Chapter

Embryo Transfer

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Abstract

Assisted reproductive technologies (ART) have made tremendous advances, in last years. Artificial insemination is a method for achieving slow genetic progress in populations of animals. Many large and small ruminants are bred by AI, and more than a half million embryos are transferred every year around the world. Most of the ruminants sires used for artificial insemination were derived from embryo transfer. Improvements of reproductive biotechnologies of controlling the estrous cycle and ovulation have resulted in more effective programs for AI, superovulation of donor, and the management of ET. In the ruminants, ET procedure is a timely alternative that can allow good conception rates to be obtained constant in a year. There have been great advances of this biotechnique with on aimed to intensify the genetic progress between generations of farm. The gains is possible with the development of advanced reproductive biotechnique. The best current strategy in applying biotechnology to farmers is to use AI with sexed semen, so farmers will enjoy and benefit. The use of ET together with cryopreserved sexed embryos has a very specific potential for donor replacement and genetic improvement of the herd. In this chapter, procedures of the MOET protocol were described step by step.

Keywords: embryotransfer (ET), ruminant, cow, estrus, IVEP, MOET, ART

1. Introduction

The ruminants sector plays an important role in global socioeconomic directions. Therefore, it is necessary to research, to discover and to innovate and transfer knowledge to the farmer, for practices and alternatives that improve ruminants reproduction and production.

Increasing the efficiency of breeding and production of a farm herd is one of the great challenges for large ruminant producers. Recently and now, genetic selection programs have sought the characteristics needed to increase milk production, with gains by increasing the quantity and quality of milk. However, reproductive efficiency was neglected. In recent years, various publications have presented strategies to further increase milk and meat production and also to increase reproductive performance, which is a key factor for the efficient growth of ruminants [1].

In farmers, importance of a sustainable, economically viable production system can be obtained by maximizing reproductive efficiency of the ruminants herd.

This reproduction management can determine the profitability from the number of offspring produced, the genetic progress and the shorter interval between lactations periods.

The essential importance of precision reproductive care is therefore highlighted. This reproductive biotechnology (ET, AI) applied is capable of produce maximum

production efficiency in animal farms with vulnerable populations, or in limited areas, in addition to improving animal welfare.

The first biotechnology of reproduction represented by artificial insemination (AI) is known as the simplest and lowest cost of reproductive biotechnology. This technique enhances the male's genetics, bringing slow genetic progress. However, dairy breeds in conditions of seasonality and climate change exhibit failures in estrus cycling and demonstration, which compromises AI results. In other words, with the discovery and description of FTAI protocols that synchronize follicular growth and induce ovulation, it is possible to achieve a high rate of inseminated animals without the need to observe the clinical and behavioral signs of estrus. [2], thus providing an increase in the conception rate and avoiding the occurrence of human errors in the detection of estrus, and calculating the optimal time for insemination. However, gametes an embryo can undergo degeneration in the extreme temperatures of summer [3]. The transfer of embryos produced in vivo (ET) became a strategy to avoid the deleterious effects of this period and provide a higher productive index than with AI [4].

With the beginning of the evolution of modern biotechnologies, the next step as major commercial progress in reproductive biotechnology was the transfer of embryos that appeared in the late 1970s. The ability to preserve, freeze and transport bovine embryos around the world has made ET an extremely useful technology for disease control, genetic rescue of valuable individuals and the development of new lines or breeds of animals.

ET is a multifactorial protocol that depends on several carefully and correctly performed sequential steps. Poor performance in any of the steps directly affects the success rate of the final result, the conception rate and the number of weaned products.

The use of embryo transfer as a breeding technique is growing throughout Europe, even in countries with less embryo transfer tradition. Historically the entire embryo transfer process was carried out at a specialist centres but now experienced reproduction vets are starting to carry out the artificial insemination (AI), flushing and searching as ambulatory procedures for transfer into a suitable recipient. The most time consuming and difficult part of the in vivo embryo transfer process is synchronizing recipients and transferring the embryo into the most suitable recipient. Receptors must be selected with with the best chance of maintaining the pregnancy [5, 6].

Embryo transfer provided a means by which the number of conception products could be multiplied rapidly, with the same origin. However, embryo transfer veterinarians have developed technology for commercial use and taken techniques from the laboratory to the farm. There have been countless practical difficulties for practitioners in uniting and setting up the International Embryo Transfer Society (IETS) in order to facilitate the discussions and steps deemed necessary for progress. Currently, the vast majority of countries in the European Union have one or more embryo transfer associations, where these actions are reported and come to support and develop ET biotechnology (eg AETE, SIET, AET-d, AETF, ARET and others).

Embryo transfer (ET) is now commonly used to produce AI sires from the top producing cows and proven bulls for the dairy industry [7]. As a perspective, the new genomic techniques presented are increasingly used for the selection of embryo donors, and genomic analysis has become essential for the selection of bull dams that will be used in embryo transfer [8]. Although the economy sometimes seems to exclude the use of embryo transfer techniques for anything other than gamete production, the commercial cattle industry benefits from the use of commercial males produced through well-designed MOET programs [9].

With the explosive development of this biotechnology, the techniques for obtaining embryos have been improved, the materials and consumables have become more efficient, the equipment more efficient, which makes the production cost of the embryo decrease and be higher quality. This desideratum is fully accepted by farmers and who apply this ET biotechnology more and more frequently [10].

Although there has been no appreciable increase the embryo production per poliovulated donor in last years, the importance of follicle wave dynamics [11] and methods for the synchronization of follicular wave emergence [12, 13], they simplified the protocols by which female poiliovulation could be achieved, leading to increased embryo production per application session. Currently, donor cows are hyper-stimulated more frequently than in the past (at an interval of 30–60 days) and thus more embryos can be produced per year, without changes in the current superstimulation protocol [14]. Other authors [15] have been interested in various factors that affect the viable production of embryos in animals and especially in dairy cows.

Potential embryo donors can be inseminated naturally or artificially (AI) and the embryos are normally collected non-surgically from 6 to 8 days after fertilization. After collection, the embryos must be identified and then evaluated morphologically. The evaluation procedure is done in an appropriate environment before the transfer. At this stage, they can be subjected to manipulations, such as splitting and sexing, and can be cooled or frozen for shorter periods or longer storage [16]. Discussion of donor superovulation, recipient synchronization, and embryo transfer should begin with a review of recent information on the physiology of female reproduction and the estrous cycle.

The reproductive genetic potential of every normal newborn calf is enormous. It is said that there are about 150,000 "eggs" or potential oocytes in a female and many billions of sperm produced by each male. We can say that through natural reproduction, only a small part of the reproductive potential of a valuable individual could be realized. The bull will be able to produce an average of 15 to 50 calves per year, and the cow will have an average calf per year. With the use of artificial insemination biotechnology, it is possible to exploit the large number of sperm produced by a genetically superior bull; however, the reproductive potential of the female with superior genetics was largely unused. It will produce on average 5–8-10 calves in its entire biological life through normal management programs. As artificial insemination has done for bulls, embryo transfer is a technique that can greatly increase the number of offspring that a genetically important cow can prove and produce. The main reason for the development of embryo transfer in cattle was to further the increase in genetic progress of the female.

2. Advantages of embryo transfer (ET)

- Increase the small population of valuable animals [17, 18].
- Helps in the genetic improvement of animal, decreases the generation interval, increases selection intensity [19].
- It helps to get more many calves from a genetically superior single female as against a few calves which can be produced naturally in its lifetime
- An possibilites tool to produce breeding bulls from a limited number of superior females for use in AI [20].

- May increase the numbers of the existing purebred herd.
- Possible to obtain offspring from the genetically valuable female that has become infertile due to disease, injury, or age [21].
- With the improvement in cryopreservation and the advancement of technologies, it is economical and easier to transport (import/export) embryos instead of living animals.
- Endangered animals can be saved from extinction by embryo production and cryopreservation [22].
- Avoids transmission of diseases from infected donors to their offspring-Genetic defects [20].
- Decreases the risk of transmitting infectious agents. Embryo collected from cows with bovine leukemia virus, blue tongue virus, FMD virus if washed properly before transfer (Trypsin treatment) to unaffected recipients does not transmit disease.
- ET allows differentiation between normal and abnormal fertilization [23].
- An important tool for disease control, genetic salvage of valuable animals, biosecurity program, development of new lines/breeds of animal.
- Helps in the proliferation of femele genetic material from dam and sire [24].
- Associated reproductive technologies (ART) such as embryo splitting, sexing of embryos, cloning, transgenesis further broadens the horizons of ET [25].
- The males produced by ET out of superior donors by using elite bull semen can be used as future elite sires on the AI network and females produced would serve as the future bull mother. Therefore the existing demand of elite sires especially in cattle and buffalo breeding programs can be largely met [24].
- Can be used to evaluate the contribution of the aging oocyte to decreased reproduction in geriatric animals [23].
- Infertility treatment, ET serves as an important tool in the treatment of infertility in cows.

3. Sexual cycle physiology

The intrinsic control of the bovine estrous cycle is coordinated by the interdependent secretion of hormones from structures such as: hypothalamus, anterior pituitary, ovaries and uterus [26]. These include gonadotropin-releasing hormone (GnRH) from the hypothalamus, folliculostimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland, estrogen, progesterone and inhibin from the ovary and prostaglandin F2a (PGF) from the uterus.

During gestation, the multiplication phase of the oogonia results in the constitution at the birth of a stock of primordial follicles, the number of which in the cow is between 200 and 250,000. These primordial follicles have a diameter of between

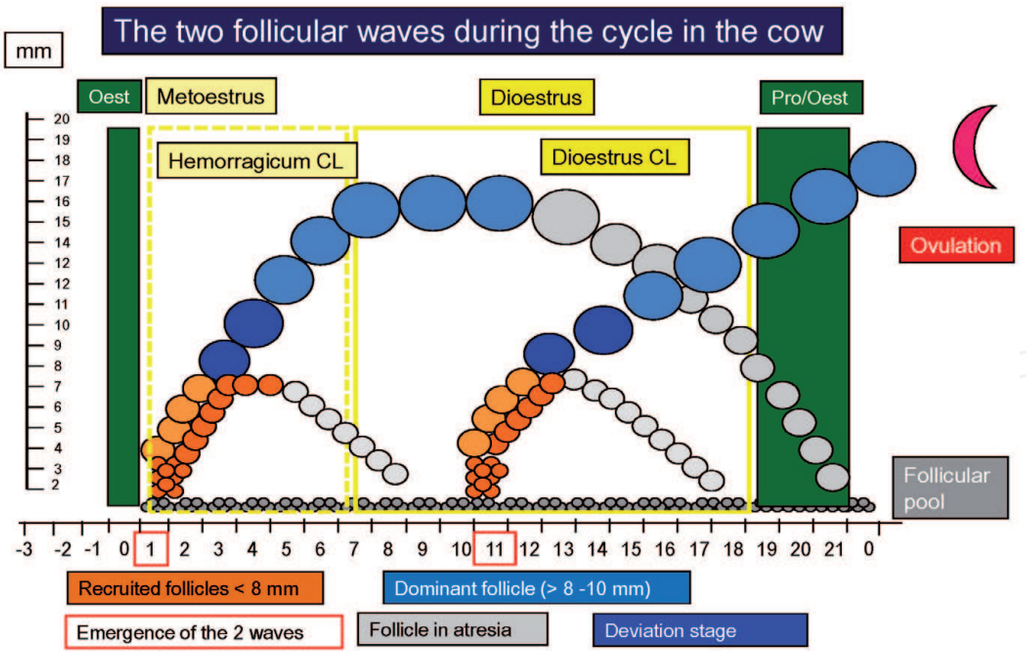

Figure 1.
Physiology of the sexual cycle in cows, with two follicular waves.

30 and 40 microns. At the secondary and especially tertiary stage, a cavity appears. It then becomes possible to identify by ultrasound these follicles with a diameter of between 2 and 4 mm.

In cows, as in many other mammals, follicular growth develops in the form of waves. In a 21-day cycle, there are two waves. The presence of a third wave is not uncommon. It has the effect of lengthening the average length of a cycle by a few days (24 vs. 21 days). When the cycle has two waves (**Figure 1**), the emergence of one wave occurs on days 0–1 and 10–11 of the cycle. Day 0 corresponds to that of estrus. By emergence we mean the moment or by ultrasound, it is possible to distinguish in the mass of follicles recruited the one which will become dominant. During the 2.5 days after the emergence of a wave, the selected future dominant and dominated follicles continue to grow. The dominant follicle reaches at this time the average diameter of 8 or even 9 mm. This moment is called "follicular deviation" and characterizes the moment when the dominant follicle will be able to clearly distinguish itself from other growing follicles. Its diameter is therefore 2 mm greater than that of the other selected follicles. The follicle continues to grow until it reaches a diameter of 10 mm. Clinically, this dominance can be identified by ultrasound, or by Doppler ultrasound to identify changes in the vascularization of the follicle or by assaying hormones such as estradiol or follicular fluid inhibin. This dominance is therefore both morphological, ie exerted by the largest follicle and physiological, because it brings about an arrest in the development of the dominated follicles which go through a static phase before settling down. This physiological dominance also implies the appearance at the granular level of LH hormone receptors which will take over from FSH to ensure further growth of the dominant follicle. The period of physiological dominance is shorter than that of morphological dominance. Clinically, the identification of more than 10 follicles with a diameter of between 3 and 8 mm makes it possible to exclude the presence of a physiologically dominant follicle. Growth of the dominant follicle will continue until it reaches a maximum diameter of 16 mm.

This is followed by a so-called static plateau phase lasting more or less 6 days at the end of which the dominant follicle will begin to regress. Due to the gradual decrease in estradiol synthesis by the dominant follicle, there is a new release of

FSH and the appearance on day 10 of new follicular emergence. This cannot be observed as long as the dominant follicle from the previous wave is in the growth or plateau phase. This new wave develops like the previous one through the dominance of a new follicle which will suppress the growth of the subordinate follicles which will become saturated. The follicle continues to grow. In proestrus he is no longer under the progesterone influence, so given the massive release of LH he can stop growing and then ovulate.

During the cycle, the follicular population is therefore distributed into several classes except that of the follicles in the reserve. A first class consists of recruited follicles. Their diameter is 2 to 4 mm. A second class is made up of growing follicles. These follicles can potentially become the ovulatory follicle. Their diameter is between 6 and 10 mm. The third class refers to the dominant follicle. Its diameter is between 10 and 16 mm. Finally, can we also identify the preovulatory follicle with a diameter greater than 15 mm. It will persist on the ovary for 5 to 6 days before regressing or ovulating (**Figures 1** and **2**) [5, 6, 10, 27].

In a cycle with three waves (**Figure 2**), the emergence of cows occurs on days 1, 10 and 17 respectively, with day 0 being estrus and therefore day 1 ovulation. The general wave pattern is comparable to that described for a cycle with two waves. Waves 1 and 2 are anovulatory. Only the third is normally ovulatory. It will be seen that the luteal phase like the cycle is of longer duration than for a cycle with two waves. Likewise, the interval between the onset of the ovulatory wave and ovulation is shorter (7 vs. 11 days).

The main mechanism of synchronization of the estrous cycle is ovulation, when the first follicular wave occurs [11]. A new hormone-secreting endocrine gland is formed instead of the ovulatory follicle and is called the corpus luteum (CL) it is formed in the following days (3–5) and in the absence of pregnancy, it wraps around day 16 or 17 of the cycle [26]. The most common hypothesis for CL regression is that the non-pregnant uterus secretes a luteolytic factor into the uterine venous blood. This substance is transferred through a local blood (veno-arterial) pathway to the ovarian artery through which it reaches the ovary and causes luteolysis [27].

After CL regression, a rapid decrease in serum progesterone concentrations to values lower than 1 ng/ml results, at the same time, the frequency of LH pulse increases and follicular development is further stimulated. The growth and maturation of the follicle that becomes preovulatory results in increased estradiol secretion, which causes local estrogenic changes in the oviduct and uterus, behavioral estrus and a preovulatory release of LH (around the time of estrus manifestation). The preovulatory LH peak results in the resumption of the oocyte meiosis process, and ovulation 24 to 32 hours later and the luteinization of the ovulated follicle to form a secretory corpus luteum hemoragicum. The growth and development of the hemorrhagic corpus in a fully functional CL results in progestative changes in the oviduct and uterus that are favorable for embryonic development and pregnancy. If pregnancy does not occur, the cycle resumes again with the disappearance of CL [6, 11, 23, 27].

3.1 Estrus synchronization, superovulation

Estrus synchronization and superovulation are critical components of an embryo transfer program. These techniques involve the manipulation of the basic endocrine patterns, presented and described in this document [28]. The key to successful estrus synchronization is synchronous growth and ovulation of a viable dominant follicle and closely synchronized, rapid declines in circulating progesterone to values <1 ng/ml [29]. If properly implemented, within the physiological constraints of their mechanism of action, current techniques for synchronization of estrus and ovulation are highly successful [30]. However, the variation in the

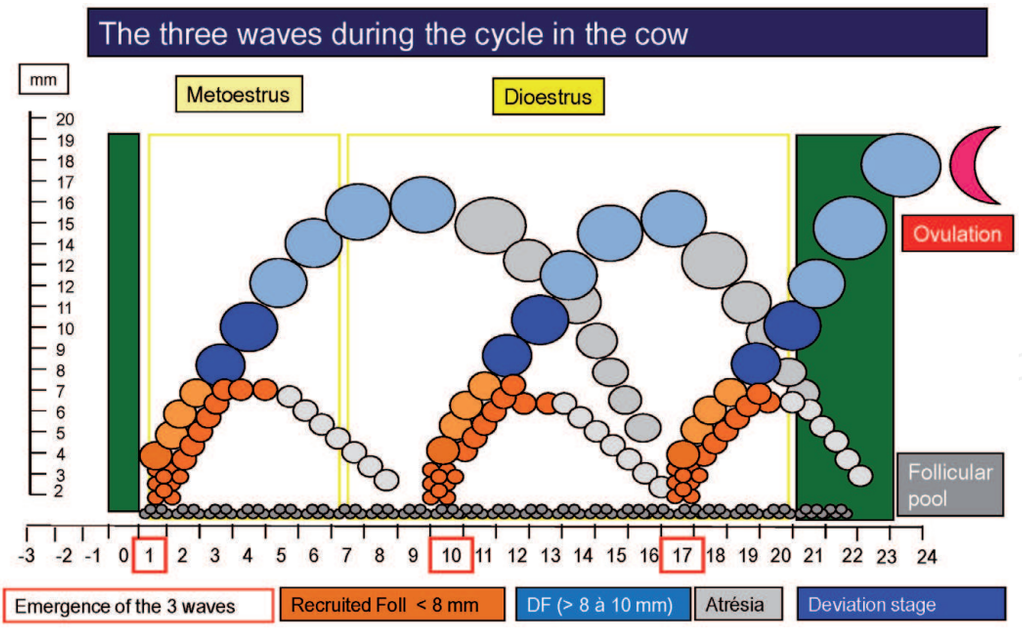

Figure 2.
Physiology of the sexual cycle in cows, with three follicular waves.

dynamics of ovarian follicular waves makes it difficult to control the exact time of estrus and ovulation.

The goal of superstimulation treatments in cows is to obtain the maximum number of fertilized and transferable embryos with the highest possible probability of inducing and sustaining a pregnancy.

The variability of the ovarian response was related to differences in superstimulatory treatments, such as gonadotropin preparation, gonadotropin type, duration of treatment, timing of previous estrus treatment, total gonadotropin dose, and use of additional hormones in superstimulation. Protocol [4]. Additional, equally important, sources of variability are factors inherent in the animal and its environment. These factors may include nutritional status, reproductive history, age, season, breed, ovarian status at the time of treatment and perhaps most importantly, inherent numbers of antral follicles [5]. While considerable recent progress has been made in the study of bovine reproductive physiology, factors inherent to the donor animal that affect superovulatory response are only partially understood [13, 25, 30].

4. Embryo transfer procedures

In farm animals, fertilized ova is removed from the uterus of their dam (the donor) and transferred to the uterus of other females (recipients) for development to term. Almost all commercial embryo transfers now use nonsurgical methods to recover the embryos rather than surgical methods (only for small ruminants). The procedure requires multiple steps (**Figure 3**), a large amount of time, and a variable cost.

The stages of a direct/in vivo ET protocol are highlighted in the following mandatory steps [31]:

1. Donor cows, selection of embryo donors.

2. Poliovulatory treatment of donors,

3. Artificial insemination/mounting,

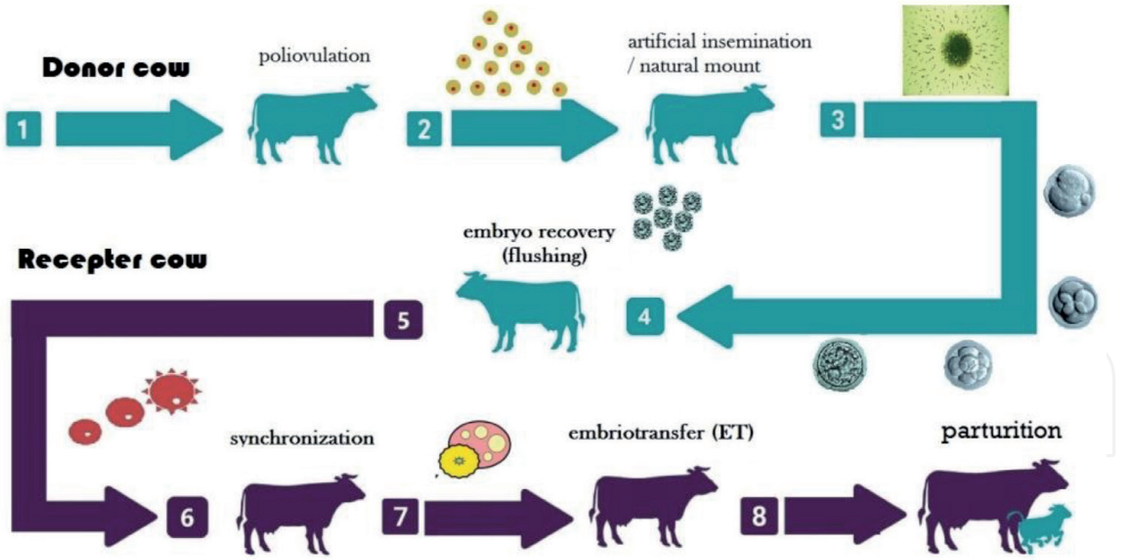

Figure 3.
Stages of in vivo embryo transfer in large ruminants.

4. Collection of embryos and classification,

5. Selection and preparation of receptors,

6. Synchronization of estrus and ovulation with the donor,

7. Direct transfer/preservation,

8. Gestation and parturition.

4.1 Donor selection

The selection of the embryo donor candidate is based on two major criteria: (1) the genetic merit, generally evaluated by the owner and based on performance, and (2) the reproduction criteria interpreted and evaluated by the veterinarian. The donor must be in good physical condition, an average but growing BCS. It should be free of underlying conditions, be at least 50 to 60 days after calving and have a regular cycle. In general, cows with a history of reproductive problems, even minor ones, do not make good embryo donor animals.

Donors are further evaluated by careful examination of the cervix, uterus, and ovaries per rectum to determine if they lack adhesions to neighboring organ structures, and the presence of other palpable lesions. It is recommended to test the permeability of the cervical canal with a cervical dilator, especially if the donor is before the first calving - heifer. This prevents the occasional of being unable to negotiate the cervix after a series of costly hormonal injections.

Single or multiple embryos can be collected from ovulating or naturally superovulated cows. For optimal efficiency, 2 to 4 donors should be treated and synchronized with their recipients for each attempt/session; this allows the sharing of the recommended potential of 8–10 recipients per donor.

4.2 Superovulation

Superovulation is and remains one of the least anticipated steps in the process of embryo production. The objective of superstimulation treatments in the cow is

to obtain the maximum number of fertilized and transferable embryos with a high probability of producing pregnancies [32].

In the bovine tremendous variation in response occurs with age, breed, lactational status, nutritional status, season, and stage of the cycle at which treatment is initiated. Follicle stimulating hormone (FSH), which has a short half-life (Pluset, Folltropin-V, and others), necessitates twice-daily injections over a period of 4 to 5 days. Synthetic hormones with a long half-life (like PMSG), are administered in a single dose, but have other drawbacks. Treatment is start in the mid-luteal phase (day 8 to 12) of the donor's estrus cycle and white use of prostaglandins (PGF) to synchronize the estrus of the donors and the recipients. Alternatively, treatment may be induced on day 16 or 17 (day 0 = estrus) of the donor's natural estrous cycle, or with progesterone administration (which mimics a progesterone phase). Ultrasonography and palpation of the ovaries per rectum have been shown to have similar accuracy for determination of the number of follicles (in estrus fase) or CL (at the time of embryo recovery). However, the number of anovulatory follicles can be counted more accurately [1, 4, 33, 34].

4.3 Artificial insemination/mounting

Donors should be artificially inseminated twice with a 10–12 hour interval, beginning 6 hours after the occurrence of oestrus, to ensure the time interval in which ovulation occurs. Depending on the quality of the frozen/sexed semen, a dose with a higher sperm concentration, even a double dose, can be used for each insemination. Ultrasonography is helpful in assessing the potential superovulatory response on the day prior to ovulation or at the time of AI.

4.4 Collection of embryos and classification

The donor animal is kept in a standing position in a trevis. The first step in the non-surgical recovery of embryos is to determine the numbers of corpus luteum in the ovary [21]. This step is important to rule out that the superovulation response in the animal; if less or no CL is found-indicates the poor response of superovulation-flushing not to be done in such animals. The donor was given an epidural anesthesia, then a wash and disinfection of the ano-vulvar region (**Figure 4**).

A two-way round tip balloon catheter (Fr. size 16 to 24) with a tul inflatable balloon is used. Once the instruments has been made ready (two/tree-way catheter), the vulvar lips are parted and the catheter with stylet is inserted into the vagina and advanced towards the lumen of the cervix. It is further advanced to the horn of the uterus until the balloon is situated at the base of the uterine horn. By blowing air, a dam is created with the tip of the uterine horn, there are located the embryonic formations between days 5–8 after ovulation. The amount of air used depends upon the size of the uterus. Basically, there are two methods of embryo collection [35]: the continuous or interrupted flow, closed-circuit system and the interrupted-syringe technique. The most commonly used medium for embryo recovery is Dulbecco's phosphate buffered saline (PBS), but there are many others ready to use (Euroflush, Vigro). Uterine horn is flushed with 30–60 ml of media and repeated until 300–800 ml of media is used up. The same process is repeated for the other horn as well [36].

Embryos are found with a 10 X magnification stereoscope after filtering the collection/washing medium through a pore filter with a diameter between 50 and 70 μm. The identified embryos are usually transferred as soon as possible, sometimes if desired it is possible to keep the embryos in that environment for a few hours at room temperature. It is also possible to cool the bovine embryos in storage

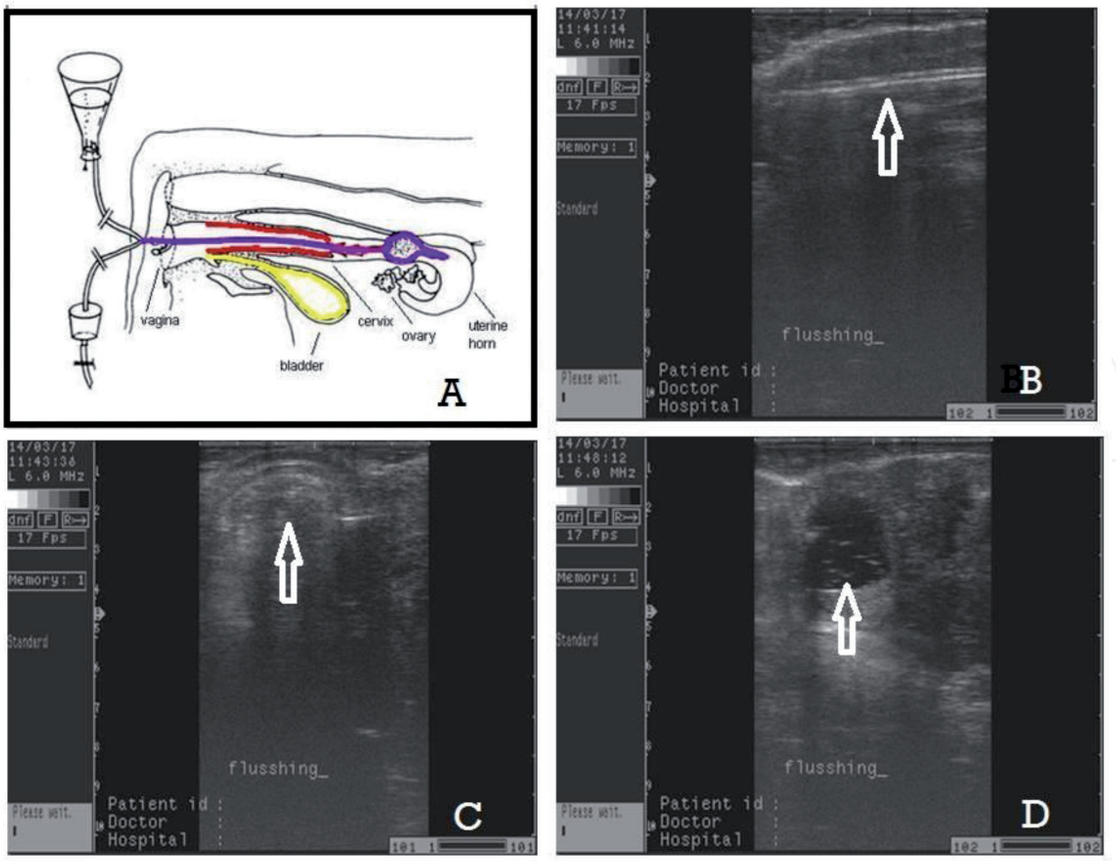

Figure 4.
Recovery of bovine embryos by flushing method. A. Scheme of catheter placement and reservoir-uterus-filter fluid flow, ultrasound images with catheter guidance through the cervix and visualization of uterine lavage, see white arrow. B. Inserting the stylus through the cervical lumen, C. inflating the balloon from the catheter and obtaining the dam at the top of the uterine horn, D. flow of flushing fluid and recovery of embryos.

medium and store them in the refrigerator for 2 or 3 days. Most often, embryos can be frozen for use at a later date.

A good response and an appropriate recovery rate results in getting a 4–5 embryos are recovered with each flush. This can lead to 50 freezable embryos per donor per year resulting in the birth of 30 calves after the transfer of the embryo to a recipient [24].

After the fecundation, the single-celled embryo now called the zygote undergoes rapid mitotic divisions (cell number increases, cytoplasm remaining same) called cleavage [37]. Bovine embryo descends into the uterus around day 4.5 days (estrus day 0) [38]. According to the standards, embryos are recovered from six to eight days after the onset of estrus (day 0). Embryos can be recovered even earlier from four days when the embryos arrive from the salpinx in the uterus, but before day 6 the recovery rates are lower than on days 6–8 (**Table 1**).

Species	Days from estrus
Cattle	7
Buffalo	6
Sheep	3–6
Goat	3–4

Table 1.
Day of collection of embryo.

However, embryos can also be recovered on days 9–14, although they leave the pellucid area on days 9–10, making them more difficult to identify and isolate from cellular detritus and more susceptible to infection [39].

Identification and evaluation of embryos is one of the most important and delicate stage, the practitioner needs experience to get used to the procedure. Embryo quality and poor handling techniques can directly affect pregnancy rates. A step-by-step procedure for looking for embryos is presented in the content of this section.

Evaluation of the embryo in the uterine effluent is based on identification of several morphologic features of the embryo using light microscopy. These methods are subjective and depend on experience. The embryo is spherical and is composed of blastomeres surrounded by a gelatin-like shell and zona pellucid (**Figure 5**).

Embryos recovered 5 to 8 days after estrus are classified morphologically into the following groups, based on their stage of development. Proper evaluation requires rolling of the embryos along the bottom of the dish.

Morula. The blastomeres are round and not tightly joined together. Individual blastomeres are difficult to identify with each other. The blastomere cell mass of the embryo occupies most of the perivitellin space.

Compact morula (tight morula). The shape and appearance of a tight mill is similar to a golf ball, in that the outer edge is slightly wavy (curly) given due to compaction. Individual blastomeres grow and become indistinguishable. The cells on the surface of the mass have a polygonal shape. The embryonic cell mass occupies 60–70% of the perivitellin space.

Early blastocyst. A tiny transparent (clear) space that contains fluid is visible. This area is the beginning of the blastocele (cavity). The embryo occupies 70–80% of the perivitelline space [6, 14, 37].

Blastocyst. The blastocele cavity becomes prominent and represents more than 70% of the embryo's volume. Inside, two groups of cells are separated and differentiated. They can be clearly recognized as a trophoblastic layer below the pellucid area and the darker cell mass occupying part of the embryo. The perivitellin space is still visible, but it is very small.

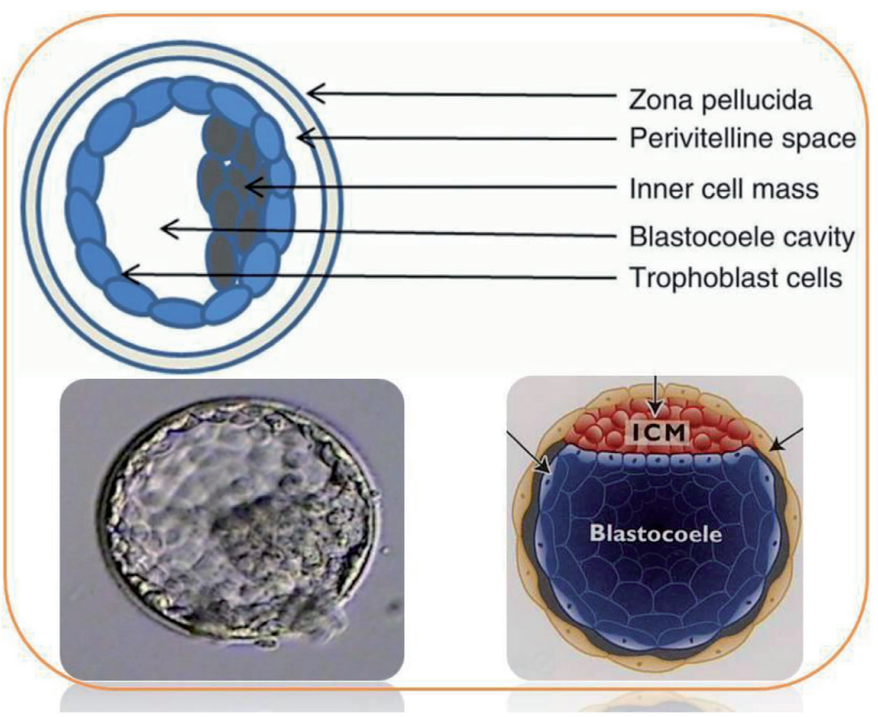

Figure 5.
Schematic diagram of a transferable embryo (expanded blastocyst phase).

Expanding or expanded blastocyst. There is no more perivitelline space between the trophoblastic cell layer and the interior of the area. The area of the pellucida stretches becomes thinner as the blastocyst expands. A small, well-compacted internal cell mass is observed positioned in one part of the embryo. The color of the embryo becomes pale to clear and is due to the large amount of fluid present inside.

Collapsed blastocyst. A perivitelline space can be identified together with a very thin pellucid area. The blastocyst may be partially collapsed, with a smaller blastocyst cavity, or completely collapsed and have the appearance of a compact morula.

Hatched blastocyst. After a continuous growth, the blastocyst expands to rupture and the embryo escapes from the pellucid area. From this moment the embryos pass into the gastrulation phase. The hatched blastocysts can be spherical with a well-defined blastocyst or they can be collapsed, similar to cellular detritus. Identifying embryos at this stage is especially difficult for the inexperienced operator.

When blastocysts/gastrules without areas or hatching are collected, there is a higher risk of damage due to handling. In addition, hatched blastocysts are sticky and can adhere to micropipette handling tubes. Therefore, the use of embryonic filters is not recommended when there is a suspicion that hatched embryos will be recovered (> day 7.5).

Embryos are then classified according to quality based on morphologic appearance. Excellent/good, fair, and poor quality embryos are considered transferable into recipients. Excellent or good quality embryos (Code 1) are freezable (**Figure 5**).

4.4.1 Codes for embryo quality

Code 1: ***Excellent or good***. The mass of the embryo is symmetrical and spherical with individual blastomeres (cells) they are uniform in size, color and density. The embryo was in accordance with the expected stage of development (collection day). The irregularities are usually minor and more than 85% of the cellular material should be a compact and intact embryonic mass. This is based on the observation of the percentage of embryonic cells represented by the extruded material in the perivitelline space. The pellucid area should be smooth and smooth and could adhere to a micro-plate or a straw.

Code 2: ***Fair***. Some irregularities can be observed in the general shape of the embryo mass or in the size, color and density of individual cells. At least 50% of the cellular material must be an intact, viable mass of embryos.

Code 3: ***Poor***. Some major irregularities in the shape of the embryo mass, or the size, color and density of blastomeres, are identified. At least 25% of the cellular material should be like an intact, viable mass of embryos.

Code 4: ***Dead or degenerating***. Degenerate embryos, oocytes, or I-cell embryos are nonviable.

Embryos of fair quality can be transferred fresh, if the receptors are available and synchronized. The category of good and excellent quality embryos have a high probability of surviving cryopreservation. The EITS considers that the export of embryos of poor and fair quality is inadequate [40]. The assessment of bovine embryos has recently been revised and is constantly improving [41], but the IETS manual has the most comprehensive library of embryonic images useful to practitioners.

Loading the Straw. Immediately before direct transfer, the embryos are individually aspirated into sterile 0.25 ml French straw. The embryo is usually loaded from the support vessel into the straw using a 1 ml syringe attached to the end of the straw stopper. First, a 3 cm medium column is aspirated into the straw, then a 0.5 cm air column is aspirated, then a 3 cm medium column containing the embryo, followed by another air bubble.

Selection and preparation of receptor. The recipient should be non-pregnant, cyclic (minimum of two normal cycles), should have CL on at least one of the ovaries. The embryo stage should match the uterine age of the recipient for a successful pregnancy to occur. While perfect synchrony is desirable, recipients that are 1 day out of phase can be considered acceptable; this means that a 7-day embryo can be transferred into a recipient who showed heat 6–8 days earlier. The lower quality embryo is more sensitive to asynchrony. The recipient should not have any pathological condition which can hinder its pregnancy. Pregnancy rates following embryo transfer are best when the recipient is in estrus from 36 hours before to 12 hours after the donor [42].

Synchronous recipients can be produced in three ways:

1. Selection from a large pool of cycling females. This strategy limits the number of embryos and time when embryos can be collected. Approximately five percent of the herd will be in heat on any given day.

2. Estrous cycles of any number of recipients can be synchronized with PGF2alpha or its analogues, or with CIDR devices, to exhibit heat the same day as or just ahead of the donor.

3. Timed ET, analogous to timed AI (Ov-Sync), can also be used. The importance of close synchrony between the age and the stage of development of the embryo, and the endocrine status of the endometrium of the recipient must be emphasized. Pregnancy rates following embryo transfer are best when the recipient is in estrus from 36 hours before to 12 hours after the donor [43].

Embryo transfer to the recipient can be done surgical or non-surgically. However non-surgical is more ethical to use. The recipient is secured in a Travis and the vulvar area is cleaned. As the animal is in the luteal phase. Epidural anesthesia is induced to prevent straining and defecation. The insertion of the tip of the instrument into the desired uterine horn should be done quickly, and smoothly. Trauma to the delicate endometrium causes bleeding, and blood (complement in the serum) is embryocidal. Ruminants embryos are transferred to the uterine horn and the same procedure as A.I. is followed except that in ET embryos are deposited deep in the horn ipsilateral to CL [44].

Pregnancy rates for IVP embryos were lower in commercial embryo transfer programs than for in vivo embryos [45].

Pregnancy rates are 10% lower in frozen embryos than the fresh ones [37]. Using heifers as recipients, there have been reports that in some 10% of such animals (heifers) it is difficult, if not impossible, to carry out ET via the cervix.

Any kind of stress to the recipient should be avoided. Any other routine treatments scheduled (eg antiparasitic) must take place at least 3 weeks before the transfer; also changes in the feeding regime should be prohibited for 3–4 weeks before and after embryo transfer. Beneficiaries must be accommodated where they can be easily and quietly handled on the day of transfer [23]. Any stressors should be removed.

5. Embryonic mortality

It is said that about 25–40% of embryonic losses are produced in the first few days after transfer to the cow [46, 47]. It has been observed that most of these females return to heat at an interval after 20–22 days, presenting a complete and

normal sexual cycle [48]; Therefore, it is believed that embryonic mortality (EM) could occur between days 7 and 17, the period from embryo transfer (ET) until it settles at maternal recognition of pregnancy [49]. In a lower proportion, but just as important, is the pregnancy losses that occur between days 28 and 98, after the transfer and the percentages between 7% and 33% have been reported [50].

The critical nature of the period and the phenomenon of recognition and survival of the embryo at the maternal uterine endometrium during implantation requires a very careful synchronization between the transferred embryo and the recipient. Thus, the importance of both the biochemistry of the uterine environment and the signals of the embryo that generates the recognition and implantation is highlighted [51, 52]. These embryonic signals must be released at the time and concentration necessary to maintain CL morphology and maintenance of function, thus generating a continuous production of P4. Progesterone levels play an essential role in maintaining the embryotrophic environment and supporting the normal development of the concept (the embryo and all adjacent cell structures) [48].

In connection with the influence of P4 (progesterone) on certain events related to pregnancy maintenance from the early stages and the ability of PGF2α to trigger luteolysis, a number of hormonal strategies for maintaining pregnancy have been researched, developed and supported [53, 54]. These strategies tend to be based on the increased efficiency and secretion capacity of P4 by CL: secretion must occur in a timely manner, thus ensuring a suitable uterine environment for the development of the embryo transferred to the recipient bovine female. All these strategies aim to increase the load rate in ET programs [48].

In order to prevent the mortality of the transferred embryos, and the loss of the pregnancies during embryo transfer sessions, two main objectives are considered: - Maintaining the corpus luteum function, even inducing a new one; and Inhibition of the appearance/secretion of luteolytic factor. All procedures apply to female embryo recipients.

In the first case, it is recommended to administer a treatment with Gn-RH, more precisely HCG to develop and support the luteal tissue, or even to form another CL (by causing ovulation of the follicle, if any). In the second case, the administration of non-steroidal anti-inflammatory drugs is considered, which is said to block the synthesis of PGF.

6. Embryo production biosecurity and contamination risks

The procedures for embryo production, in MOET programs, include several steps where contamination with pathogen agents may occur. For instance, the first source of potential contamination comes from the donor itself. Before ovulation, an oocyte could be contaminated by its contact with a given pathogen shed in granulosa cells or follicular fluid during infection (viremia or bacteremia). For example, in bovines, viruses were detected in follicular fluid a few days after experimental exposure to bovine viral diarrhea virus [55]. Hence, the recovery of cumulus–oocyte complexes at this moment might lead to production of contaminated embryos [6, 37].

Disease Risk Management. Success in embryo production by either MOET or IVEP relies on the capacity to correctly perform all technical steps, eliminating or reducing factors recognized to have negative effects. It is essential to select donors and recipients with good general health and adequate nutrition. In addition to those issues, considering that the first source of potential contamination comes from the donor itself, an important measure is to select these females, taking into account their sanitary status. When incorporating animals into the flock, their health status

should be checked before and quarantine should be respected. Vaccination and deworming must be employed as prescribed, depending on the location and system of production, but always before their use as donors. Testing should be conducted for some infectious diseases, and those positive should be culled. All technicians in direct contact with the animals must be careful and well trained to ensure familiarity with and safety in the procedures. The technique must be aseptic and all labware sterile. The equipment should be cleaned and all devices that are in contact with the animals should be sterilized before reuse. Clothing should be completely cleaned before reuse [56].

In general, in IVEP, the risk of potential hazards associated with oocyte collection from slaughterhouses are higher than those collected by laparoscopic ovum pickup. Consequently, when using these ovaries, it is important to determine their origin, particularly whether ovaries came from a herd depopulated for any health cause [57]. Care must be taken in the transportation of this material to the laboratory to avoid any external contamination. For media preparation and gamete or embryo manipulation or culture, all biological products should be avoided. These reagents could be replaced by those derived from plant origin or amino acids. When cell culture is preferred for IVEP, the use of controlled cell lines, confirmed to be pathogen-free, is recommended. From a sanitary point of view, safer strategies include the use of chemically defined media that do not contain serum or somatic cells [57].

For MOET, pathogens could be present in the female genital tract and can adhere to either oocytes before fertilization or embryos before collection. Intact zona pellucida is a natural barrier to penetration of pathogen into the oocyte or prehatching embryos. However, some pathogens may adhere to the zona pellucida of oocytes and embryos; thus, the zona pellucida represents a vector for disease transmission to recipients and to embryos after hatching (once transferred). For IVEP, the magnitude of this risk may vary according to the source of ovaries or oocytes that are being used: either from laparoscopic ovum pickup when the donor health status is well known or from the slaughterhouse [57].

Follicular aspiration by laparoscopy, instead of transvaginally, practically eliminates the chance of contamination by microorganisms being carried into the follicle from the vagina via the collection needle, as has been reported in humans [58].

On the other hand, ovaries collected from slaughterhouses provide an inexpensive and abundant source of oocytes, which is usually helpful for research projects and cloning. However, considering that these ovaries are generally transported in containers together, the presence of just a few ovaries from infected animals could represent a potential source of contamination. Other general sources of possible contamination involve the presence of environmental pathogens associated with the technician, slaughterhouse, equipment for laparoscopic ovum pickup or embryo collection, or even in the laboratory, such as glassware, culture dishes, media, and incubators. Regarding media, it is known that any biological product such as fetal calf serum and bovine serum albumin used in the recovery, culture, and cryopreservation of oocytes, sperm, and embryos may constitute a risk of contamination [6, 37, 55–58].

The semen used in a ET protocol (MOET or IVEP program) must be collected from males managed under appropriate sanitary protocols that ensure their good health status. Although AI represents a useful tool for disease control when best practices are applied, a further source of risk in an embryo production program is the semen. Numerous viral, bacterial, and parasite agents may be present in semen, which may adhere to the surface of spermatozoa or they could be present in the seminal fluid or in the semen extender.

In general, the studies are in agreement when the sanitary procedures recommended by IETS are correctly implemented. The risk of disease transmission from donor to recipient and to offspring for most pathogens is negligible or, at least, is much lower than that associated with live animals. These facts confirm that embryo transfer represents a safe strategy for global genetics trade and a valuable tool for the control and eradication of several diseases in small ruminants [59, 60].

Various publications [57] describe the possibility of transmitting diseases and the management of prevention procedures. The procedures for managing these risks have been described in the ***OIE Terrestrial Code*** [61], which explicitly refers to the specifications published in the IETS manual. These procedures are included in national regulations for the transfer of embryos between herds.

Adherence to these procedures ensures that embryo transfers contribute to improving the animal health of a population by controlling the movement of genetic material between herds. The basic concept behind these regulations is the official validation of embryo transfer teams. This approval is a very important method of veterinary regulations, as they are usually based more on animals coordinated in protocols and their products. However, in this case, the safety of embryo transfer procedures is based on the correct ethics and technique of the head of the embryo transfer team [62].

The criteria used by national veterinary services for the approval of embryo transfer teams are based on the *Terrestrial Code*. For example, in Chapter 4.7. it is stated that: "the embryo collection team is a group of competent technicians-operators, including at least one veterinarian, who carry out the production, collection, processing and storage of embryos".

It is recommended that the following conditions be met:

a. the team should be approved by the competent national authority;

b. the team should be supervised and ordered by a team veterinarian;

c. the team veterinarian should be responsible for all operations of his team, including:

- checking the health of the embryo donor
- implementation of appropriate disease control measures when handling or operating donors
- disinfection and hygiene procedures;

d. team staff should be regularly trained appropriately in disease control techniques and principles. High standards of hygiene must be practiced to prevent the spread of infection;

e. the embryo collection team should have adequate equipment for:

- induction and collection of embryos
- processing and treatment of embryos in a permanent laboratory or in a mobile laboratory
- conservation and storage of embryos;

These facilities do not necessarily have to be in the same location;

f. the embryo collection team must draw up a correct record of its sessions, which should be kept for verification by the Veterinary Authority for a period of at least two years after the export/movement of the embryos;

g. the embryo collection team should be periodically inspected and checked at least once a year by an official veterinarian, to ensure compliance with the procedures and sessions for the proper collection, processing and storage of embryos.

7. Conclusion

Embryotransfer *in vivo*, (IVD by MOET) is a procedure that can significantly increase the amount of offspring a genetically significant ruminants can carry. This it is a multistep procedure involving superovulation, synchronization of donor and recipient, insemination of donor, collection, isolation, evaluation, genetic testing freezing and transfer of embryo. This is the shortest path to genetic progress on economic efficiency in large and small ruminant farms.

Conflict of interest

The authors declare no conflict of interest.

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