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Bovine *In vitro* Embryo Production and its Contribution in Improving Animal Products and Productivity: Review

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Abstract: *In vitro* embryo production (IVEP) of bovine embryos has become implemented in cattle breeding in many countries for the production of embryos in the laboratory which has several advantages as compared with other conventional methods of embryo production. The various aspects of *in-vitro* maturation, fertilization and embryo culture in domestic farm animals are the major components of IVEP where to be considered due to *In vitro* embryo development being strongly influenced by events occurring during these steps of the techniques. Many factors can affect the success of IVEP by affecting all the steps of the techniques or at least one step. Therefore, this review aimed to focus on the factors that influence the efficiency of *In vitro* embryo production. So, for improving the efficiency of IVP and making the systems more important for routinely producing blastocyst stages of embryos for high genetic merit, factors affecting *In vitro* maturation, fertilization and embryo culture should be considered.

Key words: *In vitro* · Embryo · Media · Oocyte

INTRODUCTION

The world food crisis increases the demand for food of animal origin. The competitive global markets of such foods and the negative effects of climatic change, especially in the marginal tropical areas [1] demand innovative strategies and interventions are urgently employed [2]. The dairy sector in developing countries has the potential of providing cost-effective animal protein requirements especially in low-input production systems. In order to provide high-quality meat and dairy products, the enhancement of domestic production is the most permanent method. Thus, the implementation of advanced animal improvement programs is essential to make animal agriculture sustainable and more profitable.

Overall, advanced animal biotechnologies are essential tools to accelerate the improvement of genetics and allow the selection of high-producing, fertile and healthy animals in developing countries. These reproductive biotechnologies include artificial insemination (AI), semen technologies and evaluation, embryo transfer, *In vitro* fertilization (IVF), ovum pickup and combination with IVF, estrus synchronization, superovulation and pregnancy monitoring [3].

Over the past years, *In vitro* production (IVP) of bovine embryos has become implemented in cattle breeding in many countries. Compared with conventional superovulation and ET, the production of embryos in the laboratory has several advantages. First, IVEP can be used in donors those fail to respond to superovulation treatment. Second, IVEP can be used to salvage the genetic potential of terminally ill females that could not respond to conventional ET. Third, semen from different bulls can be used to fertilize oocytes harvested from a cow resulting in embryos with different sires being produced at the same time. Fourth, oocytes for IVEP can be obtained from the ovaries of the live donors using Transvaginal Oocyte Recovery (TVOR), or from the slaughter ovaries [4].

The development of techniques for the effective production of bovine preimplantation embryos from oocytes matured and fertilized *In vitro* is important for embryo transfer and basic scientific research. Techniques for producing pre-implantation embryos by *In vitro* maturation/*In vitro* fertilization (IVM/IVF) are being used in many laboratories worldwide [5].

In the laboratory, embryos can be routinely produced and developed up to the blastocyst stage using three

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subsequent techniques: *In vitro* maturation (IVM) of oocytes, followed by sperm capacitation and *In vitro* fertilization (IVF) of matured oocytes and then *In vitro* culture (IVC) of the fertilized oocytes up to the blastocyst stage. *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 are more important for routinely producing blastocyst stages of embryos for high genetic merit [3].

Therefore, the Objectives of this review are:

- To review the optimal condition required for IVM, IVF, IVC and factors affecting their success
- To review the different semen preparation techniques and their effects on In-vitro fertilization of the matured oocyte
- To review the types of media used in each step of IVEP

Invitro Maturation: Oocyte maturation is a long process during which oocytes acquire their intrinsic ability to support the subsequent stages of development in a stepwise manner, ultimately reaching activation of the embryonic genome. This process involves complex and distinct, although linked, events of nuclear and cytoplasmic maturation. Nuclear maturation mainly involves chromosomal segregation, whereas cytoplasmic maturation involves organelle reorganization and storage of mRNA, proteins and transcription factors that act in the overall maturation process, fertilization and early embryogenesis [6]. The maturation of the oocytes includes two aspects: Nuclear and cytoplasmic maturation [7].

Nuclear Maturation: Meiosis, a Greek word meaning reduction, consists of two successive cell divisions following one round of DNA replication. Meiosis gives rise to four haploid cells from a single diploid cell. This type of cell division is characteristic of germ cells.

Meiosis, up to the diplotene stage, occurs in the foetal ovary. During the first meiotic division, maternal and paternal genes are exchanged before the pairs of chromosomes are divided into two daughter cells. The second meiotic division occurs without being by DNA synthesis and nuclear reformation. The two meiotic divisions of the oocyte are asymmetrical, resulting in the expulsion of polar bodies. Meiosis in each female germ cell results in a single egg and two polar bodies [8]. In addition to observation of oocytes, this stage is a more reliable means for defining the *In vitro* maturation progress [9, 10].

The Mammalian oocytes begin meiosis during foetal life but do not complete meiosis-I until ovulation and meiosis-II was only completed upon fertilization [11].

The maturation of follicular oocytes is normally arrested at the prophase-I of the first meiotic division and the oocyte remains in the dormant stage, called a dictate nucleus. At this stage, nuclear material is enveloped and the resulting structure is called a germinal vesicle. The oocytes remain at this stage until the onset of puberty. Under the influence of gonadotropins and particularly in response to the LH surge, oocytes resume meiosis just before ovulation and nuclear maturation refers to the progression of the oocyte nucleus from the germinal vesicle to the metaphase II stage [6].

Nuclear maturation involves germinal vesicle breakdown (GVBD), condensation of chromosomes, metaphase I spindle formation, separation of the homologous chromosomes with extrusion of the first polar body and arrest at metaphase-II [12]. The nuclear membrane starts to fold, the nuclear pores disappear and then the nuclear membrane undergoes fragmentation and rapidly disappears [13]. It appears that nuclear maturation follows the same pattern in vivo and *In vitro*. Nuclear maturation involves changes in protein synthesis patterns [14].

The ability of the oocyte to complete meiosis is known as meiotic competence. Meiotic competence is acquired gradually during follicular growth. Oocytes firstly acquire the capacity to undergo GVBD and chromosome condensation and then further follicular development is required to acquire the ability to progress to metaphase I [13] and finally, they acquire the ability to reach metaphase II [6]. The ability to complete the MI to MII transition coincides with the achievement of full size and with the process of nucleolar compaction [15].

Meiotic competence is closely correlated with oocyte size, which in turn is correlated with follicle size [16]. Bovine oocytes acquire the ability to complete GVBD and meiosis by the time the antral follicle reaches 2-3 mm in diameter [17].

Cytoplasmic Maturation: Cytoplasmic maturation could be defined as the unity of metabolic, molecular and ultra-structural processes that modify the oocyte cytoplasm for normal fertilization and acquisition of developmental competence [18, 19]. It encompassed a

wide array of syntheses for oocyte-specific developmentally regulated protein, relocation of cytoplasmic organelles and alteration to the membrane transport system in the oocyte [20, 21].

Some elements of cytoplasmic maturation could be visualized as the line-up of cortical granules and increase in number and a change in the morphology and location of mitochondria, whereas, many other elements were molecular and very challenging to visualize or monitor [22]. For that, the cytoplasmic maturation was often divided into three major processes: organelle redistribution, cytoskeleton dynamics and molecular maturation and it could be used indirectly and retroactively to assess the ability of the mature oocyte to undergo normal fertilization, cleavage and blastocyst development [23, 24]. Failure to complete cytoplasmic maturation could block the development at fertilization, embryonic genome activation, blastocyst formation, or even post-implantation [25]. Other indirect morphological parameters that could be taken into account to evaluate the cytoplasmic maturation were cumulus cell expansion, extrusion of the first polar body and increased perivitelline space [26].

Glutathione (GSH) has been shown to play an important role in oocyte maturation. The process of oocyte cytoplasmic maturation involves numerous molecular events, including the synthesis of biochemical compounds, protein phosphorylation and activation of particular metabolic pathways [27, 28]. These changes are a prerequisite for normal fertilization and embryo development. The synthesis of intracellular glutathione is a critical part of oocyte cytoplasmic maturation [27].

Oocyte Selection for *In vitro* Maturation: For the most successful species, it was only possible to obtain the development of a maximum of 40 to 50% of the zygotes to the blastocyst stage [18]. This limited success had been attributed to: the reduced oocyte developmental competence which was suggested to be the primary reason for the reduced potential of *In vitro* produced embryos or due to the heterogeneous population of oocytes obtained from the follicles [29, 30].

Bovine oocyte selection for IVM was performed on the basis of visual assessment for the morphological features [31]. Cumulus cells establish intimate contacts together as well as with the oocyte to allow cell communication through gap junctions, which were necessary for efficient maturation of the oocyte [32].

The criteria for oocyte selection depend not only on the morphology of the oocyte-cumulus complex but also upon other factors such as the follicular size [33]. Where, as a lot of researches show that the developmental capacity of the bovine oocyte is affected by the follicular size [34]. The capacity of the bovine oocyte derived from different follicular sizes to undergo normal fertilization and early embryonic development *In vitro* and reported that the follicles could categorize into three groups: > 4 to 8 mm (large), > 2 to 4 mm (medium) and 2 to 1mm in diameter (small) were, there were high percentages of embryonic development and blastocyst rate recovered from the large follicles. Similarly, there was an effect for the follicular size on the oocyte developmental competence where the percentage of the oocytes reached M-II from large follicles (=1.5 mm) was 55% compared to 20% recovered from small follicles (<1.5 mm). Gupta *et al*. [35] added that the oocytes obtained from 6-7mm follicles usually had many layers of cumulus cells and significantly produced a higher proportion of morula/blastocyst stage embryos (65.9%) compared to those obtained from 2-6 mm follicles (34.3%).

Overall, the morphological criteria are still routinely used for a rough selection of the oocytes in most laboratories as there was no real breakthrough with respect to increased blastocyst rates that had been achieved yet.

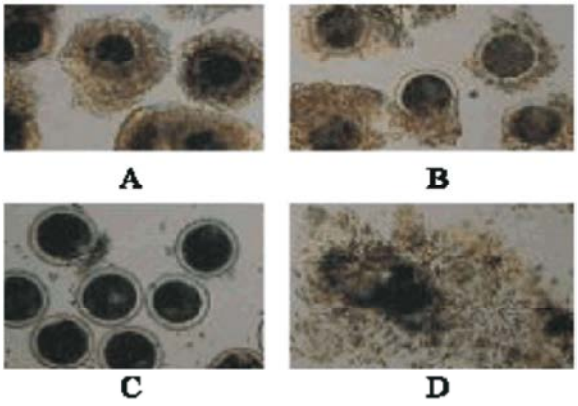

Fig. 1: Representative photographs of the four grades of COCs

Grade A- homogenous COCS, Grade- B: COCs not homogenous, Grade- C: COCs were not found at all and Grade- D: expanded COCs

Factors Affecting *In-vitro* Maturation: *In vitro* maturation is the most critical step *In vitro* embryo production. There is a constant need to emphasize the fact that effective oocyte maturation is the foundation of embryo production. Identifying these factors will improve the *In vitro* embryo production systems in bovine [3].

The recovery of a large number of oocytes with high developmental competence remains an ultimate goal for the mass production of embryos in cattle. At the same time, the origin of the oocyte can play an important role in their IVF and subsequent developmental competence.

Effects Follicular Size: Oocytes for IVM are generally selected using the following criteria: follicle size, cytoplasmic appearance, the appearance and a number of cumulus cells around the oocytes (COCs). Cumulus expansion can importantly be used to microscopically assess the *In vitro* maturation rate of oocytes and also showed a relationship between follicle size and oocyte quality [35 37].

In bovine, the oocyte first acquired competence to develop into blastocysts *In vitro* system at a follicular size of 2-3 mm. When follicles were pooled according to size, it was shown that large follicles (10 mm diameter) contain oocytes with a higher potential to become embryos [17]. Some studies described the fate of individual oocytes according to the exact follicular size and it confirmed an increased competence with follicle size, i.e., bovine oocyte complexes (COCs) isolated from ovaries carrying follicles of 2-5 mm in diameter showed lower rates of maturation and blastocyst formation than those from ovaries carrying follicles of >10 mm in diameter. These indicate that large follicles (6 mm diameter) provide the oocyte with a microenvironment that improves its quality [38].

It has been recorded that the follicular size was not the only criterion determining the oocyte competence to develop, as there were some oocytes originating from large follicles failed to produce embryos, while others from small ones already have this capacity; this simply because the oocyte capacity to mature, fertilize and to develop into blastocysts was acquired in a stepwise fashion during oogenesis and folliculogenesis [27].

The Existence of Cumulus Cells: The presence of cumulus cells was necessary for the cytoplasmic and /or nuclear maturation of oocytes in cattle [39]. Cumulus cells benefit oocytes development either by secreting soluble factors, which induced developmental competence or by removing an embryo development suppressive component from the medium [40]. Cumulus cells supported the IVM of oocytes to the MII stage and were involved in the cytoplasmic maturation needed for optimal developmental competence, such as male pronucleus formation and development to the blastocyst stage. Cumulus cells might be a good indicator for an oocyte's ability to undergo meiosis I *In vitro* and that the developmental problems of denuded oocytes were due to deficient cytoplasmic maturation [41].

Various roles of cumulus cells include prevention of the hardening of zona pellucida, the provision of energy for oocyte maturation and production of cytoplasmic maturation factors and the uptake of nutrients for oocytes during maturation in culture medium [42]. In addition, the cumulus cells are also important for fertilization, for example, such as the trapping of spermatozoa, guiding spermatozoa to the oocyte, protection of the oocytes against zona hardening and prevention of changes in the oocyte that are unfavourable for subsequent fertilization [32, 43]. Moreover, factors like chemokines secreted from COCs induce sperm capacitation and enhance fertilization, providing evidence for a regulatory loop between sperm and COCs during fertilization [44]. It has been demonstrated that chemokine signalling facilitates both sperm attraction to the COC and COC compaction by the cumulus extracellular matrix assembly [45].

Oocyte Quality: Naturally, the oocyte quality is determined by the oocyte's ability to mature, be fertilized and give rise to normal offspring [19, 46]. The quality of the oocyte is also related to the oocytes' follicular environment, as well as several factors: such as the age of the donor animal, stage of follicular development and the media used for maturing the oocytes [47, 48].

Cumulus cells morphology and the microscopic aspect of the ooplasm are generally considered as the two main parameters to assess the quality of the cumulus-oocyte complex (COC). The criteria employed by various authors for the selection of oocytes for IVM include the presence of a multilayer compact cumulus oophorus and homogeneous cytoplasm [49].

Duration of Maturation: The success of IVEP is temperature-dependent. Changes in temperature expose oocytes to temperature shock, which induces chromosome abnormalities. As a result, *In vitro* conditions during maturation need to be optimal. That is, during incubation, the temperature should be between 38°C and 39°C, CO2 gases at a level of 5% and the time should not exceed 48 hours which may cause denature of oocytes or hinder maturation [50]. Oocytes remained in the germinal vesicle (GV) stage from the onset to 6-8 h of culture. The germinal vesicle breakdown (GVBD) occurred between 7-9 h and the metaphase-I became established within 12-18 h. Finally, most oocytes reach the metaphase-II stage after 27 h at 38.5°C [6].

The most suitable maturation period in both cattle and buffaloes is 24h [51]. The attainment of the MII stage commenced after 18 h of maturation but the majority of oocytes completed nuclear maturation between 21 and 24 h [52]. There is a large variation in the timing of the *In vitro* oocyte maturation process in cattle [53, 54].

Effect of Culture Media: A wide variety of media has been used for IVM in domestic animals, ranging from simple physiological solutions to complex culture media containing amino acids, vitamins, purines and other compounds regarded as essential for general cell culture. The culture employed in IVM not only affects the proportion of bovine oocytes that reach metaphase II (M II) and become capable of undergoing *In vitro* fertilization but can also influence subsequent embryonic development [55]. *In vitro* maturation medium can be broadly divided into simple and complex. Simple media are usually bicarbonate-buffered systems containing physiological saline with pyruvate, lactate and glucose and they differ in their ion concentration and in the concentrations of the energy sources. Complex media contains in addition to the basic components of simple media, amino acids, vitamins and purines.

Most IVF laboratories routinely use M-199 as the basic IVM medium in cattle and there have been few reports suggesting that other media may be more appropriate. In one comparison of complex media for IVM, the scientists concluded that under their conditions, the F-10 medium is superior to M-199 and B2 media [56]. Another comparison of IVM commercially available complex chemically defined media showed that TCM-199 was superior to RPMI-1640 [57].

Oocytes matured in medium leading to poor developmental competence have depressed levels of glycolysis that are necessary for the completion of maturation, the reduced level of glycolysis may reflect the reduced activity of the pentose phosphate pathways, which plays an important role in the meiotic maturation of bovine oocytes [28].

For the energy source, the excessive glucose in the media used for oocyte maturation impairs the development of bovine oocytes to the blastocyst stage, possibly due to the increase of Reactive Oxygen Species (ROS) and the decrease in the intracellular glutathione content of bovine oocytes [58].

***In vitro* Fertilization:** The IVF is the 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 [59]. 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 co-incubation [60]. Co-incubations are usually conducted in 50 to 100 fill micro droplets with 5 to 25 Oocytes/drop-in Petri dishes covered with mineral oil. The sperm concentration for *In vitro* fertilization ranges from 1 to 20 x 10s motile sperm/ml of medium and oocytes are usually co-incubated with sperm for 6 to 24 hours. Finally, it was washed several times to remove extra sperm cells and then transferred into embryo development culture medium [61].

Factors Affecting *In vitro* Fertilization: Fertilization is a complex process, which results in the union of two gametes, the restoration of the somatic chromosome number and the start of the development of a new individual. Successful cattle IVF requires appropriate preparation of both sperm and oocyte, as well as culture conditions that are favourable to the metabolic activity of the male and female gametes [62].

Methods of Sperm Preparation: Mammalian spermatozoa, matured in the epididymis and ejaculated, are not immediately capable of fertilizing oocytes. The sperm must undergo a period of capacitation, which normally occurs in the female reproductive tract. preparing sperm for fertilization of the bovine oocyte involves a sequence of events in which the sperm: One Is motile (to reach the oocyte and move through the Zona Pellucida (ZP), two the ability to undergo capacitation and express the Acrosome Reaction (AR), Third is the capacity to bind to the zona pellucida and vitelline membrane by acquiring the correct binding proteins during maturation and exposing these binding sites to the oocyte at the appropriate time and fourth is the ability to fuse with the oolemma and be incorporated into the oocyte.

It is clearly important to have highly motile bull sperm available for IVF. This may be achieved by applying various procedures for isolating motile samples. There are also a number of chemical agents which may be employed to stimulate motility and AR of bull sperm and to maintain motility [3].

Percoll density gradient method: in the procedure of this method 1.5 ml of 90% Percoll was mixed with 1.5 ml of sperm- Tyrode's-Lactate (Sp-TL) stock to obtain 3 ml of 45% Percoll in a 15 ml tube. With a transfer pipette, 90% Percoll (3 ml) was carefully deposited under 45% Percoll

in the bottom of the tube. Semen (0.25 ml) was deposited on the top of the Percoll gradient. This was centrifuged at 400G for 30 minutes at room temperature (25°C). A sperm pellet formed at the bottom was removed and mixed with Sp-TL to make a final volume of 5 ml which was again centrifuged for 10 minutes. After discarding supernatant, the pellet was examined for sperm concentration and diluted with Tyrodealbumin- lactate-pyruvate (TALP: NaCl 99.0 mM, KCl 3.1 mM, NaHCO3 25.0 mM, NaH2PO4 0.35 mM, bovine serum albumin 6.0 mg/ml, sodium pyruvate 1.0 mM, DL-lactic acid 21.6 mM, Hepes 10 mM) medium to have final concentration of 1x106/ml of TALP [63].

Swim-up method: Swim-up method was performed with two types of media separately; i) Modified Ca2+ free Tyrode's medium and ii) Tyrodealbumin- lactate-pyruvate (TALP) medium. Each medium (pH 7.3-7.4) was incubated in an atmosphere of 5% CO2 in air at 38.5°C for 2 hours prior to use. A 0.25 ml of fresh semen was deposited at the bottom of 1.5 ml of each medium separately. The tubes were incubated in an atmosphere of 5% CO2 in air at 38.5°C for 30 minutes and supernatant from the same media tubes was pooled separately in a sterile conical tube and centrifuged at 100G for 10 minutes. The supernatant was discarded, saving 100 µl sperm suspension at the bottom of each tube. This sperm suspension in each tube was diluted with 1 ml of the respective medium. This preparation was equilibrated at room temperature for 5 minutes. After adding 5 ml of more medium, it was again centrifuged for 10 minutes at 100 G. The supernatant was again discarded and the remaining 100 µl of sperm suspension in each tube was diluted with the same medium containing heparin (21.87 IU/ml) and incubated finally for 15 minutes in CO2 incubator at 38.5°C [64].

Sodium citrate washing: In the sodium citrate washing method, 0.25 ml of fresh semen was mixed with 2.9% sodium citrate to make a final volume of 5 ml. The suspension was centrifuged at 300G for 10 minutes. The supernatant was discarded and the pellet containing sperms was dissolved in 3 ml of 2.9% sodium citrate and centrifuged again for 10 minutes. After discarding the supernatant, the concentration was determined in the sperm pellet and sufficient Tyrode solution was added to obtain a final sperm concentration of 1x106/ml of medium [65].

Fertilization Medium: Treatment of semen with a medium of High Ionic Strength (HIS) like Brackett and Oliphant (BO) medium (osmolarity 360-390 mOsm) is described for capacitation of fresh and frozen bovine semen [66, 67]. Many authors used TALP-medium for *In vitro* capacitation of bovine spermatozoa [68]. In this respect, a group of scientists observed that a significantly higher proportion of bovine oocytes developed to blastocyst stage after insemination with spermatozoa prepared by swim-up in Fert-TALP supplemented with heparin than by centrifugation in BO supplemented with 10 mM caffeine-sodium benzoate [69].

Use of heparin and caffeine: many Studies support the view that capacitation of bull sperm by heparin probably reflects the in vivo mechanism [70]. Heparin dosage and incubation period for sperm capacitation are important factors affecting bovine IVF and subsequent embryo development [71]. Heparin induces changes in the calmodulin (cAMP)-binding properties of sperm proteins and induces a reduction in Ca2+ concentrations during capacitation [72]. The capacitation of bovine sperm with heparin requires extracellular calcium, the maximal kinetics of heparin-induced capacitation occurs when extracellular calcium exceeds 10 µMI91. Changes in calcium trigger subsequent increases in cAMP; pH and tyrosine are known to be essential for capacitation [73]. Also, another study reported a synergistic effect of 20 µg mLG1 heparin and 10 mM caffeine in their capacitation treatment of frozen-thawed bull sperm. It was evident that the optimum dose of the agent was 100µg mLG1 [74]. The fertilization rate may be rapidly improved by adding heparin to the IVF medium at values varied between 0.5-5.0 µg mLG1. A pre-incubation period of 15 min was found to be satisfactory [75].

Effect of glucose in fertilization medium: In cattle, glucose inhibits the role of heparin in inducing sperm capacitation. On the other hand, for cattle oocytes inseminated in a chemically defined medium, glucose is required for stimulating *In vitro* fertilization of the bovine oocyte [76].

***In vitro* Embryo Culture and its Development:** The fertilized oocytes are cultured *In vitro* to the blastocyst stage when such embryos may be transferred into a recipient or frozen and stored in liquid nitrogen. Development to the morula and blastocyst was found to be dependent on the gas phase environment during *In vitro* culture. The best gas-phase observed was 5% CO2, 5% O2 and 90%N2. The IVC lasts for 7 days in desirable defined sequential media to satisfy the energy needs of the pre-implantation embryo [77]. During IVC, the embryo is passing through 4 important developmental stages: the first mitosis, activation of the embryonic

genome, compaction in morula stage, the formation of the blastocyst with differentiation of their cells. Unfavourable conditions during IVC may affect any of the mentioned stages and negatively influence the quality of cultivated blastocyst. The quality of the embryo has further influenced the success of freezing as well as the percentage of conception after transferring to the recipient animal. Although numerous methods are used to evaluate embryo quality, the assessment of this quality presents a challenge for embryologists in selecting the best quality embryos to transfer to the recipient. The simplest and most widespread method to determine the embryo quality is a morphological evaluation, which is the most subjective method. Mammalian embryos have been cultured in a variety of chemically defined and undefined media. A chemically defined medium has been described as a liquid prepared from bench chemicals and containing four or less basic components: inorganic salts, amino acids, vitamins and an energy source at known concentrations for lists of ingredients of several common defined media [59].

Obviously, the study continued to improve methods of maturation and fertilization but embryo culture stood as the final great frontier to *In vitro* embryo production. The benefits offered by IVC are easy to see, such as the ability to observe development in real-time, as well as the ability to non-surgically transfer embryos at a later stage of development. The latter is absolutely essential for the widespread commercial application of *In vitro* embryo production.

Although the media choices of the initial attempts at *In vitro* culture were logical as they supplied protein, nutrients and growth factors, embryo culture in them was relatively unrewarding. Many researchers set out to find exactly what type of fluid embryos were cultured in vivo. The first scientists elucidated the ionic and protein contents of the oviductal fluid of sheep. Appropriately, the resulting media was called Synthetic Oviductal Fluid (SOF) and forms the basis for many ruminants' *In vitro* production protocols of today [78]. The SOF was used for the first time in bovine embryo culture [79]. The in vivo produced embryos that started at the eight-cell stage proceeded to the morula stage in 30% of embryos, which represented only the second time that a morula had been produced in culture. In the same study, oxygen tension was varied to better approximate the conditions of the oviduct [80]. When O2 levels were reduced to 5% (from the 21% found in the atmosphere) with 5% carbon dioxide and the balance nitrogen, the development of 8 cells in vivo produced embryos proceeded appropriately and the world saw for the first time a bovine blastocyst as the result of *In vitro* culture. The lower oxygen tension is thought to reduce the load of reactive oxygen species present in the media [81].

Early successes in bovine embryo development were often seen either from 1-2 cell embryos to the 8-cell stage or from the eight-cell stage to morula/blastocyst. Thus, the term "8 cell block" was coined to describe the insufficiency of culture systems of the day to support development through the fourth cell cycle. Interestingly, this "Block" occurs at the same point as Embryonic Genome Activation (EGA) or the shift in the embryo from the embryo's dependence on maternal mRNA to embryonic. The application of low oxygen tension in bovine embryo culture was the first step in solving the 8-cell block [79]. The embryos in the third and fourth cell cycle were most affected by *In vitro* culture conditions and would not proceed in development *in vivo* after *In vitro* culture for 24 h, even though embryos cultured for short periods *In vitro* at other points in development could proceed to the blastocyst stage after *in vivo* culture. In addition to low oxygen tension, somatic cell co-culture has also been shown to overcome this "Block" [82].

Cellular Co-Culture: The presence of a somatic cell monolayer in the culture medium during *In vitro* culture of the developing embryo was found to be very important in enhancing its development potential. The provision of cell basement support provides the developing zygotes with a comfortable environment and secretes some growth factors supportive to further development *In vitro*. Another investigator chose to focus on a co-culture of oviductal cells in bovine embryo culture [82]. It was the first to show the beneficial effect of oviductal co-culture (or oviductal conditioned media) on bovine embryo development and it was the first to demonstrate a fully *In vitro* system of embryo production which resulted in the birth of 5 normal, healthy calves. With these promising results, somatic cell co-culture was soon adopted by many groups studying the embryonic development of a wide variety of species. The benefits seen included faster cleavage, higher blastocyst rates, increased cell numbers, better hatching rates, increased resultant pregnancies and most importantly, increased live births [83].

Serum Added to Culture Media: Many Researchers across parts of the world used bovine serum to provide an ample source of protein, growth factors and other hormones. One study compared embryos cultured in SOF with those cultured in SOF+10% Foetal Bovine Serum (FBS) showed that the development to blastocyst was faster and approximately doubled by the addition of serum 16 vs. 30% oocytes developed to blastocyst [84]. This effect was maintained even when the SOF was supplemented with glucose 0.67 mM 15% of oocytes developing to blastocysts or the FBS was de-salted (components less than 5 kDa removed) 29% of oocytes developing to blastocysts suggesting both that the increased development was due to components of serum which were over 5 kDa in mass and not simply the added glucose. A similar increase in development with SOF supplemented with serum 34.4 vs 47.5% of cleaved embryos to blastocyst [85].

Use of Modern Media: Modern embryo culture is trending toward cell-free, serum-free conditions. This results in more defined protocols which are potentially easier to replicate in different laboratories around the world. However, finding the right constituents of media which successfully support embryos through the 8-cell block and help them to form healthy blastocysts has proven a considerable task. Media additives for group culture which have shown some promise include defined amino acids, transforming growth factor and basic fibroblast growth factor and polyvinyl alcohol, insulin, transferrin, selenium, Myo-inositol and epidermal growth factor [86, 87].

Assessment of *In vitro* Produced Embryo Quality: There are several ways to objectively measure the health and quality of *In vitro* produced embryos. The most obvious measure is to transfer them to synchronized recipients and assess pregnancy and calving rate compared to in vivo produced embryos transferred to another group. Indeed, although this might serve as the "Gold standard" test of embryo health, it is often not feasible or practical due to using oocytes of unknown genetic quality or financial constraints of research. There is a validated method of evaluating in vivo produced embryos which were developed by members of the International Embryo Transfer Society. These guidelines concern the percentage of cellular material which forms the embryonic cell mass as well as the stage of development compared to the expected stage. They serve to inform decisions about transferring, freezing and discarding in vivo produced embryos [88].

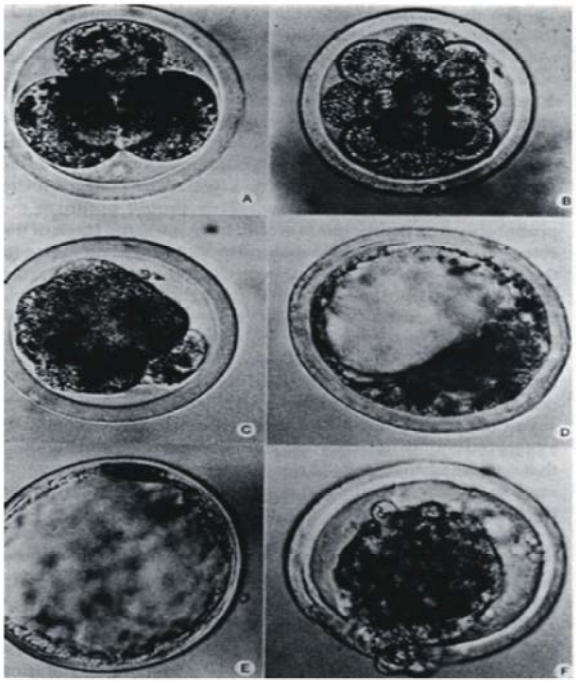

Fig. 3: Cattle embryos at various stages of development
A. 4-cell egg, day 3.
B. 16-cell egg, day 5.
C. Morula, day 6. Cells have compacted and lost individual outlines.
D. Early blastocyst, day 7.
E. Blastocyst fully expanded within the zona pellucida, day 10.
F. Hatching blastocyst, day 10.

Important Considerations for IVF: The cleanliness of the glassware is of utmost importance. Used glassware is rinsed thoroughly with tap water and soaked in biological detergent overnight. The following day, glassware is rinsed thoroughly at least 20-40 times in tap water and then submerged in a 3-5% HC1 for a minimum of 2 hours. It is then rinsed in running water for about 15 minutes and rinsed again 20 - 40 times in tap water. Thereafter, glassware is rinsed with distilled water and placed upside-down to dry by heating it at 120°C for 2 - 4 hours. Glassware is then packed separately in aluminium foil and sterilized by heating at 120°C for 4 hours.

All ultrasonic cleaner is also used for heavily soiled and difficult to wash glassware such as volumetric flash, test tube, Pasteur and volumetric pipettes. Cleaning by sonication takes around 15 minutes in hot water. After sonication, glassware is rinsed 20 - 40 times with tap water and finally rinsed with distilled water. Thereafter, the drying and sterilization protocol described above is practiced.

CONCLUSION AND RECOMMENDATION

The development of techniques for the effective production of bovine preimplantation embryos from oocytes matured and fertilized *In vitro* is important for embryo transfer. However, *In vitro* embryo development is strongly influenced by events occurring during the three subsequent steps of the techniques: oocyte maturation, fertilization and development of fertilized oocytes. Therefore, based on the above conclusion the following recommendations are forwarded:

- Factors affecting oocyte maturation should be considered before starting maturation of with recommended quality of oocytes.
- Appropriate semen preparation methods should be used for *In vitro* fertilization.
- The optimal condition required for embryo culturing should be considered to minimize loss of fertilized zygote like that of eight-cell blocks.
- The types of media and its composition or ingredients used for each step should be considered with great attention for the amounts of ingredients.

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