APPLIED REPRODUCTIVE TECHNOLOGIES IN PRACTICE: YOU CAN DO IT!

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Knowledge of herd immunity and herd health monitoring in your client's herd of super-ovulating donors, for the purpose of transferring fresh, frozen or in vitro produced embryos is at the crux all success. Efforts to multiply progeny using these technologies from elite females without regard for the health status of the herd increases the likelihood of poor pregnancy results. An inadequate vaccination program, the presence of a single Bovine Virus Diarrhea Persistently Infected (BVD-PI) calf, or Leptospirosis sp. infection, etc. will result in poor pregnancy results from embryos transferred or increased pregnancy loss.

Embryo Transfer

The first successful embryo transfer in mammals is credited to Walter Heape in 1890 with rabbits [1]. In 1951, more than 70 years later the first embryo transfer in cattle was reported [2]. The first frozen thawed embryo resulting in a live calf was reported in 1973[2].

In 1970 embryo transfer was described as a technique that allowed us to propagate the top 1% of the females. Using the top 1% of the males, this technique allowed a much faster genetic progress that could have been achieve until then. As an associate in a large dairy practice in Central California in 1980, it was natural that my first clients as an embryo transfer (ET) practitioner were dairies. A new technology, capable of being performed on farm with a modicum of success allowed dairy clients to add marketing of genetics to their repertoire of income generation.

Beef clients quickly followed, in 1982 with cow calf producers leading the way. Soon the club calf producers realized the benefits of ET by producing multiple show steers per year out of their donor dams instead of just one.

Application

The applications of embryo transfer are many. The genetic advancement of embryos from superior females not only increases the production of superior females in the herd but also decreases the genetic influence of the poor production females. This is multiplied exponentially when the poorer performing females are used as recipient females. The economic impact of multiple offspring from superior females in one calving season is most obvious. In females, where the genetic value is the combination of a show quality phenotype and EPDs (Expected Progeny Differences), it is possible that marketing offspring from the herd can be enhanced by their performance in the show ring. Sales of bulls, heifers, embryos, or pregnant females carrying sexed embryo calves can be quite rewarding. Functional phenotype with balanced EPDs would be more applicable to the seed stock producer [3].

In cases of restocking herds or countries in which disease has caused a catastrophic loss of livestock, embryos may be the best way to repopulate the area. Risk of reintroduction of disease

is lowered along with the expense of repopulation, when embryos are used to repopulate instead of live animals [4].

Storage of frozen embryos from select families can act as a reservoir in the event of an unexpected loss, or a poor producing mating.

The Process

The process of embryo transfer is not difficult, but involves multiple sequential steps.

- Selection of the donor and recipient females
- synchronization of the donor and recipient follicular waves
- superovulation of the donor female
- detection of estrus in the donor and the recipient females
- breeding of the donor
- recovery of the embryos from the donor
- transferring embryos to the recipients
- freezing of the embryos

Producers generally know which genetics they want to propagate with embryo transfer. A physical examination of the donor including reproductive Ultrasonography should be performed. Incidence of dystocia, retained placenta, caesarian section, pyometra, cystic ovarian disease or anestrus may have a negative impact on the embryo production. Ovarian size may be correlated to follicular wave size and may provide some insight of productivity [5].

Donors should have had all pre breeding processing completed at least 30 days and preferably 45 days prior to embryo transfer and be a minimum 50 days post partum and cycling. Pre breeding processing should include a good quality modified live Bovine Virus Diarrhea (BVD), Infectious Bovine Rhinotracheitis (IBR), Parainfluenza 3 (PI3), and Leptospirosis sp. (Lepto), Clostridium sp., and pink eye, vaccines as a minimum. Treatment with a broad spectrum Anthelmentic, selenium bolus, and copper bolus are preferred.

Donor nutrition should include a balanced mineral supplement with or without added protein determined by type of feed. Upon presentation, the donor with a body condition of 2.0-3.5 is acceptable.

Beef recipients should be selected based on their production history, mothering ability reproductive history, and absence of periparturient disease and of course soundness. It is most desirable for recipients to receive the same pre breeding processing as donors. Ideally, this processing is completed between 30- 45 days prior to embryo transfer. Additionally, recipients should be 60-65 days post partum at the time of ET. The plane of nutrition should include a balanced mineral supplement with or without added protein determined by type of feed. Females with body condition score of 2.0-4.0 and $\leq$ 7 years old are acceptable. Preferably, recipients should be the best cows in the herd reproductively. As opposed to the not all too common cows that were open after being AI-ed twice and have now spent two estrus cycles with a herd bull.

It is advisable to engage in a candid discussion with the client regarding reasonable production expectations prior to beginning a superovulation protocol. Their expectations may be unrealistic

and based on a neighbor producer whose outlier donor produced 25 quality grade one embryos in each of three flushes. An example of realistic expectations are based on data collected from 2048 recoveries where the average superovulation resulted in 11.5 embryos of which 6.2 are of a transfer or freeze quality [6]. It is equally important to inform the client that 25% of all superovulations yield zero embryos that can be transferred or frozen [6].

There are several ways to initiate superovulation of donors. Using a reference estrus with FSH (Follicle Stimulating Hormone) injections beginning 9-12 days post estrus, which coincides with follicular wave two and three emergence, is common. A less common method is using FSH injections that start the day after estrus with the emergence of the first follicular wave. Application of a CidR (Progesterone-Releasing Intravaginal Device) along with stimulation of follicular wave emergence by injecting Gonadatropin Releasing hormone (GnRh) or estradiol benzoate is widely used. Ideally, FSH injections should coincide with follicular wave emergence 2 and 4 days, respectively[4]. This use is extra label use for both products. FSH injections twice a day, ten to twelve hours apart for three to six days is ample time for follicular growth. A prostaglandin (PGF) injection included with the last two FSH injections causes luteolysis of corpus lutea present on the ovary. The CidR is removed with the last injection. Forty eight hours after the first prostaglandin injection the donor should show estrus.

Activity that the cows display prior to the onset of estrus such as, licking, rubbing necks, butting, two cows circling each other, walking nose to tail in a line, and attempted mounts are clues to an impending standing estrus event. The only true sign of estrus in cattle is standing to be mounted [7]. Identification of the timing of the first standing event in donors determines the optimal time to breed [4]. The timing of the standing event in recipients determines the degree of synchrony between the stage or age of the embryo to be transferred with the days since estrus or age of the post heat period[4].

Commercially available computer aided estrus detection products such as Heat Watch® and AFI Act II® are effective in detecting cows in estrus. Heat Watch® uses a radio transmitter attached to the tail head of a cow with glue that is activated when the cow is mounted. AFI ActII® uses pedometers attached to a leg of the cow. The pedometer then measures the number of steps a cow takes as well as resting bouts. The relationship between movement and rest periods are used to accurately identify the time of first time stand of a cow in estrus [8]. Although these products are expensive, over time, they become an economically viable tool. One of these systems shared by multiple producers at a centrally located breeding facility is realistic. The author is using AFI ACT II® for heat detection of donors and recipients at the embryo transfer facility.

Breeding of the donor should take place 10-12 hrs after the first standing event. A second service 10-12 hrs later is suggested. Two units of good quality semen are used with each service.

Proper semen handling is important [9]. Proper thaw temperature and time, minimal light exposure, minimal temperature shock, proper deposition, and rate of deposition of semen are extremely important [10] [11]. Sanitation during the process is pivotal to success [10] [11]. Handling the semen at or below the frost line of the semen tank is often disregarded and a major problem [12]. An inexpensive head lamp and a card board canister holder are helpful. Data shows that AI gun covers or" cow condoms" increase conception [13] and are inexpensive.

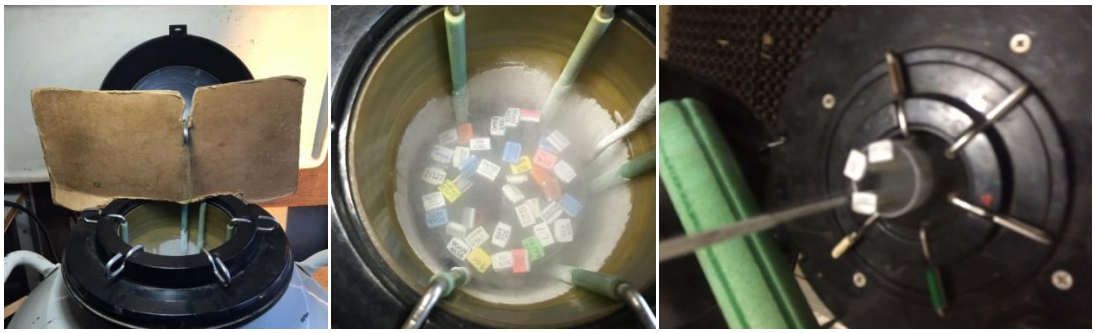

Figure 1. Frost line and visibility of semen cane tops at a proper working height with the canister in working position and the all too familiar improper handling.

Two common non-surgical methods of embryo recovery are gravity flow and the syringe method. In both methods a Foley type catheter is placed either in the body of the uterus or each horn individually and the uterus is lavaged with several exchanges of flushing solution. PBS (Phosphate Buffered Saline) with added antimicrobials and a surfactant is a flush commercially available. An alternative is Lactated Ringer's Solution with a surfactant added just prior to use. Following the actual flush, the recovered flush fluid from the uterus is filtered. The filter is then meticulously rinsed and the embryos are transferred to a Petri dish. This dish is carefully examined under a stereomicroscope and all embryos are removed with a pipette. The embryos are then placed in a commercially available embryo holding media, rinsed minimally three times, and held until transfer or freezing.

Freezing embryos is a slow process; most commonly accomplished today using a computer-controlled apparatus. The typical program starts with a 10-minute hold at -6.0 ºC. While at this temperature, the embryos are seeded in 1/4cc straws to induce ice crystal formation above the normal cryoprotectants' freeze temperature. The common cryoprotectants used today are 1.5 molar ethylene glycol or 10% glycerol. Freezing is accomplished at a rate of -0.5 to -0.6 ºC/minute until the embryos are cooled to -32.0 ºC. Once this temperature is achieved, each embryo containing straw is quickly plunged into liquid nitrogen at -193°C. Finally, the labeled straw containing the embryo is placed in a labeled cane [4]. Canes with the embryos are stored in a "semen tank" (Liquid Nitrogen Dewar) until they are ready to be transferred. Such canes may be stored indefinitely provided they have been kept properly filled with liquid nitrogen.

Conversely, vitrification is a fast freezing cryopreservation method utilizing 3.5 M ethylene glycol as the cryoprotectant. Due to the high concentration of the ethylene glycol, a glass forms instead of the more common crystal formations from slow freezing. This *glass* formation is a much safer environment for the embryo than ice *crystals* which are damaging to the embryos [4].

The process of non-surgical transfer of fresh embryos requires that the recipient be restrained in a chute and epidural anesthesia performed. The recipient is examined per rectum for the presence of a corpus luteum, which is the ovulation site from the previous estrus 6.5-7.5 days earlier in the recipient. The embryos are loaded into ¼ cc straws in holding media. The straws containing the embryo are placed into ¼ cc Cassou type guns. The vulva is cleaned and the gun introduced with the embryo being deposited in the horn ipsilateral to the corpus luteum. The date, donor, sire, recipient ID, horn (right or left) stage and quality of the embryo are recorded. Pregnancy

diagnosis is performed as early as 28 days with tests such as Biopryn® or by manual palpation or ultrasound 40-45 days after estrus of the recipient.

Transferring previously frozen embryos differs from fresh transfer of embryos only by the thawing process. Ethylene glycol frozen embryos are commonly air thawed 5-10 seconds, placed in a 20-27 ºC water bath for 10-25 seconds, wiped dry then transferred immediately. This is the direct transfer method of frozen embryos [6] Glycerol frozen embryos are air thawed for 15- 25 seconds, thawed in 25-28 ºC for 15-25 seconds. Glycerol is then removed by an osmotic process that requires passage through multiple solutions of varying dilutions of glycerol and sucrose and then rehydrated with ovum culture media (OCM). This requires the use of a microscope and needs to occur in a clean environment. Transfer of glycerol-frozen embryo should occur within 30 minutes of thawing.

Recipient handling has been shown to affect the embryo pregnancy. Hot shots, dogs, blaring music, and essentially any loud activities are counterproductive. Poor facilities that do not flow well can set the stage for counterproductive methods being used [14]. Chute scores can influence outcomes by causing bruising that increases the risk of prostaglandin release and is counterproductive. Simply narrowing the chute can minimize this [15].

Ultrasonography

Without U/S (Ultrasound) the existence of follicular wave dynamics and their ensuing manipulation into the estrus synchronization protocols would not be available. U/S can be used for pregnancy diagnosis, fetal sex determination, donor reproductive evaluation, and superovulation response evaluation and in vitro fertilization [16, 17] U/S can also be used for Breeding Soundness Examinations and diagnosis of infertility in bulls [18].

The absence of fetal heart beat, visible with U/S is a diagnostic tool for fetal mortality , [16, 17, 19]. Age determination by measurement of fetal length, circumference, head width, nose to crown length, cotyledon size [16, 17] etc. is reasonably accurate. The diagnosis of twins can be made with or without the presence of the twin line using U/S. Fetal sexing [16, 17] is useful information for the producer in culling decisions, feed inventory projections and sorting of pre calving cows into two groups to help prevent pregnant heifer calves at weaning. There is another advantage in using U/S to evaluate the superovulatory response prior to insemination. This can influence the determination to use a particularly expensive bull, or even whether to proceed with insemination. The presence of foreign body's, scarring or calcification in the scrotum and testis due to trauma or infection can diagnose unexplained acute infertility in breeding bulls.

Ovum Pick up and In Vitro Fertilization

Donor selection for In Vitro Fertilization (IVF) should be no different than donor selection for conventional embryo transfer (CET). Often though, the IVF donor is a non responding CET (NRCET) donor. Frequently NRCET donor oocyte recoveries are high and of varying quality [20]. The pre implantation IVF embryo production surpasses the CET historical embryo recovery of NRCET donors. In productive CET donors IVF production can surpass CET production primarily due to the frequency interval difference between CET and IVF.

OPU of donors can be performed with or without pre OPU FSH stimulation. Similar to CET, follicular wave stimulation and the beginning of FSH injections should coincide. Dosage of FSH

can vary from 7-20+ cc s dependent on donor response variability. A coast period follows the FSH injections, allows for follicular growth, and can vary from donor to donor. Most donors' coast period length falls between 24-32 hours prior to OPU. Restricting movement of the donor can increase oocyte recoveries. Epidural anesthesia, tail restraint and cleaning of the perineal area is performed. Critical to oocyte survival is the time from recovery to maturation media immersion. All equipment that comes in contact with the dishes that contain the oocytes must be maintained at 36.5 ºC and work performed in a dust free warm area. The time from oocyte recovery to maturation media immersion, if prolonged, will negatively affect oocyte quality. On the contrary, CET embryos held in OCM for 4-6 hours yield acceptable pregnancy results.

Oocytes are graded and grouped by quality, incubated for approximately 24 hrs at which time semen dilution, capacitation and IVF occur. Oxygen and carbon dioxide incubators are used for the maturation of the embryos until day 7 at which time embryos are either transferred into recipients or frozen.

Stem cell oocyte production

Quite possibly the next ART is oocyte production from isolated cultured ovarian germ cells[21]. Current research reports that isolated purified oocyte germ cells capable of proliferating in vitro can be transplanted into ovaries of recipient mice and generate fully functional oocytes that fertilize to produce viable embryos and offspring in mice [22, 23]. Artificial insemination, conventional embryo transfer and in vitro fertilization are all technologies that have had an immense impact on the genetic advancement of cattle. This new technology may commercially produce cattle embryos. This will likely replace the current "new technology- IVF" which is very tedious, labor intense, and expensive to perform, with a technology that can offer much greater results with less invasiveness, improving animal welfare at the same time that it will improve animal reproduction.

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