EMBRYO TRANSFER IN BEEF CATTLE

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Introduction

Embryo transfer is a multi-step process that involves the production and collection of preimplantation embryos from genetically superior females (called donors) and the subsequent transfer of the harvested embryos into reproductively healthy females (called recipients) for the purpose of establishing pregnancies and producing live offspring. The first successful embryo transfer in mammals was performed with rabbits in 1890 (Heape, 1891), but it was more than 70 years later that the first successful embryo transfer in cattle was reported (Willett et al., 1951). Today, more than ¾ million bovine preimplantation embryos are transferred each year throughout the world (Thibier, 2006).

The embryo transfer process involves the following steps:
1. identification of genetically superior donor females
2. identification of reproductively healthy recipient females
3. synchronization of estrus in donor and recipient females
4. superovulation of donor females
5. detection of estrus in donor and recipient females
6. mating of superovulated donor females
7. recovery of preimplantation embryos from inseminated donor females
8. transfer of viable preimplantation embryos into recipient females

If the transfer of preimplantation embryos from donor females can not be performed immediately after embryo harvest, it is also possible to cryopreserve (freeze) the embryos for transfer at a later date. As an alternative to the above process, it is also possible to produce preimplantation embryos *in vitro* (in the laboratory).

The objective of this paper is to provide readers with an overview of embryo transfer, embryo cryopreservation, and *in vitro* embryo production in beef cattle.

Embryo Transfer

Reasons to Perform Embryo Transfer

The primary reason most cattle producers utilize embryo transfer is to enhance genetic improvement in their herds. By collecting embryos from genetically elite females and transferring the harvested embryos into females of lesser genetic merit, it is possible to produce more calves from genetically superior females and fewer calves from genetically less valuable females. The result is an increase in the rate of genetic improvement. Transfer of embryos harvested from genetically elite donor females also enables those genetically elite females to produce more calves in a single year (by transfer to recipient females) than they would produce in their normal reproductive

lifetime. Just as artificial insemination capitalizes on genetically outstanding bulls by enabling them to sire more calves than they could via natural mating, embryo transfer capitalizes on genetically outstanding females by enabling them to produce more calves than they could via natural mating.

Embryo transfer technology may also be implemented in a cattle breeding program for other reasons. Embryo transfer may be used to perform a progeny test on females in order to determine whether or not they are carriers of recessive genetic defects for which a DNA test is unavailable. Embryo transfer may be used to rescue the genetics of a diseased herd in circumstances where disease was inadvertently introduced and there is no underlying genetic control of disease susceptibility. This "genetic rescue" may be accomplished by strictly following the embryo washing protocol adopted by the international embryo transfer industry (Stringfellow, 1998). Embryo transfer may be used as a form of "genetic insurance" to enable cattle producers to create a bank of cryopreserved embryos obtained from their valuable bloodlines before implementing new genetic selection criteria that could potentially result in a decline in productivity in certain economically important traits. Embryo transfer may be used to produce calves from clinically infertile females if the cause of infertility is not of genetic origin. Most often, however, embryo transfer is used to introduce new genetics into a herd or to generate income via the sale of embryos from genetically valuable females. Introducing new germplasm resources into a herd as embryos poses much less biosecurity risk than does the introduction of live animals (Wrathall et al., 2004).

Donor Selection

The factor which typically limits the amount of genetic improvement obtainable via embryo transfer is the genetic value of the selected donor. The genetic characteristics of any embryo are determined when the sperm unites with the egg at the time of fertilization. Because most cattle producers have access to the same bull semen (and hence the same genetic material contributed by the male to the embryo), it is the genetic contribution by the egg which dictates the genetic merit of the embryo.

When making genetic selection decisions, most beef producers can evaluate up to three different sources of information. One source of information is an animal's pedigree. An animal's pedigree typically will show the parents (sire and dam), maternal and paternal grandsires and granddams, as well as maternal and paternal great grandsires and great granddams. Although multi-generation pedigrees for registered breeding stock are readily available through breed associations, there is little value in examining the pedigree beyond three generations except when trying to determine the origin of a genetic defect. The theory behind the use of pedigree information as a genetic selection tool is some animals have a tendency to beget genetically superior offspring because their genetic make-up consists mostly of favorable genes for traits of economic importance.

A second source of information evaluated by beef producers is an animal's phenotype. The phenotype of an animal includes how it looks (e.g., conformation, breed character) as well as raw (unadjusted) production records. Although producers will not knowingly select animals with poor feet and legs or other structural defects, selection of animals that look nice is no guarantee their progeny will have desirable conformation or be productive. An animal that happens to be selected one day by a judge as the champion at a cattle show may or may not produce genetically desirable offspring. This is because

the heritability of phenotype (e.g., breed type) typically is low. The environment exerts a large effect on lowly heritable traits, making selection of genetically superior individuals quite challenging. Even moderately heritable traits have a fairly large environmental influence on phenotypic expression of traits, potentially leading to some incorrect genetic selection decisions. For example, a cow with a slightly lower production record raised in a poor environment may actually be genetically superior to a cow with a slightly higher production record raised in an excellent environment which enabled that animal to fully express its genetic potential.

A third source of information used by beef producers when making genetic selection decisions is genetic evaluations obtained from breed associations. Most breed associations employ the latest, most sophisticated statistical methodology available to segregate environmental versus genetic influences on an animal's phenotype. For quantitative traits of economic importance, estimated breeding values (EBVs) are calculated for each animal in the herd. When animals in two or more herds have a common genetic ancestor (e.g., a common sire), it is possible to calculate expected progeny difference (EPD) values. The calculated EPD value takes into consideration how environment influenced the raw production record and indicates how the progeny of the individual are expected to perform relative to breed average. Producers are strongly encouraged to use EBVs and/or EPDs as the major basis of genetic selection of embryo transfer donor females. In the future, donors may also be selected with the aid of DNA markers for traits of economic importance.

Once genetic selection of donors has occurred, it is also important to evaluate the reproductive health of the prospective donors. Donors should be free of any reproductive problems such as ovarian cysts, reproductive tract adhesions, and freemartinism. Heifers should have reached puberty and should be exhibiting normal length estrous cycles. Donors should not be repeat breeder animals, and they should be at least 50 days post-partum. They should not have been superovulated within the past 30 days.

Recipient Selection

The selection of cows and heifers suitable for use as recipient females is a two-stage process. The initial selection of recipients should be done at the same time or earlier than selection of donor females, but the final selection of recipients should not be done until the day of embryo transfer. This section of the paper will focus on initial recipient selection, and the final selection of recipients will be described later.

A variety of factors should be considered when identifying females suitable for potential use as recipients. Firstly, females should be in the bottom half of the herd from a genetic standpoint. Given the overall goal of embryo transfer is to facilitate genetic improvement, it seems unwise to use genetically above average females as recipients. Instead, use the genetically below average females as "incubators" for embryos possessing high genetic merit. Secondly, females should be reproductively healthy. Given the time and cost associated with the production of embryos from donor females, it seems unwise to transfer embryos into the uterus of a reproductively compromised female. Suitable recipient females should be free of reproductive anatomical abnormalities (e.g., they should have two functional ovaries plus normal oviducts, uterus and cervix), free of reproductive tract adhesions (created as a result of dystocia and/or a caesarian section),

and free of ovarian follicular cysts. Females should be consistently exhibiting estrous cycles of normal length.

One of the perennial debates regarding selection of recipient females pertains to parity. Some embryo transfer technicians advocate the use of heifers as recipients because their reproductive tracts are easier to handle. However, others argue use of heifers is unwise because they are reproductively unproven and because heifers should be of higher genetic merit than many cows in the herd. If a producer decides to utilize heifers for recipients, they are advised to perform reproductive tract scoring at least 30 days prior to intended use. Reproductive tract scoring involves assessment of the diameter and tone of the uterine horns, as well as ovarian morphology and function. A strong correlation exists between reproductive tract score (scale of 1= reproductively immature to 5 = sexually mature) and response to estrous synchronization protocols and pregnancy rate (Andersen et al., 1991). Only reproductive tract score 4 and 5 heifers are considered suitable as embryo transfer recipient females.

Overall health status of the recipient should be considered. Animals should be free of signs of infectious disease (if desired, serological testing for diseases such as bovine leukosis, bovine viral diarrhea, and neospora can be performed), they should be healthy and alert, they should have a good appetite, and they should be of intermediate body condition score (score of 4, 5, or 6 [on a scale of 1=emaciated to 9=obese]). Females that are emaciated, obese, or exhibiting rapid weight loss should be avoided. Structurally sound animals are highly desirable.

Finally, recipients should be of adequate frame size and should possess adequate milking ability to gestate and raise the embryo transfer calf. It would be unwise to transfer an embryo of a large-framed, rapid growth potential calf into a small-framed recipient with low genetic potential for milk production (Humes et al., 1987).

Synchronization of Estrus in Donors and Recipients

One of the factors greatly influencing the pregnancy rate following embryo transfer is the degree of synchrony of estrus between donor and recipient females. Estrus (heat) is the period of sexual receptivity of females to breeding males, and estrus must be exhibited in donor and recipient females at approximately the same time in order to obtain high pregnancy rates following embryo transfer.

There are three fundamental approaches used to synchronize estrus in donor and recipient females. One approach involves the administration of exogenous prostaglandin $F_{2\alpha}$, another approach involves the administration of exogenous progesterone, and the third approach involves administration of a combination of prostaglandin $F_{2\alpha}$ and progesterone. Other methods to synchronize estrus exist, but they are less commonly used in beef cattle embryo transfer programs. Some protocols involve products not approved for use in beef cattle in the U.S., and these will not be discussed.

Prostaglandin $F_{2\alpha}$. Prostaglandin $F_{2\alpha}$ is a fatty acid-derived hormone produced in many body cell types, but especially in the endometrium (lining of the uterus). One of its major reproductive functions is to lyse (destroy) the corpus luteum during the latter part of the estrous cycle if a female is not carrying a viable embryo (i.e., is not pregnant). The corpus luteum (CL) is the structure that forms on the ovary at the site from which the egg was released at the time of ovulation. The CL produces progesterone, and progesterone

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binds to receptors in the hypothalamus, anterior pituitary, and uterus to help regulate estrous cycles. It is also the hormone produced in very high quantities during pregnancy.

Injections of prostaglandin $F_{2\alpha}$ (PGF) may be given to females to control the timing of estrus. A number of reproductive factors will influence the efficacy of PGF. Firstly, females receiving exogenous PGF must have already reached puberty in order for PGF to exert its intended impact on reproductive cyclicity. The reason for this is because the manner in which PGF acts is to lyse the CL, allowing for a decline in progesterone levels in the females' bloodstream and subsequent increase in other reproductive hormones that lead to estrus. If a female has not reached puberty, she has not yet ovulated and she will not possess a CL. Thus, do not use PGF in heifers unless puberty has been reached and cyclicity has been documented.

Cows in the early post-partum period behave similarly to pre-pubertal heifers. Early post-partum cows do not exhibit estrous cycles and usually do not ovulate or possess a functional CL on their ovaries. Thus, response to exogenous PGF will be minimal to non-existent. Producers are advised not to use PGF in post-partum cows unless cyclicity has been documented (by palpation, ultrasound, or visual observation of estrus).

Females must be at the appropriate stage of the estrous cycle (diestrus) in order for PGF to synchronize estrus. If females are early in the estrous cycle (estrus or metestrus) or late in the estrous cycle (proestrus), exogenous PGF will have no effect on reproductive cyclicity.

Females receiving exogenous PGF should not be pregnant. Although producers will not knowingly select pregnant females as either donors or recipients, animals assumed to be non-pregnant occasionally receive exogenous PGF and termination of the pregnancy occurs. Any potential donor or recipient female artificially inseminated or exposed to a bull for natural service should be examined for pregnancy status before receiving PGF.

If giving PGF to a group of randomly cycling females, it is expected approximately 65% of them will exhibit estrus within 2 to 5 days after receiving PGF. Unless a producer has an extremely large pool of recipient females, this response would not be adequate for use in embryo transfer. However, by administering two injections of PGF at the label-approved interval of 9 to 12 days between injections, it is possible to elevate the response to exogenous PGF to 80% or more. Thus, this double-injection system for synchronization of estrus is the one most commonly used for PGF-based synchronization.

There are five commercially available PGF products. Three of them are a "natural" PGF product (known as dinoprost): Lutalyse®, ProstaMate™, and In-Synch™. The typical cattle dose for dinoprost is 5 cc. Two of the PGF products are PGF analogues, which are compounds similar in chemical structure and function to the "natural" PGF. The PGF analogue used in the U.S. is cloprostenol, and the available products are Estrumate® and estroPLAN™. The typical cattle dose for cloprostenol is 2 cc.

Progesterone. The second fundamental approach used to synchronize estrus in donor and recipient females involves the administration of exogenous progesterone. Progesterone is a steroid hormone that has a major impact on the regulation of estrous cycles. When concentrations of progesterone in the blood are high, females will not exhibit estrus and will not exhibit estrous cycles. Progesterone may not be administered orally and retain its biological activity because it is broken down as it passes through the digestive system. Thus, if producers desire to administer progesterone to synchronize estrus, it must be given via daily injections (not practical) or via an intravaginal device

such as a CIDR® (controlled internal drug releasing device). Fortunately, progesterone analogues are available that may be given orally and still retain biological activity. One such analogue is melengestrol acetate (MGA) fed at a rate of 0.5 mg MGA® per head per day for 14 days.

Producers can administer progesterone (or progesterone analogues) to a group of randomly cycling females, and it will block estrus in any female that does not possess a viable CL. Once the exogenous source of progesterone is removed, any female without a viable CL will exhibit estrus in 2 to 7 days (depending on whether progesterone or a progesterone analogue is used). Typically, 80% or more of treated females will exhibit estrus in response to treatment with progesterone.

Progesterone-Prostaglandin $F_{2\alpha}$ combination. The third fundamental approach used to synchronize estrus in donor and recipient females involves administration of a combination of exogenous progesterone and PGF. The primary advantages of this combination treatment are the avoidance of reduced fertility often noted in females treated for 14 days with progesterone/ progesterone analogues and a reduction in the total length of time needed to synchronize estrus. Progesterone prevents any female without a viable CL from exhibiting estrus until the progesterone is removed, and PGF lyses the CL of any female in diestrus.

The most widely utilized progesterone-PGF combination treatment used to synchronize estrus is the CIDR®-PGF combination. The label-approved protocol involves insertion of a CIDR® on any random calendar day (Day 0), administration of PGF 6 days later (Day 6), and removal of the CIDR® one day after PGF (Day 7). The proportion of females exhibiting estrus response rates ≥80% are typical.

Superovulation of Donors

Superovulation, as the name implies, is a process designed to elicit release of a greater than normal number of eggs at the time of ovulation. The efficiency of the overall embryo transfer process can be greatly increased by administering gonadotropic hormones (hormones that act on the ovary) to donor females in order to induce superovulation (the prefix "super-" means more than usual).

There are two glycoprotein hormones that may be administered to induce superovulation. The hormone FSH (follicle stimulating hormone) is the hormone "naturally" responsible for recruitment and growth of ovarian follicles during the estrous cycle. The other hormone is PMSG (pregnant mare serum gonadotropin; more recently termed equine chorionic gonadotropin [eCG]). However, because eCG is not approved for use in cattle in the U.S., superovulatory protocols using eCG will not be described.

Superovulatory treatments of donor females with FSH are typically initiated on Days 8-10 of the estrous cycle in order to stimulate follicles at the start of the second follicular wave when a dominant follicle is not present. Research has clearly documented fewer transferable quality embryos will be obtained when superovulation is initiated in the presence of a dominant follicle (Guilbault et al., 1991; Bungartz and Niemann, 1994). Because the half-life of FSH is short (~ 4 to 6 hours) in cattle, FSH must be given as a series of multiple injections administered over a period of several days. A typical superovulation regimen for beef cattle involves a series of eight injections of FSH administered at 12-hour intervals over a 4-day period. The FSH may be given as a constant dose (all eight injections are the same amount of FSH) or as a descending dose

(FSH doses get smaller over time). An injection of PGF should be given in conjunction with the 6th (or 7th or 8th) dose of FSH in order to induce luteolysis and enable the donor to exhibit estrus.

There are four superovulatory products commercially available in the U.S. Two of them are derived from the anterior pituitary glands of pigs (Folltropin®, Pluset®), and two of them are derived from the anterior pituitary glands of sheep (Ovagen™, Embryo S®). There are two consequences of using porcine and ovine pituitary glands as the raw material for FSH isolation. The first consequence is the pituitary glands also contain the hormone LH (luteinizing hormone), and studies have shown excessive LH can lead to poor superovulatory responses and/or poor fertilization rates (Donaldson and Ward, 1986; Donaldson et al., 1986). The second consequence is use of FSH from one species (pigs or sheep) in another species (cattle) may lead to an immune system reaction (i.e., formation of antibodies against FSH). When this happens, superovulatory responses are poor. Interestingly, clinical observations suggest switching from one species' FSH to another can sometimes improve superovulatory response.

As a general guideline, one vial of FSH is the dose to be used for one donor cow. However, many embryo transfer technicians will vary from this guideline based on the age, body weight, breed, and stage of production of the donor female. For example, heifers generally receive a lower total dose of FSH for superovulation than do mature cows. Animals of *Bos indicus* breeding typically receive a lower total dose of FSH for superovulation than their *Bos taurus* counterparts.

Results from superovulation are highly variable (Kafi and McGowan, 1997), and the reasons for variation in response are not well understood. Recently, it was reported superovulated beef cattle in the U.S. produced an average of 6.76 transferable quality embryos in calendar year 2005 (Stroud and Reyher, 2006).

One common mistake made during superovulation is the use of too much exogenous FSH. Many inexperienced embryo transfer technicians are under the mistaken impression if use of 100% of the label dose results in a reasonable superovulatory response, use of 150% or 200% of the label dose should result in an extremely good superovulatory response. This is not true for a variety of reasons. Firstly, if too much FSH is given for superovulation, it can cause the donor cow to produce the ovarian hormone inhibin which inhibits the release of FSH from the anterior pituitary. This reduces the amount of endogenous FSH secreted by the anterior pituitary, leading to a lower basal level of FSH and a poorer superovulatory response. Secondly, administration of increased levels of the superovulatory compound means an increased administration of LH. Although the FSH:LH ratio of the product remains the same, the total amount of LH given to the donor increases, possibly to a level that reduces superovulatory response due to luteinization of follicles and/or premature ovulation of the dominant follicle (Callesen et al., 1987)

Detection of Estrus in Donors and Recipients

As stated previously, one of the most important factors influencing the overall success of the embryo transfer process is the degree of synchrony of estrus between donor and recipient females. Hence, it is vital to the success of any embryo transfer program to give great attention to detection of estrus. The time of onset of estrus impacts the schedule for insemination of donors and dictates the proper time for transfer of embryos into recipients.

Many persons engaged in the practice of embryo transfer believe visual observation of standing heat is the best method for detection of estrus. Clinical observations strongly suggest donors will have an excellent superovulatory response if they exhibit a very intense estrus soon after receiving the luteolytic dose of PGF given as part of the superovulatory process. In contrast, donors that exhibit a very weak estrus, a delayed estrus, or no estrus probably will not produce many transferable quality embryos.

Visual observation of estrus should be performed a minimum of twice per day for a minimum of 30 minutes per observation period, preferably near dawn and dusk. However, many persons would argue this guideline is far too conservative. If infrequent observation of animals leads to failure to detect estrus in superovulated donors, money will be wasted. Similarly, failure to detect estrus in recipients could lead to unexpected expense for embryo cryopreservation. This author recommends detection of estrus 3 to 4 times per day to minimize the chance for missed heats and to provide more precise information regarding the timing of onset of estrus.

Factors such as ambient temperature, type of housing, and number of pen mates influence estrous behavior of cattle and the ability of producers to detect estrus. Furthermore, most producers are unable to continuously monitor their females for signs of estrus. Thus, many producers choose to use one or more aids for detection of estrus.

Aids for detection of estrus may be categorized in several ways. Pressure sensitive mounting devices are those which are placed on the tailhead of females and which undergo a physical change or record data in response to pressure (i.e., the weight of one female mounting another). Pressure sensitive mounting devices include Kamar®, Bovine Beacon™, HeatWatch®, HeatWatch® Xpress™, MountCount™, Show Heat™, TattleTale™, and Estrotect™. Potential drawbacks of these devices include loss from tailhead, false negatives (due to poor placement on the female), and false positives (due to rubbing on trees, oiler chains, etc.). A second category is physical marking devices. Physical marking devices include tail chalk/paint, chin ball markers, and painting of the brisket of a teaser male. A third category is activity monitors (such as pedometers), and a fourth category is physiological indicators such as a vaginal mucus electrical resistance probe (e.g., Ovatec®). Most beef producers use only pressure sensitive marking devices because aids in the latter categories are often impractical, costly, and/or labor intensive to use.

Insemination of Superovulated Donors

Once estrus has been detected, it is important to artificially inseminate the estrual donors with excellent quality semen from a genetically outstanding bull. However, it is equally important not to breed the recipient females because it is undesirable for a recipient to be carrying her own embryo at the time the donor embryo is transferred into her uterus (this creates a competitive environment for the transferred donor embryo).

It has been known for decades a suitable method for mating of estrual cows is the "a.m./p.m. breeding rule" (Trimberger, 1944). With this method, females observed in estrus in the morning are inseminated in the evening of that same day, whereas females observed in estrus in the evening are inseminated in the morning of the following day. The goal of this method is to have capacitated sperm in the oviduct at the time of ovulation. Given that ovulation occurs approximately 27 hours after the onset of estrus, sperm transport and capacitation requires approximately 6 hours, the life span of the

ovulated egg is approximately 8 hours, and the true onset of estrus is often not known with precision, the time of mating using the a.m./p.m. method works well.

It seems logical to use the a.m./p.m. breeding rule for insemination of superovulated donors, except superovulated donors typically ovulate multiple eggs over a span of more than 12 hours (Yadav et al., 1986). Because of the prolonged ovulatory process, the insemination schedule for superovulated donors should include at least one (if not two) additional breedings. Typical industry practice for insemination of superovulated donors is to use one unit of excellent quality semen at 12 hours after the onset of estrus, followed 12 hours later by a second insemination with one unit of semen (Donaldson, 1985; Schiewe et al., 1987). However, depending on the time of onset of estrus, some technicians choose to perform three inseminations -- one soon after estrus is detected and two others approximately 12 hours apart at times of the day convenient for the technician. There is no advantage to using more than one unit of semen per insemination. Semen is deposited in the uterine body as occurs with conventional artificial insemination (Hawk and Tanabe, 1986) because semen deposition in the uterine horns may lead to tissue trauma and/or increased microbial contamination. Be certain to use semen from the same bull at each insemination so parentage of the resultant embryos is not in question.

Recovery of Embryos from Donors

After donors have been artificially inseminated, fertilization will occur and preimplantation embryonic development will begin. The embryos reside in the oviduct for approximately four days before migrating to the tips of the uterine horns (Winters et al., 1953). Once embryos are found in the uterus, it is possible to recover them using non-surgical embryo recovery techniques. Typically, embryos are recovered non-surgically on Day 7 (Day 0=onset of estrus).

Non-surgical embryo recovery methods emerged in the mid 1970s (Drost et al., 1976; Elsden et al., 1976) and revolutionized the bovine embryo transfer industry. To perform this procedure a device known as a Foley catheter is passed through the vagina, through the cervix, into the uterine body, and up into one of the uterine horns of a donor that has received caudal epidural anesthesia (to deaden the nerves of the rectum and tail). There is a small balloon on the outside of the Foley catheter inflated to create a physical seal between the catheter and the uterus. A sterile flushing medium is introduced into the tip of the uterine horn, and the uterine horn is gently massaged to suspend the embryos into the flushing medium. The flushing medium then passes through the catheter, and medium containing the embryos is collected in an embryo filtration device. The uterine horn is usually flushed at least three times. After multiple flushing of one uterine horn, the catheter is removed and then reinserted into the opposite uterine horn for flushing. Harvested embryos are recovered from the filtration device and are examined using a microscope.

There are two different non-surgical embryo recovery methods. One method is the gravity flow method. With this method, the sterile flushing medium is suspended above the donor cow and the force of gravity introduces medium into the uterine horn. Advantages of this method are over-filling of the uterine horns seldom occurs and the system is "closed", minimizing likelihood of contamination of the flushing medium and embryos. The second method, known as the syringe method, utilizes 60 cc syringes to introduce and remove medium from the uterine horns. Advantages of this method include

knowing the exact volume of medium introduced and recovered from the uterus, as well as the ability to more forcefully extract flushing medium from the uterus. One of the disadvantages is it is an "open system" (the syringe is connected and disconnected from the catheter multiple times for each uterine horn), providing an opportunity for potential contamination of the embryos. This may be of particular concern for embryos destined for export.

Transfer of Embryos into Recipients

Prior to transfer, harvested embryos must be evaluated using a microscope in order to assess their developmental stage and quality. The International Embryo Transfer Society has adopted a uniform standard for evaluating and describing cattle embryos (Robertson and Nelson, 1998). Each embryo is assigned a two-digit code. The first digit is a code to describe the embryonic stage of development (e.g., 4= compact morula, 5=early blastocyst, 6=blastocyst, 7=expanded blastocyst), and the second digit is a code to describe the quality of the embryo (e.g., 1= excellent/good, 2=fair, 3=poor). Typically, only embryos of quality grades 1 and 2 are considered to be of transferable quality. Similarly, embryos typically are not transferred if they are less developed than a compact morula or more developed than an expanded blastocyst.

Embryonic stage of development has little influence on pregnancy rates following transfer to synchronous recipients if embryos are IETS stage codes 4 to 7 (Spell et al., 2001). Contrarily, there is a strong correlation between IETS embryo quality grade and pregnancy rates following embryo transfer (Lindner and Wright, 1983). Quality grade 1 embryos typically yield higher pregnancy rates than quality grade 2 embryos which usually produce higher pregnancy rates than quality grade 3 embryos.

As stated previously, one of the most important factors influencing pregnancy rates after embryo transfer is the degree of synchrony of estrus between donors and recipients. As a general guideline, the recipient females must be in estrus within 24 hours (either before or after) of the donor exhibiting estrus. Some technicians, however, prefer a tighter degree of synchrony. Spell et al. (2001) showed pregnancy rate was not affected by the degree of synchrony of donors and recipients if falling within the 48-hour time span described earlier.

Skill of the embryo technician obviously plays an important role in post-transfer pregnancy rate. Embryos typically are loaded into ¼-cc straws and placed into an embryo transfer gun (usually 3" longer than an artificial insemination gun). A sterile specially designed embryo transfer sheath is placed over the gun, and a protective chemise is placed over the sheath. Once the technician reaches the cervix, the chemise is pulled back over the embryo transfer gun, exposing the sheath. The gun is carefully guided through the cervix and into the uterine horn adjacent (ipsilateral) to the ovary that possesses a CL. The gun is guided as far up the horn as is reasonably possible without excessive manipulation, and the embryo is deposited.

Pregnancy rates obtained with fresh embryos should be ≥ 60% under good management conditions. If the transfer was difficult or required extensive manipulation of the uterus, some technicians will administer a PGF synthesis inhibitor such as aspirin (Pugh et al., 2004).

Embryo Cryopreservation

Embryo cryopreservation is the preservation of embryos by freezing (the prefix "cryo" refers to freezing). The first successful cryopreservation of mammalian embryos was achieved in 1971 by researchers working with mouse embryos (Whittingham et al., 1972; Wilmut, 1972), and that breakthrough was soon followed by successful cryopreservation of bovine embryos (Wilmut and Rowson, 1973). Much of the initial impetus for research on cryopreservation of embryos stemmed from the desire to move "exotic" breeds of cattle from one part of the world to another. Transporting germplasm resources as embryos rather than as live animals is cheaper, poses less risk for disease transmission, and enables embryo transfer calves to develop in a recipient female whose colostrum provides maternal antibody protection against diseases endemic in that area.

There are a number of other reasons why embryo cryopreservation may be employed. It prevents wasting of "surplus" embryos when donors produce a greater than expected number of embryos or when recipients do not exhibit estrus synchronously with the donors. Cryopreservation may be utilized when recipient females are unavailable, inadequate, or in instances when it may simply be more convenient to perform embryo transfer at a later time (e.g., when embryos from a particular donor are being stockpiled). Embryo cryopreservation is used by many purebred breeders as a secondary avenue for marketing of their breeding stock, as embryos from a genetically valuable donor can be harvested and sold without having any change in ownership of the donor. Recently, embryo cryopreservation also has become an integral part of germplasm preservation efforts.

The primary obstacle to overcome during embryo cryopreservation is the high water content within the embryo. When water freezes, ice crystals form. These ice crystals have sharp, jagged edges capable of cutting cell membranes and/or cell organelles. If the membranes or organelles of a substantial number of cells in an embryo are damaged, death of the embryo will occur. Hence, the primary challenge in embryo cryopreservation is the "dehydration" of the embryo prior to freezing. This is accomplished by placing embryos into a hypertonic solution containing a cryoprotective agent, which forces water out of the cells of the embryo (Leibo, 1992). Historically, glycerol was the predominant cryoprotective agent used for embryo cryopreservation. However, glycerol is now being rapidly replaced by ethylene glycol.

Conventional embryo cryopreservation

The first step in the conventional embryo cryopreservation process is to place embryos into a cryoprotectant solution for equilibration. The equilibration period allows some of the permeating cryoprotectant to enter the cells of the embryo while at the same time allowing water inside the cells of the embryo to exit in an attempt to obtain an equal concentration of cryoprotectant both inside and outside the cell (i.e., to attain an osmotic equilibrium). After equilibration (10 minutes for glycerol, 5 minutes for ethylene glycol), embryos are loaded individually into ¼-cc straws and are subsequently sealed in the straws.

The second step is to place the embryos directly into a controlled rate biological freezer at a reduced temperature (either 0°C or ~ -6.5°C). If embryos are placed into the freezer at 0°C, they must be cooled slowly (at a rate of ≥ 1°C/min) to -6.5°C. Once the

embryos reach -6.5°C, the technician purposefully induces ice crystal formation in the straw containing the embryo using a process called "seeding". When ice crystals form in the medium surrounding the embryo, it increases the concentration of the cryoprotective agent surrounding the embryo, resulting in further dehydration of the embryo.

Embryos are held at seeding temperature for approximately 10 minutes, and then slow cooling resumes at a rate of 0.5°C/min down to a temperature of approximately -35°C. Embryos are held for approximately 10 minutes before direct plunging into liquid nitrogen at -196°C. Excellent quality embryos cryopreserved and stored in an appropriate manner should maintain their post-thaw viability indefinitely.

Cryopreserved embryos must be thawed in an appropriate manner to be viable post-thaw. Typically, straws are removed from the liquid nitrogen tank, held in room temperature air for 3 to 5 seconds (to reduce incidence of cracked zona pellucidae and exploding straws), and placed into a 35 to 38°C water bath for 25 to 30 seconds. Embryos are removed from the straws for placement into a 1.0 Molar (34.2% w/v) solution of sucrose, a non-permeating compound that will cause the cryoprotectant to exit the embryo. After 10 minutes in sucrose, the embryo is transferred into holding or transfer medium for a few minutes prior to being loaded into another straw for transfer into a recipient. Alternatively, some people use a step-wise dilution in decreasing concentrations of cryoprotectant (1.5, 1.0, 0.5 Molar). As a general rule, only quality grade 1 and 2 embryos should be cryopreserved with this method.

Direct transfer. One modification to the standard embryo thawing method is called direct transfer (Voelkel and Hu, 1992). As the name implies, embryos are thawed and then directly transferred into the recipient female. Direct transfer (DT) embryos are cryopreserved in the same manner as described above except ethylene glycol <u>must</u> be used as the cryoprotective agent. Similarly, DT embryos are thawed in the same manner as described above (3 to 5 seconds in air; 25 to 30 seconds in a 39°C water bath), but they are <u>not</u> placed into sucrose solution. Instead, they are transferred as quickly as possible after thawing into the uterus of a synchronous recipient female. Because ethylene glycol is a small molecule that rapidly crosses cell membranes, it is assumed ethylene glycol will quickly exit the embryo after placement into the uterus of the recipient female (where no ethylene glycol exists) in an attempt to reach osmotic equilibrium.

There is a voluntary industry standard any embryo cryopreserved for direct transfer should be frozen in an amber-colored (yellow) straw. Many straw manufacturers imprint the initials "DT" on the straw as an extra indication of the type of embryo contained in the straw. Care should be exercised, however, when purchasing DT embryos because unscrupulous vendors of embryos could, in theory, market unfertilized eggs or degenerate embryos (they assume no one will thaw the embryos, place them in sucrose, and then look at the thawed embryos under a microscope).

A second very important consideration when using DT embryos is the time interval from embryo thawing to embryo transfer. Although controlled scientific studies have not been conducted to test this point, field observation suggests an inverse relationship between pregnancy rate and interval from thawing to transfer (i.e., pregnancy rates will decrease as the length of time from embryo thawing to embryo transfer increases). This seems to be particularly true during the summer months in the southern United States.

Vitrification. One method of embryo freezing seeing limited use in the bovine embryo transfer industry is a non-equilibrium method called vitrification. Although research on

vitrification has been conducted for more than 50 years, the initial success with vitrification of mammalian embryos was not reported until 1985 (Rall and Fahy, 1985). Vitrification is a fundamentally different method for cryopreservation. In contrast to the conventional embryo cryopreservation described earlier, vitrification is a procedure that does not rely entirely on equilibration. The overall goal of vitrification is to use an ultra-rapid freezing rate so water inside the cells of the embryo goes from liquid to solid without having a chance to form ice crystals. Instead, the cryoprotectant solution forms a glass-like solid. Vitrification technology has recently been reviewed (Vajta and Nagy, 2006).

The general approach to vitrification involves placement of embryos into one or more permeating cryoprotective agents at a slightly higher than normal concentration (e.g., 2.0 Molar). Embryos are placed into this "equilibration" mixture for the initial (and major) dehydration of the embryos. Immediately thereafter, embryos are placed into a much higher concentration (e.g., 7.0 Molar) cryoprotectant solution for no more than 30 to 45 seconds, after which time they are promptly plunged into liquid nitrogen.

Several research groups have investigated cryopreservation of bovine embryos via vitrification. Studies have been performed with *in vivo* derived as well as *in vitro* produced embryos. However, the majority of investigations have been conducted using *in vitro* produced embryos because vitrification has been touted as a potentially better alternative to conventional embryo cryopreservation for *in vitro* produced embryos (which are more sensitive).

Much of the research has been conducted using the open pulled straw (OPS) method (Vajta et al, 1997); however, a growing number of studies has been published where ordinary ¼-cc straws have been used (for review, see Seidel and Walker, 2006). One of the most encouraging studies published to date reported a 43% pregnancy rate when vitrification was performed using a high concentration (46% or ~ 6.2 Molar) of glycerol (van Wagtendonk-De Leeuw et al., 1995).

Although results obtained with this experimental cryopreservation technique at present are less than those obtained following conventional cryopreservation, vitrification offers several distinct advantages: less time-consuming procedure, easier to perform, eliminates need for expensive embryo freezing machine (although a vitrification machine is now commercially available from at least two different companies), and practical for use on any farm. As research on this technique continues, success rates will undoubtedly improve.

In Vitro Embryo Production

There are two methods to produce embryos for transfer to recipient females. One method is the *in vivo* production method described earlier in this paper, and the other is a method for laboratory production of embryos using the so called "test-tube" procedure of *in vitro* fertilization. The world's first calf produced via *in vitro* methods was born in 1981 in Pennsylvania (Brackett et al., 1982), and development of commercially viable methods took an additional 8 to 10 years. However, *in vitro* production of bovine embryos is quite common today, as evidenced by the transfer of more than ¼ million *in vitro* produced embryos throughout the world in calendar year 2005 (Thibier, 2006).

In vitro embryo production is a multi-step process that involves harvesting an egg (oocyte or ovum) from a female, maturing it in the laboratory (*in vitro* maturation; IVM), co-incubating it with sperm (*in vitro* fertilization; IVF), and culturing the fertilized egg (*in vitro* culture; IVC) until it is developmentally advanced enough to transfer into a recipient female.

Identification of oocyte source

The initial step in *in vitro* embryo production is identification of an oocyte source. Although much of the research on *in vitro* production of bovine embryos was conducted with oocytes obtained from the ovaries of females harvested at an abattoir, one must question the genetic potential of any offspring produced from oocytes obtained from abattoir females. Most cull females presumably are of poor genetic quality or have some other problem such as disease or structural deformity which prevents farmers from keeping them in their herds. Females to be used as oocyte donors for *in vitro* embryo production should be of outstanding genetic merit. If they are not, great time and expense will be spent producing offspring that do not increase the average genetic merit of animals in the herd. Oocyte donor females also should be free of infectious diseases and genetic abnormalities.

Harvest of oocytes

The second step in *in vitro* embryo production is the harvest of oocytes. Oocytes are typically collected directly from the ovaries of living animals by either laparoscopic (Lambert et al., 1983) or ultrasonographic procedures (Pieterse et al., 1988). With the laparoscopic procedure, a fiber optics instrument (laparoscope) is inserted directly into the abdominal cavity to allow visualization of the ovaries. An assistant positions the ovaries (via the rectum) while the operator guides an aspiration needle directly into the ovarian follicles. Gentle suction removes the follicular fluid and oocyte from each follicle.

In the ultrasonographic procedure, an ultrasound machine is used to visualize the ovaries. The transducer is placed into the vagina of the female, and the aspiration needle is placed through the channel in the ultrasound probe, through the vaginal wall, and into the ovarian follicle. Oocytes may be collected from either superovulated on non-superovulated females, and repeated oocyte collections can be made on each female. There is essentially no discomfort for the animal, and the incidence of post-operative adhesions is almost non-existent.

Perhaps the most interesting application of this ultrasound-guided oocyte retrieval (also called ovum pick-up or OPU) is the collection of oocytes from pregnant females (Meintjes et al., 1995), enabling them to produce embryos during a stage of production where they ordinarily do not make any reproductive contribution. Pregnant females may serve as a source of high genetic quality oocytes during early to mid-pregnancy, but care must be taken not to harm the corpus luteum during oocyte harvest. A second novel application of OPU-IVF technology is with early postpartum females (Perez et al., 2000). It is possible to obtain oocytes from these females prior to the time they could produce and sustain their own pregnancy.

If oocyte donor animals are dead or are euthanized, special care must be given to maintain the ovaries (and hence oocytes) at a stable temperature (30 to 35°C). Good

sanitation practices should be observed when handling the ovaries, and follicles may be aspirated using a sterile needle and syringe or by slicing open with a small razor blade. Alternatively, the entire ovary may be minced with a home-made slice-and-dice apparatus (razor blades slid onto nails with thin washers in between the razor blades). It is important to harvest the oocytes within 6 hours of death, but harvesting the eggs as soon as possible after death/euthanasia will lead to better results.

Maturation of oocytes

Although many early studies on *in vitro* embryo production utilized *in vivo* matured oocytes obtained from the oviducts of donor females (i.e., ovulated oocytes), this practice is seldom used today because it is time consuming and requires surgery. Instead, oocytes are matured *in vitro*. Harvested oocytes are evaluated morphologically, and cumulus-oocyte complexes (COC) exhibiting a compact cumulus mass and even cytoplasmic pigmentation are placed into a tissue culture medium such as Tissue Culture Medium 199 (TCM 199), modified by the addition of fetal bovine serum and exogenous hormones (LH and FSH). The bovine oocyte IVM process typically takes approximately 22-24 hours. Upon completion of IVM, cumulus cells should be greatly expanded.

Sperm capacitation

Ejaculated sperm can not readily fertilize eggs without first undergoing some morphological and biochemical changes known as capacitation. Proteins and other compounds such as cholesterol must be removed from the sperm cell before it can undergo the acrosome reaction (release of the enzymes which assist the sperm in penetration of the egg). Sperm capacitation normally occurs inside the female reproductive tract, but capacitation must be induced *in vitro* during *in vitro* embryo production by placing sperm in a specially formulated medium containing compounds such as heparin, caffeine, or calcium ionophore.

In Vitro Fertilization

Oocytes are removed from the maturation medium and are placed into a fertilization medium along with the sperm. The sperm and oocytes are left together for 6 to 24 hours depending upon the specific protocol being followed. Sufficient time must be given for the sperm to penetrate (through the cumulus cells, zona pellucida, and vitelline membrane) the egg. As with oocyte maturation and sperm capacitation, a special medium is used for fertilization. The concentration of sperm cells is carefully controlled, as is the temperature and gaseous environment for incubation.

In Vitro Culture

Fertilized eggs are placed into a specialized embryo culture medium to facilitate development to the blastocyst stage of embryonic development (Godke et al., 2002). This process requires approximately one week. During this time, a number of fertilized eggs will fail to develop normally. The reason(s) for this have not been fully elucidated. Nonetheless, blastocyst production rates of 40-50% are now common.

Applications of in vitro embryo production technology

Production of live offspring from IVM/IVF/IVC procedures is now a fairly routine procedure. A number of private companies has been started based upon the IVM/IVF/IVC technology, and some embryo transfer firms have added this procedure to their list of commercially available services. The impetus for using these procedures in beef cattle include producing hundreds of offspring from a single genetically superior female, producing embryos from prepubertal or pregnant females, producing offspring from clinically infertile females, and ultimately obtaining more productive livestock at a lower cost.

Although the ovaries of most farm animals contain thousands of oocytes, only a small portion of those can be harvested for IVF. Of the follicles present on the ovary, not all will yield oocytes when aspirated. Of the harvested oocytes, not all will have a compact cumulus mass or even cytoplasmic pigmentation. Of the COC placed into maturation medium, not all will mature. Of the matured oocytes, not all will become fertilized and cleave. Of cleaved embryos, not all will develop to the blastocyst stage of development. Of transferred embryos, not all will result in live offspring. Even though there are multiple opportunities for things to go wrong, approximately 15 - 20% of the oocytes recovered will yield live calves on the ground.

Pregnancy rates with quality grade 1 bovine *in vitro* produced embryos usually run 5 to 10% below the pregnancy rates achieved following the transfer of *in vivo* produced embryos. In addition, the ability to routinely cryopreserve *in vitro* produced embryos lags behind that for *in vivo* derived embryos. However, with further research the gap in success rates will undoubtedly narrow.

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