CHAPTER 24

BOVINE IN VITRO EMBRYO PRODUCTION (IVEP)

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INTRODUCTION

Efforts to increase animal based foods have been going on for centuries. *In vitro* fertilization (IVF) is known as the general definition of obtaining an embryo *in vitro* condition. For this purpose, breeding studies are carried out and biotechnological techniques are frequently used in these studies. Embryo production with *in vitro* fertilization in bovine is one of the biotechnological methods (Alkan 2021; Satılmıs and Guler 2021). The first live offspring from embryo transfer was obtained from an Angora rabbit by Walter Heape in 1890 (Gordon 2004). However, success with the IVF technique in bovine and other mammal animals could not be accomplished for a long time. The first successful application of IVF for cattle was reported in 1977. Iritani and Niwa (1977) proved that calves can be produced with the IVF technique as a result of their long term studies. The first live calf was obtained by the IVF method by Brackett et al. (1982). Then, the first twin calf was obtained by culturing zygote in the mouse oviduct by Hanada et al. (1986). In Ireland, the first calf was obtained by performing all of the steps in the laboratory (*in vitro* maturation, *in vitro* fertilization, and *in vitro* culture) in 1987. In 1980s, IVF took place only as a research technology among embryo transfer technologies. *In vitro* fertilization studies in bovine have gradually increased. *In vitro* fertilization studies were supported by OPU in the 2000s, and recently *in vitro* fertilization studies are being used for genetic selection (Hasler et al. 2017).

Embryo transfer studies at the cellular and molecular level in bovine are not common. Because the applicability of embryo transfer studies is limited and difficult. However, it is known that the required experimental material can be obtained at a low cost by *in vitro* fertilization method. Superovulation in embryo transfer studies is very expensive and causes excessive manipulation on animals. (Erdem et al. 2020; Alkan 2021). This situation provides an occasion for *in vitro* fertilization studies both financially and ethically. The demand to maintain the genetic existence of high-yielding animals that have been slaughtered in the last 10-15 years has contributed to the increase in IVF studies (Galli et al. 2003; Gordon 2004).

The State of Bovine *In Vitro* Fertilization Today

According to the data collected from International Embryo Technology Society (IETS), it has been reported that *in vitro* fertilization studies have increased gradually in the last 15 years, especially in the last 4 years. According to IETS 2019, data of *in vitro* production of embryos from abattoir-derived oocytes by region are given in Table 1 and transfer of *in vitro* produced bovine embryos by region are given in Table 2 (Viana 2020).

In Vitro Embryo Production in Bovines

Embryo production by *in vitro* fertilization is an assisted reproductive technique, all stages of which are performed in the laboratory (Satılmıs 2019). Stages of embryo production by *in vitro* fertilization are listed below.

- Obtaining oocytes from the ovary;
  - From abattoir-derived ovary: Obtaining oocytes from abattoir-derived ovaries with Aspiration, slicing, and dissection technique.
  - By ovum pick up (OPU) technique: Obtaining oocytes from OPU from live animals.
- Maturation of oocytes,
- Fertilization,
- Embryo culture (Gordon 2003; Saleh 2017; Ferré et al. 2020).

Obtaining Oocytes from Ovary

Immature oocytes from ovaries is collected from abattoir derived ovary or from live animals by the OPU technique (Karaşahin 2021). To collect oocyte from abattoir derived ovaries; aspiration technique, slicing technique and dissection technique are used. However, aspiration technique is the the most commonly used method for this purpose (Gordon 2003).

Aspiration Technique

One of the most commonly used techniques in bovine is follicle aspiration. Antral follicles on the ovary can be aspirated with the help of pipette, vacuum aspiration needle or injector. The aspiration technique is advantageous as it is faster than other methods. Fast oocyte collection is a very important criterion for embryo production units. With this method, antral follicles of 2-8 mm in size can be aspirated and 10-20 mL injectors with 18–22-gauge needle without gasket are recommended for aspiration (Gordon 2003).

Slicing Technique

Slicing is another method of obtaining immature oocytes from abattoir derived ovaries. During slicing, the ovary is held with the help of forceps and the follicles are cut with the help of a scalpel. This process is carried out in an already prepared 90 mm petri dish containing the washing solution. The oocytes collected from the follicles are poured into the washing

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Table 1: *In vitro* production of embryos with abattoir-derived oocytes by region (Vianna 2020).

Region/	Donors			Oocytes			Transferrable embryos		
Country	Dairy	Beef	Total	Dairy	Beef	Total	Dairy	Beef	Total
Africa	0	143	143	0	2,343	2,343	0	373	373
Asia	0	0	0	0	0	0	0	0	0
Europe	662	204	866	7,499	2,017	9,516	1,449	649	2,098
N America	0	60	60	14,316	5,650	19,966	3,013	1,085	4,098
Oceania	0	0	0	0	0	0	0	0	0
S America	0	2,860	2,860	0	71,590	71,590	0	14,318	14,318
Total	662	3,267	3,929	21,815	81,600	103,415	4,462	16,425	20,887

Table 2: Transfer of *in vitro* produced (IVP) bovine embryos by region (Vianna 2020).

Region/	Embryos transferred							
Country	OPU				Abattoir			
	Fresh	Frozen		Total	Fresh	Frozen		Total
		Domestic	Foreign			Domestic	Foreign	
Africa	1,257	1,976	0	3,233	0	0	0	0
Asia	0	0	0	0	0	0	0	0
Europe	24,554	22,103	322	46,979	2	29	0	31
N America	183,229	150,412	53	333,694	84	1,980	0	2,064
Oceania	7,134	2,897	0	10,031	0	0	0	0
S America	222,302	169,900	0	392,202	8,751	205	0	8,956
Total	438,476	347,288	375	786,139	8,837	2,214	0	11,051

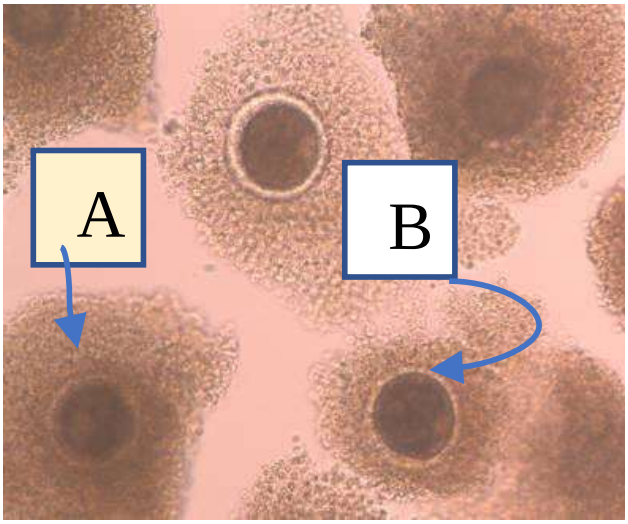

Figure 1. A and B quality oocytes (Satılmıs 2019)

solution and examined under a microscope (Saleh 2017). The slicing technique is faster than aspiration and more oocytes are collected, but the collected oocytes are of poorer quality (Wang et al. 2007). Sometimes, slicing method is used in combination with the aspiration technique. In this method the follicles on the ovary are aspirated first and then sliced. It is reported that the number of collected oocytes by this method increases significantly (Hoque et al. 2011).

Dissection Technique

In the dissection method, the follicles on the ovary are dissected and separated. The dissected follicles are poured into previously prepared petri dishes that contain collection solutions. In this way, the cumulus cells of the collected oocytes are minimally damaged. It is reported that the number and quality of collected oocytes by the dissection method is quite high. With the dissection technique, an average of 32-44 oocytes per ovary can be collected. The most important advantage of this technique is that atretic and non-atretic follicles can be more easily identified. However, this method requires a lot of experience (Carolan et al. 1994; Gordon 2004).

Ovum Pick Up (OPU) Technique

In the ovum pick-up method, oocyte is collected by aspirating the follicles on the ovaries of live animals. OPU is a systematic non-invasive technique that can be performed with help of an ultrasound probe, aspiration pump, and aspiration needle. The use of OPU in humans has also predicted its usability in bovine. It has been determined that OPU is a flexible and repeatable method after bovine applications (Pieterse et al. 1991). Unlike OPU technique is advantageous over Multiple Ovulation Embryo Transfer (MOET) because donors can be used more frequently (Faber et al. 2003; Pontes et al. 2010). OPU is a conventional and practical technique because it can be used in the postpartum $2^{nd}/3^{rd}$ week, pregnancy, and calves from 6 months of age (Galli et al. 2001; Besenfelder et al. 2012; Qi et al. 2013). In addition, the OPU technique has proven that the oocyte is not only collected from abattoir derived ovary. The OPU technique has also increased the success of *in vitro* fertilization studies and the number of collected embryos (Galli et al. 2004).

Quality Evaluation of Oocytes

The selection of bovine oocytes for the *in vitro* maturation process is based on some basic morphological evaluations (Satılmıs and Guler 2021). Classification schemes of oocytes were made with the help of a microscope. Cytoplasm density of oocytes, the density of cumulus cells and some visible morphological features are included in this scheme. Bovine oocytes are classified according to some morphological features from 1 to 4. Grade 1 oocytes have compact and multi layered cumulus, homogeneous ooplasm, cumulus-oocyte complex (COC) clear and transparent. In Grade 2 oocytes, the cumulus structure is multi layered, the ooplasm is homogeneous, there is darkening of the zona, the POPs are darker and less transparent. Grade 3 oocytes are those in which the cumulus structure is less layered, the ooplasm is irregular and has dark clusters, rather dark compared to COC

grades 1 and 2. In Grade 4 oocytes, the cumulus cells are enlarged and scattered, ooplasm is irregular, COC is dark and irregular (Gordon 2004). In addition, immature oocytes can be classified as A, B, C, and D according to the distribution of cumulus cells (Cetica et al. 1999) where cumulus cells appear as 5 or more layers around the zona are categorised as A Quality, cumulus cells cover more than 1/3 of the circumference of the zona in 2 or fewer layers or a small part of them may not be cumulus are categorised as B Quality, oocytes are usually completely bare or have much fewer cumulus cells than B grade oocytes are categorised as C Quality and the cumulus cells look like a spider or swollen are categorised as D Quality.

Some researchers reported that most of the grade A oocytes (87.7%) are in the germinal vesicle (GV) stage. In addition, it was determined that the meiotic maturation of grade A oocyte was higher (76.5%) compared to other grades (Cetica et al. 1999). For this reason, A and B quality oocytes are matured in IVF studies. It is known that C and D quality oocytes are not used for maturation. The maturation capability of oocytes is affected by factors such as oocyte size, presence of cumulus cells and characteristics of the animal. Another factor affecting the maturation quality of oocytes is the size of the aspirated follicle. Therefore, aspiration of follicles with a size of 2-8 mm on the ovary is recommended (Gordon 2003). It has been reported that low-quality embryos are collected from cultured oocytes as a result of aspiration of follicles smaller than 2 mm or larger than 8 mm (Katska and Simorak 1984; Carolan et al. 1994).

In Vitro Maturation (IVM)

The oocyte is a special cell of the reproductive system as it is a pool where the genetic information of two organisms is combined. Oocytes undergo a series of molecular and conformational changes for development. Oocytes are formed as a result of mitosis of embryonic germ cells in the ovarian cortex during the embryonic process. Mitotic proliferation is completed by 7.5 months of fetal life. This event indicates that germ cell mitosis is completed before birth. Afterward, mitotic activity ceases. After this stage, germ cells enter the prophase of meiosis and transform into oogonia. During subsequent growth, oogonia develop into mature oocytes (Alexander 2012).

Oocytes mature both cytoplasmically and cellularly. For germ cell formation, the diploid somatic cell transforms into a haploid cell. During fertilization, as a result of the fusion of the sperm to the oocyte, two haploid cells combine to form a mixed-genome diploid cell (zygote) obtained from both parent cells. During the meiotic split in the transformation of somatic cells into germ cells, a decrease in the number of chromosomes is observed. In this process, changes occur in the cytoplasm depending on the accumulation of substances. Due to the unequal divisions seen in females, they have more cytoplasm than males (Alexander 2012).

It has been reported that the rate of oocyte maturation *in vitro* is lower than *in vivo*. The reason for the slow maturation of the oocyte in *in vitro* conditions is that the follicular fluid content is not known exactly. In addition, maturation success is greatly affected by the quality of the used oocyte. For this purpose, it has been reported that metabolic analysis of follicular fluid and characterization of oocyte quality may be beneficial to better perform oocyte maturation *in vitro* (Wrenzycki 2018).

Nuclear Maturation of the Oocyte

Nuclear maturation takes place during meiosis of the oocyte and includes the process from the germinal vesicle stage to metaphase-II. This phase takes place similarly to the mitotic division of somatic cells and consists of some phases. These include synthesis phase (S), Mitotic or meiotic phase and Developmental or GAP phase (G). DNA replication takes place in synthesis phase (S). The maternal cells are required for the synthesis of molecules, and genetic distribution takes place between the two female cells. In mitotic or meiotic phase (M) chromosomes and cytoplasm divides into two and disperse into female cells. Developmental or GAP phase (G) is formed between M and S phases. The difference of this process from mitosis is that it involves two successive S phases and as a result, diploid cells are formed. As a result of the miotic cell cycle, cells become diploid and then meiosis takes place. Meiosis consists of two parts, meiosis-I and meiosis-II, and these parts are separated from each other by a gap called interkinesis. The stages of meiosis are named as those of mitosis. Names of stages are prophase, metaphase, anaphase, and telophase. Diplotene is the first division phase of meiosis, the chromatins are scattered and despiralized. Germinal vesicle is available. Diakinesis is the end of the prophase of meiosis-I. Chromosomes were divided into four uniform copies and fragmentation of the germinal vesicle (GVBD) occurs. The GVBD event occurs as the nuclear membrane begins to fold during diakinesis, the nuclear pores disappear, and then the nuclear membrane fragments are separated from the small double-walled sacs and disappear rapidly. In bovine, GVBD occurs after follicular development or ovulatory LH release. At the metaphase-I stage, chromosomes are localized with maximum density at the equator. As a result of GVBD, the cumulus cells and their connections around the oocyte are destroyed. In anaphase-I, condensed chromosomes are pulled to the poles. In the telophase-I stage, a group of chromosomes degenerates to form polar body-I between the oocyte and the perivitelline space. In metaphase-II, the second meiosis begins and the chromosomes travel and reach at the equator of the oocyte. And finally in anaphase-II/telophase-II, spermatozoa enter the oocyte cytoplasm (Monniaux et al. 2009). In the first meiosis, the chromosomes match and exchange by recombination. The number of cells is doubled, but the number of chromosomes does not decrease to half of the number of chromosomes per cell. In meiosis-II, mitosis-like division takes place to reduce the number of cells (Monniaux et al. 2009; Tripathi et al. 2010; Brunet and Verlhac 2011; Alexander 2012). As a result of the meiosis of the spermatozoon, four haploid cells are produced from the diploid cell at the beginning, while only one oocyte is obtained from a diploid cell in the meiosis of the oocyte. It is reported that in each meiosis, one of the cell matches completes its development in the body of the other cell in the form of a primary polar body. The meiotic distribution of cytoplasm among female germ cells is not equal. After completing its maturation, the cell receives most of the cytoplasm to form the polar body. Degenerated chromosomes of the primary polar body are dysfunctional and secretly expelled from the surface. It is known that during and after all these events, the chromosomes are despiralized in the pronucleus. The haploid female germ cell formed during fertilization infuses with the male haploid germ cell to form a diploid cell and forms the zygote, which is a diploid organism (van den Hurk and Zhao 2005; Kimura et al. 2007).

Cytoplasmic Maturation of the Oocyte

Cytoplasmic maturation refers to oocyte growth, changes in the structure and distribution of oocyte organelles and the accumulation of biologically active substances necessary for further development of the oocyte. Complex changes occur in the position and structure of all organelles, especially mitochondria, until metaphase-II. In the cytoskeletal stage, the cytoplasm containing microfilaments and microtubules greatly assists nuclear maturation during the separation of chromosomes. Ribosomes in the cytoplasm produce peptides necessary for oocyte maturation and subsequent steps. Thus, cytoplasmic maturation involves the storage of ATP, mRNAs, proteins, and transcription factors (Brevini et al. 2007; Miyano and Manabe 2007; Ferreira et al. 2009). After the resumption of nuclear maturation, protein synthesis is rapidly activated and a significant part of the mRNA is degraded and dies (Aerts and Bols 2008). Storage of metabolic substances in the cytoplasm induces oocyte growth. Mammalian oocytes grow in two different characteristic structures. In the first phase, growth is temporarily associated with the follicle from which it develops while in the second phase, the oocyte maintains its current size, although the follicle continues to grow until ovulation. Organelles stored in oocytes during the first growth phase are responsible for the progression of meiosis. In addition, these macromolecules are responsible for condensation of penetrating spermatozoa, formation of a male pronucleus, shaping of fertilization, formation of zygote, and reaching the 6-8 cell stage (van den Hurk and Zhao 2005). The growth and development of the oocyte in the follicle also depends on the somatic cells around the cumulus and granulosa layers. Connection is established between these cells and the oocyte by gap junctions. Energy and substrates (nucleosides, amino acids, phospholipids, and ions) required for oocyte development are transported through this relationship (Feng et al. 2007; Mermillod et al. 2008; Sirard 2016). Defects in somatic cells or junctional errors between gap junctions prevent oocytes from reaching optimal size, maturation, and fertilization (Hutt and Albertini 2007). Synchronization of cytoplasmic and nuclear maturation is very important for the development of oocytes and also in *in vitro* embryo production (Eppig et al. 2004).

In Vitro Maturation Environment and Affecting Factors

As a result of successful *in vitro* maturation, the oocyte should be able to fertilize and provide the development of the embryo. However, the maturation success of the oocyte is affected by some factors such as the diameter of the follicle and the oocyte, the estrous cycle, the medium used for the maturation of the oocyte and its content, maturation time, temperature, humidity and environmental gas components, osmotic value of maturation media, the technique of collection of oocyte and the characteristics of the animal. Bovine oocytes are generally incubated for 20-24 hours at 38.5°C in an environment containing 5% CO_2 for *in vitro* maturation. The appearance of cumulus expansion or any of the primary polar body formations after incubation is considered maturation (Alexander 2012). For *in vitro* maturation, there are many different preferred media such as Tissue Culture Medium (TCM-199), Synthetic Oviductal Fluid (SOF), North Carolina State University (NCSU-37), and Ham's F10a. Hormones, bovine serum albumin, follicular fluid, growth factors, vitamins, serums and different antioxidant substances can be added to these mediums. Supplement to the maturation medium is intended to improve the medium (Grøndahl 2008; Strejček and Petrovičová 2012).

In Vitro Fertilization (IVF)

In vitro fertilization consists of a complex mechanism involving oocyte maturation, semen separation and capacitation. IVF is the process of bringing the capacitated sperm and mature oocyte together in the laboratory and fusing the nuclei of these two cells. One of the most important steps in IVF technique is the selection of semen to be used. The selected sperm must go through a maturation process to fertilize the oocyte. Spermatozoon shows capacitation, hyperactivation and acrosome reaction during maturation. In *in vivo* conditions, hyperactivation and capacitation of spermatozoa take place in the female genital tract before encountering the oocyte. The acrosome reaction begins during the penetration of the oocyte into the cumulus cells and zona pellucida. Some biochemical changes in spermatozoa inside the female genital tract are called capacitation. In *in vitro* fertilization studies, capacitation is provided with ingredients that mimicking the female genital tract environment. Hyperactivation is a necessary step for spermatozoa to enter the zona pellucida (ZP) and cumulus cells. At this stage, Ca^{+}, ATP and cAMP are used. Acrosomal Reaction (AR) involves the distribution between the acrosome membrane and the surrounding plasma membrane so that hydrolytic acrosomal enzymes can be released (Gordon 2003). IVF is considered successful if one of the criteria listed below is identified like presence of the tail of the sperm in the ooplasm or the head of the spermatozoon in the ooplasm, the presence of male and female pronuclei in the ooplasm, the detection of first and second polar bodies, the presence of sperm in the zona pellucida or perivitelline space, and the detection of the telophase stage of the second meiosis (Donnay et al. 2002).

In Vitro Fertilization Media and Used Medium

It is reported that the optimal condition for fertilization is 38.5-39°C, environments containing 5% CO_2 and maximum humidity (Hammam et al. 2010). The most commonly used media for IVF are Tirode's Albumin Lactate Pyruvate Solution (TALP), Synthetic Oviduct Fluid (SOF), Brackett and Oliphant Medium (BO) and Potassium Simplex Optimization Medium (KSOM) (Nedambale et al. 2006). Also, SOF (synthetic oviductal fluid) is used instead of TALP medium. In addition, substances such as heparin, caffeine, epinephrine, penicillamine, taurine, hypotaurine, BSA, glycine and hyaluronic acid can be added to assist fertilization media (Gordon 2003).

Preparation of Semen for *In Vitro* Fertilization

In IVF, "Separation" is applied to the sperm to increase the fertilization ability. With this process, the ones with the highest motility are selected among the available spermatozoa (Ohnami et al. 2012; Wrenzycki 2018). Some techniques have been developed to remove substances (bacteria, enzymes, abnormal spermatozoa, chemicals used during freezing) that adversely affect IVF success in straws. These are migration-sedimentation, a density gradient centrifugation and filtration technique (Kaymaz 2012). Swim-up and percoll gradient

techniques are mostly used for the separation of sperms. Percoll gradient is among the centrifugal technique. It has been reported that the acrosome integrity of spermatozoa collected by the separation method with the percoll gradient technique is higher than the swim-up technique (Somfai et al. 2002). Important tips during the application of the separation technique should be kept in mind. It should be fast and easy, obtain a large number of motile spermatozoa, be economical, separate toxic substances, leukocytes and bacteria, allow to work in high volume semen, and most importantly, it should not cause damage to spermatozoa (Kaymaz 2012).

In Vitro Culture (IVC)

It is the stage of washing the zygote with a culture medium after *in vitro* fertilization and culturing it for 6-9 days until it completes the developmental period. It is very important for embryo development that the zygote is cleared of denatured cells, spermatozoa residues and cells that may contaminate the culture medium before being placed in the culture medium. For embryo culture, simple media were used in the first days of culture. Following considerations regarding culture media should be kept in mind to enhance success of In vitro culture: 1. Preparation of the medium considering the needs of the embryo at the developmental stage, 2. Additions to the culture medium should be made as early as possible in the early developmental period of the embryo, 3. Adding components such as growth factors to the culture to obtain a high rate of the blastocyst, 4. If BSA is to be added to the culture medium, support should be obtained from non-acidic oil preparations, 5. Examination of multi-step culture systems for embryos, 6. Frequent use of embryo culture medium to control the standard (Gordon 2003).

Embryo Culture System Medium

It is desired that the oxygen in the *in vitro* culture medium should be below 10%. The use of 5% O_2, 5% CO_2 and 90% N_2 gases is generally preferred in culture systems. CO_2 stabilization is an important factor in culture systems. It is desired that the pH of the environment should be between 7.2-7.6. It is reported that the ideal temperature in the culture environment is between 38-39 °C, and the most ideal light is filtered yellow light (Garcia et al. 2006).

At the start, Bovine Oviduct Epithelial Cells (BOEC) were used as embryo culture medium, and then mediums such as Tissue Culture Medium (TCM-199), Synthetic Oviduct Fluid (SOF), Hamster Embryo Culture Medium-6 (HECM-6), Chatot Ziomek Bavister Medium (CZB), CR1aa (Charles Rosenkrans), KSOM and Complete Defined Medium (CDM) were introduced. Complex embryo culture media such as TCM-199 are designed for *in vitro* development of somatic cells rather than early embryonic development of mammals. Bovine embryos and somatic cells develop a large number of embryotrophins in the serum, which play different roles in embryo development (Gordon 2003; Feugang et al. 2009). Today, SOFs have become the most widely used mediums as embryo culture medium. SOFs were designed as a result of biochemical analyses of oviduct fluid (Gordon 2003). In recent years, successive culture medium systems have been developed for embryo development. In this system, the embryo culture medium mimics the physiological changes of the oviduct environment *in vivo*. It is reported that the application of these culture media systems is more complicated, but the results obtained are more successful (Wrenzycki 2018). Donnay et al. (2002) reported that excess glucose can have a toxic effect in the early embryonal period, but more glucose is needed after the morula stage. This indicates that it may be advantageous to use a sequential culture medium system.

In the use of the sequential embryo culture medium system, the development of embryos is decelerated as a result of the freezing-thawing process (Garcia et al. 2006). To prepare a successful embryo culture medium, oviduct biochemistry should be analyzed and simulated well and optimum conditions should be provided for some parameters. It has been reported that the oviductal fluid has an osmolarity of approximately 245-290 mOsm / kg and a pH of 7.2-7.6. At the same time, concentrations of Na^+, $Ca^{2+,}$ Mg^{2+}, and Cl^- are low in the oviductal fluid, while potassium (K) and bicarbonate ($HCO3^-$) levels are high. In the *in vitro* environment, lactate, pyruvate and glutamine are preferred as energy sources in the early embryonal development period, while glucose is preferred after the morula stage. Amino acids and macromolecules are used as protein sources in culture systems (Gilchrist et al. 2015). To increase the success in embryo culture medium systems, somatic cells have been used. This situation forms the basis of co-culture systems. Mostly granulosa cells, oviduct epithelial cells, rat liver cells and uterine cells are used for co-culture systems. Co-culture systems have a positive effect on embryo development by eliminating certain toxic components in the environment and reducing the O_2 concentration (Lonergan et al. 2001; Lonergan and Fair 2016).

Evaluation of Embryo Quality

Interest in studies on morphology, ultrastructure, cryo resistance and gene structure of embryo have been increased recently. There are many factors that affect embryo quality such as the color of the blastocyst, its compactness, and the size of the hatched blastocyte. It is not possible to detect abnormal chromosome distribution or chromosomal disorders in bovine embryos with current morphological methods. However, genetic analysis can be performed by ultracentrifugation or multiphoton laser microscopy while in the zygote stage. Another option is the biopsy method. During the pre-implantation period, the viability of the embryo is mostly affected by a normal morphology, rapid division rate and early pregnancy factors (EPF, cytokines and luteotrophic factors) released from the embryo. These factors have a direct effect on the transport and activity of the ovum (Makarevich 2012). To evaluate the quality of embryos in bovine, examinations from different perspectives are made under the microscope with 50X or 100X magnifications. The size of the bovine embryo varies between 150 and 190 µm. Structural features of an ideal embryo are the compact and spherical zona pellucida, the distinguished perivitelline cavity, any granules and vesicles containing cytoplasm, distribution of the color, and the uniform zona pellucida (Bo and Mapletoft 2013). Following points should be considered while evaluating the morphology of embryos:

1. Presence or absence of cellular fragments is easy to assess during early embryonic development. Evaluation becomes more difficult in later stages. The cells are based on the zona pellucida and the perivitelline gap cannot be seen. For these reasons, evaluation becomes very difficult.

2. The degree of compaction; Generally, the roundness and sharpness of the outer periphery of the morula are evaluated.
3. Color and texture of the blastomere varies from light to dark. Many factors affect this change. Embryos collected from *in vitro* fertilization are darker in color than embryos collected *in vivo*.
4. Irregular division occurs due to time-synchronous divisions (Makarevich 2012).

Different developmental stages of embryos are:

1-cell: unfertilized oocyte (UFO)

2-12 cells: contains 2-12 cells

Early morula: These contain 16-32 cells, the large non-compact cluster of blastomeres,

Morula: These contain 32-64 cells, compact cluster of small blastomeres

Early blastocyst: blastoceles < 50%

Blastocyst: blastoceles > 50%, expanded blastocyst

Embryo increases in size and the zona pellucida wall becomes thinner and in result, zona pellucida rupture. The blastocyst is then hatched, the embryo is discarded and expanded hatched blastocyst is completely discarded and increase in size resulting in empty zona pellucida (Makarevich 2012).

Embryo evaluation is one of the most critical stages of the embryo transfer procedure. The quality and developmental stages of the embryos are evaluated with a standard scale determined by IETS. Embryos are categorised into 9 according to their degree of development and into 4 according to their quality (Erdem et al. 2020; Dursun and Karasahin 2021). Quality-I embryos are excellent or good consisting of more than 85% living cells. The degree of development of embryos is compatible with their development period. Cell distributions are diffuse and zona pellucida is present. Irregularities within the cell are minimal. A high rate of pregnancy is obtained from perfect-quality embryos. Embryos at this stage are suitable for freezing. Quality-II embryos are evaluated as a medium, consisting of 50% living cells. The color is slightly lighter and there is a partially irregular cell mass. They are not suitable for freezing as they lose their viability during freezing and thawing process. For this reason, 2nd quality embryos are used for fresh transfer if suitable recipients are found. Quality-III embryos are weak or of poor quality consisting of 25% living cells and have a more irregular structure. These are not suitable for freezing, and the chances of survival in fresh transfers are very low. Quality-IV embryos are known as degenerated oocyte or 1-cell zygotes (Bo and Mapletoft 2013).

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