Inland Norway University of Applied Sciences

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Master's Thesis

Exploring the impact of cryoprotective agents on bovine oocytes

A comprehensive investigation into lipid damage and its implications for cryopreservation success

Experimental Biotechnology

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Mehrdad Ganji

Abbreviations

- **ART:** Assisted reproductive technology
- **ATP:** Adenosine triphosphate
- **BHT:** Butylated hydroxytoluene
- **CASA:** Computer-assisted sperm analysis
- **COC:** Cumulus-oocyte complex
- **CPA:** Cryoprotective agent
- **DMSO:** Dimethyl sulfoxide
- **EG:** Ethylene glycol
- **FITC:** Fluorescein isothiocyanate
- **FSH:** Follicle-stimulating hormone
- **GLY:** Glycerol
- **GV:** Germinal vesicle
- **GVBD:** Germinal vesicle breakdown
- **IVC:** *In vitro* culture
- **IVF:** *In vitro* fertilization
- **IVM:** *In vitro* maturation
- **IVP:** *In vitro* production
- **LH:** Luteinizing hormone
- **MeOH:** Methanol
- **MII:** Metaphase II
- **PBS:** Phosphate-buffered solution
- **PFA:** Paraformaldehyde
- **PG:** Propylene glycol
- **PVP:** Polyvinylpyrrolidone
- **ROCK:** Rho-associated coiled-coil kinase
- **ROS:** Reactive oxygen species
- **RT:** Room temperature

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Abstract

Cryopreservation has increased the possibilities in animal genetic breeding and reproduction technologies. In recent years, vitrification has emerged as a promising substitute for conventional cryopreservation techniques, especially for oocytes and embryos. The rapid cooling/warming rates associated with vitrification prevent the formation of intracellular ice crystals and improve the survivability of oocytes. However, this method relies on the exposure of oocytes to high concentrations of cryoprotective agents (CPAs) that can cause irreversible damage to the oocyte structure. Lipid droplets are a vital part of energy metabolism in oocytes and are highly sensitive to CPA toxicity and low temperatures. This study aimed to identify the optimal concentrations of CPAs that cause the least visible change to lipid droplets in bovine oocytes before vitrification. Additionally, the survival rate of bovine oocytes that are exposed to selected CPAs after the vitrification process was examined. In order to determine the optimal CPAs, a CPA stress test was performed on bovine oocytes (n = 985) using ethylene glycol (EG), propylene glycol (PG), glycerol (GLY), methanol (MeOH), and dimethyl sulfoxide (DMSO) as CPAs. Two concentrations of each individual CPA were used in the stress test in addition to different combinations of these CPAs. From each treatment group, five oocytes were stained with BODIPY 493/503 to visualize lipid droplets and approximately 20 oocytes were fertilized to evaluate the development. Four treatments of PG 20%, GLY 20%, DMSO 7.5% + EG 7.5%, and EG 7.5% + PG 7.5% were selected as optimal CPAs due to their high embryo development and low impact on lipid droplets. Vitrification was performed (n = 153) using the droplet method with the selected CPAs and 0.5% sucrose. Upon thawing, approximately eight oocytes were stained with BODIPY 493/503 to visualize lipid droplets and the remaining oocytes were fertilized to evaluate development post vitrification. All oocytes appeared degenerated after fertilization. Confocal imaging of lipid droplets showed significant damage to the oocytes incubated with PG, GLY, and DMSO + EG. Another vitrification process was conducted (n = 35) with EG + PG which yielded the same cleavage result of 0.0%. However, out of 35 vitrified/warmed oocytes, 29 appeared degenerated and six appeared not cleaved. The conclusion of this study was that the irreversible damage to cellular structures caused by CPAs and low temperature lead to a decreased survival rate. The addition of more equilibrium stages and increased exposure to vitrification media in future experiments may result in oocyte survival post-thaw.

1. Introduction

1.1 Background

The conducted study is 60 credits and serves as a master's thesis project for the Experimental Biotechnology program at Inland Norway University of Applied Sciences (INN). The study is a component of the larger Zygote project, which involves collaboration between various academic and industry partners, including SINTEF, OsloMet, SimulaMet, Oslo University Hospital, Geno, Norsvin, and SpermVital. The project aims to establish a strong research and knowledge base in sustainable breeding and applied biotechnology in the Inland region, fostering international competitiveness and providing local businesses with enhanced expertise and research capabilities, by investigating the physiological mechanism regulating gamete production, developing novel methods and tools to improve fertilization utilizing assisted reproductive technologies (ARTs), generating new insights into the characterization and identification of oocytes and embryos, and enhancing existing preservation techniques. This master's project is part of the research on bovine oocytes and embryos.

1.2 Bovine follicle formation and ovulation

In placental mammals, germ cells in the developing ovaries begin the first meiotic division by entering the prophase stage and they remain dormant at the diplotene stage until shortly before ovulation. Millions of oocytes are produced in cows and remain dormant until ovulation, however, over the years a vast number of oocytes will perish. Around the time of ovulation, oocytes get surrounded by somatic cells of ovaries and form follicles. The oocytes are first enclosed in a single layer of pregranulosa cells (Dunning et al., 2014) and form the primordial follicles. Inside these follicles, oocytes increase in size from approximately 10 μm to 120 μm in diameter to become developmentally competent (Aguila et al., 2020; Roelen, 2019). The largest follicles, known as preovulatory of Graafian, are composed of roughly 5 – 10 outer layers of mural granulosa cells and 2 – 3 layers of cumulus cells (Jaffe & Egbert, 2017).

Increases in the production of follicle-stimulating hormone (FSH) after puberty leads to an ovulatory follicle. The mural granulosa cells of the dominant follicle triggered by luteinizing

hormone (LH), initiate oocyte meiotic resumption and progress to the MII stage (Dadarwal et al., 2015). The oocytes then enter a second period of meiotic arrest and are released from the ovary by ovulation (Dunning et al., 2014; Jaffe & Egbert, 2017; Roelen, 2019). Both maintaining the arrest and re-initiating meiosis, require signaling between granulosa cells and the oocyte via extracellular space and gap junctions (Jaffe & Egbert, 2017).

Only a small portion out of thousands of oocytes present in the ovaries at birth are ever ovulated during the lifespan of a female mammal. This is due to ovulations occuring post-puberty and ceasing during pregnancy, primarily because progesterone suppresses the LH pulsatility. Additionally, the reproductive lifespan of female animals is finite. The number of ovulations in bovine species can be as low as 4 to 5 times per year, and pregnancies in cows lasts 9 months (Lonergan & Fair, 2016). Moreover, typical dairy cows does not live beyond 5 to 7 lactations. In contrast, bulls can produce 10 billion sperm per ejaculation which can result in hundreds of thousands of offspring. Hence, the ability to retrieve oocytes fated for degeneration, either for research or to generate offspring, holds significant potential advantages for both animal breeding operations (Lonergan & Fair, 2016) and animal models intended for advancements of human reproduction.

1.3 Oocyte structure

A variety of internal (breed, age, reproductive health, nutritional and metabolic state, hormone levels, and estrous cycle stage) and external factors (timing between slaughter and aspiration, collection method, ovary storage temperature, and operator skills) can affect the quality of the Cumulus-oocyte-complexes (COCs) (see 1.3.3). Morphology of the COCs is easy to assess using microscopes (Aguila et al., 2020).

1.3.1 Cellular membrane

The structural components of the plasma membrane are phospholipids, specifically phosphatidylcholine and sphingomyelins. The composition of phospholipids controls the majority of the physio-chemical characteristics of the cellular membrane, such as permeability, and fluidity (Sprícigo et al., 2015).

At physiological temperatures, lipids in biological membranes are typically in liquid-disordered phase, and their high flexibility inhibits membrane rupture (Amstislavsky et al., 2019). The membrane fluidity of the oocytes and their sensitivity to low temperatures is

determined by the cholesterol to phospholipid ratio of the oocyte membrane (Díez et al., 2012; Horvath & Seidel, 2006). High-cholesterol membranes are more flexible at low temperatures and are therefore less prone to cooling-related damage (Mogas, 2019). There is a close relationship between the chilling sensitivity, lipid phase transition temperature, and lipid composition of the cellular membrane (Horvath & Seidel, 2006).

1.3.2 Cytoskeleton and meiotic spindle

The cytoskeleton acts as an internal scaffold to preserve oolemma shape, control organelle positioning, and support the inner surface of the oolemma (Albertini, 2015). Cytoskeletal components like microtubules and microfilaments control the distribution, anchoring, and to some extent, even the function of cytoplasmic organelles including mitochondria (Somfai et al., 2015).

The primary structure involved in the segregation of chromosomes during the anaphase of meiosis I and meiosis II is the meiotic spindle (Albertini, 2015) which oversees the chromosome's spatial organization (Mogas, 2019). The meiotic spindle has a direct correlation with the developmental competence of the oocyte and can be assessed by polarized light microscopy (Rojas et al., 2004). Imbalances in the meiotic maturation and possible damages to the meiotic spindle may result in aneuploidy.

1.3.3 Cumulus cells

The structure that envelops the mammalian oocyte is called the cumulus oophorus (Figure 2). It has been demonstrated that cumulus cells release essential components for oocyte maturation, growth, fertilization, and early embryo development (Kato et al., 2022). Cumulus cells are physically and functionally connected to the oocytes, and their interactions are essential for the maturation and developmental competence of oocytes. Cumulus cells also preserve the oocyte's ability to be fertilized by reducing the release of cortical granules and preventing the hardening of zona pellucida (Díez et al., 2012). The oocyte cortex is pierced by cumulus cells, often forming junctional complexes with the plasma membrane (de Loos et al., 1989). Cumulus cells facilitate bidirectional paracrine signaling through these gap junctions (Auclair et al., 2013; Memili et al., 2007).

The amount of cumulus cell expansion following maturation indicates the quality of the oocyte. Cumulus cells have a significant role in fertilization by inducing acrosome reaction

(Aguila et al., 2020) and regulate chromatin remodeling and RNA synthesis in the oocytes (Auclair et al., 2013).

Oocytes have a limited ability to metabolize glucose, which is a primary energy source for oocytes and plays a critical role in the maturation and early development of embryos; therefore, the oocytes rely on the cumulus cells for the metabolism of glucose via pentose-phosphate route and glycolysis to generate pyruvate for oocyte energy synthesis (Auclair et al., 2013). The bidirectional exchange of molecules such as ions, oxidative substrates, and adenosine triphosphate (ATP) contribute to the metabolic crosstalk between oocytes and cumulus cells (de Andrade Melo-Sterza & Poehland, 2021).

1.3.4 Cortical granules

Cortical granules are present in abundant clusters located around the plasma membrane (de Loos et al., 1989). Meiotic maturation culminates in the development of the oocyte's capacity to release cortical granules upon fertilization (Wang et al., 1997). The secretion of cortical granules upon fertilization blocks the rest of the sperms to avoid polyspermy (Albertini, 2015). The prevention of polyspermy is one of the paramount events in fertilization of mammalian oocytes (Wang et al., 1997).

The cortical granules are widely scattered as amorphous particle aggregates throughout the peripheral cytoplasm; however, as oocyte maturation advances, the aggregated cortical granules break down and redistribute throughout the cortex of the cytoplasm (Hosoe & shioya, 1997).

1.3.5 Zona pellucida

As follicles form, a coating of glycoproteins known as the zona pellucida is formed between the oocyte and the somatic tissue, creating a physical barrier (Albertini, 2015) between the germ and the granulosa cells. Zona pellucida shields the oocyte and early embryo, and has a significant role in the initial sperm binding and the prevention of polyspermy (Albertini, 2015; Roelen, 2019). The zona pellucida (Figure 2) has a complex and porous network structure and due to the maturation process, zona pellucida in bovine oocytes has a spongy and lattice-like structure. Methods of analysis, conditions of *in vitro* maturation (IVM), and the source of oocytes (ovum pick-up vs. slaughterhouse) contribute to the differences in the average diameter of zona pellucida pores (Báez et al., 2019).

1.3.6 Lipid droplets

The physical appearance of the ooplasm is impacted by the lipid content. Lipids are signaling molecules (McKeegan & Sturmey, 2012) that have a significant role in oocyte maturation and competence acquisition by acting as an energy source in the form of droplets. (Aguila et al., 2020; Genicot et al., 2005). It is believed that lipid droplets are active organelles that are essential to maintain the balance of cell energy. Lipid droplets work in tandem with mitochondria, endoplasmic reticulum, and other subcellular components, forming metabolic units (Amstislavsky et al., 2019).

Additionally, fatty acids are intracellularly stored as triacylglycerides in lipid droplets, which can be a powerful source of energy when needed (de Andrade Melo-Sterza & Poehland, 2021; Sturmey et al., 2009). Triglycerides can be metabolized throughout oocyte maturation, fertilization, and the first embryonic cleavage (Genicot et al., 2005). Fatty acids have exhibited major importance in promoting early embryo development (Dunning et al., 2014; McKeegan & Sturmey, 2012).

Previous studies have demonstrated the dynamic lipid profile of the oocytes which is potentially influenced by the environment in which the oocyte develop (McKeegan & Sturmey, 2012). Lipid droplets are often observed as small in diameter and dispersed in abundance throughout the oocyte ooplasm (de Loos et al., 1989; McKeegan & Sturmey, 2012). The larger lipid droplets cluster around mitochondria (de Loos et al., 1989) and endoplasmic reticulum (Sprícigo et al., 2017) following maturation *in vitro* or following the LH surge *in vivo* (Sturmey et al., 2009). Lipid droplets grow during maturation (Figure 1) and demonstrate a small increase in the number of droplets during the maturation to the MII stage (Dunning et al., 2014), and a decrease in the number of lipids droplets in lipid-rich oocytes such as bovine, often indicates oocyte degeneration (Amstislavsky et al., 2019).

The lipid droplets can be observed as dark aggregates distributed homogenously in the ooplasm (Genicot et al., 2005). In previous studies it has been concluded that oocytes with higher lipid accumulation in the ooplasm have better developmental competence (Aguila et al., 2020; Dunning et al., 2014).

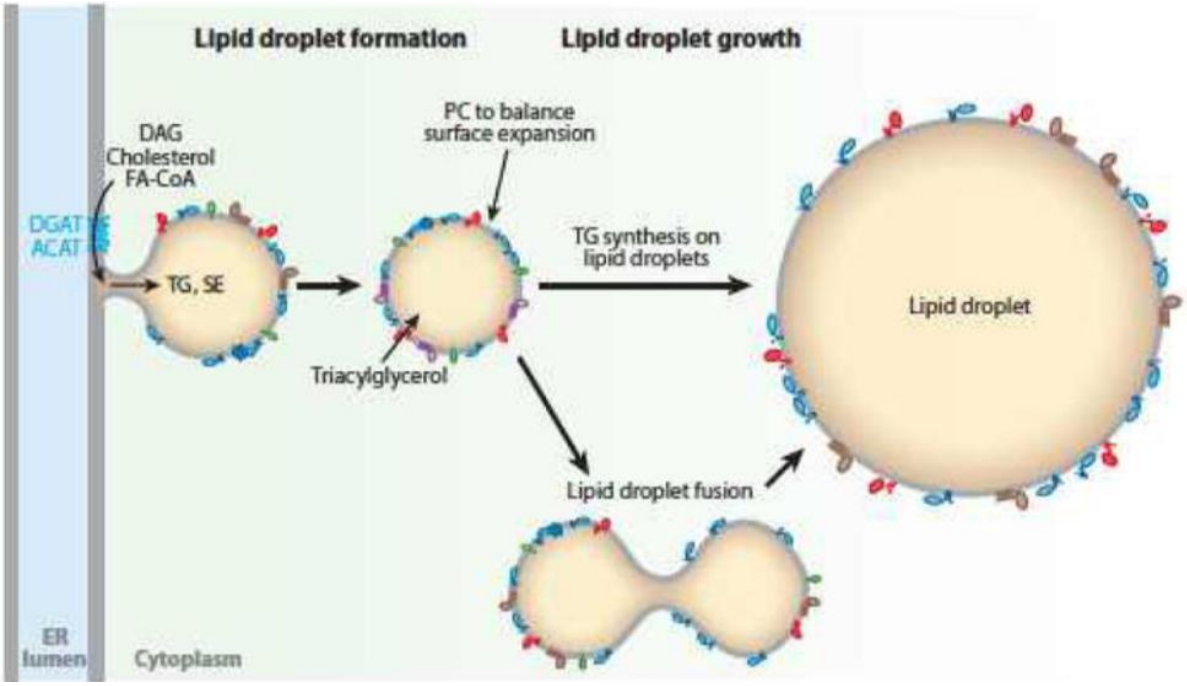


***Figure 1**: Illustration of lipid droplet formation and expansion. The formation of lipid droplets occurs by budding from the endoplasmic reticulum. The lipids that fill the core are formed by the neutral lipid synthesis enzymes, acyl-CoA cholesterol acyl transferase (ACAT) or diglyceride acyltransferase (DGAT). The expansion of lipid droplets is shown by fusion and local synthesis of triacylglycerol (TG) and sterol ester (SE). The surface expansion is balanced by phosphatidylcholine (PC). Abbreviations: DAG, diacylglycerol; FA-CoA, fatty acyl-coenzyme* (Walther & Farese, 2012, License ID: 1487847-1).

Another important factor in oocyte quality is the lipid content of the lipid droplets (Figure 2). Research has shown that higher quantities of oleic acid can be observed in high-quality oocytes (Amstislavsky et al., 2019), while low-quality oocytes demonstrate higher levels of stearic acid (de Andrade Melo-Sterza & Poehland, 2021). However, the abundance of lipid droplets in the oocyte complicates the assessment of meiotic spindle in the oocyte selection process (Dujíčková et al., 2021).

1.3.7 Nucleus and polar body

One of the most noticeable characteristics of the mammalian oocyte is the germinal vesicle (GV) (Albertini, 2015). At the GV stage of development, the nucleus has a non-centrical position inside the oocytes (de Loos et al., 1989). Rich in nuclear pores, the GV's nuclear envelope mediates the high degree of RNA trafficking that takes place during the development phase (Albertini, 2015).

A biological marker of meiotic maturation in oocytes is the extrusion of the polar body (Aguila et al., 2020). Upon the breakdown of the GV during oocyte maturation, homologous chromosomes are separated, and the oocyte will mature to the MII stage after the extrusion of the first polar body (Bevacqua et al., 2011). Evaluation of nuclear maturation is performed by the assessment of polar body (Figure 2), where its absence indicates immature oocyte or age-related degradation. However, the presence of the polar body does not imply complete maturation and developmental competence as cytoplasmic maturation in bovine species occur several hours after nuclear maturation (Aguila et al., 2020).

As a result of the spatial restriction of contractile forces needed to create both polar bodies, a single set of chromosomes is retained in the ooplasm, and one is eliminated during the anaphase I and anaphase II chromosomal segregation. Due to the restricting size of the polar bodies, the oocyte can hold onto the majority of the molecular and organellar components that will be used and consumed by the zygote (Albertini, 2015).

Figure 2: *Images taken during this experiment, all depicting mature oocytes at the MII stage. Different structures have been shown by arrows. Lipid droplets have been stained and visualized using BODIPY 493/503 fluorescent dye and imaged under confocal microscope. The metaphase plate has been visualized using Hoechst 33342 dye and imaged from the ocular lens of confocal microscope.*

1.4 *In vitro* maturation and fertilization of bovine oocytes

The physiological process known as oocyte maturation is necessary for successful fertilization and the development of embryos. Oocytes maturation is inhibited in the follicles

until the process of ovulation occurs, however, upon removal of the oocytes from ovarian follicles *in vitro*, meiosis can resume spontaneously resulting in nuclear maturation progression until the MII stage (Dadarwal et al., 2015).

The nuclear maturation of oocytes is characterized by the completion of the first meiosis, chromosomal condensation, GV breakdown (GVBD), and an additional stop in growth during the metaphase of the second meisis (MII) (Figure 3). Furthermore, these nuclear maturation-related activities involve substantial cytoplasmic alterations, including modifications to organelle structure, and extensive translational activities which result in the synthesis of many new proteins while terminating the production of other proteins (Memili et al., 2007). Cytoplasmic maturation will also involve redistribution of several cell organelles, including cortical granules in order to prepare for fertilization (Dadarwal et al., 2015; Lonergan & Fair, 2016).

Oocyte quality is determined by certain patterns of mRNA and protein expression. It is recognized that the *in vitro* culture conditions employed to mature oocytes might affect gene expression and, consequently, developmental competency in this regard (da Costa et al., 2016). The optimization of the maturation media, therefore, has been a major research priority.

The oocytes extracted from a particular batch of bovine ovaries show various degrees of competence and resume meiosis at different rates depending on their GV status (Le Gal & Massip, 1999). Therefore, the use of meiotic inhibitors in the maturation media can allow the oocytes to achieve cytoplasmic mturation by production of necessary mRNAs and proteins which in turn, increase the developmental competence of the oocytes (Díez et al., 2012).

Many metabolic pathways including active lipid synthesis, transport, storage, and degradation occur during oocyte maturation. In the cumulus cells, lipase activity decreases after maturation, but the glycolysis and pentose phosphate pathways remain unaffected. The lipase activity provides the oocyte with fatty acids at the beginning of maturation and as a result influences oocyte survival and maturation success (de Andrade Melo-Sterza & Poehland, 2021). The crucial function of β oxidation in oocyte nuclear maturation and acquisition of developmental competence has been verified by multiple studies employing pharmaclogical inhibitors. Meiosis and nuclear maturation cannot resume without β

oxidation in bovine oocytes (Dadarwal et al., 2015; Dunning et al., 2014), and an inhibition in β oxidation results in a decreased embryo viability (McKeegan & Sturmey, 2012).

The oocyte cannot sustain fertilization until it has finished the maturation process during which complementary binding and fusion mediating molecules necessary for fertilization are synthesized (Wheeler et al., 1996). During fertilization, sperm attaches to the oocyte, which is next engulfed by the egg and results in the creation of male pronuclear structure. At the same time, a portion of the oocyte's metaphase II chromosomal plate is activated to generate a female pronucleus (Figure 3). The second polar body is formed from the remaining portion of the metaphase plate. A single-cell embryo that can be genetically distinguished from its parents is created when the two pronuclei unite (Ball et al., 1984). The oocyte's plasma membrane aids in sperm-oocyte fusion and binding by promoting the adhesion of sperm to oocyte via integrin-like molecules (Wheeler et al., 1996).

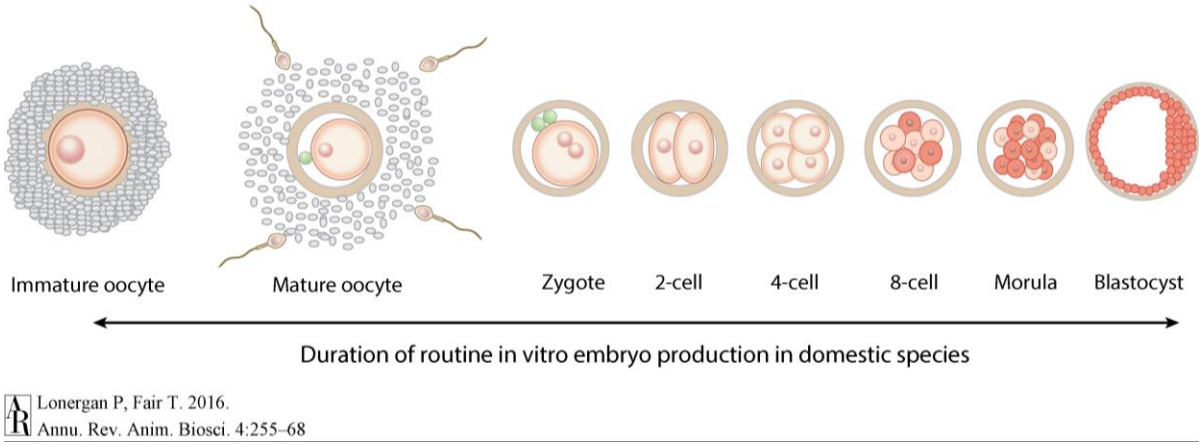


Figure 3: *Ilustration of the maturation and fertilization processes, showing oocyte maturation, cumulus expansion, extrusion of polar body, formation of pronuclei, and the development progress of the embryo to the blastocyst stage* (Lonergan & Fair, 2016, License ID: 1483511-1).

1.5 Parthenogenesis

Parthenogenesis, which happens spontaneously in many invertebrates and some vertebrates, is the growth and development of embryos from oocytes that have not been fertilized by sperm (Bevacqua et al., 2011). Parthenogenesis can be induced chemically using Ca^{2+} ionophores and ethanol. The chemical induction of parthenogenesis can be easier achieved in aged bovine oocytes compared to younger oocytes (Susko-Parrish et al., 1994).

Parthenogenetic embryos can be used as embryonic stem cells. An efficient method to induce parthenogenesis is preventing the extrusion of the second polar body, which results in the maintenance of diploidy and the pseudo-zygote developing into an embryo (Kim et al., 2007).

1.6 Cryopreservation

Cryopreservation is an essential tool for assisted reproduction and long-term storage of genetic material (Dujíčková et al., 2021). The effective freezing of embryos and oocytes has greatly broadened the possibilities for treating infertility (Chen et al., 2003; Lai et al., 2015; Vincent et al., 1990), and advancements in genetic breeding and reproduction technology (Zeron et al., 1999). Cryopreservation also lowers maintenance costs and offers protections against genetic drift, infection, and disease (Zhou & Li, 2013). Furthermore, the ability to effectively cryopreserve bovine oocytes allows for their preservation following ovum pick-up and aspiration from slaughtered cow ovaries, removing the requirement for an IVF facility close to the aspiration sites and maximizing the *in vitro* production (Yamada et al., 2007).

Although frozen semen is widely accessible, it only ensures preservation of half of the animal's genetic resources. Given that oocytes are scarce and susceptible to cryopreservation methods, preserving the other half of the genetic resources of the animal is more difficult. The conservation of female genetics depends on the development of a dependable procedure for maintaining the viability of mammalian oocytes following long-term preservation (Prentice et al., 2011).

Cryopreserved oocytes can be kept until the female exhibits her genetic potential. Effective oocyte cryopreservation would also ease the use of assisted reproduction techniques with oocytes recovered from dead females. This might allow better management of animals with higher production and scientifically important genes (Agca et al., 1998). Conversely, the ethical issues associated with embryo cryopreservation would be minimized (Chen et al., 2003) if female gametes were successfully preserved and stored, as this would enable the advancement of assisted reproductive technologies and medicine in humans (Agca et al., 1998; Díez et al., 2012).

Oocyte cryopreservation has been accomplished using three major techniques of slow-freezing, classical vitrification in straws, and ultrarapid vitrification (Díez et al., 2012). Cryopreservation of bovine oocytes is still limited since routine calf production from frozen oocytes is rare, however, the practicability of this method is proven by the birth of live calves from cryopreserved oocytes (Zhou & Li, 2013).

Slow freezing is dependent on extracellular and intracellular fluid exchange to dehydrate the cells in controlled cooling rates. However, this method risks extensive cell shrinkage and expansion during the addition and removal of CPAs which might result in irreversible damage to oocytes in the form of osmotic shock and intracellular ice crystallization (Díez et al., 2012; Yamada et al., 2007). Other risks associated with this technique are formation of intracellular ice crystals which happens if the cells are cooled too rapidly, not allowing proper dehydration of the oocytes (Díez et al., 2012), and affecting the lipid phase transition in low temperatures, resulting the loss of functionality of the membrane. (Zeron et al., 1999).

Regarding the cryopreservation of mammalian embryos, particularly oocytes, vitrification has emerged as a feasible and promising substitute for conventional methods and is has proven to be more successful than slow freezing (García-Martínez et al., 2020). The toxic and osmotic damage caused by crystallization are avoided by vitrification (García-Martínez et al., 2021). Crystallization is impossible if the specimen is chilled below the “glass transition temperature”. The ultrarapid vitrification process has taken the place of the traditional vitrification in straws for oocytes and embryos, due to higher success rate following increased cooling/warming rates, circumventing the chilling sensitivity of oocytes (Díez et al., 2012; Le Gal & Massip, 1999; Vieira et al., 2002).

1.7 Vitrification

Vitrification is a process that involves immediate submergence of material in liquid nitrogen to achieve rapid cooling rates, hence reducing the amount of time the material is exposed to CPA solutions (Yamada et al., 2007). Vitrification involves a series of steps where oocytes are exposed to escalating concentrations of permeable CPAs, often combined with non-permeable CPAs to prevent ice crystallization both inside and outside the cells, followed by rapid cooling in liquid nitrogen (Marques et al., 2018). The high cooling and warming rates of vitrification resolves both ice crystal formation and membrane phase transition (Arav et

al., 2018). Conversely, oocyte warming involves gradually reducing concentrations of non-permeable CPAs to manage hypoosmotic shock during rehydration (Marques et al., 2018).

Vitrification of bovine embryos is simple, efficient, and widely used (Sprícigo et al., 2015). However, vitrification of bovine COCs has shown limited success since the COCs are more sensitive to chilling injuries and have a complex structure (Gutnisky et al., 2020; Prentice et al., 2011). The sudden exposure of oocytes to high concentrations of CPAs is toxic to the cells. Therefore, the optimal exposure time of oocytes to CPAs is important to avoid physical and chemical stresses to the cells, therefore oocytes are often exposed to equilibrium media which has a lower concentration of CPAs compared to the vitrification media (Díez et al., 2012; Prentice et al., 2011).

Many vitrification techniques have been used over the years to minimize the toxicity and harmful effects of the CPAs. Out of these techniques, reduction of the vitrified volume has been of major interest (Buschiazzo et al., 2017). The application of minimum volume techniques, such as open pulled straws, cryoloop, electron microscopy grids, and droplet-based vitrification has proven to be the most successful recent advancements in vitrification methods, as the small sample size allows a significant increase in cooling and warming rates, resulting in increased survival of oocytes (Zhang et al., 2012).

The permeability of the plasma membrane has a significant role in establishing the ideal circumstances for oocyte vitrification (Jin et al., 2011). The movement of water and CPAs through the oocyte membrane can occur by simple diffusion through the lipid bilayer, or facilitated diffusion through channels (Díez et al., 2012; Jin et al., 2011). Permeability of water and CPAs in bovine oocytes is lower than in morulae and blastocysts, and flow rates are dependent on temperature for simple diffusion of water and CPAs through the membrane (Díez et al., 2012).

1.8 Cryoprotective agents

Many studies have been conducted on permeating and non-permeating CPAs to reduce osmotic, toxic, and other damages during cryopreservation. The main permeating CPAs utilized in the cryopreservation process include ethylene glycol (EG), propylene glycol (PG), glycerol (GLY), and dimethyl sulfoxide (DMSO). The most common combination used for vitrification of gametes is EG + DMSO. Oocytes have selective tolerance to different CPAs

at different meiotic stages. Different CPAs have different transition temperature, penetration rate, and toxicity. Exposure temperature is also an important factor in the osmotic effects of the CPAs, as exposure to CPAs in higher temperatures shows less shrinkage; however, high temperatures increase the toxicity of CPAs (Díez et al., 2012). These osmotic and chemical stresses can directly influence the developmental capacity of oocytes by damaging cellular structures such as cytoskeleton and meiotic spindle. Therefore, an optimization of the CPA concentrations before vitrification can prove beneficial to the success of cryopreservation (García-Martínez et al., 2022; Park et al., 2015).

Different routes allow CPAs to enter the oocyte; these pathways rely on the CPA itself, as well as the developmental stage of the cell (Mogas, 2019). The movement of CPAs through the plasma membrane through simple diffusion occurs regardless of the temperature. The process of simple diffusion is slow due to low permeability of oocyte cell membrane to water and CPAs; however, this process happens at a higher rate upon movement of water and CPAs by facilitated diffusion through channels (García-Martínez et al., 2022; Jin et al., 2011). In previous studies, it has been demonstrated that permeability of the oocytes to CPAs and water varies among different stages of maturation and oocytes at the MII stage have a higher permeability to CPAs and water compared to oocytes at the GV stage (Magnusson et al., 2008).

The addition of non-toxic and non-permeating CPAs such as sugars (sucrose, glucose, trehalose, sorbitol) decreases the detrimental effects of CPAs on oocyte survival. The addition of sucrose as a non-permeating CPA has proved beneficial to cellular membrane stability (Dujíčková et al., 2021). Thus, incorporation of sucrose in the vitrification media mitigates the osmotic impact (Díez et al., 2012). In recent studies use of a combination of permeable and non-permeable CPAs has been suggested to mitigate the toxic effects of a sole highly concentrated CPA (Dujíčková et al., 2021; García-Martínez et al., 2022).

1.9 Cryodamage

Cell viability and functionalities can be preserved for a long time by subjecting the oocytes to very low subzero temperatures, but during the cooling and warming process, some physical stresses can damage the cells (Gutnisky et al., 2020; Hwang & Hochi, 2014). The cryopreservation of mammalian oocytes is usually halted by disadvantages such as low permeability coefficient of plasma membrane and the presence of the zona pellucida slowing

the movement of water and CPAs (García-Martínez et al., 2020), escalation of chilling sensitivity due to the high lipid content and low surface-to-volume ratio of the oocytes (Le Gal & Massip, 1999), susceptibility of the meiotic spindle to cryoinjuries at the MII stage, and the induced premature cortical granule exocytosis following freezing/thawing (Díez et al., 2012).

1.9.1 Damage to cellular structures

Cryopreservation of female gametes can result in various forms of damage, including disorganization of oocyte cytoskeleton, abnormalities in spindle and chromosome structure, changes in cortical granule distribution (Stachowiak et al., 2009), increased rates of polyspermy, and alterations in gene expression (García-Martínez et al., 2020; Marques et al., 2018). The disorganization of the meiotic spindle and depolymerization of microtubules are the main risks of oocyte cryopreservation at the MII stage (Díez et al., 2012; Men et al., 2003; Rojas et al., 2004; Zeron et al., 1999), which may result in aneuploidy (Vincent et al., 1990). Cytochalasin is a suggested chemical which mitigates the disruption of microfilaments by preventing cytokinesis without compromising karyokinesis (Magnusson et al., 2008).

The cytoskeleton is one of the biological elements that is frequently harmed during cryopreservation (Díez et al., 2012; Prentice et al., 2011). Shrinkage and alterations in the oocyte shape caused by high concentrations of CPAs result in irreversible changes in the cytoskeleton structure.

Additional negative consequences of cryopreservation include the breakdown of intracellular coupling via gap junctions between cumulus cells and the oocyte, and fracture damage in zona pellucida (Hwang & Hochi, 2014; Stachowiak et al., 2009). Therefore, the main factors used to evaluate an oocyte's post-thaw viability are assessment of membrane degeneration, cytoplasmic abnormalities, and fractures in zona pellucida (Hwang & Hochi, 2014).

Cryopreservation of oocytes at the GV stage is difficult due to lower permeability to CPAs and water and high sensitivity of the cytoplasmic membrane and gap junctions which leads to a disruption of connections between the oocyte and cumulus cells (Díez et al., 2012; Magnusson et al., 2008; Rojas et al., 2004). The existence of these gap-junction-based intercellular connections is crucial for the oocyte's metabolic interaction with cumulus cells during growth and development of the oocytes (Rojas et al., 2004). However, freezing of

oocytes at this stage overcomes the risk of damage to the meiotic spindle as it is not organized yet at the GV stage (Magnusson et al., 2008; Vieira et al., 2002), and at this stage, the nuclear envelope contains the genetic material (Le Gal & Massip, 1999; Rojas et al., 2004). Although presence of cumulus cells in cryopreserved oocytes at the GV stage show an increase in developmental competence (Aguila et al., 2020), no observable difference has been recorded for oocytes at the MII stage (Díez et al., 2012).

Vitrification can damage the cumulus cells by causing necrosis in the nuclei and creating large numbers of cytoplasmic vacuoles that damage the plasma membrane (Zhou & Li, 2013). The alterations in plasma membrane's physical characteristics during the *in vitro* growth indicate a probability of oocyte maturation influencing their sensitivity to low temperatures. Additionally, it is suggested that small lipophilic molecules like antifreeze proteins and butylated hydroxytoluene (BHT) protect membrane lipids against low temperature damage by acting as antioxidants, agents that could alter the membrane lipids' physical characteristics directly, or inhibitors of recrystallization (Zeron et al., 1999).

Previous studies have concluded that low temperatures have negative effects on the integrity and structure of DNA. In the studies performing Comet assay on the cryopreserved oocytes, DNA fragmentation was relatively common in vitrified bovine oocytes (Men et al., 2003; Stachowiak et al., 2009).

1.9.2 Lipid damage

Chilling injuries can be divided in two different categories of direct and indirect chilling (Arav et al., 1996). The primary locations where direct chilling injury occurs are the cytoplasmic membrane, namely the lipids within the membrane. The high lipid content of the oocyte cells is a major factor in hindering cryopreservation (Prates et al., 2014) due to its ability to intensify morphological and functional damages to cryopreserved oocytes (Sprícigo et al., 2015). However, not many studies have been conducted on the correlation of CPAs and lipid droplets (Amstislavsky et al., 2019).

Low temperatures can result in lipid phase transition by transition of lipids from liquid crystalline phase to gel phase (Mogas, 2019), which can have detrimental effects on membrane integrity and cause cell death if irreversible alterations take place (Horvath & Seidel, 2006). Numerous studies have linked lipid peroxidation to decreased embryo quality and halted development (McKeegan & Sturmey, 2012).

It has been observed that the abundance of cytoplasmic lipid droplets results in difficulties in successful vitrification (Dode et al., 2023). Numerous lipid droplets are found in the cytoplasm, and it has been proposed that a high lipid-to-protein ratio can significantly impact the sensitivity of oocytes to cryopreservation (Mogas, 2019; Rojas et al., 2004). Over the years, numerous attempts have been made to improve bovine oocyte vitrification efficiency (Sprícigo et al., 2015). Modification of the lipid membrane composition prior to cryopreservation either by incubation with cholesterol-rich media or addition of linoleic acid albumin or modification of the lipid phase transition temperature has proved beneficial to the success of the freezing procedures and preventing chilling injuries (Díez et al., 2012; Horvath & Seidel, 2006). The potential role of intracellular lipids in oocyte sensitivity to cold temperature has been investigated by polarization and removal of granule lipids by centrifugation which shows no beneficial result regarding oocyte quality and cryosurvival (Zeron et al., 1999). Damage to the lipid content can be decreased by ultra rapid vitrification which minimizes the time spent at the lipid phase transition temperature (Horvath & Seidel, 2006).

Oxidative stress can also affect the lipid droplets by lipid peroxidation which generates diverse lipid metabolites including lipid peroxyl radicals. Lipid peroxyl radicals remove hydrogen atoms from nearby polyunsaturated fatty acids, which destabilizes the plasma membrane, affecting the membrane integrity and fluidity (Bollwein & Bittner, 2018).

1.9.3 Damages caused by cryopretective agents

CPA damages refer to damages caused by osmotic swelling, CPA toxicity, and intracellular ice formation as related to the permeability of the cellular membrane (Jin et al., 2011; Yamada et al., 2007). Assorted parameters to CPA toxicity are CPA concentration, exposure length, and exposure temperature (García-Martínez et al., 2021). A significant issue in cryobiology is the potential harmful mechanical stresses that cells undergo when they are subjected to hypertonic solutions facilitating the exchange of intracellular water with cryoprotective agents (CPAs). This issue is especially prominent in the vitrification procedure as high concentrations of CPAs are used to prevent ice crystallization of intracellular structures. Oocytes and zygotes are highly susceptible to osmotic stress, since these cells have a much larger diameter compared to normal mammalian cells (120 μm vs. 10μm on average) resulting in a high surface to volume ratio (Le Gal & Massip, 1999; Yamada et al., 2007) and a larger volume of water that needs to be replaced with CPAs

before cryopreservation. To preserve the largest possible cell volume, exposure to non-permeable solutes – which are essential for the completion of vitrification but prevent any cell re-expansion – is saved until the last step before vitrification. (Lai et al., 2015). Furthermore, the osmotic shock affecting the oocytes during equilibration leads to contraction and contortion of oocytes, potentially damaging the cytoskeleton (Mogas, 2019).

Cortical granule exocytosis is a calcium-dependent occurrence (Mogas, 2019), hence an increase in the intracellular Ca^{2+} concentrations during cryopreservation, resulting from the effects of CPAs such as DMSO (Vincent et al., 1990), and the drop in temperature, would trigger the cortical granule exocytosis (Zhou & Li, 2013). In previous studies, many vitrified oocytes showed signs of polyploidy after development which can be a result of retention of the second polar body (Arav et al., 1996; Horvath & Seidel, 2006) or polyspermic fertilization following premature cortical granule exocytosis (Arav et al., 1996).

In non-freezing conditions, exposure to DMSO can increase the zona pellucida's hardening and resistance to sperm penetration, additionally, DMSO can cause disruptions to the cytoskeleton and metaphase plate organization (Vincent et al., 1990).

1.9.4 Oxidative stress

A disruption in the balance of prooxidants and antioxidants leading to a higher level of prooxidants is known as oxidative stress which leads to potential damage. The presence of an immoderate amount of reactive oxygen species (ROS) due to an imbalance in the generation and elimination of ROS results in oxidative stress (Olexiková et al., 2022; Poljsak et al., 2013).

The damages resulted from the vitrification-warming procedure mostly cause oxidative stress (Olexiková et al., 2022). Vitrification can lead to increased levels of ROS, affecting the morphological integrity of the oocyte's mitochondria and oocyte competence. The presence of excessive amounts of ROS can cause mitochondrial damage, chromosome misalignment, and disassembly of the meiotic spindle. ROS can also activate the caspase cascade, resulting in apoptosis in oocytes and early developed embryos (García-Martínez et al., 2020). The occurrence of osmotic stress during the process of cryopreservation can also lead to oxidative stress by increasing the oxidative metabolism of mitochondria (Figure 4). Oxidative stress has been suggested as one of the main unfavorable factors in fertilization (Olexiková et al., 2022).

ROS also remove the hydrogen from deoxyribose carbons in the DNA that causes DNA damage in the form of strand breaks and base releases. These radicals also attack bases leading to base alterations such as oxidation of guanine and consequently cause mutagenic lesions (Bollwein & Bittner, 2018).

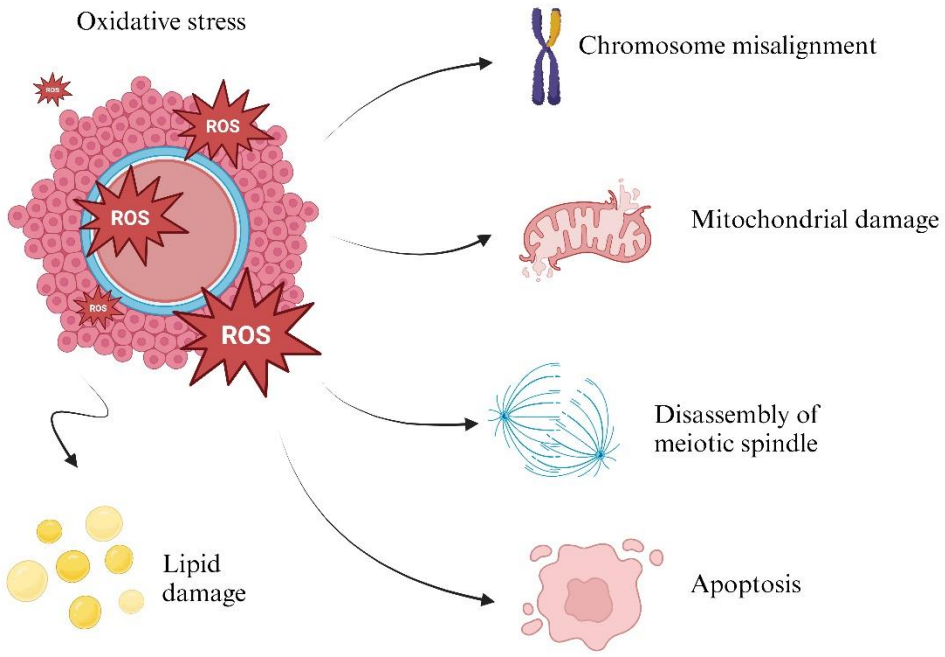


***Figure 4**: Simple illustration depicting the effects of oxidative stress on the oocyte. Created with Biorender.com*

1.10 Confocal microscopy

Confocal microscopy relies on the basic premise of focusing both the lighting and detection lenses on the same diffraction-limited spot. This spot is then moved across the subject to create the entire image on the detector. Confocal imaging provides optical sectioning and reduces the haze seen in normal light microscopy with thick and highly scattering samples by illuminating the full field of view while leaving much of the picture beyond the focal plane (Elliott, 2020). This is accomplished by utilization of the illumination and detection-side pinhole apertures in the same conjugate image plane, focusing the beam of light on a spot inside the cell and blocking the lights outside the focal plane through the detection pinhole (Elliott, 2020; Nwaneshiudu et al., 2012) (Figure 5).

In fluorescence confocal microscopy, the excitation light is often produced by a laser at a wavelength that will also excite a particular fluorophore. In certain cases, more than one

fluorophore can be utilized simultaneously, and distinct sections of the material can be identified by varying the emission wavelength or the excitation light (Nwaneshiudu et al., 2012).

1.10.1 BODIPY 493/503 staining

BODIPY 493/503 is a cell permeable lipophilic fluorescent dye that exhibits intense green fluorescence. This dye can be used to visualize neutral lipid droplets in live or fixed cells, and it can be combined with various staining or labeling techniques (Warzych et al., 2017).

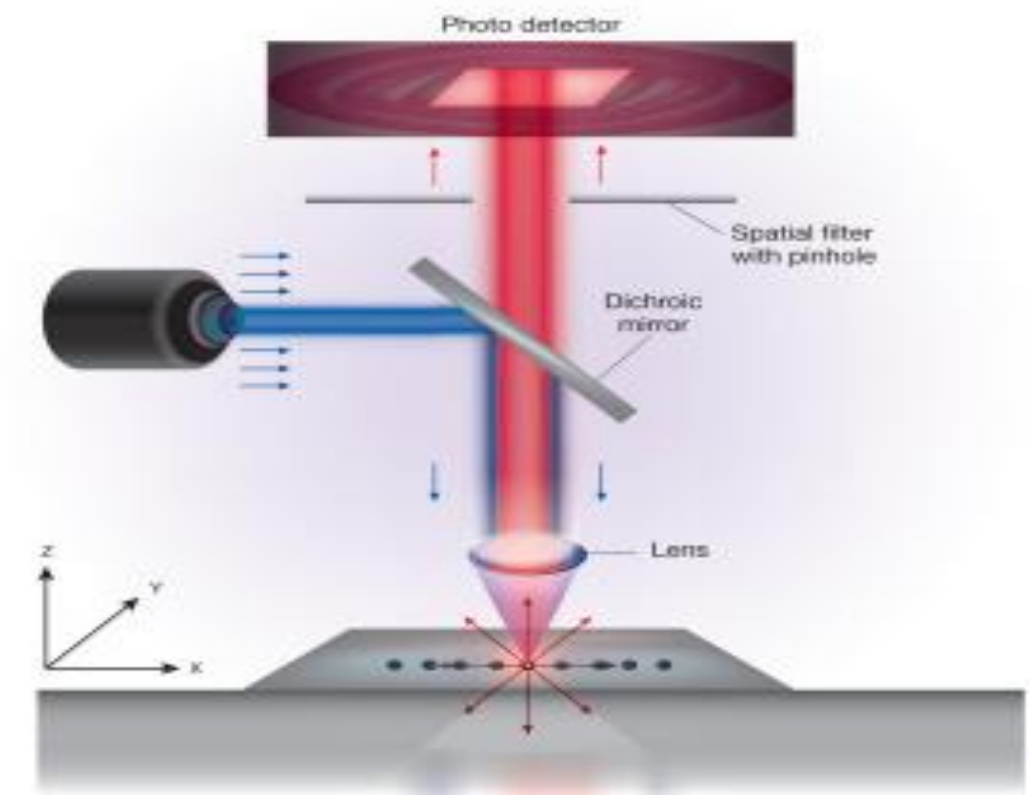


Figure 5: *Simplified view of confocal microscopy, depicting the laser source, dichroic mirror, lens, excitation and emission of the fluorophores and the detection filters with pinholes* (Nwaneshiudu et al., 2012, License Number: 5787120463008)

1.11 Animal modeling

In recent years, oocyte vitrification has shifted from an experimental procedure to a clinical approach. Oocyte vitrification is currently advised for young women who are delaying motherhood or who are receiving cytotoxic drug treatments for cancer or other illnesses that

may result in a loss of reproductive abilities (Mogas, 2019; Smith et al., 2011). However, a lack of proper vitrification protocol and proper training of embryologists leads to varying success rate in human oocyte vitrification (Arav et al., 2018).

One of the most studied animals for assisted reproductive techniques is cattle, and human oocytes can be cryopreserved more effectively by using bovine oocytes as a model (Kuwayama et al., 2005). The majority of the development and implementation of this methodology focused on enhancing genetic selection and reducing generation intervals, primarily using oocytes sourced from slaughterhouses and frozen semen. This has led to a highly effective model for clinical use in both cattle and humans (Sirard, 2018).

1.12 Aim of the study

The long-term objective of the Zygote project is to establish an efficient method for cryopreservation of bovine oocytes and embryos. Therefore, the aim of this study was to find an optimal concentration and mixture of CPAs for the vitrification of bovine oocytes. To study the effects of different CPAs, lipid damage was chosen as the main point of focus due to its importance in the success of the cryopreservation.

To reach these goals the following tasks were conducted:

- Assessing the development of oocytes into blastocysts after exposure to different concentrations of CPAs
- Staining and visualization of lipid droplets after exposure to different concentrations of CPAs
- Vitrification of oocytes at the MII stage after exposure to the selected CPAs and evaluation of lipid droplets and development competence post-thaw

2. Material and Methods

2.1 Experimental design

The study was divided into two separate experiments. Bovine oocytes were aspirated from ovaries obtained from slaughtered unknown cows and heifers. COCs were selected and incubated until they reached the MII stage. In the first experiment, bovine oocytes were exposed to different concentrations of five CPAs for 10 min. CPA-treated oocytes were fertilized and the development of the embryos was recorded. Small groups of CPA-treated oocytes (3-5 oocytes per treatment) were stained using BODIPY (493/503) to observe and evaluate the effects of CPAs on the integrity and distribution of lipid droplets in the oocytes. The data obtained from the development of embryos and staining of oocytes was used to select four optimal CPAs for cryopreservation.

The second experiment was focused on cryopreservation using the droplet vitrification method. The oocytes were treated with the four selected CPAs, vitrified, and thawed. The vitrified-warmed oocytes were separated into two groups, 5 oocytes for staining with BODIPY (493/503) and 15-20 for IVF. The development of fertilized oocytes was recorded. Results from staining and fertilization were used to determine the effects of CPAs on oocytes during the vitrification process. Figure 6 shows a summary of the workflow of the study.

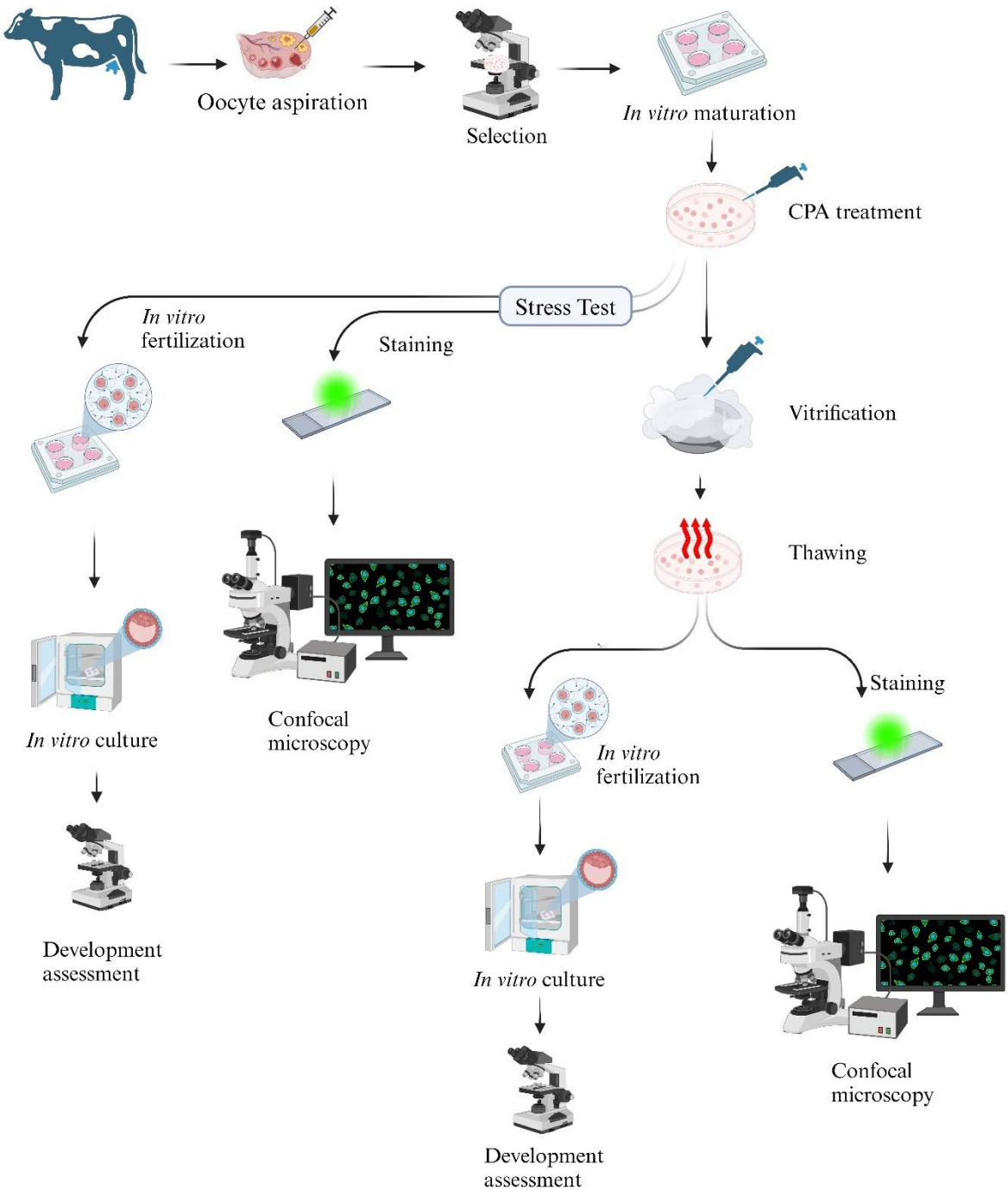


***Figure 6**: Schematic illustration of the experimental design and workflow of the study. Figure created with BioRender.com.*

2.2 Chemicals and solutions

All chemicals have been purchased from Sigma-Aldrich (Oslo, Norway) unless otherwise stated. All media used for *in vitro* production (IVP) of bovine embryos were acquired from IVF Biosciences (Falmouth, UK). Physiological saline solution (0.9% NaCl with 0.1 mg/ml kanamycin) was used for transportation and washing of bovine ovaries. Heparin (5000 IU/ml) was used during COC aspiration to decrease the blood clotting. BO-Wash (washing medium) was used for all the washing steps of COCs, denuded oocytes, and fertilized oocytes. BO-IVM medium (oocyte maturation medium) was used for the *in vitro* maturation (IVM) of oocytes to the MII stage. Fertilization was performed using BO-IVF medium (fertilization medium), and BO-SemenPrep (semen preparation medium). BO-IVC medium (embryo culture medium) was used along with BO-Oil (oil for medium overlay) for *in vitro* culture (IVC) of the embryos.

Ethylene glycol (EG, ethane-1,2-diol), propylene glycol (PG, propane-1,2-diol), glycerol (GLY, propane-1,2,3-triol), dimethyl sulfoxide (DMSO), and methanol (MeOH) were used as CPAs in different concentrations, acquired by diluting the CPAs in phosphate-buffered saline (PBS). The PBS solution was prepared using the following recipe: 137 mM NaCl, 2.7 mM KCL, 8.1 mM $Na_2HPO_4.2H_2O$, and 1.76 mM KH_2PO_4, with the pH set to 7.4. The solution was sterilized by autoclaving and stored at 4 °C.

BODIPY™ 493/503 dye (10 mg/ml, Thermo Fisher Scientific, Waltham, MA, USA) was used for the staining of lipid droplets, along with triton X-100 (12 mg/ml, Thermo Fisher Scientific, Waltham, MA, USA), polyvinylpyrrolidone (PVP, 7 mg/ml). oocytes were fixed in 4% paraformaldehyde (PFA) and mounted on slides using a fluorescence mounting medium (Dako, Glostrup, Denmark).

2.3 Bovine oocyte collection and *in vitro* maturation

Over a period of 5 weeks, bovine ovaries from unknown cows and heifers were collected from a local slaughterhouse weekly and transported in 0.9% saline to the laboratory where they were washed three times by gentle massaging in a solution of 0.9% saline and 0.1 mg/ml kanamycin, then kept at 33 °C during the aspiration process. COCs were aspirated from follicles on the surface of the ovaries with an approximate diameter of 3 – 8 mm, using an 18-gauge needle (Figure 7).

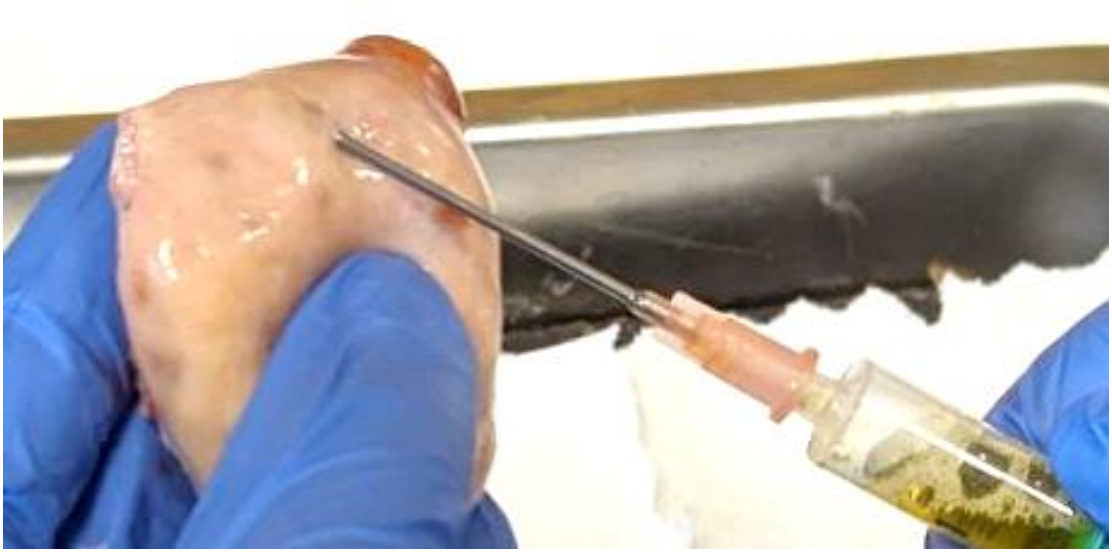


Figure 7*: Aspiration of cumulus-oocyte complexes from visible follicles on bovine ovaries. Image taken during the current study.*

The aspirated fluid containing COCs was incubated at 36 °C for 10 min in tubes containing 140 µl heparin (5000 IU/ml). The settled COCs were transferred to a 90 mm petri dish (Thermo Fisher Scientific, Waltham, MA, USA) containing 6 ml of pre-warmed BO-Wash media. COCs with 3 – 5 layers of cumulus cells (Figure 8) were selected using a stereo microscope and washed three times in 35 mm NUNC IVF dishes (Thermo Fisher Scientific, Waltham, MA, USA) with 2 ml of BO-Wash media. The washed COCs were selected in groups of 23 – 25 and rinsed in 100 µl droplets of BO-IVM media. COCs were then moved into each well of a 4-well NUNCLON dish (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 µl of BO-IVM media. Afterwards, the plate was incubated at 38.8 °C and 6% CO_2 in a humidified environment for 21 h for the oocytes to mature until the MII stage (Figure 8).

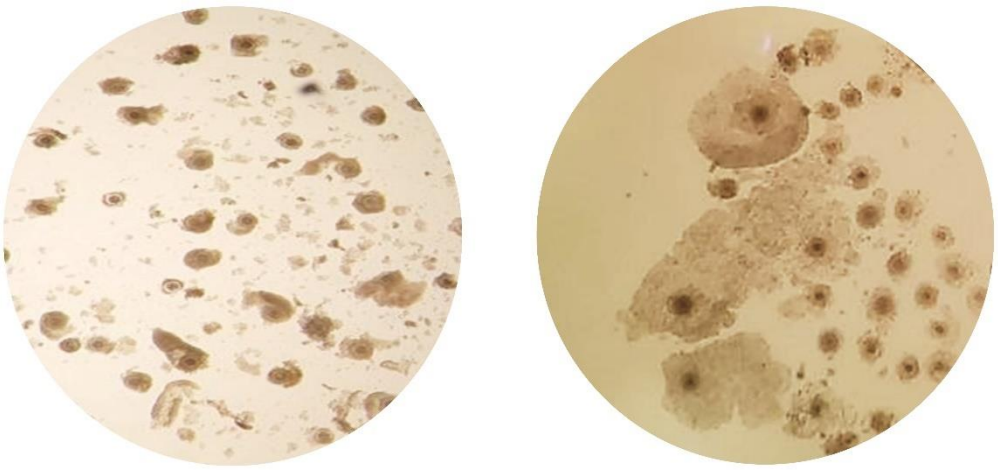


Figure 8*: Selected cumulus-oocyte complexes with 3 – 5 layers of cumulus cells before the wash step (left). Matured cumulus-oocyte complexes after 21 h of incubation in IVM media (right). Images taken during the current study.*

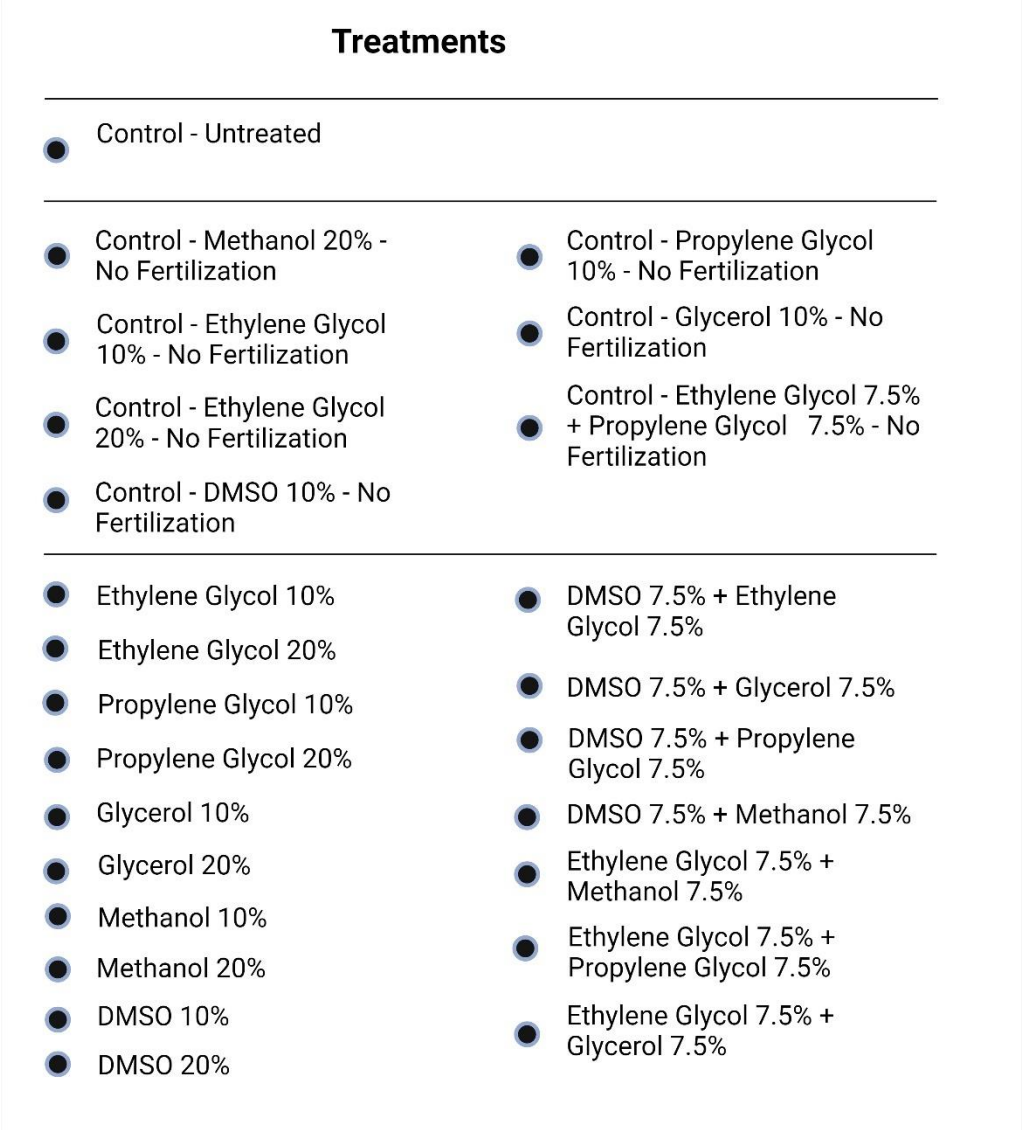
2.4 Cryoprotective agents stress test on bovine oocytes

The CPAs were diluted with PBS and prepared as follows:

- EG 7.5%, 10%, and 20%
- PG 7.5%, 10%, and 20%
- GLY 7.5%, 10%, and 20%
- MeOH 7.5%, 10%, and 20%
- DMSO 7.5%, 10%, and 20%

A total of 25 wells were prepared containing the treatments shown in Table 1. For each treatment, matured COCs were moved to the well containing the respective CPA and incubated for 10 min at room temperature (RT). For the samples from the negative control with no treatment and no fertilization, the COCs were incubated in PBS for 10 min at RT. The COCs were rinsed once in 100 µl droplets of BO-IVM medium before being returned to the IVM wells. Seven CPA concentrations were selected for parthenogenesis control treatments where samples were treated but not fertilized.

***Table 1**: List of all treatments used for the cryoprotective agents stress test.*



2.5 BODIPY 493/503 staining

From each well 3 – 5 COCs were selected for staining. The COCs were moved to a 35 mm plate containing warm BO-Wash media and the cumulus cells were removed by gentle pipetting. The denuded oocytes were washed twice in 2 ml of BO-Wash media and rinsed in 100 µl droplets of 0.2% PVP. The oocytes were thereafter fixed in 50 µl droplets of 4% PFA under oil overnight, stored at 4 °C.

The fixed oocytes were rinsed in 100 µl droplets of 0.2% PVP and moved to 400 µl of 0.1% Triton X-100 for 30 min at RT to permeabilize the cell membrane. After 30 min of

permeabilization, oocytes were moved to 400 µl of BODIPY 493/503 20 µg/ml for 1 h at RT.

The stained oocytes were mounted on microscope slides using 6 µl of Dako mounting medium and incubated at RT for 24 h before being stored at -20 °C until use. The slides were imaged and analyzed using a Leica DM-i8 fluorescence microscope (Thermo Fisher Scientific, Waltham, USA) with the LAS X program (Leica Microsystems, Wetzlar, Germany) with a fluorescein isothiocyanate (FITC) filter using a 488 nm laser at 5% intensity and average gain of 380 V to study the effects of CPAs on the distribution and integrity of lipid droplets in the oocytes.

2.6 *In vitro* fertilization of bovine oocytes

The matured COCs were rinsed in 100 µl droplets of BO-IVF media and a volume of 60 µl containing groups of approximately 20 COCs was transferred to each well of a 4-well plate containing 400 µl of BO-IVF media and incubated at 38.8 °C and 6% CO_2 under humidified air until the preparation of semen.

Cryopreserved semen samples for all experiments were obtained from a single ejaculate of a Norwegian red bull. Two frozen semen straws were thawed in a water bath at 37 °C for 1 min. The semen was transferred to 4 ml of pre-heated BO-SemenPrep and washed twice by centrifugation at 330 x g for 5 min. The supernatant was removed after each wash and the pellet was diluted 1:4 with PBS in preparation for CASA (Microptics, Barcelona, Spain). Upon evaluation of the motility and concentration, a final volume of 2×10^6 cells / ml in 500 µl IVF media was added to each well of the IVF plate containing oocytes. The oocytes were co-incubated with the sperm cells at 38.8 °C and 6% CO_2 for 18 hours in a humid environment.

2.7 *In vitro* culture of embryos

The presumptive zygotes were transferred from each IVF well to individual falcon tubes containing 2 ml of preheated BO-wash media (35 °C) and vortexed vigorously for 2 min to remove the cumulus cells of the oocytes. Denuded fertilized oocytes were transferred to 35 mm plates containing 2 ml of BO-wash media and were washed three times before being rinsed in 100 µl droplets of BO-IVC media.

The fertilized oocytes were subsequently moved in groups of approximately 20 to individual wells of a 4-well NUNCLON dish containing 500 µl of preheated (38.8 °C) IVC media, covered with 350 µl of oil. The dishes were incubated at 38.8 °C, 6% O_2, 6% CO_2, and 88% N_2, under humid air for eight days. The cleavage rate of the embryos was assessed on day 2 and the blastocyst rate of the embryos was evaluated and recorded on day 7 and 8 of the incubation period using a Leica DM-IL inverted microscope (Ortomedic AS, Lysaker, Norway) shown in Figure 9. The cleavage and blastocyst rates were calculated by dividing the number of cleaved embryos, and blastocyst respectively, by the total number of cultured oocytes.

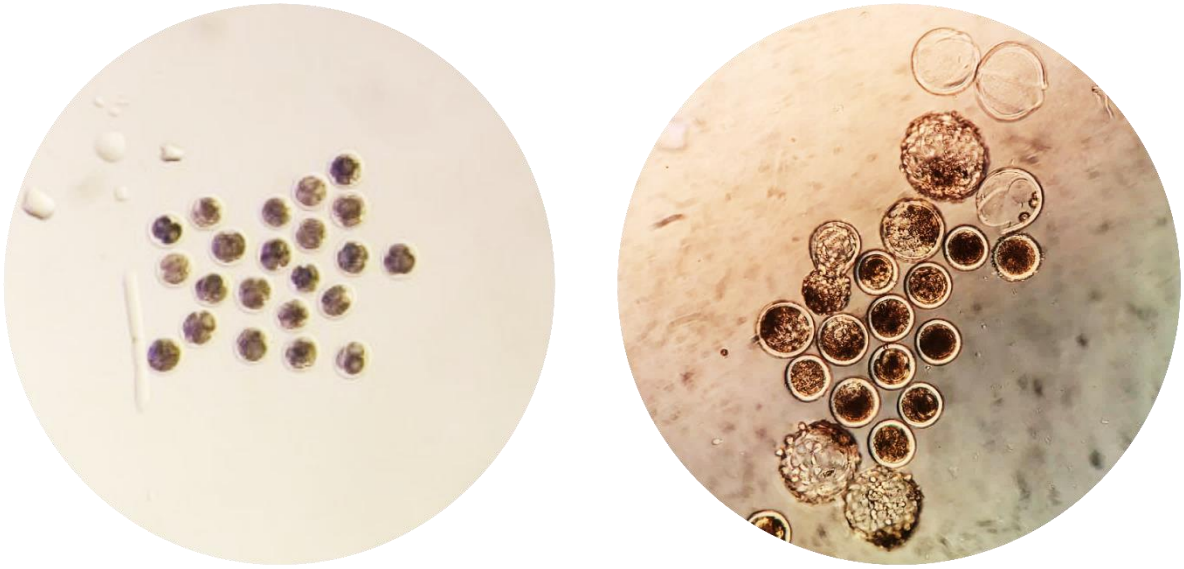


Figure 9*: Cultured bovine embryos visible in different stages of development from 2-cell stage on day 2 (left) (4X magnification), to hatched embryos on day 8 (right) (10X magnification). Embryos were incubated at* 38 °C, 6% O_2, 6% CO_2, and 88% N_2 *after fertilization. Images taken during the current study.*

2.8 Vitrification and thawing

The COCs were collected and prepared using the same method as previously described (see 2.3) and incubated at 38.8 °C and 6% CO_2 under humid air in BO-IVM media for 22 h to mature until the MII stage. COCs were denuded in warm (36 °C) BO-wash media and kept at 38.8 °C and 6% CO_2 under humid air until exposure to the selected CPAs.

The CPAs were diluted with PBS as follows:

- PG 10% (equilibration), and 20% (vitrification)
- GLY 10% (equilibration), and 20% (vitrification)
- DMSO 7.5% + EG 7.5% (equilibration), and DMSO 15% + EG 15% (vitrification)
- EG 7.5% + PG 7.5% (equilibration), and EG 15% + PG 15% (vitrification)
- Sucrose 0.1%, 0.2% (thawing), and 0.5% (vitrification and thawing)

The denuded oocytes were moved to separate equilibration media containing 500 µl of each of the equilibration CPAs and incubated for 8 min at RT to avoid excessive osmotic stress. From the equilibration media, oocytes were transferred to their respective vitrification media containing 500 µl of the vitrification CPAs and 200 µl of 0.5% sucrose for 30 sec. The concentrations were revised for a second experiment and the oocytes were incubated in 500 µl of a mixture of EG 15% + PG 15% and 0.5% sucrose for 30 sec before vitrification.

Oocytes were then dropped one by one in 2 µl droplets into a small container filled with liquid nitrogen to be vitrified. The vitrified oocytes from each treatment were moved to separate cryotubes and stored at -196 °C until thawing.

The vitrified oocytes were thawed in 500 µl of preheated (37 °C) 0.5% sucrose and subsequently washed in 100 µl droplets of 0.2% and 0.1% sucrose for 1 min in each concentration. The oocytes were rinsed in 100 µl droplets of BO-IVM medium and moved to individual wells in a 4-well NUNCLON dish containing 500 µl of BO-IVM media. The dishes were incubated at 38.8 °C, 6% CO_2 for 3 h under humid air.

After 3 h of recovery, 5 oocytes from each sample were stained with BODIPY 493/503 (see 2.5) to be analyzed for possible lipid damage during the vitrification procedure. Further, 18 – 20 oocytes from each sample were fertilized and cultured (see 2.6 and 2.7). Development data was evaluated and recorded on day 2 of incubation.

2.9 Statistical analysis

The dataset comprised records of treatments administered to oocytes and the corresponding outcomes in terms of blastocyst formation. Each record included information on the treatment administered and the total number of oocytes, along with the number of blastocysts and failures observed. Treatments encompassed different concentrations and combinations of cryoprotectants, including DMSO, EG, GLY, PG, and MeOH.

Logistic regression analysis was applied to evaluate the correlation between the treatments and the probability of blastocyst development. Treatment variables were included as predictors, and the outcome variable, blastocyst development, was modeled as a binary response (presence or absence of blastocysts). To take into consideration the binary nature of the response variable, the model was fitted using the “glm” function in R using a binomial family. The logistic regression model was formulated as follows:

glm(formula = cbind(Blastocysts, Failures) ~ Treatment, family = binomial, data = data)

where Blastocysts and Failures represent the counts of blastocysts and non-blastocyst outcomes, respectively, and Treatment denotes the categorical variable indicating the treatment administered.

The goodness-of-fit of the logistic regression model was evaluated using the deviance statistic, with lower values indicating better model fit. Additionally, an analysis of deviance was conducted to compare the full model with treatment effects to a null model with no treatment effects. Post-hoc tests, specifically Tukey's Honestly Significant Difference (HSD) test, were performed to examine pairwise differences between treatment levels.

All statistical analyses were conducted using R version 4.4.0. The following R packages were utilized: “lme4” for fitting the logistic regression model, “car” for performing the analysis of deviance, and “multcomp” for post-hoc testing.

3. Results

3.1 Embryo development post oocyte stress test

As shown in Table 2, the development of the control and treated samples was recorded 48 h after IVF. The negative control with no treatment was set as a point of comparison, with an 80% cleavage rate, and 34% of the cells at the 4-cell+ stage, calculated by dividing the number of cleaved embryos by the number of total oocytes fertilized. The large number of oocytes in the untreated control sample is due to the repetition of this sample in each replicate of four experiments that led to this final result. The same applies for samples treated with PG 10%, GLY 10%, DMSO 10%, and EG 7.5% + PG 7.5%, as these samples had a third replicate compared to two replicates for the rest of the samples.

Table 2 shows seven negative controls treated with different CPAs, but not fertilized. These were included to measure possible parthenogenesis among the CPA-stressed samples. Parthenogenesis was observed in two samples, EG 10% (7.14%), and EG 7.5% + PG 7.5% (5.26%). One embryo from the unfertilized EG 10% sample developed until the morula stage before degenerating. The cleaved embryo from the unfertilized EG 7.5% + PG 7.5% sample degenerated at the 3-cell stage.

Table 3 shows the recorded development of the treated samples on day 8 after IVF. The untreated control sample with a 47% blastocyst rate and 20% of the cells at the hatched stage is set as a point of comparison. The highest blastocyst rate amongst the treated samples was obtained with EG 7.5% + PG 7.5% (40%). The higher percentage of embryos at the hatched stage indicates a faster development, as hatched embryos are at the final stage of embryo development and are ready to be attached to the uterine wall.

All seven unfertilized and CPA-treated negative controls showed an absence of development, indicating no parthenogenesis at this stage. The embryos produced by parthenogenesis degenerated before reaching the blastocyst stage.

Table 2. Cleavage rates*: Recorded data on day 2 after IVF. Total count of embryos at each development stage, and cleavage rate for each treatment. Untreated negative control is shown in the top row, followed by seven unfertilized parthenogenesis controls. Created with BioRender.com.*

Treatments	Total Oocytes	Not Cleaved	2-Cell (%)	3-Cell (%)	4-Cell+ (%)	Cleavage Rate %
Control - Untreated	102	20	17 (16.7%)	30 (29.4%)	35 (34.3%)	80.39%
Control - Methanol 20% - No Fertilization	34	34	0	0	0	0.0 %
Control - Ethylene Glycol 10% - No Fertilization	42	39	2 (4.8%)	0	1 (2.4%)	7.1%
Control - Ethylene Glycol 20% - No Fertilization	21	21	0	0	0	0.0%
Control - DMSO 10% - No Fertilization	20	20	0	0	0	0.0%
Control - Propylene Glycol 10% - No Fertilization	20	20	0	0	0	0.0%
Control - Glycerol 10% - No Fertilization	19	19	0	0	0	0.0%
Control - Ethylene Glycol 7.5% + Propylene Glycol 7.5% - No Fertilization	19	18	0	1 (5.3%)	0	5.3%
Ethylene Glycol 10%	37	13	4 (10.8%)	15 (40.5%)	5 (13.5%)	64.9%
Ethylene Glycol 20%	35	11	5 (14.3%)	17 (48.6%)	2 (5.7%)	68.6%
Propylene Glycol 10%	60	11	14 (23.3%)	21 (35.0%)	14 (23.3%)	81.7%
Propylene Glycol 20%	38	8	6 (15.8%)	16 (42.1%)	7 (18.4%)	76.3%
Glycerol 10%	58	13	17 (29.3%)	20 (34.5%)	8 (13.8%)	77.6%
Glycerol 20%	40	14	8 (20.0%)	14 (35.0%)	3 (7.5%)	62.5%
Methanol 10%	39	18	8 (20.5%)	12 (30.8%)	1 (2.6%)	53.8%
Methanol 20%	33	19	3 (9.1%)	11 (33.3%)	0	42.4%
DMSO 10%	56	18	10 (17.9%)	13 (23.2%)	3 (5.4%)	46.4%
DMSO 20%	34	15	9 (26.5%)	6 (17.6%)	4 (11.8%)	55.9%
DMSO 7.5% + Ethylene Glycol 7.5%	40	8	8 (20.0%)	5 (12.5%)	19 (47.5%)	80.0%
DMSO 7.5% + Glycerol 7.5%	42	16	8 (19.0%)	9 (21.4%)	9 (21.4%)	61.9%
DMSO 7.5% + Propylene Glycol 7.5%	35	9	9 (25.7%)	8 (22.9%)	9 (25.7%)	74.3%
DMSO 7.5% + Methanol 7.5%	37	15	7 (18.9%)	3 (8.1%)	12 (32.4%)	59.5%
Ethylene Glycol 7.5% + Methanol 7.5%	38	21	5 (13.2%)	11 (28.9%)	1 (2.6%)	44.7%
Ethylene Glycol 7.5% + Propylene Glycol 7.5%	52	14	10 (19.2%)	15 (28.8%)	13 (25.0%)	73.1%
Ethylene Glycol 7.5% + Glycerol 7.5%	34	8	9 (26.5%)	11 (32.4%)	6 (17.6%)	76.5%
Total	985					

***Table 3. Blastocyst rates**: Recorded data on day 8 after IVF. Total count of bovine embryos at each stage of development, and the blastocyst rate for each treatment. Untreated negative control is shown in the top row, followed by seven unfertilized parthenogenesis controls. Created with BioRender.com.*

Treatments	Total Oocytes	Young (%)	Normal (%)	Expanded (%)	Hatching (%)	Hatched (%)	Blastocyst Rate %
Control - Untreated	102	2 (2.0%)	12 (11.8%)	11 (10.8%)	3 (2.9%)	20 (19.6%)	47.1%
Control - Methanol 20% - No Fertilization	34	0	0	0	0	0	0.0 %
Control - Ethylene Glycol 10% - No Fertilization	42	0	0	0	0	0	0.0%
Control - Ethylene Glycol 20% - No Fertilization	21	0	0	0	0	0	0.0%
Control - DMSO 10% - No Fertilization	20	0	0	0	0	0	0.0%
Control - Propylene Glycol 10% - No Fertilization	20	0	0	0	0	0	0.0%
Control - Glycerol 10% - No Fertilization	19	0	0	0	0	0	0.0%
Control - Ethylene Glycol 7.5% + Propylene Glycol 7.5% - No Fertilization	19	0	0	0	0	0	0.0%
Ethylene Glycol 10%	37	0	2 (5.4%)	0	0	6 (16.2%)	21.6%
Ethylene Glycol 20%	35	1 (2.9%)	5 (14.3%)	3 (8.6%)	0	4 (11.4%)	37.1%
Propylene Glycol 10%	60	0	4 (6.7%)	6 (10.0%)	1 (1.7%)	6 (10.0%)	28.3%
Propylene Glycol 20%	38	0	2 (5.3%)	5 (13.2%)	4 (10.5%)	4 (10.5%)	39.5%
Glycerol 10%	58	0	6 (10.3%)	9 (15.5%)	2 (3.4%)	6 (10.3%)	39.7%
Glycerol 20%	40	0	5 (12.5%)	3 (7.5%)	3 (7.5%)	2 (5.0%)	32.5%
Methanol 10%	39	1 (2.6%)	0	1 (2.6%)	2 (5.1%)	4 (10.3%)	20.5%
Methanol 20%	33	0	0	1 (3.0%)	0	2 (6.1%)	9.1%
DMSO 10%	56	1 (1.8%)	7 (12.5%)	9 (16.1%)	2 (3.6%)	3 (5.4%)	39.3%
DMSO 20%	34	0	6 (17.6%)	3 (8.8%)	0	2 (5.9%)	32.4%
DMSO 7.5% + Ethylene Glycol 7.5%	40	3 (7.5%)	3 (7.5%)	5 (12.5%)	0	4 (10.0%)	37.5%
DMSO 7.5% + Glycerol 7.5%	42	0	0	3 (7.1%)	4 (9.5%)	1 (2.4%)	19.0%
DMSO 7.5% + Propylene Glycol 7.5%	35	0	3 (8.6%)	2 (5.7%)	0	2 (5.7%)	19.4%
DMSO 7.5% + Methanol 7.5%	37	1 (2.7%)	3 (8.1%)	1 (2.7)	0	1 (2.7%)	16.2%
Ethylene Glycol 7.5% + Methanol 7.5%	38	0	1 (2.6%)	3 (7.9%)	0	0	10.3%
Ethylene Glycol 7.5% + Propylene Glycol 7.5%	52	0	5 (9.6%)	7 (13.5%)	5 (9.6%)	4 (7.7%)	40.4%
Ethylene Glycol 7.5% + Glycerol 7.5%	34	1 (2.9%)	4 (11.8%)	2 (5.9%)	0	1 (2.9%)	22.9%
Total	985						

The high cleavage rate (80%) and blastocyst rate (47%) of the untreated control oocytes were expected since this sample should be unaffected by oxidative and osmotic stress. On day 2 following IVF, 7 samples showed a high cleavage rate compared to the control, with PG 10% having a higher cleavage rate (82%) than the control. DMSO 7.5% + EG 7.5% (80%), GLY 10% (77%), PG 20% (76%), EG 7.5% + GLY 7.5% (76%), DMSO 7.5% + PG 7.5% (74%), and EG 7.5% + PG 7.5% (73%) had the following high cleavage rates. The rest of the CPA concentrations showed lower cleavage rates ranging from 54% to 68% with the lowest result belonging to MeOH 20%.

The speed of development was assessed on day 2 by registration of the different stages of embryo development at this time point. The stages observed were from the 2-cell stage to the 4-cell+ stage. DMSO 7.5% + EG 7.5% showed the highest development speed of 47% with the cells being at the 4-cell+ stage compared to 34% for the control sample. The sample with the lowest development speed was MeOH 20% with 0% of the cells at the 4-cell+ stage.

Blastocyst rate was measured on day 8 following IVF by dividing the sum of all blastocysts by the total number of fertilized oocytes for each treatment. Although PG 10% had the highest cleavage rate on day 2, it had a significantly lower blastocyst rate (28%) compared to the negative control (47%). The following 5 CPAs had the highest blastocyst rate on day 8: EG 7.5% + PG 7.5% (40%), GLY 10% (40%), PG 20% (39%), DMSO 10% (39%), DMSO 7.5% + EG 7.5% (37%). As for the speed of development, EG 10% had the fastest development among the CPA treatments with 16% of the embryos being hatched on day 8.

Statistical analysis was performed on the cleavage and blastocyst result using logistic regression analysis (Table 4). The results provide insights into which treatment combinations significantly impact the blastocyst formation. Statistical significance is determined at the levels of 0.001, 0.01, and 0.05, as indicated by the corresponding symbols in the table.

Table 4. Logistic regression*: Statistical analysis on the cleavage and blastocyst results after the exposure of the oocytes to cryoprotective agents. Treatments, estimated coefficients, standard errors, z-values, and p-values for each treatment condition are included in the table. Significant coefficients (p < 0.05) are marked with asterisks to indicate their level of statistical significance. The model comparison between the null model (control) and the treatment model demonstrates the overall effectiveness of the treatments.*

Treatments	Estimate	Standard Error	z value	p value	
Control - Untreated	-0.1178	0.1984	-0.594	0.552683	
Ethylene Glycol 10%	-1.1701	0.4459	-2.624	0.008690	**
Ethylene Glycol 20%	-0.4083	0.4022	-1.015	0.309962	
Propylene Glycol 10%	-0.8102	0.3485	-2.325	0.020070	*
Propylene Glycol 20%	-0.3097	0.3866	-0.801	0.423198	
Glycerol 10%	-0.3021	0.3338	-0.905	0.365449	
Glycerol 20%	-0.6131	0.3916	-1.566	0.117387	
Methanol 10%	-1.2368	0.4434	-2.789	0.005283	**
Methanol 20%	-2.1848	0.6372	-3.429	0.000606	***
DMSO 10%	-0.3175	0.3380	-0.940	0.347444	
DMSO 20%	-0.6198	0.4168	-1.487	0.137012	
DMSO 7.5% + Ethylene Glycol 7.5%	-0.3930	0.3821	-1.029	0.303690	
DMSO 7.5% + Glycerol 7.5%	-1.3291	0.4402	-3.019	0.002532	**
DMSO 7.5% + Propylene Glycol 7.5%	-1.2685	0.4668	-2.717	0.006581	**
DMSO 7.5% + Methanol 7.5%	-1.5244	0.4881	-3.123	0.001790	**
Ethylene Glycol 7.5% + Methanol 7.5%	-1.9924	0.5654	-3.524	0.000425	***
Ethylene Glycol 7.5% + Propylene Glycol 7.5%	-0.2717	0.3453	-0.787	0.431393	
Ethylene Glycol 7.5% + Glycerol 7.5%	-1.0609	0.4503	-2.356	0.018489	*

	Degrees of Freedom	Deviance Residuals	p value
Null	17	51.926	
Treatments	17	0.000	0.00002112

Significance. codes : '***' 0.001 '**' 0.01 '*' 0.5 '.' 0.1 ' ' 1

The logistic regression analysis revealed significant associations between the administered treatments and the likelihood of blastocyst formation ($\chi^2 = 51.926$, df = 17, $p < 0.001$). The logistic regression model provided a good fit to the data, as evidenced by the null deviance of 51.926 and the residual deviance approaching zero. This indicates that the model explains a significant proportion of the variance in blastocyst formation rates among the treatment groups.

Several treatments exhibited statistically significant effects on blastocyst formation rates compared to the reference group (Table x). Treatments such as DMSO 7.5% + GLY 7.5% ($\beta = -1.3291$, $p = 0.002$) and DMSO 7.5% + MeOH 7.5% ($\beta = -1.5244$, $p = 0.002$) were associated with significantly lower odds of blastocyst formation, suggesting potential detrimental effects on embryonic development. Conversely, treatments such as MeOH 20% ($\beta = -2.1848$, $p = 0.001$) and EG 7.5% + MeOH 7.5% ($\beta = -1.9924$, $p < 0.001$) exhibited the most pronounced decreases in blastocyst rates, indicating potential toxicity or inhibitory effects on embryo development.

Post-hoc testing was conducted to compare treatment levels pairwise. However, due to computational issues, the post-hoc test results could not be obtained. Further investigation is warranted to address this issue and provide additional insights into the differences between treatment groups.

3.2 Staining results

Figure 10 shows the imaging results of the staining procedure on the samples from the stress test. Images were selected from a pool of 3-5 oocytes per treatment. All samples were at the MII stage of development and were acquired after 22 h of incubation in the IVM medium at 38.8 °C. One negative control and three positive controls were set as points of comparison. The negative control was not treated with any CPAs. The three positive controls were acquired after three separate treatments, one heated to 65 °C for 7 min, one incubated at RT for 48 h, and one stressed with H_2O_2 30% for 10 min (see Appendix 1 and Appendix 2 for all control samples).

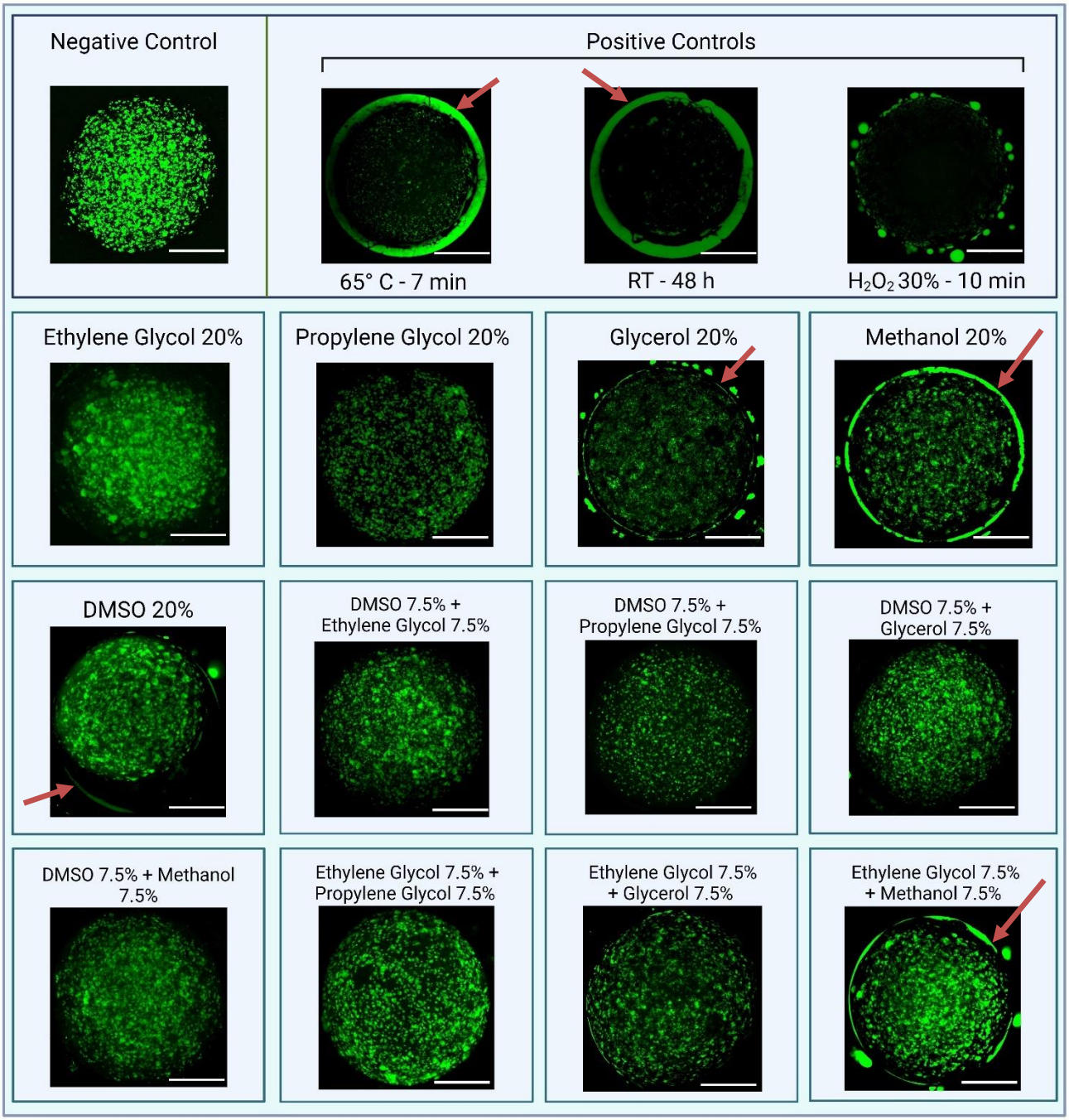


***Figure 10**: The stress test staining results following the exposure of bovine oocytes to different cryoprotective agents. All samples were stained with BODIPY (493/503) and imaged by exposure to a 488 nm laser at 5% intensity with 370-390 V gain. Controls are shown in the top row. Treatments are shown in the rows below. The white line indicates a 50 µm scale. Orange arrows indicate the localization of lipids around the zona pellucida. Figure Created with BioRender.com*

The size of the oocytes, measured as diameters, was consistent in all treatments ranging between 120 μm and 140 μm except for the DMSO 20% treatment which had a size of approximately 105 μm which indicates osmotic stress. The size of the lipid droplets in all samples, however, varied significantly between each treatment, which indicates oxidative stress caused by CPAs in the form of lipid peroxidation.

The even distribution of small lipid droplets (2.5 μl – 5 μl) indicates that the treatments with EG 7.5% + PG 7.5%, and DMSO 7.5% + GLY 7.5% have exposed the oocytes to less oxidative stress compared to the rest of the samples before cryopreservation.

Positive control treatments of 48 h (RT) and 7 min (65 °C) showed that the localization of lipids around the zona pellucida and diminution of lipid droplets was caused by a physical stressor. The H_2O_2 30% treatment confirms that a lack of lipid droplets in the cell, possibly due to the lipids being used up for survival following the treatment, is an indication of oxidative stress. These samples confirmed the signs of physical and oxidative stress in many of the treated samples in the stress test.

The oocytes treated with DMSO showed signs of oxidative stress. However, treatments with a mixture of DMSO and other CPAs showed an even distribution of lipid droplets (2.5 μl – 5 μl) all around the cells. Therefore, DMSO alone is more toxic to the oocytes compared to its mixtures.

The treatments with MeOH showed signs of physical and oxidative stress in all the treated samples except samples treated with DMSO 7.5% + MeOH 7.5%. This sample had a normal cell size and droplet size compared to the control.

Samples treated with PG 20%, GLY 20%, DMSO 7.5% + PG 7.5%, and EG 7.5% + GLY 7.5%, had an even lipid distribution but lacked abundance and signal intensity which indicates a slight oxidative stress. The oocytes treated with GLY 20% also showed signs of physical stress by having lipid droplets localized around the zona pellucida.

Aggregated lipid droplets were visible in treatment samples of EG 20%, DMSO 20%, EG 7.5% + MeOH 7.5%, and slightly in DMSO 7.5% + EG 7.5%, which suggests that EG is a contributor to lipid aggregation (see Appendix 4 and Appendix 5 for all treated samples).

Figure 11 shows the imaging results following the staining procedure on vitrified-warmed oocytes treated with four selected CPAs from the stress test. Images were selected from a

pool of 5 oocytes per treatment. All CPA-treated oocytes were denuded and vitrified at the MII stage. The same controls as the stress test staining were selected as comparison points to measure the effects of vitrification on CPA-treated oocytes.

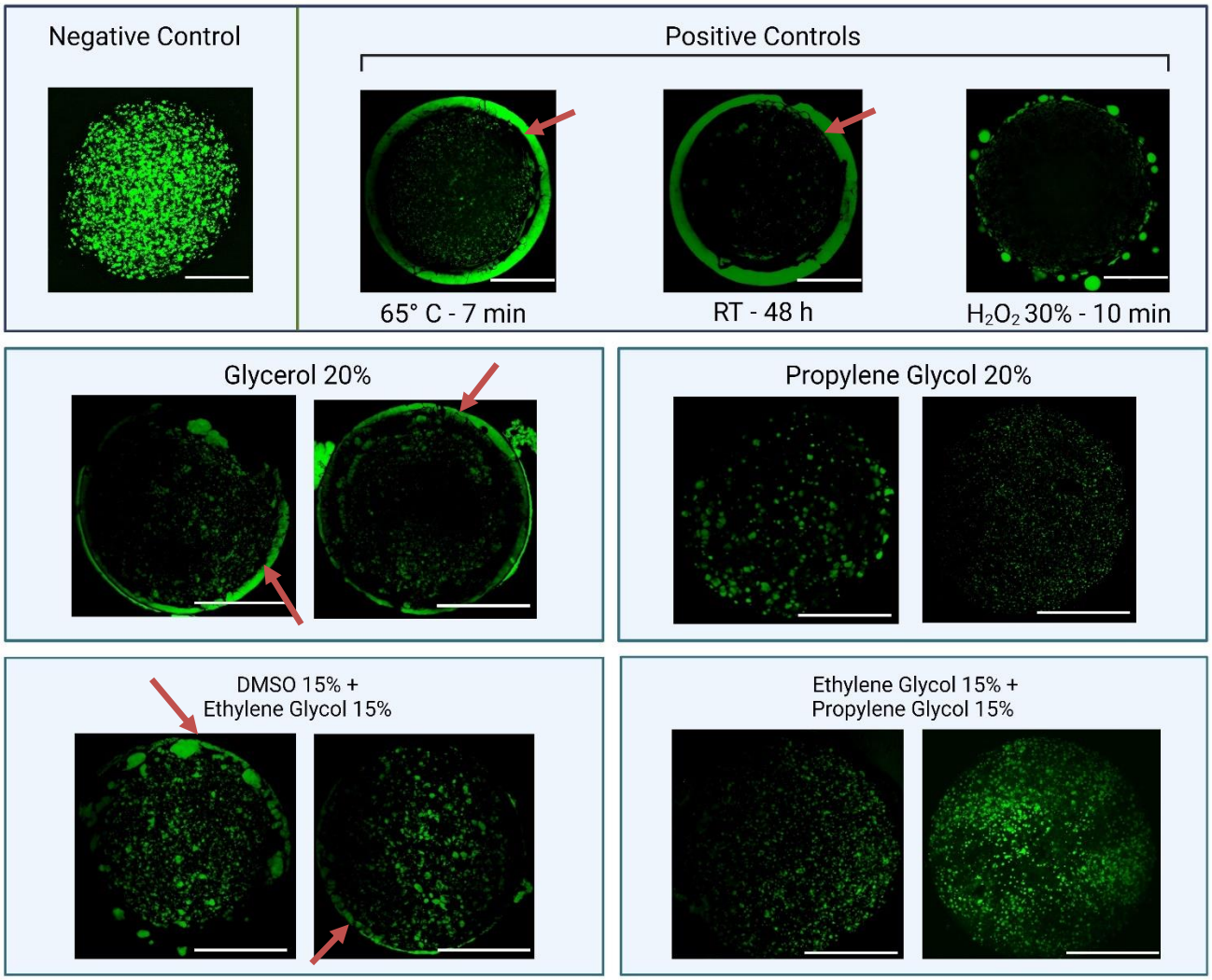


***Figure 11**: Staining results following vitrification of bovine oocytes using the droplet method. All samples were stained with BODIPY (493/503) and imaged by exposure to a 488 nm laser at 5% intensity with 370-390 v gain. Controls are shown in the top row. The white line indicates a 50 µm scale. Orange arrows indicate the localization of lipids around the zona pellucida. (Figure Created with BioRender.com)*

Staining images of the vitrified samples (Figure 11) showed that out of the 4 CPA treatments used before vitrification, two showed signs of physical stress, with GLY 20% showing a pattern of lipid droplet localization around the zona pellucida in most of the vitrified oocytes. Stained samples from the DMSO 15% + EG 15% treatment also showed a similar pattern with less intensity. However, samples from the PG 20% and EG 15% + PG 15% treatments

showed less signs of physical damage, but more signs of oxidative stress by having less abundant lipid droplets inside the oocytes, with several samples completely lacking lipid droplets in most parts of the cell. This correlates with the positive control treatment of H_2O_2 30% which confirms the lipid peroxidation in these samples. The vitrified samples also had a smaller size than controls (100 μm – 120 μm vs. 120 μm – 140 μm in control) (see Appendix 6 – 9 for all vitrified/warmed stained samples) .

3.3 Development of vitrified-warmed oocytes

As shown in Table 5, all the vitrified-warmed samples degenerated after IVF. Two replicates and a total of 153 oocytes were used for this experiment. Development result for fertilized vitrified-warmed oocytes was recorded to be 0.0%.

***Table 5**: Development results of fertilized vitrified-warmed oocytes. Data is recorded 48 h after IVF. Created with BioRender.com*

Treatments	Total Oocytes	Degenerated
Propylene Glycol 20%	39	39
Glycerol 20%	39	39
DMSO 15% + Ethylene Glycol 15%	37	37
Ethylene Glycol 15% + Propylene Glycol 15%	38	38
Total	153	

Figure 12 shows images of vitrified-warmed oocytes after 3 h incubation in the IVM medium and 18 h after IVF. Morphologically normal oocytes could not be assessed immediately after thawing, however, the recovery of the oocytes was visible after incubation in the IVM medium with dark and granulated cytoplasm, however, all cells appeared degenerated 18 h after IVF, before and after centrifugation in preparation for IVC.

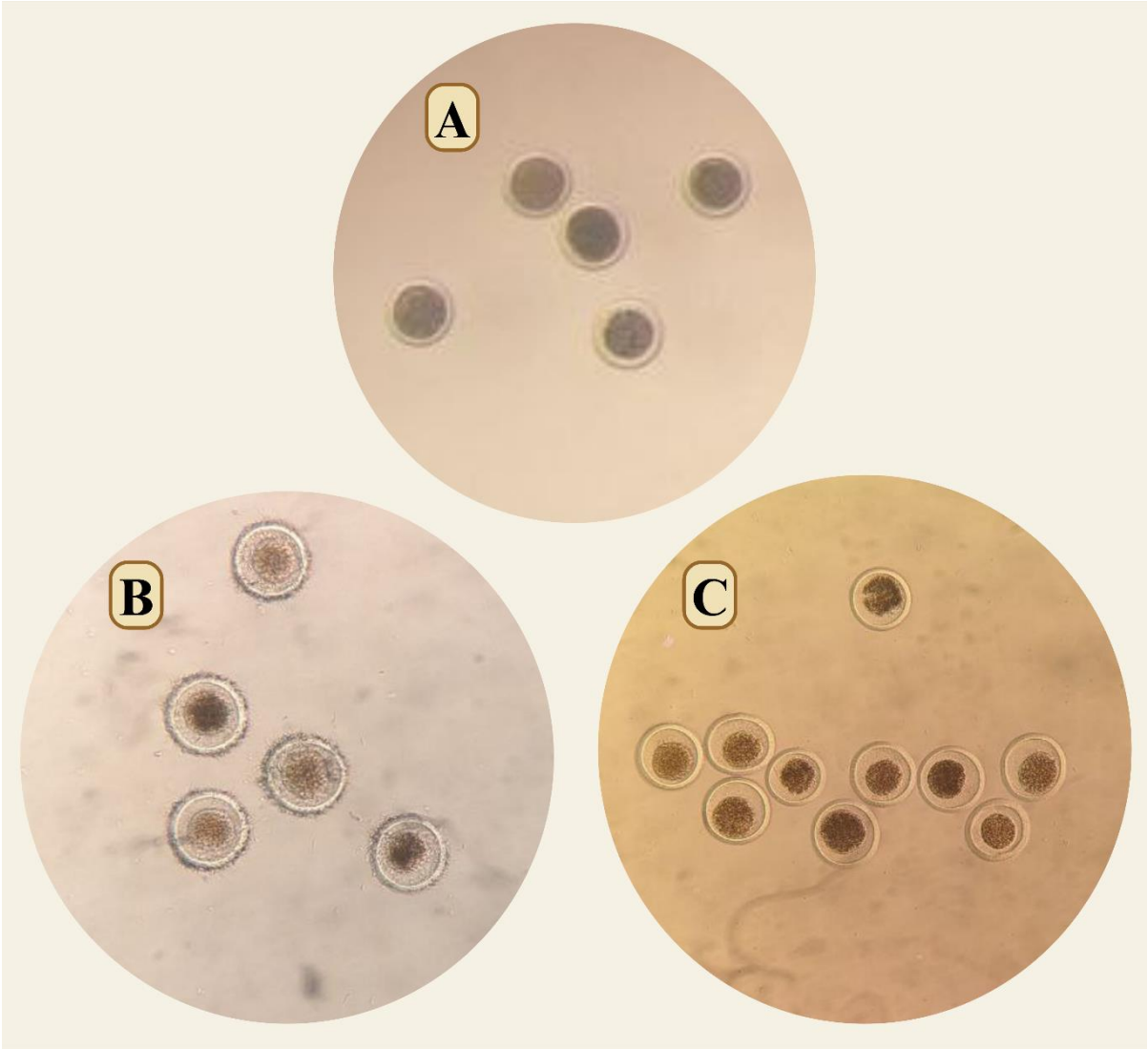


***Figure 12**: Vitrified-warmed bovine oocytes imaged under stereo microscope. A) after 3 h incubation at 38 °C in IVM medium. B) 18 h after IVF, before centrifugation. C) 18 h after IVF, after centrifugation in preparation for IVC. All images were taken at 10X magnification. Figure created with BioRender.com.*

As shown in Table 6, after revision of the concentrations, out of 35 vitrified oocytes, the development results remained at 0.0%. However, not all oocytes were degenerated in this experiment and six oocytes were not cleaved 48 h after IVF.

***Table 6**: Development results of fertilized vitrified-warmed oocytes after revision of the vitrification media concentration. Data is recorded 48 h after IVF. Created with BioRender.com*

Treatments	Total Oocytes	Degenerated	Not-Cleaved
Ethylene Glycol 15% + Propylene Glycol 15%	35	29	6
Total	35		

Figure 13 shows images of the vitrified warmed oocytes after revision of vitrification media concentration. Oocyte degeneration was observed after 3 h of recovery in IVM media, however almost half of the oocytes appeared to have recovered to their original size. On day 2 of the culture post-IVF, more oocytes were recorded as degenerated, and the remaining oocytes appeared not cleaved.

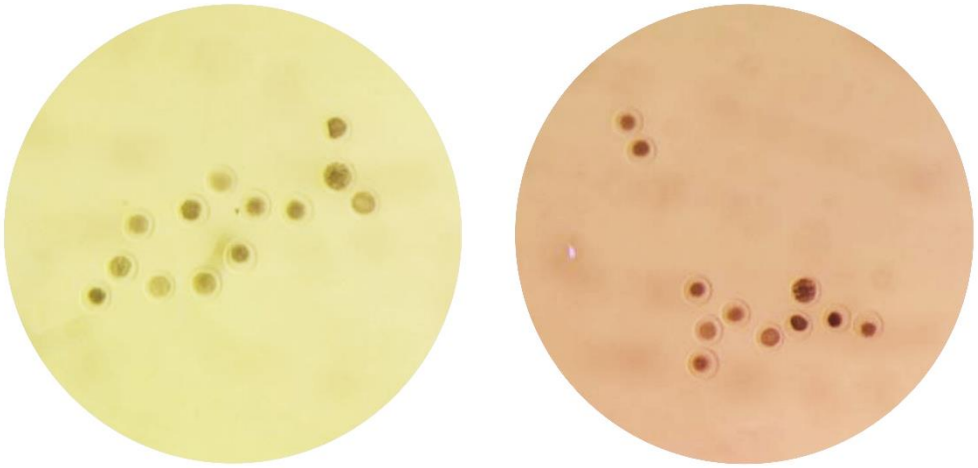


***Figure 13**: Vitrified-warmed bovine oocytes imaged under stereo microscope after 3 h of incubation in IVM media (left), and 48 h after IVF (right). Images were captured at 4X magnification.*

4. Discussion

The findings of this study provide valuable insights into the impact of various treatments on blastocyst formation rates in an IVF setting. The significant associations observed between treatment variables and blastocyst formation highlight the importance of selecting appropriate cryoprotectants and concentrations for optimizing IVF outcomes.

Finding optimal concentration and exposure time is a challenging process since the CPAs are inherently toxic to the cells. It is important to provide enough CPA to the oocytes to avoid ice crystal formation without inducing oxidative stress, and an adequate exposure time is necessary to reduce osmotic stress (Lai et al., 2015). Each CPA will have a range of temperatures, concentrations, and exposure times that are ideal for minimizing cell damage (García-Martínez et al., 2022). High concentration of CPAs is necessary for vitrification solutions; however, higher CPA concentrations may be hazardous to oocytes. (Yamada et al., 2007). A study performed by Szurek & Eroglu, (2011), concluded that the CPA toxicity is more dependent on the CPA type and concentration, in contrast to the exposure time and temperature. Our analysis revealed several treatments that were significantly associated with altered blastocyst formation rates compared to the untreated control group. Treatments such as DMSO 7.5% + GLY 7.5% and DMSO 7.5% + MeOH 7.5% were found to have significantly lower chances of blastocyst formation, suggesting potential detrimental effects on embryonic development. Conversely, treatments such as MeOH 20% and EG 7.5% + MeOH 7.5% exhibited the most pronounced decreases in blastocyst rates compared to the rest of the treatments, indicating potential toxicity or inhibitory effects on embryo development. Furthermore, these findings underscore the need for further research to elucidate the mechanisms underlying the observed treatment effects and to develop improved protocols for IVF treatment.

Hence, our study was mainly focused on the CPA type and concentration. The goal of the stress test was to find an optimal concentration of CPAs during 10 minutes of exposure. The oocytes were exposed to two different concentrations of CPAs, and their vitality and developmental competence were checked after exposure, on day 2, and on day 8 post IVF. The shrinkage and recovery of the oocyte can be observed during the CPA exposure, with all cells going back to their normal size after 10 minutes with no sign of degeneration. The development results, however, differ significantly between different treatments. The results

of the present study show a high blastocyst rate in four treatments as follows: EG + PG, GLY, PG, and DMSO + EG. These CPAs were therefore selected for cryopreservation.

A possible explanation for the differences in embryo developments following CPA stressing on oocytes can be the damage to the meiotic spindle and DNA fragmentation. This has previously been investigated by García-Martínez et al., (2022), evaluating the CPA cytotoxicity at different exposure times and temperatures, showing a higher degree of DNA fragmentation in oocytes exposed to CPAs at temperatures lower than 38.5 °C. In future experiments, immunofluorescent staining can be applied to investigate the possibility of meiotic damage after CPA exposure.

Parthenogenesis can occur naturally or being induced by chemicals or physical means (Santos et al., 2023). Previous studies have shown that parthenogenesis activation can occur upon exposure of oocytes to chemicals such as ethanol (Szurek & Eroglu, 2011). Parthenogenesis was observed in our study in the treatments with EG 10%, and EG 7.5% + PG 7.5%. However, since the parthenogenesis in these specific treatments was not repeated in the replicates, it cannot be concluded with certainty that these CPA concentrations directly cause parthenogenesis activation and cleavage, as this could have been caused by the natural attributes of the selected oocytes in the aforementioned samples.

Oocytes are more susceptible to osmotic stress due to their smaller size compared to blastocysts (100 – 120 µm in oocytes vs. 150 – 200 µm in normal blastocysts), hence a gradual increase in concentration of permeable CPAs, and a single exposure to impermeable solutes can be beneficial to their survival. Previous studies have shown that multi-step techniques, involving increasing doses of permeable CPAs resulted in reduced osmotic damage and enhanced developmental competence (Marques et al., 2018). In the present study lower concentrations of CPAs were tested with the goal of minimizing toxicity and osmotic damage. Addition of CPAs in a multi-step method allows shrinkage and recovery of the oocytes to happen in stages, avoiding lethal damage (Lai et al., 2015; Marques et al., 2018).

The high surface-to-volume ratio of oocytes increases their vulnerability to physical and chemical stressors. Lipid droplets increase the chance of chilling injury by reducing the cryotolerance of the oocytes. These lipid droplets are commonly located at the periphery of the plasma membrane and close to organelles such as endoplasmic reticulum and

mitochondria which are sensitive to cryodamage (Hwang & Hochi, 2014). Bovine oocytes have a large number of lipid droplets and a diminution in these droplets at the early stages of development results in a decreased sensitivity to low temperatures (Zhou & Li, 2013). Hence, the treatment samples observed in this study with less abundant and large aggregates of lipid droplets such as MeOH 20% and DMSO 10% are more susceptible to cryodamage.

In the second part of this study, bovine oocytes were vitrified using the droplet method to try to establish a cost-efficient and successful method of cryopreservation. The results of this experiment show that this method combined with the selected concentrations of CPA did not yield a successful result. Oocyte vitrification remains a difficult process in cattle as well as other animal species, and studies on the survival of bovine oocytes have had varying degrees of success (García-Martínez et al., 2022). Certain elements that impact the process of vitrification, IVF, and subsequent embryo development make the optimization of the technology challenging. The outcomes of embryo development following oocyte vitrification are affected, even when identical procedures and culture systems are employed (Dujíčková et al., 2021).

The death of all cultured samples post-thaw in our study can be caused by many factors. In a previous study conducted by Yamada et al. (2007) reduced maturation rates were observed after 60 sec exposure to the CPA solution of EG + DMSO, compared to 30 sec exposure in our study. This implies that these CPAs are harmful to oocytes at the 60 sec exposure duration. It can be hypothesized that in the present study, the vitrification process damaged the oocyte cell membrane, specifically the cortical granules, which may have caused polyspermy and in turn, lead to the death of the samples. A significant consequence of CPAs is the early elevation of intercellular Ca^{2+} concentrations, leading to cortical granule exocytosis, and subsequent hardening of the zona pellucida, hampering fertilization (Marques et al., 2018). These effects can be a possible explanation of the impaired oocyte development.

The damage to the oocyte membrane was clearly visible in most of the stained samples in our experiments. It has previously been confirmed that bovine oocytes have a low tolerance for low temperatures, and freezing and thawing along with the effects of CPAs alter the zona pellucida, cortical granules, cytoskeletal elements, and particularly the meiotic spindle (Otoi et al., 1997). This hypothesis could be evaluated by fluorescent staining of the pronuclei to observe if more than one pronuclei is formed in each oocyte.

Another possible explanation for the death of the cells can be damage to the meiotic spindle resulting in its disorganization, which leads to chromosomal disruption. Bovine oocytes are less resistant to temperature fluctuations due to the irreversibility of temperature-induced spindle disruptions (García-Martínez et al., 2022). Depolymerization of microtubules and microfilaments also affects the movement and formation of lipid droplets which in turn affects the cryosurvivability of oocytes (Zhou & Li, 2013). This could explain the pronounced difference in lipid droplet formation of the stained post-thaw samples in comparison to the stained samples stressed with the same CPAs observed in our study. In certain species such as human and bovine, some oocytes have a lack of pericentriolar materials which results in a failure in reforming of MII spindles after warming (Men et al., 2003). This partially formed spindle will cause chromosome misarrangement resulting in aneuploidy in some oocytes or the retention of the second polar body at the time of fertilization, leading to polyploid zygotes and embryos.

DNA damage can also be a factor in the death of thawed samples, as vitrification also affects the nuclear components. DNA damage in frozen-thawed bovine oocytes at the MII stage is known to be significantly higher than in unfrozen samples. This can be due to the formation of ice crystals during the warming process (Zhou & Li, 2013). Damage to the nuclear components can cause an up-regulation of apoptotic genes in vitrified bovine oocytes which is related to the reduced development and death of these cells. In a study performed by Men et al. (2003), it was concluded that unrepaired damaged DNA at the haploid stage before fertilization can be lethal to oocytes and may trigger oocyte degeneration by apoptosis mechanism. Damages occurring in haploid DNA cannot be promptly repaired by endogenous mechanisms compared to diploid cells, and by the formation of diploid state post-fertilization, it is too late for the repairing mechanism to mend the damaged DNA since other chromosome abnormalities will arise. Therefore, TUNEL assay or Comet assay can be used in future experiments to evaluate the possible DNA fragmentation in the oocytes after CPA exposure.

Removing lipid droplets prior to vitrification can have a significant improvement in oocyte survivability during the freezing process (Hwang & Hochi, 2014). In recent studies, the use of L-carnitine has proven beneficial to the success of oocyte cryopreservation (McKeegan & Sturmey, 2012; Sprícigo et al., 2017). L-carnitine can enhance lipid metabolism and ATP production in animal cells by increasing β oxidation (Dunning et al., 2014). The addition of L-Carnitine to the IVM medium can reduce the amount of lipid droplets and their

distribution from the cortex to the medulla of oocytes, which in turn reduces the chance of lipid damage and helps the success of the vitrification procedure (Hwang & Hochi, 2014). Therefore, it can be considered in future experiments.

Lipid metabolism is pivotal for the developmental competence of oocytes due to its ability to produce large amounts of ATP from low levels of lipid substrate (Dunning et al., 2011). In the study performed by Dunning et al. (2011), the addition of L-carnitine demonstrated an upregulation of β-oxidation during IVM of COCs which resulted in higher levels of ATP generation within mitochondria from lipid droplets, and higher blastocyst rates.

The significant alteration in nutritional demand for energy metabolism can affect the morphology of lipid droplets. The lipid droplets increase in size as they prepare to store additional neutral lipids, which will be needed as energy consumption increases. Enlarged lipid droplets also provide a greater surface area for interaction with other organelles, promoting lipid exchange and replenishment (Ibayashi et al., 2021). The enlargement of lipid droplets can occur due to compaction of small and dispersed droplets, or individual growth of the droplets. In a study conducted by Aizawa et al. (2019), it was proven that a reduction in the number of lipid droplets leads to developmental impairment. It was also shown that overabundance of lipid droplets has an adverse impact on embryonic development. The data obtained in this study indicated a substantial decrease in IVF success following delipidation of oocytes, suggesting a possible correlation between depletion of lipid droplets and hardening of zona pellucida leading to the suppression of sperm penetration through the zona pellucida. It is hypothesized that an overabundance of lipid droplets affects cleavage speed during early development. This was somewhat visible in the results of this experiment as treatments with DMSO + EG, DMSO + MeOH, and EG + PG showed the most abundance of small lipid droplets and had the fastest cleavage speed in early development.

Increased apoptosis after oocyte vitrification results in decreased developmental competence. Rho-associated coiled-coil kinase (ROCK) can regulate apoptosis of cells, along with growth, metabolism, migration, and adhesion. Therefore, use of a ROCK inhibitor in the IVM media following the thawing procedure improves the developmental competence of vitrified oocytes by reducing the chance of apoptosis during the recovery time of the cells (Hwang & Hochi, 2014). Facilitating this inhibitor in future experiments may help the success of the IVF procedure for vitrified/warmed oocytes.

The staining results of this study confirmed the possibility of damage to membrane and cortical granules, so a higher concentration of GLY, and DMSO + EG may help reduce the chilling injury and result in a successful IVF after vitrification. However, signs of oxidative stress in the PG and EG + PG indicate that the high concentration of these CPAs is toxic to the cells. Therefore, in future experiments, a shorter exposure time or lower concentration of these CPAs can be tested. This can be challenging since proper exposure time is required to avoid osmotic stress.

The staining results also showed that the oocytes had not fully recovered in the thawing and IVM media, since they had slightly smaller diameters (100 μm – 120 μm) compared to unfrozen samples (120 μm – 140 μm). Consequently, osmotic stress needs to be accounted for in future considerations for this experiment. This is especially visible in the samples treated with GLY 20%, and DMSO 15% + EG 15%.

Temperature and duration of CPA exposure are critical factors in the vitrification process. Oocyte shrinkage decreases with increasing temperatures; however, CPA toxicity may increase at higher exposure temperatures (García-Martínez et al., 2022). In a study performed by Smith et al. (2011), EG 15% + PG 15% was used, and the oocytes were exposed to vitrification media in a 3-step procedure for 5 s, 5 s, and 90 s. The exposures in this procedure were performed at 22 °C and the oocytes were frozen in vitrification containers. Considering the success of this procedure a longer exposure period to these CPAs, and a more extensive step-by-step exposure to gradual increases of concentration for these CPAs before vitrification and a lower exposure temperature in a future experiment, may result in the survival of oocytes after vitrification.

5. Conclusion

In conclusion, the treatments used in the experiments, regardless of the developmental results of the oocytes in the stress test, resulted in the degeneration of vitrified-warmed oocytes post-IVF. Considering the high blastocyst rate of the oocytes treated with CPA solutions of EG + PG, GLY, PG, and DMSO + EG in the stress test, possible reasons for the impairment of oocyte fertilization can be damage to the membrane, cortical granules, DNA, meiotic spindle, and lipid integrity of the oocytes, due to vitrification using the droplet system. The staining of lipid droplets using BODIPY 493/503 also confirmed the changes in lipid droplet distribution and localization of lipid droplets around the zona pellucida post-vitrification. Addition of steps in the equilibration preparation before vitrification and a longer exposure of the oocytes to the vitrification solution may result in a successful procedure in future experiments.

6. References

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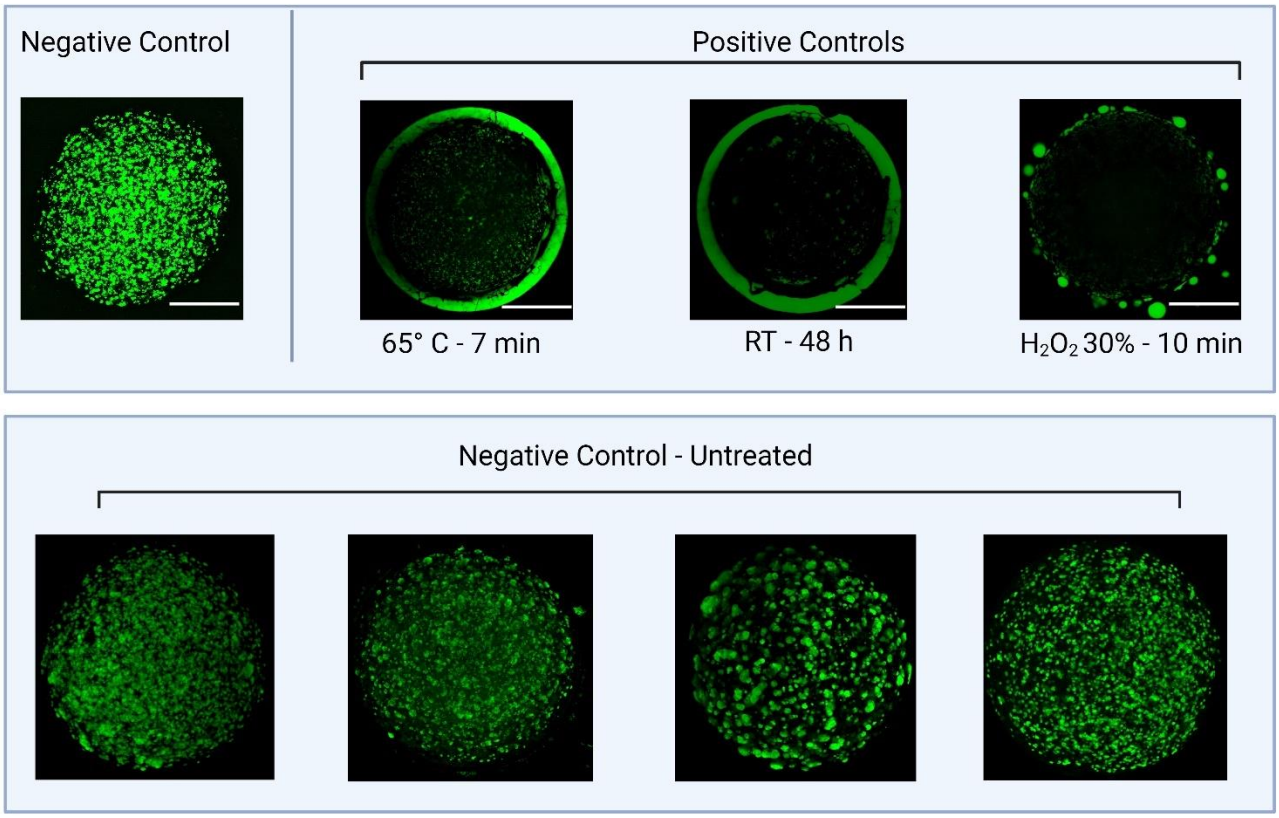
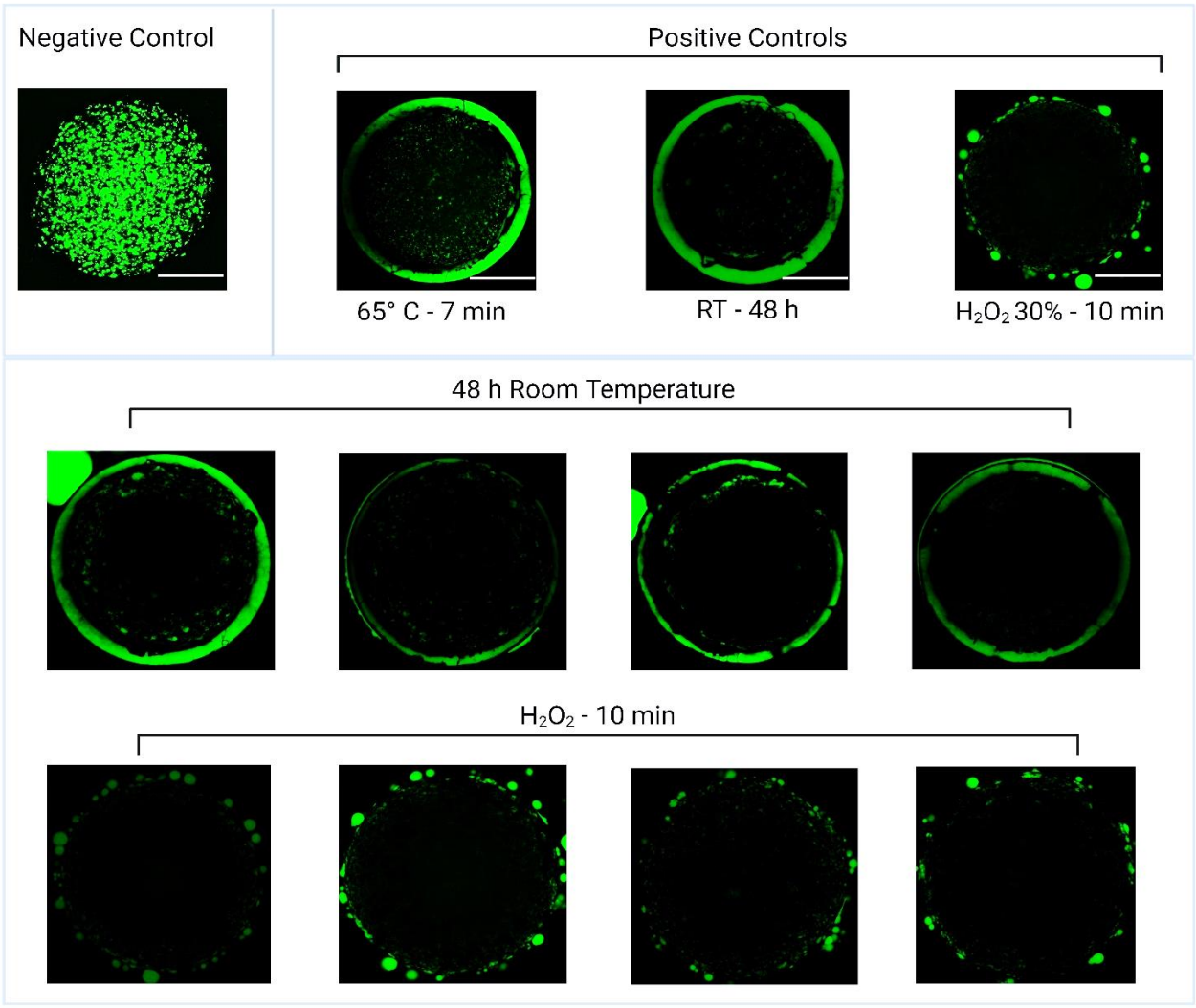
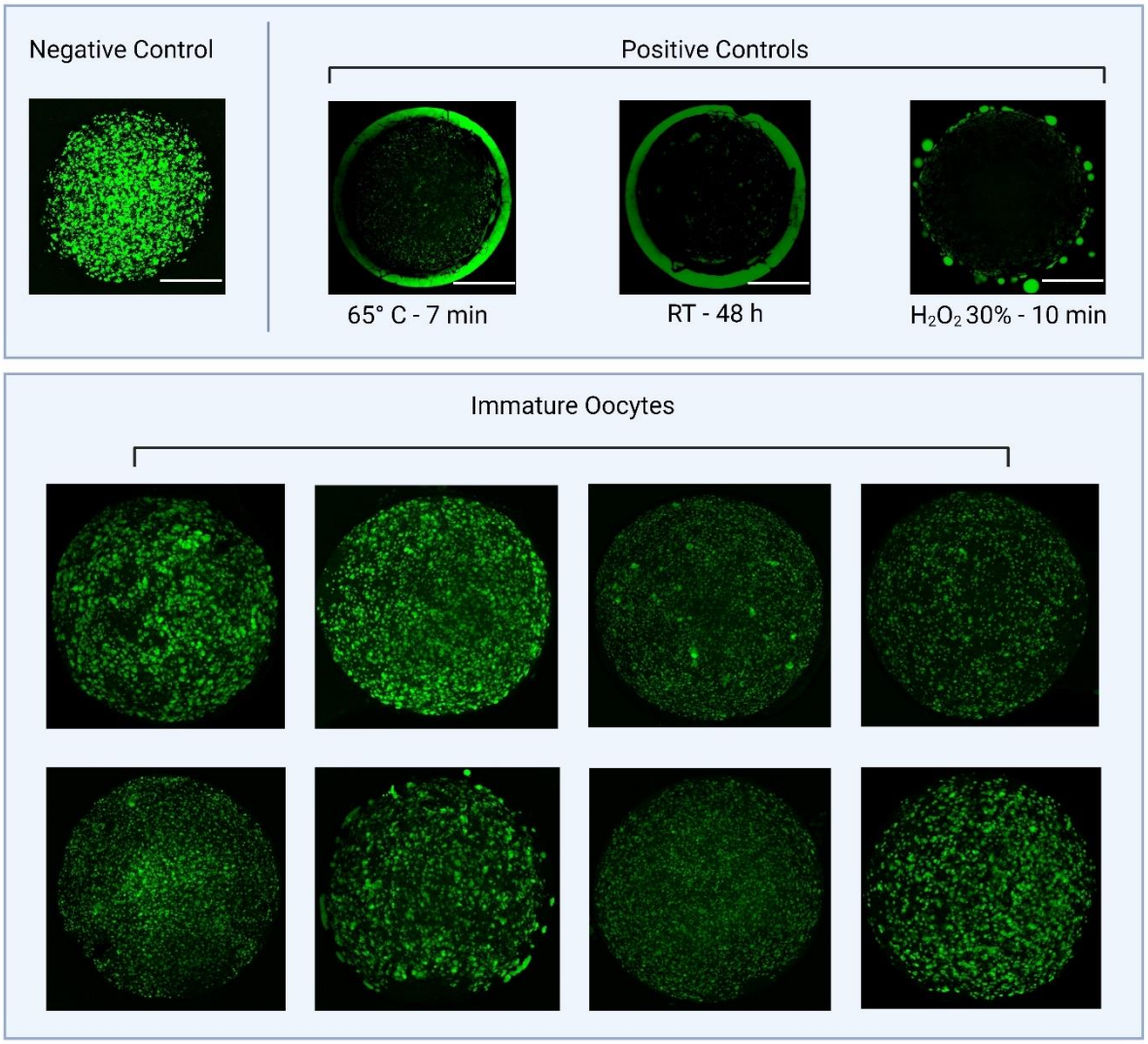
Appendix



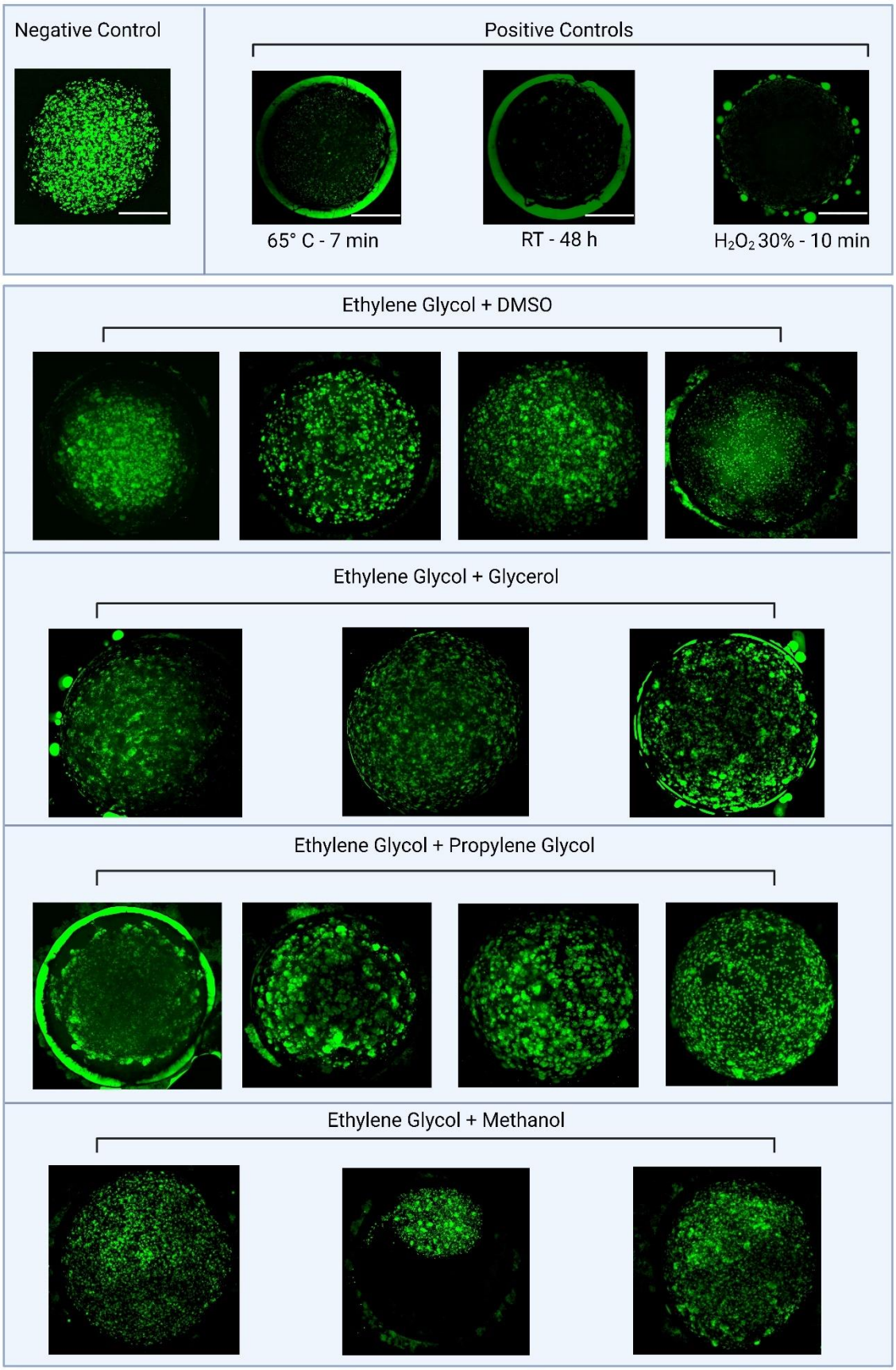
Appendix 1: Bovine oocyte BODIPY 493/503 staining results of negative control samples.



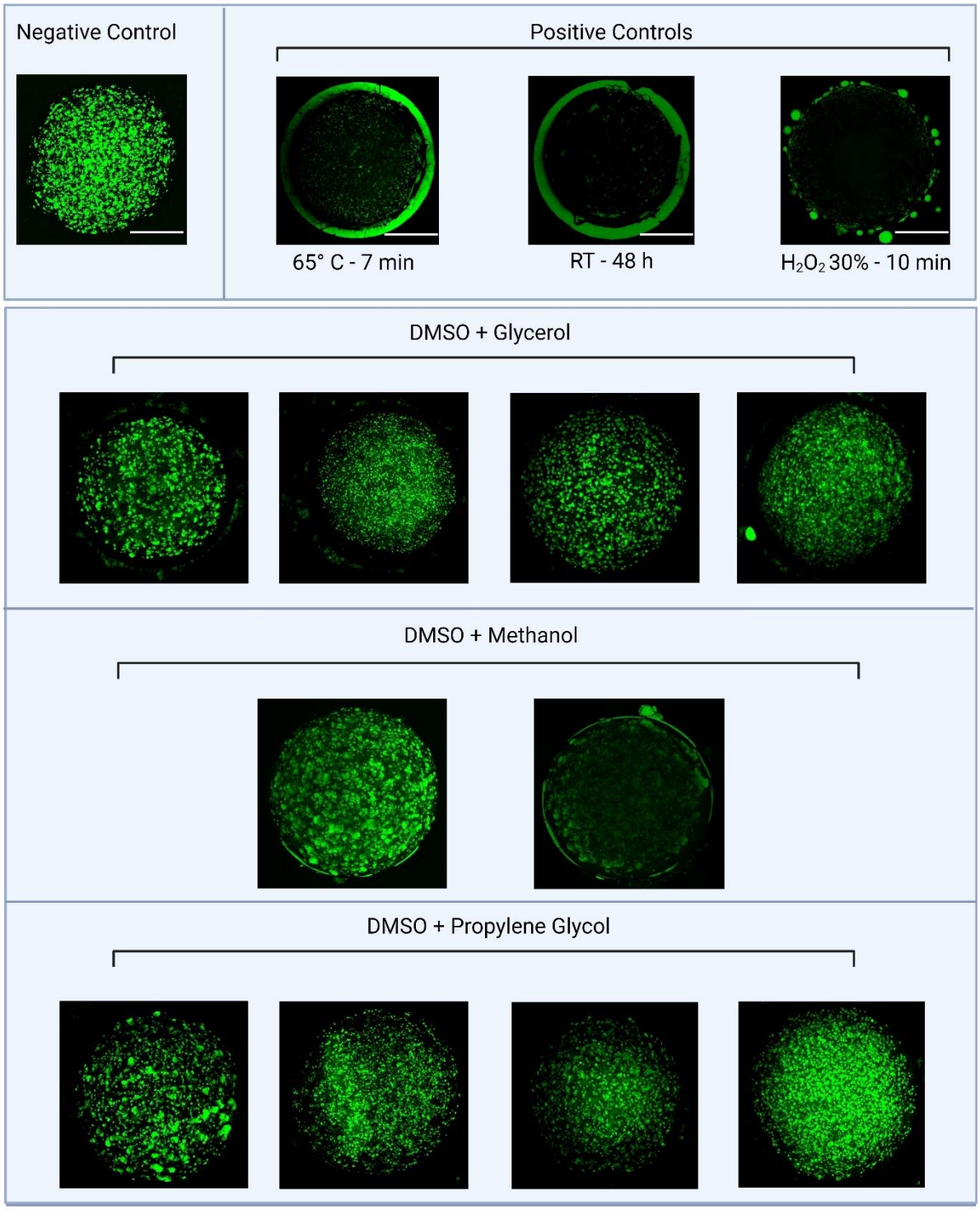
Appendix 2: Bovine oocyte BODIPY 493/503 staining results of positive control samples.



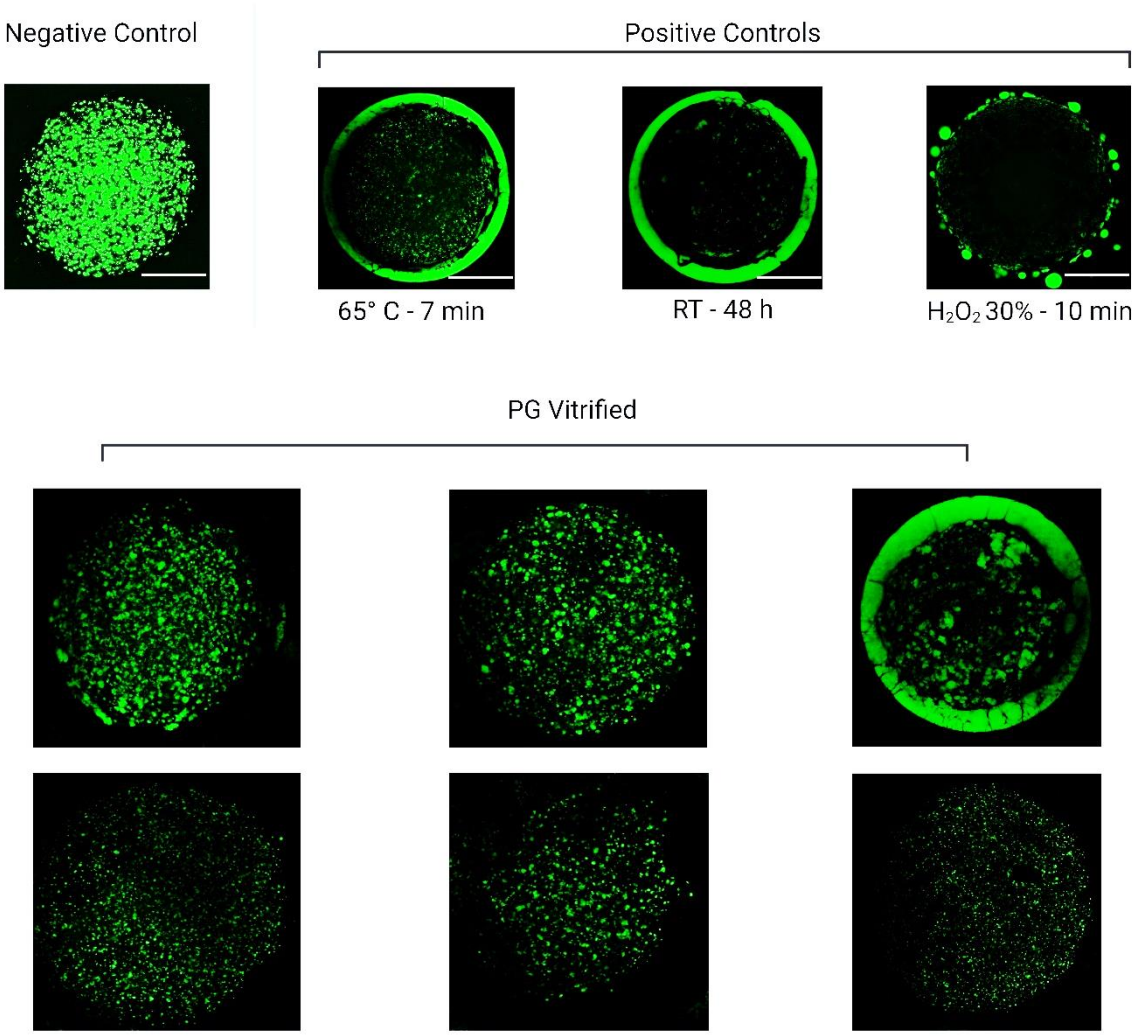
Appendix 3: Bovine oocyte BODIPY 493/503 staining results of immature oocytes. The oocytes are untreated, and at the GV stage.



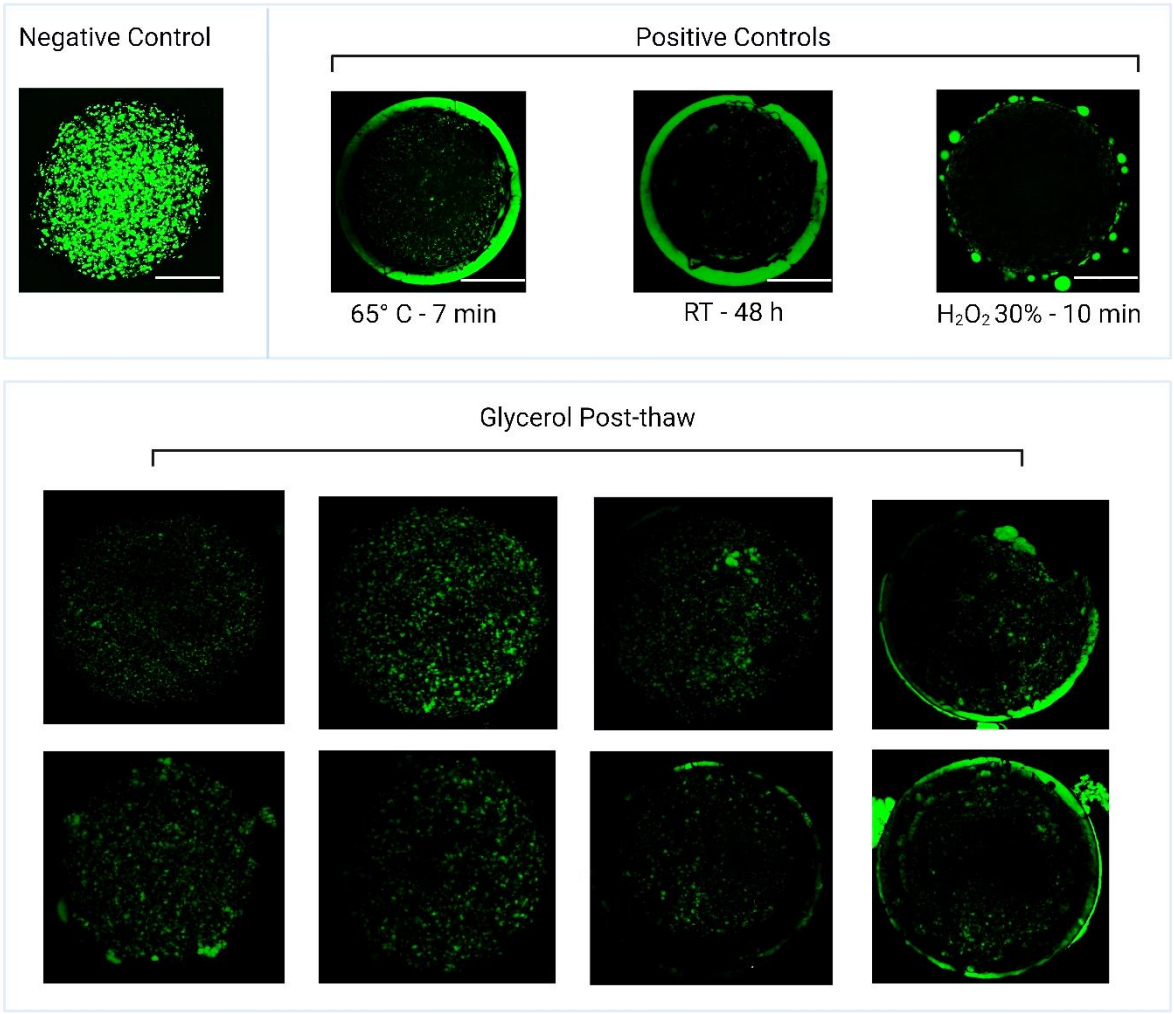
Appendix 4: Bovine oocyte BODIPY 493/503 staining results of cryoprotective agent combinations with ethylene glycol.



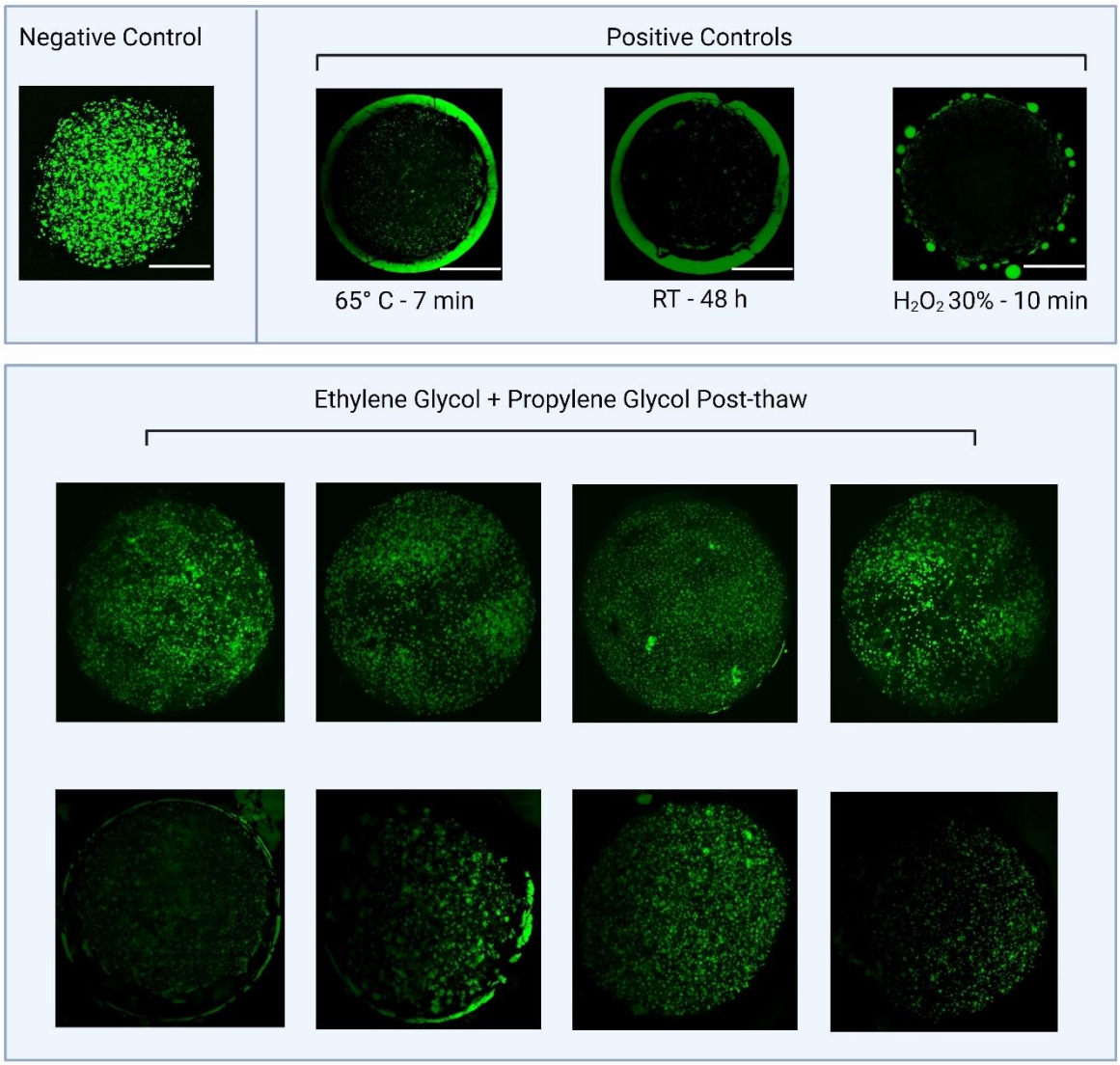
Appendix 5: Bovine oocyte BODIPY 493/503 staining results of cryoprotective agent combinations with dimethyl sulfoxide.



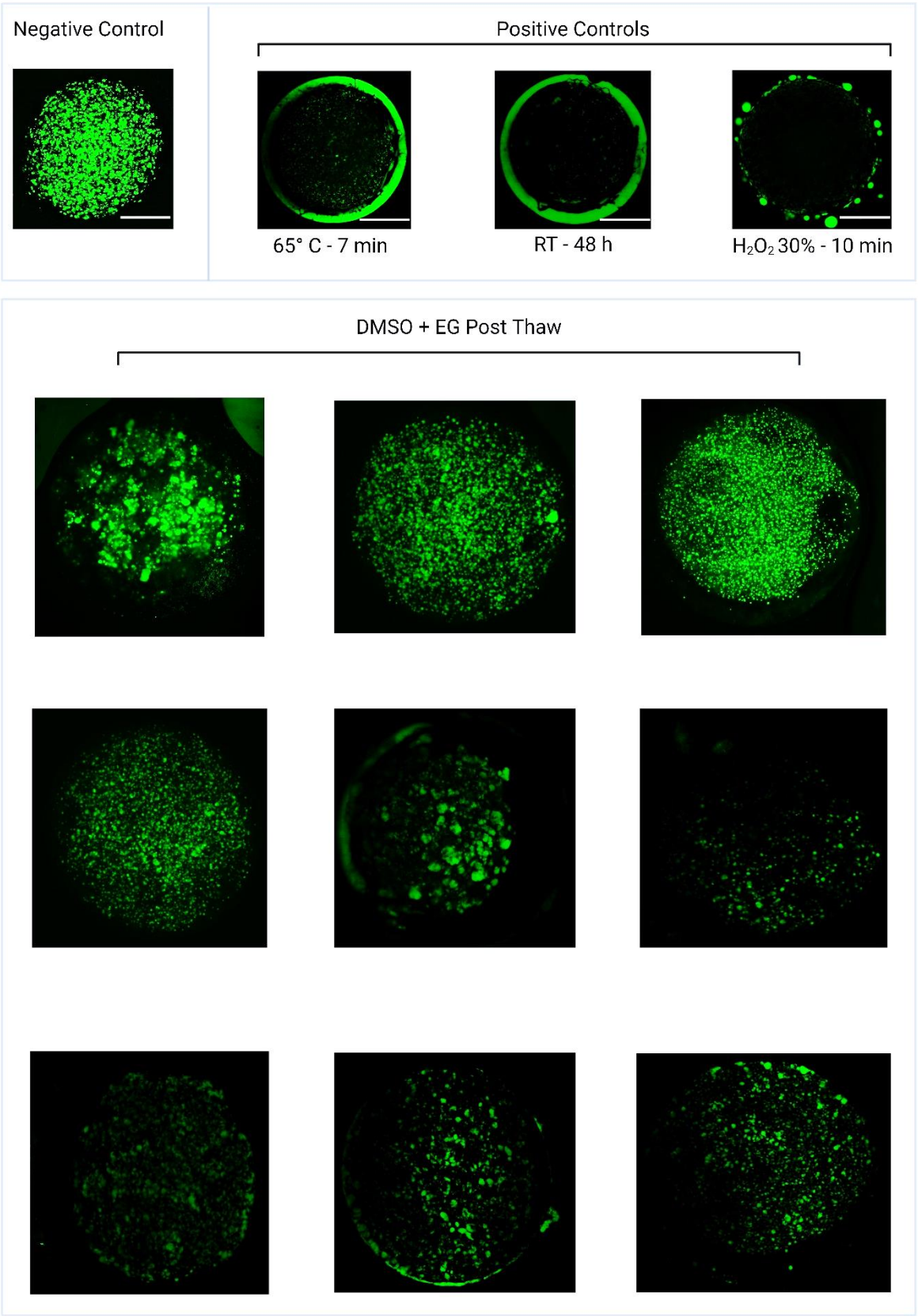
Appendix 6: Bovine oocyte BODIPY 493/503 staining results of vitrified/warmed samples treated with propylene glycol 20%.



Appendix 7: Bovine oocyte BODIPY 493/503 staining results of vitrified/warmed samples treated with glycerol 20%.



Appendix 8: Bovine oocyte BODIPY 493/503 staining results of vitrified/warmed samples treated with a combination of ethylene glycol 15% + propylene glycol 15%.



Appendix 9: Bovine oocyte BODIPY 493/503 staining results of vitrified/warmed samples treated with a combination of ethylene glycol 15% + dimethyl sulfoxide 15%.