UNIVERSITÀ DI PARMA

Dipartimento di Scienze Medico Veterinarie

Corso di Laurea Magistrale a ciclo unico in

Medicina Veterinaria

EVALUATION OF THE USE OF EMBRYO TRANSFER AND EMBRYO FREEZING IN THE REPRODUCTIVE MANAGEMENT OF DAIRY FARMS

Valutazione dell'impiego dell'embryo transfer e del congelamento embrionale bovino nel management riproduttivo aziendale

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Anno Accademico 2021-2022

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ABSTRACT

Embryo transfer is an assisted reproductive technology, largely used in cattle, that involves the recovery of embryos, usually from an elite female donor, and the subsequent transfer to recipient females. This technique can find various applications: it can be used to progressively improve the genetics within the herd; to transport embryos instead of live animals, which is much more practical and prevents the spread of infectious diseases; to safeguard livestock breeds that may be in danger of extinction; to solve fertility problems that affect the herd, in particular repeat breeder cows and conditions that reduce dairy cattle fertility, such as heat stress (so-called embryo therapy). Selection of the donors is based on genetic merit and reproductive adequacy, which is determined by the veterinarian also through the use of ultrasonography. The reproductive competence can also be assessed by measuring the concentrations of Anti-Müllerian hormone, which can be considered a viable endocrine marker of antral follicle count in cattle. The recovery of embryos includes the induction of multiple ovulation by hormonal treatment. To reach superovulation, the common protocol requires intramuscular administration of pFSH twice daily for 3-4 days after manipulation of the follicle wave emergence (which can be performed in various ways). Recently, some scientists have analysed alternative routes of administration of FSH and different pFSH formulations with the aim of simplifying the management and of ensuring the welfare of animals, with variable results. After the superovulation treatment the donors are artificially inseminated at a fixed time with high quality semen, preferably coming from high fertility bulls. In fact, recent studies showed that the quality of the semen and the fertility of the bull can influence embryonic development and quality. To perform embryo transfer both conventional semen and sex-sorted semen can be used but when using sex-sorted sperm the moment of AI should be adjusted. Recipients are usually selected among reproductively healthy cows that display calving ease and have good milking and mothering aptitude. Embryos obtained *in vivo* can be collected and transferred surgically or non-surgically. Oftentimes the non-surgical techniques are highly preferred. The flushing procedure involves epidural anaesthesia, introduction of a

Foley catheter through the cervix of the donor cow 7 or 8 days after estrus, flushing of a medium (usually D-PBS) into and out the uterus (or each uterine horn at time) to pick the embryos, which are separated from the medium through a filter. The non-surgical transfer is performed with a simple transfer gun, containing the straw with the embryo, put into the uterine horn on the same side of the ovary with an active *corpus luteum*. Many factors can affect the pregnancy rates: cervix transfer score (difficult, moderate or easy), location where the embryo is transferred, duration of the transfer (because of the release of chemical inflammatory mediators), embryo quality, diet, chromosomal abnormalities, cryopreservation, uterine environment. Embryos standard classification is based on a number code system for their stage of development, ranging from 1 (an unfertilized oocyte or a 1-cell embryo) to 9 (expanding hatched blastocyst) and for their quality (1: excellent or good; 2: fair; 3: poor; 4: dead or degenerating). Evaluation of bovine embryos is usually done with a stereomicroscope and requires experience and competence. Only Code 1 embryos can be used for international trade and only Code 1 and 2 embryos are eligible for freezing. The three main strategies used to prevent the transmission of pathogens from donors to embryo recipients are: donor testing, embryo processing or treatment and recipient testing. An intact *zona pellucida*, IETS recommended washing procedures with trypsin, aseptic technique, sterile equipment and labware are enough to avoid the spread of the main pathogens and infectious diseases. Cryopreservation simplifies trades between countries and allows the storage of high value genetics for an unlimited time. It can be performed with a slow process or with an ultra-fast process (so-called vitrification). To avoid or at least minimise cell damage cryoprotectants must be used, the most widely used are Glycerol and Ethylene glycol. Instead, vitrification is still a technique under development. Ultimately, prior to the introduction of embryo transfer in a breeding program, the costs associated with this technique, its economic feasibility and the cost-benefit ratio should be carefully evaluated.

RIASSUNTO

L'embryo transfer è una tecnica di riproduzione assistita ampiamente utilizzata nella specie bovina che prevede la raccolta di embrioni, solitamente provenienti da una bovina di elevato valore genetico, e il conseguente trasferimento degli stessi in bovine riceventi. Questa tecnica può trovare varie applicazioni, può essere utilizzata per: aumentare progressivamente il valore genetico all'interno della mandria; trasportare embrioni anziché animali vivi, la qual cosa risulta molto più pratica e permette di prevenire la diffusione di malattie infettive; salvaguardare particolari razze a limitata diffusione; far fronte a problemi di fertilità che possono affliggere la mandria, in particolare il problema delle cosiddette repeat breeder e particolari condizioni che possono ridurre la fertilità delle bovine da latte come lo stress da caldo (in questo caso l'utilizzo di questa tecnica viene definito embryo therapy). La selezione delle donatrici si basa sul valore genetico e sull'adeguatezza riproduttiva la quale viene stabilita dal medico veterinario anche grazie all'uso dell'ultrasonografia. La competenza riproduttiva può essere determinata anche misurando la concentrazione di ormone Anti-Mülleriano che può essere considerato un valido marker endocrino di riserva ovarica di follicoli antrali. La raccolta degli embrioni prevede l'induzione di un'ovulazione multipla attraverso un trattamento ormonale. Per ottenere questa superovulazione il protocollo più utilizzato richiede somministrazioni intramuscolari di pFSH due volte al giorno per 3-4 giorni non prima di aver manipolato l'insorgenza delle ondate follicolari e ciò può essere realizzato in vari modi. Recentemente alcuni ricercatori hanno valutato vie di somministrazione alternative dell'FSH e diverse formulazioni di pFSH con lo scopo di semplificare il management e di assicurare il benessere animale, ottenendo risultati variabili. Dopo il trattamento per ottenere la superovulazione le donatrici vengono inseminate artificialmente in un momento prestabilito e utilizzando seme di alta qualità, proveniente preferibilmente da tori molto fertili. Infatti recenti studi hanno mostrato che la qualità del seme e il livello di fertilità del toro possono influenzare sia lo sviluppo embrionale che la qualità degli embrioni. Per attuare l'embryo transfer si possono utilizzare sia il seme convenzionale che il seme sessato a patto di modificare il momento

dell'inseminazione artificiale quando si impiega il seme sessato. Le riceventi vengono solitamente selezionate tra vacche sane dal punto di vista riproduttivo e che mostrano facilità di parto, buona attitudine lattifera e materna. Gli embrioni ottenuti *in vivo* possono essere raccolti e trasferiti sia chirurgicamente che non chirurgicamente. Spesso le tecniche non chirurgiche sono da preferirsi. La procedura di flushing prevede anestesia epidurale, introduzione di un catetere Foley attraverso la cervice della vacca donatrice 7 o 8 giorni dopo l'estro, lavaggio dell'utero (o prima di un corno e poi dell'altro) con un medium (solitamente D-PBS) che viene prima introdotto e successivamente aspirato allo scopo di raccogliere gli embrioni, che vengono poi separati dal medium tramite il passaggio attraverso un filtro. Il transfer non chirugico viene effettuato con una semplice pistolet contenente la paillette con l'embrione che viene rilasciato nel corno uterino corrispondente all'ovaio che presenta un corpo luteo attivo. Diversi fattori possono influenzare il pregnancy rate: il punteggio riferito alla facilità di passaggio della cervice (difficile, moderato, semplice), il punto dove viene collocato l'embrione, la durata del transfer (a causa del rilascio dei mediatori chimici dell'infiammazione), la qualità dell'embrione, la dieta, le anomalie cromosomiche, la crioconservazione e l'ambiente uterino. La classificazione standard degli embrioni si basa su un sistema di codificazione numerico che indica lo stadio dello sviluppo embrionale, che va da 1 (oocita non fertilizzato o embrione con una cellula) a 9 (blastocisti espansa in hatching) e la qualità dell'embrione (1: eccellente o buono; 2: discreto; 3: scarso; 4: morto o in degenerazione). La valutazione degli embrioni bovini viene effettuata solitamente con uno stereomicroscopio e richiede esperienza e competenza. Solo gli embrioni di Grado 1 possono essere utilizzati nel commercio internazionale e solo quelli di Grado 1 e 2 sono idonei al congelamento. Le tre strategie principali applicate per prevenire la trasmissione di patogeni dalle donatrici alle riceventi sono: test sulle donatrici, processamento o trattamento degli embrioni, test sulle riceventi. Una *zona pellucida* intatta, le procedure raccomandate dalla IETS di lavaggio con tripsina, asepsi, equipaggiamento e materiali da laboratorio sterili sono misure di prevenzione sufficienti ad impedire la diffusione dei principali patogeni e malattie infettive. La criopreservazione semplifica gli scambi internazionali e permette la conservazione di

materiale genetico di alto valore per un periodo di tempo illimitato. Può essere effettuata tramite un processo lento o ultra-rapido (la cosiddetta vitrificazione). Per evitare o quantomeno limitare danni cellulari vi è la necessità di adoperare dei crioprotettori tra cui i più frequentemente impiegati sono il Glicerolo e il Glicole Etilenico. La vitrificazione è invece una tecnica ancora in fase di perfezionamento. Infine, prima di introdurre l'embryo transfer in un programma di allevamento, dal punto di vista manageriale è opportuno valutare attentamente i costi associati a questa tecnica, la sua fattibilità economica e il rapporto costi-benefici che potrebbero derivare dal suo impiego.

1. INTRODUCTION

Embryo transfer is an assisted reproductive technology, largely used in farm animals, that involves the recovery of embryos, usually from an elite female donor, and the subsequent transfer to recipient females. The recovery of embryos includes the induction of multiple ovulation by hormonal treatment.

The first effective transfer of mammalian embryos was accomplished by Walter Heape in 1890. Heape transferred two four-cell Angora rabbit embryos into an inseminated Belgian doe, which afterwards gave birth to four Belgian and two Angora young. Warwick and colleagues did significant work on embryo transfer in ship and goats in the 1930s and 1940s, but it was Umbaugh in 1949 who performed the first efficient embryo transfer in cattle. He obtained four pregnancies from the transfer of cattle embryos, but all the pregnancies ended before full term. In 1951, the first embryo transfer calf was born in Wisconsin after the surgical transfer of an abattoir-derived day-5 embryo. It was Rowson and colleagues who developed much of the technology that thereafter found commercial use, and for this reason Rowson is considered the founding father of embryo transfer in farm animals. In 1972, Rowson arranged the first international course on bovine embryo transfer in Cambridge that brought together 13 veterinarians from around the world. Many of these applicants became the founding members of the International Embryo Transfer Society (IETS) and practitioners of commercial embryo transfer (Mapletoft, 2013).

The bovine embryo transfer industry as it is known today emerged in North America in the early 1970s where was used to quickly increase the number of cattle of worthwhile continental breeds imported into Canada.

In the early 1970s all embryo recoveries and transfers executed were conducted surgically. Consequently, the first commercial embryo transfer programmes depended on mid-ventral surgical exposure of the uterus and ovaries with the donor under general anaesthesia. This necessitated surgical facilities and limited the use of the technology in the dairy industry because the udder of dairy cows impeded mid-ventral access to the reproductive tract. It was not until mid-1970s that non-surgical embryo recovery became enough established to be used in commercial practice.

In the early 1980s, non-surgical embryo transfer techniques were adopted, enabling embryo transfer to be performed on the farm and making it particularly appealing to dairy farmers.

The embryo transfer industry expanded fastly in the late 1970s, both in relation to the number of practitioners and the count of donors flushed. Nowadays North America has remained the centre of commercial embryo transfer activity and in South America this technique is widely spreading. Europe and Asia reported 19% and 14% respectively of the total number of embryos collected in 2012 (Thibier, 2016).

The IETS was founded in 1974 and was significantly involved in the diffusion of basic and applied information, supporting the rapid growth of the embryo transfer industry in the 1980s and 1990s, especially with the founding of regional embryo transfer organizations. The Health and Safety Advisory Committee (HASAC) has been pivotal in collecting and diffusing scientific information on the possibility of disease control through the use of bovine embryo transfer. Sustained close cooperation between the IETS and the World Organization for Animal Health (OIE), have made the international movement of cattle embryos feasible. In this respect, the *Manual of the IETS* has become the referral source for disease control methods applied in export protocols.

Regional embryos associations also collaborate straightly with breed associations, producer groups and international groups. Their aim is to set up standards of practice to ensure confidence in the use of embryo transfer technology for disease control. Their certification programmes are important to guarantee that embryo transfer practitioners are technically and ethically skilled to handle embryos for international trade.

There has been no significant increase in the number of embryos yield per superovulated donor over the past twenty years. Nevertheless, acknowledging the relevance of follicular wave dynamics and elaborating methods for the synchronization of follicular wave emergence have facilitated the way in which superovulation is achieved, leading to increased embryo production per unit of time. Donor cows are being superstimulated more repeatedly than in the past, and more embryos are being produced per year. Applying comparable procedures to the

recipients has made oestrus detection, and the necessity to wait for animals to ‘come into heat’ unneeded, simplifying the management of commercial embryo transfer programmes (Mapletoft, et al., 2005).

In conclusion, considering that about one million bovine embryos are transferred worldwide each year, it can be affirmed that nowadays this assisted reproduction technology clearly has a significant impact on the cattle industry (Thibier, 2016).

2. OUTLINE OF CATTLE REPRODUCTIVE CYCLICITY

Each oestrus cycle in cattle, which duration is normally of 21 days in cows with two follicular waves while duration is 23-24 days in milk/beef heifers and beef cows with 3(4) follicular waves, consists of a follicular phase and a luteal phase. The follicular phase includes proestrus and oestrus and the luteal phase consists of metestrus and diestrus.

The follicular phase is dominated by the hormone estradiol produced by ovarian follicles. The luteal phase is dominated by the hormone progesterone produced by the corpus luteum, that prepares the reproductive tract for pregnancy.

The follicular phase begins after luteolysis which leads to a marked reduction in progesterone. Consequently, the negative feedback by progesterone on hypothalamus is eliminated and GnRH (gonadotropin releasing hormone) is released at higher amplitude and frequencies. This causes FSH (follicle stimulating hormone) and LH (luteinizing hormone) to be secreted from the anterior lobe of the pituitary. These gonadotropins induce the production of estradiol by the ovarian follicles. A positive feedback on the neurons of the hypothalamic surge center takes place and, as a consequence, the GnRH neurons secrete a burst of GnRH. In fact, at hypothalamic level there are two centers that produce GnRH, the surge center and the tonic center. This last one releases small quantities of GnRH in a pulsatile way continuously throughout the reproductive life, while the surge center is sensitive to estrogen positive feedback and releases high pulses of GnRH when estrogen arrives at certain levels. In brief, the preovulatory rise of GnRH in influenced by the combination of high estradiol and low progesterone.

The process of follicular growth and degeneration happens continuously throughout the entire oestrus cycle. Antral follicles of different sizes grow in reply to tonic levels of FSH and LH and are present all the time. These antral follicles have been classified as small, medium or large according to their diameter. The dynamics of these follicles involve four processes: recruitment, selection, dominance and atresia (degeneration). During recruitment a cohort of small antral follicles start to grow and produce estradiol and some of the recruited follicles become atretic. In cattle only a single

follicle is selected and as it moves towards dominance, it continues to produce rising quantities of estradiol along with the hormone inhibin. Inhibin is a protein hormone produced by the antral follicle that suppresses the secretion of FSH. The dominant preovulatory follicle applies an inhibitory effect on the other antral follicles caused by the production of inhibin and estradiol in association with a reduced blood flow to some follicles. In fact, only those follicles receiving a high blood supply (and consequently higher amounts of gonadotropin) proceed to grow and ovulate.

During metestrus takes place the first follicular wave during which a group of follicles is recruited. These follicles are not in the adequate endocrine conditions to continue their development so they undergo atresia within the ovary. Also the follicles of the second follicular wave, which occurs during diestrus, undergo atresia. These two first follicular waves start and terminate during a period of time in the cycle when progesterone level rises or is at its highest. Thus, total follicular development and ovulation are not possible under progesterone dominance. Anyway, the dominant follicle of each wave can ovulate if luteolysis occurs.

After luteolysis there is a third wave of follicles and one of these follicles will become the dominant and preovulatory follicle. To summarize, during recruitment FSH increases and stimulates antral follicle growth, during the selection phase inhibin and estradiol inhibit FSH secretion and at this point FSH secretion is at its lowest while LH secretion increases, during dominance the largest follicle produces high levels of estrogen inducing the preovulatory center to release a surge of LH, and FSH secretion stays low because the dominant follicle produces high levels of inhibin and estradiol too. Once the preovulatory surge of LH occurs, estrogen secretion by the dominant follicle suddenly declines (Senger, 2003). Anyway, even though the stimulation of emergence of a follicular wave by a surge in FSH is well defined, it is not clear which are the proper concentrations of inhibin and estradiol that must be present in the circulation to lead to the initial declining portion of the surge. The dominant follicle maintains the basal concentrations of FSH between surges and despite the suppression of FSH, it is able to develop through a shift in primary gonadotropin dependency from FSH to LH. The resulting cellular LH-driven mechanism supports

the dominant follicle throughout its post-deviation growth phase (Ginther, et al., 1996).

During follicular growth, LH binds to LH-specific membrane receptors localized on the cells of the theca interna of the developing follicle. Thecal cells secrete testosterone that diffuses into the granulosa cells that present FSH receptors. FSH binds to these receptors causing the production of enzymes that converts testosterone to estradiol. This happens until levels of estrogen increase to a maximum that provokes the preovulatory LH rise.

After the LH peak, the cells of the theca interna begin to produce progesterone rather than testosterone. Progesterone promotes the synthesis of the enzyme collagenase by the theca interna cells. Collagenase causes the rupture of collagen, which is a component of the tunica albuginea that covers the ovary. At the same time the follicular fluid volume inside the follicle increases. In this way the so-called stigma (apex of the follicle) begins to protrude above the ovary. At this point prostaglandin $F_{2\alpha}$ and prostaglandin E_2 are synthesized and spread in the area; the first one provokes contractions of the myoid components of the ovary increasing the pressure on the stigma, the second one helps the follicle to transform itself into a corpus luteum by the activation of plasminogen.

Over the course of ovulation, the rupture of small blood vessels causes local haemorrhage which appears in the form of a blood coagulum on the surface of the ovary, this structure is called *corpus hemorragicum*. During ovulation the follicle implodes and the cells of the theca interna and the granulosa start mixing and the basement membrane creates the substructure of the corpus luteum. The corpus luteum is made of large luteal cells and small luteal cells that are both steroidogenic, meaning that they can produce steroids, in particular progesterone. The production of progesterone requires the presence of basal LH and cholesterol.

The luteal phase starts suddenly after ovulation. Throughout the early luteal phase, the corpus luteum develops and progesterone starts rising. During the mid-luteal phase the corpus luteum is completely functional and progesterone is at its highest concentration. The functional capability of the corpus luteum depends on the number of luteal cells and on the degree to which it becomes vascularized.

Progesterone is an extremely important hormone in the endocrine control of reproduction because it reduces basal GnRH release and frequency, prevents behavioural estrus, blocks the preovulatory LH surge and reduces myometrial tone.
Over the last 2-3 days of the luteal phase, corpus luteum is demolished (luteolysis) and the luteal phase ends. The hormones regulating luteolysis are oxytocin and progesterone synthesized by the luteal cells and $PGF_{2\alpha}$ produced by the endometrium. Throughout the first half of the oestrus cycle progesterone inhibits the secretion of $PGF_{2\alpha}$ by blocking the development of oxytocin receptors in the uterus, but after 10-12 days progesterone loses this inhibitory effect. Thus, during the late luteal phase oxytocin provokes the secretion of $PGF_{2\alpha}$ by the uterus. In cattle, $PGF_{2\alpha}$ goes from the uterus to the ovary through a vascular countercurrent diffusion system which avoids dilution in the systemic circulation. Although the specific mechanism through which $PGF_{2\alpha}$ induces the regression of corpus luteum still hasn't been defined properly, it is thought that $PGF_{2\alpha}$ leads to vasoconstriction of arterioles supplying the luteal tissue, thus provoking ischemia. In addition, it is supposed that $PGF_{2\alpha}$ binds to specific receptors on large luteal cells and activates a cascade of events resulting in the death of these cells.
The knowledge of the described follicular dynamics allows the manipulation of the timing of ovulation for management and convenience purposes. The main procedures formulated are the hormonally induced ovulation and superovulation. The first one necessitate a premature luteolysis which can be achieved through the administration of Prostaglandin $F_{2\alpha}$. In fact, injections of $PGF_{2\alpha}$ between day 7 and 18 of the oestrus cycle cause the manifestation of oestrus in about three days (60-80 hours after the administration). Moreover, it must be taken in consideration that the corpus luteum of the cow is not sensitive to $PGF_{2\alpha}$ between days one and 6 of the cycle.
Secondly, the concept of superovulation consists in providing the donor with higher than normal levels of FSH in order to recruit and select greater number of follicles (Senger, 2003).

3. APPLICATIONS OF EMBRYO TRANSFER TECHNOLOGY

3.1 Genetic improvement

With the evolution of commercial embryo transfer in the 1970s, its prevalent use in animal production programs was the spread of desirable phenotypes. Nevertheless, the University of Guelph developed the MOET approach (multiple ovulation and embryo transfer) in 1987, a method of increasing reproductive potential. They displayed that MOET programs could lead to improved selection intensity and reduced generation intervals, resulting in enhanced genetic gains that approached twice those reached with traditional progeny test schemes. Embryo transfer is now routinely used to generate artificial insemination sires from the best productive cows and tested bulls. Furthermore, newfound genomic techniques are being applied more and more to select embryo donors. Genomic analysis has become indispensable for the selection of bull dams to be used in embryo transfer. Even though economics would seem to exclude the use of embryo transfer techniques for anything but semen production at this time, the commercial cattle industry took advantage in the use of commercial bulls generated through well-conceived MOET programs. The effectiveness of MOET programs has also turned to the use of this technology to genetically test AI sires; bulls were tested through production records from siblings instead of offspring. It was feasible to genetically test a bull in 3.5 years instead of 5.5 years through traditional progeny testing schemes, which also effected in reducing generation intervals (Mapletoft, 2013). It is now acknowledged that significant limitations to the efficiency of MOET programmes in cattle can be the poor average and high variability of embryo numbers (Simianer, 2016). Anyhow, it is now plausible to think, in order to overcome such limits, of using the non-surgical collection of oocytes (ovum pick-up technique), with following *in vitro* maturation and fertilization (Gordon, 2017).

3.2 Embryo import-export

The capacity to employ embryos to avoid the transmission of infectious diseases makes them perfect for the international transportation of animal germ plasm. The intercontinental transfer of live animals also implies high costs, while a whole herd can be transported in the shape of frozen embryos, for less than the price of a single flight ticket. Further advantages of embryos for the international flow of animal genetics includes lower risk of disease transmission, less quarantine costs, a broader genetic base from which to choose, the preservation of the original genetics within the exporting country , the ability of the animals to adapt. Adaptation is significant especially in tropical and subtropical environments, where the resulting calf would have the chance to adapt first while in the uterus and then while suckling a recipient cow native of the area.

Even though handling procedures suggested by the IETS make it possible to export *in vivo*-derived embryos without risk, it is different with embryos obtained *in vitro*. In fact the *zona pellucida* of *in vitro*-produced bovine embryos is different from that of *in vivo*-derived embryos and it has been demonstrated that pathogens tend to stay more tied with *in vitro*-produced embryos after washing than with *in vivo*-derived embryos. This is likely to have significant consequences for international movement, and protocols may subsequently be modified (Mapletoft, 2013).

3.3 Disease control

Many large studies have now reported that *in vivo*-produced bovine embryos do not transfer infectious diseases. Indeed, the IETS has classified disease agents based on the risk of transmission by a bovine embryo. Category 1 diseases involve disease agents for which enough evidence has been gathered to prove that the risk of transmission is insignificant, under the condition that embryos are properly manipulated between collection and transfer. Proper handling includes: microscopic inspection of the *zona pellucida* at >50X magnification to guarantee that it is undamaged and free of adherent material; ten washes of the embryo with at least 100-

fold dilution of each wash; in some cases two trypsin treatments to separate viruses that tend to adhere to the *zona pellucida* (Mapletoft, et al., 2005). Category 1 diseases include Enzootic bovine leucosis, Foot and mouth disease (cattle), Bluetongue (cattle), Brucella abortus (cattle), Infectious bovine rhinotracheitis, Pseudorabies in swine and Bovine Spongiform Encephalopathy (BSE).
Category 2, 3 and 4 diseases are those for which not enough data investigation has been provided yet. Nevertheless, it is significant that none of the infectious diseases analysed have been transferred by *in vivo*-produced bovine embryos. Therefore, it has been proposed that embryo transfer could be used to save genetics in view of a disease outbreak, which could be a valuable alternative in establishing disease-free herds (Mapletoft, 2013).

3.4 Embryo-therapy

Repeat breeding is one of the main problems in dairy cows. Repeat breeders are cows without anatomical or infectious malfunctions that do not become gravid after three or more reproduction attempts or many artificial inseminations. Repeat breeding reduces dairy earnings because of wasted semen and insemination costs, extended inter-calving periods and higher veterinary expenses, culling and replacement costs.
The causes of repeat breeding are multifactorial: fertilization can fail because of poor oocyte quality or issues related to artificial insemination, one of which is insemination at an inadequate time. Fertilization failure can occur also because of chromosomal abnormalities, heat stress and endocrine issues caused by high milk production or unbalanced alimentation. There are many studies reporting that oocytes in repeat breeder cows are inferior in quality compared to those of healthy cows and that it is this oocyte quality deficiency that interferes with fertilization or causes embryo mortality.
Oocytes generated by high-producing cows during lactation turned out to evolve into embryos of lower quality than oocytes from non-lactating cows, cows with average milk production or heifers. Other researchers discovered the hormonal asynchrony suprabasal progesterone levels and postponed LH peaks around oestrous in repeat

breeder cows, and these alterations extended the lifespan of preovulatory follicles and prejudiced the final maturation of oocytes. Fertilization failure can also occur because of deficient oestrus detection leading to inappropriate time of AI, erroneous AI technique, poor semen quality and obstructed oviducts.

Embryo death is the main cause of reproductive failure. In high-yielding dairy cows, the utmost embryo loss happens during the first week post conception. The factors leading to early embryo mortality to day 7 post conception focus on the early embryo's incapacity to develop as a result of poor oocyte quality or an unsuitable uterine environment related to nutritional factors, heath stress, endocrine imbalance and uterine disorders. A surplus of dietary protein has a deleterious effect on the uterine environment as it increases the concentration of ammonia in the blood and uteri of cows. A low progesterone level due to its enhanced metabolism in high-producing cows impoverishes the uterine environment such that it is incompetent to support early embryonic evolution. Endometritis may lead to embryo death by endometrial harm, bacterial toxins and inflammatory mediators such as prostaglandins, nitric oxide, reactive oxygen species and cytokines; indeed a high incidence of endometritis has been reported in repeat breeder cows (Nowicki, 2021). Many studies have evaluated the use of embryo transfer to improve fertility in repeat breeder cows.

Tanabe et al. confronted the fertility of normal and repeat breeder cows as embryo recipients. Fresh embryos were placed surgically into uteri and groups were checked for pregnancy. There were no substantial differences in pregnancy rates on day 60 between normal and repeat breeder recipients (82% and 70%, respectively) (Tanabe, et al., 1985). Rodrigues et al. compared pregnancy rates in repeat breeder Holstein cows after artificial insemination and cows after embryo transfer. Pregnancy rates were higher after ET (41,7%) then after AI (17,9%) (Rodrigues, et al., 2007). To bypass oestrus detection during ET process, an option is timed artificial insemination (TAI) in superovulated donors and timed embryo transfer (TET) in embryo recipients. Son et al. assessed pregnancy rates after controlled internal drug release (CIDR) TAI or TET protocols confronted with the rates post AI after a single $PGF_{2\alpha}$ injection in the luteal phase (8-13 days after oestrus) and AI post oestrus in lactating

repeat breeder dairy cows. Cows at casual stages of the oestrus cycle received the CIDR device and 2 mg estradiol benzoate (day 0), a $PGF_{2\alpha}$ injection at the moment of CIDR removal on day 7 and a 1 mg estradiol benzoate injection on day 8 for ovulation synchronization. The cows then received TAI on day 9, 30 hours after the second estradiol benzoate injection or TET on day 16 using defrosted embryos. The pregnancy rate was remarkably higher in the ET group (53,8%) than in the group for AI at detected oestrus (18,5%) or the TAI group (7,7%) (Son, et al., 2007) . Anyway, estradiol benzoate is prohibited in EU countries.

Furthermore, embryo transfer was especially successful in infertile cows that had already experienced multiple failed breeding efforts. The cows bred more than three times had a considerably lower rate of pregnancy outcome than cows bred less than three times if the cows were inseminated, but not if the cows received a fresh or vitrified embryo.

Some studied displayed that embryo transfer following artificial insemination enhanced pregnancy rates in repeat breeder cows compared with artificial insemination alone. It is supposed that greater pregnancy rate of ET after AI in repeat breeder cattle is a result of the higher release of interferon tau from the added embryos. In fact, a higher amount of interferon tau could help maternal recognition of pregnancy (Nowicki, 2021).

The results of the described studies are promising and indicate that ET enhances the pregnancy rate in repeat breeder cows by reducing the impact of poor oocyte quality and inappropriate uterine environment on fertilization and embryo evolution during the first 7 days after artificial insemination (Nowicki, 2021).

Another aspect that has a negative effect on dairy cattle fertility (including reduction in the follicular growth, postpartum anoestrus, oocyte quality, implantation and viability of the blastocyst and fetal development) is heat stress. In fact, heat stress has detrimental effects on oocyte maturation and on the developmental competence of preimplantation embryos because of the elevated maternal body temperature (Nowicki, 2021).

Heat stress is defined as a misbalance between the proportion of heat acquired by different sources as the body metabolism and the environmental conditions, against

the heat dissipation system by the body that triggers an increase in body temperature of the animal (Brown-Brandl, et al., 2018) (Interrelationships of Heat Stress and Reproduction in Lactating Dairy Cows, 2010) (Bernabucci, et al., 2014) (Collier, et al., 2017) (Leese, et al., 2019). Especially in tropical climates, cattle production systems are mainly grazing, and animals are persistently exposed to solar radiation, high environment temperature, high humidity and wind speed, increasing the effective ambient temperature. Accelerated respiratory frequency, sweat rate and peripheral vasodilation are multiple effector reactions toward heat stress, establishing the internal body temperature. Moreover, these responses may be inadequate at high temperature and high environment humidity conditions, reducing the body ability to dissipate heat. In cattle, a short-term rise of the temperature during oocyte maturation has a serious influence on embryo production and quality, leads to modifications in oxidative balance among the oocyte and the surrounding cumulus cells, and creates important alterations in gene expression in the oocyte, cumulus cells and blastocysts. Nevertheless, physiological adaptations to heat stress can prejudice other systems. For example in the cardiovascular system the redistribution of blood flow from the viscera to the periphery during heat stress provides the dissipation of body heat, but it also provokes a decreased perfusion of the placental vessels and a delay in fetal growth. Heat stress can also reduce the production of the hormones that control ovarian function including GnRH, LH and FSH, causing poor follicle maturation. Reduced ovarian function leads to a decrease in the number of ovulations and the quantity of viable embryos quantified in the embryo recovery rate, pregnancy and conception rate. Both dairy and beef cattle subjected to heat stress manifest a decrease in health and productivity performance, resulting in a reduction in the conception rate between 20% and 30%.

Firstly, the embryo's environment alludes to the conditions in the oviduct, considering that the fertilization happens there thanks to the changing in the composition of the medium in order to enable sperm capacitation, transport of mature sperm in the ampulla until arriving at the oocyte, and early embryonic development in the isthmus. The bovine oviduct is a small stretched organ, which links the ovaries to the tip of the uterine horns; gametes enter the oviduct and meet in the ampulla,

where fertilization takes place. Bovine embryos stay in the oviduct from 3,5 to 4 days and migrate to the distal end of the isthmus before they enter the uterus.

The oviductal fluid includes simple and complex carbohydrates, ions, lipids, phospholipids and proteins which act as substrates to generate lactate, pyruvate, glucose and amino acids, to preserve the embryo. Some of these proteins such as glucodelins and lattoferrin are implicated in the interaction with gametes, and others like oviductin (OVPG1), osteopontin and complement protein C3 play a role in early embryonic development. OVPG1 stimulates sperm capacitation while preserving mobility and viability and it is considered a protein with a shielding effect on the early embryo. Oviductin, which is localized in the perivitelline space and membrane of embryos before implantation, has then a protective activity toward the embryo. Consequently, modifications in the constitution of the oviductal fluid and gene expression indicate the capacity of the oviduct to suit the environment in various events from fertilization to early embryonic development.

It has been shown that an increase in the environment temperature of the donor females caused by the season change, immediately reduces the proportion of the viable embryos.

The consequence of heat stress on embryo quality can derive from environmental exposure; it has been demonstrated that heat stress can interfere with the oocyte RNA, heat shock proteins and other elements including antioxidants, undermining the later phases of its development. Similarly, alterations in sperm conditions can change the outcome of embryo development. In fact, it has been shown that an increase of the body temperature can diminish the total sperm count and motility, including modifications in the morphology of the spermatozoa and a rise in the ROS (reactive oxygen species) level.

Embryonic death has been linked to heat stress as a consequence of the cow internal temperature (over 39°C) which happens within the first 6 days of embryo growth, due to the scarcity of heat-tolerant proteins that protect the embryo in the uterus. Moreover, it probably reduces the capacity of the embryo to become transcriptionally competent. When it comes to the 2-cell embryo stage, high temperature induces alterations in microfilament and microtubule network, depolarization of the cell due

to higher number of swollen mitochondria and production of ROS. Later embryos, such as blastocyst, elaborate thermotolerance by the storage of antioxidants such as reduced glutathione (GSH), in reply to heat-inducible production of ROS and the capability to synthesize heat shock protein 70, HSP70 (Naranjo-Gòmez, et al., 2021).

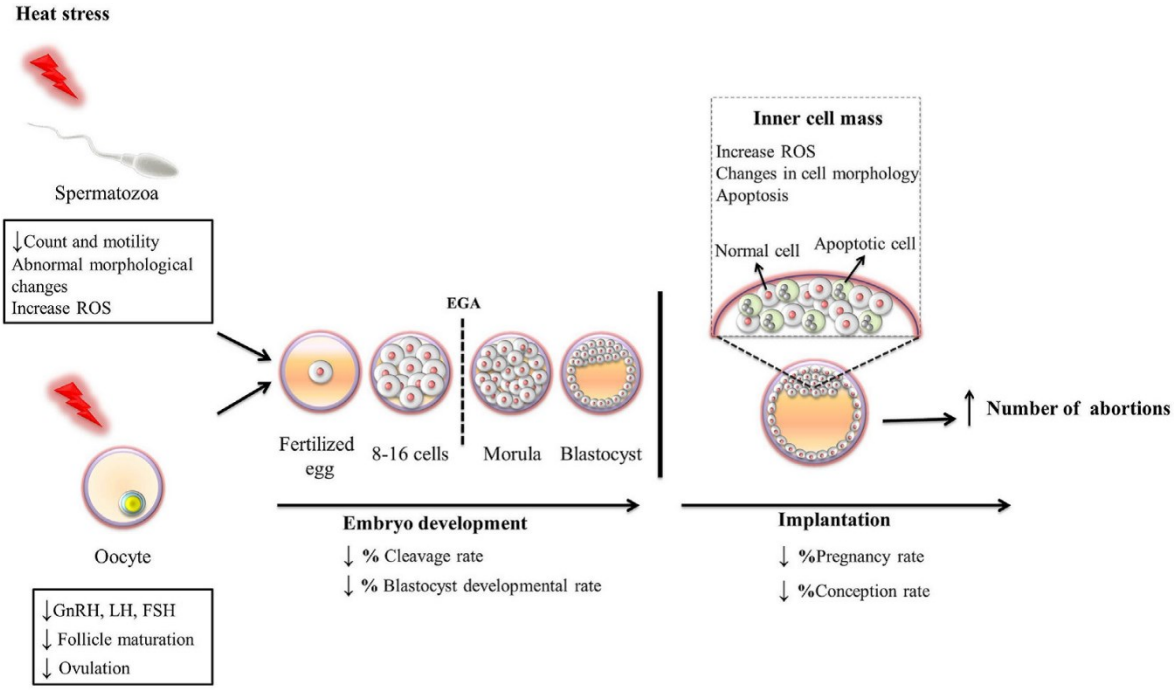

Figure 1. Effects of heat stress in the different stages of embryo development (Naranjo-Gòmez et al., 2021).

Considering all this information, it is plausible to think to embryo transfer as a therapy to avoid the occurrence of all the above conditions related to heat stress consequences. In fact, thanks to the application of the embryo transfer technique, it is possible to bypass all the delicate phases that precede the implantation of the embryo. Because oocytes and early embryos are seriously injured by hyperthermia, the transfer of embryos at the morula or blastocyst stages, when they have achieved resistance to high temperature, helps reestablishing fertility during heat stress periods (Baruselli, et al., 2020).

In this regard, as a management strategy to maintain high pregnancy rates throughout the year, it would be a useful idea to create embryo banks in order to be able to transplant embryos when necessary (in case of repeat breeder cows, during hot and

sultry periods or to improve fertility in very young animals). For instance it could be possible to produce embryos during cooler months, when oocyte quality is superior, and use them to obtain pregnancies during periods of heat stress, when oocyte quality is negatively affected.

During the warmer months, one approach to enhance the efficiency of using fresh embryos produced *in vivo* or *in vitro* is to use donor categories less affected by the compromising effects of heat stress on oocyte quality and embryo production, such as heifers and non-lactating cows. A further alternative is to select animals with higher thermotolerance as donors, such as well adapted breeds that are naturally more resistant to heat stress or *Bos taurus* females genetically ascertained as more efficient to thermoregulate. Moreover, the application of timed embryo transfer protocols, in which oestrus detection in recipients is not needed, has simplified the management and improved the success of embryo transfer programs during heat stress (Baruselli, et al., 2020).

3.5 Preservation of endangered breeds

There is significant interest in plans for the safeguard of livestock breeds that, according to the Food and Agriculture Organization, may be at risk of extinction. The FAO's Global Data Bank for Domestic Livestock indicated 221 cattle breeds in danger of disappearing, most of these (60%) in developed countries. Intensification of agriculture in the Western world resulted in an increased dependence on a small number of breeds, with subsequent disregard of the others. In developing countries, menace to genetic variability usually takes the form of enhanced use of AI and thoughtless crossbreeding of indigenous breeds. The expansion of farming in these countries can imply that indigenous breeds are in danger to be driven to extinction because native farmers, aspiring to higher yield, utilize exotic breeds such as Holsteins and Friesians. The FAO reports that it is important to use the wider number of animal species for breeding with the aim of preserving genetic resources. There are those who consider it as a duty for humans to conserve biological diversity, and this applies to both farm animals and wild species; aside from scientific aspects,

probably there are also cultural and ecological reasons. Even though humans were responsible for creating several breeds of farm animals over the centuries, in the past 50 years they are also chargeable for the extinction of many of these same breeds. The vanishing of many breeds typically occurred in the name of progress, led by intensification of food production systems that have promoted the most yielding breeds. For instance, in Ireland and the UK, the brown-and-white of the Dairy Shorthorn has been completely substituted by the black-and-white Holstein-Friesian. Embryo transfer technology is now considered an essential instrument for genetic preservation of endangered species and breeds; in fact it allows the creation of embryo banks and the possibility to transfer embryos into populations with reduced biodiversity. In native breeds of cattle embryo transfer can be used to preserve genetic lines with good maternal characteristics (Gordon, 2017).

4. DONORS SELECTION AND MANAGEMENT

Selection of the donors is based on two main factors: genetic merit, usually assessed by the owner and based on performance, and reproductive adequacy, determined by the veterinarian. The donor must be in proper healthy condition, free form primary diseases, a minimum of 50 to 60 days post partum and cycling regularly. Normally, cows with past reproductive issues do not become good donors.

Additionally, donors are evaluated by checkup of the cervix, uterus and ovaries per rectum through ultrasonography, also to secure that they are free from adhesions and other palpable lesions. It is advisable to test the openness of the cervical canal with a cervical dilator for adequate internal diameter to allow the transit of a collection catheter, particularly if the potential donor is a heifer or of *Bos indicus* breeding. This avoids the sporadic frustration of being incapable of dealing with the cervix after a series of expensive hormonal treatments.

Vaccinations should be current for local diseases. Blood typing of both donor and sire before or at the time of embryo transfer for successive recognition of progeny is highly recommended and usually required before export (Youngquist, et al., 2007).

Recent studies observed that the number of follicles in ovarian follicular waves in cattle were extremely variable among animals. For example, Singh et al have reported divergences in superstimulatory response between cows that had more than 30 follicles (3 to 5 mm) versus those with fewer than 30 follicles (Singh, et al., 2004). Consequently, it might be possible to select donor cows based on the number of follicle present at the time of follicle wave emergence detected using ultrasonography (Mapletoft, 2013). As just said, the antral follicle count (AFC; follicles $\geq$3 mm in diameter) is an extremely varying trait among animals, but with significant repeatability in the same individual. Therefore, females can be classified into low, intermediate or high AFC. Many studies in *Bos taurus* report a positive association between AFC and fertility parameters, such as enhanced quantity and quality of embryos, higher pregnancy rates and progesterone levels. This persistence in AFC in the same individual becomes a tactical resource to classify an animal by the AFC through a single ultrasound examination. Nevertheless, other elements such as

genetics, maternal environment, nutritional status and health also seem to influence the AFC. The AFC appears to have an impact on the production of cattle embryos, both *in vivo* and *in vitro*. In a study, for *indicus-taurus* animals, the average number of embryos per collection was greater for females with high in comparison with low AFC animals (6.9 ± 5.3 *vs* 1.9 ± 2.1) (Morotti, et al., 2015).

Most recently, it has been documented that the follicle numbers were related to circulating concentrations of Anti-Müllerian hormone (AMH) (Ireland, et al., 2011) (Monniaux, et al., 2013), a glycoprotein belonging to the transforming growth factor-β family that is produced by the granulosa cells of all growing follicles. It could be possible to measure the ovarian supply of gonadotropin-responsive follicles by detecting the concentrations of AMH in the circulation. Rico et al have reported a connection between AMH production and superovulatory response in Holstein donors and have recommended values that could be used to discriminate between cows producing more or less than 10 transferable embryos (Rico, et al., 2012). Thus, AMH can be considered a viable endocrine marker of AFC in cattle. In fact, it has been demonstrated that bovine females with high AFC (>25 follicles) have higher circulating AMH concentrations compared to the females with low AFC (<15 follicles) and also that high AFC was favourably associated with endometrial thickness. Furthermore, augmented endometrial thickness was related with greater embryonic implantation rates. The low AFC in dairy *taurus* females (Holstein) was correlated with many unfertility factors, such as smaller ovaries, lower chances of pregnancy at the end of the breeding season, inferior reactivity to the superovulation treatment, less viable embryos, lower levels of progesterone and AMH and reduced endometrial thickness. Regarding these considerations, it is assumed and documented that in Holstein cows there is a straight correlation between high AFC and reproductive efficiency markers. Anyway, it is not possible to use the *Bos taurus* AFC criterium for reproductive selection also for *Bos indicus* females because in *indicus* cattle contradictory results were found. Thus, the variability of the results indicates the need to always give priority to genetic merit in the choice of a donor, and not AFC (Morotti, et al., 2015).

Furthermore, in order to evaluate if a cow has the potential to become a good embryo donor it is possible to measure progesterone levels in blood serum. In a study it was reported that both the total number of embryos and their quality increased with increasing concentrations of progesterone at 5 days after the superovulatory oestrus. Inadequate superovulatory response, lower production and ability of transfer of embryos in lactating cows compared to the heifers were related to lower concentrations of progesterone at diestrus and before and after the superovulatory treatment. The cows with lower progesterone level at the time of insemination produced more embryos of higher quality and did not differ substantially from donor heifers. The yield and quality of embryos of cows with high serum levels of progesterone at insemination was considerably lower compared to the heifers (Novotny, et al., 2005).

It must also be considered that the number and quality of embryos can be affected by the nutritional and metabolic status of donors before, during and after the superovulatory treatment and recovery of embryos. In fact, it is widely known that nutritional and metabolic condition of females is strictly related to reproductive success. Diet can affect ovarian activity through effects at different levels of the hypothalamus-pituitary-ovarian axis. Modifications in the plane of nutrition can influence follicular growth by causing alterations in plasma metabolites and metabolic hormones, including insulin and IGF1, or in hormones and growth factors in follicular fluid. Diet can also influence oocyte morphology and developmental ability and embryo yielding. It has been reported that over-feeding can be detrimental for the developmental quality of oocytes and embryos produced *in vivo* and *in vitro*. Moreover, limited feeding can have a favourable effect on oocyte quality (Ponsart, et al., 2014). Freret et al demonstrated that an increase in insulin levels during a short time period has a positive effect on small follicle growth before superovulation (Freret, et al., 2006). Anyway, in the heifer, it has been reported that hyperinsulinaemia negatively affects the quality of oocytes. These results sustain the idea that it may be feasible to regulate insulin concentrations temporarily to enhance reproductive success, i.e. rise insulin during the phase of follicle growth and then return to pre-stimulated levels just before ovulation so as not to have a deleterious

effect on oocyte quality. Thus, it is possible to improve fertility and embryo quality by using diets or dietary supplements (like propylene glycol, which increases plasma glucose and insulin and decreases non esterified fatty acids and β-hydroxybutyrate) that induce a programmed sequence in circulating insulin concentrations (Ponsart, et al., 2014).

Furthermore, Colour-Doppler ultrasonography (CDU) can be used for a detailed analysis of the ovary and uterus, particularly to investigate local blood flow in ovarian follicles and the *corpus luteum*. Even though CDU is mainly used for the diagnosis of early pregnancy or the selection of recipients, this tool can also be useful for the selection of oocyte donors according to blood flow to the ovary. The idea is that more blood flow in the ovary leads to a healthier intraovarian environment with higher progesterone levels and removal of reactive oxygen species, producing higher quality oocytes. This application of CDU on the selection of oocyte donors has been evaluated in a recent study that, considering the blood flow to the ovary, showed that although low blood flow donors had more total recovered oocytes, blastocyst rates were increased in high blood flow and median blood flow donors (Villasenor-Gonzàlez, et al., 2021).

5. SUPEROVULATION

5.1 Historical perspectives

The purpose of the treatments used to induce superovulation in embryo transfer programs is to reach the maximum number of transferable embryos with a high probability of producing pregnancies.

Initially practitioners used eCG to induce superovulation in cattle. In 1978 Elsden reported a greater superovulatory response after treatment with a crude pituitary extract containing FSH plus 20% LH, compared with the use of eCG (Elsden, et al., 1978). Also Monniaux observed that after the use of a pituitary extract containing FSH rather than eCG the ovulation rate and the percentage of cows with more than three transferable embryos tended to be higher (Monniaux , et al., 1983).

The majority of practitioners changed from eCG to FSH mainly because of the problems associated with the prolonged stimulation of the ovaries after a single injection of eCG. In fact the half-life of eCG has been recognized to be 40 hours in the cow and its persistance in the bovine circulation has been shown to be up to 10 days. For these reasons the long half-life of eCG caused continued ovarian stimulation, unovulated follicles, abnormal endocrine profiles and reduced embryo quality. These problems associated with the persistance of eCG were largely overcome by the intravenous injection of antibodies to eCG at the time of the first insemination, 12 to 18 hours after the onset of oestrus. Unluckily, the pharmaceutical company that developed the commercial monoclonal antibody to eCG (Neutra-PMSG; Intervet, The Netherlands) interrupted its production in the 1990s.

Nowadays most cows are superstimulated with pituitary extracts containing FSH. However, these crude pituitary extracts also contain a large amount of LH.

Purified pituitary extracts with low LH contamination have been reported to enhance the superovulatory response in cattle. Chupin superstimulated three groups of dairy cows with 450μg of pure FSH and various amounts of LH and demonstrated that the average ovulation rate and the number of recovered and transferable embryos increased as the quantity of LH decreased (Chupin, et al., 1984). Several experiments

also supported the hypothesis that the damaging effects of high doses of pituitary gonadotropins on ova and embryo quality are caused by an excess of LH.
Despite it is generally believed that some LH is necessary for successful superovulation, endogenous LH levels should be adequate to promote the final follicle growth (Bò, et al., 2014).

5.2 Current superstimulation treatment protocols in cattle

The approximate half-life of FHS in the cow is 5 hours or less so it must be injected twice daily to efficiently induce superovulation. The usual treatment is twice daily intramuscular administration with FSH for 4 or 5 days with a total dose of 20 to 50 mg (Armour) of a crude pituitary extract or 400 mg NIH-FSH-P1 of a partially-purified pituitary extract (Folltropin-V; Bioniche Animal Health Inc., Belleville, Ontario, Canada). 48 or 72 hours after the beginning of the treatment, $PGF_{2\alpha}$ is administered to induce luteolysis. Oestrus takes place in 36 to 48 hours, with ovulations beginning 24 to 36 hours later.
Many practitioners prefer to decrease the amount of FSH schedules and others use constant-dose schedules. Some of them treat with $PGF_{2\alpha}$ on the third day of the treatment protocol, others prefer to treat with $PGF_{2\alpha}$ on the fourth day and many don't treat with FSH on the day after the administration of $PGF_{2\alpha}$. Even though there are no studies supporting one approach over the other, recent experiments have suggested that ovulation rate can be enhanced in at least some donors if FSH is dispensed over 6 or 7 days. In all cases, inseminations are commonly done 12 and 24 hours after the onset of oestrus.
Thanks to the information obtained by monitoring the follicular development through ultrasonography it is known that the estimated time of emergence of the second follicular wave is 8 to 12 days after oestrus (corresponding to 7 to 11 days after ovulation), and a cohort of growing follicles would be present around that time. Nevertheless, the day of emergence of the second follicular wave has been shown to be 1 or 2 days earlier in the three-wave cycles compared to the two-wave cycles and it also differs between individual animals.

In this respect, it has been demonstrated that superovulatory response was maximized when superstimulatory treatments were initiated at the time of the follicular wave emergence. Starting gonadotropin treatments at least 1 day before or after wave emergence considerably reduced the superovulatory response compared with initiating treatments on the day of wave emergence.

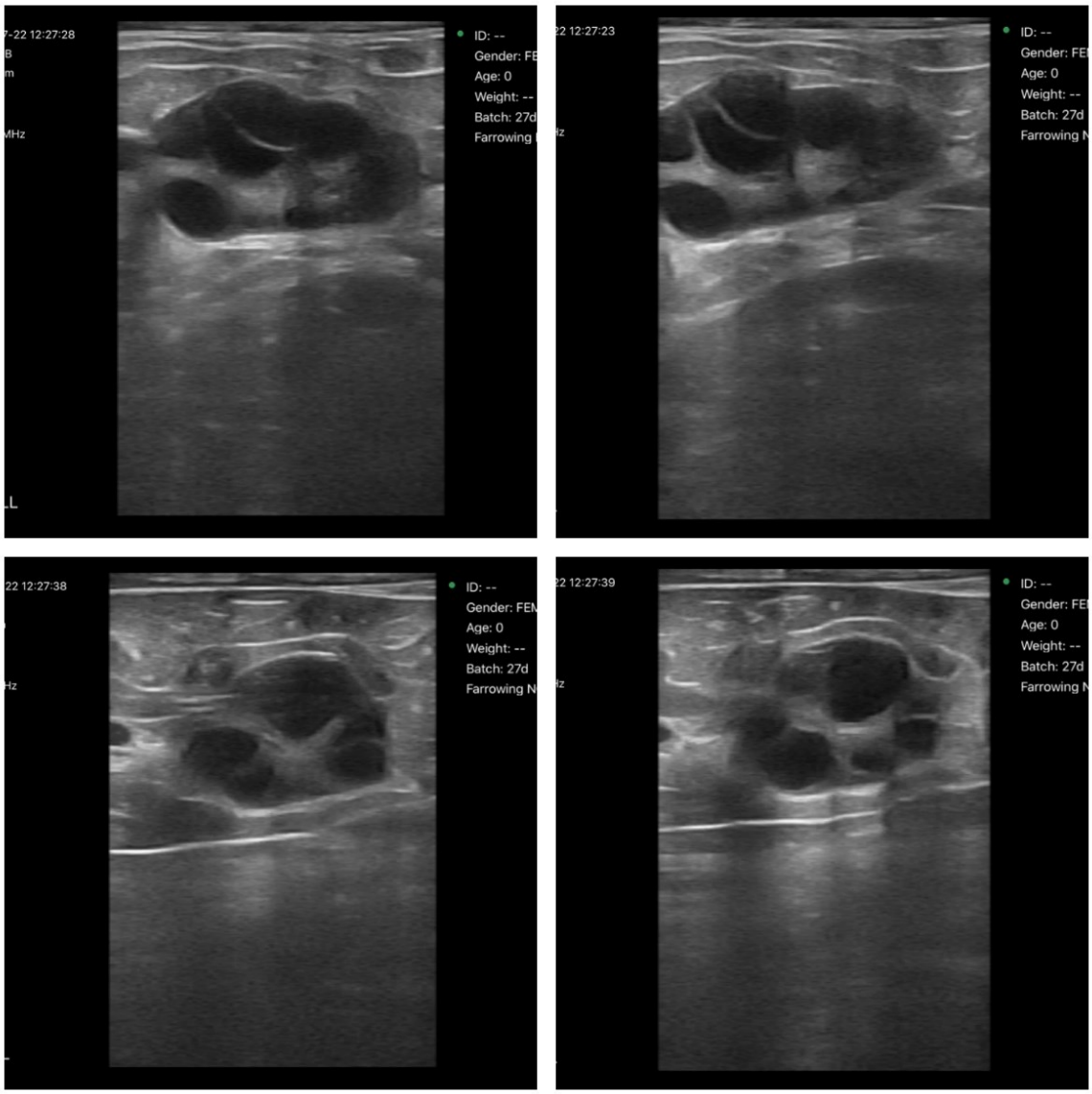

Figure 2 Ultrasound appearance of ovary with multiple follicles after superstimulatory treatment (Courtesy of Professor G. Morini)

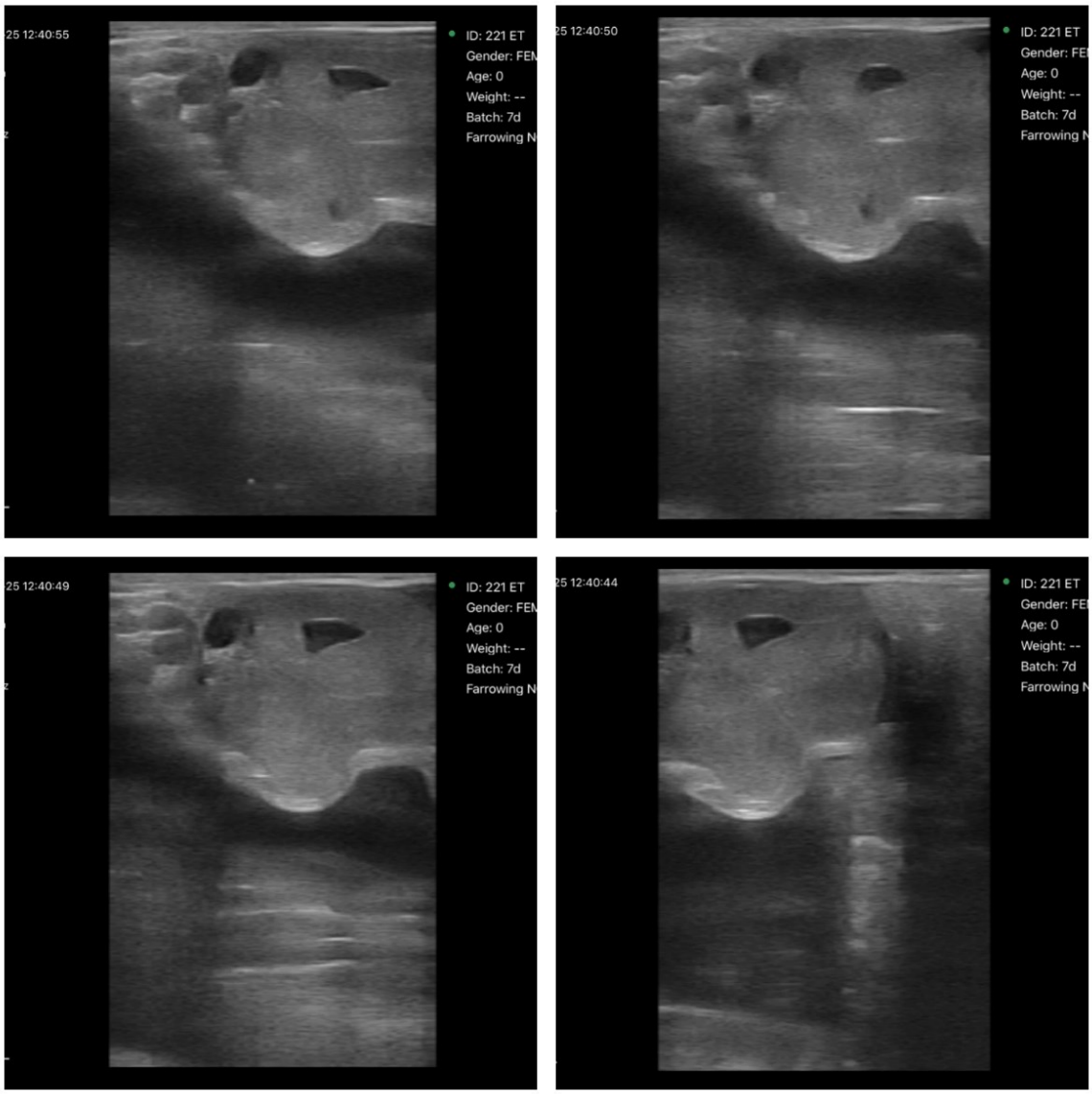

Figure 3 Ultrasound appearance of the subsequently developed corpora lutea, a week after superovulation (at the time of flushing) (Courtesy of Professor G. Morini)

Considering the length of the developmental phases of the dominant follicle in two-wave and three-wave interovulatory intervals, the probability at any given time that the dominant follicle is not functionally dominant is nearly 30% for two-wave animals and 35% for three-wave animals. Consequently, only 20% (4 or 5 days) of the oestrus cycle is accessible for starting the treatment at the time of the follicular wave emergence. Thus, the 80% of the oestrus cycle is not suitable to obtain an optimal superovulatory response. Since most treatment protocols used today imply the initiation of superstimulatory treatments after the exogenous control of follicular

wave emergence, there is no longer the necessity to wait until midcycle (which implies monitoring oestrus and an obligatory delay).
The manipulation of the follicle wave emergence can be pursued in many ways:

- **Estradiol and progestin**: the most frequent protocol used to synchronize the emergence of a new follicular wave for superstimulation requires the administration of 2,5-5 mg of estradiol-17β or 2-2,5 mg of estradiol benzoate plus 100 or 50 mg of progesterone by intramuscular injection on the day of insertion of an intravaginal progestin device. The treatment with estradiol inhibits the release of FSH and provokes follicle atresia. When estradiol has been metabolized, FSH increases and a new follicular wave emerges, in general 4 days after treatment. At that point gonadotropin treatments begin. Consequently, this treatment provides for a new cohort of 3 to 5 mm follicles to develop at the same time. Even though the number of transferable embryos has not always been higher than when cows were superstimulated between 8 to 12 days after oestrus, the fertilization rate in donor cows superstimulated after estradiol and progestin medication was remarkably higher than in the control cows in at least two studies. The outcome of estradiol and progestin treatments was the growth of a new follicular wave and as a consequence a more homogeneous group of viable follicles with competent oocytes at the time of gonadotropin treatment. It has been demonstrated that subordinate follicles can be preserved from atresia by the ablation of the dominant follicle or with FSH treatments at the moment of dominant follicle selection. In addition, Goulding et al. have proved that premature superstimulatory treatment in the luteal phase in heifers with a progestin device resulted in poor embryo quality, which allegedly happened because subordinate follicles that were already undergoing atresia were stimulated (Goulding , et al., 1994). Thus, estradiol and progestin treatments not only eliminated the necessity of oestrus synchronization and detection prior superstimulation, but also managed to enhance ova and embryo quality (Bò, et al., 2014).

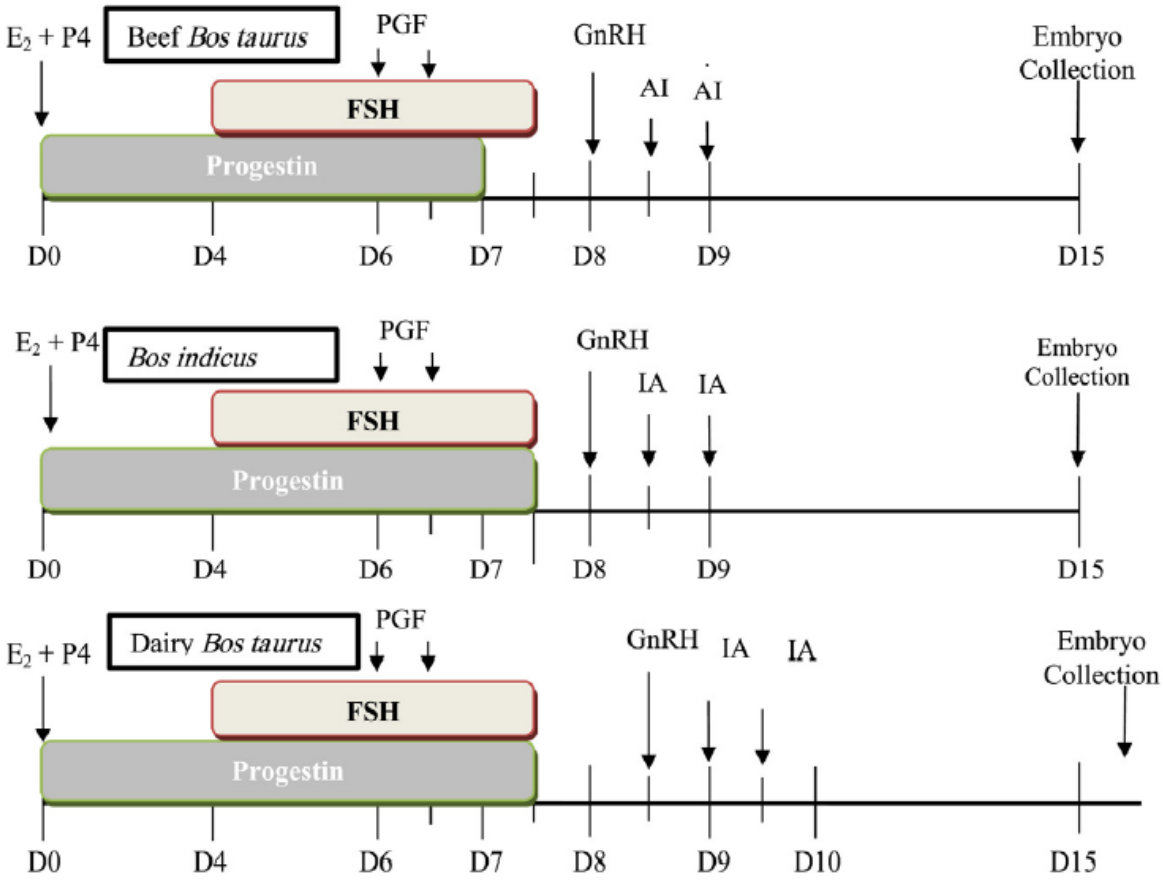

Figure 4 Recommended estradiol-based superstimulatory treatments. D: Day; E_2: 2.5-5 mg estradiol-17β or 2-2.5 mg estradiol benzoate; P_4: 50 or 100 mg progesterone; PGF: $PGF_{2\alpha}$ (Bò et al., 2014).

- **Follicle ablation**: another option to obtain the synchronization of follicular wave emergence is to remove the inhibitory effect of the dominant follicle using ultrasound-guided follicle ablation. Early studies involved the ablation of all follicles ≥ 5 mm but later it was showed that only the ablation of the two largest follicles was needed to guarantee that the dominant follicle was removed. Superstimulatory treatments are then started 1 to 2 days later, at the time of emergence of a new follicular wave. Even though follicle ablation has been demonstrated to be highly successful, ultrasound equipment and trained personnel are needed. Follicle ablation is difficult to use in the field, particularly when donors are distant from the embryo production center, so this technique is most suitable for embryo production centers, where the equipment and the staff are present and donors are kept (Bò, et al., 2014).

- **GnRH**: it has been demonstrated that after GnRH induced ovulation, a new follicular wave will emerge about 1,5-2 days after treatment. Nevertheless, follicular wave emergence takes place only when GnRH induces ovulation, and recent studies have displayed that the injection of GnRH at casual stages of the estrus cycle leads in ovulation in 44% to 54% of dairy cows, 56% of beef heifers and 60% of beef cows. Consequently, the gap from GnRH treatment to wave emergence might be too inconsistent for superstimulation. Actually, Deyo et al. reported poor embryo production after synchronization of follicular wave emergence with GnRH (Deyo, et al., 2001). Although recent results have showed more promising results. A progestin device has been inserted at casual stages of the oestrus cycle and GnRH has been injected 2 or 3 days later with superstimulation treatments initiating 1,5 to 2,5 days later. Ovulatory response might have been enhanced because the presence of the progestin device has inhibited ovulation of mature follicles and as a result it improved the efficiency of GnRH.

  Most fixed time artificial insemination (FTAI) protocols using GnRH to synchronize follicle wave emergence use a form of presynchronization to enhance the ovulatory response to the first administration of GnRH. Bò et al. recently displayed a series of experiments with the aim of formulating a protocol for superstimulation after ovulation induced by treatment with GnRH (Bò, et al., 2010). The recommended superstimulation protocol requires the administration of $PGF_{2\alpha}$ at the moment of insertion of a progestin device. Seven days later, with the device still implanted, GnRH is injected to induce ovulation of the persistent follicle and synchronization of follicle wave emergence. FSH treatments begin 36 hours after the administration of GnRH. Despite this protocol was developed for 4 days of FSH treatment, a 5-day superstimulation protocol can be achieved easily by postponing the removal of the progestin device by 1 day. In general, in these experiments, more than 95% of animals ovulated to the first GnRH injection and superovulatory response and ova/embryos quantity and quality were comparable to that achieved using estradiol to synchronize follicular wave emergence (Bò, et al., 2014).

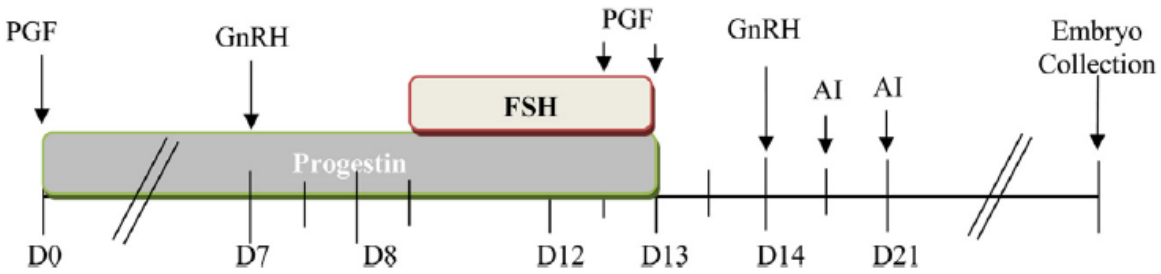

Figure 5 Recommended GnRH superstimulatory treatment for Bos taurus donors. D: Day; PGF: $PGF_{2\alpha}$ (Bò et al., 2014).

- **Subordinate follicle development**: during a normal follicular wave there is a regression of the subordinate follicles caused by the decreasing concentrations of circulating FSH. This happens because the dominant follicle secretes estradiol and inhibin. Small follicles necessitate FSH to develop, and follicles as small as 1 mm in diameter will start growing under the influence of FSH. It has been hypothesized that, in order to recruit follicles for superstimulation, small antral follicles have to grow to a diameter of 3 or 4 mm at which time the regular 4 or 5 day superstimulatory treatment protocol could be started. It should take 2 to 3 days for a 1 mm follicle to reach 3 or 4 mm and this implies adding 2 or 3 days to the superstimulation treatment protocol. Beef donors were efficiently superstimulated using this procedure at casual stages of oestrus cycle and without consideration of the presence of a dominant follicle. Small doses of FSH were injected twice daily for 2 days and then the usual FSH treatment protocol was started with no increment in the total quantity of FSH administrated. Alternatively, the 2 days of FSH pretreatment could be substituted with a single injection of eCG. Indeed, it was hypothesized that the eCG recruited extra follicles into the wave prior to the beginning of the FSH treatments (Bò, et al., 2014).

- **Follicular waves generated exogenously**: it has been recently investigated that prolonging the FSH treatment protocol to 7 days, with no enhance of the total quantity of FSH injected, increases the number of ovulations and the synchrony of ovulations, and tends to rise the average number of fertilized ova and transferable embryos. In brief, the extended superstimulatory treatment protocol

outcome is a higher number of follicles reaching an ovulatory size and gaining the capacity to ovulate with an increased number of ovulations, and with no reduction of oocyte and embryo quality. Thus, extended FSH treatment protocols might be a successful strategy to recruit small follicles in the follicular cohort eligible for superstimulation, and to give the extra time needed for these follicles to reach an ovulatory size and acquire the capacity to ovulate. Moreover, these conclusions indicates that the usual 4-day superstimulatory treatment protocols might not provide the necessary time for all follicles among the cohort to obtain the capacity to ovulate (Bò, et al., 2014). Undoubtedly this requires further study.

The necessity of FSH and LH at various times during superstimulation has been evaluated. Basic studies on follicular growth have displayed that FSH is needed for follicle recruitment and growth, unless the dominant follicles achieves 8,5 mm in diameter in *Bos Taurus* breeds of cattle and 6,2 mm in *Bos indicus* cattle. After selection, dominant follicles have LH receptors and become LH-dependent. For these reasons, follicles of superstimulated cattle might take advantage from the introduction of LH close to the end of the treatment protocol. Equine chorionic gonadotropin is a gonadotropin with FSH and LH effect and could produce a regular stimulus for the LH receptors of the growing follicle toward the end of a standard FSH superstimulation treatment protocol (Bò, et al., 2014). Recently Barros et al. documented a comparable betterment in embryo production when 1 mg pLH was added to each of the last two Folltropin-V injections in Angus donors (Barros, et al., 2013).

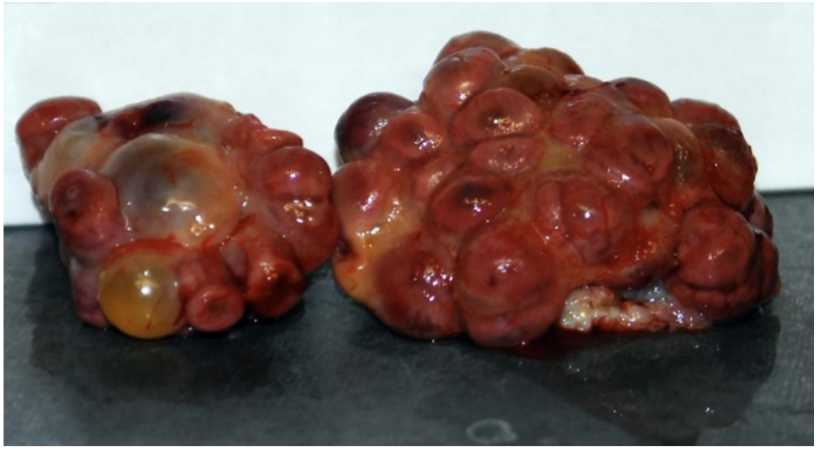

Figure 6 Macroscopic aspect of superovulated ovaries (Courtesy of Professor G. Morini)

Donor cows commonly undergo embryo collection at about 60 day intervals. Anyway, current studies indicate that cows can be superstimulated as often as every 30 days. In fact, when the cow reovulates, follicular wave patterns are re-established. Thus, the cow could be superstimulated the day after the expression of oestrus, 8 to 12 days later, or after synchronization of follicle wave emergence. By doing so, donor cows can be superstimulated in groups at about 30 to 35 days intervals. In recent studies, no variations were identified in embryo production when cows were superstimulated at intervals ranging from 28 to 30 days to more than 90 days (Bò, et al., 2014).

5.3 Fixed-time AI of donors

As it has already been mentioned, in addition to protocols formulated to regulate follicular wave emergence, recent protocols have been developed to control also ovulation and allow initiation of supertimulatory treatments and the insemination of donors at a self-appointed time (FTAI). The time of ovulation can be controlled through postponing the extraction of the progestin/progesterone implant and administration of GnRH/LH at the end of the superovulation protocol. Furthermore, delaying the LH peak with regard to $PGF_{2\alpha}$ treatment provides the growth of more follicles that achieve the capacity of ovulation, consequently resulting in more embryos.

Studies have been conducted to determine a superovulation protocol that enables FTAI in *B. taurus* and *B. indicus* cattle treated with P4-releasing devices and EB on the first day of the protocol (day 0). Protocols are entitled in accordance with the time of the first $PGF_{2\alpha}$ treatment to the time of P4 source removal, which takes place before the induction of ovulation to prevent the detrimental effect of high P4 concentration on embryo quality during the ovulation period. Consequently, when the $PGF_{2\alpha}$ is administrated on day 6 AM and the P4 device is removed on day 7 AM, the treatment is called “P-24” (a 24 hours interval between $PGF_{2\alpha}$ and P4 removal), while when the P4 device is removed on day 7 PM the treatment is called “P-36”.

No considerable differences in the number and quality of transferrable embryos have been identified between the P-24 and P-36 treatments. Both treatments can hence be used to superovulate *B. taurus* and *B. indicus* cattle with FTAI.

	Donors		Recipients
Treatment Day	**AM**	**PM**	**AM**
0	P4 device insertion + $PGF_{2\alpha}$		
4			P4 device insertion + GnRH
7	GnRH/LH		
8		FSH (20%)	
9	FSH (20%)	FSH (15%)	
10	FSH (15%)	FSH (10%)	
11	FSH (10%)	FSH (5%) + $PGF_{2\alpha}$	P4 device removal + $PGF_{2\alpha}$
12	P4 device removal + FSH (5%) + $PGF_{2\alpha}$		
13	GnRH/LH	FTAI	GnRH/LH
14	FTAI		
20	Flushing		FTET

Table 1 Timed embryo transfer programs using GnRH plus progesterone combinations in B. taurus donor cows and embryo recipients (Baruselli et al., 2011)

Further researches were conducted to establish the proper time to induce ovulation for FTAI in superstimulated *B. indicus* (Nelore) and *B. taurus* (Holstein) donors. Since the diameter of the dominant follicle at deviation and the diameter at which it achieves ovulatory capability are smaller in Nelore than in Holstein cows, it is logical that the suitable time to induce ovulation may diverge. As a consequence, treatment with GnRH or pLH to induce ovulation for FTAI in superstimulated *B. indicus* and *B. taurus* donors should be performed at 12 and 24 hours respectively, after the last FSH treatment (Baruselli, et al., 2011).

5.4 Commercial formulations

Nowadays many products containing FSH are available on the veterinary market (United States, Europe and Japan) to provoke superovulation in cattle. Each product contains a standardized amount of pFSH to induce superovulation with an established protocol. Among the residual impurities, it is important to regulate the concentration of LH because, as has already been said, a negative influence of LH has been demonstrated. The FSH/LH proportion must be appropriate to induce an effective and reproducible superovulation, but a small amount of LH enhances the ovarian response in cows. pFSH powder in marketed products is not pure but is quite high-purified against LH (Deguettes, et al., 2020).

The 1st generation of veterinary products made with pig pituitary extract were merchandised in the 1970s (e.g. FSH-P®) and the 2nd generation (current pFSH powder vial) with a monitored pLH concentration appeared in the 1990s (Bò, et al., 2014).

Excluding Antrin-R10 Al®, these products are dispensed in two units: one containing pFSH in powder form and the other for the solvent used to reconstitute the pFSH at the time of the first injection. The volume after reconstitution of the total dose of pFSH is 10 mL (Super-OV®, Stimufol®), 20 mL (Folltropin-V®, Pluset®) or 25 mL (Antrin-R10®) and, in the regular protocol, each injection contains 1-3 mL. The solvent is made of a saline solution including a preservative to avoid, after reconstitution, microbial contamination during the time of administration (3-4 days). To reach superovulation, the common protocol requires, after reconstitution of the lyophilized powder with the saline solution, intramuscular administration twice daily for 3-4 days.

Antrin-R10® is the most recent product on the market (3rd generation) and it is similar to none of the other products. In fact, the superovulation is achieved after just one subcutaneous injection versus 6-8 IM ones. pFSH powder is primary dissolved in a saline solution and then diluted with the sustained-release solution which contains an aluminium oxyhydroxide gel (Al-gel). The documented electrostatic interaction between the positively charged Al-gel and the negatively charged pFSH at

physiological pH leads to the absorption of 99,9% of the FSH. Thus, the release is protracted over 4 days as a result of the electrostatic interactions instead of the viscosity of the solution. Aluminium salts are generally used as adjuvants in vaccines and can stimulate the occurrence of granuloma but, at the low concentration used in Antrin-R10® and with SC injection, the inflammatory response in minimal.
Available on the market only in Japan, a worldwide licence application was filed for commercial use in Europe and America (Deguettes, et al., 2020).

Product	API	Origin	Excipients and solvent	Standard protocol
Super-OV® (United States)	FSH (powder)	Porcine	NaCl 0.9%, preservative (ND), water for injection (10 mL)	1 injection (1.6 mL) / 12 h 3 days (6 injections) Intramuscular
Folltropin-V® (Europe and United States)	FSH (powder)	Porcine	NaCl 0.9%, benzyl alcohol, NaOH, HCl, water for injection (20 mL)	1 injection (2.5 mL) / 12 h 4 days (8 injections) Intramuscular
Pluset® (Europe)	FSH (powder)	Porcine	NaCl 0.9%, chlorocresol, water for injection (20 mL)	1 injection (1–3 mL) / 12 h 4 days (8 injections) Intramuscular
Stimufol® (France)	FSH (powder)	Porcine	NaCl 0.9%, methyl parahydroxybenzoate and propyl parahydroxybenzoate, water for injection (10 mL)	1 injection (1.25 mL) / 12 h 4 days (8 injections) Intramuscular
Antrin-R10® (Japan)	FSH (powder)	Porcine	NaCl 0.9%, preservative (ND), water for injection (25 mL)	1 injection (1–3 mL) / 12 h 4 days (8 injections) Intramuscular
Antrin-R10 Al® (Japan)	FSH (powder)	Porcine	Solvent: NaCl 0.9%, water for injection (2.5 mL) + Sustained-release solution: aluminium oxyhydroxide 0.059%w, water for injection (0.5 mL)	1 injection (3 mL) Subcutaneous

Table 2 Marketed products containing FSH available in the USA, Europe and Japan to induce SOV in cattle. API: active pharmaceutical ingredient; ND: not determined (Deguettes et al., 2020).

5.5 Alternative routes of administration and pFSH formulations

Some scientists have analysed the induction of superovulation by Folltropin-V® or Antrin-R10® reconstituted in a saline solution in a single SC injection as an alternative to several IM injections (Deguettes, et al., 2020). In fact, the use of the

standard method of inducing superovulation by repeated administration of FSH is time-consuming and stressful for cattle. Research on alternative methods has been performed for many years due to a growing awareness of the above and thus a general obligation to ensure the welfare of farm animals (Kulus, et al., 2019).

Indeed, SC tissue is widely known to prolong the release by slowing the rate of diffusion of molecules. The single SC injection behind the shoulder of pFSH in Rubia Gallega, Fleckvieh or cross-breed beef cows revealed that the superovulation was not remarkably different from those using the protocol with 8 IM injection for 4 days. Nevertheless, the same SC injection in Holstein cows produced a lower superovulation efficacy than the multi-injection protocol. In Holstein cows, administering the SC injection in two steps (75% of the total dose at day 0 and 25% at day 2) enhanced the hormonal stimulation but superovulation was still somewhat lower compared with the treatment with 8 IM injections. These results show that there is considerable variability in pFSH response between different species of cows with a single SC injection.

Moreover, variability in the superovulation response has also been demonstrated within breeds depending on the site of injection. Indeed, SC injection in the neck (low-fat content) of cross-breed cows does not induce superovulation systematically whereas injection behind the shoulder (high-fat content) induces a reproducible hormonal response. The presence of SC fat tissue grants a slower diffusion of pFSH from the injection site and consequently a better efficacy. Superovulation is influenced by the body condition score of the cow (BCS). The BCS is a rating of the body condition and is established by visual and/or tactile analysis of fat deposits in the SC tissue. Using a five points BCS (1: very thin, and 5: very fat), a single SC injection into the neck will induce superovulation only in cross-breed cows with a BCS greater than 2. A single SC injection in the neck in Japanese Black cows with a BCS of about 5 (1: very thin, and 9: very fat) showed a superovulation induction comparable to that of the multi-injection protocol. The results were similar by injecting the total dose in 10 or 50 mL. The intermediate BCS indicates that fat of the SC tissue in the neck slows the release of pFSH. Given the previous results, SC injection of the total dose of Folltropin-V® or Antrin-R10® (saline solution) in one

injection induces a superovulation in cows in some specific cases being influenced by many parameters as the species of the cow (different sensitivity to pFSH), the percentage of SC fat tissue and the SC injection site (neck versus shoulder). Treatment with 2 IM injections per day for 4 days decreases the variability of response and is, consequently, more universal. This explains why treatment in one SC injection of the total dose of commercial products only in saline as the vehicle is not present in the veterinary market.

To decrease response variability while reducing the number of injections, studies were also performed by modifying pFSH formulation. The aim was to maintain the release of pFSH over 4 days either by changing the solvent vial such as in the case of Antrin-R10 Al® or by modifying the vial containing pFSH powder (Deguettes, et al., 2020).

Several articles have been published on the establishment of a sustained-release dosage form of pFSH to provide superovulation in cows after 1 or 2 injections simply by adding different types of excipients in the solvent vial. Superovulation in cows in only 1 injection was achieved by adding hyaluronic acid (HA, 2%w) (Tribulo, et al., 2011) (Tribulo, et al., 2012), polyvinylpyrrolidone (PVP, 25-50%w) (Takedomi, et al., 1995) (Yamamoto, et al., 1995), gelatin (3,2%w) (Hill, et al., 1985) or polyethylene glycol (PEG, 3-140%w) supplemented with novocaine (Kosovskij, et al., 2016). These products are proposed in the same way to that of commercial products with the presence of a powder vial (natural pFSH) and a solvent vial (sustained-release solution) for the reconstitution before injection. PEG or PVP permits injection both in SC or IM, while HA shows satisfying results only following IM administration. Gelatin was only tested by SC in the neck. The limitation of gelatin is associated to its animal origin. Marginal differences in the purification process and set production of gelatin may also modify FSH-releasing properties and cause alterations in hormonal response.

The use of injectable polymers improves the controlled release in comparison with the single SC injection of a saline solution of pFSH and consequently, a decrease of the variability of pharmacological response associated to the species and BCS. However, with this type of formulations, new limitations has been identified. At the

concentrations used, the viscosity of PVP (25-50%w) and HA (2%w) solutions is too high to permit easy handling (Kimura, 2016). The homogenization of the pFSH powder and the injection of the solution become difficult. Therefore, even though the induction of SOV in a single SC/IM injection by adding a viscous agent in the solvent vial has been demonstrated, the viscosity must be low enough to allow easy handling of the pFSH formulation but high enough to have a release over 4 days. If this equilibrium cannot be reached, an injection in two steps of the total dose can be used to induce a SOV similar to the multi-injection protocol.

The strategy of modifying only the solvent vial composition has the advantage of keeping production costs under control, the main contributor being pFSH in powder form. Alternative approaches have also been analysed by implementing controlled release technologies directly on pFSH formulations and also by using chemical modification or recombinant technology (Deguettes, et al., 2020).

A new generation of products could be based on recombinant FSH (rec-FSH). Despite a high production cost, the use of rec-FSH has the benefit of avoiding pituitary impurities associated with the extraction/purification process and thus the transmission of diseases between animals. The bovine rec-FSH is produced from the unmodified DNA sequence and has the same properties as the natural FSH (e.g. half-life of a few hours). Recombinant technology is also able to create FSH with a modified structure and new biological properties. Adding some glycosylation sites (CTP) and/or a covalent bond between the FSHα and the FSHβ to modify pharmacokinetics parameters results in prolong-acting FSH analogs (Hesser, et al., 2011). In Angus beef cows, SOV was achieved with a single injection of BoviPure-FSH^{TM} containing a recombinant hyper-glycosylated FSHαβ single chain (rec-bFSHβCTPα).

Future formulations could be based on *in situ* forming implant, microparticles, chemical modification of pFSH or directly through the production of rec-FSH with best possible pharmacokinetics parameters. Commonly, the choice to develop a veterinary product is made according to the complexity of the manufacturing process, the cost and the easy handling of the final product (Deguettes, et al., 2020).

6. RECIPIENTS SELECTION AND MANAGEMENT

6.1 Recipients selection

Selection and identification of high-quality recipients is not easy. Cows that are reproductively healthy, that display calving ease, and that have good milking and mothering aptitude are potential recipients (Mebratu, et al., 2020).

Many prefer the use of virgin heifers, whereas others choose cows with a known history of high fertility. When heifers are used as recipients, the selection criteria should be the same as for high quality replacement heifers. Heifers need to be cycling, which can be evaluated indirectly by using reproductive tract scores, on a high plane of nutrition, have an adequately-sized, normally shaped, pelvic canal and have no history of receiving growth implants.

Lactating recipients have the advantage of a known reproductive history. Recipients that carry an embryo transfer calf to term but do not raise a normal calf to weaning should be re-evaluated as potential recipients. In the same way, open cows with an unknown reproductive history need to be scrupulously examined prior to join a recipient herd or program. The reproductive tract needs to be carefully examined via rectal palpation or trans-rectal ultrasonography for pregnancy, or uterine irregularities such as fluid or fetal remnants or evidence of metritis or endometritis and the ovaries examined for normal follicular or luteal structures. Furthermore, recipients should have good teeth and eyes, a good udder, be less than 8 years of age, and be structurally healthy. Also, highest fertility occurs in herds where handling facilities are conceived to secure that cattle are handled with a minimum of stress (Lamb, et al., 2016).

It is difficult to know how many recipient cows are needed per each donor flushed. To determine an average figure for the number of embryo transfer calves from a single donor cow in a year is not simple. Variations in conditions are wide, but if a cow is flushed every 90 days over a 12-month period and five pregnancies result per collection, an average of 20 pregnancies per year could be obtained. Some cows have produced more than 50 pregnancies per year by embryo transfer and probably could have produced more if economically feasible (Troxel, 2013).

Pregnancy rates have been demonstrated to vary with the synchrony of the donor and recipient. Higher pregnancy rates were recorded when recipients were in oestrus coinciding with the donor or 12 h before the donor. Pregnancy rates decreased in recipients in oestrus 12 h after the donor, but not until 24 h (Lamb, et al., 2016).

One of the factors evaluated in the selection of the suitable recipient is transrectal palpation to determine the presence of the CL on one of the ovaries. It seems that to properly evaluate the morphological structure of the CL, the ovarian structures must be visualised with the use of ultrasound. The ultrasound examination offers a precise description of ovarian structures, including objective determination of the position, number, dimensions and structure of the *corpus luteum*. In cattle there are two types of *corpus luteum*: homogeneous (CL_{hom}) and cavitary (CL_{cav}). Despite they are reputed equal in their hormonal activity, the function of the CL_{cav} is questioned by many veterinarians. As a consequence, females with the CL_{cav} are considered less valuable for assisted reproductive techniques such as embryo transfer, where recipients with CL_{hom} are preferred. Due to the fact that the presence of the cavity inside the CL might translate into the survival of the embryo after ET, it seems important to define the relationship between the confirmed morphological form of the CL and the pregnancy rate after ET in cattle. A recent study intended to compare the two types of CLs in relation to morphological endpoints, serum progesterone concentrations and final pregnancy rate in recipient heifers after embryo transfer (Jaskowski, et al., 2021). In this study clinical and transrectal ultrasound examinations of ovaries were performed on the day of ET. The presence of the CL_{hom} or CL_{cav} was determined, and the CL diameter and cross-sectional area were measured. If present, measurements of the cavities were also taken. Embryos were transferred regardless of the CL morphology and in addition, from arbitrarily selected heifers, blood samples for serum P_4 concentration analysis were collected. The mean serum P_4 concentration was 8.84 ng/ml, higher for females with the CL_{cav} than for those with the CL_{hom}. The pregnancy rate was 36.1%, higher for recipients with the CL_{cav} compared to CL_{hom}. The presence of a CL_{cav} in the recipient heifers did not adversely affect the ability of the CL to maintain pregnancy. On the contrary, the CL_{cav} may give the embryo better chances of surviving the time of pregnancy

recognition and consequently, may have a positive effect on pregnancy rate in heifers (Jaskowski, et al., 2021).

Moreover, it is known that the fluctuation in progesterone concentrations in recipients mirrors a combination of different rates of CL development and the variability of progesterone secretion during the early luteal phase. The optimum circulating concentration of progesterone to establish pregnancy was reported to range between 2.0 and 5.0 ng/mL. However, a recent study has revealed that the minimum threshold progesterone concentration on the day of embryo transfer necessary for the establishment and maintenance of pregnancy may be lower than previously reported. In fact no differences were observed in pregnancy rates when progesterone concentrations were as low as 0.58 ng/mL or exceeded 16.0 ng/mL. In addition, the diameter and volume of the CL varied among recipients that received embryos from 6.5 to 8.5 days after oestrus, increasing as days post-oestrus increased. Although, pregnancy rates did not differ among recipients receiving embryos 6.5 to 8.5 days after oestrus (Lamb, et al., 2016).

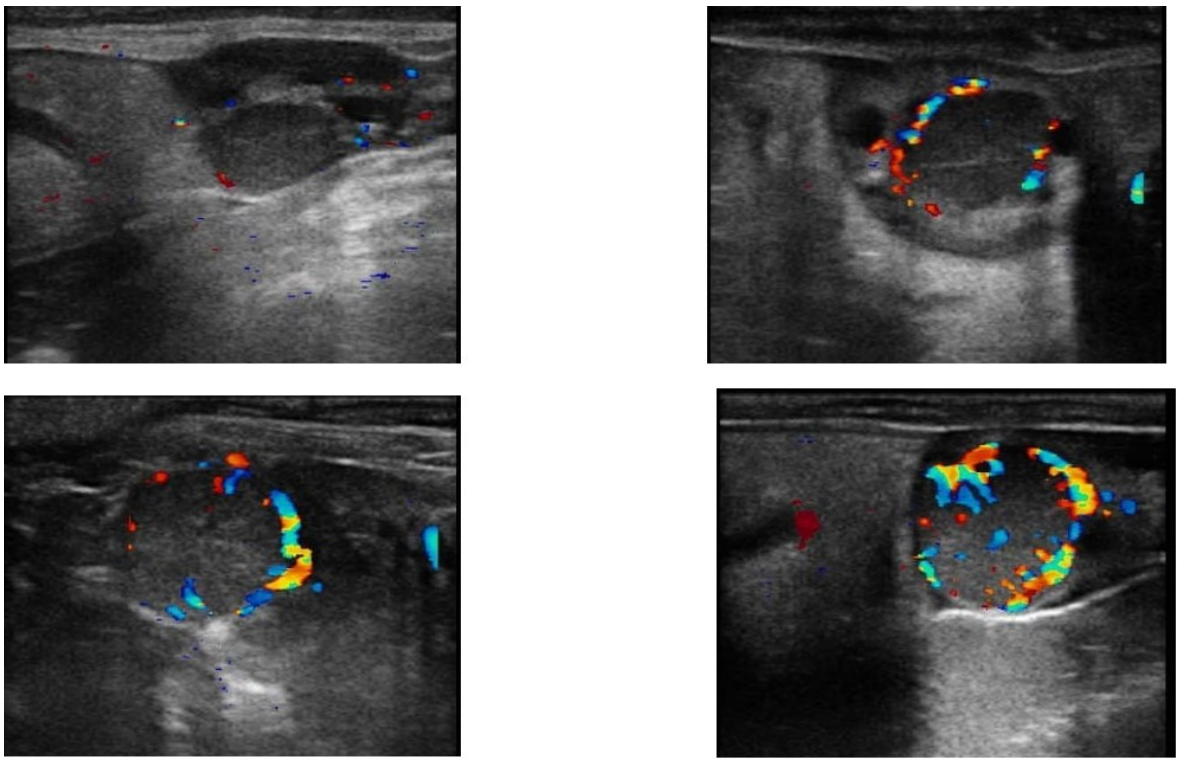

Figure 7 Color-Doppler ultrasound evaluation of recipients' corpora lutea before embryo transfer (Courtesy of Professor G. Morini)

6.2 Recipients management

To increase embryo survival in the recipient female following transfer, conditions in the recipient reproductive tract should faithfully look like those in the donor. This requires synchronization of the oestrus cycles between the donor and the recipients, ideally within one day of each other. Synchronization of the recipients can be performed in similar way and at the same working time as the donor cows. There are many different oestrus synchronization protocols with advantages and disadvantages for each protocol. The critical point concerning recipient cow oestrus synchronization is that the timing must match the time of insemination of the donor cow so that the donor and the recipients have a similar uterine environment seven days later when the transfer occurs. Synchronizing products are more efficacious on recipient females that are already cycling. Anestrus or noncycling cows that are too thin or too short in days postpartum will not be useful recipients (Troxel, 2013).

The most valuable alternative to increase the number of animals receiving embryos is to use protocols that allow for embryo transfer without the need of oestrus detection, commonly called fixed-time embryo transfer (FTET) protocols. Although, much of the research about the systems currently used in embryo transfer programs were formulated for fixed-time artificial insemination (FTAI) rather than FTET. Transfer of embryos into oestrus synchronized cows has been most efficacious when embryos were transferred 6 to 8 days after detected oestrus or GnRH injection. Early oestrus synchronization systems centred on modifying the oestrus cycle by inducing luteolysis with an injection of $PGF_{2\alpha}$ followed by oestrus detection. After systems involving a single $PGF_{2\alpha}$ treatment became successful, researchers focused on multiple injections of $PGF_{2\alpha}$ to further reduce days needed for oestrus detection. The next generation of oestrus synchronization systems involved the use of exogenous progestins, such as an intravaginal progesterone release insert (CIDR) or melegestrol acetate (MGA), which were utilized to postpone the time of oestrus following natural or induced luteolysis and lengthen the oestrus cycle.

Not till the discovery that growth of follicles in cattle happens in distinct wave-like patterns were scientists able to embark on the third generation of oestrus

synchronization systems. Regulating follicular waves with a single injection of GnRH at casual stages of the oestrus cycle involves release of an LH surge, which provokes synchronized ovulation or luteinization of dominant follicles. Therefore, a new follicular wave is initiated in most (>60%) cows within 1 to 3 days of GnRH administration. Luteal tissue that forms after GnRH administration will undergo $PGF_{2\alpha}$-induced luteolysis 6 or 7 days later. A disadvantage of this method of oestrus synchronization is that nearly 5 to 15% of cows are detected in oestrus on, or before, the day of $PGF_{2\alpha}$ treatment, reducing the proportion of females that are detected in oestrus during the synchronized period.

Efficient management of a recipient herd necessitates getting the recipient ready to receive an embryo and identifying and preparing open cows to be resynchronized and re-used or inseminated. In any group of synchronized recipients, a small percentage will not be detected in oestrus and not all detected in oestrus will receive an embryo, either because of an asynchronous oestrus or lack of a proper CL at the time of transfer. If 80% of the synchronized recipients are detected in oestrus and 90% of those receive embryos and 60% become pregnant, then less than 45% of any group of recipients will become pregnant. Consequently, it is important to design a strategy to resynchronize recipients as soon as possible.

Re-insemination of non pregnant cows at the first suitable oestrus can be made easier by resynchronization of the oestrus cycle, which has a wide application in intense embryo transfer programs. Resynchronization strategies differ depending on the resources and capabilities of the ranch. With the use of ultrasonography, non-pregnant recipients may be identified and resynchronized as early as 3 weeks after embryo transfer. However, to most efficiently reduce the calving season, the second round of oestrus synchronization should begin before the pregnancy status of the animals is known. Even though resynchronization with a progestin enhanced synchronized return rates of non-pregnant females, resynchronization with CIDR devices and estradiol cypionate or estradiol benzoate decreased subsequent conception rates to AI. In contrast, further studies did not observe a decrease in fertility when estrogens were used for resynchronization with a CIDR. Moreover, insertion of a CIDR for 13 days on the day of embryo transfer, 7 days after oestrus or

from 5 days after TAI until day 21, was efficacious in resynchronizing oestrus in non-pregnant cows. Thus, resynchronization of oestrus is an approach that increases the number of times a female can be exposed to biotechnologies such as artificial insemination and embryo transfer; therefore, increasing its possibilities of becoming pregnant and producing a genetic superior offspring (Lamb, et al., 2016).

7. BULL FERTILITY RELATED TO MALE CONTRIBUTION TO EMBRYO QUALITY AND OFFSPRING HEALTH

7.1 New potential biomarkers for bull fertility

Selecting the male is generally more critical than selecting the donor female because males will normally be bred to many females and can be selected more properly than females. Additionally, it is necessary to select fertile bulls and fertile semen which makes it particularly important to use high quality semen (Hamco cattle co., 2016).
Fertility, the competency of sperm to fertilize and activate the egg to sustain embryo development, has significant economic impact on agri-food industry. Fertility is influenced by a number of factors including genetics, epigenetics, environment and management.
Bull fertility is crucial for the overall cattle operation because a single ejaculate from a bull distributed by breeding companies can be used to inseminate thousands of cows around the world. Even though bulls produce large amounts of sperm with normal morphology or motility, some animals may still suffer from infertility or subfertility. These substantial differences in fertility between individual bulls cause large reproductive losses. Thus, studying critical aspects of male fertility will shed light on the infertility secrets and could have positive impacts on the AI protocols.
Many studies have been performed over the past few years to discover the effects of semen quality parameters on sire fertility. The early standards for evaluation of bull fertility were defined by a society known as “Rocky Mountain Society for the Study of Breeding Soundness”, and this organization is currently known as “Society for Theriogenology” (SFT).
Breeding soundness examination (BSE) is a systematic method to evaluate the reproductive potential of a given bull, and current standards of BSE were adopted by STF. According to BSE requirements, a bull is tested for four categories including physical examination, scrotal circumference, sperm progressive motility and sperm morphology. A bull should be capable to pass all these four evaluation criteria to be reputed a satisfactory breeder.

The current research is focused on the definition of new markers to estimate bull fertility. Evaluation of arterial blood flow to the testes through an ultrasonography is one of the potential methods to properly assess male fertility.

According to the current literature, it is clear that scientists have great knowledge of male reproductive physiology but there is no single method or parameter that can precisely estimate bull fertility. As a consequence, novel fertility markers are required, as well as more comprehensive methodologies and updated techniques for evaluation of sperm quality and viability. Novel biomarkers (proteins, small noncoding RNAs, lipids, metabolites or epigenomic markers) coupled with computational analyses can be used as integrated approaches to better understand spermatogenesis and sperm quality, and to predict male fertility.

Sperm cells may not have all the RNA polymerase and the transcription machinery to fully transcribe genes like active diploid cells. However, pieces of experimental evidence show that sperm may have some ability to make proteins from RNAs already present in the cells after the completion of spermatogenesis. Alternatively, sperm cells, at some point during their development, maturation and beyond may have some transcription activity as well. Sperm collect proteins from the milieu where they are maintained in the epididymis. Thus, the protein composition of spermatozoa includes intracellular proteins, membrane proteins of the cell, and proteins attached to them coming from the epididymal and accessory sex gland fluid. It is complicated to predict how many proteins ejaculated bull sperm have but it is plausible to presume that the total number of proteins present in the male gamete is similar across mammalian species.

For the bovine species, coverage of the global proteome of sperm has achieved significant accomplishments but it is still limited, with almost 3000 proteins being identified so far. Analyses of bull sperm have shown empirical relationship among sperm proteins, parameters of both fresh and frozen-thawed sperm, and fertility indexes (Ugur, et al., 2022). Recent research results reveal that the proteome of sexed and non-sexed bovine sperm are distinct and that Y- and X-chromosome bearing sperm have different protein signatures (Mostek, et al., 2020). This information supports the idea that sperm quality and even the actual fertility of the bulls can be

potentially tracked back to the proteome of the sperm cells. In the bull sperm, Protamine 1 (PRM1), and postacrosomal assembly of sperm head protein (PAWP) and outer dense fiber of sperm tails 2 (ODF2) have been identified as potential fertility markers. In the years ahead, further studies and standardized proteomic approaches will possibly help to interpret the reliable molecular indicators of sperm quality and bull fertility. These biomarkers could then be used to help the selection of superior sires.

Another relevant aspect of studies focused on sperm proteins is the fact that the population of sperm cells in a single ejaculate is not homogeneous. Each sperm cell may differ in size, morphology, kinetics, metabolism, and protein composition, among other criteria, and these different characteristics influence how sperm protein markers of fertility are scrutinized.

For many years, scientists supposed that the great majority of the genome that is noncoding part of DNA was useless because no noticeable function had been identified for this portion. However, recently it has been demonstrated that approximately 20,000 protein-coding genes are actually regulated by such noncoding regions. The discovery of non-coding RNAs (ncRNA) in biomarker studies intensified as well. Small noncoding RNAs (sncRNA) are short noncoding sequences. A number of sncRNA classes have been reported, including microRNA (miRNA), small interfering RNAs (siRNA), small nucleolar RNAs (snoRNA), small nuclear RNA (snRNA), PIWI-interacting RNA (piRNA). Despite sperm are reputed transcriptionally inactive, increasing evidence indicates that mature sperm carry several long-coding RNAs and sncRNA.

The specific role of sperm borne sncRNA is not well understood. Nevertheless, recent evidence suggests that sperm borne miRNAs are transferred into oocytes during fertilisation and potentially play a role in early embryonic development.

A total of 959 miRNA candidates were detected in bovine sperm using high throughput sequencing technology. Also, variations exist in the miRNA profiles of sperm from high and low fertility bulls and differential expressions of seven sperm-borne miRNAs have been identified for bulls with moderate and high non-return rate. In recent times, higher expression of miR-15a and miR-29 in low fertility compared

to high fertility bulls were reported as well. In this regard, individual or global expression of sncRNAs could be considered as potential fertility biomarkers for the diagnosis of male infertility.

The membrane of the sperm is essential for fertilisation because of its role in spermatozoon-oocyte cross-talk and membrane fluidity. The most abundant component of the sperm membrane is phospholipids (70%), followed by neutral lipids (25%) and glycolipids (5%).

The bilayer of the sperm plasma membrane is mostly made of phospholipids and fatty acids, and saturation levels of fatty acids could be a potential indicator for sperm parameters. Polyunsaturated fatty acids (PUFAs) are susceptible to lipid peroxidation as opposed to saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). A high abundance of PUFA makes sperm more vulnerable to lipid peroxidation caused by reactive oxygen species (ROS) and may damage sperm functional parameters. It has been demonstrated that Arachidic acid, Oleic acid, Myristic acid have differential abundances in sperm of bulls with contrasting freezability phenotypes. More recently, it has been shown that supplementation of frozen-thawed bull sperm with saturated long chain fatty acids, including myristic acid, palmitic acid, margaric acid and stearic acid, enhanced linear motility of sperm. Consequently, the composition of lipid fractions in the membrane could be a potential determinant of sperm fertilization ability.

Metabolomics is an emerging technology for the discovery of biomarkers and offers a picture of biological processes through the measurement of metabolites and investigation of metabolomics pathways. Since metabolites are the end products or intermediates of metabolic reactions, they could give a wide understanding of phenotypic traits.

More recent metabolomics studies in animal reproduction aimed at profiling seminal plasma and sperm cell composition to detect both multivariate and single biomarkers (Ugur, et al., 2022). Kumar et al (Kumar, et al., 2015) profiled seminal plasma from high fertility bulls using proton nuclear magnetic resonance and showed that taurine, isoleucine, and leucine of seminal plasma are potential biomarkers of bull fertility. Similarly, Velho et al. (Velho, et al., 2018) examined the seminal plasma from high

fertility and low fertility Holstein bulls using gas chromatography-mass spectrometry. These authors identified a total of 63 metabolites in seminal plasma samples of Holstein bulls, and demonstrated that fructose, citric acid, lactic acid, urea, and phosphoric acid were the most abundant ones. Univariate analysis of metabolomics data showed that the abundance ration of 2-oxoglutaric acid, ornithine, L-leucine, and D-mannitol were greater in low fertility bulls than those in high fertility bulls. Moreover, it has been reported that abundance ratios of gamma-aminobutyric acid, carbamate, benzoic acid, lactic acid, and palmitic acid were different in sperm of high fertility and low fertility bulls. More recently, Ugur et al. reported that plentifulness of phenylalanine in seminal plasma is a potential biomarker of Holstein bull sperm freezability because of its antioxidant effect (Ugur, et al., 2020).

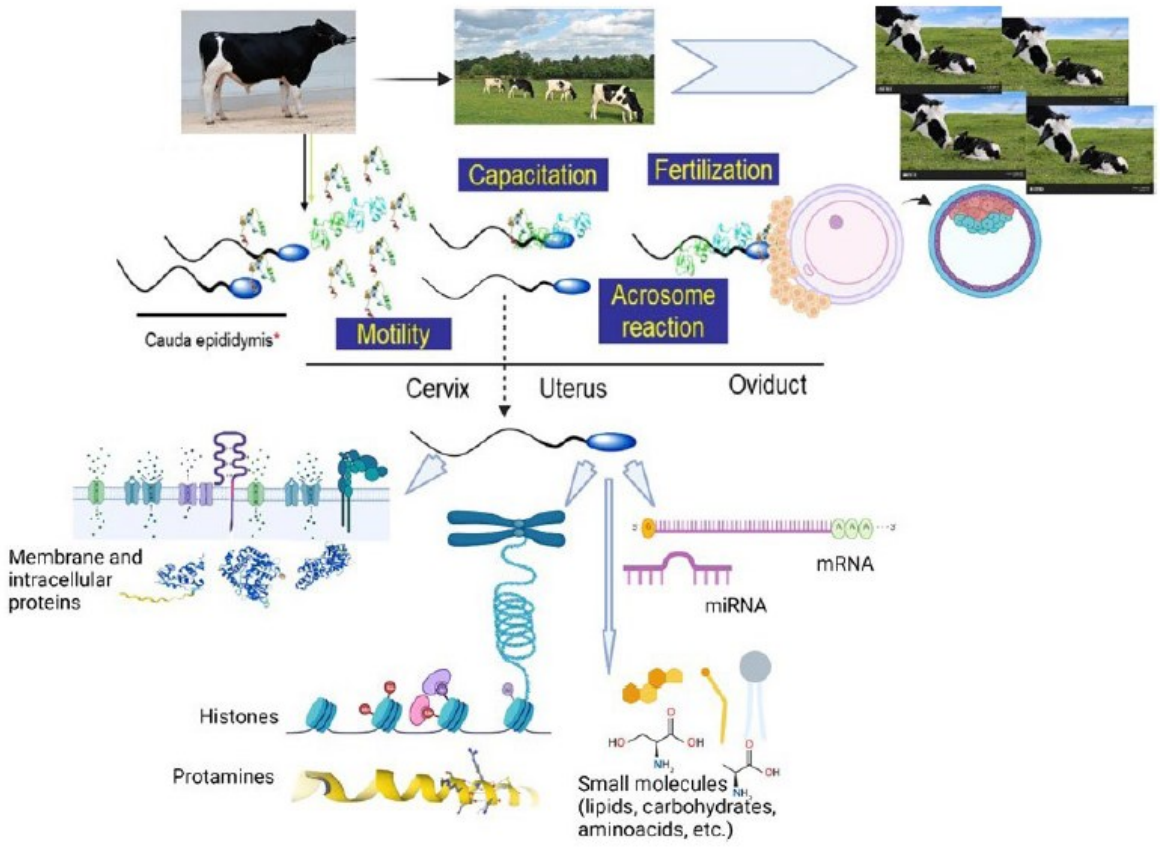

Figure 8 Intrinsic factors of the male gamete that play central roles in the process of fertilization and early embryo development (Ugur et al., 2022)

A property of sperm cell DNA is that it is more condensed than the one of somatic cells, which is important for fertilization because sperm cells are exposed to chemical and physical damage moving through the female reproductive tract. Additionally, sperm head shape and size affect velocity and hydrodynamics of the sperm cell: a more elongated and condensed sperm head makes sperm cells more motile and protects the paternal genome from chemical damage in the female reproductive tract. The abundance of both retained histones and protamines is indispensable for sperm physiology, chromatin integrity and embryo development because of their essential role in sperm maturation. Several studied reported that the ratio of protamine 1 (PRM1) to protamine 2 (PRM2) in mature sperm is related to DNA fragmentation, sperm motility and sperm morphology. In addition, the relative abundance of PRM1 and PRM2 influences the outcomes of assisted reproductive techniques. Abnormal amounts and localization of PRM1 were linked to deficiency in sperm chromatin structure and reduced fertility in bulls. The potential roles of acetylated H3 lysine 27 (H3K27ac), acetyl-Histone 4 (H4 acetyl) and methylated H3 (H3K27me3) were studied and showed that bull fertility is modulated by H3K27me3 and H4 acetyl (Ugur, et al., 2022).

7.2 The paternal influence on semen quality, embryo development and offspring health

Although associations between maternal health and offspring wellbeing are well-defined, the relevance of paternal health for the quality of his semen and the post-conception development of his offspring have been widely ignored.

In fact, since the original findings by David Barker, which shaped the foundation of the current Developmental Origins of Health and Disease (DOHaD) hypothesis, much of the subsequent epidemiological, clinical and fundamental research has focused on the relevance of maternal nutrition and well-being during defined periods of periconception development, gestation and neonatal life. According to the DOHaD hypothesis, the maturing gametes and preimplantation embryo respond to changes in their immediate environmental conditions (both *in vivo* and *in vitro*), resulting in

alterations in post-fertilisation, rates of embryo development, metabolic homeostasis, blastocyst lineage allocation and epigenetic remodelling (DNA methylation, histone modifications, RNA populations), affecting long-term offspring development and health.

In recent years, a new focus into the role of the father, the effect his health has on semen quality and how this may influence the long-term health of his offspring has emerged. Earlier, the general consensus was that the sperm were just a vehicle carrying the paternal genetic material into the egg, and that the seminal plasma was simply a medium to support and transport the sperm. However, we are beginning to understand the epigenetic complexity of the mature sperm, the interaction of the seminal plasma with the maternal uterine environment and the effect that paternal health has on these key reproductive and developmental processes.

Since the use of sub-fertile males can have important economic and sustainability consequences (such as increased time to conception, reduced rates of ongoing pregnancy, prolonged seasons of calving and reduced weight of offspring), understanding how environmental factors affect semen quality, and delineating the potential post-fertilization consequences of using semen from 'poor' reproductive fitness, is important to guarantee commercial viability, the success of conservation programmes and the long-term health and well-being of offspring.

In many large animal species, sperm abnormalities are categorised into 'compensable' and 'uncompensable'. Compensable abnormalities can be overcome through the increase in the number of sperm used during artificial insemination. Such abnormalities are related to an inability for the sperm to reach and fertilize the oocyte, and so are commonly perceived as defects in sperm motility. On the other hand, uncompensable defects are associated with an inability to sustain pregnancy and the ongoing development of the embryo/fetus. Such defects are probably attributable to chromosomal defects, increased DNA fragmentation and impaired epigenetic status (Morgan, et al., 2020). Interestingly, some studies show that standard semen processing procedures such as cryopreservation or sex-sorting of sperm by flow cytometry may increase the rates of uncompensable defects (Inaba, et al., 2016). The cryopreservation of sperm has been of fundamental benefit to the ability to increase

genetic diversity within populations and the spread of favourable/superior traits around the world. Nevertheless, during the cooling and freezing process, sperm can be exposed to several damaging factors which can negatively affect genomic integrity, membrane composition and metabolic stability. During cryopreservation, sperm are susceptible to modifications in membrane protein localisation due to lipid phase separation. Moreover, sperm shrinkage during cryopreservation may result in high levels of reactive oxygen species being released from the mitochondria which may detrimentally affect DNA integrity. Numerous studies have analysed the impact of the sorting process on sperm and the embryos they generate (Morgan, et al., 2020). Some studies report reduced rates of embryo development with sex-sorted sperm (Inaba, et al., 2016) (Steele, et al., 2020) which may occur in a bull-dependent manner (Morgan, et al., 2020). Additionally, some studies showed changes in the number and structure of organelles like mitochondria, rough endoplasmic reticulum and the nuclear envelope in blastocysts derived from sex sorted sperm (Palma, et al., 2008).

As in many mammalian species, scrotal temperature is a critical factor in regulating spermatogenesis. High scrotal temperature has been related to increased amounts of abnormal sperm, increased amounts of sperm cytoplasmic droplets, lipid peroxidation and levels of reactive oxygen species (Morgan, et al., 2020). Recent studies have reported that sperm collected from bulls in the spring display higher indices of sperm quality (intact acrosome status, lower reactive oxygen species production, intact mitochondrial membrane potential) then when collected at other times of the year (Sabes-Alsina, et al., 2017). Underlying these effects may be seasonal-dependent changes in semen lipid composition. Sperm collected during the summer had higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids and cholesterol than in the winter associating with decreased proportions of morphologically normal sperm. In addition, exposure of bulls to a high temperature-humidity index increased rates of sperm death and decreased rates of blastocyst development. Moreover, high scrotal temperature in the bull has also been associated with poor fertilisation capacity of the sperm and perturbed paternal genome DNA demethylation in the zygote. Diet can also play an important role on testicular temperature via its influence on the levels of scrotal fat. Bulls receiving high amounts

of dietary energy intake between 6 and 12 or 24 months of age showed increased amounts of scrotal fat associated with increased scrotal temperature. Daily sperm production and epididymal sperm reserves in these males were remarkably reduced when compared to males receiving a control diet.

On a macro nutrient scale, many studies in ruminants have analysed the role of dietary fatty acid supplementation for semen quality. As motility, capacitation and ability to penetrate the oocyte *zona pellucida* are all affected by the lipid composition of the sperm plasma membrane, dietary levels of n-3 and n-6 polyunsaturated fatty acids (PUFAs) are important for male reproductive activity. Dietary supplementation with appropriate amounts and ratios of PUFAs can have favourable effects of sperm lipid composition and male fertility. Lower levels of PUFAs (namely Docosahexaenoic acid; DHA) have been reported in semen from older bulls. These findings indicate that as males age, testicular fatty acid metabolism may change, reducing sperm membrane fluidity and in the end the capability of the sperm to undergo cryopreservation and/or fertilisation. Because of the high commercial importance of many breeding males, and with the advances in sequencing technologies, detailed molecular and epigenetic profiling of sperm from males of differing fertility is now being performed in livestock. Unlike the "biological determinism" paradigm (in which phenotypic characteristics are determined purely by genes), epigenetics offers a molecular mechanism to better interpret the long-term effects the environment has on the appearance of certain phenotypes. Epigenetic mechanisms regulate patterns of gene expression in response to environmental factors and thus act as a link between the environment and an organism's physiology. Confrontation of sperm DNA methylation levels in high fertility and subfertile bulls showed differential methylation at genes for transcription, spermatogenesis, sperm maturation, capacitation and embryo development. Analyses of sperm transcript levels between bulls of low or high fertility have revealed deficits in genes associated to gene transcriptional and translational regulation. In addition to sperm transcript levels, studies have identified levels of sperm histone methylation (H3K27me3) and acetylation (H3K27ac) as markers of male fertility in Holstein bulls. Moreover, sperm from bulls with low fertility have been shown to display less DNA condensation,

altered protamine exchange and increased DNA damage in comparison to sperm from higher fertility bulls. The age at which semen is collected from a bull may also influence the epigenetic status of the sperm. DNA methylation profiles have been displayed to vary in sperm collected from early pubertal, late pubertal, and pubertal bulls. Individually, semen samples collected from bulls younger than 1 year of age have been shown to have lower sperm motility profiles than bulls older than 1 year.

As in humans, embryo implantation and fetal development can take place without seminal plasma in cattle (Morgan, et al., 2020). Anyhow, some studies indicate that TGFβ infusion at the time of insemination can enhance pregnancy rates in cows, particularly in low fertility herds (Odhiambo, et al., 2009). In ruminants, proteins which can attach the sperm and can both stimulate and inhibit sperm function have been identified in seminal plasma. In addition, characteristics of sperm quality including motility and chromatin integrity have been shown to be altered in response to different compositions of bovine seminal plasma, while analysis of seminal plasma composition between bulls of high fertility and bulls of low fertility have detected variations in the profiles of specific proteins.

Although these technologies have improved rates of conception and the number of offspring produced, attention must still be given to the health of the offspring generated. A large number of paternal factors that can affect offspring health have now been identified, including age, environmental exposures and nutritional status (Morgan, et al., 2020). In cattle, a recent study identified 25 paternal candidate genes and differential profiles of sperm DNA methylation that are related to maternal gestation length (Fang, et al., 2019). Such effects could potentially affect fetal growth and weight at birth, factors known to influence adult risk for cardio-metabolic diseases (Morgan, et al., 2020).

7.3 Use of sex-sorted sperm in embryo transfer programs

The selection of offspring from the desired sex can be one of the determining factors to increase the genetic progress and farmer's profit in either beef or dairy cattle. In fact, sex-sorted sperm has been employed worldwide combined with artificial

insemination. Sex-sorting of sperm cells by flow cytometry is a settled method that has been commercially used in cattle. This technology is an important tool for the dairy and beef industry, leading to greater supply of replacement heifers and the consequent accelerating of genetic gain.

Especially in beef farms, the use of sex-sorted semen could increase the incidence of male calves, product of greater interest due to the increased meat production potential. The separation of sperm bearing X and Y chromosomes is practicable thanks to the differences on the DNA content of these cells (X bearing sperm has about 4% more genetic material than Y bearing sperm) identified by flow cytometry.

Several researches have been accomplished with the aim to investigate the use of sex-sorted sperm during timed artificial insemination programs and for insemination of superstimulated donors for *in vivo* embryo programs.

The pregnancy per artificial insemination (P/AI) of females inseminated with sex-sorted sperm may be affected by their reduced lifespan in the uterus, reduced number of sorted sperm per straw and bull-related fertility. The reduced lifespan of the sex-sorted sperm in the female reproductive tract, as a consequence of mitochondria modification and DNA fragmentation, could modify the optimal interval to perform AI relative to ovulation. Despite lower P/AI described in the literature in cattle inseminated using sex-sorted sperm, there is general agreement that fertility of heifers inseminated upon oestrus detection using sex-sorted sperm is about 70 to 80% of the P/AI obtained following the use of conventional semen. The P/AI detected following the use of sex-sorted sperm depends on the P/AI commonly observed following the use of conventional semen. Thus, similarly to what is observed when conventional semen is used, P/AI of females inseminated with sex-sorted sperm depends on fertility of the bulls, animal categories (lactating cows or cyclic heifers), and management across different farms. Therefore, the main commercial recommendation for the use of sex-sorted sperm still has been in heifers after detection of oestrus, especially due to their higher fertility.

The optimal time at which insemination should occur according to ovulation depends mainly on the lifespan of spermatozoa and the viability of the oocyte in the female genital tract. In a study, Jersey heifers were inseminated following oestrus detection

using radio telemetry (Heat Watch®) in different intervals from onset of oestrus to insemination (12 to 16 h; 16 to 20 h; 20 to 24 h and 24 to 30 h). The P/AI of heifers inseminated from 12 to 16 h after the onset of oestrus was lower than those inseminated from 16.1 to 20 h and 20.1 to 24 h. No differences were noticed on P/AI for heifers inseminated from 24.1 to 30 h when compared to the other interval groups (Sà Filho, et al., 2014). Consequently, prolonging the interval from onset of oestrus to AI may increase pregnancy rates when using sex-sorted semen. Moreover, it is important to observe that the effect of timing of insemination on pregnancy rate could be more marked when using sex-sorted sperm from bulls less tolerant to the sorting process.

A chance to improve P/AI following the use of sex-sorted sperm is to control the variation in the time of ovulation through the application of ovulation synchronization protocols. For example, in beef and dairy females, P4/E2 based synchronization protocols induce ovulation 70-72 h after the P4 device removal (Sà Filho, et al., 2014).

Because sex-sorted sperm displays lower viability on the reproductive tract than conventional semen (Maxwell, et al., 2004), improvement on P/AI with delayed time of artificial insemination is possible, and seemed achievable when breeding is performed 60 h after progestin implant removal compared with the standard 54 h normally used in TAI protocols (Sà Filho, et al., 2014).

The size of the dominant follicle at the end of the synchronization of ovulation protocol and the occurrence of oestrus from progesterone source removal to the TAI have been reported to affect P/AI (Perry, et al., 2005) (Perry, et al., 2007) (Sà Filho, et al., 2010b) (Sà Filho, et al., 2011) (Sà Filho, et al., 2012). Thus, the specific use of sex-sorted sperm in females with larger follicle diameter at TAI and those manifesting oestrus following the synchronization protocol could be important instruments to optimize the use of sex-sorted sperm in TAI synchronization protocols. Sà Filho et al. (Sà Filho, et al., 2012) reported an interaction between the type of sperm and LF diameter at the time of TAI: non sex-sorted $\geq$ 9 mm = 59.8%; non sex-sorted < 9 mm = 49,5%; sex-sorted $\geq$ 9 mm = 56,8%; sex-sorted < 9 mm = 31,2%. However, recent researches (Thomas, et al., 2014) (Karakaya, et al., 2014) indicated

that, in lactating dairy cows, sex-sorted sperm produced lower fertility results when compared to conventional semen, even when applying some selection criteria for the most potentially fertile cows. Consequently, at least for beef cattle, the LF diameter at TAI and the occurrence of oestrus can be used as a selection criteria to identify cows with greater chance of pregnancy to receive sex-sorted sperm in TAI programs. Further studies are needed to precisely evaluate these strategies in lactating dairy cows.

The possibility to choose the sex of the offspring in a herd in species of economic interest is a much desired goal in animal production, particularly in the context of embryo transfer programs.

One of the first studies to evaluate the feasibility of using sex-sorted sperm in superovulation programs was performed by Sartori et al (Sartori, et al., 2004). The advantage of this study was the use of equal amounts of sperm by treatment (sex-sorted sperm and non sex-sorted). At the end of the superovulation protocol, Holstein heifers were casually allocated to one of three treatments and inseminated once with sex-sorted sperm containing 20 millions of sperms 12 h after oestrus detection (S20-1X); twice with sex-sorted sperm containing 10 millions of sperms, 12 and 24 h after oestrus detection (S10-2X); and twice with non sex-sorted sperm containing 10 millions of sperms, 12 and 24 h after oestrus (NS10-2X). When sex-sorted sperm was employed, number and percentage of fertilized and viable embryos recovered per flush was similar between the S20-1X and S10-2X group, but lower than in the NS10-2X group. Additionally, heifers bred with X-sorted sperm had an increase in the percentage of degenerate embryos when only fertilized structures were included in the analysis. Also Soares et al. (Soares, et al., 2011) conducted a study evaluating different times of insemination (12 and 24 h or 18 and 30 h after ovulation induction) and two types of sperm (sex-sorted = 4.2 millions of sperm/AI; non sex-sorted = 40 millions of sperm/AI) in Nelore and Holstein superstimulated cows. The purpose was to evaluate the delay in 6 h in the insemination time of the donors, which took place at a fixed time after induction of ovulation with porcine LH administration. The delay of TAI from 12/24 h to 18/30 h after pLH administration enhanced the number of embryos yielded in supertimulated cows inseminated with sex-sorted sperm.

However, the results are still lower than those achieved with the use of non sex-sorted sperm. These data advise the possibility of increasing the success of embryo production with sex-sorted sperm by delaying the TAI in 6 h after pLH treatment for ovulation induction.

In another study conducted by Baruselli et al (Baruselli, et al., 2007), superstimulated Nelore cows were timed artificially inseminated using sex-sorted sperm and non sex-sorted sperm 12 and 24 h after the ovulation induction with GnRH. Donors inseminated with sex-sorted sperm displayed lower number of transferable and freezable embryos and higher number of unfertilized embryos.

The real practical point in using this biotechnology is strictly related to the generation of offspring in satisfactory quantities and cost. In an experiment performed by Baruselli et al (Baruselli, et al., 2007), part of the embryos were transferred immediately after collection (fresh) at fixed time into synchronized recipients. Similar pregnancies per embryo transfer were reported at 30 and 60 days of gestation after transfer of embryos yielded with sex-sorted and non sex-sorted semen. After sexing by ultrasound, it was noticed that sex-sorted semen resulted in 90.0% females and conventional semen resulted in 52.7% females.

In conclusion, the adjustment in the moment to perform the AI using sex-sorted sperm, closer to the predicted moment of ovulation, improves reproductive outcome in terms of P/AI or embryo production per flushing. However, despite the improvements reached in the last decade, the main concern with the use of sex-sorted semen is associated with low fertility and the drastic variability in fertility among bulls undergoing sexing process. The commercial feasibility of this technique relies on the establishment of a methodology that minimizes the sperm loss during the sex sorting process, with no detrimental influence on the fertilizing potential. Also, an increased resistance to the cryopreservation process would be advisable (Sà Filho, et al., 2014).

8. EMBRYO RECOVERY AND TRANSFER

Both surgical and non-surgical techniques of embryo recovery and transfer can be fit for purpose. Under most circumstances, non-surgical recovery and transfer are highly preferred, despite the fact that surgical transfer can be performed quite rapidly, even in rather primitive circumstances.

8.1 Surgical technique

The first successful cattle ET studies obtained the embryos by a surgical procedure. The donor, which had been tranquilized, was anaesthetized by intravenous knockdown injection with a halothane/oxygen mixture. This technique of recovery was done by executing a Laparotomy (flank or midline abdominal incision) to expose the reproductive tract.

A clamp or the thumb and forefinger can be used to block the distal one-third of the uterine horn, so that fluid injected into that segment can be forced through the oviduct with a gentle milking action and collected in the infundibulum.

An alternative procedure is to occlude the uterine horn at the body of the uterus. The uterine horn itself is very fragile so it must be manipulated with great care. Culture medium is inserted through a puncture at the uterotubal junction or through the oviduct until the uterus is turgid. The uterus is punctured with a blunted needle attached to a flexible catheter.

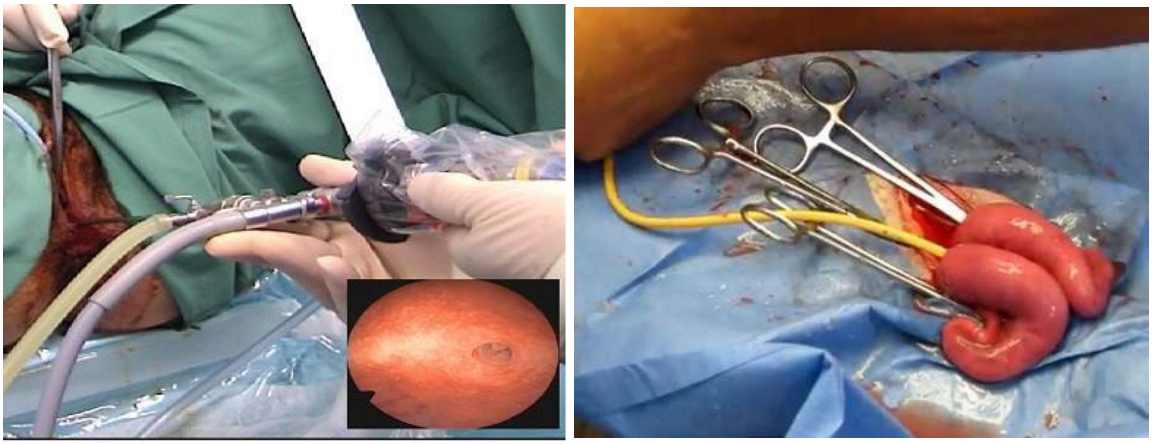

Figure 9 Surgical recovery of embryos (Patel, et al., 2018)

The pressure will cause the medium to flow through the catheter, with enough turbulence to carry the embryos into a collection tube. The incision is then closed, using two layers of sutures.

These procedures allow for the recovery of a high percentage of embryos. However, because of the surgical trauma and resulting adhesions they can be repeated only a few times (Patel, et al., 2018).

8.2 Flushing technique

Epidural anaesthesia is recommended for non-surgical recovery procedures. The tailhead should be clipped, then scrubbed with iodine soap and cleaned with 70 percent alcohol to prevent infection of the spinal column. It is recommended to inject 5 ml of a sterile 2 percent solution of procaine in water using a new 18-gauge needle each time. Recovery procedures are carried out by manipulation per rectum (Patel, et al., 2018).

To collect the embryos nonsurgically, a small synthetic rubber catheter (Foley catheter) is introduced through the cervix of the donor cow, and a special medium is flushed into and out of the uterus to pick the embryos seven or eight days after oestrus. This collection procedure is quite simple and can be completed in 30 minutes or less without harm to the cow. A presterilized stylet is positioned in the lumen of the catheter to give rigidity for passage through the cervix into the body of the uterus. Vulvar area is scrubbed with tamed iodine soap and an assistant gently opens vulvar labia to avoid contamination of cervical expander or collection catheter during insertion. When the tip of the catheter is in the body of the uterus, the cuff is slowly filled with approximately 2 ml of normal saline. The catheter is then gently pulled so that the cuff is seated into the internal os of the cervix. Additional saline is then added to the cuff to completely seal the internal os of the cervix. A Y-connector with inflow and outflow tubes is attached to the catheter. A pair of forceps is attached to each tube to regulate the flow of flushing fluid. The fluid is sequentially added and removed by gravity. The fluid in the uterus is shaken rectally, especially in the upper one-third of the uterine horn. The uterus is finally filled with medium to about the size of a 40 day

pregnancy. One liter of fluid is used per donor. Many operators use a smaller volume and flush one uterine horn at time. Each uterine horn is filled and emptied five to ten times with 30 to 200 ml of fluid each time, according to the size of the uterus. The embryos are flushed out with this fluid and collected in a filter with the fluid. The pores in the filter are smaller than the embryos, so excess fluid drains out of the filter without losing the embryos. Embryos are separated from the flush media and examined under a microscope to determine their quality and stage development (Selk, 2010) (Troxel, 2013).

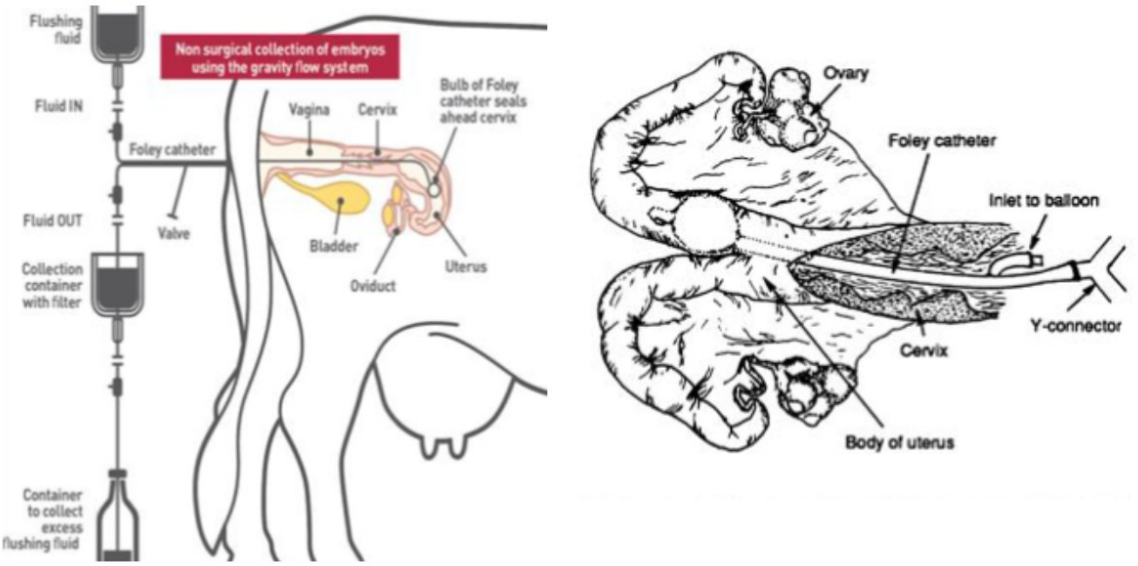

Figure 10 Left: Diagram of the embryo flushing and recovery procedure (Patel, et al., 2018); Right: Position of Foley catheter for uterine horn flush (Seidel, Jr, et al., 1998)

Given that the approximate minimum cost per embryo transfer pregnancy is estimated at $250, it goes without saying that it is necessary to increase productivity and reduce costs. The flushing procedure involves costs related to the solution used in embryo collection. In cattle, it is usual to use Dulbecco's phosphate-buffered saline (D-PBS) solution due to the satisfactory responses obtained in this species. Thus, to improve the cost-benefit ratio, alternatives should be evaluated to lower costs and preserve embryos during the flushing procedures, as well as to achieve a good rate of embryos recovered per number of ovulations. Ringer's lactate solution (RL) is exclusively used in horse for embryo collection, and there is no interference with embryo quality. A recent study intended to determine the efficacy of RL with or without 1% fetal

bovine serum (FBS) for uterine flushing of female beef cattle to recover embryos, to evaluate the quality and quantity of the recovered embryos, and to compare the recovery efficacy with that of D-PBS (de Moraes, et al., 2021). This study was performed on 22 Wagyu females aged 2-7 years, 14 heifers and 8 cows. All females displayed body condition scoring between 3.75 ± 0.13 to 3.86 ± 0.24 points, classified on a scale of 1 to 5 points. Females were divided in three groups in a completely casual design. Each experimental group consisted of superovulated females. Seven days after the first artificial insemination, their uteruses were flushed with the following solutions: RL (n=8), RL + 1% FBS (n=7), and D-PBS (n=7). After flushing embryos were collected on a 70-μ filter, kept in about 30 mL of medium, and then transferred to square Petri dishes with grids for 30X magnification stereomicroscope analyses. Afterwards, the collected embryos were placed in D-PBS enriched with 0.4% bovine serum albumin (BSA), with the formulation described by Dulbecco and Vogt. In the maintenance solution, embryos were classified under 70X magnification, according to quality grade, considering morphology and age, as recommended by the IETS. The number of total embryos collected per group were as follows: 23 in the group with uterine flushing with RL, 21 in the group with uterine flushing with RL + 1% FBS, and 32 embryos in the group with uterine flushing with D-PBS. The numbers of transferable embryos were 13, 19, and 20 in the RL, RL + 1% FBS, and D-PBS groups, respectively. Mean results of live weight, body condition scoring, number of viable embryos, frozen embryos, and number of total embryos obtained per female of a single superovulation showed no differences with the means used for uterine flushing. The data collected with this study indicate with 95% confidence that to perform uterine flushing with RL with or without FBS is practicable, because statistically, the number of total, viable, and frozen embryos obtained in this experiment did not differ among flushing procedures. In conclusion, Ringer's lactate solution or Ringer's lactate solution + 1 % fetal bovine serum are as efficient as Dulbecco's phosphate-buffered saline solution with respect to the quality and quantity of embryos. Therefore, both solutions could be used as alternatives for embryo collection.

8.3 Embryo handling and storage

Embryos are usually held in the same or a similar medium to that in which they were collected. Careful handling of embryos between collection and transfer is needed to prevent the transmission of pathogens. The use of aseptic techniques, sterile solutions, and sterile equipment is crucial.

PH	**7.2-7.6**
Osmolality	270-310 mOs M/kg
Humidity	100 %
Temperature	Room temperature(15-25^{o}C) or 37^{o}C in incubator
Buffer	Phosphate or bicarbonate ion (later must be maintained under 5% CO_2 atmosphere)

Figure 11 Recommended culture condition for bovine embryo (George et al., 2008)

8.4 Transfer of bovine embryos

Embryos can be transferred via mid-line abdominal incision to cows under general anaesthesia, but through flank incision is far more practical. About 60 ml of 2 percent procaine is given along the line of the planned incision. A skin incision about 15 cm long, high on the flank is made just anterior the hip. The ovary is located usually about 25 cm posterior to the incision and CL is visualized or palpated. The uterine horn is exteriorized by grasping. A puncture wound is made with a blunted needle through the wall of the cranial one-third of the exposed uterine horn. Using about 0,1 ml of medium in a small glass pipette (<1.5 mm outside diameter), embryo is drawn up from the storage container. The pipette is then inserted into the lumen of the uterus and the embryo is expelled (Marahall, et al., 2002). It takes some experience to be confident that the embryo has been deposited in the lumen.

Nonsurgical transfer is preferable, because it is less expensive, it is quicker and does not involve surgical procedures (Patel, et al., 2018). It requires loading of the embryo into a 1/4-ml insemination straw. This is done with the Petri dish containing the embryo placed under microscopic viewing, with the aid of a 1-ml syringe and requires considerable practice, patience and dexterity. Degenerated or embryos of very low grade need not to be loaded and can be discarded. Just prior to embryo transfer, the

ovaries of the recipient are palpated/visualised rectally to determine which ovary has ovulated. With the aid of an assistant to hold open the vulva of the recipient cow, the transfer gun or insemination rod is then allowed to slide into the horn on the same side of the ovary with an active corpus luteum. The embryo is gently expelled in the forward tip of that uterine horn (Troxel, 2013). The most commonly used instrument for non-surgical transfer is the standard Cassou Insemination gun for French straws, because it is inexpensive and easy to use correctly (Patel, et al., 2018).

Great care is taken not to cause damage to the lining of the uterus. Such inflammation and scarring would considerably reduce the probability of the pregnancy being established. Embryo flushing and embryo transfer are both done after an epidural anaesthetic has been given to block contractions of the digestive tract and aid in the ease of manipulation of the cervix and the uterine horns. Embryos can be transferred immediately upon recovery and evaluation or may be stored frozen in liquid nitrogen and transferred at a later date. The freezing and thawing process also is very intricate and usually results in an approximate 10-20% reduction in pregnancy rates from those observed with fresh embryos (Troxel, 2013).

8.5 Factors that can affect the pregnancy rates during embryo transfer

A recent study by Alkan et al. performed on recipient beef heifers (n = 561) valued the effects of specific factors on pregnancy rate after transfer of fresh *in vivo* produced bovine embryos (Alkan, et al., 2020). It was ascertained that the side of the transfer (right or left horn) and cervix transfer score (difficult, moderate or easy) had no statistically considerable effect on the pregnancy rates following embryo transfer. Nevertheless, the pregnancy rates and the odds ratios were higher in the recipients with easy access to the cervix during embryo transfer. Furthermore, difficult passage of the cervix during transfer of Code 1 quality embryos reduced the rates of pregnancy. However, in heifers with an easy cervix transfer score, the highest pregnancy rates were found when the embryos were transferred to the cranial third of the uterine horn. Also, as cervix transfer score became simpler, the rate of pregnancy increased in heifers where transfer location was cranial third (Alkan, et al., 2020).

	Not pregnant	Pregnant	Total	Pregnancy rate (%)	Odds ratio	95% confidence interval	p
Embryo quality							
Code I (Excellent or good)	165	133	298	44.66	1.39	1.090–1.670	<.05
Code II (Fair)	176	87	263	33.07	Reference	–	
Transfer Side							
Left	137	100	237	42.2	1.27	0.550–1.113	>.05
Right	204	120	324	37.0	Reference	–	
Cervix Transfer Score							
Easy	254	178	432	41.2	1.49	0.438–1.355	>.05
Moderate	41	22	63	34.9	1.29	0.377–1.186	
Difficulty	46	20	66	30.3	Reference		
Transfer Location							
Cranial third	277	193	470	41.06	1.37	0.473–1.266	<.05
Middle third	64	27	91	29.67	Reference	–	

Table 3 Pregnancy rates based on the transfer side, the cervix transfer score and the transfer location in the study performed by Alkan et al. (Alkan, et al., 2020)

Roper et al. stated that protracted duration of the transfer had a negative effect on pregnancy rate and that the pregnancy rate decreased particularly when the transfer took longer than 14 minutes (Roper, et al., 2018). Excessive uterine manipulation during embryo transfer, laborious passage of the cervix and long-lasting application leads to the release of chemical inflammatory mediators such as $PGF_{2\alpha}$. It was demonstrated that $PGF_{2\alpha}$ may cause pregnancy losses by precluding the survival and development of the embryo (Purcell, et al., 2005) (Scenna, et al., 2005). In fact, non-steroidal anti-inflammatory drugs are normally used at the time of embryo transfer to increase pregnancy rates in cows. A study by Besbaci et al. displayed that non-steroidal anti-inflammatory drugs treatment was generally related to a 15% higher pregnancy per embryo transfer compared to no treatment. The results of this study also emphasized that the use of these drugs at the time of embryo transfer was especially successful in cows with difficulty in passing the catheter from the cervix during embryo transfer, with 71% more likely pregnancy per embryo transfer with the use of non-steroidal anti-inflammatory drugs for these cows compared to other cows. However, the data were too small to investigate the influence of non-steroidal anti-inflammatory drugs molecules (flunixin meglumine and meloxicam), cyclooxygenase inhibitor type (non-selective COX inhibitor [both COX-1 and COX-2] and selective COX inhibitor [only COX-2]), embryo processing (production, conservation and quality), stress, synchronization, breed and parity on the

relationship between non-steroidal anti-inflammatory drugs and pregnancy per embryo transfer (Besbaci, et al., 2021).

In Alkan et al. study, as well as in many previous studies, it was observed that pregnancy rate decreases as embryo quality decreases. The explanation for this was thought to be the reduced rate of viable cells with decreasing embryo quality. Nevertheless, Code 1 embryos have a higher possibility to survive compared to Code 2 quality embryos. In fact, well developed embryos produce higher levels of interferon tau during the maternal recognition period of pregnancy and thus keep developing by inhibiting $PGF_{2\alpha}$ release.

It was also reported that the region where the embryo is positioned in the uterus (cranial or middle third of the uterine horn) affects the pregnancy rates. Pregnancy rates resulted higher when the embryo was positioned in the cranial part of the uterine horn. Moreover, when Code 1 quality embryos were transferred to cranial third of uterine horn, the highest pregnancy rates were observed (Alkan, et al., 2020).

Ultimately, also diet can have an effect on pregnancy rate and embryonic survival. In fact, it is widely known that high-producing dairy cows are subfertile. According to Thatcher et al., an increase in the number of days feeding prepartum diets with a negative dietary cation-anion difference (DCAD) to minimize the quick decline in blood Ca (i.e., -100 to -150 mEq/kg of dry matter intake), combined with adequate energy, protein, amino acids and trace/macrominerals, increases the consequent pregnancy rate. Similarly, supplementation of organic Se in the transition period and lactation improves immune defence, uterine health, and subsequent reproductive performance under conditions of Se insufficiency. A general understanding of the regulatory mechanism between nutrient partitioning and reproduction has led to the development of dietary strategies that benefit both lactation and reproduction. Postpartum addition of dietary non-structural carbohydrates (i.e., glucogenic diets) enhances ovarial activity in either intensive or extensive systems. Moreover, sequential feeding of glucogenic-lipogenic diets increases the proportion of cows pregnant by 120 days of lactation. Fatty acids of the n-6 and n-3 families function as nutraceuticals, modifying innate immune responses and subsequent gene expression

within the uterus to complete the progressive processes of follicle and embryo development and survival of the embryo and fetus. (Thatcher, et al., 2011)

8.6 How to achieve improvements in pregnancy success after embryo transfer

Birth of a live, healthy calf necessitate ovulation of an oocyte capable of being fertilized and supporting the development of the resulting embryo (1 in Figure 12), deposition of sperm in the reproductive tract capable of fertilizing the oocyte (2), development of an embryo with the genetic and nongenetic inheritance from the oocyte and sperm that enable it to develop to term (3), and an adequate reproductive tract able to sustain gamete transport, fertilization and development of the conceptus to term (4). For any given mating opportunity experienced by a female, one or more of these prerequisites can be deficient so that pregnancy is either not established or subsequently fails. Transfer of an embryo into the uterus at day 7 of development can prevent pregnancy failure caused by problems resulting in fertilization failure or early embryonic mortality, including those caused by intrinsic deficiencies in the gametes or embryo (5) as well as an incompetent reproductive tract (6). Transfer of an embryo does not avoid pregnancy losses caused by the inherent incapacity of the transferred embryo for development to term (7) or by the inability of the reproductive tract to sustain development after day 7 (8) (Hansen, 2020).

Achieving the objective of enhancing the reliability of embryo transfer for pregnancy success will rely on the detection of causes of failure of an embryo transferred into a recipient to develop to term. Embryos for transfer are either produced *in vivo* (multiple ovulation embryo transfer [MOET]) or *in vitro* (*in vitro* produced [IVP]) and either transferred fresh or after cryopreservation. Embryos are produced *in vitro* by aspiration of oocytes from follicles (usually by ultrasound-guided transvaginal aspiration but sometimes by collecting oocytes from ovaries ex situ), causing nuclear and cytoplasmic maturation of the oocytes in culture, incubating the matured oocytes with capacitated sperm to obtain fertilization, and then culturing the resultant zygotes to the morula or blastocyst stage for transfer (Tribulo, et al., 2019).

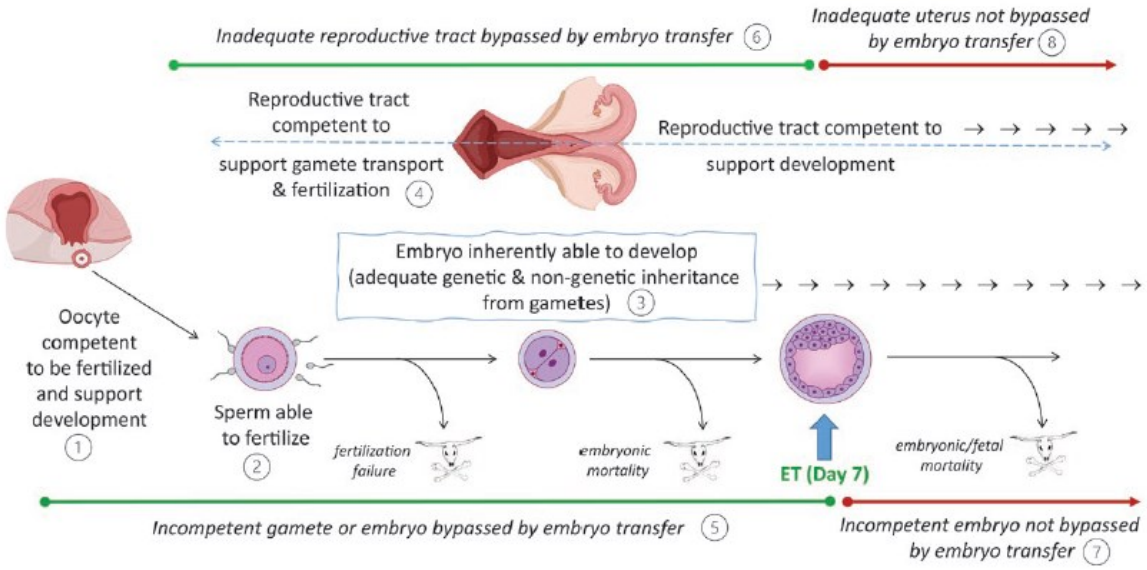

Figure 12 Requirements for a successful pregnancy prone to failure including those that can be alleviated by ET and those that cannot (Hansen, 2020).

In general, the IVP blastocyst has a high number of features that differ from that of a blastocyst produced *in vivo* by superovulation, including oxygen consumption, accumulation of intracellular lipid, other ultrastructural characteristics, gene expression, and DNA methylation. The occurrence of abnormal chromosomes is also higher than for MOET embryos. As many as 72% of IVP blastocysts were showed to exhibit mixoploidy (cell lines with different chromosomal set within the same individual). Following transfer, there are reports of impaired trophoblast elongation, loss of the embryonic disk, alterations in placental function, and dysfunction in fetal development. In particular, a smaller percent of transferred IVP embryos result in pregnancies than when MOET embryos are transferred. Evidently, technical procedures to produce a transferrable embryo *in vitro* have not yet been optimized. It can be reasonably assumed that the MOET embryo is also abnormal when compared with an embryo recovered from a non-stimulated female. Superovulation can induce modifications in the follicle and follicular fluid, oocyte, oviduct, and endometrium. The effects of superovulation on the reproductive tract could reflect the elevation in circulating concentrations of progesterone related to the process. High concentrations of estradiol in the oviduct and blood plasma can also result in cows superovulated with combined treatment with FSH and equine chorionic gonadotropin. Perhaps,

there are direct effects of FSH on the reproductive tract due to the expression by the oviduct and uterus of gonadotropin receptors and can be modified by luteinizing hormone and human chorionic gonadotropin. Studies of gene expression differences indicate that the two types of embryos (*in vivo* and *in vitro* produced) may not be identical. Examination of a limited set of expression of eight genes showed that transcript abundance for GRB10, an adapter protein for insulin and insulin-like growth factor receptors, was highest in IVP blastocysts, intermediate in MOET blastocysts, and lowest in blastocysts obtained by natural mating after regular estrus (Hansen, 2020). Using microarray analysis, Gad et al. detected 454 genes that were differently expressed between MOET embryos that developed in the uterus until day 7 vs. MOET embryos that were transferred to the oviduct of a non-stimulated heifer at a day 2 of development. On the differentially expressed genes, 429 were more numerous in blastocysts that developed in superovulated females (Gad, et al., 2011). These findings, in which all embryos were produced by superovulation but where development occurred in the uterus of a stimulated or non-stimulated donor, underline the relevance of alterations in endometrial function due to superovulation as a cause for modified features of the blastocyst. Though rarer than for IVP embryos, there is a high prevalence of chromosomal abnormalities in MOET embryos. A total of 25% of MOET blastocysts showed mixoploidy. The significance of mixoploidy for embryo survival is not known. It will be important to evaluate if the occurrence of chromosomal abnormalities in embryos produced by superovulation is any different than for embryos produced by non-stimulated females (Hansen, 2020).

Moreover, cryopreservation is related to a decrease in pregnancy per embryo transfer. An overview of the results from six large-scale studies comparing pregnancy results for recipients receiving fresh embryos vs. those receiving either frozen or vitrified embryos shows that pregnancy per embryo transfer for cows receiving cryopreserved embryos was 7.4% points lower that for cows receiving fresh embryos (reported by (Hansen, 2020)).

In addition, pregnancy outcome also depends on uterine environment. An inadequate uterine environment could not only lead to embryonic death before day 7 in an

inseminated cow but, if the uterine environment continues to remain poor, could also cause the death of a healthy embryo transferred into the uterus at day 7.

The embryo has a very noticeable effect on maternal physiology between day 15 and 17 of pregnancy when trophoblast-derived interferon-τ stops luteolysis. Pregnancy can result when an embryo is transferred into the uterus until day 16 after estrus but not when the transfer is on day 17. The release of interferon-τ is not, though, the earliest regulatory signal the embryo sends to the mother. In fact, the bovine embryo can send signals to the mother as early as day 7 of gestation. The local presence of the embryo causes modifications in many specific endometrial transcripts and in the metabolome of the uterine lumen. An example of embryo-induced modifications in the uterine fluid metabolome and related changes in uterotubal gene expression is illustrated in Figure 13. Of 205 metabolites analysed in the uterine flushings from the cranial portion of the uterus ipsilateral to the side of ovulation, 22 diverged between pregnant and non-pregnant cows. The lypoxygenase products 12(S) and 15(S)-hydroxyeicosatetraenoic acid (HETE) were higher in pregnant cows and the other 20, including specific phospholipids, acylcarnitines, glycine, and sarcosine, were lower. Expression of one lypoxygenase gene, ALOX12, was enhanced in the uterotubal junction, whereas another, ALOX15B, was decreased (Hansen, 2020).

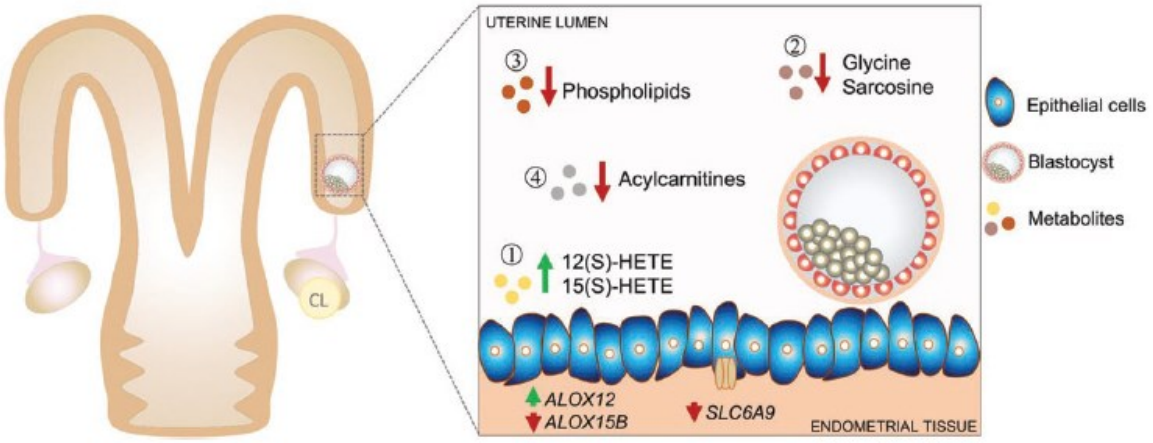

Figure 13 Representation of local effects of the bovine blastocyst on the endometrium at day 7 of pregnancy (Hansen, 2020).

The reduction in glycine content of the uterine lumen was coherent with decreased expression of the glycine transporter SLC6A9. The effects of local actions of the blastocyst on the endometrium for long-term function of the blastocyst remain to be determined (Hansen, 2020).

Enhancements in pregnancy success after embryo transfer will rely upon producing a better embryo, improving uterine receptivity and developing new tools for production and transfer of embryos. To make a better embryo, as it concerns the IVP embryo, there are probably more possibilities to change the production system than for MOET, but in this discussion the processes of production of IVP embryos nor the strategies to obtain better IVP embryos won't be analysed. MOET will remain an important technique for producing embryos for transfer because little laboratory resources are needed and pregnancy per embryo transfer is superior to that reached with IVP embryos. Research keeps improving superovulatory protocols with the aim to increase blastocyst production, and some of these procedures may also affect embryo competence for pregnancy. Moreover, new formulations of gonadotropins, including long-acting FSH, could lead to new protocols that change the kind of follicle recruited or limit perturbation in reproductive tract function. Regulation of the reproductive environment of embryo donors could also lead to embryos with better capability to establish pregnancy (Hansen, 2020). One approach, as already mentioned in the previous paragraph, might be nutraceutical. Pregnancy rates were higher when MOET embryos were obtained from cows fed with a supplement loaded with linoleic acid and β-carotene than when embryos were from control cows. A different approach could be hormonal. The pregnancy per embryo transfer was higher for recipients receiving embryos collected from donors treated with bovine somatotropin than from control donors (Hansen, 2020).

Treatments to enhance endometrial function would be gainful for ET recipients. Thus, many hormonal treatments have been tested for efficaciousness in improving recipient capacity to maintain pregnancy after ET. The methods utilized include administration of progesterone through intravaginal devices and injection of gonadotropins or GnRH or its analogs. Occasionally, positive effects on pregnancy per embryo transfer have been observed whereas, in others, treatment had no effect

or had a negative effect. There are reports of other hormonal treatments that increase pregnancy per embryo transfer, including injection of bovine somatotropin, feeding a supplement filled with sunflower seed, and infusion of peripheral blood mononuclear cells into the uterus at the time of transfer. More studies should be performed to confirm these effects and establish other treatments that can improve recipient receptivity (Hansen, 2020).

However, advancement in improving pregnancy per embryo transfer will be slow unless reliable methods for assessing embryo competence for the establishment of pregnancy and female ability to support pregnancy not dependent upon ET can be determined. The prevalent method to determine embryo quality is morphological evaluation. Nevertheless, there are probably many embryos whose grade does not reflect their ability to establish pregnancy. A method utilized in human-assisted reproduction to detect embryos with high competence to establish pregnancy is to ascertain ploidy. Some studies show improvements in pregnancy rate by using polymerase chain reaction or next-generation sequencing to establish the number of chromosomes (Sciorio, et al., 2020). A problem is the high incidence of mixoploidy in the preimplantation human and bovine embryo. Recently, live offspring have been generated from bovine embryos that were biopsied to ascertain the occurrence and paternal origin of aneuploidy with karyomapping (Turner, et al., 2019). One possible strategy to identify embryos with ability to establish pregnancy is to define a transcriptome signature linked to embryo survival. The approach has been to determine the genes whose expression is related to embryo survival in bisected embryos, where one demi-embryo is transferred into a recipient and the other is used for examination of the transcriptome. This kind of analysis has been executed for both MOET embryos and IVP embryos. In each experiment, a panel of genes was identified whose expression was linked to embryo survival after transfer. Unfortunately, there is low consistency between studies in the specific genes associated with embryo survival. Maybe specific features of the blastocyst including breed and sex are responsible for some of the variability between experiments. In addition, false positives are a characteristic of transcriptomics and their occurrence would reduce repeatability (Hansen, 2020).

Two genes have been associated frequently with pregnancy per embryo transfer, in particular ND1, which was downregulated in embryos that survived after transfer, and EEF1A1 that was downregulated in embryos that survived transfer. ND1 encodes for one of the nicotinamide adenine dinucleotide dehydrogenase subunits of complex I in the electron transport chain, and EEF1A1 encodes for the alpha subunit of the elongation factor-1 complex that takes part in protein synthesis, signal transduction, organization of the cytoskeleton, and export of nuclear proteins. Downregulation of these two genes in embryos that survived transfer is coherent with the quiet-embryo hypothesis that affirms that embryos are more healthy when they are not metabolically active (Hansen, 2020).

Transcriptomics has also been used to establish molecular signatures of uterine ability of supporting embryonic development. Divergences in mRNA transcripts abundance have been detected at days 3, 7, 6 to 8, and 14 of the estrous cycle between heifers that subsequently became pregnant or did not after ET. Differences in endometrial gene expression have also been described between cows that differ in genetic estimates of fertility. There is an urgent need to determine a molecular signature of a receptive uterus. Recently, Mazzoni et al. illustrated the utility of such an approach. Endometrial biopsies were collected from day 6 to 8 of the estrous cycle prior a cycle in which cows received a single IVP embryo. A total of 111 genes were expressed differentially between cows that became pregnant after transfer in comparison to those that did not. Afterwards, the authors used the set of differently expressed genes and logistic regression to foresee the clustering of cows from two other studies in which endometrial gene expression was determined. The precision in classifying endometrial samples as being from progesterone-treated or control animals was 66.6%, and the accuracy of classifying animals as having high genetic potential for fertility versus low genetic potential for fertility was 78.5% (Mazzoni, et al., 2020). These results produce optimism that useful tools can be created to classify cows based on the ability to support pregnancy.

9. EVALUATION AND CLASSIFICATION OF BOVINE EMBRYOS

One of the most important elements related to the success and widespread application of embryo transfer technology is the evaluation of the embryos before freezing and/or transfer to a recipient. Embryos standard classification is based on a number code system for their stage of development (1 to 9) and for their quality (1 to 4).

Evaluation of bovine embryos is usually done with a stereomicroscope at 50 to 100X magnification, with the embryo in a small holding dish. It is also necessary to 'roll' the embryo on the bottom of the dish to be able to view the embryo and its zona pellucida from different perspectives. The overall diameter of the bovine embryo is 150 to 190 μm, 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 color 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.

It is important to be able to recognize the various stages of development and to compare them with the developmental stage that the embryo should be for the day of the oestrus cycle during which donors are collected (i.e. usually day 7 after standing oestrus). The decision as to whether an embryo is eligible for transfer or freezing and whether the embryo is suitable for export will depend on the skill and experience of the person that evaluates the embryo. (Bò, et al., 2013). The main criteria for evaluation include: regularity of shape of the embryo, compactness of the blastomeres (the dividing cells within the boundaries of the embryo), variation in cell size, color and texture of the cytoplasm (the fluid within the cell wall), overall diameter of the embryo, presence of extruded cells, regularity of the *zona pellucida* (the protective

layer of protein and polysaccharides around the single-celled embryo), presence of vesicles (small bubble-like structures in the cytoplasm) (Troxel, 2013).

Standardized coding systems for use in assessing the stage of development and quality of the embryo are described in Chapter 9 and illustrated in Appendix D of the IETS Manual.

The code for stage of development is numeric, ranging from "1", an unfertilized oocyte or a 1-cell embryo to "9", expanding hatched blastocyst. Generally, embryos are collected 7 days after oestrus for cryopreservation or transfer.

Stages:

- **Unfertilized** (Stage code **1**)
- **2 to 12 cells** (Stage code **2**)
- **Morula** (Stage code **3**): 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.
- **Compact morula** (Stage code **4**): individual blastomeres have coalesced, forming a compact mass. The embryo mass occupies 60 to 70% of the perivitelline space.
- **Early blastocyst** (Stage code **5**): an embryo that has formed a fluid-filled cavity or blastocele and gives a general appearance of a signet ring. The embryo occupies 70 to 80% of the perivitelline space. Early in this stage embryo may appear of questionable quality because it is difficult to differentiate inner cell mass from trophoblast cells at this time.
- **Blastocyst** (Stage code **6**): marked differentiation of the outer trophoblast layer and of the darker, more compact inner cell mass is evident. The blastocele is highly prominent, with the embryo occupying most of the perivitelline space. Visual differentiation between the trophoblast and the inner cell mass is feasible at this stage of development.
- **Expanded blastocyst** (Stage code **7**): the overall diameter of the embryo drastically increases, with a concurrent thinning of the *zona pellucida* to approximately one-third of its original thickness.

- **Hatched blastocyst** (Stage code **8**): embryos recovered at this developmental stage can be undergoing the process of hatching or may have completely shed the *zona pellucida*. Hatched blastocyst may be spherical with a well defined blastocele or may be collapsed. Identification of hatched blastocysts can be difficult unless they re-expand when the signet ring appearance is again obvious.
- **Expanding Hatched Blastocyst** (Stage code **9**)

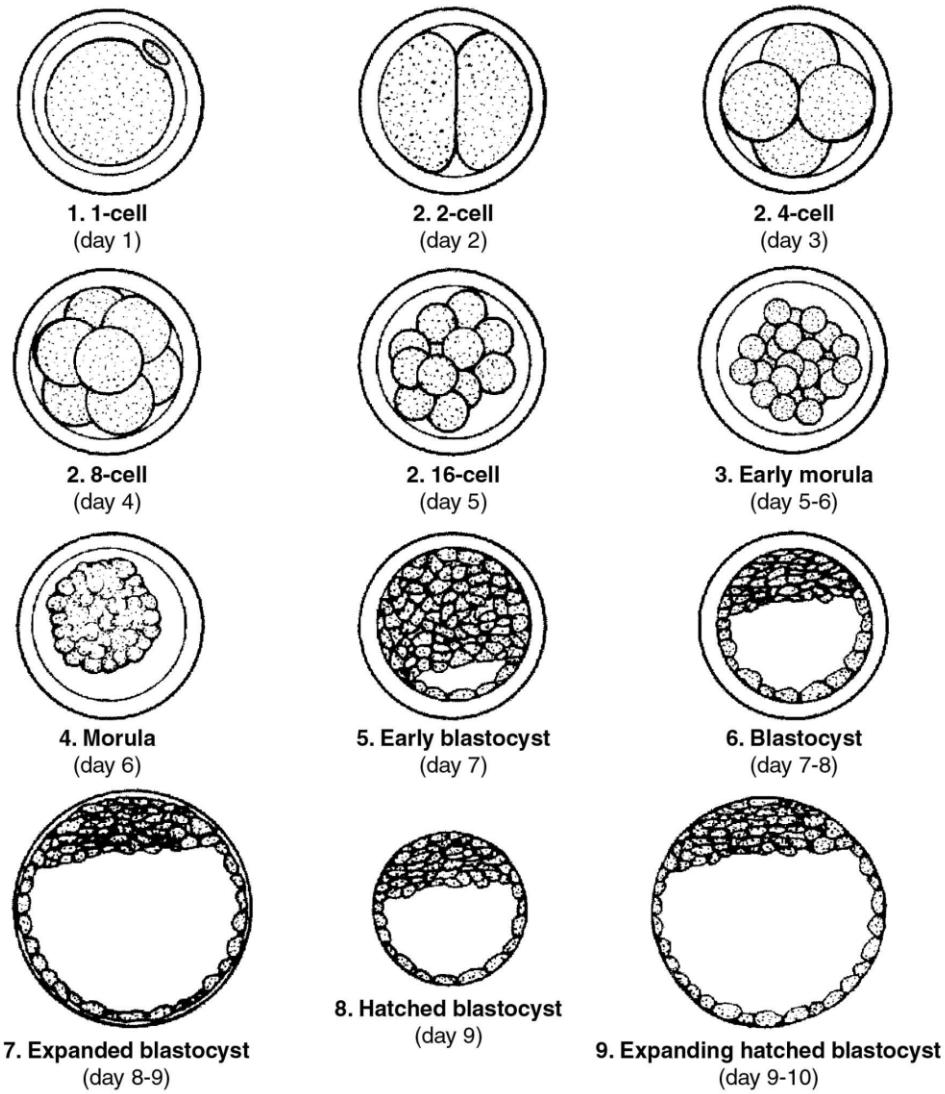

Figure 14 Numeric codes for the various stages of embryo development (IETS, 2010)

Quality: the codes for embryo quality is also numerical and are based on morphological integrity of embryos. The codes for embryo quality range from "1" to "4" as follows:

- **Code 1**: **Excellent or Good** (80-100% viable). The embryos have a symmetrical and spherical mass with individual blastomeres that are uniform in size, colour and density. This embryo is coherent with its expected stage of development. Irregularities should be relatively minor, and at least 85% of the cellular material should be an intact, viable embryonic mass. The judgment should be based on the percentage of embryonic 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 1 embryos survive well to the freezing/thawing procedure and some pratictioners call them "Freezable embryos". Grade 1 embryos are also those recommended for international trade.

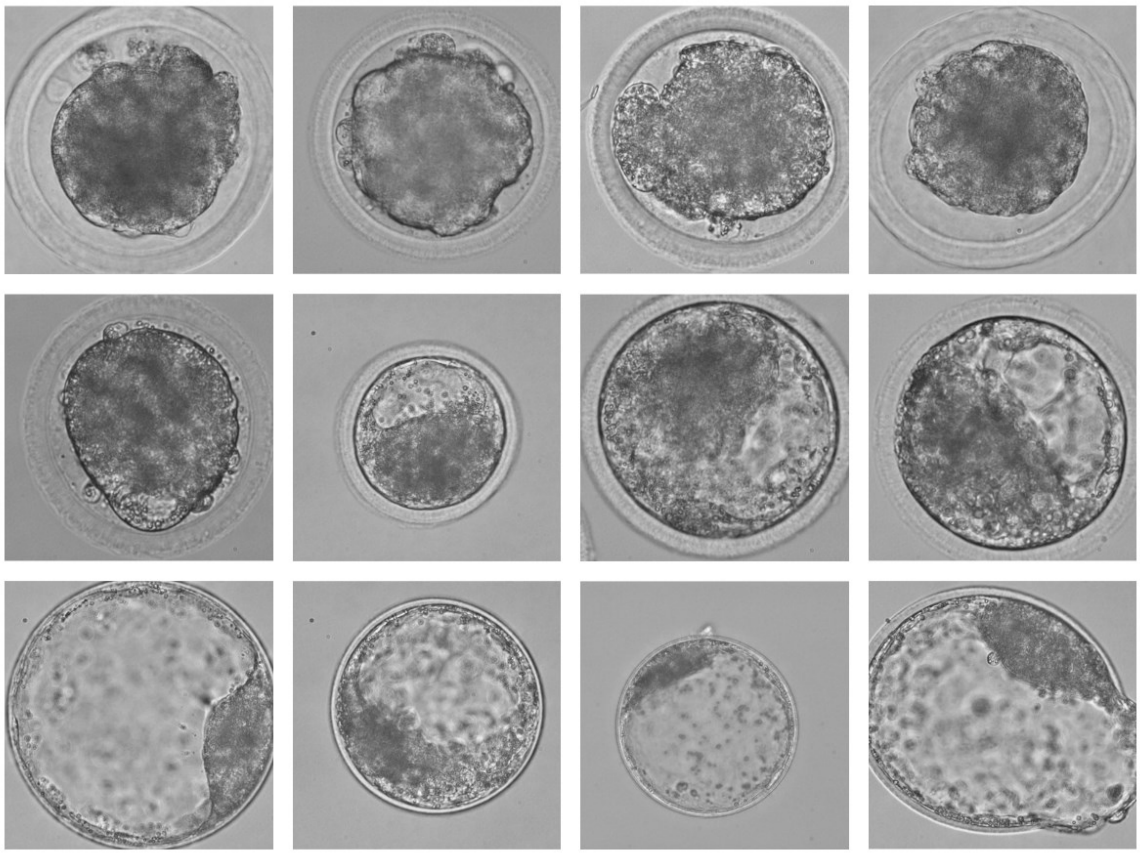

Figure 15 Code 1 (excellent or good) embryos at stages 4, 4, 4, 4, 5, 6, 6, 6, 7, 7, 7 and 8 respectively (Courtesy of Professor G. Morini)

- **Code 2**: **Fair** (50-80% viable). These embryos have moderate irregularities in the overall shape of the embryonic mass or size, colour and density of individual cells. At least 50% of the embryonic mass should be intact. Survival of these embryos to the freezing/thawing procedure is lower than with Grade 1 embryos, but pregnancy rates are adequate if embryos are transferred as fresh into suitable recipients. Consequently these embryos are often called "transferable" but not "freezable".

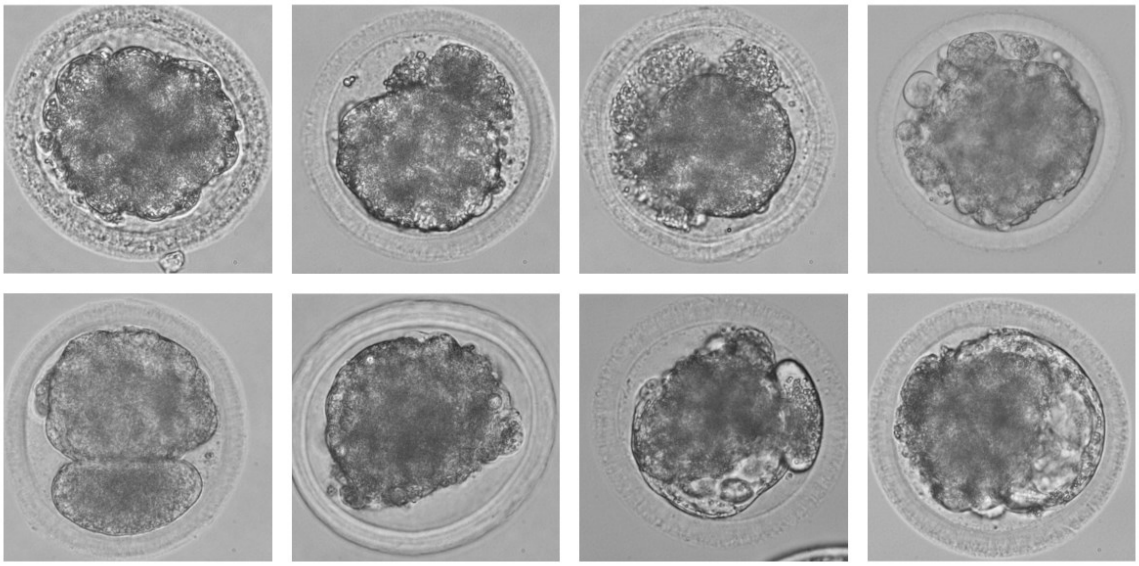

Figure 16 Code 2 (fair) embryos at stages 3, 4, 4, 4, 4, 4, 5 and 5 respectively (Courtesy of Professor G. Morini)

- **Code 3**: **Poor** (<50% viable). These embryos have major irregularities in shape of the embryonic mass or in size, colour, and density of individual cells. At least 25% of embryo mass should be intact. These embryos do not survive the freezing/thawing procedure and pregnancy rates are lower than those obtained with fair quality embryos if transferred fresh into suitable recipients.

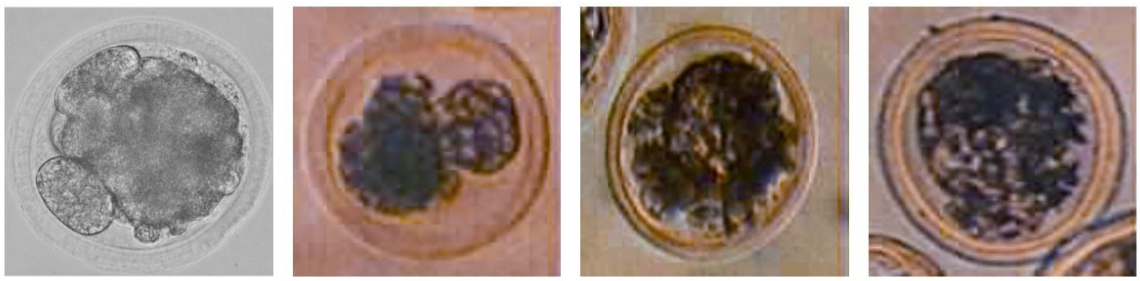

Figure 17 Code 3 (poor) embryos at stages 4, 4, 5, 5 (First image by courtesy of Professor G. Morini; last three images source (IETS, 2010))

- **Code 4**: **Dead or degenerating**. These could be embryos, oocytes or 1-cell embryos. They are non-viable and should be discarded.

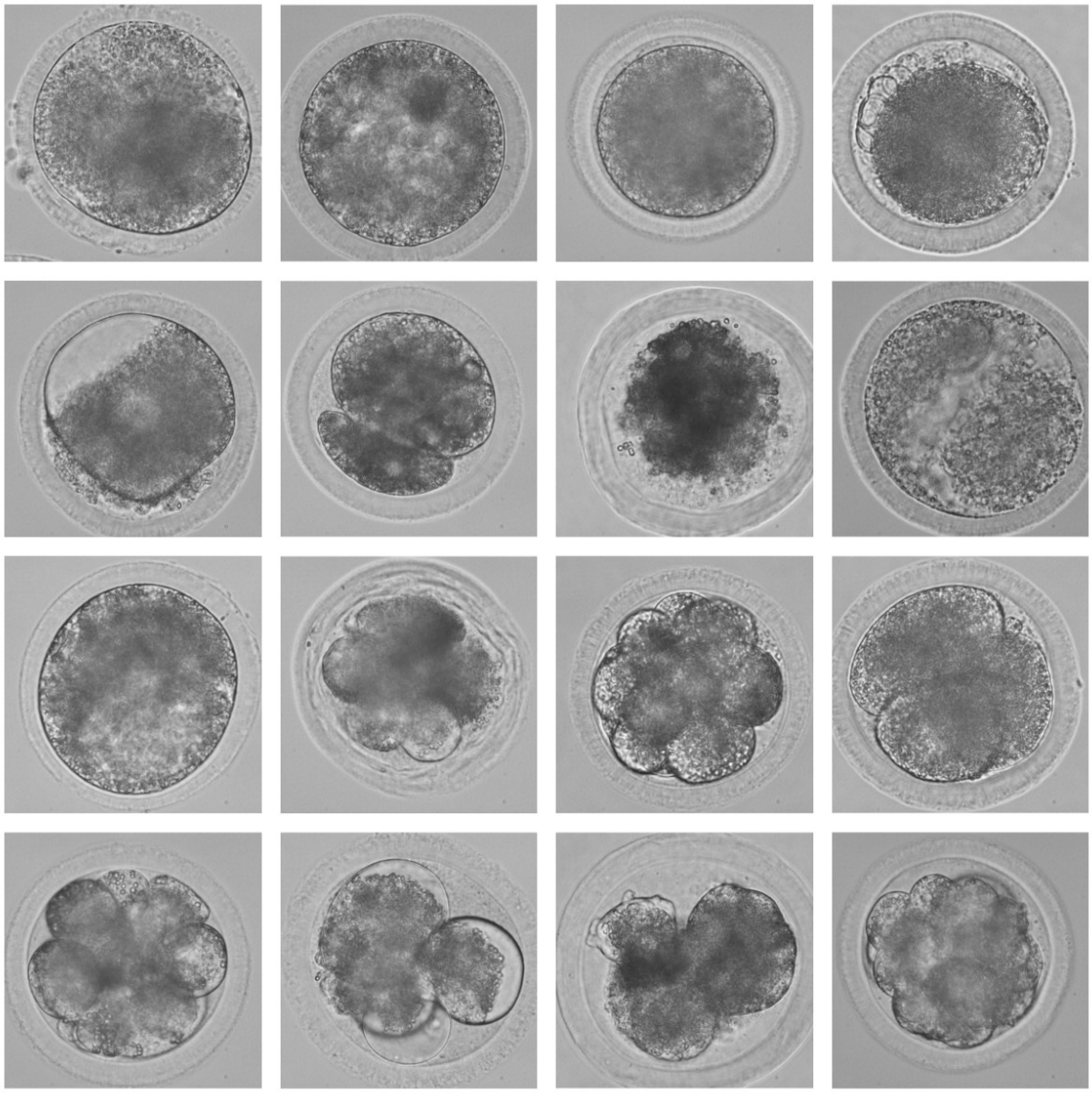

Figure 18 Code 4 (dead or degenerating) embryos at stages 1 (first 9 images) and 2 (last 7 images) (Courtesy of Professor G. Morini)

The IETS Manual also says that visual evaluation of embryos is a subjective evaluation of a biological system and is not an exact science. Consequently, pregnancy rates may sometimes be lower than predicted because of other factors such as environmental conditions, recipient quality, and technician competence. Normally, unless otherwise agreed, only Code 1 embryos should be used in international commerce.

In the superovulated cow, there is probably a considerable range of stages of development on any given day after oestrus. On day 7 after oestrus, there may be morula and hatching blastocysts into the same flush. In parallel, there may be embryos of excellent quality and also unfertilized and degenerate embryos. Normally, wide variations in embryo quality and stages of development are indicators that the existing embryos are not completely normal 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 (Bò, et al., 2013).

Other studies have evaluated pregnancy rates according to the stage of embryo development after freezing and thawing. In one study that evaluated 5,287 transfers of embryos cryopreserved in glycerol, pregnancy did not differ between morulae, early blastocysts, blastocysts, and expanded blastocysts (Hasler, 2001). In contrast, other studies found a decrease in pregnancy rates with more developed embryos (blastocysts and expanded blastocysts), but in these studies embryos were cryopreserved in ethylene-glycol prior to transfer (Dochi, et al., 1998). Fair and poor quality embryos yield poor pregnancy rates after freezing. It is recommended 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.

10. APPROACHES TO BIOSECURITY AND RISKS OF TRANSMISSIBLE DISEASES IN BOVINE EMBRYO TRANSFER PROGRAMS

Even though transfer of bovine embryos is much less likely to result in transmission of pathogens than transport of postnatal cattle, the epidemiologic risk related to bovine embryo transfer deserves examination. There are three main strategies used to prevent the transmission of pathogens from donors to embryo recipients: donor testing, embryo processing or treatment and recipient testing. The potential transmission of pathogens through the transfer of *in-vivo* derived embryos obviously depends on pathogens introduced by the embryo donor, the semen donor, reagents of animal origin (fetal calf serum or BSA) or the embryo recipient. Although environmental contamination is highly unlikely if sanitary precautions are respected, the possibility should be considered (Givens, et al., 2008).

10.1 Sanitary risks associated with semen

A lot of pathogenic organisms can be found in the genital glands or testis, in the lymphatic system, in the blood system (during a bacteremia or a viremia) or in the urinary system. For this reason, all of these microorganisms may be easily transmitted in the semen by the way of seminal liquid, sperm or urine contact. This is corroborated by the fact that nearly all of the pathogenic microorganisms listed in the OIE have been isolated from semen. For instance, there was no evidence of semen excretion of *Mycobacterium paratuberculosis* during several years, because of the dilution of semen in the extender and the low concentration of this pathogen in semen. The semen excretion could be confirmed with more sensitive tests such as polymerase chain reaction or enhanced culture systems. These microorganisms (virus or bacteria) can be present intermittently in semen either in seminal plasma and/or associated with spermatozoa. This contamination can be transmitted to the embryo or the recipient. Considering the Bovine Viral Diarrhoea Virus (BVDV), a very high viral load can be

found in semen because of the replication of the virus in the reproductive tract of the bull and as freezing does not kill the virus, an inseminated cow will be inexorably infected by a contaminated semen. This contamination has an important effect on every early embryonic mortality even if this negative effect on *in vitro* development rate may depend on the virus strain and on its cytopathic effect. The last example is *Mycoplasma bovis* or *Mycoplasma bovigenitalium* that are pathogens that can be transmitted through the IVF system and therefore infect the embryos. Experiments showed that the supplementation of the medium used for in vitro culture with standard antibiotics and the washing procedure of the embryos recommended by IETS were not efficacious in making IVF embryos free from *M. bovis* and *M. bovigenitalium*.

In conclusion, the different studies testing contamination of *in vitro* or *in vivo* produced embryos indicate that the risk of transmission of pathogenic microorganisms when using infected semen is potentially very high. In order to avoid contamination of semen and to control related sanitary risks, different measures have to be taken. The first one and the most important one is the epidemiological rule especially applied in AI centres: the only way to be sure that produced semen is pathogen free is by using a bull whose historical sanitary status is very well known: this bull is living in a pathogenic free centre where all animals are free from diseases and routinely controlled. Before entering in the AI center the bull has to remain a couple of months in the station where it has been previously tested at least two times. If this rule cannot be followed and if a bull with high genetic merit has to be used for ET, the only way to reduce the risk of contamination is to analyse each ejaculate by testing some straws with a sensitive test.

In addition to these major pathogens for which males are scrupulously controlled in AI centers, semen can be contaminated also by ubiquitous pathogens. This contamination may be limited by hygienic measures which are applied in particular on housing, collection and processing of semen. Addition of antibiotics to the extenders are also able to limit the number of bacteria present in the extended semen (Le Tallec, et al., 2002).

10.2 Sanitary risks associated with the donor female

Oocytes and embryos are surrounded by the *zona pellucida* (ZP), which protects the embryos and plays a role during all the steps of embryonic development, from maturation through fertilisation until early embryonic development. Generally, the ZP of mammalian species is composed of 3 glycoproteins, which are assembled in a complex three-dimensional structure. Changes in the structure of the glycoproteins take place during fertilisation and also during the passage of the embryo through the oviduct. As a result, pathogens can no longer bind to and/or penetrate the intact ZP after fertilisation, which becomes an efficient barrier against contamination by most of the pathogens analysed. Consequently, the major parameter is associated with the integrity of the ZP and it is usually recognised that the only way of transmission of pathogens to recipients is via adhesion to the ZP. In cattle, the *Foot-and-mouth virus*, *Bluetongue virus*, brucellosis, tuberculosis and enzootic bovine leucosis do not seem to represent any risk of transmission by ET. In contrast, the risks related to *Mycobacterium paratuberculosis*, the *Bovine herpes virus-1* and non conventional agents such as prions are more debatable.

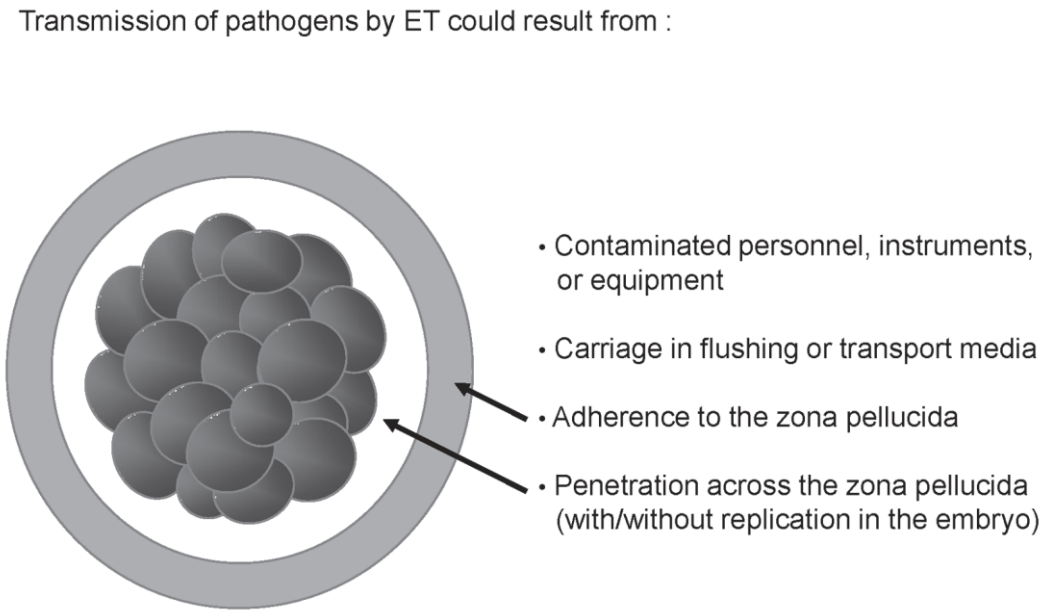

Figure 19 The various ways throughout embryo could carry pathogens during embryo transfer procedures (IETS, 2010)

As far as it concerns *Mycobacterium paratuberculosis*, only one experiment has been performed to evaluate the sanitary risk between embryos and *M. paratuberculosis*. In this experiment, 50 ZP intact embryos were incubated with 5 different concentrations of *M. paratuberculosis* during 12 hours at a temperature of 22°C. The result of the culture is that even after 10 washes, from 10 to 50% of the embryos remained contaminated according to the bacterial concentration used in the incubation. For Bovine herpes virus-1, it has been demonstrated that all viral particles are eliminated after the IETS washing procedures with trypsin. The results have led to the conclusion that ruminant ZP intact embryos do not seem to be a vehicle for the transmission of IBR. In addition, it has been shown that embryo transfer can avoid vertical transmission of scrapie. Turning to Bovine Spongiform Encephalopathy (BSE), according to the last experiments, the risk appears quite minimal provided that the IETS recommendations are respected. As a conclusion, with the sanitary risks related to *in vivo* produced embryos, ZP acts as a barrier for the majority of the diseases (enzootic bovine leucosis, foot and mouth disease, bluetongue, brucella abortus…). But since few agents, such as *M. paratuberculosis*, can attach themselves to the exterior of the ZP, maybe we should also think to an additional treatment for such a bacterium.

However, washing of the embryos may be reputed as the best way to eliminate different kinds of pathogenic or ubiquitous agents (slightly attached to the ZP or present in the flushing fluids). The essential principle expressed by the IETS is that if the ZP of the embryos is intact and if the embryos are processed according to the IETS recommendations, ruminant ZP-intact embryos do not appear to be a vehicle for the transmission of most infectious agents (Le Tallec, et al., 2002).

10.3 Sanitary risks associated with environmental conditions

Many contaminations may result from the serum or media used for the handling of embryos. Contaminations can also occur during the manipulation of embryos (collection of the embryos, washing, transfer).

In this discussion the ways of contamination of the *in vitro* produced embryos won't be analysed in detail since this dissertation aims to focus only on the *in vivo* embryo production.

Different media containing products of animal origin are used for the collection, handling, washing, cryopreservation of embryos and also represent noteworthy sanitary risk (Le Tallec, et al., 2002). Contamination of fetal calf serum is frequent and a cause of serious epidemiologic concern. Current recommendations are to use only heat-treated and gamma irradiated fetal calf serum when needed. Anyway, some data indicated that gamma irradiation was not systematically effective for inactivation of BVDV. Thus, the use of fetal calf serum should be avoided, and if necessary, irradiated and tested for pathogens of concern prior to use.

As far as it concerns BSA, its production involves heating (65-75 °C for more than 3 hours) so many infectious agents will be inactivated in the process of production (Givens, et al., 2008).

Bacterial and fungal contamination is a common risk when embryos are exposed to an artificial environment. For this reason antibiotics are added routinely to media for embryo collection or holding. However, the addition of antibiotics should not be considered a substitute for aseptic technique and high standards for cleanliness in the laboratory. Antibiotics are usually used as an extra safeguard to supplement the use of sterile lab ware and aseptic technique. When specified, treatment with antibiotics should be performed at 37-39°C to guarantee that targeted microorganisms are metabolically active and maximally susceptible to antibiotics. Penicillin G (50-100 IU/mL), streptomycin sulphate (50-100 μg/mL) and kanamycin (100 μg/mL) have commonly been used to prevent bacterial growth. Mycostatin (50 IU/mL) and amphotericin B (0,25 μg/mL) have been used for control of fungal infections. Even though an association of penicillin G (100 IU/mL), streptomycin (100 μg/mL) and

amphotericin B (0,25 µg/mL) exhibited toxicity for *in vivo* derived bovine embryos after 72 h of culture, no toxicity was observed with this concentration of antibiotics when used for 6 h of culture. When considering specific bacteria, one should notice that *in vivo* derived embryos are not likely to be exposed to *Brucella abortus*, even in an infected embryo donor. Moreover, *Brucella abortus* can be efficaciously washed from *in vivo* derived bovine embryos. Conversely, *Mycoplasma* spp. are resistant to embryo washing and conventional addition of antibiotics. Treatment with kanamycin (1000 µg/mL) or tylosin (200 µg/mL) successfully produced *Mycoplasma bovis*-free embryos when the artificially exposed embryos were washed 10 times and incubated (37°C) for an additional 4 h in media containing the antibiotics.
Although *Leptospira borgpetersenii* serovar hardjobovis was associated with washed embryos, supplementation of the media with penicillin and streptomycin guaranteed that embryos were not contaminated with infectious bacteria (Givens, et al., 2008).

10.4 Recommendations for the sanitary handling of in-vivo derived embryos

Sanitary handling of embryos between collection and transfer is a fundamental precaution to avoid transmission of pathogens from donors to recipients. Aseptic technique, sterile equipment, and sterile labware are needed. Additionally, precautions should be taken to ensure that media and solutions are not contaminated. Broad-spectrum antibiotics should be added to media and solutions to function as a deterrent to bacterial pathogens or contaminating microorganisms that might be encountered. All media and supplements to media (including sera, enzymes, and cryoprotectants) must be free of pathogens and contaminating microorganisms. When media and supplements to media are purchased as “ready-to-use” sterile preparations, they should always come from reliable sources. When sterilization is executed in the laboratory, the procedures outlined in the IETS manual should be used. Because only embryos that are *zona pellucida*-intact and free of adherent material can be washed effectively, the embryos must be carefully examined over all their surfaces, and any adherent mucus or other debris must be removed before washing.

A light microscope set at a minimum magnification of 50X is required for the examination of embryos to secure absence of defects in the *zona pellucida* and adherent material.

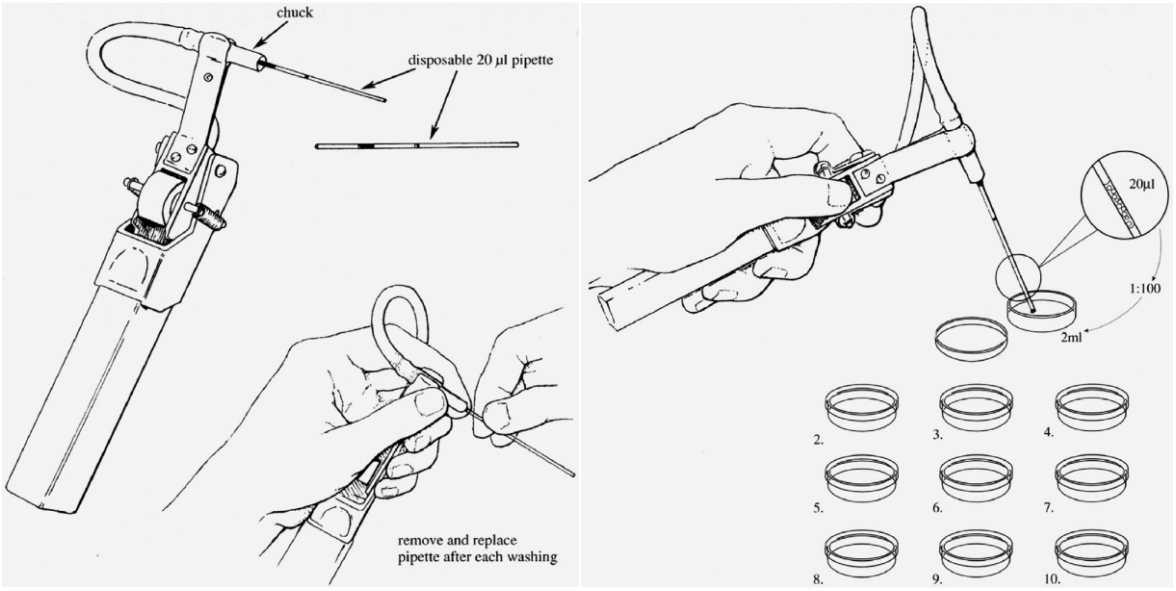

Figure 20 Left: hand-held micropipettor and disposable 20-µL pipets; Right: individual 35 mm Petri dishes for standard wash procedure. When 2 to 3 mL of wash medium is placed in each dish and separate disposable pipets are used to carry embryos between dishes, the required dilution factor of at least 1:100 is achieved (IETS,2010)

When deficiencies of the *zona pellucida* are observed or when adherent debris cannot be removed, these embryos must be discarded. If defects in the *zona pellucida* are found after washing, the defective ones are removed, and the washing procedure must be repeated for the remaining embryos using fresh washes. Because the use of separate sterile micropipets between washes is crucial for an adequate washing, it is necessary to use a pipetting device and techniques that do not permit any part of the device to touch the wash media.

When trypsin treatment is demanded, it is necessary to change the standard washing procedure, because the trypsin treatment represents a combination of both washing and exposure to trypsin (IETS, 2010). When protocols require trypsin treatment, embryos are washed five times in phosphate buffered saline, with or without calcium and magnesium, antibiotics and 0,4% BSA. This is followed with two treatments of 0,25% trypsin diluted in Hank's balanced salt solution without calcium and magnesium, for a total of 60-90 s. Then, embryos are washed five additional times with 0,4% BSA or 2% fetal bovine serum to remove any residual enzyme (Givens, et

al., 2008). It is important that trypsin is stored according to label instructions (i.e., frozen). Solutions containing trypsin should be recently prepared from the frozen stock instantly before using. Holding the trypsin-containing solution after thawing for prolonged periods will lead to a loss of enzymatic activity. As a consequence, it has been standard protocol to time the thawing and mixing of trypsin solutions to occur simultaneously with the presence of embryos for washing. It has been shown that treatment of bovine embryos with trypsin for 2 to 3 min does not reduce their viability.

Options for certification of the health of embryos before transfer include use of specific tests performed on the donor animal, washing or trypsin treatment (trypsin washing), and the testing of samples related to the embryo collection process.

Test samples that have been advised as indicators of embryonic health include 1) collection (uterine flush) fluid, 2) embryo washes, and 3) unfertilized ova and non-transferable embryos from the same collection. The reason for testing the collection fluid is that it gives some indication of pathogens to which embryos might have been exposed within the reproductive tract of the donor female. Nevertheless, it should be remarked that pathogens found in the collection fluid can be removed from embryos by adequate washing. Testing of the last few washes has been justified as an indicator of what recipient females might be exposed to when the washed embryos are transferred. Testing of nonfertile and degenerated ova that were collected from a donor is aimed to give a method for detection of any pathogen to which transferable embryos in the same collection might have been exposed. In addition, it may give an indication of the efficacy of the washing procedure. The validity of the test is based on the assumption that pathogens can be isolated from unfertilized ova and degenerating embryos as well as from transferable embryos from the same collection. The greatest utility of testing flush fluid, washing fluid, or embryos/ova might be in a scenario in which the donor is seropositive for a particular disease. It would be superfluous to test these samples if donor males and females were demonstrated to be disease free. If these samples are to be collected, they should be prepared and handled as described below. All samples should be held at 4°C if they are to be tested on the same day. If testing cannot be performed on the same day, then the samples

should be properly identified and stored at a temperature of −70°C or lower until testing can be executed. For embryos recovered by filtration, the collection (flushing) fluids should be positioned in a sterile container (e.g., graduated cylinder) and allowed to settle for at least 30 min. After embryos that have been washed from the filter are identified and removed, fluid used to wash the filter, collection fluid, and sedimented debris should be placed in sterile containers and preserved. The precise volume of each sample will be specified by the regulatory authority requesting tests of the samples. Practically, this should be a volume that is easily stored and that can be used for valuable testing protocols (e.g., viral isolation or PCR assay).

Medium used for the last 4 washes (a minimum of 10 washes is required for proper washing) of the embryos should be clustered and stored (IETS, 2010).

11. CRYOPRESERVATION OF BOVINE EMBRYOS

Cryopreservation outlines techniques that allow freezing and subsequent warming of biological samples without loss of viability. The employment of cryopreservation in assisted reproductive technology includes the freezing of gametes, embryos and primordial germ cells (Mandawala, et al., 2016).

Thanks to cryopreservation biological cells or tissues are maintained at subzero temperatures resulting in deep decrease in the rate of metabolic processes and the possibility to store samples for prolonged periods (Mandawala, et al., 2016).

There is increasing interest among agricultural breeding communities to cryopreserve oocytes and embryos of agriculturally valuable animals such as cattle, especially because of its relevant importance for meat and milk production. The United Nations indicates that the world population is predicted to grow to an expected 9.15 billion by 2050; and it has been suggested that the consumption of meat is projected to rise to supply the 20% increase in *per capita* calorific intake. Moreover, it is common practice to transport live animals that are genetically superior or that are more suitable for specific markets between countries; but the transportation of live animals is both expensive and logistically difficult (Mandawala, et al., 2016). Consequently the substantial benefits of bovine embryos cryopreservation are: the transportation of frozen embryos instead of live animals which makes much more easier the trades between countries and is considerably safer in terms of biosecurity; the storage of high value genetics for an unlimited time; the possibility to make transfers when needed with no necessity to have ready recipients; the opportunity to perform transfers during the more suitable seasons (for example summer) or over moments of good fertility of the herd; the chance to take full advantage from embryo therapy. On the contrary, the main drawbacks of cryopreservation can be: a modest decrease of the percentage of implantation compared to fresh embryos (≈ 5-10%); the time required for freezing which increases the working hours; the costs of equipment and training; the need of skills and experience for embryo handling.

As previously reported, according to the International Convention, for commercial purposes only 7 days embryos of grade 1 and 2 can be frozen. Their stage of

development must be between compact morula and expanded blastocyst. A good quality embryo, eligible for the cryopreservation process, should have a diameter of at least 150 μm, a regular, compact and spherical shape, blastomeres of analogous dimensions, homogenous colour and texture (neither too light nor too dark), absence of vesicles or vacuoles and of extruded cells, an empty perivitelline space, a spherical *zona pellucida* which should be neither wrinkled nor collapsed. The freezing process, from 20 °C to -196/-210 °C, can be slow (in equilibrium) or ultra-fast (non-equilibrium). In order to obtain proper freezing it is essential to promote a series of osmotic exchanges such as to remove all the intracellular water of the embryo, which must be replaced entirely by a cryoprotectant (Morini, 2022).

11.1 Cryoprotectants

Freezing cells inevitably causes damage. The two principal causes of cellular damage are the physical damage attributable to the formation of ice crystals and the chemical damage that derives from modifications in intracellular solute concentrations. Both of these kinds of damage can be prevented, or at least mitigated, by controlling how the temperature is reduced and by modifying the cellular conditions. For example, the mechanical damage due to the piercing action of ice crystals can be avoided by performing the freezing process very rapidly and the sharp rise in intracellular solute concentration as the formation of ice crystals increases can be circumvented by the use of cryoprotectants. Permeating cryoprotectants replace intracellular liquid and reduce ice formation; hence they need to have low toxicity, be capable of penetrating cells, and be able to withstand very low temperatures (Mandawala, et al., 2016). Examples of commonly used cryoprotectants include:

- Alcohols: ethylene glycol, propylene glycol, glycerol, ethanol, methanol;
- Amines: formamide, dimethylformamide, taurine, lysine, proline;
- Inorganic salts: ammonium sulphate;
- Macromolecules: low-fat milk, serum, PVP, PEG;
- Sugars: sucrose, maltose, raffinose, trehalose;
- Sulfoxides: dimethyl sulfoxide (DMSO 2-5%).

The cryoprotectant is soluble in water, protects the cell membranes, reduces the freezing point of the solution as it further increases the dehydration of the embryo, has a negligible toxicity for the embryo and this minimal toxicity is directly proportional to the contact time at room temperature. Moreover, the permeability of the cryoprotectant is directly proportional to its molecular weight (Morini, 2022).

Cryoprotectant	Molecular weight	Permeability
Sucrose	344	No
Glycerol	92	Slow
DMSO	78	Medium
Propylene glycol	68	Fast
Ethylene glycol	62	Fast
Methanol	32	Max

Table 4 Permeability of cryoprotectants in relation to their molecular weight (Morini, 2022)

The most widely used permeable cryoprotectants are Glycerol sol. 1,4M (10%) and Ethylene glycol 1,5M (10%) alone or with Sucrose from 0,1M to 1M. According to International Convention, embryos frozen with ethylene glycol must be packaged and stored in yellow-coloured materials (Morini, 2022).

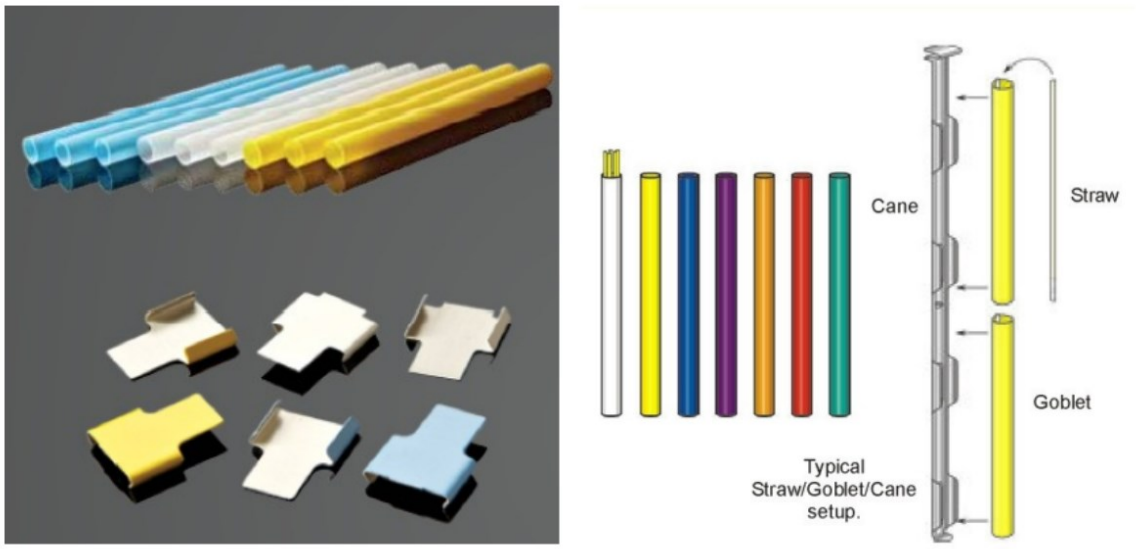

Figure 21 Left image: paillettes are identified with colour: yellow = cryoprotectant used is Ethylene glycol, white = Glycerol, light blue = vitrified. Right image: typical straw/goblet/cane setup (Morini, 2022)

11.2 Procedure of slow-freezing (in equilibrium)

The ideal laboratory temperature to execute the freezing procedure is between 18 and 22 °C. The main problem with freezing is that cells die during the process. The death of the cells can occur during freezing but also during thawing. The main determinants of cell damage and/or death are intra-cellular and extra-cellular ice crystals. In fact, the formation of crystals during freezing can damage the organelles and membranes within the blastomeres. During and after thawing the melting of crystals can cause damage due to osmotic effects. In addition, the rapid formation of ice crystals leads to an excessive concentration of cryoprotectant in the solution, which remains liquid. Excessive osmotic pressure quickly and excessively dehydrates the cells and, consequently, the cells themselves result damaged. Since in somatic cells there are from 1000 to 2000 mitochondria and in an oocyte there are up to 30000 mitochondria, the main ruptures occur on mitochondria. Collapse and increased re-expansion can be controlled by adding sucrose to the cryoprotectant. The dehydration in the following phases also seems to take advantage from this addition. With the induction to crystallization, which is called 'seeding', the balancing process continues because the water outside the embryo freezes and the solution becomes increasingly hyperosmotic. In fact, the residual water inside the embryo migrates to the outside by osmotic gradient and freezes, but only outside the blastomeres. The recommended cooling rate is 0,5 °C per minute. The process ends when all the endocellular water has migrated outside the embryo. Only when the condition of complete dehydration is achieved, the embryo can be plunged in liquid nitrogen (-194/-196 °C) (Morini, 2022). Thus, the freezing protocol foresees:

- Contact of the embryos with the cryoprotectant at room temperature in the laboratory (for Ethylene glycol min 5'/max 10' with temperature <20°C, min 3'/max 5' with temperature >24°C; for Glycerol min 10', if the room temperature of the laboratory is low (T<16°C) the time of exposure must be increased);

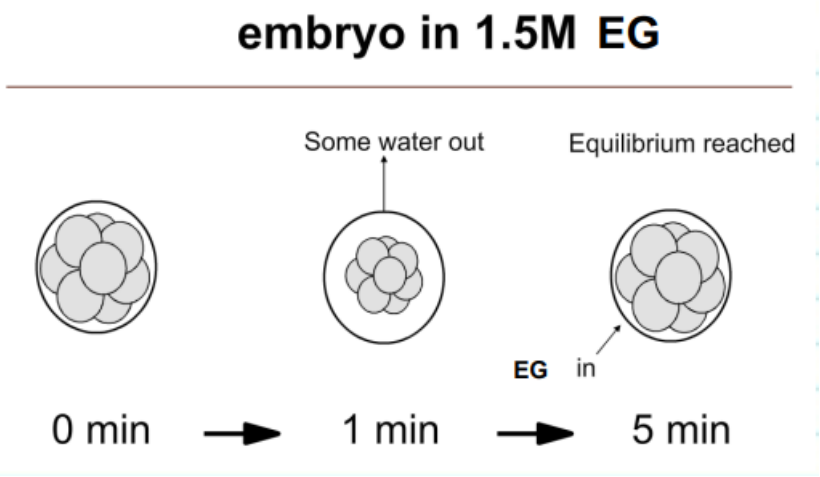

Figure 22 Modifications within the embryo after contact with cryoprotectant (Ethylene glycol) at room temperature (Morini, 2022)

- Insertion of the embryos in the freezer at -5<T<-7 °C (commonly at -6.5 °C);
- After not less than a minute (better two) seeding in a column without embryo, with pliers wrapping the paillette or the cotton bud in liquid nitrogen, for not less than 3 minutes of contact;
- Waiting for 10 minutes at the seeding temperature (checking for complete crystallization in the column with the embryo);
- Start to low the temperature until -32<T<-35 °C (-35 °C is the most applied) with a Cooling Rate of 0.5 °C/min (from -.01 to -0.8 there are no significant differences);
- Once reached the so-called Plunge Temperature, wait for stabilization for another 10 minutes;
- Quick and firm insertion of each single paillette in liquid nitrogen in a large polystyrene container with low walls or an unbreakable “Dewar”;
- Complete the insertion of the paillettes in the Canes before transferring everything in a dedicated cryogenic container (Morini, 2022).

Attention must be paid to the Cooling Rate, in fact cooling rate over -1°C per minute reduces the survival rate to less than 50%. Sperm undergo cooling rates ranging from -10 to -20 °C per minute until -100 °C before being plunged in liquid nitrogen without

suffering any particular damage. This happens because their percentage of water is around 15% against the 85% of water of which the embryo is made (Morini, 2022). There are several factors affecting the pregnancy rates obtained from frozen embryos: the quality of the embryo and of the equipment, the familiarity with the freezing protocol, the breed of the donor, the familiarity with the thawing protocol and the transfer procedure, the synchronization of the recipients and the experience in selecting suitable recipients, the geographical area, the time between collection and freezing (from 1 to 3 h: 66%; from 3 to 5 h: 54%; from 5 to 7 h: 45%) (Morini, 2022). Survival depends on the cell type and its ability to withstand various stresses caused by physical and physiochemical changes during the process, as well as rates of cooling and warming (Mandawala, et al., 2016).

Figure 23 Example of standard device used for the slow-freezing process (Morini, 2022)

11.3 Non-equilibrium ultra-fast freezing

The process of ultra fast freezing is called vitrification. The embryo is immersed in a high osmolarity solution, the operation must be completed in a maximum time of 60 seconds at a temperature of +4°C. The cooling rate of liquid nitrogen is about -20000 °C per minute. Due to the high viscosity of the solution and the ultra-fast freezing, the solution has the same physical features of glass. By the way, vitrification is still a technique under development. It has several advantages, such as cost-effectiveness, speed and ease of execution and the possibility to store more successfully also grade 2 embryos. On the other hand it requires particular paillettes called Open Pulled Straw (OPS), still not available on the market, and high defrosting temperatures (Morini, 2022).

Vitrification eliminates the damage caused by the ice crystals formation during the cooling process. In fact, as illustrated in Figure 26, during slow-freezing water flows out of the cells because of the extracellular ice crystals formation, thus causing mechanical damage to the cell by inhibiting the cell's ability to maintain its structure. Conversely, during vitrification cells are inserted into a vitrification medium with high viscosity, which avoid extracellular ice crystallization and, as a consequence, cells remain intact. In both cases, cryoprotectants prevent intracellular ice crystallization. The solid glass-like form of the solution achieved with vitrification results being amorphous, which means that it can readapt and take the shape of the cell, hence allowing the cell to keep its structure and remain intact (Mandawala, et al., 2016).

New vitrification techniques are continually being elaborated and in general can be classified as open or closed. The difference depends on whether or not the medium is in direct contact with the liquid nitrogen used during the cooling process. In an open system, the oocytes or embryos make contact directly with liquid nitrogen, whereas in a closed system, they do not. Direct comparisons among these kinds of systems have been limited; nevertheless, the available evidence indicates that the viability of oocytes and embryos after warming can be comparable (Mandawala, et al., 2016).

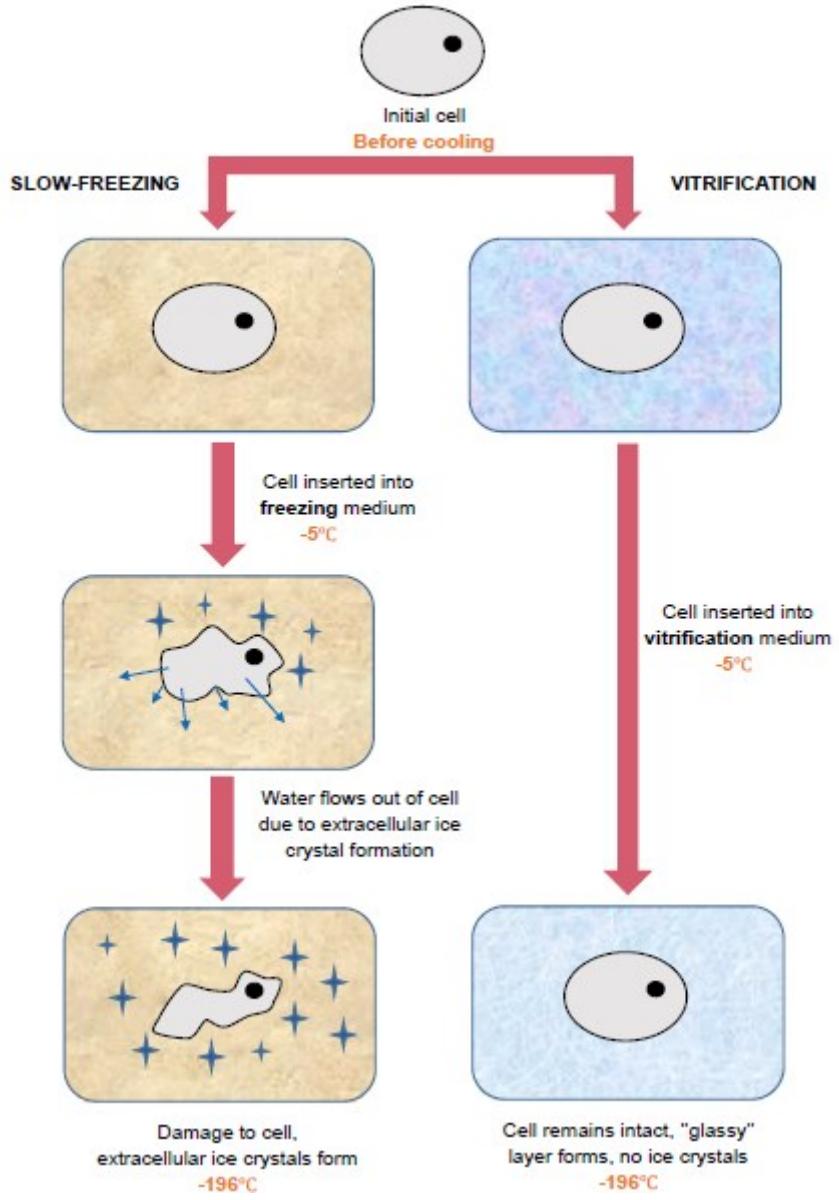

Figure 24 Comparison of rates of ice crystal formation between slow-freezing (left) and vitrification (right) (Mandawala, et al., 2016)

Cow embryos are sensitive to cooling injury at very low temperatures and at first only a few studies showed that 8- and 16-cell embryos survived vitrification at 0°C (Polge, et al., 1978). Polge and Willadsen also reported that blastocysts could better withstand cooling in comparison with 8- and 16-cell embryos or even morulae, which has led researchers to focus most of their attention to vitrifying embryos at only blastocyst stage. There has been some evidence in the literature that flushed cattle embryos produced *in vivo* show differences in their cryopreservation properties when compared with embryos obtained *in vitro* (oocytes aspirated either from ovaries

obtained from a slaughter house or by ovum pick up following super stimulation) (Polge, et al., 1978). In particular, Stachecki and Weimer noted that, in comparison to their *in vivo* produced equivalents, bovine embryos that are developed *in vitro* are more sensitive to cooling (Stachecki, et al., 2007). Although the cause of this is not yet clear; nevertheless, one reason for this could be due to the metabolic divergences which are frequent during preimplantation development (from oocyte to hatched blastocyst) of bovine embryos based on how they were developed (*in vivo* or *in vitro*) (Khurana, et al., 2000).

Techniques of vitrification of bovine embryos have remarkably advanced in recent years; in particular, a study by Park et al. (Park, et al., 1999) displayed that an electron microscopy grid could be used a valid vitrification container (instead of traditional straws), to obtain high-embryo survival rates when vitrifying bovine blastocysts. In another study, Mucci et al. confronted embryo survival (defined by blastocyst hatching rate) for bovine embryos that were either vitrified or slow frozen and noted a significant positive tendency toward vitrification (43% survival rate for vitrified embryos, compared to 12% for slow-frozen embryos) (Mucci, et al., 2006).

Hence there has been great success with vitrification of oocytes and embryos, more research is needed to obtain more data, and importantly, more reliable results particularly in agricultural animals. The creation of automated devices for vitrification is likely to be a huge step forward in this direction; however, the use of such technology in agriculturally valuable animals such as cow, whilst considering the scale of production that would be required for use in the agricultural industry, is yet to be experimented. The efficient introduction of a closed, automated vitrification system for ordinary use in cattle IVF could conceivably innovate the field and would in no doubt be in the interest of breeding companies (Mandawala, et al., 2016).

12. ECONOMICS AND COMMERCIAL APPLICATIONS OF EMBRYO PRODUCTION

Since reproduction affects both milk production and number of replacement heifers available on a farm, it remains a key component to maintain a dairy farm profitable. Consequently, reproductive performance becomes one of the essential elements to improve the economic success of dairy herds (Ribeiro, et al., 2012).

Because production constitutes more than the 88% of the gross earnings of a dairy farm, it is obvious that most attention paid to advancements in reproduction develop around altering milk production during the productive life of cows. Most often, altering milk production has to be calculated per day of calving interval, as improvements in reproduction extend the time a cow spends in the dry period, which is seen as a nonproductive stage of the lactation cycle. A reduction of the calving interval diminishes the average days in milk of the herd and, therefore, the majority of cows would be in earlier stages of lactation when peak of milk production and higher income over feed cost occurs, while a smaller proportion of cows would be in later stages of lactation producing low quantities of milk with low income over feed cost. Advancing reproduction in many cases means a greater availability of replacement animals, which increases herd turnover. Reproductive inefficiency increases cost per pregnancy, increases retentivity of low-producing cows due to their pregnancy status, and diminishes the number of replacements, which reduces the growth in genetic merit of the herd. Keeping the same replacement pressure when reproduction is inadequate becomes, oftentimes, costly and hazardous as it requires purchase of heifers that could be of lower genetic merit and could lead to breaks of biosecurity. Moreover, as days postpartum lengthen beyond a definite maximum, replacement is economically more convenient than breeding. This time interval in which breeding a cow is commercially attractive varies mainly with milk production, lactation number, and cost-efficiency during the late stages of lactation (Ribeiro, et al., 2012).

In the matter of new pregnancies, the value of a new pregnancy increases as days in milk increase until late stages in lactation when it starts to decrease (De Vries, 2006).

The economical explanation also relies on profitability of milk production at late stages of lactation and replacement policies that together drive future profits.

The value of a new pregnancy is higher for low-producing cows at early stages of lactation (De Vries, 2011). Likewise, the cost of pregnancy loss is greater for high- than low-producing cows except if it happens very early in lactation. The mean value of a new pregnancy for a Holstein cow in the United States has been evaluated at $278, while the cost of a pregnancy loss was $555 (De Vries, 2006).

The use of embryo technology has been portrayed as an option to improve pregnancy in dairy cows, particularly during periods of heat stress. In fact, as it has already been explained during this discussion, the transfer of a blastocyst to a synchronized recipient has the ability to improve fertility since it bypasses fertilization and early embryo development, which are stages of particular thermosensitivity and of increased losses of pregnancy. However, along with fertility, many other factors need to be considered to assess if the use of embryo technologies is cost-effective. The cost of the embryo, the labour force, the recipient utilization, genetic progress, and fertility to AI programs are all further important elements which cannot be overlooked (Ribeiro, et al., 2012).

Indeed, the costs that have to be taken into consideration in an embryo transfer program refer to: the hormones needed for donors and recipients, the semen (usually 3 doses of non-sexed semen or 5 doses of sexed semen), the AI technician fees, the veterinarian professional fees, costs for materials and equipment, costs due to the possible losses of embryos transferred during pregnancy and at term as a result of embryonic mortalities, abortions or stillbirth.

In Italy, according to the parameters for the settlement of professional fees indicated by the FNOVI, the average prices for veterinary services concerning bovine embryo transfer are:

- Preparation of the donor, the recipients, flushing and embryo recovery ≈ €600
- Freezing of one embryo ≈ €130
- Freezing of embryos subsequent to the first ≈ €40
- Implant of each fresh embryo ≈ €50

- Implant of each frozen embryo with the ONESTEP method ≈ €55
- Implant of the first frozen embryo with the thawing method ≈ €100
- Implant of the frozen embryos subsequent to the first with the thawing method ≈ €55 (Source: www.fnovi.it)

Taking into account the average costs of this assisted reproduction technique, before starting an embryo transfer program, each farm should independently assess the economic feasibility of the program and whether and how the farm can benefit from it, both in terms of health and genetics of the herd and in terms of financial return. This reasoning can be applied in Italy as well as in other countries depending on the costs and economic policies, which can both vary from country to country.

Sànchez et al. aimed to assess the feasibility of using embryo transfer in small community farmers by an *in vivo* study (Sànchez, et al., 2015). From the total of 59 donor cows, 62.7% were responsive to treatment, with a marked difference in the percentage of the response between breeds, being 90.5% in Holstein and 47.4% in Brahman. A total of 283 embryos were evaluated as transferable, while 141 as non-transferable, with no differences in the percentage of transferrable embryo by breed. The average of transferable embryos evaluated as Grade I and II was not different between Holstein and Brahman; as well, no differences were noted in the other grades (non-transferable). The main difference in costs, despite its quality by breed, was observed in the lower levels of probable fertility of the embryo transferred, even reaching several hundred dollars. When modelling the predicted costs for embryo produced and transferred, values can rise nearly to $2000 when the probable fertility is only 10%. Still, when the probable fertility was 60%, embryo cost was close to $300. Therefore, this technology seems to be generally practicable only on high-scale systems, having a superovulatory response between 60 and 80% with 4-6 transferable embryos. However, in small scale farming, because of the limited number of donors and/or recipients, the costs outweigh the economical feasibility of the technique (Sànchez, et al., 2015).

In addition, experienced embryo transfer practitioners acknowledge the relevance of proper husbandry and management methods on success. Well-managed cattle

procedures can make an ordinary embryo transfer practitioner appear very good, while inadequately managed operations can demean the most skilled embryo transfer practitioner. It is clear that success in bovine embryo transfer requires a combination of reproductive physiology, basic animal husbandry, and veterinary science to produce constant acceptable results. Breeding farms that use these resources with skilled staff are continuously successful, while those that do not are often unsuccessful. Many new cattle breeders seem to think that technology together with expertise on the part of the practitioner is all that is needed to succeed. The relevance of animal husbandry is oftentimes ignored and under estimated. It is up to the of the embryo transfer practitioner to make clients aware of the value of basic animal husbandry (Stroud, et al., 2006).

Moreover, according to Ribeiro et al., when embryo technologies are included to the breeding program with the purpose to improve reproductive performance, the differential in fertility has to be large compared to AI to be economically justified. In most cases, AI programs have to result in very low fertility (<15%) for the usual results from embryo transfer (40-45% pregnancy) to be economically attractive (Ribeiro, et al., 2012). After the consideration stated by Ribeiro, in order to evaluate the profitability of the application of embryo transfer, Heikkila and Peippo found an optimal breeding program that included conventional and sexed semen together with embryo donors with embryos sold to external farms for Finnish dairy herds (Heikkila, et al., 2012). Nevertheless, these studies did not consider the genetic gain from ET versus AI among the herd.

Consequently, Heikkila and Peippo urged the necessity of using dynamic models that contemplate the genetic improvement over the course of several generations through selection in the herd to study more precisely the effect of different kinds of breeding, including embryo transfer (Heikkila, et al., 2012).

Kaniyamattan et al. performed a study whose aim was to define the optimal proportions of pregnancies from an *in vitro*-produced embryo transfer system and artificial insemination (AI) in a way that the cost-efficiency is maximized over a range of prices for embryos and surplus dairy heifer calves (Kaniyamattam, et al., 2018).

In a previous simulation study, he showed that a standard dairy herd using exclusively an IVP-ET system could sell up to 50% of its dairy heifer calves and still keep an annual cow cull rate of nearly 33% (Kaniyamattam, et al., 2017). The breeding cost per cow per year was $405 for an exclusive IVP-ET system after 15 years of realization, compared with $46 for an exclusive conventional AI system (Kaniyamattam, et al., 2017).

Even after considering the greater genetic value of the dairy heifer calves obtained just with the IVP-ET system and selling the surplus of dairy heifer calves at a higher price that reflects their genetic merit, the exclusive IVP-ET system was just as profitable as the exclusive AI system in 15 years. Significant financial losses per cow per year in the first 3 years accumulated because of a delay in the return of investment in IVP embryos (Kaniyamattam, et al., 2017). According to his more recent study (2018) an improved strategy could be to use IVP-ET to obtain less than 100% of the pregnancies in the herd. Conventional or sexed semen would be utilized for animals that are not selected as embryo recipients. The hypothesis of the study is that there is an ideal proportion of IVP-ET which includes a combination of IVP-ET and AI (Kaniyamattam, et al., 2018). The optimal proportion of IVP-ET possibly depends on the prices of embryos and of surplus dairy heifer calves, and that's why the model displayed in this study could be potentially applied also to *in vivo* derived embryos, simply by recalculating the optimal proportion taking into account the differences in terms of costs of the embryos produced *in vivo*.

Kaniyamattam outlined the economic and genetic performance of dairy herds that use different proportions of pregnancies obtained by IVP-ET and AI using a stochastic, dynamic dairy model with multi-trait genetics. He executed a sensitivity analysis with 6 prices for surplus dairy heifer calves and 4 prices of embryos to define the economically best proportion of pregnancies that are produced with IVP-ET. He adapted a previously developed randomized, dynamic dairy model with multi-trait genetics that includes the genetic, technical, and financial performance of dairy herds that use various proportions of pregnancies generated by IVP-ET and AI. The 15 years profit after the initiation of the IVP-ET program was maximized when 40% of the total pregnancies in the herd came from IVP-ET.

Lower prices for IVP-ET or higher values of surplus dairy heifer calves increased the improved use of IVP-ET.

Dairy heifer calf sale price[1]		Embryo price ($)	Additional profit per cow in yr 15[2] ($) by embryo transfer conceptions[3]						Optimal ET[4] (%)	Max. add. profit[5] ($)
Base price ($)	Premium		0%	21%	42%	63%	82%	100%		
300	No	50	0	64	90	80	61	47	46	91
300	No	100	0	39	42	7	−36	−75	33	45
300	No	150	0	13	−6	−66	−133	−197	19	14
300	No	200	0	−12	−55	−139	−231	−319	3	0
300	Yes	50	0	91	155	187	209	241	100	241
300	Yes	100	0	66	107	114	112	119	100	119
300	Yes	150	0	41	58	41	15	−3	42	58
300	Yes	200	0	16	10	−32	−82	−124	28	18
500	No	50	35	107	148	150	144	142	69	158
500	No	100	35	82	99	77	47	21	41	99
500	No	150	35	57	51	4	−50	−101	28	59
500	No	200	35	32	3	−69	−147	−223	8	37
500	Yes	50	35	135	213	258	293	337	100	337
500	Yes	100	35	110	164	185	196	215	100	215
500	Yes	150	35	84	116	112	99	93	62	116
500	Yes	200	35	59	67	39	2	−29	36	69
700	No	50	70	150	205	221	228	238	84	238
700	No	100	70	125	157	148	131	116	48	158
700	No	150	70	100	108	75	33	−5	36	110
700	No	200	70	75	60	2	−64	−127	12	77
700	Yes	50	70	178	270	328	376	432	100	432
700	Yes	100	70	153	222	255	279	311	100	311
700	Yes	150	70	128	173	182	182	189	79	191
700	Yes	200	70	103	125	109	85	67	25	129

[1]Base female calf sale prices of $300, $500, or $700 at 105 d of age. The dairy heifer calf rearing cost since birth at 105 d was $375.
[2]Additional profit per cow in yr 15 for varying proportions of conceptions from IVP-ET compared with the scenario with no conceptions from IVP-ET (ET0).
[3]Scenario and actual proportion of pregnancies from IVP-ET: ET0 (0%), ET20 (21%), ET40 (42%), ET60 (63%), ET80 (82%), and ET100 (100%).
[4]The economically optimal proportion of conceptions obtained from IVP-ET.
[5]The maximum additional profit per cow per year at the optimal proportion of conceptions from IVP-ET compared with the scenario with no conceptions from IVP-ET.

Table 5 Optimal proportion of conceptions to be achieved from in vitro-produced embryo transfer such that profit per cow in 15 years in maximized, according to Kaniyamattam dynamic model (Kaniyamattam, et al., 2018).

Some use of IVP-ET was profit-making in various realistic combinations of embryo prices and surplus dairy heifer calf values (Kaniyamattam, et al., 2018).
Considering these studies, it can be suggested that dynamic models, such as the one developed by Kaniyamattam, could be designed with the aim of calculating the right combination between the use of embryo transfer (taking in consideration also the use of *in vivo* derived embryos) and AI and could be applied in the breeding program of farms in order to maximize their long-term profits.

13. CONCLUSIONS

Given all the considerations that have been made so far, it is certainly possible to affirm that commercial embryo transfer in cattle has become a well established industry with a great impact in many countries.

The annual report of data collected globally in 2021 by the IETS data retrieval committee on embryo transfer activities during 2020, indicate that in spite of the COVID-19 Pandemic and its extensive negative effects upon global economy, the world bovine embryo production increased in most regions. For the first time, more than 1,5 million bovine embryos were recorded, which represents an increase of 7.0% compared with 2019 (Joao HM Viana, Chair - IETS Data Retrieval Committee, 2021). In fact, due to the Pandemic and to the global political changes occurred in the last couple of years, the increased demand for meat and the boosting of the prices of meat and milk on the international markets rapidly created a growing demand for replacement animals and, ultimately, the use of reproductive biotechnologies.

There was a decrease in both numbers of collected and transferred IVD embryos in 2020 and this decline has been fully compensated by an increase in the number of IVP embryos produced and transferred in 2020. An opposite trend was only observed in Europe, which reported more IVD and less IVP embryos in 2020 than 2019. Europe has diverged from the trends observed in other regions probably because of the higher prevalence of low-scale dairy operations and the scattered distribution of ET activity among numerous countries (24). This consequently led to a persistency of IVD embryo collection activities. If the current trends were maintained, in a few years Europe will probably overtake North America as the region with more IVD embryos collected (Joao HM Viana, Chair - IETS Data Retrieval Committee, 2021). In consideration of this likely future scenario it is important to evaluate the strategies that could optimize IVD embryo collection techniques as well as embryo transfer itself. An adequate management of the farm remains essential to ensure the success of an embryo transfer program and this aspect should never be overlooked or underestimated. In the field of scientific research, more in-depth studies about embryo genomic evaluation could be carried out in association with further studies

on metabolomics, in order to precisely determine both male and female fertility markers in cattle, as well as markers for embryo competence and quality and for uterine adequacy. Moreover, more attention could be paid to diets and nutritional aspects in order to promote a proper uterine environment and embryonic development and to ensure dietary energy requirements for cows that have to produce not only milk but at the same time also quality embryos. In this regard, a potential suggestion could be to focus on the formulation of specific food supplements designed specifically for cows included in embryo transfer programs, maybe containing also antioxidants among other specific elements that promote fertility. Therefore, their diet should be carefully defined and integrated in accordance to the different moments of their estrous cycle and to the various stages of embryo development.

The development of more and more precise and technological tools used in the routine execution of this assisted reproduction technique, combined with an increasingly efficient managerial evaluation of its practical use, will be able to make embryo transfer a technique that goes hand in hand with the modern need to reconcile breeding industry and both economic and environmental sustainability.

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