

Perhaps the most venerable principle of embryo transfer is that the stage of the reproductive cycle of the recipient must correspond to that of the donor or physiological stage of development of the embryo. This is definitely true for cattle (see Seidel, 1981a). Two questions arise: to what extent is asynchrony of reproductive cycles tolerated, and what methods can be used successfully for synchronizing reproductive cycles pharmacologically?

Many studies indicate that pregnancy rates decline with asynchrony of donor and recipient (Seidel, 1981a, Hasler ***et al.***, 1987). In most studies with morphologically normal, unfrozen embryos, pregnancy rates were similar with perfect synchrony and asynchrony of 1 day. Several studies with large numbers per group are summarized in the following table. There is a hint that optimum pregnancy rates result when recipients are in oestrus slightly before donors, although there may be a statistical artefact in these data due to different oestrus detection practices between recipients and donors.

In any case, it appears that asynchrony of up to one and a half days does not result in marked reduction in pregnancy rates with unfrozen embryos collected six to eight days after oestrus. Pregnancy rates do not decline markedly even with + 2 days asynchrony. With embryos of lower quality asynchrony is less well tolerated, and slight negative asynchrony seems preferable to positive asynchrony for such embryos (Lindner and Wright, 1983; Hasler ***et al.***, 1987). Embryos collected three to four days after oestrus seem to be less tolerant of asynchrony than older embryos. Furthermore, several studies indicate that there is less tolerance of asynchrony with frozen embryos; more definitive data are required to substantiate this point.

METHODS OF SYNCHRONIZING OESTRUS

There are many methods of synchronizing reproductive cycles of recipients to match those of donors. In some circumstances, natural synchrony is feasible, but in most cases some recipients will need to be synchronized to augment those whose oestrous cycles match the donor's naturally. The most widely accepted procedure for synchronizing recipients is administration of a luteolytic dose of prostaglandin F_2 alpha or a suitable analogue during the luteal phase. This is probably superior to using natural oestrous cycles (Hasler ***et al.***, 1987). Injecting potential recipients with two doses of prostaglandin at 11-day intervals when stages of the reproductive cycle are unknown also works well if cattle are cycling.

TABLE 7
Percentage pregnant (No.) with varying degrees of donor-recipient oestrous cycle asynchrony

Synchrony*	Shea ***et al.,*** 1976	Nelson ***et al.***, 1982	Schneider ***et al.***, 1980	Wright, 1981	Hasler ***et al.***, 1987
+1.5		38(26)		59(27)	73(67)
+1.0	59(334)	54(100)		61(98)	75(618)
+0.5		57(277)	66(475)	68(374)	74(973)
0	62(1 126)	58(586)	67(1 488)	59(747)	73(3 340)
-0.5		61(311)	61(593)	61(620)	73(1 089)
-1.0	49(556)	57(112)		58(301)	69(707)
-1.5		52(31)		41(115)	68(132)

* + means recipient in oestrus before donor.

Various progestin withdrawal procedures also have been used successfully. Under some circumstances, one of these, Syncromate B (norgestomet) has resulted in lower pregnancy rates than prostaglandin controls (King ***et al.***, 1986). However, others have used the Syncromate B method successfully for recipients, therefore more data are required to analyse these results.

Refractoriness of cattle to repeated synchronization appears to be a nonproblem. Despite anecdotal reports of problems in the field, each of a number of experiments to study this problem systematically has indicated that no such refractoriness occurs with the prostaglandins.

EXAMPLE OF PROGRAMMING A HERD FOR EMBRYO TRANSFER

To illustrate combining the information in previous chapters in order to set up an embryo transfer schedule, key steps are listed in Table 8. In this example, it is assumed that regular oestrus detection is not being done prior to the start of an on-farm programme, therefore the system of two injections of prostaglandin F_2 alpha, or an analogue, given at 11-day intervals is used for both donors and recipients. This also synchronizes all donors and recipients,

even if the stages of their oestrous cycles are known. If a large number of donors is used at one location, it is wise to stagger them by one or two days along with recipients so that embryo collection and transfer will be done on two or three consecutive days.

TABLE 8
Example of programming donors and recipients

Day	Donors	Recipients
0	Prostaglandin injection	Palpate sample to verify that they are cycling
11	Prostaglandin injection	
12–16	Record donors in oestrus	
16		Prostaglandin injection
25 p.m.	6 mg FSH	
26 a.m.	6 mg FSH	
26 p.m.	4 mg FSH	
27 a.m.	4 mg FSH	Prostaglandin injection
27 p.m.	2 mg FSH	
28 a.m.	2 mg FSH + prostaglandin	
28 p.m.	2 mg FSH + optional prostaglandin	
29 a.m.	2 mg FSH	
28–32		Record recipients in oestrus
30 a.m.	Donors expected in oestrus	
30–31	Inseminate donors 12 and 24 hours after beginning of oestrus	
37	Collect, freeze or thaw, and transfer embryos	
37	Prostaglandin injection	
47–54		Non-pregnant recipients in oestrus
75 +		Palpate for pregnancy
105 +		Confirm palpation results

On day 0, or preferably earlier, it is wise to palpate most of the donors and a sample of recipients to determine if the majority have a corpus luteum. If fewer than half have a corpus luteum, it may be wise to postpone the programme for several weeks because prostaglandin is effective only for cows in the luteal phase. Most of Table 8 is self-explanatory, but we call attention to the following. On day 27, potential recipients are given prostaglandin one day before donors since gonadotrophin-treated cows show oestrus one-half to one day earlier after prostaglandin injection than untreated cattle. On day 30, most donors will be in oestrus, although a few may already have been in oestrus the previous evening and some may be up to a day late (these cows usually have a poor response). Actually, most will first show oestrus 40–46 hours after prostaglandin. On day 37, after embryo collection, donors are given another prostaglandin injection to prevent multiple pregnancy from occasional uncollected embryos. The interval from this injection to oestrus is unpredictable; it may occur weeks later.

Obviously, this is only one of many possible programming schemes. However, it does illustrate the principles involved.

Chapter 9
Cryopreservation of bovine embryos

Bovine embryos can be cryopreserved easily. If procedures are carried out correctly, pregnancy rates are 75–85 percent of those for unfrozen embryos transferred under similar circumstances. The following protocol has worked well in a variety of settings, but attention to detail is required.

1. Start with good to excellent quality embryos recovered six to eight days after the donor's oestrus. Embryos should be frozen within three to four hours of recovery.

2. Wash embryos through at least three changes of medium (ten washes if embryos are to be exported or if it is suspected that embryos have been exposed to infectious disease; see Chapter 16) of sterile Dulbecco's phosphate-buffered saline plus 0.4 percent bovine serum albumin (BSA) or 10 percent ***heat-treated*** serum (steer serum, newborn calf serum, or foetal calf serum are all satisfactory; however, serum should not be used if embryos are to be exported).

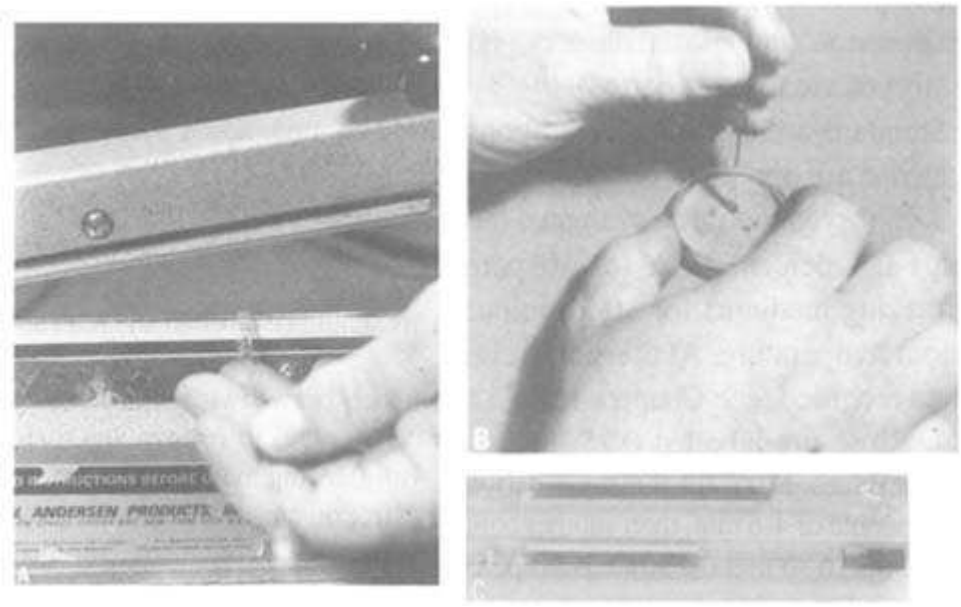
   Standard antibiotic concentrations should be used; added pyruvate and glucose are optional.

3. Embryos should be evaluated morphologically and then placed into PBS plus 0.4 percent BSA (or 10 percent serum) plus 10 percent glycerol (freezing medium) for 10–20 minutes. All of the above steps are done at room temperature. At this point, containers should be labelled and relevant data recorded (see Chapter 16).

4. Rinse pre-labelled 0.25- or 0.5-cc French straws twice with freezing medium (up to, but not including, the cotton plug) to remove any toxic residues. Next, fill the straw half-way with freezing medium, then an air bubble of 4–6 mm, then another column of freezing medium containing the embryo so that the straw is 90 percent full when the cotton end is wetted (see Figure 26). An optional step is to add 1.5–2 mm of non-toxic paraffin oil to the top of the column. The end is then sealed with heat (for example, by heating a haemostat with a cigarette lighter and then clamping the end of the straw) or polyvinyl chloride powder (PVC) (see Figure 28). The straw is placed into the freezing machine horizontally or, if a vertical system is used, with the heat- or PVC-sealed end down so that the embryo sinks and rests on the paraffin oil.

One function of the paraffin oil is to flatten the meniscus to prevent mechanical damage to embryos that get caught in the angle of the meniscus and the wall of the straw when ice forms. This is critical for the smaller mouse embryos but of minor importance for bovine embryos. Paraffin oil is of no value for this purpose if straws are frozen in a horizontal position. A second benefit of paraffin oil is to prevent embryos from entering the air space next to the heat seal. This results in death of the embryo during freezing. Without paraffin oil, embryos enter this air space easily unless straws are handled ***very gently***.

FIGURE 28
Illustration of sealing a plastic straw by heat (A) and by tamping polyvinyl chloride powder into the end of the straw before wetting (B); completed seals (C)



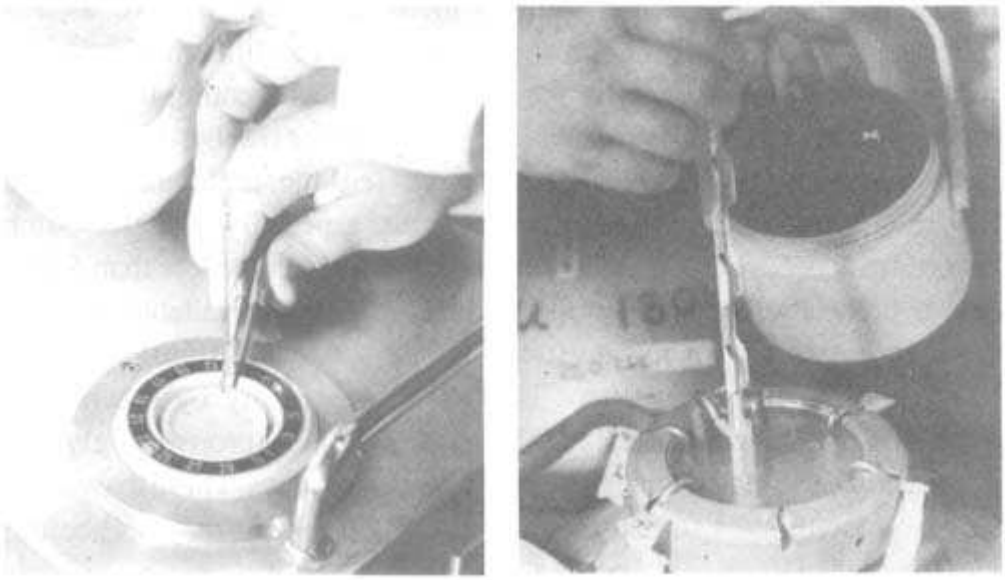
5. Cool straws to -7°C. The rate of cooling during this step can be slow or rapid.

6. Seed straws after they have been at -7°C for five minutes, and keep them at -7°C for an additional 10 minutes. Be sure that they remain seeded. Seeding is accomplished by touching the side of the embryo container with forceps dipped into liquid nitrogen (Figure 29A). Automatic seeding occurs with some freezing machines, but not all self-seeding systems are reliable in all circumstances.

7. Cool straws from -7°C to -30°C at 0.5°C/minute. When straws reach -30°C, plunge them into liquid nitrogen (within two to three minutes) and store in liquid nitrogen (Figure 29B). The equipment to cool embryos can be simple or complex. The only advantage of complex equipment is saving labour. The cooling rate should average 0.5°C/minute (it can fluctuate briefly between 0.3° and 0.7°C).

8. Thaw 0.5-cc straws by holding them quietly in the air for exactly 20 seconds followed by 20 seconds in a 37°C water bath; 0.25-cc straws should be thawed for 15 seconds in the air plus 15 seconds in 37°C water. After thawing, do all the steps at room temperature.

FIGURE 29
Inducing formation of ice crystals by touching the walls of the straw with forceps cooled in liquid nitrogen (A), and transferring a straw with a frozen embryo in an insulated container of liquid nitrogen from the freezing machine to the liquid nitrogen tank (B)



9. Next, isolate the embryo. This is done by cutting the heat-sealed end of the straw with clean scissors and expelling the embryo by pushing on the cotton plug. Glycerol may be removed from embryos in several ways. The standard method is to dilute in six steps: PBS plus 0.4 percent BSA plus 8.3 percent glycerol, 6.7 percent, 5 percent, 3.3 percent, 1.7 percent and then 0 percent glycerol, six minutes per step at ambient temperature. Theoretically, a better approach is to use unequal steps, e.g. 7 percent, 5 percent, 3.5 percent, 2 percent and 1 percent; although this is rarely done, perhaps it should be.

An alternative is four steps: (1) 6 percent glycerol plus 10 percent sucrose; (2) 3 percent glycerol plus 10 percent sucrose; (3) 10 percent sucrose (all in PBS plus 0.4 percent BSA); and then (4) PBS plus 0.4 percent BSA with no sucrose or glycerol. Instead of 0.4 percent BSA, 10 percent serum can be used. Each step should take six minutes, or five minutes in warm conditions (above 25°C). Both procedures lead to similar results, but the four-step method is faster.

Glycerol can also be removed in one step by placing embryos in 20–30 percent sucrose plus 0.4 percent BSA with no glycerol for five minutes. We have little direct experience with this method, but others have used it successfully. With some modifications in strategy, the dilution of cryoprotectant can be done directly in the straw, thus circumventing the need to manipulate the embryo between thawing and transfer (Leibo, 1988).

10. Evaluate embryo and transfer as soon as feasible, preferably within a few minutes of removing the cryoprotectant, especially if the one-step procedure is used. Discard degenerate embryos (should be less than 5 percent if procedures are done properly). If recipients are available, transfer the degenerate embryos anyway (non-surgically). A few will turn into calves.

Variations in procedures have been used successfully by others. For example, glycerol can be replaced by 1,2 propanediol (propylene glycol). Some people use glass containers instead of plastic straws. These thaw more slowly, and embryos should therefore be cooled to -35° or -38°C before plunging when glass containers are used.

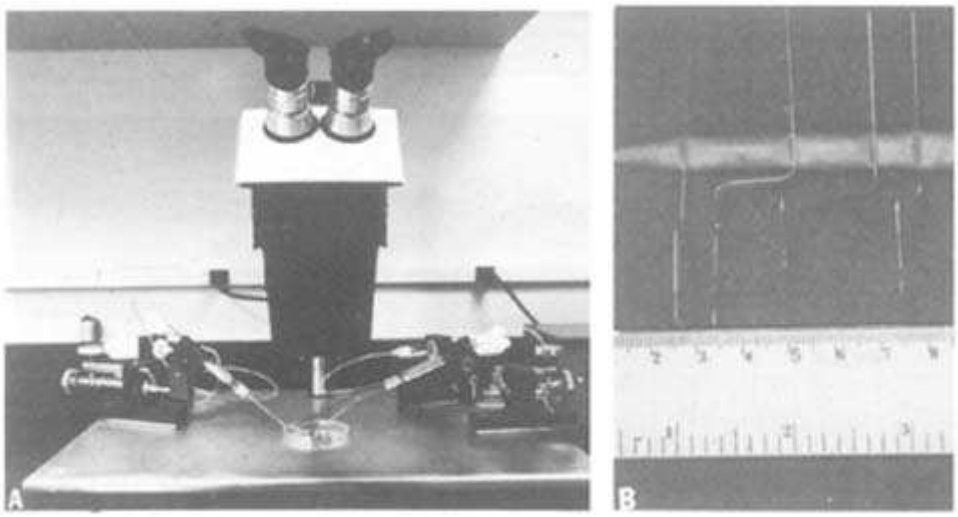
Further details about principles of cryopreservation may be found in Seidel (1988b). There are also some new approaches to cryopreservation that are much simpler, for example, vitrification. However, these cannot yet be recommended for routine cryopreservation of bovine embryos.

Chapter 10
Splitting embryos

Embryos can be bisected from the two-cell stage through the hatched blastocyst stage. However, because of logistical constraints, such as the need for surgical recovery, dividing pre-morula stage embryos will not be considered here (see Willadsen, 1982). There are two main reasons for splitting embryos. The first is to obtain identical twins, which are very useful for research as well as for certain commercial goals. The second is to increase productivity. Under above average commercial conditions, about 50 percent more calves result per two demi-embryos than per whole embryo.

Splitting bovine embryos is easy provided that the technician has patience, proper training, and appropriate equipment (Figure 30). Splitting need not involve complex technology, which means it is appropriate for less developed countries provided that there is a legitimate application.

FIGURE 30
Example of a micromanipulator system (A) and microtools for bisecting embryos (B)



Dozens of procedures for bisection of embryos have been published, from the very complex (Ozil, 1983) to the very simple (Williams and Moore, 1988). Most of these procedures consist of two stages: immobilizing the embryo and bisecting it. Immobilization can be done by applying suction to the zona pellucida, making a depression or cul-de-sac in the container, constructing a device that traps the embryo, or making the embryo stick to a surface, e.g. by roughening the surface of the container or using protein-free culture medium. The bisection is usually done with a broken fragment of razor blade or a fine glass needle (Figure 31). After the blastocyst stage, the embryo must be bisected so that both halves receive cells from the inner cell mass. After the late morula stage, there is no need to

return demi-embryos to a zona pellucida (Warfield ***et al.***, 1987).

Pregnancy rates will be high provided that: (1) the embryo is immobilized without damaging it; (2) the bisection process does not damage too many cells, and (3) embryos are bisected reasonably symmetrically. Embryos also may be divided into thirds or quarters, but this lowers success rates considerably.

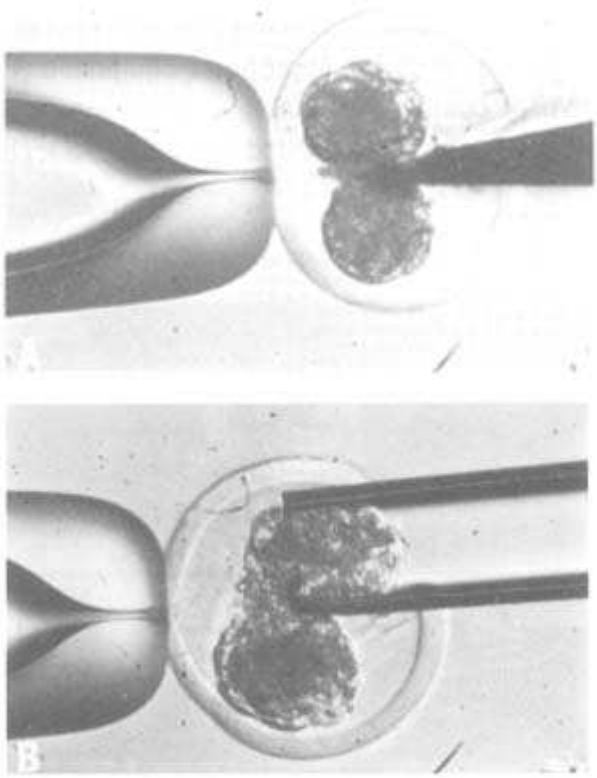


FIGURE 31
Immobilizing a blastocyst by means of suction using a micropipette and bisecting the embryo with a fragment of razor blade (A), and removing one demi-embryo from the zona pellucida. (These figures first appeared in Williams *et al., Theriogenology,* 22:524, 1984)

One or two demi-embryos may be transferred per recipient. It is probably best to place one demi-embryo in each uterine horn rather than placing both ipsilateral to the corpus luteum (Rowson ***et al.***, 1971). Twinning results in more calves per recipient. The main disadvantage is that there is increased morbidity and mortality when cows have two calves, and the calves will be slightly smaller than singles. Under most circumstances, twinning heifers is not recommended (see Chapter 2).

Chapter 11
Brief overview of emerging technologies

It took more than 80 years from the demonstration that embryo transfer was possible in laboratory animals, until it started to be used commercially. However, the interval from demonstration to application was less than a decade for cryopreservation of embryos. While, in selected cases, the pace is becoming even more rapid for the transition from basic laboratory science to application, some other seemingly practical technologies never find a use, or only a limited use. Artificial insemination is such an example: it is used widely in dairy cattle and some species of poultry, but only to a very limited extent in most countries for beef cattle, pigs, sheep, goats and horses. For that matter, embryo transfer currently has only very limited use in all species, although some of these uses are very important. This chapter deals with emerging technologies related to embryo transfer, but not in routine commercial use. Because it is impossible to predict which of these technologies will be useful in commercial agriculture in the near future, methodology will not be presented. Nevertheless, it was felt that a brief introduction to each would be appropriate.

SEXING EMBRYOS

Theoretically, the ideal method for sex control in cattle is separation of X-and Y-bearing sperm. Unfortunately, to date, there has been no clear example of a method that accomplishes this in mammals without damaging the sperm (Seidel, 1988a), although several methods have been used to sex embryos successfully and convincingly (Seidel, 1988a). As all of these methods have limitations, commercial application has been limited. Nevertheless, sexing embryos will be used commercially in the near future.

There are, however, several intrinsic constraints to sexing embryos. If the embryos of the undesired sex are to be discarded, the process is innately inefficient because of the high cost of obtaining embryos. Moreover, in many instances, embryos of either sex are valuable from the particular donors that justify the cost of embryo transfer, which makes sexing irrelevant.

Methods of sexing embryos are likely to remain imperfect. With current techniques, many embryos are not sexable at all, and some are sexed incorrectly. Procedures are clumsy, time consuming, slightly damaging to embryos and costly. Many of these problems will be solved, but it will probably be some years before most embryos collected will be sexed. The three most common procedures for sexing embryos are described briefly below. Other procedures are discussed elsewhere (Seidel, 1988a).

Karyotyping

With this procedure, a biopsy of the embryo is obtained and cultured with colchicine or a related drug that causes cells to stop dividing at the metaphase stage of mitosis. After some hours, cells are lysed osmotically, and the preparation is fixed and stained so that chromosomes can be examined microscopically. The main advantage of the method is that

it is quite accurate for the embryos that produce at least one readable metaphase set of chromosomes (about half for day-7 embryos). Another advantage is that gross chromosomal abnormalities can be detected. A third advantage is that, except for the biopsy procedure, the equipment needed, primarily a good microscope, is already part of many laboratories. Also, reagents are inexpensive and easy to obtain.

There are three main disadvantages to karyotyping: readable sets of chromosomes are frequently not obtained, particularly from embryos recovered before day 10; the embryo must be biopsied; and the procedure requires considerable training and can take as long as 12 or more hours. Because of these problems, most people feel that this approach is unsuitable for everyday use.

Antibodies to male-specific antigen

This procedure requires antibodies to cell-surface molecules specific to male tissues (sometimes referred to — probably incorrectly — as the anti-H-Y antigen method). Embryos are incubated for 30–60 minutes with antibodies, and then for an additional 30–60 minutes with an antibody to the first antibody containing a fluorescent dye. Embryos are then briefly examined with a fluorescence microscope. Male embryos fluoresce. The advantages of this approach are its speed and lack of need to biopsy embryos. The disadvantages are need for a fluorescence microscope, commercial unavailability of reagents, the subjective nature of determining what is and what is not specific fluorescence and limited accuracy (80 percent). Despite these problems, many people feel that this approach may be developed into an acceptable procedure for routine use.

Y-chromosome-specific DNA probes

This approach is based on a molecular biological technique. Pieces of DNA (probes) can be made that bind to DNA on the Y-chromosome, but not other chromosomes. Embryos are biopsied, the DNA is extracted from the cells, and an enzymatically or radioactively labelled DNA probe is incubated with the extracted embryonal DNA. If Y-chromosomes are present, the probe binds. This procedure suffers from many of the same problems as karyotyping: the need to biopsy and the long time and complex skills required. The time required can probably be shortened to several hours with further research. The main advantages are that it is highly accurate, and a higher percentage of embryos can be sexed than with karyotyping since cells need not be in metaphase. This method is offered commercially by one company for frozen embryos (which presents problems for exportation to some countries since the zona pellucida is damaged during biopsy). A slight variation on this procedure is to use the polymerase chain reaction so that a segment of DNA can be observed directly.

IN VITRO FERTILIZATION

This procedure usually comprises four separate steps ***in vitro***: oocyte maturation, capacitation of sperm, fertilization, and culture of embryos until they can be frozen or transferred to the uterus. The actual ***in vitro*** fertilization step is the easiest of the four, but success requires that the other steps work well. Oocyte maturation, capacitation, and culture of embryos can all be done ***in vivo***, but as the number of ***in vivo*** steps increases, the practicality decreases greatly. Recently, there have been significant advances in the ***in vitro*** fertilization process with cattle (Lu ***et al.***, 1987; Goto ***et al.***, 1988). A major advance has been co-culture of oocytes and embryos with cumulus cells or oviduct epithelial cells (see also Gandolfi and Moor, 1987). The references just cited provide information on successful methodology which, however, still leads to fairly variable results.

Potential applications of ***in vitro*** fertilization include supplying embryos from slaughterhouse oocytes for twinning programmes to increase calf crops without increasing the number of cows. Another obvious application is to circumvent certain kinds of infertility, rather as it is used for humans. A third possible application is as an alternative to harvesting gametes

from valuable cows by superovulation: by removing an entire ovary, recovering thousands of oocytes and allowing them to mature and be fertilized ***in vitro.*** However, current methodology with cattle is limited to maturing a few dozen oocytes per ovary at most. Oocyte maturation could also provide material for cloning by nuclear transplantation and for making transgenic animals. Despite its promise, ***in vitro*** fertilization has resulted in fewer than 150 calves to date, and has not been used commercially at all in cattle. This is likely to change soon, as has been described in Chapter 2, although ***in vitro*** fertilization techniques may not be commercially profitable for some time.

CHIMERAS

Chimeras are animals with cells of two or more different genotypes in their bodies. They are usually made either by mixing cells of two or more embryos just before compaction or by injecting cells of one embryo into the inside of another, generally at the blastocyst stage. With cattle, chimeras can also be produced by transferring embryos so that fraternal twins occur. Due to placental anastomoses of blood vessels, haemopoetic tissue of such twins contains both genotypes. Chimeric cattle have been made by several variations on the techniques just described. Some of these have been quite valuable from the standpoint of basic science. We are not aware of any agricultural applications of such technology to date.

CLONING BY NUCLEAR TRANSPLANTATION

Amphibia have been cloned by nuclear transplantation since the early 1950s. Results of studies with mice, which began in the early 1980s, have not been clear-cut until recently. It is now generally accepted that nuclei from cells of mouse embryos greater than the four-cell stage are unsuitable for nuclear transplantation into one-cell ova. However, there are recent reports of cloning sheep and cattle by nuclear transplantation from more advanced embryos (Willadsen, 1986; Robl ***et al.***, 1987). It appears that nuclei from 32-cell embryos and even the inner cell mass of blastocysts of these species can be used successfully. It is not yet clear what percentage of nuclei from 32-cell bovine embryos make suitable donors for this purpose, but it could well be more than 50 percent.

There are a number of ways of effecting the actual transplantation of nuclei, and the recipient ovum can be either an unfertilized oocyte, a one-cell embryo, or one cell of a two-cell embryo. The genetic material of the recipient cell must be removed or inactivated so that the resulting animal will have the genotype of the donor nucleus and, more important, so that it will not have excess chromosomes. Details of procedures can be found in papers by Willadsen (1986) and Robl ***et al.***, (1987).

Currently, success rates with nuclear transplantation appear to be quite low with cattle, although very little has been published. There is no doubt that success rates will eventually improve so that the procedure can be used commercially. Key technologies to make it affordable include ***in vitro*** oocyte maturation and ***in vitro*** culture of embryos to the late morula or early blastocyst stage.

Perhaps the biggest problem with this technology at present is that it is not possible to clone animals, only embryos. To circumvent this problem, one strategy is to clone 16- to 32-cell stage embryos by transplanting nuclei to one-cell ova, allow the resulting embryos to develop to the 16- to 32-cell stage, and reclone them repeatedly until sufficient viable embryos accumulate. If only three successes occurred per round on the average, the number would increase fairly rapidly after several rounds: 3, 9, 27, 81, 243, etc. Note that all embryos will be of the same sex and that diagnosing the sex of the embryo prior to cloning would be extremely important. It is not yet known how well repeated recloning of embryos will work. Most of the cloned embryos would be transferred and allowed to mature in order to measure the productivity of the resulting animals, but some would be kept frozen. If the animals proved outstanding, the frozen embryos would be thawed, and their nuclei transplanted to produce as many clones of that given individual as would be profitable. In a sense, one is cloning adults with this strategy. However, it is necessary to freeze embryos

prior to the time that the resulting adults exist. This technology can clearly work, even though it is somewhat inexpedient. Genetic progress will be much slower than it would be if adults could be cloned directly.

EMBRYONIC STEM-CELLS

In mice, it is possible to remove the inner cell mass from the blastocyst and culture these cells ***in vitro*** so that they continue to divide without further differentiation, resulting with time in millions of these embryonic stemcells. It is possible to inject such cells back into a blastocyst to form a chimeric foetus. In up to 30 percent of cases, the germ-cells of such foetuses are partly or exclusively derived from the embryonic stem-cells, which are therefore the parents of the next generation. Embryonic stem-cells can be frozen, have genes added to them, or be manipulated in various other ways, thus forming ideal ***in vitro*** parents.

TRANSGENIC ANIMALS

One of the most exciting technologies is to make transgenic animals. Genes are added to, replaced, or deleted from embryos, one gene at a time, to make interesting animals for research or more productive animals for agriculture. There are several methods of doing this (Renard and Babinet, 1987; Murray ***et al.***, 1988). As with many other new technologies, costs are high, success rates are low and results are highly variable. No two transgenic animals are alike, although once one is made, it transmits the new gene to half its offspring. The genes to be added can originate from many sources, including other species or from computer-controlled gene machines. There are huge logistical problems in making homozygous transgenic lines of farm animals. Perhaps the biggest problem is that we know so little about genetic control of development, growth, lactation, reproduction and disease resistance that few genes have been identified that could reasonably lead to improved animals. Nevertheless, this technology has potential importance in production agriculture and already has many research applications.

Chapter 12
Success rates of embryo transfer

MEASURES OF SUCCESS

For the cattle breeder, success should usually be measured in terms of marketable product or its equivalent, for example, increased net worth. However, factors such as inflation and interest rates complicate evaluations of net worth to such an extent that more broadly and internationally applicable measures of success are normally used, such as numbers of live calves at one month of age. This is a marketable product, and other measures of success can be derived from it.

For the provider of embryo transfer services, success rates are usually measured by the income received, which in turn generally depends on the number of transferable embryos recovered per donor and the pregnancy rate per recipient. Thus, success is measured at an earlier stage in the process than for the cattle breeder.

DETERMINANTS OF SUCCESS

Embryo transfer consists of a series of steps, ***all of which must be done well*** or failure will result. These steps are summarized in Table 9 along with comments concerning the degree of control that can be exercised over each step. As indicated in the right-hand column, the breeder or personnel providing the services that the breeder purchases have considerable control over embryo transfer success rates. The one factor that is nearly impossible to deal with is variability in the number of normal embryos produced in response to superovulation. This causes considerable frustration.

Several other items in this table deserve comment. One can never be sure of intrinsic fertility of donors, recipients, and semen, or of the viability of embryos, although one can certainly improve the chances of success by selecting donors and recipients from a population that has high fertility. It is probably not cost-effective to go to the lengths of testing a large batch of semen for fertility, or buying large batches of drugs for superovulation and using a batch shown to work well on hundreds of donors. Weather cannot be controlled directly, but one can avoid doing embryo transfer during seasons of the year when fertility is low.

Success rates of embryo transfer

TABLE 9
Steps in the embryo transfer process and the ability to deal with them successfully

Steps in process	Possibilities for control

Selection of fertile donors	Moderate
Purchase of high fertility semen	Moderate
Proper injection of superovulatory drugs, beginning days 9–14 of the oestrous cycle	High
Variability in superovulatory response	Low
Oestrus detection in donors and recipients	High
Proper timing of insemination, proper handling of semen, good insemination techniques	High
Recovery of most embryos from each donor	Moderate
Isolation and classification of embryos	High
Storage of embryos between collection and transfer	High
Proper cryopreservation procedures	High
Transfer of embryos to the recipient	High
Intrinsic viability of embryos from a particular donor	Moderate
Selection of fertile recipients	High
Pregnancy diagnosis, preferably after day 50, if by palpation	High
Prevention of abortion	High
Proper management at calving	High
Good management of calves	High
Nutrition: donor, recipients, calves	High
Control of disease, vaccinations	High
Batch differences in superovulatory drugs	Moderate
Weather	Moderate
Good record keeping	High

TABLE 10
Factors that may alter success rates with embryo transfer

Infertile donors will have lower responses than normal ones
Fresh semen is superior to frozen semen from certain bulls
Fewer pregnancies will result from frozen embryos
Pregnancy rates are lower with non-surgical transfer for some technicians
Fewer pregnancies will result per half embryo, but more per embryo collected, if embryos are split
Young cows may be more fertile as donors and recipients than heifers or old cows
Success rates decline after the third or fourth superovulation with some donors

Some additional factors which can affect success rates with embryo transfer are listed in Table 10. Cows with histories of infertility generally produce only about one-third as many pregnancies as normal donors, although this depends on the kind of infertility (Bowen ***et al.***, 1978). Occasionally, results with infertile donors are satisfactory. Usually one has little choice in the second factor in Table 10, fresh versus frozen semen. Either kind of semen works well if handled properly, except that some bulls do not produce semen that freezes well.

An important point is that calves from embryo transfer are normal. We studied 1 900 embryo transfer pregnancies (King ***et al.***, 1985), and found no differences from non-embryo transfer calves in abortion rates, congenital abnormalities, birth weight, sex ratio (51 percent male), neonatal death, gestation length, calfhood disease or any other characteristic studied.

CURRENT PRODUCTION AVERAGES

On average, two to four calves will result per superovulated donor under the following field conditions: normal, fertile donors being superovulated for the first or second time, excellent management, well-trained embryo transfer personnel, sufficient synchronous recipients for the majority of embryos, surgical or non-surgical transfer by experienced technicians with proven skills, and unfrozen embryos. If embryos are frozen, the average will be 15–20 percent lower.

TABLE 11
Distribution of transferable embryos produced by superovulated donors

Donor response	No. embryos	Percentage donors		Percentage embryos	
		Mean	Range	Mean	Range
None	0	25	20–30	0	0
Poor	1–2	15	10–20	5	3–10
Fair	3–5	18	15–25	15	10–20
Good	6–9	20	15–25	25	20–30
Excellent	10–20	20	15–25	45	40–50
Excessive	21–50+	2	1–3	10	5–15

Table 11 contains approximate distributions of embryos recovered per donor superovulated derived from several sources (e.g. Looney, 1986). Note that no transferable embryos are recovered from 20–30 percent of donors and that about 80 percent of embryos are recovered from the 40 percent of donors that do best. Methodology has improved slightly since the data in Table 11 were collected, so current success rates could be slightly higher at embryo transfer units. Success rates would probably be lower on the farm.

Success rates can also be examined on the basis of pregnant recipients per donor, as is illustrated in Table 12 which gives the results of 64 consecutive superovulations of normal donors of beef breeds, taken from records at the Embryo Transfer Unit at Colorado State University. Data include all donors treated, even those that did not show oestrus, did not respond to superovulatory treatment or did not yield ova. Often animals with these problems are not included in averages.

Unfortunately, it is not possible to predict which donor will have one pregnancy and which will have ten or more. Note that 47 percent of the donors had zero, or only one or two pregnancies as a result of superovulation. If one superovulates each donor repeatedly, for instance, three times at two-month intervals, the variability in total pregnancies per donor evens out somewhat. With these 64 donors, an average of 3.4 pregnancies per donor per superovulation resulted. About 90 percent of recipients pregnant at three months of gestation will produce a live calf at one month of age with excellent management.

TABLE 12
Distribution of numbers of pregnancies from 64 superovulated donors

No. pregnancies	No. donors	Percentage donors	Percentage pregnancies
0	14	22	0
1–2	16	25	11
3–4	16	25	26
5–7	11	18	31
8–10	4	7	16
11+	3	5	15

Chapter 13
Costs of embryo transfer

Costs of embryo transfer vary greatly from country to country, and within countries, depending on a variety of factors. Thus, precise costs mean little in a publication designed for many different countries. However, two generalizations can be made. First, no matter how it is done, embryo transfer programmes are relatively expensive. The costs of the actual embryo transfer services or technology may be quite low; however, labour and feed costs averaged over the number of calves produced are high since normal, healthy cows or heifers are kept out of production in order to be available as recipients. An exception to this is twinning programmes to increase beef production. Also, recipients could come from a source such as a feedlot for fattening heifers for beef. A second generalization is that costs per calf are lowest when success rates are high, because costs are spread over more calves.

Costs will be listed assuming that embryo recovery and transfer services are purchased. In many cases, these services will be provided by a government, a cooperative or the company owning the cattle, when the costs should be determined in a different way; they will still be real costs, nonetheless. Costs are listed in Table 13 and discussed below (comments are numbered to correspond with numbers in the table).

Discussion (of Table 13 on p. 102)

1. Drugs include FSH or PMSG and prostaglandin and/or progestin.
2. Many embryo transfer companies charge a set fee per donor with discounts for large numbers of donors.
3. These costs only apply when embryos are frozen.
4. Embryo transfer fees are usually charged by the pregnancy, but alternatives are so much per transfer, or a certain amount per hour or day; there are also several other approaches.

TABLE 13
Costs of embryo transfer

Actual embryo transfer services

1. Drugs for superovulation
2. Labour, equipment and supplies to collect and isolate embryos
3. Labour, equipment and supplies to freeze and store embryos
4. Labour, equipment and supplies to transfer embryos
5. Travel expenses for personnel

Other direct costs

6. Feed costs while donor is non-pregnant and not producing natural calves
7. Semen
8. Health tests and vaccinations for donor and recipients
9. Cattle transportation
10. Registration fees
11. Blood-typing fees
12. Feed and care of recipients
13. Decreased productivity of unused and non-pregnant recipients
14. Costs for synchronizing recipients, including drugs and labour
15. Costs of facilities and extra labour for embryo transfer
16. Loss of pregnant recipients' natural calves
17. Telephone, postage, etc.
18. Costs of frozen embryos, if purchased

Indirect costs

19. Interest on investment
20. Abortion and calf losses (frequently 10 percent or more)

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5. Travel expenses only apply if personnel must travel to the embryo transfer site. Sometimes travel expenses are also incurred to palpate donors when treatments are started and to inseminate donors.
6. The donor will not be able to have calves by conventional breeding while awaiting superovulation and recovering from it. The cost in feed and lost reproduction should thus be included.
7. More than one dose of semen is used per superovulated donor; however, the amount of semen per calf produced is actually less with superovulation and embryo transfer than with conventional artificial insemination.
8. Costs for health tests vary widely.
9. This is not an expense for many programmes.
10. Registration fees for pure-bred cattle frequently are higher for embryo transfer calves than naturally born calves.
11. Many breed societies require blood-typing for the donor, sire and embryo transfer calf.
12. Recipient feed and care should be included for the length of time animals are kept non-pregnant in order to be available as potential recipients, for recipients that did not become pregnant, and for recipients that did become pregnant, at least through calving, and in most cases until they become pregnant again with their own calf.
13. Recipients not pregnant, and cattle kept non-pregnant as potential recipients, will have delayed reproduction resulting in lighter calves at weaning time, which is a cost in addition to feed costs in item 12. An example with dairy cows is: if 10 cows were not bred so that they would be available as recipients for one donor, they would average a delay of 45 days in getting pregnant. In the United States, it is frequently calculated that each additional day that a dairy cow remains open costs US$2.50. This would mean an additional cost of US$1 125 over normal costs for those recipients and potential recipients.
14. Usually many more animals are synchronized than are used as recipients.
15. Sometimes facilities must be built or expanded for embryo transfer programmes; it also takes labour for sorting, injecting, record-keeping and assisting with the actual embryo transfer.
16. There will be no profit from the recipients' own calves while they are carrying the embryo transfer calf.
17. Logistics of embryo transfer frequently entail considerable communication costs.
18. Frozen embryos will be purchased for some programmes.
19. Interest on capital, possibly adjusted for inflation, should be considered as a cost.
20. Abortion and calf losses are no higher with embryo transfer, but because costs per calf are higher than with conventional breeding, losses are higher. Under excellent

conditions, one should probably plan for 10 percent losses due to abortion, death of recipient, neonatal death, and losses before calves reach breeding age. Under poor conditions, these losses can exceed 50 percent.

The costs summarized above almost always are hundreds to thousands of dollars per embryo transfer calf over and above conventional costs of cattle breeding. A number of costs have not been included in Table 13. Among these are interest on the donor's value, costs of insurance, or costs of the owner's or manager's time. In some countries, there are tax advantages to investing in these technologies, which may lower costs.

One further problem is chance. For example, an unfavourable (or favourable) sex ratio may result unless large numbers of calves are produced on each farm. Moreover, the most valuable donors may not produce the largest numbers of calves.

Although embryo transfer is generally costly, it is frequently still profitable. Obviously, one must analyse the costs and benefits. When the benefits exceed the costs, this technology should be used by all means. However, if costs are not justified by benefits, embryo transfer programmes should not be initiated in most cases.

Chapter 14
Embryo transfer and disease transmission

The risk of transmitting ***genetic disease*** via embryo transfer is the same as that involved in natural mating or artificial insemination; wise selection of dams and sires is mandatory, no matter how cattle are propagated. There is no increased incidence of abnormal offspring due to these procedures (King ***et al.***, 1985). With this technology, however, there may be greater temptation to amplify reproduction of cows with a very high market, but questionable genetic, value, both because some kinds of infertility with a genetic basis can be circumvented and because the sale of the offspring can be very profitable.

Embryo transfer procedures can be used to control large-scale transmission of genetic diseases such as syndactyly by screening young bulls for undesirable recessive Mendelian characteristics before semen is distributed for artificial insemination (see Chapter 2; Johnson ***et al.***, 1980). Also embryos from parents with abnormal karyotypes can be biopsied and karyotyped, and only the normal ones transferred.

If proper procedures are followed, the risk of transmitting ***infectious disease*** via embryo transfer is lower than with natural mating or artificial insemination (Stringfellow, 1985; Hare, 1986; Hare and Seidel, 1987). Carelessness in even the smallest of details, however, greatly diminishes the advantage.

Arguments for the inherent safety of embryo transfer procedures are based on the physical characteristics of the embryo and the ability to test the environment of the embryo for the presence of pathogens and to treat the embryo to remove pathogens. Prior to collection, an embryo is exposed only to the oviductal and uterine environment of the donor. Thus, even when the donor is infected, there is little opportunity for infection by pathogens other than those present in uterine fluid and, rarely, by blood-borne pathogens that are introduced into the uterine lumen because of injury to the endometrium. Viruses are rarely found at infective levels in uterine fluid. Furthermore, pathogens present in the uterine fluid frequently do not adhere to, or are only loosely associated with, embryos. Pathogens can be removed entirely by washing embryos experimentally infected ***in vitro***. Of great importance is the vast dilution of any pathogens by the flushing procedure. In addition to dilution and physical removal of infectious agents, antimicrobial agents can be added to the washing medium.

Primary resistance to pathogens is provided by the physical barrier of the zona pellucida. Nevertheless, some pathogens adhere to the zona pellucida (Hare, 1986); in some cases treatment with enzymes removes these agents. The presence of pathogens on or in the embryo cannot be diagnosed by examining the embryo microscopically with current techniques.

The possibility of infection of the embryo between recovery and transfer to a recipient can be controlled by ensuring a sterile environment. Again, conscientious attention to detail must be emphasized.

An issue apart from the infected or uninfected status of the embryo is whether transfer to a recipient of an embryo with adherent pathogens results in transmission of the disease. In most cases, it does not because the very few pathogenic organisms are eliminated by the body without causing a productive infection.

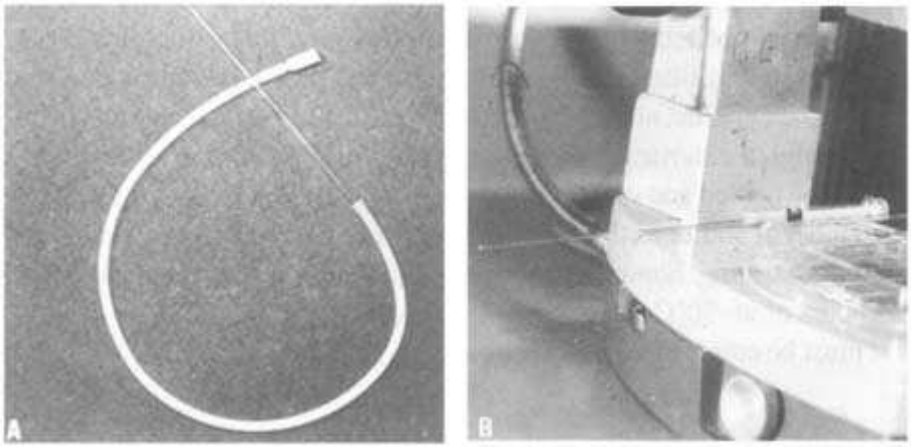
Continuously updated analyses of research on the interaction between embryos and pathogens and the transmissibility of infectious disease via embryo transfer are available (Manual of the International Embryo Transfer Society, 1987). Minimum standards for the sanitary handling of embryos are also presented in the Manual of the International Embryo Transfer Society. This manual is updated regularly to include recommendations based on the latest research and serves as a reference document of the International Zoosanitary Code. Currently recommended handling procedures are outlined below.

Embryo handling area (see Chapter 15).

Equipment and solutions must be sterile and free from contaminants (see Chapters 6 and 15). Care must be taken to avoid contamination of equipment while it is in use. For example, setting a pipette down on the bench for a moment to adjust the microscope could result in the introduction of infectious pathogens into the container of embryos if the pipette is reused (see Figure 32).

Washing embryos. As soon as embryos are isolated from the collection fluid, they should be pipetted in as small a volume of fluid as possible into a container of fresh medium. No more than 10 embryos should be placed in a single container, and embryos from different donors should ***never*** be placed in the same container. The container should be agitated gently. The embryos should then be transferred with a new sterile pipette to a second container of fresh medium. It is important to transfer as little fluid as possible from the first wash to the second wash, and the volume of medium in each wash should be sufficient to dilute the volume of fluid transferred in the pipette with the embryos one hundred times (2 ml is standard). The second container should be agitated gently before the embryos are transferred to a third container. These steps should be repeated until the embryos have been washed ten times in fresh medium. Containers should be covered to avoid contamination from dust and aerosols (see Figure 2). Containers and pipettes should not be reused without resterilization.

FIGURE 32
A mouth pipette laid on the work-bench (A) and a pipette attached to a tuberculin syringe laid on the microscope stage (B) so that the tips of the pipettes do not touch any surface. Pipettes are made of fine-drawn, fire-polished glass tubing.



Trypsin treatment. So far, two viruses (infectious bovine rhinotracheitis virus and vesicular stomatitis virus) have been found to adhere to the zona pellucida of bovine embryos so firmly that normal washing procedures do not remove them. It is recommended that

embryos exposed to these viruses be washed five times in phosphate-buffered saline to which antibiotics and 0.4 percent bovine serum albumin have been added, but without Ca^{++} and Mg^{++} (because they inhibit trypsin), then through two washes of trypsin of 30–45 seconds each, then again through five washes of saline containing antibiotics and 2 percent heat-inactivated serum (not bovine serum albumin) to inactivate the trypsin. The trypsin enzyme used to prepare the washes should have an activity such that 1 g will hydrolyse 250 g of casein in 10 minutes at 25°C and pH 7.6. The sterile trypsin solution should contain a concentration of 0.25 percent trypsin in Hank's balanced salt solution without Ca^{++} and Mg^{++}. A ready-to-use solution is available commercially.

Evaluation of embryos. After embryos have been washed, they should be examined over their entire surface area at no less than 50X magnification. For purposes of disease control, only embryos that have an intact zona pellucida and that are free of adherent material should be transferred or cryopreserved.

Collection of diagnostic samples. Although current diagnostic procedures kill embryos, an indication of the health status of embryos can be obtained by testing samples of the fluids used to recover and wash the embryos and by testing unfertilized ova and non-transferable embryos recovered from the same flush. If embryos are recovered by examining the sedimented collection fluid, the last 100 ml in the bottom of the cylinder along with any debris should be retained in a sterile, sealable bottle for testing. If embryos are recovered by filtration of the collection fluid, the fluid should be retained in a sterile cylinder, allowed to settle for 30 minutes, and the bottom 100 ml transferred to a sterile bottle for testing. The fluid from the last four washes should be pooled and stored in a sterile, sealable bottle for testing. Degenerate embryos and unfertilized ova should be pooled, washed ten times, and stored for testing. Samples may be stored at 4°C if the tests are done within 24 hours or at -70°C if the interval until testing is longer.

It must be emphasized that the above procedures must be carried out with scrupulous regard for sterility and cleanliness or they will be of little avail. Every detail is important.

Regulations for exporting and importing embryos are usually different for each set of countries, and are often determined in part by political considerations. Nevertheless, most are based on the principles discussed in this chapter.

Chapter 15
Washing procedures for work areas, glassware and equipment

EMBRYO HANDLING AREA

The area in which embryos are handled should be indoors and ideally in a room separate from the embryo collection area. The area must be isolated from exposure to aerosols arising from sick animals and people. To reduce the incidence of microbes, dust, and chemical contaminants, it is recommended that laboratory work surfaces be washed regularly with soapy water followed by, first, a thorough clear-water rinse and, second, an alcohol (70 percent) rinse. Alternatively, surfaces could be washed with a mild disinfectant that is relatively non-toxic, but is yet a good solvent (e.g. quaternary ammonium or ampholytic compounds). Surfaces must be allowed to dry thoroughly before they are used. In addition, proper techniques must be followed scrupulously when handling embryos. For example, if pipettes must be set down for a short time while manipulating embryos under the microscope, the tip must not be allowed to touch any surface (see Figure 32); likewise, containers of medium should be kept covered.

The temperature of the room should be within the range of 15–30 °C. The ideal humidity is 30–70 percent, but this is not a critical factor and need not be taken into account unless, for other reasons, it is desirable to install a special room with controlled environment. Similarly, positive-pressure air filtration reduces the risk of contaminating equipment and containers of embryos; however, unless there are unusual amounts of air-borne micro-organisms and dust, such precautions are not required. The presence of insects should be avoided, but special care must be taken to ensure that chemical control measures do not result in aerosols and surface residues that might contaminate equipment or solutions.

GLASSWARE AND METAL EQUIPMENT

Glassware and equipment made only of metal (e.g. cervical expanders or stylets for Foley catheters) should be scrubbed thoroughly on all surfaces in a basic detergent, soaked in an acidic detergent, and subsequently rinsed 12 times in tap water and 12 times in distilled, deionized water. Adequate rinsing is essential because all detergents are embryocidal. Glass- and metalware should be wrapped with aluminium foil to protect from contamination any surface that may come in contact with embryos or the reproductive tract of the cows (e.g. mouths of flasks and bottles should be covered, watch glasses should be completely wrapped, as should cervical expanders), and then sterilized by dry heat at 160 °C for two hours. An alternative is to wrap the items in cloth, paper or other material that allows steam to evaporate but that provides an effective barrier to micro-organisms, and then sterilize them by moist heat (a temperature of 121 °C at a pressure of 104 kilopascals for ***at least*** 30 minutes; Manual of the International Embryo Transfer Society, 1987).

Sterilization by dry heat has the advantage of not exposing equipment rinsed in distilled,

deionized water to possible contaminants in the water used to produce steam. Moist heat under pressure has the advantage of killing highly resistant bacterial spores, provided that sufficient time is allowed for sterilization. An indicator, such as heat-sensitive tape or dye, can be used to verify that desired temperatures have been reached.

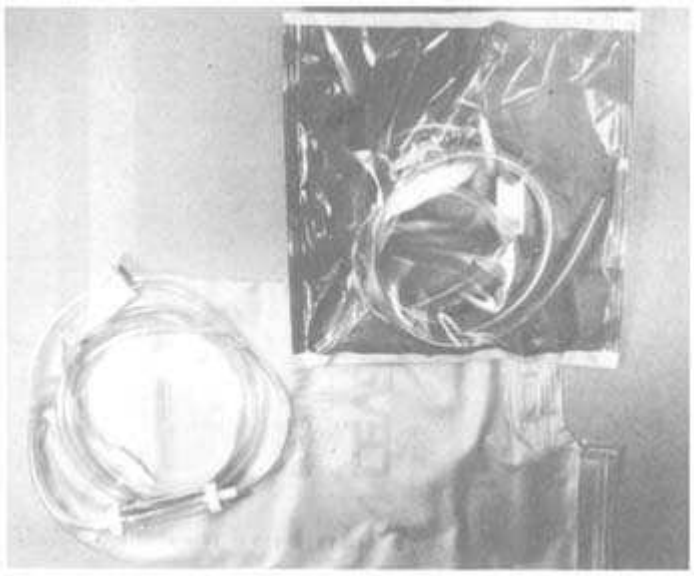
PLASTIC AND OTHER HEAT-LABILE EQUIPMENT

All reusable heat-labile items, such as catheters, tubing, or searching dishes, should be disassembled and washed in the same manner as glassware. After they have dried completely, they should be wrapped and sealed in a gas-permeable paper (Figure 33). The recommended sterilization procedure (Manual of the International Embryo Transfer Society, 1987) is exposure to at least 500 mg of ethylene oxide per 1 000 cm^3 for 24 hours. Ethylene oxide is highly embryocidal and residues can take 24 hours to months to dissipate, depending on the concentration of ethylene oxide, duration of sterilization, material sterilized, the type of packaging, and aeration conditions. Aeration for one week at room temperature should be adequate for most materials (Schiewe ***et al.***, 1985, 1988). A heated aerator or an evacuation hood are useful pieces of equipment, but are not required. Staff must take precautions to avoid contact with ethylene oxide because it is highly mutagenic.

SOLUTIONS

Solutions should be prepared with pure water. This can be obtained from sophisticated deionizing and filtration systems or from water distilled in glass stills. At least two distillations and sometimes more are required to obtain suitable water. Basic salt solutions (e.g. sodium chloride) can be decanted into 500-ml (or smaller) screw-cap bottles and sterilized by moist heat as outlined above. Bottle caps should be loosened before sterilization and tightened afterwards. More complex solutions should be sterilized by membrane filtration with 0.22-µm pore size (Figure 34), taking care to use positive pressure to avoid frothing and unacceptable changes in pH (Manual of the International Embryo Transfer Society, 1987; see also Chapter 6 on culture of embryos ***in vitro***). It may be helpful to filter solutions preliminarily using a 0.45-µm pore size to reduce the likelihood of clogging the finer membrane during sterilization.

FIGURE 33
Sterile supplies for flushing embryos: a transfusion bag, which is sterilized by the manufacturer, plus a set of outflow tubing and Y-connector wrapped in a gas-permeable packet sterilized with ethylene oxide



SILICONIZING GLASSWARE

Some workers siliconize pipettes used to handle embryos. It is especially useful to siliconize glass micropipettes for microsurgical work. Directions of the manufacturer should be followed in siliconizing equipment, which must be washed and rinsed thoroughly after

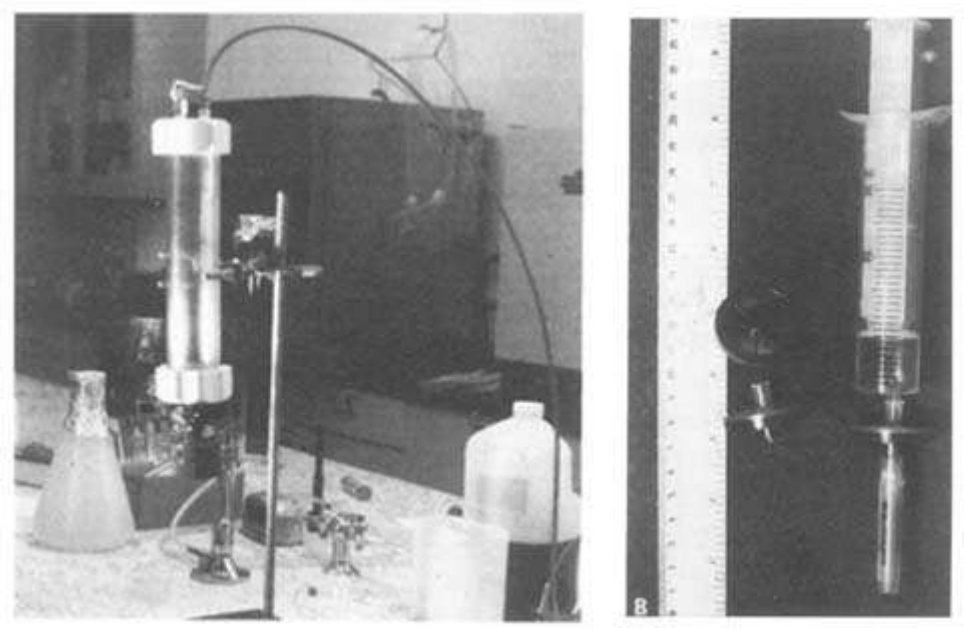
siliconization and then sterilized. The advantage of siliconization is that debris and embryos are less likely to adhere to the treated surface; this also makes cleaning easier.

RINSING PRIOR TO USE

As an added precaution, all equipment, whether taken directly from the manufacturer's package or sterilized at the embryo transfer unit, should be rinsed just prior to use with sterile medium. Under no circumstances should equipment be used for more than one cow or more than one group of embryos without first being washed and sterilized. Equipment to be sterilized and used again should be disassembled and put to soak in soapy water as soon as possible after use.

FIGURE 34

A biological filtration system for large volumes with positive pressure provided by a tank of nitrogen or air (A) and a small filter mounted on a 30-ml syringe (B). Both filters have a pore size of 0.22 µm



Chapter 16
Record keeping

Good records are essential for successful embryo transfer programmes for business and legal purposes, so that breed associations can verify parentage (it is not unusual that information is requested five or ten years after embryo transfers were done to sort out discrepancies of blood type), and so that clients can export embryos. Records frequently permit one to determine why pregnancy rates deteriorated after making a certain change in procedures or materials (see Chapter 18). In many countries licences to do embryo transfer are issued only after scrutiny of record-keeping systems. This is to protect clients. It is not unusual for courts to subpoena records. We do not provide any examples of financial records, but obviously these are also important in commercial situations to secure timely payment, and for tax and investment purposes.

The first form in this chapter is the form recommended by the International Embryo Transfer Society for registration purposes (Example 1). Many breed registries require this form to be used for offspring to be eligible for registration. The form is especially relevant for frozen embryos. The absolute minimum data are requested:

On the container of frozen embryos:

- identification of the organization that processed the embryos
- breed of embryo
- identification of the dam (sire optional)
- date on which the embryos were frozen
- identification number for the container
- number and stages of embryos in the container

On the goblets and canes:

- cane and goblet numbers
- identification of the organization that processed the embryos
- date of cryopreservation
- identification of dam and sire
- breed
- number of embryos
- kind of packaging/indication of repackaging

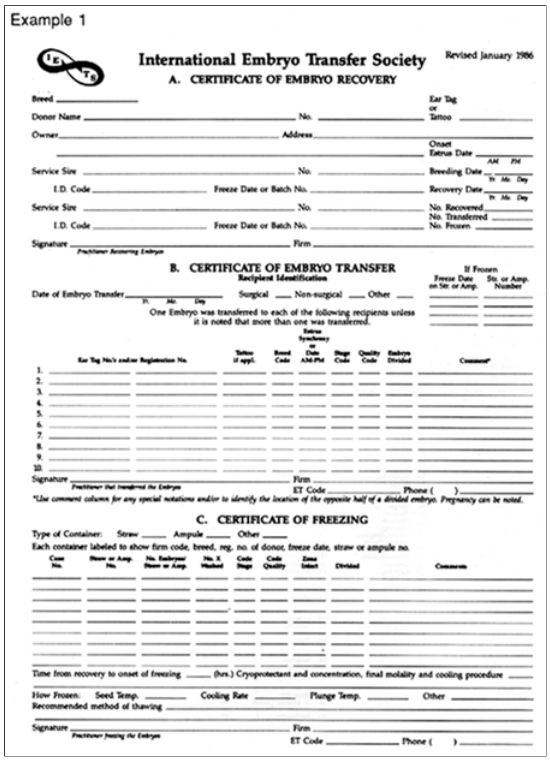
The above information must be codified in order to fit in the limited space available on ampules, straws, canes, goblets, etc. It is obvious that using internationally standardized codes will maximize an organization's flexibility and reduce the problems encountered when importing or exporting embryos from another embryo transfer group or another country.

What can be recorded on a container of embryos is clearly not enough to provide all the information necessary, for example, for determining ownership, for proper thawing and transfer, or for registration of calves born as a result of the transfer of those embryos; however, the data should be sufficient to allow recovery of all necessary information from appropriate forms on file with the embryo transfer practitioner, breed associations, and owners of the animals.

There follow examples of forms used to record identifying information, the status of health, and oestrous cycles for donors (Example 2) and recipients (Example 3), oestrus detection (Example 4), superovulatory treatment (Example 5), embryo recovery (Example 6), embryo evaluation (Example 7), and embryo transfer (Example 8). In many laboratories, coded numeric responses are recorded in order to facilitate entry of data into a computer for analysis (see key to Example 7).

Example 1

Example 1



International Embryo Transfer Society Revised January 1986

A. CERTIFICATE OF EMBRYO RECOVERY

Breed _____ Ear Tag or Tattoo _____

Donor Name _____ No. _____

Owner _____ Address _____

Onset Estrus Date _____ AM PM

Service Sire _____ No. _____ Breeding Date _____ Yr Mo Day

I.D. Code _____ Freeze Date or Batch No. _____ Recovery Date _____ Yr Mo Day

Service Sire _____ No. _____ No. Recovered _____

I.D. Code _____ Freeze Date or Batch No. _____ No. Transferred _____

No. Frozen _____

Signature _____ Firm _____

Practitioner Recovering Embryos

B. CERTIFICATE OF EMBRYO TRANSFER

Recipient Identification

If Frozen: Freeze Date on Straw or Amp. / Straw or Amp. Number

Date of Embryo Transfer _____ Yr Mo Day Surgical ___ Non-surgical ___ Other ___

One Embryo was transferred to each of the following recipients unless it is noted that more than one was transferred.

	Ear Tag No's and/or Registration No.	Tattoo if appl.	Breed Code	Estrus Synchrony or Date AM-PM	Stage Code	Quality Code	Embryo Divided	Comments*
1.								
2.								
3.								
4.								
5.								
6.								
7.								
8.								
9.								
10.								

Signature _____ Firm _____

Practitioner that Transferred the Embryos ET Code _____ Phone ( ) _____

*Use comment column for any special notations and/or to identify the location of the opposite half of a divided embryo. Pregnancy can be noted.

C. CERTIFICATE OF FREEZING

Type of Container: Straw ____ Ampule ____ Other ____

Each container labeled to show firm code, breed, reg. no. of donor, freeze date, straw or ampule no.

Cane No.	Straw or Amp. No.	No. Embryos/ Straw or Amp.	No. X Washed	Grade Stage	Grade Quality	Zona Intact	Divided	Comments

Time from recovery to onset of freezing ____ (hrs.) Cryoprotectant and concentration, final molality and cooling procedure ____

How Frozen: Seed Temp. _____ Cooling Rate _____ Plunge Temp. _____ Other _____

Recommended method of thawing _____

Signature _____ Firm _____

Practitioner freezing the Embryos ET Code _____ Phone ( ) _____

Example 1 (reverse)

CONDITIONS FOR COMPLETING EMBRYO CERTIFICATES

A. Complete one or more Certificates of Embryo Recovery for each recovery. The responsible practitioner signing this certificate is attesting to the fact that the donor dam was identified with her certificate of registration, that the service sire information was taken from a written record of services, and all the information is true and correct.

B. Certificate of Embryo Transfer will be completed to the extent that is necessary and/or appropriate to identify each recipient into which an embryo is transferred. If frozen embryos are transferred, the Certificate of Embryo Recovery will be completed by the responsible practitioner or by transferring from the original Certificate of Embryo Recovery or by attaching a copy. The practitioner signing the Certificate of Embryo Transfer is attesting to the accuracy and completeness of the identification of the embryos being transferred and the identity of the recipients into which embryos are being transferred.

   A complete Certificate of Embryo transfer, with Certificate of Embryo Recovery, will be submitted to the appropriate breed office within 120 days of transfer, and before any resulting offspring will be registered.

   Should any embryo identified hereon in recipient change ownership, such change will be documented by the seller completing an application for transfer with one copy submitted to the breed office and one copy provided the buyer which, in turn, will be submitted with the application to register the resulting offspring. One application for transfer is required for each change in ownership. The application for registration will be accepted from the person shown as the last owner of the recipient and/or the owner of the resulting calf at the time of its birth.

C. The Certificate of Freezing will be completed, with the Certificate of Embryo Recovery, whenever embryos are frozen. The practitioner signing the certificate is attesting to the identification of each embryo, with container labelling, as set forth within the Certificate, along with the accuracy of all other information.

   One copy will be sent to the breed office and one copy provided the owner.

   When a frozen embryo changes ownership the seller will submit one copy of an application for transfer to the breed office with a second copy provided the buyer from which an application for registration of the resulting offspring will be accepted on condition that properly completed Certificates A-C have been submitted to the breed office. Each change of ownership must be covered by a transfer.

   When frozen embryos are exported a special application for embryo export will be submitted to the respective breed office, with the appropriate fee.

   Use the following codes to describe the embryo, identify the breed and identify the month in all dates.

STAGE OF DEVELOPMENT

No.	Stage
1	Unfertilized
2	2 - to 12-cell
3	Early Morula
4	Morula
5	Early Blastocyst
6	Blastocyst
7	Expanded Blastocyst
8	Hatched Blastocyst
9	Expanding Hatched Blastocyst

QUALITY OF EMBRYOS

1. Excellent or Good
2. Fair
3. Poor
4. Dead or degenerating

MONTHS

January	JA
February	FE
March	MR
April	AP
May	MY
June	JN
July	JY
August	AU
September	SE
October	OC
November	NO
December	DE

BOVINE

AN - Aberdeen Angus
AB - Abondance
AF - Afrikander
AY - Ayrshire
BA - Barzona
BE - Beefalo
BF - Beef Friesian
BM - Beef Master
BB - Belgium Blue
BG - Belter Galloway
BD - Blonde D'Aquitaine
BO - Bradford
BR - Brahman
BH - Brahmental
BN - Brangus
BU - Braunvieh
SB - Brown Swiss (beef)
BS - Brown Swiss (dairy)
CP - Campine Red Pied
CN - Canadienne
CB - Charbray
CH - Charolais
CA - Chianina
DB - Danish Black & White
DJ - Danish Jersey
RW - Danish Red & White
DE - Devon
DR - Dexter
FP - East Flemish Red Pied
ER - Eringer
FA - Flamand
FL - Fleckvieh
FR - Fribourg
FB - Friesian (Belgium)
DF - Friesian (Dutch)
GA - Galloway (beef)
GD - Galloway (dairy)
GS - Gascone
GV - Gelbvieh
GR - Groninger
GU - Guernsey
HC - Hays Converter
HH - Hereford (horned)
HP - Hereford (polled)
SH - Highland (Scotch Highland)
HO - Holstein
HY - Hybrid (Alberta Hybrid)
JE - Jersey
KB - Kobe (Wagyu)
LU - Luing
LM - Limousin
LR - Lincoln Red
MA - Maine-Anjou
MR - Marchigiana
ME - Maremmana
MI - Meuse-Rhine Ijessel
MO - Montbeliard
MG - Murray Grey
NM - Normandie
NR - Norwegian Red
PA - Parthenais
PI - Piedmont
PR - Pie Rouge
PZ - Pinzgauer
RA - Ranger
AR - Red Angus
RB - Red Brangus
RD - Red Dane (Red Danish, Danish red)
WW - Red Holstein
RP - Red Poll
RN - Romagnola
RO - Rotbunte
SA - Salers
SG - Santa Gertrudis
MS - Shorthorn (milking)
SS - Shorthorn (beef - Scotch)
SP - Shorthorn (polled)
IS - Shorthorn (Illawarra)
SM - Simmental

DS - South Devon
SX - Sussex
TA - Tarentaise
TG - Tasmanian Grey
TL - Texas Longhorn
WB - Welsh Black
WF - West Flemish Red
XX - Crossbreds

CAPRINE

AL - Alpine
AG - Angora
LN - La Mancha
NU - Nubian
TO - Toggenburg

EQUINE

AS - American Saddlebred
AP - Appaloosa
AB - Arabian
BL - Belgian
CL - Clydesdale
HA - Hackney (Horse)
HK - Hackney (Pony)
HU - Hunter
MN - Morgan
PL - Palomino
PE - Percheron
PN - Pinto
QH - Quarter Horse
SE - Shetland
SI - Shire
SN - Standardbred
SF - Suffolk Punch
TW - Tennessee Walking
TH - Thoroughbred
WE - Welsh

PORCINE

YO - Yorkshire
LA - Landrace
HA - Hampshire
DU - Duroc
LC - Lacombe
PC - Poland China
BK - Berkshire
SO - Spotted
CW - Chester White
PE - Pietrain
TM - Tamworth
WS - Wessex Saddleback
LW - Large White (British)
LB - Large Black (British)

OVINE

BC - Border Cheviot
CO - Columbia
CR - Corriedale
DO - Dorset
FN - Finish Landrace
HA - Hampshire
LE - Leicester
LI - Lincoln
MT - Montadale
NC - N. Country Cheviot
OX - Oxford
RA - Rambouillet
RM - Romnelet
SB - Scottish Blackface
SR - Shropshire
ST - Southdown
SU - Suffolk

Example 2

Card for recording identifying information, status of health and oestrous cycles for donors

Name	Owner(s)				CSU No.			
Breed					Arrival			
Born	(front of card)				Departure			
Reg. No.	Vacc. Record	Date	Vaccine	Brand	RET	LET		Metal Tag
Sire								
Dam					RETT	LETT		
Date Blood Type								

HEAT DATES					DATE	BULL	VO	CODE	DATE	BULL	VO	CODE

HEALTH		TREATMENT					RECOVERY AND TRANSFER				
Date	History	Date	TRT	(R) Response (L)		Heat	Date	Recip	P/O	Recip	P/O
				(back of card)							
	HEALTH TESTS										

Example 3

Card for recording identifying information, status of health and oestrous cycles for recipients

RECIPIENT CARD

Original No.: _____ Source: _____ CSU No.: _____
Birth date: _____ Breed: _____
Name and registration no. _____
Sire: _____ Disposed: _____

Estrus and embryo transfer dates

Genital examination and health record

Date	Vaccinations, blood tests pregnancy diagnosis, etc.

Example 4

Card for recording observations of oestrus detection

Date _____ Time started _____ Finished _____
Pens checked _____
_____ Signature _____
Codes: +=standing heat; K=red KAMAR but not standing heat;
B=metestrous bleeding; M=mucus; O=other.

cow no.	breed	pen no.	code and comments

Example 5

Superovulation record

Please fill in all blanks; if not applicable, use NA

Cow's identification number: _____ Breed: _____

Semen: _____ Location: _____

DATE	TIME	HORMONE	MG OR UNITS	ROUTE

----------	----------	----------	----------	----------
----------	----------	----------	----------	----------
----------	----------	----------	----------	----------
----------	----------	----------	----------	----------

Estrus date and time:

INSEMINATION

DATE	TIME	CODE #	# AMPULES OR # MOTILE SPERM	COMMENTS*
----------	----------	----------	----------	----------
----------	----------	----------	----------	----------
----------	----------	----------	----------	----------

DATE OF RECOVERY:----------

* At first insemination, evaluate motility and give % progressively motile

PALPATION DETAILS:	LEFT OVARY	RIGHT OVARY
SIDE OF CL BEFORE TREATMENT:	----------	----------

COMMENTS: ----------

NO. FOLLICLES 96 HOURS

AFTER START OF TREATMENT:	----------	----------

COMMENTS: ----------

Example 6

Record of embryo recovery

D O N O R R E C O R D S (Front side)

DATE	DONOR IDENTIFICATION		WEIGHT	AGE	TREATMENT	SEMEN	INTERVAL TO ESTRUS	LENGTH OF ESTRUS	INTERVAL ESTRUS TO RECOVERY
	BREED	NUMBER							

DATE: Year Month Day, e.g. 88 AU 20, using numeric codes for year and day, and two-letter alpha code for month; see Example 1 for recommendations

DONOR IDENTIFICATION By breed and registration number (or ear-tag or tattoo number); see Example 1 for alpha abbreviations of breeds

TREATMENT Superovulatory treatment schedule

00 = unsuperovulated 01 = 6,6,4,4,2,2,2,2, mg FSH 02 = 2 500 IU eCG (PMSG) 03 = …

SEMEN Whether fresh (1) or frozen (2)/number of times inseminated

11 = fresh/1 time 22 = frozen/2 times 21 = frozen/1 time 12 = fresh/2 times … etc.

INTERVAL TO ESTRUS Interval to estrus from initial superovulatory injection to nearest 0.5 day, e.g. 55 = 5.5 days, 00 = recovery from unsuperovulated donor

LENGTH OF ESTRUS Length of estrus to nearest 0.5 day, e.g. 10 = 1.0 day, 00 = appointed estrus

INTERVAL FROM ESTRUS TO RECOVERY To nearest 0.5 day, e.g. 075 = 7.5 days

(back side)

RIGHT OVARIAN RESPONSE		LEFT OVARIAN RESPONSE		OVA RECOVERED		COMMENTS
No. CL	No. follicles	No. CL	No. follicles	Transferable	Abnormal	

RIGHT OVARIAN RESPONSE and LEFT OVARIAN RESPONSE Number of corpora lutea and number of follicles estimated by rectal palpation

OVA RECOVERED Numbers of transferable and abnormal embryos (including unfertilized ova)

Example 7

Form for recording data on evaluation of embryos

E M B R Y O D E S C R I P T I O N

DATE	DONOR	RECIPIENT	CONSECUTIVE NO.	TRANSFERER	AGE OF EMBRYO	CELL NUMBER	COMPACTNESS	SHAPE	VARIATION CELL SIZE	COLOR	VESICLES	EXCLUDED CELLS	PERIVITEL SPACE

DATE Year Month Day, e.g. 88 AU 20, using numeric codes for year and day, and two-letter alpha code for month (see Example 1 for recommendations)

DONOR Identification by breed and registration number (or ear-tag or tattoo number); see Example 1 for alpha abbreviations of breeds

RECIPIENT Identification by breed and registration number (or ear-tag or tattoo number); see Example 1 for alpha abbreviations of breeds

CONSECUTIVE No. (to identify embryo in data files)

TRANSFERER Name of person who evaluates and transfers embryo: 1 = Joe Transferer; 2 = Jill Transferer

AGE OF EMBRYO Days from donor estrus to embryo collection to nearest 0.5 day (donor estrus = day 0), e.g. 075 = 7.5 days

CELL NUMBER Number of cells (3 digits), e.g. 080 = 80 cells

COMPACTNESS Compactness of cells: 1 = tight (polygonal blastomeres); 2 = loose (round blastomeres)

SHAPE Shape of embryo mass: 1 = spherical; 2 = elliptical; 3 = irregular

VARIATION CELL SIZE 1 = normal; 2 = irregular

COLOR Color of cellular mass: 1 = normal; 2 = dark; 3 = light

VESICLES 1 = normal; 2 = excessively large vesicles

EXCLUDED CELLS Number of large excluded blastomeres

PERIVITELLINE SPACE Percentage of zona cavity occupied by embryo: 111 = no zona; 080 = 80 percent

STAGE OF EMBRYONIC DEVELOPMENT 1 = ≤8 cells; 2 = 9–16 cells; 3 = early morula; 4 = tight morula; 5 = early blastocyst (blastocoele just discemible); 6 = blastocyst; 7 = expanded blastocyst; 8 = hatching blastocyst; 9 = hatched blastocyst; 10 = elongated blastocyst

QUALITY OF EMBRYO 1 = perfect embryo for its stage (excellent); 2 = trivial imperfections such as oval zona, few, small excluded blastomeres, slightly asymmetrical (good); 3 = definite but not severe problems such as moderate numbers of excluded blastomeres, small size, small amounts of degeneration, etc. (fair); 4 = partly degenerate, vesiculated cells, greatly varying cell size, very small and/or similar problems (poor); 5 = severely degenerate, probably not worth transferring (very poor); 6 = unfertilized, zona only, ghost-like, 3-cell, etc.

RETARDATION Number of days

HOURS STORED To nearest 0.5 hour (e.g. 035 = 3.5 hours), frozen = 001

Example 8

Record of embryo transfer

RECIPIENT RECORD

DATE	DONOR	RECIPIENT	CONSECUTIVE No.	SYNCHRONY	LENGTH OF ESTRUS	METHOD OF TRANSFER	BODY CONDITION	TONE OF TRACT	SIDE OF CL	TYPE OF CL	FOLLICULAR TISSUE	ANESTHESIA	PREGNANCY DIAGNOSIS	AGI

DATE Year Month Day, e.g. 88 AU 20, using numeric codes for year and day, and two-letter alpha code for month; see Example 1 for recommendations

DONOR Identification by breed and registration number (or ear-tag or tattoo number); see Example 1 for alpha abbreviations of breeds

RECIPIENT Identification by breed and registration number (or ear-tag or tattoo number); see Example 1 for alpha abbreviations of breeds

CONSECUTIVE No. (to identify embryo in data files)

SYNCHRONY Degree of estrous synchrony between donor and recipient: 0 = unknown; 1 = exact; 2 = -0.5 day; 3 = +0.5 day; 4 = -1 day… etc. Note: + = recipient in estrus after donor

LENGTH OF ESTRUS 1 = >12 hours; 2 = 12–24 hours; … etc.

METHOD OF TRANSFER 1 = non-surgical; 2 = flank surgery

BODY CONDITION 1 = normal; 2 = fat; 3 = thin

SIDE OF CL (Corpus luteum) 1 = right; 2 = left; 3 = none; 4 = both

TYPE OF CL 1 = normal; 2 = small; 3 = none

FOLLICULAR TISSUE 1 = absence of large follicles on ovary in addition to CL; 2 = presence of large follicles on ovary in addition to CL

ANAESTHESIA 1 = epidural anaesthesia; 2 = no anaesthesia

Chapter 17
Equipment and supplies

EQUIPMENT, SUPPLIES, DRUGS AND REAGENTS

Most embryo transfer practitioners in North America use entirely disposable supplies and purchase sterile saline and complete media. Various suppliers ship these materials with just a telephone call (credit is prearranged). This greatly simplifies operations. There is nothing to wash and sterilize; no medium need be prepared except to add macromolecular and antibiotic solutions with a sterile syringe; there is no need to purify water; there is no danger of spreading disease from farm to farm because everything is disposed of at each farm. Some practitioners do not even have a refrigerator, but depend on each farmer's household refrigerator.

This approach is inappropriate for embryo transfer in many countries because of unreliable access to suppliers, but it should be considered seriously in some situations. We have organized this chapter by listing equipment and supplies needed for basic embryo transfer, and add additional supplies for various functions, such as media preparation and cryopreservation.

Equipment

- stereomicroscope(s)
- compound microscope (optional)
- hair clippers (scissors can substitute)
- cabinet or incubator for embryos (an insulated box can substitute)
- cervical expander

Supplies

- syringes and needles
- betadine (tamed iodine scrub)
- ethanol
- plastic palpation sleeves
- sterile plastic gloves
- lubricant (K - Y jelly)
- insemination or embryo transfer guns
- sterile sheaths for guns
- 0.25-cc plastic straws
- transfusion bag to hold medium (2-litre flask can substitute)
- Foley catheter plus stylet
- tubing for flushing
- straight, tapered and Y-connectors
- clamps (haemostats can substitute)
- graduated cylinder or embryo filter

- 0.22-µ bacteriological filters
- pipettes for embryos and rubber connectors
- microscope bulbs
- searching dishes and small Petri dishes
- KaMaR oestrus-detection aids (optional)
- labelling tape and indelible marking pens
- paper towels
- blood collection tubes

Drugs and reagents

- appropriate vaccines for health programme
- prostaglandin F_2 alpha (or analogue)
- follicle-stimulating hormone or pregnant mare's serum gonadotrophin
- Dulbecco's phosphate-buffered saline
- Na penicillin G
- streptomycin sulphate
- bovine serum albumin (Fraction V) or heat-inactivated bovine serum
- procaine (2 %)
- siliconizing agent (optional)

Additional needs if washing and sterilizing capabilities are required for reuse of equipment:

Equipment

- gas sterilizer (a ventilation hood is a useful option)
- drying oven
- autoclave (optional)

Supplies

- detergent
- sterilization packaging and heat and gas indicator tape
- aluminium foil

Drugs and reagents

- ethylene oxide

Additional needs if media are to be prepared at the embryo transfer laboratory:

Equipment

- balance
- pH meter
- osmometer (optional)
- centrifuge (if serum prepared)
- water bath (if serum prepared)
- refrigerator with freezer
- still or deionizer (unless water is purchased)
- Bunsen burner or alcohol lamp
- large bacteriological filter unit

Supplies

- flasks
- sealable bottles

- weighing paper

Drugs and reagents

- $CaCl_2.2H_2O$
- $MgSO_4.7H_2O$
- NaCl
- KCl
- Na_2HPO_4
- KH_2PO_4
- glucose
- Na pyruvate
- distilled or deionized water (unless made in the laboratory)

Additional needs if embryos are to be cryopreserved:

Equipment

- liquid nitrogen tank (can use farmer's)
- freezing machine or apparatus
- heat sealer or haemostat (unless PVC polymer powder is used)
- small insulated container for liquid nitrogen (e.g. DeWar flask)

Supplies

- forceps (for seeding)

Drugs and reagents

- glycerol
- non-toxic paraffin oil (optional)
- sucrose
- liquid nitrogen
- polyvinyl chloride powder (unless a heat sealer is used)

Additional needs for micromanipulation:
(Note: Simple bisection of embryos does not require this equipment.)

Equipment

- fixed-stage microscope
- micromanipulators (usually left and right)
- pipette puller
- microforge

Supplies

- breakable razor blades
- glass capillary tubing

Other optional equipment:

- ultrasonography apparatus
- laparoscope

Suppliers

The following list of suppliers includes companies which have exhibited at the annual conference of the International Embryo Transfer Society recently or which are listed in ***Procedures for recovery, bisection, freezing and transfer of bovine embryos*** (Elsden and Seidel, 1985). We have listed only our local suppliers; it is logistically impossible to list all suppliers worldwide. These suppliers, however, can give information on distributorships for their products in other localities. Inclusion in this list does not signify endorsement nor does exclusion signify lack of endorsement.

American Embryo Systems, 2619 Skyway Drive, Grand Prairie, TX 75051 USA. 214-641-5420. Culture media, serum, antibiotics.

H.W. Andersen Products, P.O. Box 1050, Chapel Hill, NC 27514 USA. Anpro gas sterilizer and sterilization products.

CEVA Laboratories, Inc., 10560 Barkley, Overland Park, KS 66212 USA. Transfusion bags, Syncromate B.

Colorado State University, Embryo Transfer Laboratory, Fort Collins, CO 80523 USA. 303-491-5287. Cervical expander.

Curtin Matheson Scientific, 12950 E. 38th Avenue, Denver, CO 80239 USA. 303-371-5713. Siliconizing agent, culture dishes, biological filters, pipettes, tubing, and many laboratory supplies including plastic ware.

Edwards Agri Supply, P.O. Box 65, Baraboo, WI 53913 USA. 608-356-6641. Artificial insemination equipment; oestrus-detection aids.

Emery Medical Supply. 5601 Gray Street, Arvada, CO 80002 USA. Sterilization packaging and supplies.

EM-TEX Supply Co., Inc., 2741 S. Great Southwest Parkway, Grand Prairie, TX 75051 USA. 214-660-1771; Fax: 214-660-2303. Antibiotics, antiseptics, artificial insemination equipment, catheters, dishes, flushing and freezing media, disposable flush kits, embryo filters, embryo transfer guns and straws, gloves, bovine serum albumin, sera, programmable cryopreservation unit, cervical expanders, drugs for superovulation and oestrus synchronization, connectors, sterilization packaging.

Fisher Scientific Company, 14 Inverness Drive E., Building A, Suite 144, Englewood, CO 80112 USA or Fisher Scientific International, 50 Fadem Road, Springfield, NJ 07081 USA. 201-467-6400; Cable: Fishersci, Springfield, NJ; Telex: 475 4246 or 138287; Fax: 201 379 7415. Paraffin oil, culture dishes, biological filters, pipettes, tubing, microscopes, and many laboratory supplies including plastic ware.

GIBCO, 3175 Staley Road, Grand Island, NY 14072 USA. Culture media.

Mobay Corp., P.O. Box 390, Shawnee, KS 66201 USA. 913-631-4800. Estrumate (cloprostenol).

IMV, 10, rue Georges Clemenceau, B.P. 76, F-61300 L'Aigle, France. 333-324-0233 or 6870 Shingle Creek Parkway, Suite 100, Minneapolis, MN 55430 USA. 612-560-4986. Artificial insemination equipment, transfer guns, sheaths, straws and polyvinyl chloride powder.

Intermed, Inc., Newfoundland, NH 07435 USA. 201-697-3818. Foley catheters.

Kamar, Inc., P.O. Box 26, Steamboat Springs, CO 80477 USA. 303-879-2591. KaMaR oestrus-detection aids.

M & M Company, 1120 Industrial Ave., Escondido, CA 92025 USA. 619-746-0800; Telex: 607 950. Micromanipulator.

PETS, Professional Embryo Transfer Supply, Inc., 27221/2 Garden Valley Road, Tyler, TX 75702 USA. 216-595-2047; Telex: 205997-PETSUR; Fax: 214-592-1525. Antibiotics, artificial insemination equipment, catheters, dishes, flushing and freezing media, disposable flush kits, embryo filters, embryo transfer guns and straws, gloves, bovine serum albumin, sera, polyvinyl chloride powder, sterile water, microscopes, cervical expanders, drugs for superovulation and oestrus synchronization, connectors and sterilization packing.

Reproduction Resources, Inc., P.O. Box 135, Hebron, IL 60034 USA. 815-648-2431. Sani-Shield Protector.

Research Instruments, Ltd., Kernick Road, Penryn, Cornwall, TR10 9DQ, UK. Micromanipulator.

Rocky Mountain Microscope, 440 Link Lane, Fort Collins, CO 80524 USA. 303-484-0307. Microscopes.

Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 USA. 314-771-5750. Reagents for culture and freezing media.

The Upjohn Company, P.O. Box 108, Kansas City, MO 60901 USA. 616-323-4000. Lutalyse (prostaglandin F_2 alpha).

VWR Scientific, P.O. Box 39396, Denver, CO 80239 USA. 303-371-0970. Latex tubing, culture dishes, biological filters, pipettes, and many laboratory supplies including plastic ware.

Veterinary Concepts, 100 McKay Avenue, Spring Valley, WI 54767 USA. Antibiotics, antiseptics, artificial insemination equipment, catheters, dishes, flushing and freezing media, disposable flush kits, embryo filters, embryo transfer guns and straws, gloves, bovine serum albumin, sera, programmable cryopreservation unit, cervical expanders, drugs for superovulation and oestrus synchronization, connectors and sterilization packaging.

United States Biochemical Corporation, P.O. Box 22400, Cleveland, OH 44122 USA. 216-765-5000. Bovine serum albumin.

MICROSCOPES

It is essential to have a stereomicroscope of good quality to search for embryos, and most programmes should have at least two. Most people use a magnification of 8X to 15X to locate embryos. Higher magnifications are unsuitable for this purpose because the field of view is too small, which greatly increases the time required for searching as well as the likelihood that embryos will be overlooked. However, a 30X to 50X magnification is essential for evaluation of embryos once they are located. Thus, one needs a stereomicroscope with at least two magnification settings. In practice, there is usually some zoom or step arrangement to vary magnification from lowest to highest settings.

Stereomicroscopes of good quality are priced in the range of US$1 200–1 500. Sometimes good used instruments can be purchased for much less. Unless required for some other purpose, such as splitting embryos, stereo-microscopes costing US$3 000 and higher are a luxury; they are not any better for routine embryo transfer work than the less expensive ones. Conversely, the microscopes marketed for US$200–300 (price when new) simply are

not good enough.

Appropriate stereomicroscopes that we have used include the Olympus Zoom model SZ-111-100 with transmitted light-base illuminator; American Optics (now Reichert-Jung) Stereostar 561B or 561C with Starlite illuminator; and Bausch and Lomb (now Cambridge Instruments) BVB-73 with Nicholas illuminator. Eyepieces of 10–20X magnification are available for most of these. Similar models from other companies are usually satisfactory. Always be sure to obtain a base/stand designed for transillumination of transparent specimens and a good light source (with spare bulbs).

Advice on purchasing a compound microscope for embryo evaluation purposes is similar to that for a stereomicroscope: a sturdy, easy-to-use bright-field microscope without complex accessories is best, generally in the range of US$1 500–2 000. It should be borne in mind that a compound microscope is not absolutely essential, but that a small percentage of embryos cannot be evaluated properly without one, and evaluation of progressive motility of semen requires a microscope with 100–200X magnification. Also the process of learning to evaluate embryos is easier with the improved resolution of a compound microscope.

Many laboratories have compound microscopes for other purposes such as semen evaluation or microbiology studies. Any of these can be used for embryos as well. If a new microscope is to be purchased just for embryos, an inverted type should be considered. This is easier to use for embryos because the objective is below the stage, which reduces the risk of contaminating or spilling embryos. Inverted microscopes, however, are more expensive and generally have slightly poorer resolution. In purchasing a compound microscope, one also should take into account needs such as micromanipulation, for which a fixed stage is required, whether the microscope is inverted or not. Obviously, in some cases it is best to have more than one compound microscope, for example, one with differential interference phase-contrast (Nomarski) optics or phase-contrast optics and one that is less expensive with simple bright-field optics. Note well, however, that embryos can be evaluated perfectly well with a 10X bright-field objective, and for this purpose more sophisticated systems are of little additional value. A 2X or 4X objective is useful for locating embryos prior to examination with the 10X objective.

FREEZING MACHINES

More than 20 models of freezing machines are currently being manufactured by approximately 12 companies in eight countries. Nearly all of these machines work satisfactorily. All machines require repairs from time to time, so arrangements for service are important, particularly in remote areas. A particularly good approach is a system of shipping a replacement machine on loan while the malfunctioning machine is being repaired. An obvious generalization is that more can go wrong with complex machines than with simple ones; however, this does not always apply because some of the more complex machines are particularly well made.

The reason for purchasing more complex freezing machines is that they are easier to use; most have automatic functions so that little or nothing need be done except to add the straws or ampoules to the freezing chamber at the beginning of the process and remove them prior to plunging into liquid nitrogen. A somewhat incongruous situation is that companies and organizations in developing countries tend to purchase complex and expensive freezing machines. Success rates are not usually improved with more complex machines; they just save (and replace) labour. This is especially ironic since capital is short and labour is in excess in many countries.

There are two important criteria for evaluating performance of freezing machines. The first is whether the machine cools embryos at the assigned rate. The smoothness of the cooling curve is frequently overemphasized. Fluctuations in temperature of 0.5-1℃ from a perfect, straight-line cooling curve are not of much consequence as long as the average cooling rate is correct. The latter capability is essential, however. The second important criterion is

whether the temperature being recorded in the freezing chamber is, in fact, correct. Temperatures at the time of seeding and plunging are critical, and drifts in thermometer readings of 2-3℃ can lead to catastrophic results. In fact, it is a good idea independently to check temperatures in freezing chambers on a regular basis, perhaps every few months, as a quality control measure.

TABLE 14
Information on some commercially available freezing machines

Brand name	Address of company	Source of coolant	Description of chamber
Bio-Cool	FTS Systems, Inc. P.O. Box 158 Stone Ridge, NY 12484 USA; 914-687-7664	Mechanical refrigerator	Alcohol bath
Cryoembryo-PSP	Hoxan Hoxan Bldg. 2 Nishi 1-chome Kito 3-jo Chuo-ku Sapporo 060 Japan	Vessel of liquid nitrogen	Slots for straws only
Cryo Genetic	Cryo-Genetic Technology 400 Hoover Rd. Soquel, CA 95073 USA	Liquid nitrogen vessel	Straws lowered into vapour
Cryo-Med	Cryo-MedN 49659 Leona Dr. Mt. Clemens, MI 48045 USA; 323-371-5713	Liquid nitrogen tank	Large chamber
CTE	Labortechnik Postfach 1107 D-3406 Bovenden—Göttingen Fed. Rep. Germany; (0551) 82835	Liquid nitrogen tank	Open vessel
Freeze Control	Freeze Control USA 3377 Solano Ave. Suite 303 Napa, CA 94558 USA	Vessel of liquid nitrogen	Slots for straws only
Glacier Technology	Glacier Technology 404 Europe St. Baton Rouge, LA 70802 USA	Peltier effect/electricity	Small chamber
Mini Cool	CFPO B.P. 15 F-38360 Sassenage France	Liquid nitrogen tank	Large chamber
McDonald	Veterinary Concepts 303 South McKay Ave. Spring Valley, WI 54767 USA	Neck of liquid nitrogen tank	Small chamber
Planer	Planer Products, Ltd. Windmill Road Sunbury-on-Thames	Liquid nitrogen tank	15 cm diameter × 20 cm high cylinder

	Middlesex TW16 7HD UK		
RPE	Peter Elsden & Assoc. P.O. Box 9677 Fort Collins, CO 80525 USA; 303-223-6665	Neck of liquid nitrogen tank	Slots for straws only

One other criterion for selecting a freezing machine is ease of use. Things to consider are weight, if it must be moved from place to place, ease of access to the cooling chamber, simplicity of programming, systems for holding straws, vials or ampoules, depending on which is to be used, and ease of diagnosing problems and fixing them. Cost, reliability of service, reputation of the manufacturer and dealer, and similar factors also need consideration. A brief description of freezing machines is given in Table 14. We have no way of knowing about all possible models of freezing machines available and have provided information on those companies that have contacted us in recent years.

One final point is that embryos can be cooled perfectly adequately with dry ice and alcohol in DeWar flasks (see Maurer, 1978, for examples). This is more labour intensive and requires conscientious personnel, but if done correctly results will be as good as with freezing machines.

Chapter 18
Quality control

Many things can go wrong in an embryo transfer programme. The concept of quality control is to carry out tests on a regular basis to avoid problems or at least to identify them early. Quality control procedures need not be elaborate and, if worked into regular routines, may be effective at a cost of only a small percentage of the budget of a programme.

Frequently we rely on quality control procedures of manufacturers by assuming, for example, that purchased drugs are efficacious and sterile, or that disposable plastic ware is not contaminated with micro-organisms or embryo toxins. Quality control concepts become more critical in an embryo transfer programme as personnel at the embryo transfer unit carry out more steps, such as making culture media from the constituent reagents rather than purchasing complete media, or washing and reusing disposable Foley catheters instead of using new ones each time.

RECORDS

Good records are the foundation of a quality control programme. Recording data as suggested in Chapter 16 is a good start. Being able to correlate events in time by comparing dates is especially useful. For instance, the cause of an unexplained decrease in pregnancy rates may be traced by checking personnel schedules or dates of purchase of lots of reagents or supplies. Records need not be complicated but they need to be complete. It is good to date supplies when the container arrives or when it is opened. This is especially important for drugs and chemicals. Lot numbers should be recorded for products like FSH or paraffin oil that vary from batch to batch with regard to potency or toxicity. For certain procedures, the person doing the work should be recorded, for example, for artificial insemination, oestrus detection, embryo evaluation and embryo transfer.

A number of checks should be scheduled on a regular basis, such as levels of liquid nitrogen in cryogenic storage containers and inventories so that supplies can be ordered as needed. Indicators of proper function of equipment and proper execution of procedures are discussed below in more detail, and a recommended schedule of quality control measures is given in Table 15.

CULTURE MEDIUM

If culture media such as modified Dulbecco's phosphate-buffered saline are made up correctly, they will be suitable for embryos. However, since many steps are involved, it is easy to use an incorrect chemical, make a tenfold error in weighing, use an instrument or container with toxic residues, or fail to effect sterility. If media are made at the embryo transfer unit, they should be checked at the least for pH and osmolality. The latter can be done with an osmometer or, more crudely, by observing shrinking or swelling of red blood cells. If the medium is suspect, the notes recorded when weighing the ingredients should be

consulted to see if a weighing error occurred. If the problem is not resolved at this point, the batch of medium should be discarded. It is better to discard an occasional good batch of medium that deviates slightly from acceptable pH and osmolality standards than to have an occasional batch that kills embryos. Note that a somewhat aberrant pH indicates that something is wrong, but pH may not be the factor that kills the embryos. Put another way, adjusting the pH will not solve the problem.

Sterility can be checked by incubating a sample at 37°C overnight and examining it for micro-organisms the next day. Toxicity is more difficult to deal with. Some laboratories incubate two-cell mouse embryos in each new batch of medium to verify that it will support normal development. Paraffin oil, if used, can be checked similarly for sterility and toxicity. If circumstances warrant extra precautions, a sample of each batch of culture medium can be labelled and stored in the freezer until pregnancy rates are available.

Determining water quality is especially difficult. Resistance to electric current is commonly used to measure absence of ions, but this is of little value in detecting organic compounds such as endotoxins. The best course is to pay scrupulous attention to detail in preparing water, cleaning deionizing or distillation systems regularly to prevent micro-organisms from colonizing components, and otherwise following directions. Culture of twocell mouse embryos in medium made with the water in question is probably the best overall test of water quality.

EQUIPMENT AND SUPPLIES

It is convenient to divide equipment and supplies into two groups for purposes of quality control: those that come into contact with embryos and those that do not. Anything that contacts embryos should be non-toxic and sterile. Once a routine has been established, it should not be necessary to check equipment for toxicity and sterility each time. However, it is wise to check various items for sterility every three or four months by rinsing with culture medium followed by incubation of the rinse.

Toxicity of most items contacting embryos, except plastic culture dishes (manufacturers generally exercise stringent control over quality of these items), should be handled in a different way: assume that there is toxic residue and rinse with sterile medium or 0.9 percent saline before use. From time to time toxicity has been demonstrated with syringes, Foley catheters, bacteriological filters, most kinds of rubber tubing and straws. Accordingly, all of these should be rinsed before use. For example, discard the first few ml of medium coming through bacteriological filters, rinse straws before loading embryos, etc. The rubber plungers of certain syringes have been proven to be extremely toxic in the past. Some toxicity is due to sterilization procedures, e.g. with ethylene oxide. If one gets into the habit of always rinsing these items before use, risks of potential toxicity will be markedly lowered. It is of course extremely important not to compromise sterility during rinsing.

Filters for collecting embryos should be examined with a stereomicro-scope before each use, especially if filters are reused. Sometimes they are damaged during the washing and sterilizing procedures, which results in loss of embryos.

Temperature controls on incubators, freezers, warming plates, microscope stages, etc. should be verified on a regular basis. Deviations of only a few degrees can be lethal. For example, incubators set at 38°C that are actually at 40° or 41°C have killed many embryos. Freezing machines set at -6°C for seeding that actually were at -3° or -4°C so that seeding was ineffective have resulted in disastrous pregnancy rates. Refrigerators that should be at 5°C but are actually at 9° or 10°C lead to marked increases in microbial growth. Warming plates and microscope stages can cook embryos. Temperature settings on items of equipment can rarely be trusted, and many laboratory thermometers, especially dial thermometers, are incorrect by several degrees and get worse with age. Always check any thermometer against a second or even a third one of high quality. Further, we suggest that all equipment be checked every three months to verify that the desired temperature is, in

fact, the actual temperature.

ANIMALS

Appropriate herd health programmes are critical and deserve particular focus when cattle are constantly moving in and out of a facility. Health status can be determined and controlled as described in Chapter 3. Each animal should be observed daily for indications of disease or injury when oestrus detection is carried out. Excellent quality control information on nutrition programmes can be obtained by weighing cattle periodically. This may not be necessary in all embryo transfer programmes, but weights every three or four months will indicate if animals are, in fact, gaining weight. This does not provide information on all aspects of nutrition, but it is the single most important indicator of a good nutrition programme.

One other item worth checking is possible presence of abortifacient or teratogenic weeds in corrals and pastures. This is especially a problem when animals are moved to areas of new plant growth at certain times of the year.

The reproductive status of donors and recipients can be checked by palpation of the ovaries and by studying the intervals between oestrus, when appropriate. Periodic measurement of concentrations of progesterone in milk or blood may be helpful when conditions of management or climate are less than ideal. For example, if heat stress has reduced the efficacy of oestrus detection, screening donors before beginning superovulatory treatment and recipients before transfer on the basis of progesterone may improve success rates.

SEMEN HANDLING AND ARTIFICIAL INSEMINATION

While most bull studs exercise good control over the quality of semen processing, control over conditions of shipping and storage of semen is often in the hands of individuals who have no knowledge of how to handle semen and no vested interest in its quality. Therefore, it is recommended that a drop of semen from one straw from each lot of semen be examined microscopically for the percentage of progressively motile sperm. This should be done carefully and the result should be recorded. In most cases, it is not necessary to examine sperm for intact acrosomes or morphological abnormalities.

Correct procedures for thawing semen are straightforward, but it is amazing how frequently this is done incorrectly. The main error with artificial insemination is semen placement. Quality control for this is best accomplished by inseminating animals about to be slaughtered with a thick dye, and then examining the reproductive tracts. If this can be arranged easily, it is well worth the effort to check the insemination technique.

EMBRYO TRANSFER PROCEDURES

The most important overall quality control information comes from studying records, particularly returns to oestrus for the earliest indication of pregnancy rates. These records should be scrutinized monthly. A good quality control of oestrus detection procedures is to take blood or milk samples to determine if progesterone levels are, in fact, low at presumed oestrus. An even simpler procedure is to study intervals between oestrus of cows that are not resynchronized or used as recipients. If the majority of intervals are not 17–24 days, there is a problem.

Ultrasonography is a marvellous tool for quality control of skills in palpation per rectum, and may replace palpation when equipment becomes less expensive. Ultrasonography can be used for pregnancy diagnosis and definitively locating corpora lutea. Errors in palpation of corpora lutea are frequent, and the incidence of such errors can be determined.

Reflushing a random subset of donors periodically will establish whether most embryos are

being recovered. Efficacy of isolating embryos from the collection fluid can be evaluated by accumulating the contents of every container throughout the day in a 2-litre cylinder after presumably all embryos have been found and siphoning off the top and re-examining the remaining fluid as described in Chapter 5. An embarrassing number are missed, even by conscientious personnel, but this loss is costly and should be kept to a minimum.

TABLE 15
Suggested schedule of quality control measures

Item or procedure	Quality control measure	Frequency
Culture medium, if made at embryo transfer centre	Measure pH Check sterility by culture overnight	Each batch Every 3 months
Serum, if processed at embryo transfer centre	Check sterility by culture overnight Heat-inactivation by culturing mouse embryos or sparing use until pregnancy data available	Every batch Every batch
Sterility of equipment and supplies	Rinse with sterile medium and culture overnight	Every 3–6 months
Temperature controls	Independent thermometer	Every 3–6 months
Embryo filters	Stereomicroscopic examination	Every use
Cycling of new cattle	Palpate sample for CLs	Every group
Nutrition of cattle	Weigh cattle	Every 3–4 months
Semen	Determine percentage progressive motility	Each code of each bull
Site of semen deposition	Placement of dye in reproductive tract of slaughter cattle	Annually
Efficacy of oestrus detection	Study inter-oestrus intervals, measure blood or milk progesterone	Every 3–6 months
Efficacy of non-surgical embryo recovery	Reflush donors	5 cows every 6 months
Efficacy of isolating embryos	Collect and examine medium from 5 cows	Every 6 months
Accuracy of CL palpation	Ultrasonographic evaluation	Check until proficient
Non-return to oestrus after ET	Study records	Monthly
Pregnancy rate	Study records	Monthly
Accuracy of pregnancy diagnosis	Ultrasonographic confirmation	Check until proficient
Technician differences in percentage fertilized embryo recovery pregnancy rates	Study records	Every 6 months

With some instruments, transfer of the embryo can be verified by rinsing the transfer device and examining the fluid under the microscope for presence of the embryo. The reproductive tract of a cow that received a sham transfer just before slaughter can be examined to see if undue trauma of the endometrium has been caused. The site of deposition of the embryo is more critical than that of semen, and this can be checked in a similar way by transfer of 0.25 cc of dye.

Performance of technicians may vary because of innate ability, attitude, training and other factors. Attitude problems may be due to health, personal problems, tension in the laboratory, etc. In any case, workers should be sufficiently professional that they will agree

to sign their name to their work. Differences among persons can be determined by studying such records as pregnancy rates after non-surgical transfer. When marked, consistent differences occur among technicians, retraining is indicated. This has been highly successful with artificial insemination technicians. If retraining is unsuccessful or inappropriate, reassignment of responsibilities is often a good option.

Not all of the procedures in Table 15 are carried out at every embryo transfer facility, so some measures of quality control are irrelevant. In many cases, the frequency of quality control checks should be increased when people are learning techniques; likewise, some steps may not be necessary at all with very experienced personnel. If the volume of embryo transfer at a given facility is very low, checking at the suggested frequencies may be misleading because of inadequate sample size; checking less frequently would give a more representative summary.

Chapter 19
Training programmes

The scope of training programmes in bovine embryo transfer depends primarily on the background of the people to be trained and the extent of the training. Training can range from a one-day seminar to a two-year master's programme. At the present time, there are a number of training programmes ranging from one week to one month in length. Such programmes are usually sponsored by private companies, most of which have extensive experience in commercial bovine embryo transfer, and usually provide considerable “hands on” experience with collection and transfer of embryos with cattle. They are expensive, generally costing from US$1 000 to US$10 000 each, depending on the number of animals involved, the extent of personalized training, the length of the programme and whether room and board is included.

An alternative is to invite one or several experts to a particular site to carry out training. This is an effective approach if the right kinds and numbers of animals are available, and if appropriate supplies and equipment are provided.

Almost universally, a prerequisite for these training programmes is proficiency in artificial insemination with cattle. People accomplished in this area can usually make great progress in learning to recover embryos non-surgically within a week or two. Those without proficiency in artificial insemination waste a great deal of time becoming comfortable in manipulating instruments through the rectal wall. A veterinary or other advanced degree is also helpful for trainees, but is less useful in achieving technical proficiency than experience in artificial insemination.

Below is an outline of a training programme that has been successful for us at Colorado State University. This programme is suitable for training three to six students simultaneously. It requires one full-time plus one half-time instructor, and the trainees must provide considerable help. Depending on the number of students, the programme requires 25–40 head of cattle, which must be available for some weeks before training begins, and which will be unsuitable for other uses for one to two months afterwards.

TABLE 16
Example of four-week training programme in embryo transfer

Week 1	
Monday	Introduction, tour facilities, receive written information
Tuesday	Practice passing Foley catheters through slaughterhouse tracts, demonstration of non-surgical recovery
Wednesday	Practice non-surgical recovery in cull cows, study slides of embryos, lecture on oestrus detection
Thursday	Ultrasound demonstration, lecture on ultrasonography, superovulation and programming donors

Friday	Practice non-surgical recovery on superovulated cows, manipulate and evaluate embryos, lecture on preparation of drugs and making up media
Week 2	
Monday	Practice non-surgical recovery, study embryos, lecture on microscopes
Tuesday	Practice ultrasonography, practice loading straws, lecture on cryopreservation principles and procedures, practicum in making up media
Wednesday	Practice non-surgical recovery, study embryos, freeze embryos
Thursday	Lecture on washing and sterilizing, make glass pipettes, wash dishes
Friday	Further lectures on cryopreservation, thaw and study frozen embryos, transfer frozen embryos
Week 3	
Monday	Non-surgical recovery of transferred embryos, study and retransfer embryos
Tuesday	Practice non-surgical recovery, freeze and transfer embryos
Wednesday	Help with ***in vitro*** fertilization experiments, lecture on ***in vitro*** fertilization
Thursday	Lecture on purchase of equipment; afternoon off
Friday	Recover transferred embryos from recipients, lecture on micro-surgery and sexing
Week 4	
Monday	Non-surgical recovery, lecture on pure water and avoiding toxins
Tuesday	Thaw and evaluate embryos, transfer them
Wednesday	Help with ***in vitro*** fertilization experiment, lecture on ordering supplies
Thursday	Recover transferred embryos, lecture on genetics and success rates
Friday	Answer questions
Daily	Help with injections, insemination, oestrus detection, and clean up, read and study extensive written material provided

Note: Each time cattle are used, students practice palpation, ultrasonography, epidural anaesthesia; each time embryos are available, students evaluate them, manipulate them, etc.

Upon completion of such a course, a student will not be competent in all areas of embryo transfer, but will be in a position to become competent with sufficient practice. Differing backgrounds and innate abilities make the concept “sufficient practice” hard to quantify. One set of arbitrary objective measures is used by the Canadian Embryo Transfer Society in screening practitioners for admission to its certification examination: the candidate must have superovulated and flushed at least 50 donors, have transferred at least 200 embryos, and have maintained a pregnancy rate of 50 percent or better during the year before making application. Another estimate is that one to three months' practice is required to master each step. Even after a student has become proficient, constant practice and attention to detail are required.

Chapter 20
A note to administrators

Embryo transfer programmes consist of a series of relatively simple techniques. However, each step in the process must be done correctly for the programme to succeed. The end result will only be as good as the ***weakest*** step in the process. Thus, attention to detail is essential.

KEY INGREDIENTS FOR SUCCESSFUL EMBRYO TRANSFER PROGRAMMES

Sometimes embryo transfer programmes are failures, usually because pregnancy rates are very low. Probably the main reason for failure is insufficient investment in training personnel. The second most common problem is insufficient animal resources. Unless large numbers of healthy, thriving cattle are available, embryo transfer will not work well, particularly when personnel are developing skills.

Facilities and equipment are also important, but are frequently over-emphasized. A clean laboratory work area is needed; mobile vans can be used for this purpose. Obviously, cows must be kept separate from bulls. An unusually common error is that recipients become pregnant from natural service rather than embryo transfer, which is not discovered until calves are born one oestrous cycle late or are of the wrong breed. It is clearly necessary to be able to catch animals for injections, insemination, embryo recovery and embryo transfer. Simple, well-designed pens, runways and head catches close to where animals can be fed are essential. Implicit in such a facility is the need for intensive management based on feed supplementation.

A generally successful approach is to build embryo transfer on a programme that has been successful for artificial insemination. Facilities and logistics of handling animals are similar for both techniques. Also, the skills of good oestrus detection and passing catheters through the cervix are an excellent foundation for embryo transfer. In fact, we do not recommend training people in techniques of embryo recovery and transfer until they are proficient in artificial insemination (meaning that they have inseminated well over 100 animals with good pregnancy rates).

TABLE 17
Examples of reasonable goals of embryo transfer programmes

To meet commercial objectives, i.e. to make a profit by providing services where commercial demand exists

To train personnel who are in demand to meet other goals

For research purposes, where embryo transfer is deemed the best approach to testing a hypothesis

For preserving genetic material of indigenous breeds in danger of extinction by cryopreservation of embryos

For importing embryos to provide new genetic resources and then increasing the numbers of animals of the new breed quickly

For national livestock improvement programmes such as MOET schemes in which embryo transfer fits into a well-thought-out overall programme

To test otherwise outstanding males and females suspected of being carriers of undesirable recessive genetic traits

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APPROPRIATE GOALS

A final thought is that many embryo transfer programmes suffer from not having clearly-thought-out goals. Frequently the goal is simply to establish a successful embryo transfer programme for reasons of prestige, or because it seems the wave of the future. Embryo transfer should be thought of as a technique such as oestrus synchronization or artificial insemination, rather than as an end in itself. Only rarely is it the method of choice for reproducing cattle. Even in so-called developed countries, use of embryo transfer has plateaued at about one per 500 calves born. Obviously this may change as new technologies such as sexing and cloning become inexpensive but except for a few special cases, we predict that embryo transfer will be used to produce fewer than 1 percent of the births of calves in any given country for the remainder of this century.

Examples of reasonable goals of embryo transfer are listed in Table 17.

Note that for some of these goals it is much less expensive to hire someone to do the work than to develop an embryo transfer programme ***de novo***. In calculating the cost effectiveness of an embryo transfer application, administrators frequently fail to define the end product accurately, and thus misjudge actual costs. For instance, it is misleading to use cost per viable embryo or cost per pregnancy when a first-calf heifer entering the milking herd is the desired product. For a more thorough discussion of uses of embryo transfer, see Seidel and Seidel (1989).

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