Embryo Transfer 101 with a Technical Slant

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Introduction

The commercial embryo transfer industry in North America developed in the early 1970's with the introduction of continental breeds of cattle. The use of embryo transfer technology in cattle breeding has continued to increase over the past 30 years with the movement toward genetic improvement as opposed to the production of desirable phenotypes. Throughout the world over the past year, more than 100,000 donor cows were superstimulated and more than 500,000 in vivo-derived bovine embryos were transferred [68]. More than 50% of the world's bovine embryo transfers are being done in Canada and the USA. In Canada, approximately 80% of the embryo transfer work is now being done on dairy cattle, whereas only about 35 % of the donors in the USA represent dairy breeds. Approximately 15,000 embryos are being frozen annually for export (Canadian Embryo Transfer Association Statistics, www.ceta.ca). This technology is influencing the direction of cattle breeding industries; the numbers are small but the impact is high.

General Procedural Steps

Donors are typically subjected to a superovulation protocol. Collection of single embryos from donors following natural estrus, spontaneous ovulation and mating or AI is also possible but is not frequently utilized. The donor may be inseminated naturally or artificially and embryos are normally collected non-surgically 6 to 8 days after breeding. Following collection, embryos must be identified, evaluated and maintained in a suitable medium prior to transfer. At this point, they may be subjected to manipulations, such as splitting and sexing, and they also may be cooled or frozen for longer periods of storage. Successful embryo transfer also requires transfer to recipient(s) with cycles that are synchronous to that of the donor, either during natural cycles or following a cycle-synchronization protocol.

Superovulation

The objective of superstimulation treatments in the cow is to obtain the maximum number of fertilized and transferable embryos with a high probability of producing pregnancies. Wide ranges in superovulatory response and embryo yield have been detailed in several reviews of commercial embryo transfer records [22,37]. These reports demonstrate the high degree of unpredictability in superovulatory response that creates problems affecting both the efficiency and profitability of embryo transfer programs.

Variability in ovarian response has been related to differences in superstimulatory treatments such as gonadotrophin preparation, batch of gonadotrophin, duration of treatment, timing of treatment with respect to the estrous cycle, total dose of gonadotrophin and the use of additional hormones in the superstimulation protocol [3,8,18,35,42,61]. Additional, and perhaps more important sources of variability are factors inherent to the animal and its environment. These factors may include nutritional status, reproductive history, age, season, breed, ovarian status at the time of treatment and the effects of repeated superstimulation. While considerable recent progress has been made in the field of bovine reproductive physiology, factors inherent to the donor animal that affect superovulatory response are only partially understood [10,42].

Superovulation-inducing treatments are usually initiated between Days 8 and 12 of the estrous cycle (estrus = Day 0) [40,41,42]. It was clearly demonstrated that the superovulatory response was greater when gonadotrophin treatments were initiated on Day 9 of the estrous cycle (Day 8 post-ovulation) as compared to Days 3, 6 or 12 [36]. This observation has been supported by more recent ultrasonographic evidence showing the second follicle wave beginning 8.5 days post-ovulation (Day 9.5 of the cycle) in 3-wave cows and 9.5 days post-ovulation (Day 10.5 of the cycle) in 2-wave cows [1,20,53]. In a practical sense, it is noteworthy that two-wave cows tended to have shorter cycles (18 - 20 days) than three-wave

cows (21 - 23 days). Therefore, the length of the previous estrous cycle can provide a clue as to the best time to initiate superstimulatory treatments.

Moor et al. [46] suggested that both ovulation rate and number of viable embryos produced are relatively consistent within individual cows; animals that responded poorly in one trial did so in subsequent trials, and animals that responded well initially continued to do so. Although there was considerable variability between cows, the number of follicles was shown to be similar between ovaries in the same cow. Further, the number of follicles > 1.7 mm in diameter in an ovary was positively correlated with ovulatory response to gonadotrophin treatments. More recently, Singh et al. [64] showed that the numbers of follicles present at the time of follicular wave emergence was predictive of superstimulatory response. Collectively, these data suggest that some of the variability resides in genetic or physiologic makeup of the animal rather than in exogenous factors. Indeed, cows and heifers selected for a high incidence of twinning had higher superovulatory responses than unselected controls (reviewed in [42].

Hormone Profiles in Superovulated Cows

Low progesterone concentrations at the time of initiation of gonadotrophin treatment have been shown to be related to a reduced superovulatory response, indicating the importance of a functional CL [42]. Normally, the decline in progesterone is rapid after treatment with PGF [5,26,60] and levels of progesterone dropped to less than 1 ng/ml of serum within 10-32 h. Ovulation was shown to occur approximately 72 h after PGF treatment in superstimulated cows [11]. At the onset of estrus, progesterone concentrations were lower in superstimulated cows that yielded good quality embryos than in cows that yielded unfertilized ova [3]. A high level of progesterone at the time of estrus may affect luteinizing hormone (LH) release and sperm transport and capacitation. After ovulation, the increase in serum progesterone concentration occurred earlier and increased more rapidly as the number of CL increased in superovulated cattle.

Gonadotrophins and Superovulation

Three different types of gonadotrophin preparations have been used to induce superovulation in the cow: semi-purified or purified FSH gonadotrophin fractions from extracts of domestic animal pituitaries (FSH), equine chorionic gonadotrophin (eCG) purified from plasma of pregnant mares, and human menopausal gonadotrophin (hMG) [3,42]. PGF has been used for the induction of luteolysis in ovarian superstimulation regimens to allow for precise timing of onset of estrus and of ovulation. As the biological half-life of pituitary FSH in the cow has been estimated to be 5 h or less, it must be injected intramuscularly twice daily to induce superovulation. The usual regimen is 3 or 4 days of twice daily treatments of FSH in decreasing doses. Forty-eight or 72 h after initiation of treatment, PGF is injected to induce luteolysis. Estrus and preovulatory LH release occurs between 36 and 48 h later, with subsequent ovulation in 24 - 36 h. Purified domestic-animal pituitary extracts (with most of the LH removed) are available in most countries today.

Manipulation of the follicular wave for superstimulation

The conventional protocol of initiating ovarian superstimulation during mid-cycle was originally based on anecdotal and experimental information in which a greater superovulatory response was reported when superstimulatory treatments were initiated 8 to 12 days after estrus [reviewed in 42]. However, none of these early studies evaluated follicular status at the time that gonadotrophin treatments were initiated.

Through information generated by ultrasonography, it is now known that 8 to 12 days after estrus (equivalent to Days 7 to 11 after ovulation) would be the approximate time of emergence of the second follicular wave in 2- or 3-wave cycles and a cohort of growing follicles would be present. However, the day of emergence of the second follicular wave differs among individuals and is 1 or 2 days later in two- versus three-wave cycles. In this regard, it has been clearly shown that superovulatory response was higher when superstimulatory treatments were initiated at the time of wave emergence; as little as 1 day asynchrony significantly reduced the superovulatory response compared to initiating treatments on the day of wave emergence [47].

Based on duration of the developmental phases of the dominant follicle in two- and three-wave interovulatory intervals, approximately 20% (4 or 5 days) of the estrous cycle is available for initiating treatment at the time of follicular wave emergence. Therefore, 80% of the estrous cycle is not conducive to an optimal superovulatory response [1]. The necessity of waiting until mid-cycle to initiate superstimulatory treatments implies monitoring estrus and an obligatory delay. To obviate these problems, an alternative approach is to initiate superstimulation treatments subsequent to the synchronization of follicular wave emergence. There are basically three methods of synchronizing follicle wave emergence for superstimulation.

Follicle Ablation

One approach to the synchronization of follicle wave emergence involves transvaginal ultrasound-guided follicle ablation of all follicles ≥5 mm, regardless of stage of the estrous cycle [6,19]. This removes the suppressive effects of follicle products (estradiol and inhibin) on FSH release; as a results FSH surges and a new follicular wave emerges 1 day later. Superstimulatory treatments are then administered, beginning 1 day after ablation, and PGF is administered 48 or 72 h later [7]. The timing of estrus was more synchronous when a progestin device was inserted for the period of superstimulation and 2 injections of PGF were administered on the day of progestin removal. Combined over 2 experiments, there was no difference in the superovulatory response between the ablated and non-ablated control groups. Transvaginal ultrasound-guided follicle ablation of all follicles [7] or just the dominant follicle [13,62] during mid-diestrus, followed in 2 days by superstimulation, also resulted in a higher superovulatory response than cows in which the dominant follicle was not ablated. Conversely, in a retrospective analysis of superovulatory responses of lactating dairy cows, follicle ablation resulted in a significantly higher number of ova/embryos, but a comparable number of transferable embryos compared to cows superstimulated 7 to 13 days after estrus [29]. In a more recent study, ablation of the 2 largest follicles at random stages of the cycle was as efficacious as ablating all follicles ≥5 mm in synchronizing follicular wave emergence for superstimulation [4]. However, it is always advised that a progestin device be used during superstimulation.

GnRH

Another method of synchronizing follicular wave emergence for superstimulation involves the use of GnRH or pLH to induce ovulation of a dominant follicle followed by emergence of a new follicle wave 2 days later [38,44,54,67]. However, the administration of GnRH or pLH does not always induce ovulation, and if ovulation does not occur, follicle wave emergence will not be synchronized [44]. Therefore, the reported asynchrony in follicular wave emergence (range, 3 days before treatment to 5 days after treatment) suggests that GnRH-based approaches may not be feasible for superstimulation [44]. In a study involving 3 different experiments [14], GnRH or pLH treatments consistently resulted in a lower number of embryos than when follicular wave emergence was synchronized with other methods. Therefore, we do not recommend using GnRH or pLH to synchronize follicular wave emergence prior to superstimulation.

Estradiol and Progesterone

Traditionally, estradiol has been administered near the beginning of progestin treatment to induce luteolysis and allow for shortened progestin treatment periods [8,48,71]. However, we have shown that the benefit of estradiol in shortened progestin treatment protocols may also be associated with the fact that it causes follicular regression [8,9]. The mechanism involves suppression of FSH and possibly LH. The initial suppression of FSH and LH results in regression of FSH- and LH-dependent follicles. Once follicle regression begins and the exogenously administered estradiol is metabolized, FSH surges and a new follicle wave emerges 1 day later. The use of a short acting estradiol-17β in progestin-implanted cows was followed by the emergence of a new wave, approximately 3 to 5 days later regardless of the stage of follicular growth at the time of treatment [8,9]. Estradiol-17β is normally injected with 50 to 100 mg of progesterone at the same time as placement of a progestin device [8,9,12,43]. The progesterone prevents an estrogen-induced preovulatory-like LH surge in those animals that do not have a functional CL, and appears to cause regression of LH-dependent follicles.

Many practitioners are now utilizing estradiol-17β and progesterone injections along with one of the many progestin-releasing devices that are now available to synchronize follicle wave emergence for superstimulation of donors [reviewed in 42]. On Day 0 of treatment (random stages of the estrous cycle; try to avoid the last 2 or 3 days of the cycle), donor cows receive a progestin device and an injection of 5 mg of estradiol-17β and 100 mg progesterone. On Day 4, FSH treatments are initiated. On Day 6 or 7 cows receive two injections of PGF 8 to 15 h apart and the progestin device is removed with the second PGF injection. Estrus is expected to occur approximately 36 to 48 hours after the first injection of PGF. Artificial insemination is normally done 12 and 24 hours after onset of estrus, or 60 and 72 hours after the first injection of PGF. In this way, the full extent of the estrous cycle is available for superstimulation and the need for detecting estrus or ovulation and waiting 8 to 12 days to initiate gonadotrophin treatments is eliminated. Data from experimental [9] and commercial [10] superovulation programs have shown that the superovulatory response of donors given and injection of estradiol-17β plus progesterone at unknown stages of the estrous cycle was comparable to that of donors superstimulated 8 to 12 days after observed estrus.

This is a fairly robust protocol in terms of steroid doses and duration of FSH administration. And modifications have been successful. For example, it is possible to use only 2.5 mg estradiol-17β and 50 mg progesterone with no apparent effect on results. Furthermore, FSH is often given for 3 days before PGF is administered and several practitioners remove the progestin device 24 h after PGF treatment to avoid early expression of estrus. In addition, FSH is often not administered on the last day of the protocol i.e., Day 7 above; we have shown that it is not necessary to administer FSH on the day after PGF is given [11,42].

Traditionally, donor cows were not subjected to embryo collection any more frequently than once every 2 months. However, the elective synchronization of follicular wave emergence has resulted in cows being superstimulated successfully every 25 days, without regard to expression of estrus [11]. Once multiple CL have been induced to regress by the administration of PGF, and the cow reovulates, normal follicular wave patterns are reestablished and the cow can be superstimulated again. The following protocol shows how this approach can be used to superstimulate cows every 30 days, without the need for estrus detection and without compromising results (Table 1).

Table 1. Protocol for follicle synchronization and superovulation that can be used to superstimulate cows every 30 days, without the need for estrus detection and without compromising results.

Day 0 - Insert CIDR and inject 5 mg estradiol-17β plus 100 mg progesterone
Day 4 - FSH b.i.d. by deep intramuscular injection
Day 5 - FSH b.i.d. by deep intramuscular injection
Day 6 - AM - FSH by deep intramuscular injection; PGF - PM – FSH by deep intramuscular injection; remove CIDR
Day 7 - FSH b.i.d. by deep intramuscular injection
Day 8 - PM - AI
Day 9 - AM - AI
Day 15 - Embryo collection, freezing and/or transfer; PGF
Day 30 - Insert CIDR and inject 5 mg estradiol-17β + 100 mg progesterone

Collectively, the above mentioned studies and their results demonstrate that exogenous control of follicle wave emergence offers the advantage of initiating superstimulatory treatments at a time that is optimal for follicle recruitment, regardless of the initial stage of the estrous cycle. The treatment is practical, easy to follow by farm personnel and, more importantly, it eliminates the need for detecting estrus or ovulation

and waiting 8 to 12 days to initiate gonadotrophin treatments. Synchronization of follicular wave emergence by follicle ablation or estradiol + progesterone treatments has resulted in comparable superovulatory responses. Furthermore, the "estradiol + progesterone injection plus progestin implant" approach to synchronizing cows for superstimulation makes it possible to superstimulate cows that are not cycling, have silent or unobserved estrus, or have abnormal ovarian function e.g., follicular cysts [reviewed in 42].

Practical Considerations

Donor cows should be a minimum of 50 days post-partum, cycling normally, and on an increasing plane of nutrition with no specific nutritional deficiencies. Some authors recommend trace mineral supplementation before superstimulation, and although there are apparently no supporting data, beyond clinical impressions, the use of chelated minerals is recommended to improve superovulatory response and embryo yield. There should be no history or physical evidence of infertility. It is noteworthy that cows (or daughters of cows) with a previous history of superovulatory success or of twinning are likely to be most responsive.

Superstimulatory treatments are normally initiated on Days 8, 9 or 10 of the estrous cycle. The length of a cow's cycle (interestrous interval) may provide a clue as to the most appropriate time to start superstimulatory treatments. A cow with a 21 - 23 day cycle should be started on Day 9, whereas, a cow with an 18 - 20 day cycle should be started on Day 10. If a cow is not cycling normally or the day of estrous cycle is unknown, the use of a progestin releasing device along with an injection of estradiol-17β or estradiol benzoate and progesterone can be used to synchronize follicular wave emergence. Superstimulatory treatments are usually initiated after 4 days and the progestin releasing device is removed 60 to 84 h later (12 to 24 h after PGF treatment). This is a reliable means by which superstimulatory treatments can be initiated at predetermined times without consideration of the stage of the cycle.

PGF may be administered on either the third or fourth day of FSH treatment; i.e., 48 or 72 h after initiating FSH treatment, and the dose of PGF is often repeated over two FSH administrations. Cows are normally in estrus 36 to 48 h after PGF and are inseminated with a single straw of high fertility semen at 12 and 24 h after the onset of estrus or at 60 and 72 h after the initial or single PGF administration. Cows of Bos indicus breeding may require a lower dose FSH, whereas older or lactating dairy cows may require a higher dose of FSH. If the superovulatory response is poor, it may be necessary to increase the dose of gonadotrophins in subsequent treatment protocols. If embryo quality is poor in the face of a high superovulatory response, it may be necessary to reduce the FSH dose or to initiate treatments earlier in the follicle wave. In either case, it may be advisable to use a purified preparation of pituitary extract.

Several gonadotrophin preparations are available for use. Within optimal dose ranges, they all work well. Evidence indicates that gonadotrophins should be administered by deep intramuscular injection [42]. Avoid fat deposits unless the purpose is to administer a single subcutaneous injection. The single subcutaneous injection of a pituitary extract may be advisable if stress of handling could be an impediment to successful superovulation. Regardless of whether a single subcutaneous injection or multiple intramuscular injections are administered, stress and overdosing must be avoided. If all else fails, one may consider single embryo collections at 10 or 21 day intervals. One high quality embryo is superior to any number of poor quality embryos.

Embryo Recovery

Non-surgical embryo recovery involves the passage of a cuffed catheter through the cervix and into the uterus on Days 6 to 8 after estrus [15,16,40,41,57]. The catheter may be placed either into a uterine horn or inserted just inside the cervix. Once the catheter is in place, the cuff is inflated with saline or flushing medium. When inserted into a uterine horn, care must be taken not to over-distend the cuff as the endometrium may split causing loss of collection medium and embryos. There are two basic types of catheters used for non-surgical embryo collection. Original reports on non-surgical collection involved the use of two and three-way Foley catheters. Although Foley catheters are inexpensive and readily available, the distance from the cuff to the catheter tip is short, sometimes interfering with drainage of the

collection fluid. In addition, their rubber or latex composition prevents them from being conveniently sterilized. Autoclavable silicone catheters, specifically designed for embryo recovery, are now widely available in sizes from 12 to 20-gauge and 5 or 30 ml cuffs. Some practitioners prefer using the two-way Rusch catheter, which has Luer-Lok fittings. These long catheters, 67 cm in length, are available in 14- or 18-gauge. The tip in front of the cuff measures 5.5 cm and has four holes. All recovery catheters are stiffened for passage through the cervix by a stainless steel stiletto.

There are basically two methods of embryo collection [41]: 1) the continuous or interrupted flow, closed-circuit system, and 2) the interrupted-syringe technique. However, any combination of these techniques is possible. It must be recognized that each system has advantages and disadvantages relative to the other. For closed-circuit collection, a bag or flask of collection medium is suspended above the donor female and gravity provides the pressure for the inflow of medium. A total of 1 to 2 l of medium is often used for this technique and the catheter may be positioned either in the uterine body or into each uterine horn separately. The tubing carrying the outflow of collection medium is usually connected directly to an embryo filter with pores that are approximately 50 to 70 μm in diameter [41].

A large 50 or 60 ml syringe provides inflow pressure for the interrupted-syringe technique. The syringe also provides negative pressure for recovering the medium. This medium is then either filtered or placed directly into a large Petri dish so that embryos can be located. Disposable equipment for all methods of embryo collection is commercially available.

Collection media containing no animal products and not requiring refrigeration is commercially available in 1 and 2 liter disposable IV bags. Some practitioners prefer to prepare Dulbecco's phosphate-buffered saline (PBS). This can be prepared and stored ready for use in 500 to 1000 ml containers. If syringes are used in the flushing procedure, it is recommended that those with rubber plungers be washed and heat sterilized before use. Holding media prepared in the lab are normally passed through a disposable 0.22 μm Millipore filter prior to use. Although commercially-prepared embryo collection and holding media are now available, they must be kept refrigerated if they contain animal products e.g., serum or BSA.

Within a reasonable range, temperature is not critical to bovine embryo survival. Holding embryos during the period from collection to transfer in temperatures ranging from cool (10° C) to room temperature has proven to be very satisfactory. However, holding embryos at extremely high temperatures above 38° C should be avoided. Embryo collection and transfer cannot be conducted under strictly sterile conditions, but every attempt should be made to be as clean as possible. Sterilization with chemicals often may be as likely to kill embryos as bacterial contaminants. Thorough washing of embryos with sterile medium has been shown to remove most infectious agents. As a routine, embryos should be passed through 10 washes of fresh medium prior to transfer or freezing.

Embryo Handling

Following collection and filtration of the collection medium, embryos are located under 6-10X magnification with a stereoscope. Although embryos are usually transferred to recipients as soon as possible after collection, it is possible to maintain embryos for 12 to 18 hours at room temperature in holding medium (23). It is also possible to cool bovine embryos in holding medium and to maintain them in the refrigerator for 2 to 3 days. For long term storage, embryos must be cryopreserved.

Embryos are normally held in a medium similar to that in which they were collected. Media must be buffered to maintain a pH of 7.2 to 7.6 and have an osmolarity around 275 mOs. Dulbecco's PBS or commercially-available media with Hepes buffer, BSA and antibiotics are normally used in the field. More complex media with a bicarbonate buffer generally yield superior results for long term culture of bovine embryos, but they must be maintained in an incubator with an elevated CO_2 atmosphere. As indicated earlier, embryo collection, holding and freezing media which are free of animal products have recently become available, avoiding the need for refrigerated storage and increasing biosecurity.

Biosecurity

To date, none of the infectious diseases studied in the bovine species have been transmitted by embryos when embryo handling procedures were conducted correctly. Several large studies have now shown that

zona intact, washed bovine embryos do not transmit infectious diseases [63,66,72]. Embryos must be examined at a magnification of at least 50X to ensure that the zona pellucida is intact and free of adherent material, and embryos are washed (and trypsin-treated) according to the recommendations of the IETS Manual [65]. In addition, every effort possible is made to ensure that collection and holding media are not contaminated by the environment. Recommendations to ensure that embryos are not contaminated by the environment follow.

Laboratory

In those embryo transfer practices with a laboratory, it should be separated from animal holding areas and embryo collection and transfer areas. Only personnel involved in embryo production/manipulation procedures should have access. Floors and sinks should be decontaminated routinely with disinfectant which has been shown to be non-toxic to embryos (e.g. Savlon) and immediate working areas, such as the bench-top and the laminar flow hood should be cleaned with 70% alcohol. Under field/farm conditions, efforts should be made to use clean (preferable sterile) paper sheet covers on the embryo handling surfaces. Eating, drinking or smoking should be prohibited in all embryo handling areas, and personal hygiene (use clean lab coats, shoes and clean hands, disinfected before embryo handling with alcohol) must be strictly in place.

Equipment

Only necessary equipment, microscopes, warming stage, incubator, refrigerator, biological freezer, scale, pH meter and osmometer should be in the laboratory. Special care should be taken to keep the exterior surfaces of all equipment clean; dust collectors e.g., cooling units and vents in refrigerator which should be cleaned weekly. Covers should protect other equipment such as microscopes and micromanipulator and the stages of microscopes should be cleaned with 70% alcohol with adequate time allowed for the alcohol to evaporate before use. Special attention should be given to the incubator. The elevated temperature and high humidity provides ideal conditions for bacteria to grow. Routine daily quality control and aseptic working habits will prevent contamination of the incubator.

It has been known for some time that some types of equipment may be toxic to various cell types, especially after certain methods of sterilization. Therefore, it has become widely accepted practice to test for toxicity prior to using materials that come in contact with gametes or embryos. The rubber plunger of certain types of plastic syringes has been found to be problematic for both semen and embryos and therefore, plastic on plastic syringes are commonly used to hold the handling medium for embryos.

Washing and sterilization of equipment

Most equipment is now disposable and can be purchased, pre-sterilized from a number of suppliers. All glassware and other reusable equipment used for embryo production/handling should be rinsed once in the distilled water and then soaked for 24 hours in a 1% solution of non-toxic detergent (e.g., Alconox; Alconox Inc. 9E 40th St, New York, N.Y 10016), rinsed 5 times in distilled water, dried and wrapped for sterilization. Sterilization is the process of destroying microorganisms (bacteria, fungi, and viruses) by heat or chemical reaction. There are many methods of sterilization such as gas, radiation, and chemicals, but saturated steam is the most effective and complete method. All non-disposable items should be sterilized with one of the following alternatives:

Dry heat: items should be held at 160^0C for 2 hours.

Steam sterilization: items must be held at 121^0C and 104 kilopascals pressure for 30 min.

Ethylene oxide vapor (gas sterilization): items should be wrapped in material that is readily permeable to ethylene oxide and then exposed to a minimum of 500 mg of ethylene oxide per 1000 cm^3 for 30 min. Sterilized equipment should be disassembled in order to allow better gas penetration. Adequate time must be allowed for aeration of sterilized equipment (up to 30 or 40 days) or residual gas will kill embryos. However, the amount of aeration required depends on varying circumstances. Various studies indicate that ethylene oxide sterilization has the potential of killing embryos. Under these experimental conditions, a period of 144 hours aeration was insufficient to render straws non-toxic to mouse embryos [21].

Membrane filtration: culture media should normally be filtered through a membrane with pores of 0.22 mM (Millipore filter) using positive pressure prior to use.

Serum substitutes and surface tension

The surface tension of culture media has been shown to be an important factor in the in vitro culture of mammalian embryos [52]. This has traditionally been met by the addition of serum or BSA. However, biological products are potentially infectious and they are variable in constituents and function. Although synthetic polymers can successfully replace surface-active properties, they will not replace the embryotrophic properties of serum or BSA in culture media. These must be substituted for by growth factors in chemically defined media.

The effectiveness of serum or BSA in embryo collection, short- or long-term culture and freeze/thaw media (very different biological and physicochemical processes) suggests that both serum and BSA possess common properties, other than nutritional components. At the same time, polyvinylpyrrolidone (PVP) [32,59], polyvinyl alcohol (PVA) [28], sodium hyaluronate (SH) [25,49] and ET surfactant (pluronic) [50] have been successfully used as a replacement for serum/BSA in collection, culture and freezing media for mammalian germ cells. Although some of these defined macromolecules may resemble albumin, they do not provide for the potential reactions that can occur in serum; they are physiologically inert (The Merck Index, 1989).

Embryo Evaluation

Bovine embryos should be evaluated in small Petri dishes at 50 to 100 X magnification. It is important to be able to recognize the various stages of development and to compare these with the developmental stage that the embryo should be, based on the days from estrus (Table 2). Often a decision as to whether an embryo is worthy of transfer will depend on the availability of recipients. Fair quality embryos should be transferred fresh, if recipients are available. The International Embryo Transfer Society (IETS) considers the export of poor and fair quality embryos to be improper [39].

Table 2. Stages of Embryo Development

Morula: A mass of at least 16 cells. Individual blastomeres are difficult to discern from one another. The cellular mass of the embryo occupies most of the perivitelline space. Day 6.
Compact Morula: Individual blastomeres have coalesced, forming a compact mass. The embryo mass occupies 60 to 70% of the perivitelline space. Day 7.
Early Blastocyst: An embryo that has formed a fluid-filled cavity or blastocoel and gives a general appearance of a signet ring. The embryo occupies 70 to 80% of the perivitelline space. Early in this stage of development, the embryo may appear of questionable quality. Day 7 to 7.5.
Blastocyst: Pronounced differentiation of the outer trophoblast layer and of the darker, more compact inner cell mass is evident. The blastocoel is highly prominent, with the embryo occupying most of the perivitelline space. Visual differentiation between the trophoblast and the inner cell mass is possible at this stage of development. Day 7.5 to 8.
Expanded Blastocyst: The overall diameter of the embryo dramatically increases, with a concurrent thinning of the zona pellucida to approximately one-third of its original thickness. Day 8 to 8.5.
Hatched Blastocyst: Embryos recovered at this developmental stage can be undergoing the process of hatching or may have completely shed the zona pellucida. Hatched blastocysts may be spherical with a well defined blastocoel or may be collapsed. Identification of embryos at this stage can be difficult unless it re-expands. Day 9.

Classification

Embryos are evaluated and classified (i.e., as degenerate, poor, fair or good and excellent) by morphological examination at 50 to 100 X magnification according to the Manual of the International Embryo Transfer Society [39]. The overall diameter of the bovine embryo is 150 to 190 um, including a zona pellucida thickness of 12 to 15 μm. The overall diameter of the embryo remains virtually unchanged from the one-cell stage until blastocyst stage. The best predictor of an embryo's viability is its stage of development relative to what it should be on a given day after ovulation. An ideal embryo is compact and spherical. The blastomeres should be of similar size with even density and texture. The cytoplasm should not be granular or vesiculated. The perivitelline space should be clear and contain no cellular debris. The zona pellucida should be uniform, neither cracked nor collapsed and should not contain debris on its surface. Embryos of good and excellent quality and at the developmental stages of late morula to blastocyst yield the highest pregnancy rates. It is advisable to match the stage of embryo development to the day of the cycle of the recipient as much as possible.

Quality Evaluation

Excellent: An ideal embryo, spherical, symmetrical and with cells of uniform size, color and texture.

Good: Small imperfections such as a few extruded blastomeres, irregular shape and a few vesicles.

Fair: Problems that are more definite are seen, including presence of extruded blastomeres, vesiculation, and a few degenerated cells.

Poor: Severe problems, numerous extruded blastomeres, degenerated cells, cells of varying sizes, large and numerous vesicles but an apparently viable embryo mass. These are generally not of transferable quality.

IETS Recommended Quality Codes [39]

The codes for embryo quality range from "1" to "4" as follows:

Code 1: Excellent or good. Symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, color and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor and at least 85% of the cellular material should be an intact, viable embryo mass. This judgment should be based on the percentage of embryo cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that might cause the embryo to adhere to a Petri dish or a straw.

Code 2: Fair. Moderate irregularities in overall shape of the embryo mass or size, color and density of individual cells. At least 50% of the cellular material should be an intact, viable embryo mass.

Code 3: Poor. Major irregularities in shape of the embryo mass or size, color and density of individual cells. At least 25% of the cellular material should be an intact, viable embryo mass.

Code 4: Dead or degenerating. Degenerated embryos, oocytes or 1-cell embryos; non-viable.

The Manual of the International Embryo Transfer Society [39] states *'It should be recognized that visual evaluation of embryos is a subjective evaluation of a biological system and is not an exact science. Furthermore, there are other factors such as environmental conditions, recipient quality and technician capability that play important roles in obtaining pregnancies from transferred embryos. It is also recognized that many different systems are used for "grading" embryos and that some are more sophisticated than are others. The criteria for assigning a "quality code" in the standardized forms were simplified to be "user friendly". Generally, unless otherwise specified, only Code 1 embryos should be utilized in international commerce".*

In the superovulated cow, there is likely to be a considerable range of embryo stages on any given day during development. On Day 7 after estrus, there may be morulae and blastocysts within the same flush. At the same time, there may be embryos of excellent quality and unfertilized and degenerate embryos. Generally, wide variations in embryo quality and stages of development are signals that normal-appearing

embryos may be stressed or compromised and that pregnancy rates may be disappointing. Embryos of excellent and good quality, at the developmental stages of compact morula to blastocyst yield the highest pregnancy rates, even after freezing. Fair and poor quality embryos yield poor pregnancy rates after freezing and should be transferred fresh. It is advisable to select the stage of the embryo for the synchrony of the recipient. It would also seem that fair and poor quality embryos are most likely to survive transfer if they are placed in the most synchronous recipients.

Estrus Synchronization

Transfer of embryos in the cow will result in a high pregnancy rate providing the preceding estrus in the donor and recipient occurred within 24 h of each other [23]. Alternately, recipients must be synchronous with the stage of development of embryos that had been previously frozen. Recipients can be made available by maintaining a large herd from which to select cattle after natural heats or by estrous cycle synchronization in a smaller herd, which is much more economical. Today, most recipients are synchronized regardless of whether or not embryos are transferred "on farm".

The key to successful estrus synchronization is obtaining synchronous declines in circulating progesterone to values <1 ng/ml and synchronous growth and ovulation of a viable follicle [1,2]. It follows, therefore, that PGF is effective only when a fully developed CL is present (Days 7 to 18 of the cycle) and that withdrawal of exogenous progesterone is only effective if either natural or induced regression of the CL has occurred. If properly implemented, within the physiological constraints of their mechanism of action, current techniques for estrus synchronization are highly successful. However, variation in ovarian follicular wave dynamics makes it difficult to control the time of estrus and ovulation precisely.

Acceptable pregnancy rates following embryo transfer are partially dependent upon the onset of estrus in the recipient being within 24 hours of synchrony with that of the embryo donor [23]. Recipients can be selected for an embryo transfer program by detection of natural estrus in untreated animals or after drug-induced estrus synchronization. Regardless of the method of synchronization used, timing and critical attention to estrus detection are important. Recipients synchronized with PGF must be treated 12 to 24 hours before donor cows because PGF-induced estrus will occur in recipients in 60 to 72 hours post-PGF [26] and in FSH-primed superovulated donors in 36 to 48 hours post-PGF [10,11,42]. Although pregnancy rates do not seem to differ in recipients with natural versus PGF-induced estrus, pregnancy rates were higher in PGF-synchronized recipients in at least one study [23]. It must be remembered that exogenous steroid hormones will induce estrus and even ovulation in post-partum cows and prepubertal heifers [43]. Therefore, post-partum interval, nutrition and body condition in cows, and age, weight and body condition in heifers must be closely monitored. A prospective recipient can be culled from a herd because of one or more of these factors.

The success of estrus synchronization programs is dependent on an understanding of three general areas: 1) estrous cycle physiology in the cow, 2) pharmacological agents and their effects on the cow's estrous cycle, and 3) herd management factors that reduce anestrus and increase conception rates. The use of pharmacological agents to synchronize recipients typically involves the use of PGF to induce luteolysis and/or administration of a progestin to simulate a luteal phase.

Prostaglandin

PGF has become the most commonly used treatment for estrus synchronization in cattle [17,30,48,60]. Importantly, PGF is not effective in inducing luteolysis in the first 5 or 6 days following estrus, and when luteolysis is effectively induced by PGF, the ensuing estrus is distributed over a 6-day period [26]. This variation is due to follicular status at the time of treatment. In a "two-dose PGF" synchronization scheme, an interval of 11 days between doses has been used because the time of the second dose of PGF represents the mid-point of the estrous cycle in animals in which the initial PGF induced luteolysis, and also in animals that were between 0 and 5 days after estrus or had already undergone spontaneous luteolysis at the time of the initial injection. Thus, theoretically, all cows should have a PGF-responsive CL at the time of the second PGF treatment. However, a higher conception rate has been reported with a 14-day interval [17], probably because a growing dominant follicle is more likely to be present 14 days

after an initial treatment with PGF. Stage of the cycle during which PGF treatment is given also affects fertility; pregnancy rates are usually higher when cattle are treated with PGF after mid-cycle (e.g., after Day 12) compared to early in the cycle (e.g., Day 7 or 8).

Progestins

Various progestins (progesterone-like compounds) have been utilized for estrus synchronization. Progestin treatment for >14 days will synchronize estrus upon progestin withdrawal, but fertility at the induced estrus will be reduced due to the development of a persistent follicle in some animals [43,56]. These effects are transitory, and fertility at the following estrus is normal. Vaginal inserts (e.g., CIDR) are now commonly used to synchronize estrus. The official CIDR protocol involves the insertion of a CIDR at random stages of the cycle. Six days later, PGF is administered and on the seventh day the CIDR is removed. Estrus is displayed 60 to 72 hours later. Most administer the PGF at the time of CIDR removal [43]. The CIDR protocol is also easily adapted to protocols in which the follicular wave emergence and ovulation are synchronized e.g., GnRH or estradiol [10,45]. Follicle wave emergence is synchronized by treatment at the time of CIDR insertion and ovulation is synchronized by treatment 24 to 48 hours after CIDR removal.

Management Factors

The two management factors that determine the success or failure of an estrus synchronization program are nutrition and post-partum interval. If cows lose weight during pregnancy, the onset of estrous cycles after calving will be delayed. Cows that are fed adequately during pregnancy but fail to gain weight between calving and breeding will cycle but have been shown to have reduced conception rates and may also have reduced pregnancy rates after receiving a viable embryo by embryo transfer [12]. In a field study, recipients were body condition-scored at the time of embryo transfer on a scale of 1 (thin) to 5 (fat). Pregnancy rates were significantly higher in recipients scoring 3 and 4 than in those scoring 1, 2 or 5 [41]. Therefore, the nutritional status of recipients must be evaluated before setting up an embryo transfer program. Other nutrients important to reproductive efficiency are phosphorus and trace minerals. Although effects of mineral deficiencies can be profound in affecting reproductive function, a much more common and dramatic effect on reproduction occurs with energy deficiencies.

Estrus Detection

The estrous cycle in cattle averages 21 days, with 84% lasting from 18 to 24 days. Behavioral estrus lasts approximately 12 to 16 hours; ovulation normally occurs 24 to 36 hours after the onset of estrus [27]. Estrous behavior waxes and wanes, but nearly all cattle will be detected in estrus if observation is continuous. Therefore, the incidence of true silent estrus is negligible. Causes of anestrus (lack of observed estrus) include pregnancy, cystic ovaries, ovarian atrophy, pyometra, embryonic death, free-martinism and white-heifer disease. Most anestrous dairy cows that are non-pregnant are cycling and have a normal genital tract. Dairy heifers and postpartum suckled beef cattle often have a prolonged interval of anestrus due to ovarian inactivity. Increasing energy intake and/or a 7 to 10 day treatment with progestins will hasten resumption of ovarian activity.

The primary sign of estrus is a cow standing firmly when mounted. Secondary signs of estrus include mounting other cows, mucus discharge, swollen vulva, hyperactivity, and bellowing. It is recommended that >80% of inseminations be based on "standing estrus" behavior.

The two principal causes of estrus-detection problems are missed estrus and estrus detection errors. Indicators of missed estrus include prolonged intervals from calving to breeding, prolonged intervals between breedings, 10 to 15% non-pregnant at pregnancy examination, and <50% of potential estrous periods detected. Several factors can contribute to missed estrus. Often the observer does not spend adequate time observing the cattle for estrus, does not observe frequently enough during the day, or tries to combine estrus detection with other activities (e.g., feeding). If many cattle are in estrus at the same time, they will congregate and form a 'sexually active group', which facilitates estrus detection. However, if only a single animal is in estrus, mounting activity will be much less frequent. Slippery or hard surfaces will also reduce mounting activity. Indicators of estrous detection errors include high concentrations of

progesterone in milk or blood at breeding and interbreeding intervals <17 d or >25 d. In some studies, up to 20% of cattle had high progesterone concentrations at the time of insemination, and therefore were not in estrus. Factors contributing to estrus detection errors include misinterpretation of signs of estrus, misinterpretation or misuse of estrus detection aids, and standing estrus in pregnant cows. Means by which estrus detection can be improved include inducing estrus at a predetermined time, allocating adequate time for observation, using estrus detection aids, and predicting the next estrus.

Estrus detection aids include mounting detectors such as tail-head devices, chalk or paint, chin-ball markers on teaser bulls or androgen-treated marker animals, pedometers, and electronic estrus detection systems [27]. These methods should be utilized in addition to, and not as a substitute for, visual observation of estrous behavior. Marker animals are typically given several treatments with testosterone to initiate mounting activity, followed by periodic treatments to maintain activity. It has been reported that freemartin heifers implanted with Synovex-H (four implants in each ear) were effective marker animals. The duration of effectiveness of the implants was approximately 3 months. This is an extra-label use of these implants and the appropriate withdrawal period prior to slaughter is unknown.

Embryo Transfer

Non-surgical embryo transfer techniques utilized today involve the use of specialized embryo transfer pipettes [40,58,73]. After confirming synchrony of estrus, the recipient is restrained and the rectum is evacuated of feces. At the same time, the presence and side of a functional CL is confirmed. Care is taken to prevent ballooning of the rectum with air. An epidural anesthetic is administered and the vulva is washed with water (no soap) and dried with a paper towel. The embryo is loaded in 0.25 ml straw between at least two air bubbles and the straw is loaded in the embryo transfer pipette. Care must be taken to insure that the straw engages the sheath tightly so as to avoid leakage. The sheath is coated with sterile, non-toxic obstetrical lubricant and the sheathed pipette is passed through the vulvar labia while avoiding contamination. The embryo is placed in the uterine horn adjacent to the ovary bearing the CL by passing the pipette through the cervix, very similar to artificial insemination. However, an attempt is usually made to pass the transfer pipette at least half-way down the uterine horn. The uterine lumen should be lined-up prior to transfer so as to prevent trauma to the endometrium during passage. The embryo is deposit slowly and firmly while slightly withdrawing the tip of the transfer pipette. Practice and dexterity seem to improve one's ability to achieve high pregnancy rates suggesting that trauma to the endometrium may be a limiting factor with this method of embryo transfer. Stimulation of the cervix and inadvertent introduction of bacterial contaminants do not seem to be major determinants of pregnancy rates under normal circumstances. With practice and attention to detail, pregnancy rates with non-surgical transfers can equal those of surgical transfers.

In summary, with existing technology, an average of 8 to 10 ova/embryos will be collected from each superstimulated donor cow and 5 to 6 embryos will be transferred to as many recipients, resulting in 3 to 4 pregnancies. It must be emphasized that very few donor cows are average. Pregnancy rates are generally around 60% with fresh embryos and range from 50% to 60% with frozen embryos. One can anticipate a fetal loss rate of 10% from the time of pregnancy diagnosis until the calf is six months old. It is worthy of note that this is not different from that of the normal cattle population and that embryo transfer procedures have been shown to result in no increase in calf mortality or abnormalities.

Embryo Freezing

Basic Principles

The freezing of a living cell constitutes a complex physiochemical process of heat and water transport between the cell and its surrounding medium. There exists an optimum cooling-rate for each type of cell. It is dependent on the size of the cell, its surface to volume ratio, its permeability to water, and the temperature coefficient of that permeability [33,51].

Normally, the medium that contains the embryos cools below its freezing point without ice crystal formation, a phenomenon referred to as super-cooling. Then, at some lower temperature ice nucleation occurs, followed by a rapid rise in temperature due to the release of latent heat of fusion. To avoid

extensive super-cooling, crystallization is induced in the extracellular medium some 2°C below its freezing point (4 to 7°C) by seeding the medium with an ice crystal [51]. Water in the cells of the embryo and between the ice crystals outside the embryo does not freeze at this temperature because of solutes lowering its freezing point. During further cooling and enlargement of ice crystals, the solute concentration rises and the embryo responds osmotically by losing water into the extracellular unfrozen medium.

Cells are injured during freezing and thawing primarily by solution effects and intracellular ice formation [33,51]. The latter is especially detrimental when relatively large amounts of large ice crystals form. To avoid intracellular freezing, embryos must be cooled at 1°C/min or slower. However, very low cooling rates can also damage cells by what has been referred to as the solution effect. This is especially harmful if cells are not allowed to rehydrate during very rapid thawing [51].

The required thawing rate depends on the freezing regimen used. When embryos are cooled slowly to temperatures between -27 and -40°C and then rapidly to -196°C (liquid nitrogen), thawing must be rapid, e.g., about 200°C/min. Cells treated in this way may contain some intracellular ice, and thawing has to be rapid to prevent injury from the recrystallization of that ice. On the other hand, if embryos are cooled slowly for a longer time and to a lower temperature, below -60°C, before transfer to liquid nitrogen, then thawing is normally done slowly at about 20°C/min [33]. Although both systems result in similar rates of embryo survival, the techniques of faster freezing and rapid thawing are preferred in the field.

Embryos are normally stored in liquid nitrogen at -196°C. The only reactions that occur at -196°C are direct ionizations from background radiation. Consequently, storage times of more than 200 years are unlikely to produce any detectable reduction in survival or cause genetic change of frozen embryos.

Cryoprotectants such as glycerol and ethylene glycol in concentrations ranging from 1.0 to 2.0 M in the freezing medium are required to ensure embryo survival during and after freezing. It is thought that cryoprotectants act by reducing the amount of ice present at any temperature during freezing, thereby moderating the changes in solute concentration. Recommended criteria for a cryoprotectant include high solubility, low toxicity at high concentrations, and a low molecular weight both for easier permeation and to exert a maximum colligative effect [51]. Glycerol has largely been replaced by ethylene glycol, which has gained preference because it can be used for "direct transfer" i.e., transfer into a recipient without prior removal of the cryoprotectant from the embryo [34,70].

During the addition and dilution of a permeating cryoprotectant, the cell undergoes osmotic changes resulting in swelling or contraction [51]. Consequently, if the initial addition, or particularly the dilution following thawing, is carried out inappropriately, the viability of cells can be affected. Glycerol can be added to embryos in a single step but there is clear evidence that the rate of glycerol removal post-thaw is more critical. The standard empirical method was to dilute it by the step-wise addition of PBS or to move the embryos through decreasing concentrations of glycerol, e.g., 0.25 M steps (51). However, Leibo and Mazur [33] suggested a modification in the procedure of cryoprotectant removal by including non-permeable solutes like sucrose into the dilution medium. The sucrose acts as an osmotic counterforce to restrict water movement across the membranes. As the cryoprotectant leaves the embryo, the embryo will shrink in response to the extracellular hypertonic dilution medium. It regains its normal volume when at the end of the process the embryo is placed in normal isotonic culture medium. Using this information, practical methods of quickly removing glycerol from thawed embryos have been devised. As a result, a "one-step straw" was developed so that embryos could be thawed, solutions mixed within the straw and transfer to the recipient done non-surgically, all in the field. In one field study, 476 frozen embryos thawed and processed in sucrose prior to transfer, without microscopic evaluation, resulted in a 42.4% pregnancy rate [31]. More recently, this method has given way to "direct transfer" utilizing highly permeating cryoprotectants, such as ethylene glycol, which do not osmotically harm the embryo if not removed prior to transfer. Recent pregnancy results for "Direct Transfer" in Canada, with more than 19,000 embryos, were not different from those achieved with glycerol [34].

Freeze - Thaw Procedures Utilizing Glycerol

The following protocol has been proven successful for the cryopreservation of Day 7 bovine embryos in PBS supplemented with 0.4% BSA and 1.5 M glycerol [51]. Embryos are pipetted into the freezing medium at room temperature (20°C) and left for eight to 10 minutes to permit the glycerol to equilibrate within the embryo cells. During this equilibration period the embryo(s) are transferred in volumes of 0.25 or 0.5 ml of freezing medium into French straws that are then securely sealed. The samples can be immediately transferred into the freezing chamber at -6 or -7°C and held for 5 min. Ice crystallization (seeding) of the extracellular medium is initiated by touching the outside wall of the straw with a forceps pre-cooled in liquid nitrogen (do not touch the column of media that contains the embryo(s). The samples are held at the seeding temperature for an additional 10 min to allow the crystallization of the medium to progress to equilibrium. Next, embryos are cooled at 0.3 to 0.8°C/min to a temperature between -30 and -40°C, at which time they are immersed into liquid nitrogen (-196°C) and stored.

Thawing is carried out by placing the straw into a water-bath at a temperature between 20 and 35°C. It has been reported that the incidence of cracked zona pellucida was reduced in an air-thaw or when straws were thawed in air for 10 to 15 seconds prior to being submerged into a 35°C water bath; the thaw rate should be around 200°C/minute.

Glycerol, which is a slowly penetrating cryoprotectant, must be removed without causing osmotic damage. The method of choice is the use of sucrose solution between 1.0M and 0.5M in a single step for 10 min or 0.3M in a 3-step dilution of 5 min each (0.75M glycerol and 0.3M sucrose; 0.375M glycerol and 0.3M sucrose; 0.3M sucrose) {40]. The embryos are then transferred into PBS culture medium, washed and evaluated prior to transfer.

Direct Transfer of Frozen-Thawed Embryos

Recently, the use of highly permeating cryoprotectants such as ethylene glycol has allowed the direct transfer of bovine embryos without the necessity of microscope examination and cryoprotectant removal [34]. With this approach, the embryo straw is thawed in a water-bath, much like semen, and the contents of the straw are deposited into the uterus of the recipient, much like artificial insemination. There is no need of a microscope or complicated dilution procedures. The cryoprotectant leaves the embryo in the uterus. As indicated earlier, the direct transfer of 19,000 bovine embryos in Canada resulted in an overall pregnancy rate of 58% which was not different to that achieved by regular cryoprotectant dilution techniques. The transfer of frozen/thawed bovine embryos is now becoming very similar to the use of frozen/thawed semen in AI and is being conducted by technicians in many cases.

Vitrification Freezing Procedures

The freezing of bovine embryos is now commonplace and pregnancy rates are only slightly less than that achieved with fresh embryos [34]. However, freezing and thawing procedures are time consuming and require the use of biological freezers and a microscope. These steps can be replaced by a relatively simple procedure called vitrification [55. High concentrations of cryoprotectants are used and the embryo in its cryoprotectant solution is placed directly into liquid nitrogen. Because of the high concentration of cryoprotectants, ice crystals do not form; the frozen solution forms a glass. As ice crystal formation is one of the most damaging processes in freezing, vitrification has much to offer in the cryopreservation of "had to freeze" oocytes and embryos. However, its greatest advantage is its simplicity in application. Vitrification procedures are now widely used experimentally and it is only a matter of time before they find commercial application. Recently, a procedure for the direct transfer of vitrified bovine embryos with pregnancy rates that did not differ from that of traditional techniques was reported [69]. Clearly, vitrification of bovine embryos in commercial bovine embryo transfer is on the horizon.

Identification, Certification and Registration of Offspring

Records for the accurate identification of parentage and of embryo transfer offspring is of vital importance for both domestic and international application of embryo transfer technology. The

International Embryo Transfer Society (IETS) has developed three forms for certification of embryo recovery, embryo freezing, and embryo transfer. In addition, a fourth form (certificate D) is recommended for use in embryo exports [39]. The IETS also allocates embryo freezing codes that must appear on all embryo containers and all documentation so that the organization freezing embryos can be identified. Finally, standard procedures for labeling embryo freezing containers are also recommended e.g., embryos frozen for direct transfer are to be frozen in yellow straws and placed in yellow goblets. Examples of the above forms and specific instructions on their use, the labeling of embryo freezing containers and the identification of embryo developmental stages and quality grades are available in the Manual of the IETS [39].

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