EAS Journal of Veterinary Medical Science
Abbreviated Key Title: EAS J Vet Med Sci
ISSN: 2663-1881 (Print) & ISSN: 2663-7316 (Online)
Published By East African Scholars Publisher, Kenya

OPEN ACCESS

Volume-6 | Issue-2 | May-Jun, 2024 | DOI: 10.36349/easjvms.2024.v06i02.003

Review Article

Promise of *In vitro* Embryo Production Technology for Improvement of Cattle Reproductive Potential

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Article History
Received: 13.04.2024
Accepted: 18.05.2024
Published: 30.05.2024

Journal homepage:
https://www.easpublisher.com

Quick Response Code

Abstract: *In vitro* embryo production is a process of creating a live animal through a combination of different procedures by oocyte collection from the female donor animal, maturation of oocytes *in vitro*, fertilization of oocytes *in vitro* and culturing embryos under a controlled laboratory environment. Production of embryos *in vitro* plays a role for improvement of cattle reproductive potential along with other technologies such as Ovum pick-up, Embryo transfer, Artificial insemination, gamete sexing, cryo preservation and genetic selection. Major advantages of *in vitro* production of embryos is that oocytes can be retrieved from slaughtered, juvenile, pregnant and old cattle. This helps to utilize a female cattle reproductive potential fully and produce greater number of embryos in a less amount of time unlike Multiple Ovulation and Embryo Transfer program which takes a long day. Another advantage of production of embryos *in vitro* is utilization of less semen. It takes only one straw of semen to fertilize more than two hundred oocytes. *In vitro* Production of embryos can be used to genetically select high potential female and male animal to produce genetically proved animal with a good performance for improving dairy and beef production. *In vitro* production of embryos will only be successful if factors such as breed of donor animal, age, body condition and nutritional management for the donor and recipient animals, laboratory equipments, bull effect, cryopreservation and semen preparation method can be controlled and managed. *In vitro* production of embryos along with ovum pick up technology can be taken as one of conservation strategy to increase the number of endangered animals. In general *In vitro* Production of embryos can be regarded as the economic gain of the country if employed in a large scale program.

Keywords: Cattle, embryo, *In vitro*, potential, production, reproductive.

1. INTRODUCTION

Animal production depends heavily on reproduction, and productivity is the secret to growth. Reproductive inefficiency is commonly acknowledged to be one of the main causes of financial losses in the animal industries (Verma *et al.,* 2012). Recent years have seen a significant advancement in the development of biotechnologies in the fields of breeding, reproduction, and molecular genetics, which have emerged as the most notable (Symbolic) outcomes of completed research in the fields of conservation, livestock development, and global genetic exchange. Artificial insemination (AI) and gamete freezing are the first generation of reproductive biotechnologies (Yitayih *et al.,* 2017).

Estrus synchronization comes to picture to facilitate the success of AI and involves manipulating the estrous cycle or inducing estrus in order to cause a significant number of females to go into estrus at a brief, predefined time (Odde, 1990). Followed by the second generation, multiple ovulation and embryo transfer (MOET), have a significant impact on livestock improvement programs in developed countries, is relatively a more sophisticated reproductive biotechnology that is suited to exploit and utilize the genetic potential of female animal. Bovine embryo transfer technique comprises the harvesting and transferring of embryos along with the selection and management of donor and recipient animals (Mapletoft & Hasler, 2005).

The advantages that previous generations were unable to provide led to the development of the third generation, known as the in vitro embryo production (IVEP) technology. Its two main advantages over the second generation are (i) it does not have the variability issue with the response to super-ovulation treatments when collecting embryos in vivo, and (ii) it allows for

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much higher oocyte yields per female than the previous technique because it can be repeated often (Thibier, 2005; Bertolini, 2009). These technologies facilitate the regulation of the import and export of genetic materials, the characterization and conservation of indigenous genetic resources for sustainable use, the acceleration of genetic progress, the improvement of performance, the reduction of disease transmission risk, and the expansion of the number of animals that can be bred from the superior parent. They are also an advantageous means of conserving ex situ genetic collections of species that face extinction (Foote, 2002; FAO, 2007).

IVEP was initially created to develop a large number of embryos for study and to look into the fundamental physiological occurrences during early embryonic development. Researchers then discovered that this reproductive technique may be applied to increase embryo production for use in livestock genetic improvement initiatives (Looney *et al.,* 1994).

Three key stages are combined to create *in vitro* embryos (IVEP): *In vitro* maturation (IVM) involves collecting oocytes from slaughterhouse ovaries or from live animals using the OPU technique, followed by *in vitro* fertilization (IVF), in which mature oocytes are fertilized by spermatozoa from sexed semen or semen from elite bulls, and *in vitro* culture (IVC), in which fertilized oocytes are cultured in suitable media and fed to develop up to the blastocyst stages. For cows, the culture period typically lasts seven days. The length of cultivation varies by species. The recipient animal has a higher chance of becoming pregnant when the embryo transfer is done at the right time (Sarkar *et al*., 2021).

IVF is favorable in the dairy sector in particular when employing sexed semen, as most dairies do (Anderson, 2020). Because sex-sorted sperm can fertilize more oocytes *in vitro* than would be possible with AI, the use of sexed semen as a genetic tool can be increased (Hansen, 2006). One pregnancy per straw of sexed semen is possible when AI is used. In contrast, utilizing sexed semen for IVF increases the likelihood of having many calves from a single straw of semen (Anderson, 2020).

The generation of bovine embryos *in vitro* (IVP) has attracted interest from all around the world as an assisted reproductive technology (ART) to enhance genetic gains in beef and dairy cattle. In some situations, combining IVP with multiple ovulation and embryo transfer (MOET) to create high genetic merit embryos is a preferred option to artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) (van Wagtendonk-de Leeuw *et al.,* 1998).

The progressive development in the adoption of IVEP was assisted by improvements in the methods for collection of cumulus-oocyte complexes (COC) by trans vaginal ultrasonic guided follicle aspiration (OPU) (Galli *et al.,* 2001), together with advances in systems and protocols for *in vitro* oocyte maturation, fertilization, and embryo culture.

The success of commercial IVP has considerably increased over the past ten years as a result of reported increases in blastocyst rates, enhanced cryotolerance, pregnancy rates, pregnancy loss rates, and the incidence of large offspring syndrome. About 80% of immature bovine oocytes go through nuclear maturation *in vitro*, 30%–40% reach the blastocyst stage, and 50% of the transferred embryos succeed in establishing pregnancy (Wrenzycki, 2016). However, compared to *in vivo* counterparts utilized for ET, the pregnancy rates of *in vitro* generated (IVP) embryos are still lower (Pontes *et al*., 2009; Ferraz *et al.,* 2016). It takes careful planning to produce a live calf from an IVP embryo. An oocyte of good quality, capable of maturation and successful fertilization, must be provided by the donor. The embryo must be properly chosen, loaded, and transferred by a trained technician and must develop *in vitro* until day 7 following fertilization. Semen, laboratory tools, IVP media, quality control procedures, and laboratory staff will all play a role in how well the process goes. Pregnancy outcomes are influenced by the measures performed both before and after embryo production (Sirard, 2018; Demetrio, 2019; Hansen, 2020). Despite the fact that embryos produced *in vitro* leads to the establishment of a smaller number of pregnancy success, it is still an effective technique to produce millions of embryos per year that exceeds the number produced via *in vivo* embryo production. This indicates that *in vitro* embryo production technology has a promise in increasing the reproductive potential of cattle in worldwide perspective. Therefore the objective of this seminar is to review the Promise of *In vitro* Embryo Production Technology for Improvement of Cattle Reproductive Potential.

2. *In vitro* embryo production (IVEP) in cattle

The phrase "*in vitro* production" (IVP) refers to the processes of obtaining immature oocytes (eggs) from donor animals, maturing those oocytes (IVM), *in vitro* fertilization (IVF), and culturing embryos in a controlled laboratory environment (IVC). IVF combined with embryo transfer has a lot of potential for genetic improvement in cattle (Nandi *et al.,* 2006).

2.1. Source and technique of bovine oocyte recovery for IVEP

Oocytes can be extracted from live animals using ultrasound-guided follicular aspiration (OPU) or from slaughterhouses (abattoir-derived ovaries) for use in IVEP. In all situations, oocytes are aspirated from a wide pool of antral follicles that range in size from 2 to 8 mm, come from both ovulatory and non-ovulatory follicular waves, as well as from dominant and subordinate follicles within these waves (Hyttel *et al.,* 1997). The first *in vitro* fertilization procedure was recovering oocytes from the ovaries of dead animals,

which yields a high quantity of oocytes (Nandi *et al.*, 2002). The technique of oocyte recovery from the ovary is required to obtain oocytes. Another technique is slicing, which involves placing the ovaries in a petri dish with normal saline solution and using a blade to cut them into tiny pieces (Das *et al.*, 1996; Kumar *et al.*, 1997). Slashing technique involves repeatedly slicing the follicles' surface to remove all of the follicular fluid (Saleh, 2017). It was found that the aspiration process improved the quantity and quality of oocytes as compared to the earlier techniques (Wani *et al.*, 2000; Saleh, 2017).Oocytes from slaughterhouse ovaries are a vital source for mass production of genetically enhanced embryos (Nagai *et al.*, 2014).

Despite the fact that slaughter ovaries are inexpensive and allow the harvesting of all follicles visible on the surface of the ovaries, the oocyte population is quite varied, which caused a variation in their developmental competence during *in vitro* maturation (Karadjole *et al.*, 2010). Most of the time, the ability to produce an animal embryo in a laboratory depends on the availability of oocytes, which are currently routinely obtained from donors of cattle using transvaginal ultrasound-guided collection techniques. By using this technology, oocytes from antral follicles in living animals can be removed without any harm being done to them. OPU has been regarded as the most adaptable and repeatable method to create embryos from a genetically valuable donor, along with the IVEP of oocytes (Galli *et al.*, 2001). This technique is performed using a scanner that has a suitable endo vaginal (or vaginal use-adapted) sector probe with a guided needle. Using a vacuum pump and a test tube attached to the needle, the follicular fluid and the oocyte that are inside are aspirated. A scanner with good resolution and a probe of at least 6 MHz is used in order to see follicles as small as 2-3 mm in size and to be able to see the needle during follicle aspiration. OPU has almost no side effects for the donor and, in some infertile donors with ovarian cystic syndrome or other diseases that affect reproductive function may even have a therapeutic effect (Alberio *et al.*, 2021). Any female could serve as a donor, including young cyclic heifers, cows in the third trimester of pregnancy, and even cows within two to three weeks of calving (Qi *et al.*, 2013).

Figure 1: Slicing technique for oocyte collection
(Source: Saleh, 2017)

Figure 2: Aspiration technique for oocyte collection
(Source: Saleh, 2017)

Figure 3: Slashing technique for oocyte collection
(Source: Saleh, 2017)

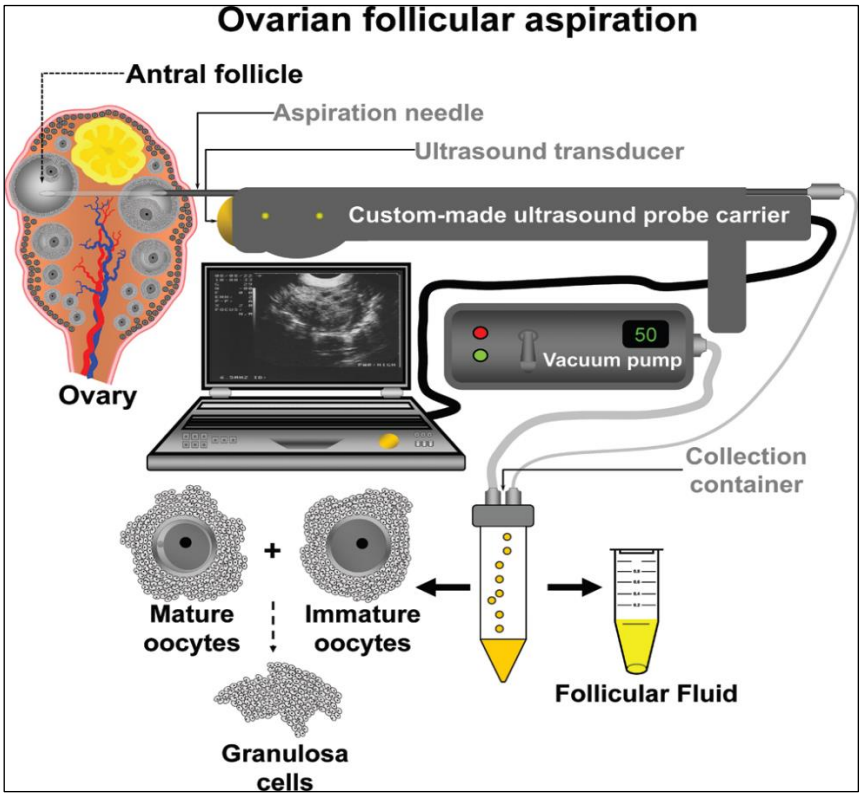

Figure 4: Schematic representation of the setup in transvaginal ultrasound guided follicular aspiration (OPU) in live cow
(Source: Velazquez, *et al.*, 2014)

2.2. *In vitro* maturation of oocyte

Immature antral follicles from the Germinal Vesicle (GV) stage are grown in a controlled environment until they reach (Meiosis two (MII) and are prepared for fertilization and embryonic development. The term "*in vitro* maturation" describes this procedure. During oocyte maturation, growth factors, hormones, and enzymes are activated and inhibited, which causes nuclear and cytoplasmic maturation (Gilchrist and Thompson, 2007). Nuclear and cytoplasmic maturation are the two phases of oocyte maturation (Ayman *et al.*, 2016). Meiotic competence acquisition, meiotic resumption, completion of meiosis I, and maintenance of metaphase-II arrest are developmental events that take place during nuclear maturation (Tukur *et al.*, 2020). The cytoplasmic processes that prepare the oocyte for fertilization and developmental competence are referred to as cytoplasmic maturation, which takes place throughout oocyte development and concurrently with nuclear maturation (Watson, 2007). Various media are available for oocyte maturation *in vitro* and the selection of media needed for maturation is different in various laboratories, however, the most used media are tissue culture medium 199 and Ham's F-10a (Gandhi *et al.*, 2000). The following serums can be added to maturation media: bovine serum albumin (BSA), oviduct synthetic fluid, fetal calf serum, and newborn calf serum (Gandhi *et al.*, 2000); hormonal preparations like follicle

stimulating hormone, equine chorionic gonadotropin, human chorionic gonadotropin; and other growth factors like insulin, insulin-like, epidermal plus fibroblast growth factor, and transferrin (Galli *et al.,* 2001).

2.3. *In vitro* fertilization of oocytes

The process of fertilization involves the sperm activating the oocyte, which unites the male and female gametes and causes the development of the pro nucleus (the nucleus of sperm or egg cells) and zygote. Fertilization medium, such as Brackett and Oliphant (BO) medium or Tyrode's modified medium, is used to co-incubate *in vitro* matured oocytes and frozen thawed *in vitro* capacitated spermatozoa for 24-48 hours at 38.50 °C in 5% CO2 (Nandi *et al.,* 2006). Typically, dead spermatozoa, seminal plasma, debris, and freezing media are removed from the spermatozoa and the more motile fraction is chosen using swim-up or density gradient centrifugation techniques. In order for the spermatozoa to develop the capacity to enter the zona pellucida (ZP) of the egg, capacitating substances must also be administered to them (Parrish *et al.,* 1986). Due to the wide variety in bulls and breeds, the minimum amount of spermatozoa needed per oocyte is not well defined; however IVF typically uses a concentration of 1 to 2 million spermatozoa (Ward *et al.,* 2002). The success of IVF of bovine oocytes 48 hours after fertilization is indicated by the number of cleaved embryos, identification of male and female pro nuclei, which may be visible after 18 to 22 hours of co-incubation (Leibfried *et al.,* 1989), extrusion of the second polar body, and sperm entry (Makita *et al.* 2016).

2.4. *In vitro* culture of embryos

Presumptive zygotes are cultivated in a specific culture medium for seven days. During the *in vitro* cultivation period, the embryo goes through various developmental phases. To the blastocyst stage, the fertilized oocytes are cultured *in vitro*, at which point the embryos can either be transplanted into a recipient or frozen and kept in liquid nitrogen (Samardzija *et al.,* 2015). The embryo is undergoing four crucial developmental phases during IVC, including mitotic cell division, activation of the embryonic genome, compaction in the morula stage, and the formation of the blastocyst with cell differentiation (Samardzija *et al.,* 2015).

3. Advantage of *in vitro* embryo production for genetic improvement and conservation

Genetic improvement is effective technique for increasing the sustainability of animal agriculture because the results are long-lasting and cumulative. Genetic advancements made in one generation are passed on to the following, unlike nutritional and animal health interventions, which call for ongoing inputs. Additionally, genetic approaches to animal health and welfare problems frequently demand less work and resources than chemical or mechanical ones. For instance, polled or hornless genetics can eliminate the need for physical dehorning of animals, which is done to ensure the safety of both workers and animals, and can save livestock producers time and money while also addressing an issue with animal welfare (Gottardo *et al.,* 2011; Tompson *et al.,* 2017).

Livestock genetic improvement programs, which started with selective breeding using statistical prediction techniques like estimated breeding values (EBVs) and more recently genomic selection (GS), in conjunction with assisted reproductive technologies (ART), have made it possible to more precisely select and heavily utilize genetically superior parents for the following generation in order to accelerate rates of genetic gain. Genetic gain is the annual increase in performance or improvement in average genetic value that selection produces in a population. Fewer animals are needed to generate the same amount of product due to improved animal performance based on genetic improvement, which lowers the environmental impact per unit of livestock product. Therefore, raising genetic gain rates can enhance the productivity of cattle and, eventually, the viability of animal agriculture. Reproductive and molecular biotechnologies, such as ART and GS, can be used within a structured breeding program to further accelerate rates of genetic gain by affecting one or more elements in the breeder's equation (Mueller & Van Eenennaam, 2022).

In order to increase selection intensity and speed up the rate of genetic gain, ART techniques such as AI, cryopreservation of sperm or embryos, estrus synchronization, MOET, OPU, and IVEP have been incorporated into cattle breeding schemes. IVEP is important because it has the benefit of allowing donors to be frequently gathered throughout the majority of the year, even when they are pregnant, keeping them in time with the yearly calving cycle (Mueller & Van Eenennaam, 2022). IVP embryo production has grown significantly over time throughout the world and in 2018, more than a million bovine IVP embryos were created globally (Baruselli *et al*., 2019).

The potential for genetic improvement can be greatly increased by combining GS with IVEP production technology, much exceeding what can be accomplished by individual approaches alone (either by GS or by IVEP). First of all, the majority of genetic investigations conducted to date have shown that the ability of donor cows to produce a good quality and high-quality oocytes varies heritably (Merton *et al.,* 2009) and ability of the recipient cows to maintain their pregnancy to term and give birth to IVP calves (König *et al.,* 2007; Spell *et al.,* 2001).

Merton *et al*., (2009) examined data from the OPU-IVP program from January 1995 to March 2006 using CRV (formerly Holland Genetics) and reported a heritability of 0.25 for the number of cumulus-oocyte complexes, 0.09 for the quality of cumulus oocyte

complexes, 0.19 for the number and proportion of cleaved embryos at day 4, and 0.21 for the number and proportion of total and transferable embryos at day 7. These heritability estimates are comparable to those of some characteristics related to milk and meat output that exhibit excellent GS response. As previously said, reducing the generation interval will result in the greatest genetic gain. The genetic merit of an unborn animal is foreseen at the embryonic stage before implantation into recipient cows in the most straightforward application of GS in IVP. Because selection is based on an animal that was never born, the genetic gain is consequently improved quickly by a significant reduction in generation interval (Fisher *et al.,* 2012; Ponsart *et al.*, 2014). A few animals are chosen from a vast pool of animals (in their embryonic stage) based on genetic merit (GEBVs), and the remaining embryos are eliminated since IVP embryos will be produced in enormous quantities compared to live-born animals. This quickly raises the selection intensity. Rapid genetic improvement will result from both a shorter generation gap and more intense selection. The cost-benefit ratio of genomic screening of preimplantation embryos (GSE) to commercial manufacturers determines its wide-scale application. In fact, the cattle sector will change as a result of using sexed semen in IVEP and mixing it with GSE (Kadarmideen *et al.,* 2015).

IVEP along with Ovum pick-up is also considered to be the most competitive technology in conservation strategies for producing a large number of embryos from live donors, ensuring the growth of endangered animal populations. Ovum pick-up enables the retrieval of immature oocytes from antral follicles (Comizzoli *et al.,* 2000; Velazquez, 2008). This is mostly owing to its repeatability, as oocyte collection can be done even twice weekly for a prolonged period of time without affecting the donors' reproductive status (Pieterse *et al.,* 1991; Kruip and Den Daas, 1997; Broadbent *et al*., 1997; Galli *et al.,* 2001).

OPU is a viable substitute for MOET for the creation of embryos in cattle since it may be effectively performed on donors with any reproductive state, including acyclic and hypo fertile cows, animals with patent tubes, and those who are unresponsive to MOET treatments (Galli *et al*. 2014). This is especially crucial for breed preservation programs, which are constrained by the scarcity of pure donors (Merton *et al.,* 2003). Presicce *et al.,* (2019) showed that it is possible to produce embryos of the endangered Podolic cattle of the Italian breed through repeated OPU procedure along with *in vitro* embryo production technology and suggested that OPU could be used as conservation strategy for Podolic cattle to enhance their reproductive performance. It has also been showed that successful *in vitro* fertilization (IVF) attempts have been made to produce embryos from the endangered Hungarian Grey cattle breed (Solti *et al.,* 1992).

4. Factors affecting *in vitro* embryo production in cattle

Many factors affect the effectiveness of the IVEP technology in cattle but the most important are the physiological parameters of the donor and the culture procedures for oocyte maturation and fertilization as well as for embryo culture from zygote to blastocyst. Therefore, a combination of more specialized parameters like reproductive soundness and ovarian cyclicity and more general factors like age, physical condition, and herd management are crucial (Galli *et al.,* 2004).

4.1. Effect of donors breed and age on *in vitro* embryo production

Breed of donors have an effect on the production of bovine embryos *in vitro*. For example it has been mentioned that the wide variation in donors' oocyte recovery, oocyte quality, blastocyst production, and pregnancy rate is a significant barrier to maintaining a constant embryo production in dairy breeds (Pontes *et al.,* 2010; Monteiro *et al.,* 2017). This variability is particularly evident when *Bos Indicus* cattle are compared with *Bos Taurus* cattle. According to some researches, Bos taurus and Bos indicus have different reproductive traits including those on antral follicle population (Baldrighi *et al.,* 2014; Silva-Santos *et al*., 2014), pre ovulatory follicle size (Baldrighi *et al.,* 2014), oocyte quality (Sales *et al*., 2015), gene expression in the cumulus-oocyte complex (Lopes *et al.,* 2017; Ticianelli, 2017; Sales *et al.,* 2015), and plasmatic levels of reproductive hormones (Baldrighi *et al.,* 2014; Sales *et al.,* 2015). Variations in the quantity of oocytes collected by OPU and in embryo formation may be caused by these differences. Lacerda *et al.,* (2020) explained that donor breed affects the rate of oocyte retrieval, the number of viable oocytes recovered and finally rate of *in vitro* produced embryos. The result of this study showed that Gir donors that are a Bos indicus breed showed higher percentage of embryos and number of pregnancies per OPU session compared to Holstein donors that are a Bos Taurus breed. Similarly, the study of Guimarães *et al.,* (2020) stated that Bos Indicus breed has a greater oocyte recovery, number of viable oocytes, and production of viable embryos than the Bos Taurus cows but the pregnancy rate was not affected by the donor breed. Another study also showed that the breed of origin of the cattle oocytes collected from slaughterhouse affected early embryo development during *in vitro* embryo production (Abraham *et al.,* 2012).

Studies also showed that Oocyte donor age affects the developmental competence of oocytes leading to the differences in the number of produced embryos *in vitro.* Su *et al.,* 2012 said that donor age of oocytes could affect developmental competence of oocytes recovered by OPU through the action of steroid hormonal balance on follicle development where younger cows have gained higher cleavage rates (CR) and blastocyst rates (BR) than those in the middle-aged and old cows. This

study explained that a lower plasma progesterone concentration and higher plasma oestradiol concentration in heifers in the course of the OPU sessions may account for a significantly higher oocyte developmental potential leading to higher cleavage and blastocyst rates.

4.2. Effect of bull and sperm preparation on *in vitro* embryo production

Bull effect has a significant limiting factor on *in vitro* fertilization (i.e., the high level of individual variation in bulls' capacity for fertilization) (Naib *et al.*, 2011; Lone *et al.*, 2017). Although there are many different IVF techniques, sperm penetration techniques, and capacitation techniques that are extremely effective, there are variations between bulls in terms of IVEP success (First & Parrish, 1987). Akyol *et al.*, 2014 studied the effect of bull on *in vitro* embryo production and found that there was variation among bulls in cleavage and blastocyst development. This study showed that some bulls had lower cleavage rates and blastocyst development than the others. This is due to the insufficient capability (low fertility rate) of some bulls not suitable for *in vitro* fertilization. Oppositely Galli & Lazzar, 1996 and Schneider *et al.*, 1999 evaluated the rates of cleavage in bulls during their IVF experiments and they did not see a statistically significant difference.

Sperm preparation method has an important role and affects *in vitro* embryo production. This method of spermatozoa selection separates motile from nonmotile sperm, as well as removing seminal plasma, infectious pathogens, cryo protective substances and also in the same time initiates the capacitation of sperm (Centola *et al.*, 1998). Samardzija *et al.*, 2006 compared the two sperm preparation methods such as Bovi pure and Swim-up and found that the percentage of cleavage and the percentage of hatched blastocyst embryos were similar for both methods but embryo production rate was significantly higher using Bovi Pure than the swim-up method and concluded that for the IVP of bovine embryos, Bovipure is an improved replacement for the swim up approach in the separation of bull spermatozoa from frozen or thawed semen. Samardzija *et al.*, 2006 compared Percoll method with Bovi pure and the results indicated that the cleavage and blastocysts rates were significantly higher for the Bovi Pure group compared to the Percoll group.

4.3. Effect of body condition and nutritional management on *in vitro* embryo production

The selection of viable oocytes for IVM is the first and most crucial stage in IVEP. Local breeds of cattle typically have low levels of follicular population, oocyte yield, and quality, and a variety of factors, including breed, age, weight, BCS, season, and the physiological, dietary, and pathological status of females, have been identified as the causes of the variations in the results (Manik *et al.*, 2003; Kouamo *et al.*, 2016). Proteoenergetic rate, vitamins, and trace elements required to maintain various reproductive processes, such as follicular growth, oocyte production and quality, as well as age, are included in the nutritional influence (Atherton, 1994). Dorice *et al.*, 2019 studied the effect of nutritional status and body condition score of zebu cattles on follicular population, oocyte yield and quality and found that both nutritional and body condition status affected the variations in the number and quality of oocytes and it was suggested in this study that body condition is the indication of nutritional status and zebu cattles with a body condition of 3 have a higher number and good quality oocytes and could be the best choice to improve IVEP. Oppositely Sales *et al.*, 2015 reported that different in dietary energy in take between the two breeds such as *Bos indicus* and *Bos taurus* breed did not affect the quality of oocyte and *in vitro* embryo production rate but Higher oocyte number observed in Bos Indicus cattles and greater rate *in vitro* embryo production rate. The results in this study are biased and the author explained the reason for this is the diets in the present study were formulated based on the requirements of Holstein cows.

4.4. Factor affecting pregnancy success of embryos produced *in vitro*

Success in getting pregnant depends on the recipient and embryo's features. For instance, *in vivo*-produced embryos are more likely than *in vitro*-produced embryos to establish pregnancy after transfer (Ferraz *et al.*, 2016), and cultivation of embryos with embryokines can enhance the number of pregnancies per transfer (Loureiro *et al.*, 2009; Denicol *et al.*, 2014). Embryo survival may be reduced by cryopreservation (Drost *et al.*, 1999; Stewart *et al.*, 2011; Ferraz *et al.*, 2016). The prevalence of disease among receivers may potentially have an impact on ET's effectiveness (Ferraz *et al.*, 2016; Ribeiro *et al.*, 2016). Estrada-Cortés *et al.*, 2019 described that frozen/thawed embryos produced *in vitro* and recipients which had metritis in the early postpartum period reduced the success of ET in multiple-service Holstein cows. So, this study indicated that the major factor for the decrease in the establishment of pregnancy is the effect of cryopreservation on the quality of embryos produced *in vitro*.

Age of the recipient is also another factor that affects pregnancy rate as indicated in the study that transferring embryos to cows gave higher pregnancy rates compared to heifers (Putney *et al.*, 1988). Pregnancy establishment is also affected by the quality of embryo. The majority of scientific hypotheses surrounding embryo transplantation recommended transferring first- or second-type embryos when they were still in the morula and blastocyst stages. Due to the blastomeres' incomplete disclosure of their complimentary abilities in succeeding divisions, the blastocyst stage is not reached. Additionally, these embryos may be subjected to arrest and lyses, which cause disintegration. The harmonious relationship ("cross-talking") between the blastocyst and the

endometrium epithelium, which is demonstrated by the support of particular paracrine molecules and ultimately has a favorable impact on the embryonic implantation process, is another piece of evidence pointing to the possibility of embryo implantation at the blastocyst stage (Galan *et al.,* 2000; Karaki *et al.,* 2002).

5. Strategies to overcome challenges of in vitro embryo production technology

It takes careful planning to produce a live calf from an IVP embryo. An oocyte of good quality, capable of maturation and successful fertilization, must be provided by the donor. The embryo must be properly chosen, loaded, and transferred by a trained technician and must develop *in vitro* until day 7 following fertilization. Semen, laboratory tools, IVP media, quality control procedures, and laboratory staff will all play a role in how well the process goes. Pregnancy outcomes are influenced by the measures performed both before and after embryo production (Sirard, 2018; Demetrio, 2019; Hansen, 2020).

The success of commercial IVP has considerably increased over the past ten years as a result of reported increases in blastocyst rates, enhanced cryotolerance, pregnancy rates, pregnancy loss rates, and the incidence of large offspring syndrome. However, *in vitro* and *in vivo* created embryos are nonetheless distinct from one another (Hansen, 2020).

The American embryo transfer association mentioned one strategy to improve the quality of embryos produced *in vitro* through the use of follicular stimulation by injecting cows with follicle stimulating hormones (FSH) and was reported the data in 2017 and 2018 that more oocytes per OPU were recovered from beef breeds than dairy breeds, and both produced more viable embryos per OPU when FSH was used to stimulate the donor cows (Demetrio & Wehrman, 2018; Demetrio & Wehrman, 2019).

One of the challenges faced *in vitro* embryo production is improving embryonic survival after cryopreservation. Compared to embryos created *in vivo*, those created *in vitro* are less resistant to cryopreservation, which is caused by, among other things, the larger concentration of lipids in their cells. In this context, it has been suggested that altering the culture conditions, such as adding lipolytic chemicals and adjusting the amount of fetal calf serum in the medium, will reduce the amount of lipid present in the embryos (Sanches, 2017). Fetal calf serum (FCS) concentration has been found to have an impact on the quantity of cytoplasmic lipid droplets in developing embryos (Sudano *et al.,* 2012). When serum concentration was decreased to 2.5% and phenazine ethosulfate (PES) was added to the culture medium on day 4 of a trial using FCS and lipolytic chemical agents such as PES supplementation, the rate of blastocoel re-expansion following embryo vitrification improved (Sudano *et al.,* 2011).

In the world´s context, Brazil is the largest producer of bovine *in vitro* produced (IVP) embryos. Brazil used some technologies to adopt and increase the *in vitro* embryo production. The commercial availability of sex-sorted semen was the main factor supporting the rise in dairy IVEP (Pontes *et al.,* 2010), which had a significant impact on the characteristics of the Brazilian embryo market. The number of frozen thawed embryos in Brazil has increased recently, reaching 22.8% in 2015, the highest level in ten years. This increase is most likely attributable to advancements in cryopreservation techniques, such as direct transfer (Sartori *et al.,* 2016).

It's interesting to note that in the early years of IVEP (up to 2005), the Brazilian market for embryos deviated from trends seen in other nations, especially in those with relevant embryo production. Thus, the high economic values and higher oocyte yield of zebu breeds in the Brazilian internal market appeared to be the cause of IVEP's commercial success in that country. As explained by viana *et al.* (2017), the creation of efficient cryopreservation techniques, together with an agreement on sanitary guidelines and laws for the export/import of IVP embryos in Brazil are essential for the continued growth of the embryo sector.

Also in Japan, the number of embryo transfers has increased till 2008, reaching 72,126 heads. The fiscal 2012 conception rates are 52% with fresh embryo transfer and 46% with frozen embryo transfer. Fresh embryo conception rates have been kept between 50 and 52% since 1988, while frozen embryo conception rates have been kept between 45 and 46% since fiscal 1995. Although the conception rate does not consistently exceed 50%, 11 entities or embryo transfer practitioners in Japan have conception rates of 60% or more (Ministry of Agriculture, Forestry and Fisheries, 2014). It has been mentioned that to increase the *in vitro* embryo production rate in cattle, advances in cryopreservation technology such as the Cryo top vitrification-straw dilution (CVSD) method is one factor. The CVSD is a simplified procedure for warming vitrified embryos using Cryo top and diluting cryo protectants in straw to enable one-step transfer of bovine embryos without the need for a microscope or other laboratory tools. According to Sano *et al.,* 2010, by using this method, sexed embryos can achieve a 50% conception rate, while *in vitro* produced (IVP) embryos can achieve a 40% conception rate (Inaba *et al.,* 2011).

6. Advanced technologies for increasing the outcome of *in vitro* embryo production in cattle

The production of embryos *in vitro* and other forms of assisted reproduction in cattle have advanced significantly in recent years. In North America, South America, and Europe, the use of in *vitro* production

together with sexed semen and genetic selection is widespread and successful.

It's significant to note that in 2016, there were more transferable *in vivo*-produced embryos than viable *in vitro*-produced embryos (multiple ovulation embryo transfer, MOET). This trend reveals a shift in seed stock producers' preferences from conventional MOET to IVP. These commercially viable assisted reproductive techniques help practitioners and cattle producers to increase reproductive performance, efficacy, and genetic gain (Ferré *et al.*, 2020).

Over time the production of embryo *in vitro* have made a progress and according to the 2021 data of the international embryo transfer Society (IETS) data retrieval committee, North America leads on the top for the production of cattle embryos *in vitro* accounting for total number of produced embryo (764,650) followed by South America (690,856). Other countries like Europe, Oceania and Africa also reported the total number *in vitro* produced embryo in cattle (42,410, 17,681 and 5,421) respectively. For the first time in 2021 more than one million total IVP cattle embryos were transferred world widely, up to 32.8% increase compared to 2020 (1,166,034 vs. 878,181, respectively). This indicates that the production of embryo *in vitro* have made a progress overtime (Joao, 2021).

Today's major advances in IVP aim to enhance efficiency across each stage, including ovarian stimulation, oocyte recovery, maturation, fertilization, embryo growth, freezing, transfer, and pregnancy establishment (Ferré *et al.*, 2020).

6.1. Transvaginal follicular aspiration

Aspiration of the ovaries using a vaginally inserted ultrasonography probe and needle is the main technique for harvesting oocytes from live cattle. Oocytes were previously extracted surgically through a flank incision (Bederian *et al.*, 1975) or laparoscopically through the Para-lumbar fossa (Lambert *et al.*, 1986), but both methods were expensive, ineffective, and carried a risk of adhesion formation and consequent loss of fertility (Hasler and Barfield, 2014). The starting materials, the ovum and sperm, are one of the most crucial factors in the success of *in vitro* production methods. The capacity of the resulting embryo to develop is determined on the quality of the oocyte. Using non-invasive transvaginal ultrasound-guided oocyte aspiration techniques, oocytes from genetically valuable animals can be recovered. OPU sessions can be repeated on cattle without harming the animal and with little to no stress (Boni, 2012).

6.2. Reverse sorting of sperm for Fertilization

Between 70% and 85% is the typical range for the fertilization rate, which is calculated as the cleavage rate at 48 hours after insemination (and assuming parthenogenesis is not occurring). The fact that IVF uses fewer spermatozoa to fertilize the harvested oocytes than other methods, which at first look favors using Sexed Semen (SS), which normally contains around 2.1 million sperm per straw as opposed to Sexed Ultra, which contains 4 million sperm per straw. At first, SS that was offered for sale by AI centers was utilized. In order to separate normally frozen semen before use in IVF, a technique known as reverse sorting of sperm was developed (de Graaf *et al.*, 2007). Reverse sorting was employed in a large scale donor IVP program, proving its viability (Morotti *et al.*, 2014). This scientific progress made it possible for seed stock producers to produce sex-specific embryos using IVP from their best females and genetically superior sires (de Graaf *et al.*, 2007).

6.3. Oocyte micromanipulation

Micromanipulation includes all techniques requiring remote-controlled robotic manipulation of gametes or embryos through technology. Systems for micromanipulation are created to do eliminate tremor and enable exact cell manipulation even at extreme magnification. In order to increase the likelihood of fertilization, micromanipulation rigs are frequently employed during intra cytoplasmic sperm injection (ICSI), zona opening for improving or facilitating hatching and for biopsying the trophectoderm, and blastocoel collapse before vitrification. In order to maintain stable environmental conditions for gametes and embryos during micromanipulation, it is necessary to consider factors like maintaining a constant, optimal temperature, similar to controlled culture conditions; restricting exposure to light; reducing vibration to lessen cell damage and improve handling; and maintaining osmolality and pH similar to controlled culture conditions (Cohen, 2019).

6.4. Intra cytoplasmic sperm injection

Intra cytoplasmic sperm injection (ICSI's) primary goal is to select a highly motile sperm cell subpopulation while removing all traces of seminal plasma and debris with the least amount of damage to the viable cells (Cohen, 2019). This method can be used for sperm from outstanding bulls when the quality of the semen is damaged; nevertheless, it calls for specialized equipment and knowledge to be employed both in lab settings and in the field. The method is also applied to sperm vector systems for transgenic animals. Sexed semen has also been used for intra cytoplasmic sperm injection, with success rates of 80% in cattle and 48-63% in small ruminants when utilizing fresh and frozen-thawed semen (Ohlweiler *et al.*, 2013). Aseptic approach, sperm survival testing, protein supplementation, use of antibiotics, and appropriate osmolality (250-290 mOsm/kg) are among the handling procedures used in ICSI, which are similar to general tissue culture procedures (Cohen, 2019).

6.5. Embryo genomics

Through the use of novel biotechnological methods, embryo genomics studies the expression of

genes in various developmental stages of embryos in both natural and artificial environments (Graf *et al.*, 2014). The activation and development of the embryonic genome can be examined in relation to fetal development, early differentiation, and successful implantation (Graf *et al.,* 2014). The growth of various IVF laboratories in North America and other parts of the world is being driven by genomic analysis (Sirard, 2018). The genetic progress for qualities of economic interest has doubled as a result of genomic selection, which is its best effect to date. The precision of genetic merit for young animals has grown, resulting in genetic improvement (Wiggans *et al.,* 2017). The genetic value of the bull is identified early using genomic analysis, and as soon as sperm with such high genetic merit are produced, they can be used for IVF. Additionally, it has boosted the market's need for heifer and calf embryo production (Sirard, 2018).

IVF programs are currently being considerably impacted by the genomic testing of cattle. Dairy production has undergone a revolution thanks to genomic selection, which has shortened breeding intervals, improved selection accuracy, and reduced the cost of progeny testing in the past (Wiggans *et al.*, 2017). The commercial pursuit of genetic research and the collection of gametes from fetuses with desired features are growing (Moore and Hasler, 2017). IVP embryos from younger females can now be developed using the small ultrasound OPU probes that are already accessible (Sirard, 2018). Furthermore, the viability and quality of oocytes and even embryos have been assessed using genetic analysis prior to transfer procedures (Moore and Hasler, 2017). The breeding industry's desire to continuously promote genetic improvement in dairy or beef cattle, particularly since the introduction of sexed semen, is a major driving force behind OPU-IVEP. To improve the number of embryos and offspring per donor and to enable more intense selection for the following generation, biopsies are taken from IVP embryos from young heifers for genomic study. The time needed to produce replacements for elite females is reduced as a result of this method. The fundamental benefit of combining assisted reproductive technology with genomic selection is the shortening of the generation gap, which can double the pace of genetic gain compared to traditional progeny testing methods (Ponsart, 2014).

7. CONCLUSION

As *in vitro* embryo production is a technique of collecting many oocytes from both slaughtered and alive animals, maturing and fertilizing them *in vitro* to create a live calf, it is the best way to improve the reproductive potential of cattles. *In vitro* embryo production can shorten generation interval of cattle by eliminating the need to wait for a cow to give as only one calf. Through *in vitro* embryo production millions of newborn calves can be obtained from only few donors within a year. *In vitro* embryo production increases the production of large number of genetically superior animals through genetic selection strategy and has a role in keeping the endangered cattle breeds from extinction adding a value for conservation of the biodiversity. The success of *in vitro* embryo production can be improved through taking precautions by controlling the factors that may affect the procedures involved in this technology. Female and male animals should be carefully selected that can bear us good quality oocytes and semen and also suitable sperm preparation and cryopreservation method must be chosen in order to establish more pregnancy when *in vitro* produced embryos are transferred to the recipient animals. Generally, *in vitro* embryo production has been proved to be a technology to improve dairy and beef industry in developed countries and it has also a promise in developing countries if it is well adopted.

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Cite this Article: Ayda Mohammed Yusuf (2024). Promise of *In vitro* Embryo Production Technology for Improvement of Cattle Reproductive Potential. *EAS J Vet Med Sci*, *6*(2), 44-58.