# CHAPTER II

# RITERATURE REVIEWS

## 2.1 *In vitro* embryo production (IVP)

An alternate method for generating bovine embryos utilizes immature oocytes obtained from donor animals of diverse ages and physiological states. Current laboratory techniques enable the effective *in vitro* maturation and fertilization of these oocytes, followed by a culture period of roughly seven days to achieve the developmental stage appropriate for embryo transfer or cryopreservation. Originally conceived as a research technique, IVP was originally employed to recover follicular oocytes from ovaries obtained from slaughterhouses. Nonetheless, its utilization has since broadened considerably. In bovine, IVP is now frequently utilized to produce embryos from live donors, serving as either an adjunct to or replacement for multiple ovulation and embryo transfer (MOET), due to its adaptability and other practical benefits. The IVP method comprises three distinct and extensively utilized biological stages, including IVM of oocytes, IVF, and subsequent IVC of embryos (Galli et al., 2003).

### 2.1.1 *In vitro* maturation (IVM)

Assisted reproductive methods, especially IVF and somatic cell nuclear transfer (SCNT), depend on maturing oocytes from bovines in a lab. Using IVM, immature oocytes taken from the ovaries of an animal may develop in a lab setting under controlled conditions. In the 1960s, early studies showed that it was possible to mature bovine oocytes *in vitro*. But it was not until the 1980s that reliable protocols were set up that supported both nuclear and cytoplasmic maturation, which allowed for successful fertilization and embryo development (Lonergan et al., 1994; Zhang et al., 1992). Establishing successful IVM systems was a big step forward in bovine breeding because it made possible to collect oocytes from ovaries taken from animals that were slaughtered or from live donors using ovum pick-up (OPU). This made a lot more oocytes available to produce embryos. IVM is based on starting meiosis again after it

has been stopped at the germinal vesicle (GV) stage in the mature oocytes inside follicles. *In vivo*, a rise in the luteinizing hormone (LH) before ovulation starts meiotic resumption. This causes the germinal vesicle to break down (GVBD), the shift from metaphase I to metaphase II, and the oocyte to become fertile (Mehlmann, 2005). These hormonal signals can be simulated in the laboratory by adding gonadotropins like follicle-stimulating hormone (FSH) and LH to the maturation medium along with estradiol. This helps the oocytes go through a similar nuclear maturation process (Lonergan et al., 1994). But it is still hard to get the cytoplasm to mature properly, which includes moving organelles around, conserving maternal mRNAs, and rearranging cortical granules. This is because oocytes that have been matured in the laboratory often don't develop as well as their *in vivo* counterparts (Watson, 2007; Palhares et al., 2022). A big part of the success of bovine IVM is the way of culture media is put together. A lot of people have used traditional media like TCM-199 with added FBS, FSH, LH, and estradiol to help oocytes mature (Ali & Sirard, 2002). Recently, people have been using chemically defined media to make the *in vitro* environment more stable and reduce batch variability (Ali & Sirard, 2002). Adding growth factors like epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) has also been shown to speed up the maturation of oocytes and improve their ability to develop by turning on signaling pathways that act like the microenvironments inside follicles (Lorenzo et al., 1995). Oxidative stress during IVM is another thing that can hurt the health of an oocyte. ROS and high oxygen stress can harm DNA, proteins, and lipids, which can affect embryo development less than ideal (Feuchard et al., 2025). Some ways to reduce oxidative stress are to bring the oxygen level in incubators down to a healthy level (5% $O_2$) and add antioxidants like cysteamine, melatonin, and vitamin E to the media (Tamura et al., 2013). Researchers have found that these actions lead to better mitochondrial function, fewer deaths, and more blastocysts after IVF. Getting nuclear and cytoplasmic maturation to happen at the same time is another area of active study. Many oocytes make it to the MII stage, but their cytoplasmic maturation may be slower than expected, which can hurt their growth. To keep oocytes temporarily at the GV stage (called "capacitation of oocytes"), scientists have investigated delaying nuclear maturation with certain inhibitors like recovering or

colchicine. This lets cytoplasmic processes catch up, which improves the ability of the embryo to develop (Lorenzo et al., 1995). Even though they are better, embryos derived from IVM often have different gene expression patterns than embryos derived from oocytes that have matured *in vivo*. Studies that use transcriptomic studies have found that embryos made from *in vitro*-matured oocytes have problems with key genes that control metabolism, stress response, and epigenetic regulation (Watson, 2007). These differences at the molecular level probably play a part in why embryos made after IVM have lower cryotolerance and implantation rates. This shows how important it is to keep improving the culture conditions. In conclusion, the ability to grow bovine oocytes in the laboratory has opened a lot of pathways for investigation and commercial applications in reproductive biotechnology. The basic steps for nuclear maturation are well known, but work is still being done to improve cytoplasmic maturation, lower oxidative damage, and make the environment more like the follicular environment *in vivo*. Molecular biology, especially omics technologies, is making progress in finding key processes that can be targeted to improve the quality of oocytes. In the end, making IVM systems that are more biologically relevant and reliable will be necessary to get the most out of bovine IVF and other related technologies.

### 2.1.2 *In vitro* fertilization (IVF)

IVF in bovines has changed the field of reproductive biotechnology by conserving valuable genetics and enhancing reproductive efficiency. In the early 1980s, Brackett et al. recorded the first successful IVF with bovine, leading to a live calf. Technology has come a long way since then and is now an important part of both research and commercial production systems. IVF includes getting oocytes from donor cows, letting them mature, fertilizing them with sperm in a laboratory, and then developing the embryos until they are ready to be transferred. Traditional MOET cannot be equally successful as IVF because it cannot collect embryos as often and can use oocytes from both young cows and older cows (Mapletoft & Hasler, 2005). The number and quality of oocytes available are very important for the success of IVF. OPU from live donors or collected from slaughterhouse ovaries. After collecting, immature oocytes must undergo maturation *in vitro* to reach the metaphase II stage, which

means they can be fertilized. For IVF to work, the sperm must also be properly prepared. In bovine IVF, sperm are often put through a process called "capacitation," which is usually caused by heparin. This helps the acrosome react and the oocytes penetrate (Parrish et al., 1988). The use of Percoll or discontinuous gradient centrifugation methods improved sperm selection by collecting spermatozoa that were highly motile and had normal morphology (Morrell & Rodriguez-Martinez, 2010). Higher rates of fertilization and cleavage have been achieved by improving the concentrations of insemination, the times that sperm and oocytes are co-incubated, and the activation methods. Understanding how the quality of the gametes, the conditions of fertilization, and the surroundings in which the embryos develop all affect each other is still important for improving IVF outcomes and making sure that it is successful for a lot of individuals in both commercial and conservation settings.

### 2.1.3 *In vitro* culture (IVC)

Current bovine reproductive technology relies heavily on IVP, which facilitates genetic enhancement and advanced assisted reproductive techniques (Lonergan et al., 1994). A critical component of IVP is the culture medium, designed to emulate the physiological conditions of the female reproductive tract, thereby supporting oocyte maturation, fertilization, and early embryonic development (Pinyopummintr & Bavister, 1994). Traditionally, undefined systems such as co-culturing with somatic cells or serum-based media have been utilized; however, there has been a shift towards chemically defined and serum-free formulations to enhance repeatability and reduce variability (Van der Valk et al., 2010).

Prominent culture media like synthetic oviduct fluid (SOF), CR1aa, and KSOM have demonstrated efficacy in promoting development to the blastocyst stage by replicating the ionic and nutritional composition of the oviductal environment (Takahashi & First, 1992; Rosenkrans et al., 1993; Biggers et al., 1997). Serum supplementation, particularly with fetal bovine serum, has historically enhanced embryo development by providing hormones, growth factors, and antioxidants that mitigate oxidative stress (Abdel-Wahab et al., 2018). Nonetheless, concerns regarding animal welfare, variability, ambiguous composition, and potential disease transmission have propelled the development of serum-free techniques (Van der Valk et al., 2010).

The addition of amino acids to culture media has been shown to improve embryo quality through mechanisms such as protein synthesis, reduction of ammonia accumulation, and maintenance of metabolic needs (Steeves & Gardner, 1999; Lane & Gardner, 2000). Currently, bicarbonate-buffered systems under 5% $CO_2$ are standard practice, as maintaining appropriate pH and osmolarity is essential for embryonic survival (Lane & Gardner, 2000). Oxidative stress remains a significant challenge in vitro cultivation, as reactive oxygen species can damage cellular components. Supplementation with antioxidants like melatonin and L-carnitine has been found to enhance embryo development and reduce oxidative damage (Tamura et al., 2013; Li et al., 2021). Apoptosis, or programmed cell death, also contributes to early embryo loss; thus, media formulations that reduce excessive apoptosis can improve blastocyst development and post-transfer viability (Brison & Schultz, 1997).

Developing effective bovine embryo culture media necessitates consideration of various biochemical and physiological elements, including nutritional content and redox balance, to optimize embryo development and ensure consistent outcomes in reproductive biotechnology (Pinyopummintr & Bavister, 1994; Van der Valk et al., 2010).

### 2.1.4 Embryo

Researchers have been studying bovine embryos as an important part of developmental biology and reproductive engineering for many years. For artificial reproductive technologies like IVF, somatic cell nuclear transfer (SCNT), and gene editing to get more effectively, scientists must have complete knowledge of the early stages of bovine embryo development. The bovine zygote goes through a number of mitotic divisions after fertilization, including the 2-cell, 4-cell, 8-cell, and morula stages before it forms a blastocyst, which usually happens 7 or 8 days after fertilization (Farin et al., 2001). It is important for the morula stage to successfully compact and for the blastocyst stage to differentiate into the inner cell mass (ICM) and trophectoderm (TE). These are the events that affect viability and implantation potential after development (Thompson et al., 1995). As a bovine embryo changes from an oocyte to a blastocyst, its metabolism undergoes a lot of changes. Embryos get most of their energy from pyruvate and lactate in the early stages of cleavage. After compaction, glucose uptake becomes important (Gardner & Lane, 1993). The "quiet embryo" hypothesis says that

embryos with lower metabolic activity, which means they use nutrients more slowly, are better able to develop (Leese, 2002). These insights into embryonic metabolism have shaped *in vitro* culture media formulations to better mimic the physiological conditions of the female reproductive tract, aiming to support optimal development while avoiding metabolic stress. *In vitro* culture systems for bovine embryos have evolved significantly. In early methods, co-culture with somatic cells was used to provide support and lower oxidative stress (Eyestone & First, 1989). But these kinds of methods led to variation and limited standardization. Creating defined media like SOF and CR1aa and adding amino acids and antioxidants to them made the conditions more controllable and increased the number and quality of blastocysts (Takahashi & First, 1992; Rosenkrans et al., 1993). Reducing the oxygen tension in culture to normal levels (5% $O_2$) instead of atmospheric levels (20% $O_2$) has also been shown to improve embryo development by lowering oxidative damage (Goto et al., 1993). Evaluating embryo quality remains a challenge. Traditionally, morphological assessment has been the main method for grading embryos, but this approach is subjective and limited in predictive power (Lindner & Wright, 1983). As genetic markers have improved, they have become more objective ways to measure quality. It is now known that the amounts of expression of genes related to pluripotency (*OCT4*, *SOX2*), trophectoderm specification (*CDX2*), and metabolism (*GLUT1*) are good ways to tell if an embryo is still alive (Kues et al., 2008; Berg et al., 2011). Epigenetic profiling, which includes DNA methylation patterns of imprinted genes, is also being used more and more to evaluate the development of embryos, especially those made in laboratories (Reik et al., 2001). Cryopreservation of bovine embryos has become a critical component of commercial embryo transfer programs. Conventional slow freezing was initially the method of choice but often resulted in suboptimal post-thaw survival rates, especially for *in vitro*-produced embryos that typically have higher cytoplasmic lipid content (Massip et al., 1995). The use of vitrification methods, which involve very fast cooling and high levels of cryoprotectants, has made it much more likely for bovine embryos to survive and implant after being warmed up (Vajta & Kuwayama, 2006). Still, getting the best cryopreservation methods is important, especially for embryos that come from specific, serum-free culture systems and may have different membrane and cytoplasmic

features. Molecular methods have helped us learn a lot more about how cow embryos grow and develop. Different gene expression patterns have been found between embryos that were grown *in vivo* and those that were grown *in vitro*. This shows how culture factors can change how embryos develop (Wrenzycki et al., 2001). Proteomic and metabolomic profiling add to these results by giving a more complete, holistic view of embryo physiology and creating new signals for choosing high-quality embryos (Gutierrez-Adan et al., 2004). Also, recent improvements in genome editing tools like CRISPR/Cas9 have made it easier to change bovine embryos to add features that are wanted or to study how genes work during early development (Tan et al., 2016). Finally, the study of bovine embryos includes many different fields, ranging from traditional embryology to cutting-edge molecular biology. To make *in vitro* culture systems better, cryopreservation methods better, and eventually assisted reproductive technologies more successful, we need to know how embryos grow, what their metabolic needs are, and their molecular signatures. Suppose omics technologies are kept together with conventional methods of evaluating embryos. In that case, it might be possible to make bovine embryo production higher-quality, reliable, and efficient in both scientific and commercial settings.

### 2.1.5 Embryo quality

Embryo quality is also assessed using a numerical grading system, which is determined by evaluating the morphological integrity of the embryos. The quality grades range from 1 to 4, with each number reflecting a specific level of structural quality (Bo & Mapletoft, 2013).

**Grade 1 – Excellent or Good:** Embryos in this category display a well-rounded, symmetrical structure with blastomeres that are consistent in size, color, and texture. Their appearance aligns with the expected developmental stage, and any imperfections should be minimal. At least 85% of the cellular content must be intact and viable, based on the amount of cell material displaced into the perivitelline space. The zona pellucida should be smooth, without flat or indented areas that could lead to adhesion in culture dishes or transfer straws. These embryos are highly tolerant of cryopreservation and are often referred to as "freezable" or suitable for international shipment.

**Grade 2 – Fair:** These embryos exhibit some moderate deviations in shape or variability among blastomeres in terms of size, color, or density. At least half of the embryonic mass must remain viable. Although these embryos have lower survival rates after freezing and thawing compared to Grade 1, they are still considered suitable for fresh embryo transfer, which can yield acceptable pregnancy outcomes. They are generally labeled as "transferable" but not ideal for freezing.

**Grade 3 – Poor:** Embryos classified in this group show significant abnormalities in shape or inconsistencies among individual cells. A minimum of 25% of the embryonic structure must still be intact. Their ability to survive cryopreservation is minimal, and when used for fresh transfer, their potential to result in pregnancy is notably reduced compared to embryos of higher quality.

**Grade 4 – Non-viable or Degenerating:** This category includes embryos that are dead or showing signs of degeneration, which may also encompass unfertilized oocytes or single-cell embryos. These are considered non-viable and are not recommended for use, they should be discarded.

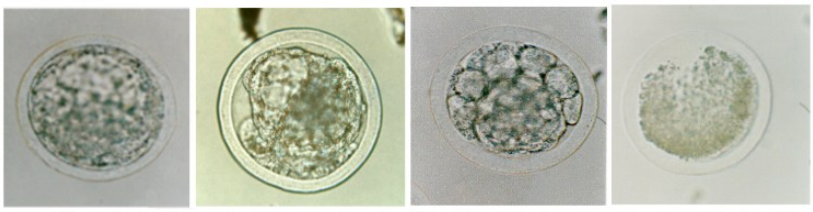


**Figure 1.** Numerical grading system used to assess embryo morphology, with quality classifications from 1 to 4 based on structural characteristics (adapted from Bo & Mapletoft, 2013).

## 2.2 Cryopreservation

In the control of bovine reproduction, cryopreservation has emerged as a crucial technology that allows genetic material to be stored and transported over extended periods of time and space. When mammalian spermatozoa were frozen using cryoprotective chemicals like glycerol in the middle of the 19$^{th}$ century, the idea of cryopreserving living cells was first proved (Polge et al., 1949). Based on these

groundbreaking findings, scientists started using cryopreservation methods on cow embryos, and in 1973, the first viable calf was born from a frozen-thawed embryo (Wilmut & Rowson, 1973). Since then, cryopreservation has transformed cow breeding and genetic improvement techniques by becoming a standard component of bovine embryo transfer (ET) programs across the globe.

Slow freezing and vitrification have historically been the two main techniques used in the cryopreservation of bovine embryos. Developed in the 1970s, the slow freezing approach includes cooling embryos gradually while including cryoprotectants such as ethylene glycol or glycerol to prevent ice formation (Leibo & Mazur, 1971). Embryos are placed in straws, chilled gradually, and then submerged in liquid nitrogen at -196°C during the slow freezing process. By allowing for some embryonic dehydration, slow freezing reduces the production of intracellular ice, which could otherwise harm cellular structures. To control cooling rates, precise equipment is needed, and the process is still time-consuming.

A more recent development, vitrification, uses extremely high concentrations of cryoprotectants and extremely fast cooling rates to completely avoid ice crystal formation (Vajta & Kuwayama, 2006). The embryos are submerged straight into liquid nitrogen, which causes the solution to solidify into a glass-like state without generating harmful ice crystals, as opposed to freezing gradually. For bovine embryos created *in vitro*, which are more prone to cryoinjury due to their higher lipid content than *in vivo*-derived embryos, vitrification has shown promise (Massip et al., 1995). Methods like Cryotop vitrification and open pulled straw (OPS) vitrification have significantly improved pregnancy outcomes and post-thaw survival rates.

Bovine embryo physiology presents cryopreservation difficulties. Compared to their *in vivo* counterparts, embryos created *in vitro* typically have a higher concentration of cytoplasmic lipid droplets, which makes them more susceptible to freezing and thawing stress (Seidel, 2006). Higher rates of intracellular ice production and freezing-induced membrane damage are linked to lipid accumulation. Using dilapidation techniques, altering culture conditions to lower lipid content, or adding metabolic regulators like L-carnitine to culture media are some ways to increase cryosurvival (Sudano et al., 2011). Furthermore, the developmental stage of the

embryo also affects cryotolerance; early blastocysts and compact morulae have a higher survival rate than extended blastocysts because of their smaller size and smaller blastocoelic cavity capacity (Dochi et al., 1998).

The selection and concentration of cryoprotectants are essential to the success of cryopreservation. Conventional cryoprotectants like glycerol and ethylene glycol work by entering cells and preventing the production of ice, but prolonged exposure can potentially have harmful consequences (Pedro et al., 2005). Because of its greater membrane permeability and decreased toxicity, ethylene glycol has become more popular than glycerol for direct transfer techniques. This allows embryos to be transferred into recipients without the cryoprotectant having to be diluted or removed after thawing. To help with osmotic dehydration during freezing and reduce the production of ice crystals, non-permeating substances such as sucrose are frequently added to freezing media (Leibo, 2004).

Assessing post-thaw embryo viability is crucial to determining how effective cryopreservation procedures are. Initial markers of embryo quality are provided by morphological evaluation as soon as the embryo is thawed, with an emphasis on membrane integrity, blastocoel re-expansion, and cellular fragmentation (Lindner & Wright, 1983). But to more accurately predict embryo competence after thawing, more sensitive methods such as detecting mitochondrial activity, membrane permeability assays, and apoptotic markers have been used. Pregnancy rates after embryo transfer are still the gold standard for determining the success of cryopreservation.

Additionally, the global movement of bovine genetics is made possible by cryopreservation, which makes it easier to export valuable embryos across continents without running the same biosecurity risks as live animal transportation. The World Organization for Animal Health (OIE) and the International Embryo Transfer Society (IETS) have established regulatory systems that guarantee exported frozen embryos adhere to strict quality and health requirements (Stringfellow & Givens, 2010). This has facilitated the quick spread of superior genetics, which has increased genetic variety and production in cow populations all over the world.

Cryopreservation of bovine embryos is, in summary, a fundamental component of contemporary genetic improvement and cattle reproductive initiatives. Post-thaw

survival and pregnancy rates have continuously increased due to ongoing developments in cryoprotectant formulations, vitrification processes, embryo culture systems, and evaluation methodologies. There are still difficulties, nevertheless, especially in improving the cryotolerance of embryos created *in vitro* and expanding the efficacy of cryopreservation to include bovine oocytes. To further improve cryopreservation techniques and optimize the reproductive capacity of cryopreserved bovine germplasm, future initiatives will incorporate molecular insights, metabolic regulation, and innovative biotechnological treatments

### 2.2.1 Slow freezing

In bovine reproduction, slow freezing has long been the mainstay of cryopreservation, allowing for the long-term transfer and storage of valuable genetic material. This technique, which was created in the 1970s, made it possible to preserve bovine embryos at extremely low temperatures without sacrificing their ability to survive after thawing. A significant advancement in reproductive biotechnology was made when Wilmut and Rowson (1973) reported the first calf born from a cryopreserved cow embryo. Since then, slow freezing has been used extensively in commercial ET operations as well as research, especially when combined with *in vivo*-derived embryos. The slow freezing method reduces intracellular ice formation, which is believed to be the main source of cellular damage during freezing, by gradually cooling embryos in a cryoprotectant solution. Usually, embryos are loaded into plastic straws, suspended in a medium that contains permeating cryoprotectants like ethylene glycol or glycerol, and then put through a programmable freezing curve that gradually lowers the temperature from room temperature to -35°C before submerging them in liquid nitrogen (Leibo & Mazur, 1971). By allowing for controlled cell dehydration, this slow temperature drop lowers the possibility of ice crystals forming in the cytoplasm.

An essential part of the slow freezing process is cryoprotectants. The first cryoprotectant to be effectively employed in the cryopreservation of bovine embryos was glycerol (Willadsen et al., 1976). By lowering the freezing point of intracellular water and penetrating the cell membrane, it reduces the generation of ice. After

thawing, glycerol must be diluted gradually since it causes osmotic stress during addition and removal. A substitute that is less toxic and has a higher membrane permeability is ethylene glycol, which makes field application easier by enabling direct transfer without dilution (Dochi et al., 1998). To improve osmotic dehydration during freezing, both cryoprotectants are frequently mixed with non-permeating substances like sucrose. The effectiveness of slow freezing depends on the source and stage of development of the embryo. Compared to expanded blastocysts or embryos created *in vitro*, *in vivo*-derived embryos, especially those at the morula and early blastocyst stages, have shown better post-thaw survival (Massip et al., 1995). Later-stage and *in vitro*-produced embryos are more vulnerable to cryodamage due to their larger blastocoel cavity and higher cytoplasmic lipid content. Selection of embryos before freezing is therefore essential. The morphological grading system created by Lindner and Wright (1983) is still commonly used to evaluate the quality of embryos before freezing; Grade 1 (excellent) embryos have the highest survival rates.

Lipid content has emerged as a significant barrier in the cryopreservation of *in vitro*-produced embryos. Because of the changed metabolic conditions *in vitro*, these embryos tend to collect more cytoplasmic lipids, which lower their cryotolerance (Seidel, 2006). Modifying culture media, dilapidation methods, and supplementing with metabolic regulators such as L-carnitine, which encourages fatty acid oxidation and decreases lipid buildup, have all been used to increase the success of slow freezing in these embryos (Sudano et al., 2011). Slow freezing has a few drawbacks despite its proven benefits. The procedure takes a long time, needs certain tools, and is subject to change depending on operator skill and protocol accuracy. Furthermore, the mechanical stress brought on by ice crystal formation and the toxicity of cryoprotectants still affect embryo viability. Slow freezing is still the preferred method for routine cryopreservation in many field settings because of its standardization and track record of success with *in vivo* embryos. To increase the post-warming survival of bovine embryos, ongoing attempts have been made to improve slow freezing procedures. To reduce intracellular ice formation, which is still a leading cause of cryoinjury, these strategies include maximizing cooling and seeding rates (Leibo &

Mazur, 1971). Osmotic stress and cytotoxicity during the freezing process have also been demonstrated to be reduced by shortening the exposure duration to cryoprotectants such as glycerol and ethylene glycol (Vajta & Kuwayama, 2006). Furthermore, incorporating post-thaw embryo cleaning and rehydration procedures enhances blastocyst viability and re-expansion (Massip et al., 1995). To reduce oxidative stress, some research has investigated adding antioxidants such as L-carnitine and cysteine to freezing or post-thaw culture conditions (Silva et al., 2015; Tamura et al., 2008). Other studies have investigated the function of mitochondrial activity and gene expression, including stress response (*HSPA1A*) and apoptotic markers (*BAX, BCL2*), as possible indicators of cryotolerance and post-thaw developmental competence (Sudano et al., 2011; Rizos et al., 2002).

Finally, ET programs still heavily utilize slow freezing, which was pivotal in the development of bovine embryo cryopreservation. The quality, origin, and developmental stage of the embryos, together with the cautious use of cryoprotectants and chilling procedures, all have a significant impact on their success. Even though newer techniques like vitrification present encouraging substitutes, slow freezing is still a dependable and practical procedure, especially for high-quality *in vivo*-derived embryos. Molecular and metabolic therapies to improve embryo cryotolerance and guarantee high post-thaw viability are probably going to be the focus of future developments in slow freezing.

### 2.2.2 Vitrification

In the field of bovine embryo cryopreservation, vitrification has become a game-changing method that provides a productive substitute for conventional slow freezing. Vitrification is a rapid-freezing method that turns cellular water into a glass-like solid without generating ice crystals, in contrast to slow freezing, which uses controlled dehydration and gradual chilling to avoid ice formation (Vajta & Kuwayama, 2006; Rall & Fahy, 1985). High concentrations of cryoprotectants and instantaneous immersion in liquid nitrogen are used to accomplish this ultrarapid chilling, which reduces cryoinjury and maintains cell structure (Vajta & Kuwayama, 2006). Interest in vitrification has increased because of slow freezing limitations, especially its decreased effectiveness with IVP bovine embryos. IVP embryos are particularly susceptible to ice

crystal injury because of their high lipid content and altered membrane permeability (Massip et al., 1995; Seidel, 2006). In these embryos, vitrification has shown enhanced post-thaw survival and developmental competency by completely avoiding crystallization (Vajta et al., 1998). Various methods have been developed to increase handling and cooling rates during vitrification, including the open pulled straw (OPS) method, Cryoloop, and Cryotop (Vajta et al., 1998; Kuwayama, 2007). The composition of the cryoprotectant solution, which typically combines non-penetrating compounds like sucrose or trehalose with permeating agents like ethylene glycol and dimethyl sulfoxide (DMSO), is a crucial component of efficient vitrification. These substances prevent ice nucleation during cooling by reducing the freezing point and increasing the viscosity of intracellular and extracellular fluids (Rall & Fahy, 1985). For this method to swiftly move across the critical temperature range of roughly 15°C to -5°C, an extremely rapid cooling rate typically surpassing 10,000°C per minute is needed.

Temperature and exposure duration are crucial factors since large doses of cryoprotectants can be harmful. Stepwise loading of cryoprotectants and shorter exposure intervals are two examples of methods that researchers have modified to reduce toxicity while preserving vitrification efficiency (Dinnyes et al., 2000). There are still issues, even if vitrification has definite benefits in terms of embryo survival and pregnancy rates. Open systems like Cryotop and OPS expose embryos to liquid nitrogen directly, which raises questions about biosafety, particularly when transporting embryos internationally. As a result, closed vitrification systems have been developed, which seek to maintain high cooling rates while lowering the possibility of contamination, albeit at the possible expense of some efficiency (Kuwayama et al., 2005). The goal of recent developments in vitrification has been to enhance the quality of embryos after warming. Antioxidants like melatonin, cysteine, and L-carnitine have been demonstrated to lower oxidative stress and enhance mitochondrial activity and developmental competence when added to post-thaw culture medium (Tamura et al., 2013). Furthermore, apoptosis (e.g., *BAX, BCL2*), oxidative stress (e.g., *SOD1*, *GPX*), and stress response (e.g., *HSP70*) genes have been identified by molecular investigations as possible biomarkers for evaluating vitrification success (Sudano et al., 2011).

In the context of oocyte preservation, vitrification has also grown in significance, especially for commercial IVP programs and genetic conservation. However, the sensitivity of the meiotic spindle and the high lipid content make oocyte vitrification more difficult. However, improvements in oocyte vitrification have also been attributed to developments in spindle imaging, cytoskeletal stabilization, and antioxidant inclusion (Arav et al., 1996). In conclusion, vitrification significantly advances bovine embryo cryopreservation, particularly for IVP embryos that exhibit low cryotolerance. Pregnancy outcomes and embryo viability have been greatly improved by the introduction of ultrarapid cooling methods, better cryoprotectant formulations, and encouraging post-warming treatments. Vitrification is still a very flexible and successful method in both commercial cattle breeding and reproductive biotechnology research, despite persistent worries about biosafety and cytotoxicity.

## 2.3 Oxidative stress

Oxidative stress is defined as a physiological imbalance between the production of ROS and the capacity of antioxidant defense systems to neutralize them, leading to potential cellular damage (Agarwal et al., 2005). This redox imbalance is a major factor in determining the quality and developmental potential of both oocytes and embryos during bovine reproduction. Normal reproductive activities, including oocyte maturation, folliculogenesis, ovulation, and early embryo development, depend on ROS, which are normally created in trace amounts during cellular metabolism (Gupta et al., 2010; Dumollard et al., 2007). However, when produced in excess, ROS can damage gametes and embryos' lipids, proteins, and DNA, therefore compromising reproductive competence (Guérin et al., 2001). With the development of ARTs, such as IVM, IVF, and IVC, the importance of oxidative stress in bovine reproduction has become even more apparent. Because of increased oxygen tension, light exposure, and a lack of maternal antioxidant factors, *in vitro* conditions frequently lack the dynamic antioxidant environment of the reproductive canal, leading to elevated ROS levels (Rizos et al., 2002; Lonergan & Fair, 2008). Reduced cleavage rates, poor blastocyst formation, and increased apoptosis in developing embryos have all been related to these increased ROS levels (Goud et al., 2008; Tamura et al., 2008).

Furthermore, mitochondria are particularly important in oocyte physiology since they are the main source of ROS during oxidative phosphorylation. Oxidative stress-induced mitochondrial dysfunction decreases meiotic competence and developmental potential by decreasing ATP synthesis and increasing the generation of ROS (Van Blerkom, 2004). Because of their high lipid content, which makes membrane structures more susceptible to lipid peroxidation, bovine oocytes and embryos are much more sensitive to oxidative damage (Sudano et al., 2011). Bovine reproductive cells use both enzymatic antioxidants (such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) and non-enzymatic antioxidants (such as reduced glutathione (GSH), vitamins C and E, and melatonin) to counteract the harmful effects of oxidative stress (Takahashi, 2012; Ali et al., 2003). The natural antioxidant defense, however, can be insufficient in *in vitro* conditions, requiring supplementation with culture media improvements (Nabenishi et al., 2012).

**2.3.1 Reactive oxygen species (ROS)**

Reactive oxygen species (ROS) are naturally occurring by products of cellular metabolism, specifically mitochondrial oxidative phosphorylation. These include hydrogen peroxide ($H_2O_2$), superoxide anions ($O_2^-$), and hydroxyl radicals (OH•) (Agarwal et al., 2005). Under normal circumstances, ROS function as signaling molecules in ovulation, embryo implantation, and folliculogenesis (Guerin et al., 2001). However, oxidative stress occurs when ROS production is excessive or antioxidant defenses are inadequate, which impairs gamete activity and embryo development (Ruder et al., 2009). When energy is produced in bovine oocytes, ROS is mostly produced by mitochondria. Increased ROS levels due to mitochondrial dysfunction can harm mitochondrial DNA (mtDNA), cause lipid peroxidation of oolemma membranes, and interfere with spindle formation, all of which can affect meiotic competence and developmental potential (Van Blerkom, 2004; Nabenishi et al., 2012). Environmental factors, including light exposure, temperature or pH changes, and increased oxygen tension (20% vs. 5% in vivo) can greatly increase ROS generation in oocytes and embryos during IVP (Rizos et al., 2002; Lonergan & Fair, 2008). According to Goud et al. (2008), the absence of maternal antioxidants in the oviductal and uterine environments in vivo increases this oxidative burden. According to Takahashi (2012) and Sudano et

al. (2011), high ROS levels in embryos can cause cytochrome c release, caspase activation, and DNA fragmentation, which can set off apoptotic pathways and lower blastocyst formation rates and developmental competence. The peroxidation of membrane phospholipids by ROS also impairs ionic transport and membrane fluidity, both of which are necessary for healthy embryonic physiology (Gupta et al., 2010). Bovine reproductive cells use both enzymatic (SOD, CAT, and GPx, GSH, vitamins C and E, melatonin) antioxidant mechanisms to reduce ROS-induced damage (Abedelahi et al., 2010; Ali et al., 2003). However, it is frequently necessary to supplement culture medium due to the absence of adequate antioxidant support under *in vitro* settings (Silva et al., 2023). According to recent research, increased ROS levels also affect the expression of genes that can be used as molecular indicators of oxidative damage in oocytes and embryos, including stress-response genes like *HSP70*, pro-apoptotic genes like *BAX*, and antioxidant genes like *SOD1* and *GPX1* (Rizos et al., 2002; Sudano et al., 2011).

### 2.3.2 Antioxidant

Oocytes include a number of defensive mechanisms against oxidative stress under normal physiological settings, including both non-enzymatic and enzymatic antioxidants (such as peroxidase, catalase, and superoxide dismutase) (Combelles et al., 2009). Through the regulation of ROS levels, these systems aid in the maintenance of redox equilibrium. A variety of internal and external factors, however, have the potential to upset this equilibrium, leading to elevated ROS production and changed intracellular antioxidant capacity (Li et al., 2016; Somfai et al., 2007; Succu et al., 2018). Numerous antioxidant compounds, such as enzymatic agents, thiol-based molecules like β-mercaptoethanol and cysteamine, vitamins like α-tocopherol and vitamin C (ascorbic acid), flavonoids like resveratrol, and other bioactive molecules like L-proline and melatonin, are used to combat oxidative damage. Notably, substances including resveratrol, melatonin, L-proline, and α-tocopherol have shown protective benefits in vitrified oocytes, reducing cryoinjury and preserving developmental potential (Chinen et al., 2020; Sovernigo et al., 2017).

### 2.3.3 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a naturally occurring polyphenolic compound classified as a stilbenoid, consisting of two aromatic rings linked by a double bond (Salehi et al., 2018). Japanese researcher Michio Takaoka isolated it for the first time in 1939 from the roots of the medicinal plant Veratrum grandiflorum (Pezzuto, 2018). According to Baur and Sinclair (2006), resveratrol is a phytoalexin, which means that plants produce it in reaction to environmental stressors, including pathogen invasion, UV radiation, or mechanical damage. According to Burns et al. (2002), dietary sources of resveratrol include peanuts, red wine, grape skins, and a variety of berries, including mulberries and blueberries. The trans isomeric form is more physiologically active than the cis isomeric form. Nevertheless, trans-resveratrol can change into the less potent cis form when exposed to UV light (Almeida et al., 2009). Due to its suggested link to the "French Paradox," which is the finding that French populations have low rates of coronary heart disease despite eating a diet high in saturated fats, a phenomenon partly ascribed to red wine consumption, the compound attracted international attention in the early 1990s (Renaud & de Lorgeril, 1992). Since then, numerous studies have shown that resveratrol has a variety of biological properties, such as anti-inflammatory, cardioprotective, neuroprotective, antioxidant, and anti-cancer benefits (Baur & Sinclair, 2006; Salehi et al., 2018). It is believed that these health advantages are mediated by processes such as sirtuin 1 (SIRT1) activation, mitochondrial function regulation, and oxidative stress signaling pathway modulation (Baur & Sinclair, 2006; Das & Das, 2007). However, because of its rapid metabolism and excretion, resveratrol has a limited oral bioavailability, which is a significant barrier to its therapeutic usage. As a result, new formulations and analogs have been created to enhance their pharmacokinetic characteristics (Walle, 2011).

About bovine reproductive technology, resveratrol, a naturally occurring polyphenolic molecule mostly found in grapes and red wine, has attracted considerable attention due to its antioxidant properties. It is a viable option for improving the results of IVP due to its capacity to reduce oxidative stress (Wang et al., 2014). Resveratrol supplementation has been demonstrated to enhance developmental competence in bovine oocyte maturation. According to Wang et al.

(2014), 1.0 μM resveratrol during IVM improved oocyte cytoplasmic maturation, activated the Mos/MEK/p42 MAPK signaling pathway, and boosted cumulus expansion, all of which led to a greater cell count and blastocyst formation. Because oxidative stress damages oocyte quality and embryonic development, resveratrol also lowers intracellular ROS and raise GSH levels in oocytes (Wang et al., 2014). According to Gaviria et al. (2019), adding resveratrol to vitrified embryos during post-warming culture increased both the number of cells overall and the rates of re-expansion and hatching. These advantages are dose-dependent, though; larger resveratrol concentrations during IVM may have detrimental effects on embryonic development, according to Sovernigo et al. (2017), underscoring the necessity for cautious optimization. According to Wang et al. (2014), the protective benefits of resveratrol are partly mediated by the activation of SIRT1, a $NAD^+$-dependent deacetylase linked to enhanced mitochondrial function and cellular stress responses. Its anti-apoptotic properties also supplement its antioxidant and mitochondrial advantages. For example, Silva et al. (2021) showed that resveratrol improved survival and developmental potential by lowering apoptotic cells in fresh and vitrified embryos.

**2.3.4 Mechanisms of Resveratrol on SIRT1**

One of the central mechanisms of resveratrol action is the activation of sirtuin 1 (SIRT1), a $NAD^+$-dependent deacetylase that regulates mitochondrial biogenesis and cellular energy metabolism. By activating SIRT1, resveratrol enhances the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), promoting mitochondrial function, a key factor for cytoplasmic maturation in bovine oocytes and energy production during early embryo development (Takeo et al., 2014).

Resveratrol supplementation during IVM has been shown to upregulate SIRT1 expression in bovine oocytes, leading to improved mitochondrial function, increased ATP production, and enhanced oocyte quality (Takeo et al., 2014). This upregulation of SIRT1 also contributes to the reduction of ROS levels and the increase of GSH concentrations, thereby mitigating oxidative stress and improving embryonic development (Wang et al., 2014). Furthermore, the activation of SIRT1 by resveratrol influences apoptotic pathways by modulating the expression of apoptosis-related

genes, decreasing pro-apoptotic markers such as BAX and increasing anti-apoptotic markers like BCL2L1 in bovine embryos, leading to reduced apoptosis and improved embryo quality (Gaviria et al., 2019). In addition, resveratrol has been found to enhance the cryotolerance of bovine embryos by reducing ROS levels, restoring GSH content, and improving re-expansion and hatching rates after warming, effects that are associated with SIRT1 activation (Gaviria et al., 2019).

The mitogen-activated protein kinase (MAPK) signaling pathway, which is involved in oocyte maturation and embryo development, is also modulated by resveratrol through SIRT1 activation. Resveratrol has been reported to activate the Mos/MEK/p42 MAPK cascade in bovine oocytes, leading to enhanced cumulus expansion, polar body extrusion, and improved blastocyst formation rates (Wang et al., 2014). *In vivo* studies have demonstrated that direct injection of resveratrol into the uterus of cows during artificial insemination increases plasma SIRT1 levels and improves conception rates, indicating the potential of resveratrol as a therapeutic agent in bovine reproduction (Kim et al., 2020).