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In-vitro embryo production and transfer technology in cattle: An updated review article

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ABSTRACT

The use of in vitro technique with domestic animal gametes and the methods of embryo manipulation for assisted reproduction is currently the most advanced method in cattle breeding with regard to scientific research and practice. In vitro embryo production (IVP) is the process of production of live embryos in the laboratory rather than in vivo by collecting oocytes from slaughterhouse ovaries as well as from live donor cows. IVP entails the completion of three biological steps that are now relatively well established in cattle: oocyte in vitro maturation, in vitro fertilization and embryo culture. Oocyte maturation is a complex process involving nuclear and cytoplasmic maturation in appropriate media. To enhance successful fertilization of matured Oocytes, sperm cells must be motile, have the ability to undergo capacitation and express the acrosome reaction. In vitro embryo culture systems depend on multiple parameters such as the composition of culture media and gases. The most common media used in culture systems are synthetic oviduct fluid (SOF), potassium simplex optimization medium (KSOM), and complement receptor 1 (CR1aa). Embryos of appropriate quality can be transferred directly to recipient cows or frozen for future use. Embryo transfer (ET) is a multi-step process that involves the production and collection of pre implantation embryos from genetically superior females and the subsequent transfer of those embryos into reproductively healthy females for the purpose of establishing pregnancies and producing live offspring. ET technology is an important tool to improve livestock at faster rate as well as gives an opportunity to utilize the genetic contribution of both male and female at the same time. Selection of recipient and proper recipient herd management are vitally important to the success of transferred cow embryos. There are two methods of transferring embryos; surgical and non-surgical. There are problems associated with in vitro embryo production. Low success rates limit the contribution that the IVP technique can make, especially to genetic improvement.

Keywords: *Bovine, Embryo transfer, In vitro, embryo production, Review*

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1. INTRODUCTION

The development and application of assisted reproduction technologies like artificial insemination(AI), estrus synchronization, embryo transfer, in vitro fertilization, transgenesis, cloning and semen sexing in animal husbandry can be expected to bring about a faster multiplication and propagation of superior genetic animal species (Manik *et al*., 2003; Mondal *et al*., 2014). The use of in vitro technique with domestic animal gametes and the methods of embryo manipulation for assisted reproduction is currently the most advanced method in cattle breeding with regard to scientific research and practice (Samardzija *et al.,* 2015).

In vitro embryo production (IVP) is the process of production of live embryos in the laboratory rather than in vivo by collecting oocytes from slaughterhouse ovaries as well as from live donor cows (Bilodeau-Goeseels and Panich, 2002; Chaubal *et al.*, 2006). IVP is not a single step process but it comprises in vitro maturation (IVM) followed by sperm capacitation, in vitro fertilization (IVF) of matured oocytes and then in vitro culture (IVC) of fertilized oocytes for 7 days till the stages of morula or blastocyst (Lojkic *et al.,* 2014). In vitro embryo development is strongly influenced by events occurring during oocyte maturation, fertilization and the subsequent development of the fertilized oocytes. So, improving the efficiency and identifying the sources of variations between IVF systems or between different laboratories are more important when routinely producing blastocysts from individuals of high genetic merits (Abd El-Aziz *et al.,* 2016).

Embryo transfer is a multi-step process that involves the production and collection of preimplantation embryos from genetically superior females (called donors) and the subsequent transfer of the harvested embryos or in vitro produced embryos into reproductively healthy females (called recipients) for the purpose of establishing pregnancies and producing live offspring (Youngs, 2007). Embryo transfer (ET) is universally recognized as the final and most critical stage in an in vitro fertilization (IVF) program (Neithardt *et al*., 2005). Recipients play a critical role in the success of ET program (Bó *et al*., 2002). Recipients must be synchronous with the stage of development of embryos that had been previously frozen (Mapletoft, 2006). Prior to transfer, harvested embryos must be evaluated using a microscope in order to assess their developmental stage and quality. The International Embryo Transfer Society has adopted a uniform standard for evaluating and describing cattle embryos (Fufa *et al.,* 2016). Therefore the objective of review is to identify the role of in vitro embryo production and embryo transfer in genetic and production improvement in cattle as well as its application.

2. OVARIAN DYNAMICS IN CATTLE

The cow is a polyestrous animal; therefore, once estrous cycles are established, they continue indefinitely unless interrupted by pregnancy. In the non-pregnant cow, ovulation occurs at approximately 21-day intervals. Cellular division of oocytes (oogenesis) begins early in fetal life.

Division is arrested at the diplotene stage of the first meiotic division, only to resume at the onset of the estrous cycle in follicles destined for ovulation. At birth, the ovaries of the female calf contain all the oocytes she will ever produce from 200,000 up to about half a million (Ball and Peters, 2004). These immature oocytes remain in a resting phase known as germinal vesicle (GV) stage (Landim-Alvarenga and Maziero, 2014). Oocytes are produced and matured, involving nuclear and cytoplasmic maturation, in the ovary (Sirard and Coenen, 2006). The process of nuclear maturation begins when meiosis resumes from the diplotene stage, signaled by chromosome condensation and germinal vesicle breakdown (GVBD). It corresponds to the reversal of the first blockage of meiosis from the germinal vesicle stage until the second blockage at metaphase II. After GVBD, the oocyte goes through the first meiotic division and then rapidly passes through metaphase II of second meiotic division where a second meiotic arrest occurs (Kubelka *et al*., 2000; Hyttel *et al.*, 2000). By the end of the second meiotic division, homologues of oocyte chromosomes (2n) have separated into two nuclei with n chromosomes each. The cytoplasm divides asymmetrically generating two cells, one keeping almost all the cytoplasm, the secondary oocyte and the other very small, first polar body. Soon after, the second meiotic division begins and remains in MII until the fertilization or parthenogenesis activation (Quetglas *et al*., 2010).

The progression of meiosis that characterizes nuclear oocyte maturation, does not by itself guarantee further embryonic development; additional cytoplasm modifications is also necessary (Ferreira *et al*., 2009). Cytoplasmic maturation includes structural and molecular changes occurring in the oocyte from the GV stage to the end of MII. The ultra-structural changes in the cytoplasm include migration of several of the organelles. The mitochondria and Golgi complex, which are located in the periphery of the oocyte in immature oocytes, move to aper nuclear distribution. The cortical granules, originating from the Golgi complex, and originally situated in the center of the oocyte migrate to the periphery and become anchored to the oocyte membrane (Landim-Alvarenga and Maziero, 2014).

At ovulation the ovum or egg is collected by the infundibula end of the oviduct or fallopian tube. It is transported down the oviduct towards the uterus possibly by a combination of cilia (hair-like) action and muscular contractions. Transport through the oviduct appears to be under the control of ovarian steroid hormones since estrogens reduce and progesterone increases the speed of passage of ova through the oviducts. Fertilization normally occurs in the ampulla section of the oviduct close to the junction with the isthmus. In the cow, the ovum enters the uterus 4 -5 days after ovulation (Hill, 2017).

Mammalian spermatozoa acquire motility and part of their capacity to fertilize the ovum, during their passage through the epididymis (Mújica *et al*., 2003). However, before spermatozoa are able to fertilize the ovum, they have to also undergo a further series of maturational changes in the female tract. These processes are known as capacitation and the acrosome reaction and are thought to require about six hours in the cow. The acrosome reaction is necessary to allow penetration of the oocyte by the sperm. Normally, only one sperm is able to pass through the zona, but when more enter, a process known as polyspermy, the resultant embryo is non-viable. Penetration by the fertilizing sperm (pronucleus) stimulates the resumption of the second meiotic division of the oocyte and the extrusion of the second polar body. Fertilization is completed with the fusion of the haploid male and female pronuclei, a process known as syngamy (Gordon, 2003).

The ovum begins to divide mitotically, a process known as cleavage, immediately after fertilization is complete. Division continues so that a solid cluster of cells or blastomeres known as a morula (mulberry shape) is formed by five or six days. From about day 6 after fertilization, the ovum begins to hollow out to become a blastocyst. By day 16, the embryo is sufficiently developed to signal its presence to the maternal system and prevent the luteolysis by mechanism of hormonal changes (Hunter, 2003).

3. IN VITRO EMBRYO PRODUCTION

The first calf from IVF, which is called "Virgil", was reported in 1982 after aspiration of an in-vivo matured oocyte, i.e. an oocyte collected immediately before ovulation, which was submitted to IVF and immediately transferred to a recipient. At that time it was restricted to research investigations and was applied to rescue follicular oocytes of slaughtered donors but it has since become an increasingly important tool employed commercially for assisted production in many farm species and leads to the beginning of IVF as a tool for production in the cattle industry (Velazquez, 2008; Parrish, 2014). In cattle, besides used as research tool in slaughtered donors, IVP has become important for the production of embryos from live donors as an alternative to or integrated with multiple ovulation embryo transfer (MOET) because of the advantages and flexibility that it offers (Bizelew and Getahun, 2016). IVP entails the completion of three biological steps that are now relatively well established in cattle: oocyte in vitro maturation, in vitro fertilization and embryo culture (Samardzija *et al.*, 2015). However, primary oocytes collection should be added upstream of these major steps and embryo management should be added downstream to give a complete overview of the whole process. Once the immature oocytes have been removed from the ovary, they are matured, fertilized, and cultured in vitro for up to seven days until they develop to a stage that is suitable for transfer or freezing (Bizelew and Getahun, 2016).

Superovulation for invitro embryo production

Superovulation is when an animal is induced (usually through use of injectable hormones) to ovulate multiple ova (the hormonal treatment for harvesting increased

number of oocytes from the ovary than normal). This is usually done on animals with superior genetics, but any cycling female can be super ovulated (Stenbak *et al*., 2001). It is important to recognize that super stimulatory protocols used for the production of embryos in vivo are different than those used to produce embryos in vitro with oocytes obtained via ovum pickup (OPU). The objective of superovulation in MOET programs is to maximize the number of ovulations without compromising embryo quality, whereas super stimulatory treatments prior to OPU are aimed at increasing the number of follicles suitable for puncture, preferentially with a diameter between 5 to 10 mm (Karadjole *et al*., 2010).

There are two glycoprotein hormones that may be administered to induce superovulation. The hormone follicle stimulating hormone (FSH) is the hormone naturally responsible for recruitment and growth of ovarian follicles during the estrous cycle. Super ovulatory treatments of donor females with FSH are typically initiated on days 8-10 of the estrous cycle in order to stimulate follicles at the start of the second follicular wave when a dominant follicle is not present (Tesfaye *et al.,* 2016). Because the half-life of FSH is short (~ 4 to 6 hours) in cattle, FSH must be given as a series of multiple injections administered over a period of several days. A typical superovulation regimen for beef cattle involves a series of eight injections of FSH administered at 12-hour intervals over a 4- day period. The FSH may be given as a constant dose (all eight injections are the same amount of FSH) or as a descending dose (FSH doses get smaller over time). An injection of PGF2 should be given in conjunction with the 6th (or 7th or 8th) dose of FSH in order to induce luteolysis and enable the donor to exhibit estrus (Youngs, 2007). The other hormone is pregnant mare serum gonadotropin (PMSG); more recently termed equine chorionic gonadotropin (eCG) (Sendag *et al*., 2008). PMSG is a glycoprotein hormone found in the blood of the mare between days 40 and 130 of gestation and is unique among gonadotrophins in possessing both FSH and luteinizing hormone (LH) biological activities within the one molecule (Gordon, 2003).

Oocyte collection

The oocytes for in vitro production of bovine embryos could be harvested from slaughterhouse ovaries as well as from live donor cows (Chaubal, 2006).

For the setup of the techniques as well as for the large scale production of average genetic merit embryos, large quantities of material can be obtained at low cost by collecting ovaries at slaughterhouses (Mermillod *et al*., 2006). Slaughterhouse derived ovaries contain a highly heterogeneous population of oocytes that are recovered regardless of follicular dynamics and may come from cattle with diverse reproductive backgrounds. Production of embryos from slaughterhouse ovaries begins with oocytes collected from all follicles visible on the surface of the bovine ovary. Oocytes obtained from slaughtered animals are variable in their developmental competence after IVM (Bilodeau-Goeseels and Panich, 2002). Bovine ovaries obtained from abattoirs should transport to the laboratory in physiological saline with antibiotics (penicillin and streptomycin) at 37 °C within 3 h of slaughter. Cumulus oocyte complexes (COCs) can be aspirated from 2 to 8 mm diameter follicles using an 18G needle attached to a

vacuum pump. Then aspirated follicular fluid should transfer from the collection tube to a perti dish (Karadjole *et al*., 2010). One of the main disadvantages of collection of ovaries from slaughter house is the lack of repeatability. Once the cow has been slaughtered there can be no further collection of oocytes from the same donor. In conjunction with in IVP, OPU technique provides an alternative means to increase the number of offspring from genetically valuable cows (Galli *et al*., 2001).

Oocyte recovery from live donors

Oocytes are more frequently recovered from live animals by transvaginal ultrasound-guided oocyte collection or OPU. The technique of ultrasound-guided transvaginal follicular aspiration is a noninvasive procedure for recovering oocytes from antral follicles in live animals. Using transvaginal OPU ova can be harvested repeatedly from the same cow, potentially providing many more ova (Qi *et al.*, 2013). According to Ball and Peters (2004) an eight-year-old cow has produced 176 embryos during 158 weekly collections and aspiration of ovum twice per week may be better than once per week as it is more likely to pre-empt the development of a dominant follicle that could have a negative effect on results. In vitro fertilization in conjunction with ovum pick-up (OPU) has become important for the production of embryos from super stimulated donors (Galli *et al*., 2001).

The equipment required for oocyte recovery in live donors consists of an ultrasound apparatus whose transducer has a built-in needle guide, echogenic needle (18G disposable needle, attached to the stainless steel guidance), sterile collection tube and suction pump. After epidural anesthesia of the donor, the transducer is introduced into the vagina and the ovary to be aspirated is moved per rectum to a position just anterior to the vaginal fornix. Thus, follicles can be imaged and the needle guide used to pass an echogenic needle through the vaginal wall into the ovary. Target follicles are positioned and aspirated by means of a foot-controlled vacuum into a sterile collection tube with aspiration medium Tissue Culture Medium 199 (TCM 199) hepes supplemented with antibiotics, heparine and bovine serum albumin attached to a suction pump to ensure constant aspiration pressure. The follicular fluid obtained by aspiration should transferred into an embryo transfer filter (Ball and Peters, 2004; Getz, 2004; Galli and Lazzari, 2005).

Table **1**: the recovered oocytes are identified under a stereomicroscope, counted and morphologically classified into four categories.

Grade	Morphology
1	Oocytes completely surrounded with more than three layers of cumulus cells and evenly granulated ooplasm that completely filled the zona pellucida
2	Oocytes with one to three cell layers and with evenly granulated ooplasm
3	Partially denuded oocytes with unevenly granulated ooplasm
4	Completely denuded oocytes, degenerated oocytes, expanded oocytes

Source: Karadjole *et al*., (2010)

Oocyte maturation

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division. In response to the preovulatory surge of gonadotrophins some of the oocytes undergo resumption of meiosis characterized by germinal vesicle breakdown, chromosome condensation, formation of the first meiotic spindle, expulsion of the first polar body and arrest in metaphase of the second meiotic division. These events are defined as oocyte maturation and lead to an ovulated fertilizable oocyte (Ball and Peters, 2004). Therefore the goal of IVM is to cause an immature oocyte from a developing follicle to resume meiosis and render itself capable of fertilization, arrested at the second metaphase step of meiosis just like in vivo matured counterpart (Gibson, 2014).

The oocytes are subsequently matured for 24-26 hour in vitro. The maturation of oocytes invitro mainly depends on medium components (cell culture medium, serum, and antibiotics) and incubation conditions. The medium component includes TCM-199 supplemented with sodium pyruvate, porcine follicular stimulating hormone (p-FSH), estradiol, epidermal growth factor (EGF), fetal calf serum (FCS) and gentamycin (Shabankareh, 2014). But oocytes are very sensitive to temperature shocks so it is important to monitor carefully the collection procedure temperatures as fluctuations can easily occur (Youngs, 2007). Thus oocytes should be cultured at 38.5°C under 5% CO2 and 95% air atmosphere with saturated humidity for 24 hours (Gibson, 2014).

Evaluation and selection of oocytes

The success of IVM of bovine oocytes is measured by morphological assessment of proliferation of cumulus oophorus and corona radiata cells, thickness and compactness of the cumulus investment and the homogeneity of the ooplasm. The degree of nucleus maturation is measured as a percentage of oocytes in the stage of metaphases II (MII) with the first polar body expelled (Lojkic *et al*., 2014). Consequently, morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to bring about full-term pregnancy. On the other hand, only 30-40% of the zygotes obtained after in vitro maturation and fertilization (IVF) will reach the blastocyst stage in culture. This is probably due to the quality of the oocytes at the beginning of maturation, and indicates the need for the development of other approaches. Different selection criteria have been used to predict the quality of these oocytes and to improve the embryo development such as oocyte diameter, brilliant cresyl blue (BCB) test and the follicle diameter (Shabankareh, 2014).

Sperm preparation and in vitro fertilization (IVF)

In vitro capacitation procedures are aimed at stimulating the sequence of events that normally occur in the cow's reproductive tract. This involves the removal of seminal proteins and other substances that coat the sperm membrane of the ejaculated semen. To do this, sperm cells are subjected to thorough washing and exposure in media with elevated ionic strength (Duran, 2000). Frozen semen is generally used and a selection step is required to enrich the sperm suspension in living and motile spermatozoa. In the swim-up method, spermatozoa are deposit at the bottom of a culture medium containing tube and allowed to swim in the medium. The top fraction, containing motile sperms is then collected after a given incubation time. Alternatively, motile sperms

may be purified by centrifugation in a discontinuous density gradient. The motile fraction is collected at the bottom of the tube after centrifugation (Mermillod *et al*., 2006).

The sperm concentration used for IVF ranges between 0.5 and 2x106 spermatozoa per ml in fertilization medium containing caffeine, heparin and bovine serum albumin (BSA). This concentration should be optimized for each male and even for each ejaculate from a given male. The fertilization rate increases together with sperm concentration. However, after a given value, an increasing proportion of the oocytes become polyspermic. A compromise has to be established for high fertilization rate with low polyspermy rate (Gibson, 2014; Pereira *et al*., 2005).

The IVF is a mixed cultivation of mature oocytes with spermatozoa in strictly controlled laboratory conditions. The co-incubation of spermatozoa with oocytes lasts for 24 hours, at 39°C with 5% CO2 in the air and 80% humidity after which the first mitosis occurs. The success of IVF of bovine oocytes is estimated 48 hours following fertilization by a number of cleaved embryos and by identification of male and female pronucleus which may be visible after 18-22 hours of coincubation (Samardzija *et al.*, 2015).

Embryo culture

The last stage of in vitro production of bovine embryos is the culturing of embryos for 7 days, from the zygote until the blastocyst stage when such embryos may be transferred into a recipient or frozen and stored in liquid nitrogen (Rynkowska *et al*., 2011). During IVC, the embryo is passing through four important developmental stages: the first mitosis, activation of embryonic genome, compaction in morula stage, the formation of the blastocyst with differentiation of their cells (Samardzija *et al.*, 2015).

In vitro embryo culture systems depend on multiple parameters such as the composition of culture media and gases (Kocyigit, 2016). An efficient culture system for in vitro embryo development should be formulated to protect from intracellular stress and include all demands of the embryo thereby embryo can maintain viability. Various approaches have been employed to improve the culture systems of bovine embryos in vitro. The plurality of research toward improving embryo development in vitro has focused on culture composition, soluble chemicals and surrounding medium components (Lonergan and Fair, 2014).

There are several articles on the composition of embryo culture media and the role of specific medium components in supporting mammalian embryo development in vitro. It is essential to appreciate that the culture media are only one part of the overall culture system. Optimal culture media for embryo production appear to require a combination of various factors such as hormones, cytokines, growth factor, antioxidants, vitamins, enzymes and macromolecules (Rezaei and Chian, 2005; Block and Hansen, 2007). The formulations of culture media are a key aspect of embryo culture (Gordon, 2003).

The most common media used in culture systems are synthetic oviduct fluid (SOF), potassium simplex optimization medium (KSOM), and complement receptor 1

(CR1aa); nevertheless, other media, such as G1.1/G2.2, complement receptor 2 (CR2aa), and TCM199, (Farin *et al*., 2001).
There are different culture systems available for in vitro fertilized oocytes. They can be classified according to their formulation as follows: In vivo, where fertilized oocytes are placed in the oviduct of a synchronized female and grown for 6-8 days and can be performed across species, e.g., bovine or equine embryos can be successfully cultured in the sheep oviduct; Co-culture, where fertilized oocytes are placed in culture drops containing cells from the oviduct to try to mimic the maternal environment (Galli and Lazzari, 2005); Semi-defined medium, where fertilized oocytes are placed in culture drops containing serum but the composition of the serum is variable and usually unknown; Defined medium, where fertilized oocytes are placed in culture drops where concentrations of all the components are known including growth factors; or Sequential culture, where embryos are placed in culture medium containing the nutrients necessary for one specific stage of development, mimicking the maternal environment (Mermillod *et al*., 2006, Feugang *et al*., 2009).

Table **2**: Supplementation with factors towards improving defined media

Factor	Action	Reference
Colony-stimulating factor 2	Blocked apoptosis in bovine embryos through regulatory actions on apoptosis genes.	Loureiro *et al.*, 2011
Epidermal growth factor	Besides its role on proliferation, migration and differentiation, EGF is involved in reduction of apoptosis during preimplantation and placentation.	Gordon, 2003
Fibroblast growth factor 2	FGF2 receptor activity were necessary for optimal development	Fields *et al.*, 2011
Preimplantation factor	Involved in modulation of local immunity, promoting decidual proadhesion molecules and enhancement of trophoblast invasion.	Stamatkin *et al.*, 2011
Insulin, transferrin, sodium selenite	Insulin exerted its actions on glucose transport and AA uptake along with antioxidant protection from transferring and selenium.	Gordon, 2003
GH and IGF-I	In combination stimulated embryonic development.	Moreira *et al.*, 2002
IGF-II, FGF2, TGFβ1, CSF2	Results from the combination were similar to culture under serum conditions	Neira *et al.*, 2010
leukemia inhibitory factor (LIF)	LIF receptors have been detected in embryos of different species that is considered essential for blastocyst development such as hatching rate, cell count and cryotolerance	Kocyigit and Cevik, 2015
Activin A	Use of activin A shortened the lag phase and was also beneficial at later stages; however, more rapid rates of development were associated with higher rates of apoptosis.	Gordon, 2003
Thyroid hormone	On the bovine embryo TH is involved in lowering apoptotic rates as well as improved hatching rates post thawing for cryopreserved embryos.	Ashkar *et al.*, 2010

In culturing of embryos the factors should be considered includes gas phase, pH, osmolality, temperature, water purity and sterility. Gas phase adjusted in embryo culture can be done using determined concentration of gases. The two most commonly used gases are 5% CO2 in air, 5% O2 and 88% to 90% N2. This gas mixture allows for better embryo development because it's thought to be more similar to the condition found in the oviduct and uterus (Sirard and Coenen, 2006; Karadjole *et al*., 2010). The pH of the culture medium should be between 7.2 and 7.4 and
Osmolality should be between 260 – 280 mOsm/kg. The culture temperature should be the same temperature found by the embryo in the oviduct and uterus. In the cow, the normal temperature ranges from 38.5 to 39 oC and so this temperature should use during culture. Resistivity of water must be less than 18 mOhm and procedures should be carried out taking care of keeping the culture sterile, using sterile techniques and antibiotics if necessary (Cevik *et al*., 2009).

EMBRYO EVALUATION

Embryos are classified and evaluated by morphological examination at 50 to 100 X magnification in small culture dish according to the manual of the International Embryo Transfer Society. The overall diameter of the bovine embryo is 150 to 190 um, including a zona pellucida thickness of 12 to 15 mm. The overall diameter of the embryo remains virtually unchanged from the one-cell stage until blastocyst stage. 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. Embryos of good and excellent quality and at the developmental stages of late morula to blastocyst yield the highest pregnancy rates. It is advisable to select the stage of embryo development for the synchrony of the recipient (Mapletoft, 2006).
Embryos are graded on a scale from one (excellent) to four (poor) with no or few extraneous degenerated cells (table 3).

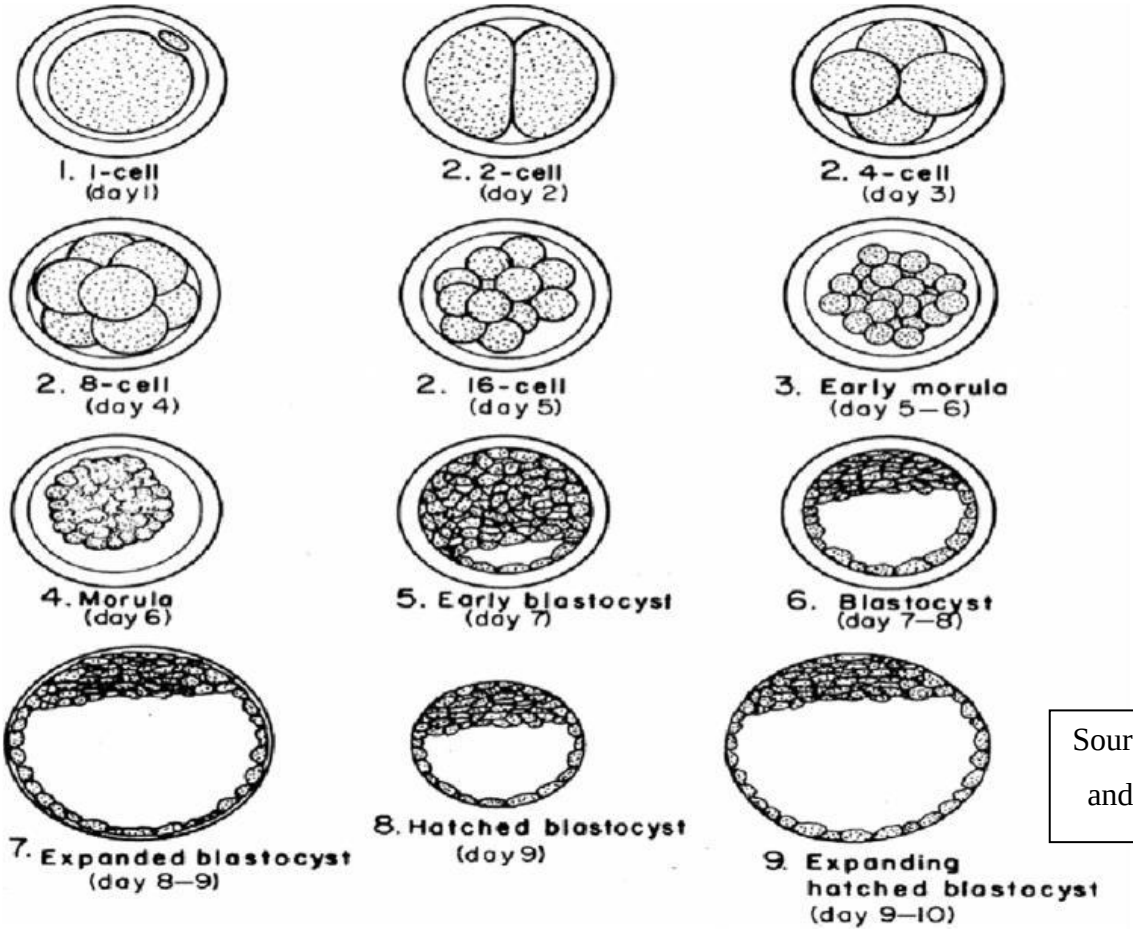
Table 3: Embryo quality grades

Grade	Description
1	Excellent or Good: Symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, color, and density. This embryo is consistent with its expected stage of development. Structural Irregularities should be relatively minor, and at least 85% of the cellular material should be intact, viable embryonic mass. This judgment should be based on the percentage of embryo cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that might cause the embryo to adhere to a Petri dish or a straw.
2	Fair: Moderate irregularities in overall shape of the embryonic mass or in size, color and density of individual cells. At least 50% of the cellular material should be an intact, viable embryonic mass.

3	Poor: Major irregularities in shape of the embryonic mass or in size, color and density of individual cells. At least 25% of the cellular material should be an intact, viable embryonic mass.
4	Dead or degenerating: Degenerating embryos, oocytes or 1-cell embryos. These embryos are non-viable.

Source: Genzebu, (2015); Mapletoft, (2006)

Embryos of appropriate quality (1 or 2 preferably) can be transferred directly to recipient cows or frozen for future use (Genzebu, 2015). The visual assessment of an embryo's morphologic characteristics is a subjective evaluation. The best predictor of an embryo's viability is its stage of development relative to the given day after fertilization (Phillips and Jahnke, 2016).



Source: Stringfellow and Seidel, (2010)

Figure 1: Illustration of normal bovine embryonic development

EMBRYO TRANSFER

Embryo transfer technology is an important tool to improve livestock at faster rate as well as gives an opportunity to utilize the genetic contribution of both male and female at the same time. With the help of ET or MOET techniques faster improve of livestock, rapid expansion of elite animals, genetic gain, accelerated herd development and conservation of rare genetic stocks could be achieved (Bizelew and Getahun, 2016).
ET does not have been only done immediately after collection of embryo. However, it can be also a frozen, either conventionally (slow freezing using ethylene-glycol) or by vitrification, which ensures safe storage and better management of the genetic resources (Saragusty and Arav, 2011). ET of in vivo (or in vitro) produced embryos to the uterus of a recipient cow is easy and reliably done by transcervical intrauterine deposition, with more than 60% of pregnancy rates. Use of OPU for collection of oocytes and the following in vitro embryo production technique make the MOET more effective since it waives superovulation and AI treatment (Presicce *et al*., 2011).

The first successful transfer of mammalian embryos was performed by Walter Heape in 1890. Heape transferred two four-cell Angora rabbit embryos into synchronized Belgian doe, which subsequently gave birth to four Belgian and two Angora young (Betteridge, 2003; Nagassa, 2016). However, the first successful cattle embryo transfer was reported 70 years later (Youngs, 2007). Today, more than ¾ million bovine preimplantation embryos are transferred each year throughout the world (Thibier, 2006).

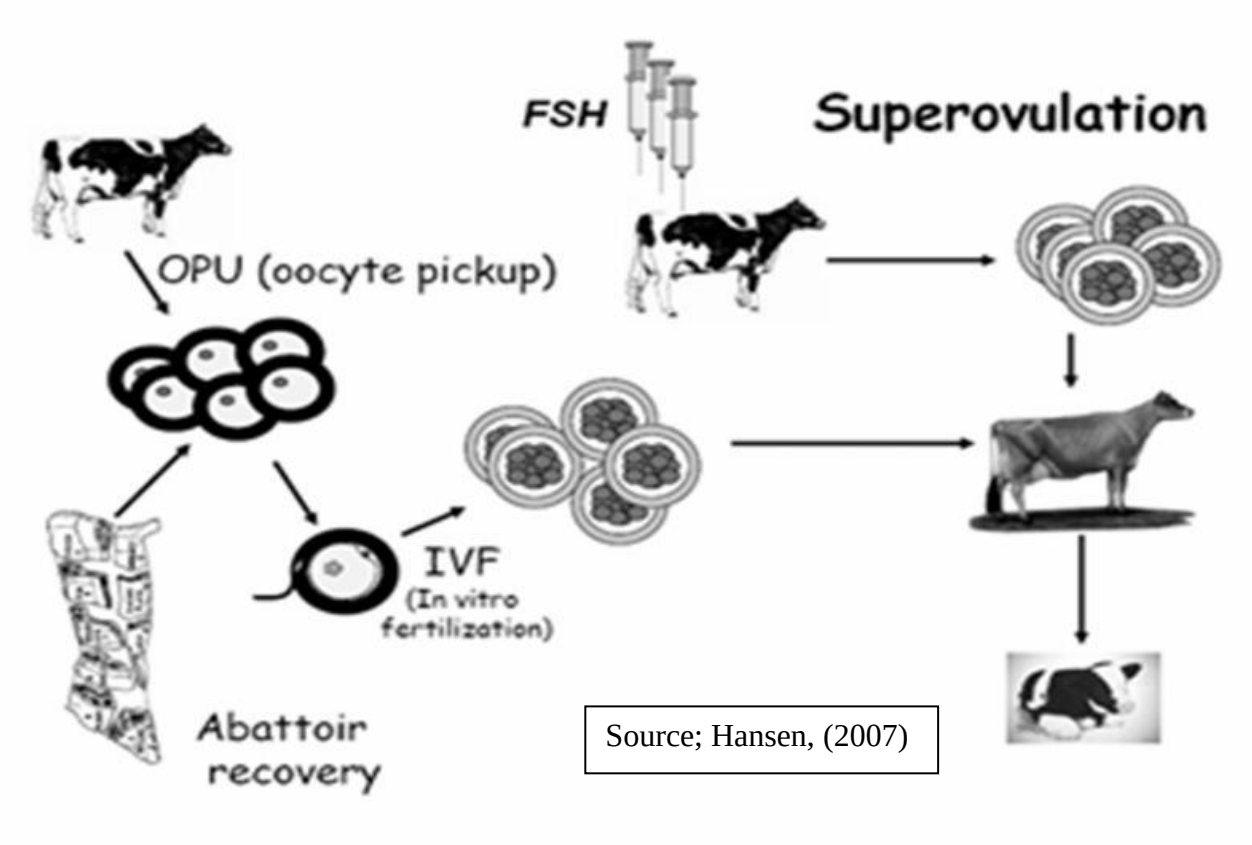


Figure 2: the production of embryo invitro (oocyte collection and IVF) and embryo transfer

Selection of recipients

Recipient (surrogate) cows are vitally important to the success of transferred cow embryos (Stroud, 2010). The surrogate contributes no genetic material to the offspring; she does exert important maternal environmental influences which affect the physical makeup (phenotype) of the calf. For example, her milk production will affect calf weaning weight. There is also a maternal environmental effect on calf birth weight. Thus she must be on a proper plane of nutrition (body condition score 6 for beef cows and dairy body condition score 3 to 4) (Marahall and Minyard, b2002). Generally females ideal as recipients are as follows; (1). Cows 3 to 8 years old make good recipients once they have a good calving record (have no history of calving problems). Cows are suited for embryos of relatively high birth weights and cows have more milk and less difficulty with calving than heifers. (2). Heifers are good recipients providing they have reached their breeding weight (around 65-70% of mature weight) and are cycling. Heifers are easier to manage than cows because they are not lactating and have higher fertility than cows. But it is more difficult to transfer embryos non-surgically in heifers than in cows. (3). First calvers are still growing and are under more stress than the rest of the herd due to the fact that they have to raise a calf. For these reasons they

should be under an intensive management and receiving enough good quality feed to use as recipients (Bova-Tech Ltd, 2012). (4). Females should be reproductively healthy (e.g., they should have two functional ovaries plus normal oviducts, uterus and cervix), free of reproductive tract adhesions, and free of ovarian follicular cysts (Youngs, 2007; Larson, *et al*., 2010). (5). One must be cautious if candidates for recipients are not lactating (and not pregnant); because this frequently means that they were culled for reasons that will make them subfertile (George *et al*., 2005).

Management of recipients

Proper recipient herd management is critical to embryo transfer success. Once elected, the animals are separate from the rest of the herd to guarantee they are away from the bulls. If animals are estrous synchronized 20 days later and an ultrasound examination should performed to reassure that the animals are not pregnant (Genzebu, 2015). A cow performs her best when she is in moderate body condition thus must be maintained on a good nutrition program (Stroud, 2010). Recipients must be synchronous with the stage of development of embryos that had been previously frozen. Recipients can be made available by maintaining a large herd to obtain natural heats or by estrus synchronization, which is much more economical. Today most recipients are synchronized regardless of whether or not embryos are transferred on farm (Mapletoft, 2006).

After transfer of embryo, the recipient should be left undisturbed as much as possible. Preferably, they should be put in a separate pasture so they are not stressed during gathering and sorting of the non-exposed recipients for further ET procedures. Make sure these cows do not lose weight during the first 90 days after transfer or have any major stressful event. The first trimester of pregnancy is the most sensitive to abortions and early embryonic death. Simply changing diet can cause early embryonic death, presumably from rumen pH changes (Stroud, 2010).

Transfer of embryo

Various methods of transferring embryos have been developed. The efficiency of transfer depends on many factors, but the most important are the experience and skill of the person(s) doing the transfer. There are two methods of transferring embryos; surgical and non-surgical. The surgical methods have in the past given higher and more consistent pregnancy rates. However, skillful technicians can now produce similar high pregnancy rates with non-surgical methods. Therefore, this method has become widely adopted today (Duran, 2000).

Surgical embryo transfer

Embryos can be transferred via mid-line abdominal incision to cows under general anaesthesia, but through flank incision is far more practical. Recipients are placed in squeeze chutes that give access to either flank. The corpus luteum is located by rectal palpation and the flank ipsilateral to the corpus luteum is clipped, washed with soap and water, and sterilized with iodine and alcohol. About 60 ml of 2 percent lidocain is given along the line of the planned incision (Tesfaye *et al*., 2016). Having scrubbed,

the surgeon makes a skin incision about 15 cm long, high on the flank, just anterior to the hip. Muscle layers are separated, and the peritoneum is cut. The surgeon inserts a hand and forearm into the incision, locates the ovary, usually about 25 cm posterior to the incision, and visualizes or palpates the corpus luteum. Then uterine horn is exteriorized by grasping and stretching with the thumb and forefinger. A puncture wound is made with a blunted needle through the wall of the cranial one third of the exposed uterine horn, because it is very fragile. Using about 0.1 ml of medium in a small glass pipette, an assistant draws up the embryo from the storage container. The pipette is then inserted into the lumen of the uterus, and the embryo is expelled. It takes some experience to be confident that the embryo has been deposited in the lumen. The incision is then closed, using two layers of sutures. With practice, the surgery takes about 15 minutes (Marahall *et al*., 2002; Nagassa, 2016).

Non-Surgical embryo transfer

Nonsurgical transfer is preferable, because it is less expensive; it is quicker and does not involve surgical procedures. This may also avoid the need for veterinary supervision, which is required for surgery in many countries (Carlos, 2014). For non-surgical transfer, the deposition of the embryos in the uterus horn in the side of corpus luteum is performed under rectal control with a transfer catheter. Care must be taken especially with young animals to avoid any injuries in the still narrow uterus (Small *et al*., 2004; Melissa, 2009). The equipment required for non-surgical embryo transfer includes petridish, stereomicroscope, transfer straw, transfer gun; transfer sheath and transfer pipette (Trasorras *et al*., 2010).

First, it is necessary to palpate ovaries accurately in order to select the side of ovulation. Pregnancy rates are markedly lowered if embryos are transferred to the uterine horn contralateral to the corpus luteum (Melissa, 2009). Also, recipients should be rejected if no corpus luteum is present or pathology of the reproductive tract is noted. The second step is to pass the embryo transfer device through the cervix. The petridish containing the embryo is placed under the stereomicroscope to load in a 0.25 ml transfer straw. The straw is then loaded into a transfer gun and covered with a transfer sheath. After the straw is loaded the transfer gun will be introduced into the vagina of recipient. The tip of the transfer gun is guided through the cervix by transrectal manipulation and positioned into the uterine horn. Heifers present a special challenge because of the small cervix. The best training prior to undertaking non-surgical embryo transfer is experience in artificial insemination. The third step with non-surgical transfer is to be able to insert the tip of the instrument into the desired uterine horn quickly, smoothly and atraumatically (Trasorras *et al*., 2010; George *et al*., 2005). The uterine lumen should be lined-up prior to transfer so as to prevent trauma to the endometrium during passage. The embryo is deposit slowly and firmly while slightly withdrawing the tip of the transfer pipette. Great care must be taken to not cause damage to the lining of the uterus (Melissa, 2009). After transfer appropriate management of the recipient is crucial for good result including definitive pregnancy

diagnosis in four to six weeks after embryo transfer and good nutrition program especially in the last three months of pregnancy (Stroud, 2010).

MERITS AND DEMERITS OF IN VITRO FERTILIZATION IN EMBRYO TRANSFER

The production of embryos using IVF presents two main advantages over the conventional approach. First, it prolongs reproductive life of cows that do not respond to superovulation treatment or produce only unfertilized ova. It is, therefore, an integral tool to keep selected cows within a breeding scheme as part of a selection program (Bousquet *et al*., 2003). Second, IVF can be applied during different stages of reproduction, including the prepubertal period and during the first 3 months of pregnancy. With the utilization of prepubertal heifers, it is possible to decrease the age of the donor female use, thereby contributing to a decreased generation interval. Using ultrasound guided trans vaginal collections, oocytes for IVF can be collected in heifers at ≤ 6 months of age (Galli *et al*., 2001). New hormonal treatments and preparation of oocyte of donor animals are promising approaches to increase oocyte competency. In a recent study, up to 80% of oocytes collected from 14 to 16 month old heifers produced blastocysts after IVF (Blondin *et al*., 2002). In addition to those merits the implementation of IVF technology allows the use of several bulls with one donor, since the control of fertilization is made in vitro. This can be realized in one session of fertilization as opposed to conventional ET, which is limited to one bull per session (Bousquet *et al*., 2003). And also IVP waives superovulation and AI treatment (Presicce *et al*., 2011).

There are problems associated with in vitro embryo production. Low success rates limit the contribution that the procedures can make, especially to genetic improvement. Specific problems include a high incidence of polyspermy during in vitro fertilization (Mizushima and Fukui, 2001) and low oocyte yield per ovary when aspiration is continuously used to increase the number of
oocytes per session or cow (Machado *et al.*, 2006). Thompson and Peterson (2000) also stated that there are significant post-day 35 fetal losses and found that much of this loss is due to failure of normal allantoic development within the conceptus. Potential welfare problems related to specifically to OPU have been referred to as a problem but do not appear to be of great concern. But much of the greater concern is the effect on offspring that are actually brought to term, and here there is indeed a welfare issue. Most serious is the birth of unusually large calves resulting from in vitro embryo production. Associated with this have been reports of breathing difficulties, reluctance to suckle and sudden perinatal death of calves (Ball and Peters, 2004).

CONCLUSION

Animal biotechnologies related to reproduction have contributed too many improvements in different important traits in livestock because reproduction lies at the heart of any livestock breeding enterprise and is vital to maintain or increase the number of animals required for production. Among the reproductive technologies,

animal breeders have made widespread use of assisted reproductive technologies to accelerate genetic improvement programs aimed at obtaining more, better and cheaper food products. Assisted reproductive technologies like in vitro embryo production and embryo transfer have contributed to animal breeding programs faster transmission of desirable trait or genetic improvement by increasing offspring of selected males and females. Successful establishment of procedures for in vitro production of bovine embryo even if IVM, IVF and in vitro embryo culture systems vary greatly among different laboratories have direct effect towards the success of embryo transfer result. However, embryo transfer and in vitro production of bovine embryos are still not widely used despite its potential benefits. In developing countries such as Ethiopia, this is mainly due to absence of the necessary facilities and infrastructure.

Therefore, on the basis of this conclusive remark, the following points are forwarded:

- Taking maximum attention in all segmented process of IVP to reach for success. Collections of oocytes, IVM, IVF and IVC all are have their own contribution in success.
- Taking maximum attention in all segmented process of embryo transfer to reach for success. Management and selection of the recipients and transfer of the embryos are all important.
- There should be well trained technician for invitro embryo production and embryo transfer technology.
- There should be of clear policy and commitment from the government about assisted reproductive technology.
- Awareness should be created in the community and companies about the profitability of invitro embryo production regulated embryo transfer and Investors should be encouraged to invest on these technologies.
- Coordination should exist between industry, universities and institutions for technology of IVP and ET.

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