Faculty of Biotechnology

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Master's Thesis

The influence of cryoprotectants on bovine embryos

Experimental Biotechnology

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Hamar, June 3rd, 2024

Sabid Ali

Abbreviations

INN – Inland Norway University of Applied Sciences

ARTs – Assisted reproductive technologies

IVM – *In vitro* maturation

IVF – *In vitro* fertilisation

IVC – *In vitro* culture

IVP – *In vitro* produced

LN_2 - Liquid nitrogen

CPAs – Cryoprotective agents

EG – Ethylene glycol

PG – Propylene glycol

DMSO – Dimethyl sulfoxide

GLY – Glycerol

MeOH - Methanol

ROS – Reactive oxygen species

ZP – Zona pellucida

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Abstract

Cryoprotective agents (CPAs) are crucial for vitrification of e.g., oocytes and embryos, aiding in assisted reproductive technologies (ARTs). CPAs protect the cell when exposed to freezing temperatures of liquid nitrogen (LN_2), forming a vitreous glass-like form. However, vitrification requires optimal exposure times and concentration of CPAs to avoid damages such as ice crystallisation, osmotic stress, DNA damage, damage to the zona pellucida (ZP) and oxidative stress. The aim of the present study was to assess the effect of CPAs on bovine oocytes and embryos and utilise these in a vitrification process while evaluating morphology by Hoechst staining. In order to evaluate the effectiveness of the CPAs on oocytes and embryos, parameters of developmental competence were used: cleavage and blastocyst rate (%). Findings showed that EG 7.5% + PG 7.5%, GLY 10%, PG 10% and EG 7.5% + DMSO 7.5% with 73.08%, 77.59%, 81.67% and 80.00% cleavage rate, respectively, performed the best amongst all selected CPAs. Measure of blastocyst rate showed that EG 7.5% + GLY 7.5% and GLY 10% with 40.4% and 39.7%, respectively, performed the best. Parthenogenesis was observed for EG 10% with 7.14% cleavage rate. However, the cleaved oocyte did not develop further. Compact morulae were vitrified using four different CPA (EG 7.5% + PG 7.5%, GLY 10%, PG 10% and EG 7.5% + DMSO 7.5%) concentrations. The post-thaw results showed that these morulae did not develop further and died. This may have been due to several factors such as osmotic stress, damage to the ZP, DNA damage or the low concentration of CPAs. Staining by Hoechst 33342 showed membrane damage and fragmentation of blastomere cells. For future studies to ensure survival and developmental competence after vitrification, higher concentration of CPAs and extra post-thaw procedure steps should be utilised. Furthermore, a TUNEL-assay would aid in providing data on the blastomere state (living/dead) following vitrification.

1. Introduction

1.1 Project background

This is a 60 ECTS accredited master's project for the experimental biotechnology program at Inland Norway University of Applied Sciences (INN). This master's project is part of a larger academic project, named Zygote. This is a project working with several disciplinaries (academic and industrial) and partners being e.g., the breeding companies Geno and Norsvin, and the academic partners Oslo University Hospital, OsloMet, SimulaMet, SINTEF, Sweden's Agricultural University to develop the science and research in the Inland region, allowing the industrial partners to provide competitively on a global scale. The main goal of the Zygote project is to focus of four areas of research; (1) effects of puberty and age for efficient breeding, (2) prediction of *in vivo* fertility, (3) *in vitro* fertilisation and successful embryo development and (4) preservation technologies for gametes and embryos. This master project is focused on research area 4.

1.2 Industrial relevance of cryopreservation

Cryopreservation is a revolutionary technology allowing for preservation of biological material and plays a large role in aiding assisted reproductive technologies (ART). It is a science that encompasses the freezing of cells and banking of biological products to ensure its longevity. As such, it provides long-term storage pertaining genetic material of endangered animals, genetic diversity of farm animals, and for sperm and eggs for fertility treatments. Initially, cryopreservation was discovered by accident in 1949 by C. Polge et al., where he identified glycerol as a cryoprotectant, capable of freezing cells without damaging their cellular components (Polge et al., 1949). As a result of this phenomenon, research into this field of science has bloomed into a wide field of disciplinaries.

1.3 Reproductive technologies in cattle breeding

In the field of ARTs, several methods are utilised to aid in fertility-related treatments wherein eggs or embryos are manipulated. In cattle breeding, the use of embryo production and transfer has increased due to the introduction of genetic selection. Therefore, the value of female heifers and cows has increased in breeding programs. Earlier, artificial insemination (AI) was the main breeding tool, based on the males. Different ARTs techniques involve *in vitro*

fertilisation (IVF), intrauterine insemination (IUI) and intra cytoplasmic sperm injection (ICSI), embryo transfer (ET) to name a few of the most commonly used currently. Historically, the first successful IVF in humans was performed in England, 1978, where a single oocyte was retrieved from the ovary following an unstimulated menstrual cycle. This was fertilised *in vitro* and transferred into the oocyte donor's uterus as an embryo (Eskew & Jungheim, 2017).

Industrially, there has been an increase in the use of *in vitro* produced (IVP) embryos over *in vivo* derived (IVD) embryos. Notably, North America and South America has seen a trend in a notable increase in the use of IVP embryos of cattle, where IVP embryos account for 71.3% and 94.0% respectively (Joao H. M. Viana, 2023). However, in Europe 137,036 of total embryos are accounted for by IVD, and 43,749 are IVP as of 2022(Joao H. M. Viana, 2023). On the other hand, on a worldwide scale, 49.1% of all cattle embryos transferred are cryopreserved indicating the change towards the technology of cryopreservation.

1.4 *In vitro* maturation and fertilisation

In vitro maturation (IVM) is a method where immature follicles are retrieved from the oocyte and matured to the metaphase II stage (MII). This procedure may be used in patients that are at risk of ovarian hyperstimulation syndrome (OHSS), polycystic ovary syndrome (POS) or in women with oestrogen-sensitive cancer (Eskew & Jungheim, 2017; Lim et al., 2013). However, the use of ARTs in humans would not be possible without the use of animal models like the bovine. This is due to the extreme scale required to study women ovarian physiology, ethical concerns of experiments that could affect future fertility potential of women and the high cost. Several ARTs are used in the dairy industry, ranging from AI, sperm sexing, IVF and ET. These tools aid in maximising the genetic enhancement of cattle breeds. Furthermore, these techniques can be readily utilised due to the low cost, ease of availability and large sample size to provide several experimental findings (Sirard, 2017). Numerous traits of human and bovine reproductive biology are comparable, including the fact that both species are mono-ovulatory, cycling constantly when not pregnant, have roughly a 9-month gestation period, and have ovaries that are similar in size and shape (Sirard, 2017). The cow has been effectively utilised to investigate several elements of human folliculophysiology and has been recommended as a good animal model to study human ovarian function (Ménézo & Hérubel, 2002; Sirard, 2017) .

Maturation of the oocyte is the first and most important step in embryo production, providing the prerequisite for further embryonic development (Naspinska et al., 2023). By doing this, it is possible to recreate an *in vivo* like environment for the oocytes to grow. As such, the maturation pertains to utilising immature oocytes and developing them to the metaphase II (MII) stage of meiosis. This makes fertilisation possible to develop the oocyte further and occurs as a result of nuclear maturation and cumulus expansion which facilitates the frequency of fertilisation (Leibfried-Rutledge et al., 1989).

IVF involves the use of sperm to fertilise the mature oocyte, allowing it to develop into an embryo that can be implanted. This can be achieved by utilising frozen sperm from desired donors to produce offspring with desirable traits. However, embryos developed *in vitro* have lower pregnancy rates and diminished freezing quality compared to those derived *in vivo* (Ferré et al., 2020). However, with the development of genetic tools to improve animal selection and culture media to best recreate *in vivo* mimicking environments for the oocytes to thrive, IVF has seen leaps and bounds of success (Hansen, 2006).

1.5 Cryopreservation techniques

There are three basic methods of cryopreservation. These are slow freezing (SF), rapid freezing and vitrification. In slow freezing, a process of freezing samples by a gradual and slow decrease in temperature is utilised. This reduces ice formation by dehydrating the cells and is widely used for single cells exposed to low concentration of CPAs.

Rapid freezing is a technique that is applied by lowering the temperature of samples to cryogenic temperatures by using liquid nitrogen (LN_2). This method lies between SF and vitrification concerning speed of cooling. The freezing of samples is achieved by utilising LN_2 vapour. This is done by freezing the samples in the vapour of LN_2 by placing the samples in a selected distance above LN_2 (AbdelHafez et al., 2010; Bearer & Orci, 1986).

However, this technique has a similar problem to SF, wherein intra-cellular ice crystal formation in the cells arise when frozen. This may be a contributor to the low pregnancy rates which arise from this method of freezing oocytes and embryos (AbdelHafez et al., 2010). On the other hand, rapid freezing is a great technique that is commonly used for semen cryopreservation. It works in tandem with non-penetrating cryoprotectants such as sucrose and

trehalose, providing better total post-thaw motility and progressive motility in straws compared to vitrification.

Vitrification is a radical approach to cryopreservation, by preventing ice formation by instantaneous freezing of the sample, providing a glass-like structure(Jang et al., 2017). This approach provides an advantage over SF wherein the solution and cells are solidified without the crystallisation of ice. A major prerequisite in cryopreservation is the use of cryoprotectants. The agents are responsible for preserving the cells to prevent damage when undergoing rapid changes during freezing. It aids to reduce the formation of ice crystals within the cytoplasm of cells, with key elements being that the CPAs should be able to penetrate the cell membrane and be distributed through the cytoplasm of cells and have low toxicity as to not damage the cells. These advantages seen in vitrification has attracted a large range of researchers into developing this technology further.

1.6 Cryopreservation principles

As mentioned, cryoprotectants are key elements needed for the survivability of cells exposed to freezing elements. However, we must first address the effect subzero temperature have on healthy tissue or cells. Exposure of cells to temperature below 0 °C without CPAs is severely detrimental to the cells. Water is a large constituent of cells, encompassing approximately 80% of tissue mass (Whaley et al., 2021). As such, when water undergoes the freezing process, both intra- and extracellular damages arise influencing structural changes of the cell. There are two theories that arise as a result of freezing unprotected cells (Figure 1). Firstly, the formation of crystals causes disruption of cellular membranes and revives the cell with its structural integrity following thawing. Secondly, the concentration of solutes increases intracellularly in the remaining liquid as ice crystals form during cooling. As a result, cooling without a protective agent for the cells results in cell death. Therefore, there is a need to associate freezing with appropriate cryoprotectants and optimal cooling and thawing rates (Pegg, 2002; Whaley et al., 2021).

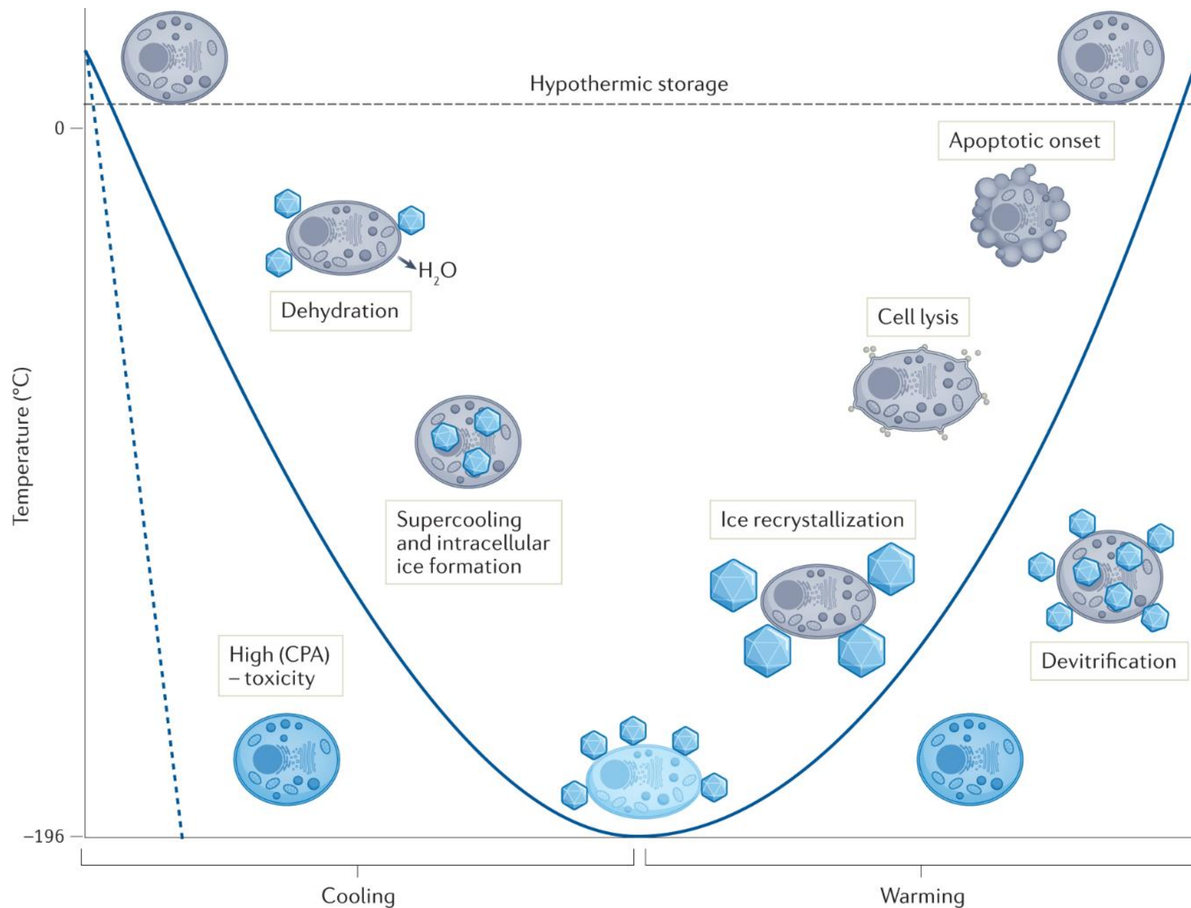

Figure 1. Cell exposed to freezing temperature. *The cell relieves itself of water during dehydration and undergoes freezing. Intracellular ice is formed, and the cell is frozen both intracellularly and extracellularly. High concentration of cryoprotective agents is used to ensure the cell can achieve vitrification, however too high and it deems itself toxic to the cells. At -196 °C, the cell is frozen and can enter the warming stage. Vitrified samples can be unstable during this, leading to further ice growth. Cryopreservation can induce apoptosis and eventual cell death post-thaw.* (Murray & Gibson, 2022), License ID: 1483548-1

1.7 Cryoprotectants

Cryoprotectants may be used in combination, utilising both intra- and extracellular protectants to ensure protection of both the inside and outside of the cell, eliminating ice formation. However, cryoprotectants are inherently toxic, therefore a very high concentration of CPAs can be detrimental to the cell.

As such, concentration, exposure times, cooling, and thawing rates are monitored and changed accordingly to ensure best possible results. The core ideas follow the quantity of ice formation, location of ice in relation to the cells, toxicity of cryoprotectants, the temperature that the

toxicity is dependent on and the ratio of osmotic changes in the volume of cryoprotectants used (Aljaser, 2022).

1.7.1 Penetrating cryoprotectants

A range of penetrating cryoprotectants exist such as glycerol (GLY), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) which work intracellularly. These must be highly soluble at lower temperature, have the ability to cross the cell membrane and be in theory, low in toxicity.

Structurally, the aforementioned penetrative cryoprotectants are considerably small, being less than 100 Daltons with amphiphilic nature allowing them to penetrate the cell membrane to exert its function inside the cell. They exert the ability to form hydrogen bonds with water found within the cell. Thus, when exposed to subzero temperature, the freezing point of water is lowered and less water molecular are able to interact upon themselves to reduce critical nucleation sites, i.e., crystal formation (Whaley et al., 2021).

The concentration of CPAs used should always be taken into consideration. However, there is evidence to showcase the properties that CPAs present. An example of this is DMSO, where low concentrations have been demonstrated to reduce membrane thickness, allowing for an increased permeability via the membrane (Gurtovenko & Anwar, 2007). This can be advantageous as intracellular water can be replaced by DMSO to elevate survival during vitrification. However, at higher concentrations (40%), lipid bilayers began to disintegrate (Gurtovenko & Anwar, 2007). Thus, there is a concern to not utilise higher concentrations as toxicity events increase the more that is used.

1.7.2 Non – penetrating cryoprotectants

On the other end of the spectrum, there are non– penetrating CPAs. These do not permeate the cell, working extracellularly to protect the outer layers of the cell. These are larger, and linked covalently in dimers, trimers, +or polymers. Examples of such agents are polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), sucrose, trehalose, and trehalose. In solution, these sugars work to decrease the viscosity of the vitrification solutions, thus raising the glass transition temperature needed to vitrify the extracellular solution (Yong et al., 2020). In warming of the cells, the agents aid in controlling the influx of water into the cells preventing osmotic imbalance and eventual cell death. Furthermore, the sugars aid penetrating CPAs when used

in tandem by reducing viscosity and tonicity of vitrification media, allowing lower concentrations and lower incidences of osmotic shock and ice formation to form (Yong et al., 2020).

1.8 Cryoinjuries

1.8.1 Ice crystallisation

Ice recrystallisation refers to the increasing size of ice crystals over time in a frozen sample/material. This might lead to cell death, where it occurs in the thawing phase of cryopreservation. Furthermore, exposure to cold temperatures also has been shown to induce disorganisation of MII spindles and retention of the secondary polar body, resulting in chromosomal deviations (Stachowiak et al., 2009). Hence, ice binding proteins inhibiting recrystallisation are available as antifreeze glycoproteins. In bovine embryo testing, AFPIII (antifreeze protein derived from Zebrafish) indicated improvement on the development of the embryo on day five of culture, however, further progress was not maintained up to blastocyst formation (Robles et al., 2019). It showed protective effects against chilling-induce injury in bovine blastocysts during vitrification. The results were obtained using 10 mg/ml AFPIII, and it was indicated that an increased concentration reduced embryo survival (Robles et al., 2019). AFPIII is a possible protective agent that may be used in oocytes and embryo preservation, however interactions with other molecules and chemicals must be taken into consideration.

1.8.2 Osmotic stress

A quick shift in the concentration of the solutes surrounding a cell result in a fast alteration in the flow of water across its cell membrane and leads to a physiological dysfunction known as osmotic shock or stress. When oocytes are exposed to high CPA concentrations, there is an osmotic tolerance limit as to how much the cell may shrink or swell beyond its volumetric limits. If the osmotic change is stressful, it can cause subsequent damage to the cell membrane (Tu et al., 2022a). This was shown in a study where MII oocytes were exposed to hypotonic solute environment, and there was a 50% decrease in development into blastocyst with 9% and 13% blastocyst rate following exposure to EG and DMSO, respectively, compared to their control at 25% (Agca et al., 2000).

In embryos, it has been shown that blastocysts in later stages such as expanding blastocysts demonstrate higher tolerance to osmotic stress compared to smaller embryos. This may be

reflective of the cell quality and makeup of the cell, indicated by higher cell numbers, more blastocoele fluid, smaller number of trophectoderm cells (Caamaño et al., 2015; Kaidi et al., 2000). On the other hand, the permeability of the cell towards CPAs or water may change in the development of the embryo.

1.8.3 DNA damage

DNA, also known as deoxyribonucleic acid, is a key that carries the genetic information for the development and function of living organisms. The double-stranded helix is composed of monomeric units called nucleotides: cytosine, guanine, adenine, and thymine which are the building blocks.

DNA damage is a possible consequence of cryopreservation of oocytes and embryos. Nuclear transfer experiments demonstrated the effects of vitrification on bovine oocytes, indicating changes being present on nuclear components (Kubota et al., 1998; Stachowiak et al., 2009). DNA damage was evaluated by comet assay in bovine oocytes exposed to varying vitrification methods. The study suggested that vitrification of MII oocytes yielded DNA fragmentation (Stachowiak et al., 2009). This has also been hypothesised in studies on humans (Lindley et al., 2001) and equine oocytes (Tharasanit et al., 2009).

A contributor to DNA damage observed in embryos has been linked to the generation of reactive oxygen species (ROS) (Refer to chapter 1.8.5 for further details on ROS). Following a comet assay of individual blastomeres from bovine blastocysts, they observed minor DNA damages amongst the majority of all cells (Sturmey et al., 2008). Furthermore, it has been seen that sperm with damaged DNA is capable of fertilising the egg, but DNA repair mechanisms are active during early development of the embryo reducing the impact. However, this resulted in reduced blastocyst growth and pregnancy rates (Musson et al., 2022; Sturmey et al., 2008).

1.8.4 Cell membrane

The zona pellucida (ZP) is the membrane around the oocytes and embryos and is the protective barrier of the egg, providing essential coverage for growth and fertilisation. This thick membrane allows for acrosome reaction adhesion and penetration by sperm cells while simultaneously functioning as a protective measure against polyspermy (Ickowicz et al., 2012). The ZP layer degenerates gradually when the blastocyst has reached the hatching stage,

being replaced by trophoblastic cells to allow for adherence to the uterine wall (Wassarman & Litscher, 2022).

There are two pathways for the movement of water and cryoprotectants across the membrane, diffusion through the lipid membrane and facilitated diffusion. It has also been suggested that permeability changes amongst the differing developmental stages of the bovine oocyte to blastocyst, with low permeability at the oocyte and early embryo stage, and highest at morulae stage(Jin et al., 2011). At the oocyte and early embryo stage, simple diffusion is the main path for movement of CPAs and water into the cell. However, aquaporin 3 and 7 (AQP3, AQP7) aid in facilitated diffusion to move CPAs, especially GLY and EG at the morulae/blastocyst stage (Casillas, 2020; Jin et al., 2011).

The membrane diffusion capacity of CPAs is not the same in all cells, however the cell membrane is the main component that undergoes the most damage during cryopreservation (Casillas, 2020). This includes loss of liquid content in their lipid components, and a reduced ability to expand during rehydration. It has been suggested that the potent interaction between water and permeable CPAs, especially DMSO, can harm proteins by dehydrating them, and has been considered the origin of the harmful effects of vitrification solutions. Any factors that modify the interaction of proteins with water, can cause instability leading to denaturation (Casillas, 2020; Gurtovenko & Anwar, 2007).

1.8.5 Oxidative stress

During oxidative metabolism, the produced component is ATP, however, an undesired product is also ROS. ROS are unstable molecules containing oxygen that is capable of reacting easily with other molecules in a cell. As they are free radicals, it may damage DNA, RNA, and other proteins (Cao et al., 2022). For example, ROS in oocytes has been shown to damage the mitochondria and endoplasmic reticulum, subsequently damaging spindles, DNA, proteins, and lipids (Cao et al., 2022; Keane & Ealy, 2024).

In embryo development, oxidative phosphorylation is the primary source of energy occurring between the 1-cell and morula stages (Lopes et al., 2010). As such, metabolically active embryos tend to produce higher levels of ROS in the earlier stages of development. They may have greater rates of development than other embryos but may not exhibit the ability to produce pregnancies at the same rate as embryos which develop with lower energy demands. Embryos that have reduced metabolism are termed as “quiet” embryos (Leese et al., 2022).

The reasoning behind this hypothesis as to why "quiet embryos" has a better competency for producing pregnancies lies behind the idea that there is less oxidative damage as a result of their lower metabolic rate. The term has been changed to the "Goldilocks hypothesis", encompassing the idea that the "perfect" embryos are ones that require neither too little nor too much energy during the earlier stages of their development (Keane & Ealy, 2024; Leese et al., 2022).

Vitrification might reduce the tolerances of the embryos against oxidative effects. The technique can induce reduction-oxidation (redox) states. As a result of this, an imbalance between ROS and cellular antioxidant defences appears, causing potential damages to the cytoskeletal structure, membrane lipids, proteins, and DNA, thus reducing the viability of the embryo (Madrid Gaviria et al., 2019). As such, antioxidants e.g., glutathione (GSH), β-mercaptoethanol (βME) and resveratrol have been used as additives to media in several studies. These antioxidants have shown improvements in embryo development and post-thaw survival (Hosseini et al., 2009), indicating greater developmental competence with the presence of βME.

1.9 Confocal microscopy for morphological assessment

Confocal microscopy, also known as confocal laser scanning microscopy (CLSM) was first built in 1955 by Marvin Minsky(Paddock & Eliceiri, 2014). It allows for imaging of fixed or living tissues labelled with fluorescence probes with utmost clarity (Figure 2), with parameters allowing it to identify targeted cellular components. This form of microscopy allows for high-resolution imaging and reconstruction of cells on a 3D level. Furthermore, the technology for 3D imaging is utilised by capturing slices of the samples on the 2D plane, allowing for details of the sample on varying planes (Elliott, 2020). This is a technique utilised in many biomedical fields of sciences.

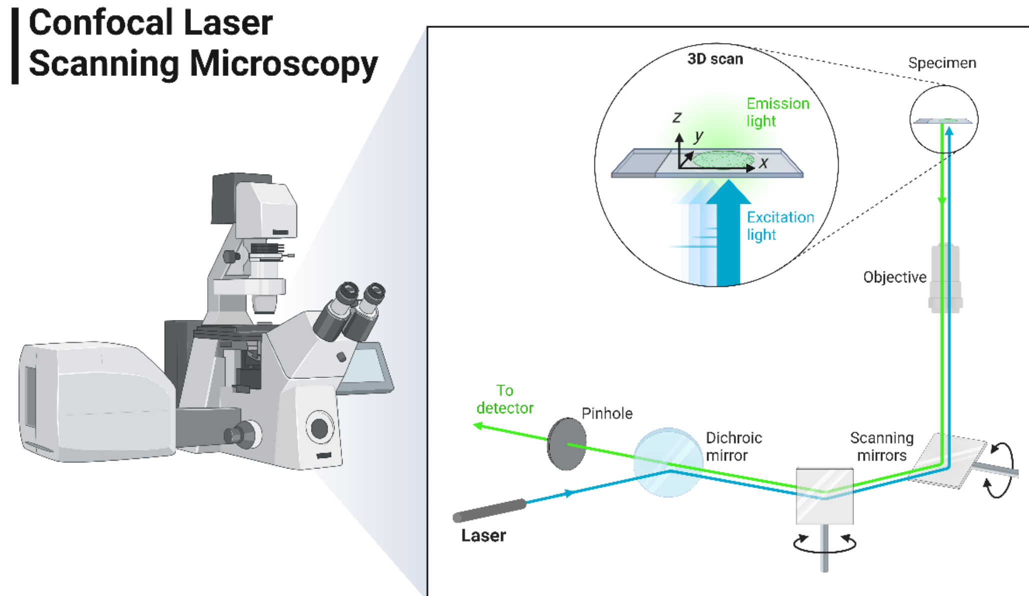

Figure 2. Confocal laser scanning microscopy method of function. *The method includes a laser which utilises several mirrors to hit the target specimen on a microscope slide. The fluorescence is excited, producing an emission light. The emission is returned to the detector as photons and the electrical signal produced returns as an image. Figure created in Biorender.com.*

1.10 Aim of the study

For the efficient use of bovine embryos in breeding programs, cryopreservation is the method of choice for storing of embryos until suitable recipient cows are available. Vitrification procedures are efficient but requires careful selection of CPAs and concentration thereof as well as exposure times, thawing rates and careful handling. Pregnancy rates after transfer of vitrified embryos are significantly lower than for fresh embryos. Blastocyst rate and developmental competence rate are two important factors to evaluate the outcome of vitrification. The main objective of this master project was to identify CPAs applied singular or in combination for compatibility with survival and developmental competence post-thaw.

To achieve the main objective, the following tasks were performed:

- Exposure of embryos to single or combination of CPAs and subsequent evaluation of cleavage and blastocyst rate for developmental competence
- Identify potential parthenogenesis created by CPAs
- Staining and visualization of blastomeres in embryos pre and post CPAs exposure
- Vitrification of compact morulae with selected CPAs and evaluation of the developmental competence post-thaw

2. Material and Methods

2.1 Experimental design

The goal of the experiments was to identify suitable CPAs and utilise these in vitrification procedures to see post-thaw embryo growth and cell viability. Two main experiments were performed, the first to identify suitable concentrations and singular/combinations of CPAs (Figure 3).

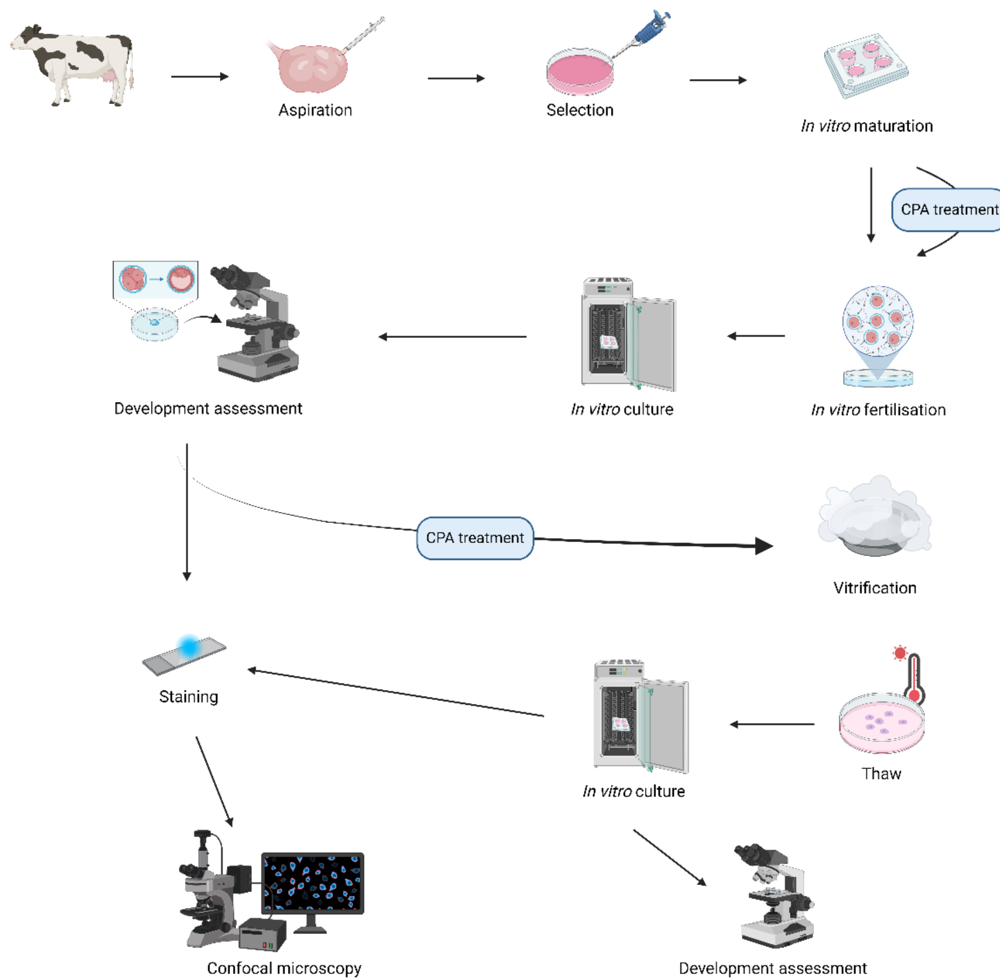

Figure 3. Experimental procedure flowchart. *Bovine oocytes were aspirated and selected for in- vitro maturation. Experiment 1: oocytes were treated with CPA and cultured for developmental assessment and stained. Experiment 2: embryos were cultured and treated at the compact morula stage, vitrified, thawed, and cultured for developmental assessment and stained. Stained samples were visualised by confocal microscopy. Figure created in Biorender.com.*

The second experiment was to utilise selected CPAs to undergo vitrification and post-thaw processing for post-thaw embryo growth and visualise blastomeres (Figure 3). In the first experiment, prevalently used CPAs such as GLY, EG, PG, DMSO and MeOH were utilised for stress testing of oocytes. Groups of 20 ~ 30 mature oocytes were exposed to singular/combination of both CPAs and 2 - 3 experimental replicates were performed. An extra experiment was performed, wherein the CPAs showed the highest cleavage and blastocyst rates were tested to monitor parthenogenesis. These were only stress tested and not fertilised.

The second experiment, four CPAs that had demonstrated the highest blastocyst yield were utilised for vitrification. Firstly, they were subjected to stress testing on compact to record development upon exposure to CPAs at this stage. Following this, the samples were subjected to vitrification and post-thaw processing.

2.2 Chemicals and solutions

All chemicals and reagents used were purchased from Sigma- Aldrich (Oslo, Norway) unless otherwise stated. Media used for bovine IVP were obtained from IVF Biosciences (Falmouth, UK). For transportation and washing of the ovaries, 0.9% saline solution (0.9% NaCl) with 1mg/ml kanamycin was used. For the collection of cumulus oocyte complexes (COCs), heparin (5000 IU/ml) was used. COCs were washed with Biowash (IVF Bioscience, Falmouth, UK). For the maturation of the oocytes, BO-IVM medium (IVF Biosciences, Falmouth, UK) was used. For *in vitro* fertilisation, BO-IVF medium (IVF Biosciences, Falmouth, UK) was used alongside BO-SemenPrep (IVF Biosciences, Falmouth, UK) to wash the semen. Cryoprotectants were made with Base B (prepared as 137 mM NaCl, 2.7 mM KCI, 8.1 mM $Na_2HPO_4 \cdot H_2O$, 1.76 mM KH_2PO_4, pH 7.4) and all cryoprotectants: GLY, MeOH, PG, EG and DMSO were from Sigma Aldrich (Oslo, Norway). For *in vitro* culture, BO-IVC (IVF Biosciences, Falmouth, UK) alongside BO-Oil (IVF Biosciences, Falmouth, UK) was used to culture the embryos. Equilibration and vitrification media were made in 1ml Eppendorf tubes with Base B. Sucrose was used in the vitrification media and post-thaw media, this was obtained from Sigma Aldrich (Oslo, Norway). Hoechst (H-33342, B2261, Sigma) was used for DNA staining. For staining, the oocytes were washed with 0.004g/ml BSA 0.01% triton X-100 in phosphate-buffered saline (PBS), and oocytes were fixed in 4% paraformaldehyde (PFA), and mounted on slides with mounting medium (Dako, Glostrup, Denmark).

2.3 Aspiration and selection of oocytes

Bovine ovaries were collected from Nortura slaughterhouse at Rudshøgda and were placed into a thermos with 0.9% saline at 37 °C and transported to the laboratory. The organs were washed and massaged three times with saline at 37 °C to remove residual blood. The organs were aspirated of cumulus oocyte complexes (COCs) avoiding lightly coloured follicular regions on the oocyte (Figure 4). Oocytes were aspirated with 5 ml syringes, using an 18-gauge needle (Figure 4), and placed into 50 ml falcon tubes with heparin held in a 33 °C water bath (Figure 4). Thereafter, the falcon tubes were placed in the incubator for a few minutes for COCs to settle. The pellet was pipetted and placed in a 90 mm scored petri dish (Thermo Fisher Scientific, Waltham, MA, USA) with Biowash and the plate was gently swirled until Biowash and pellet were mixed.

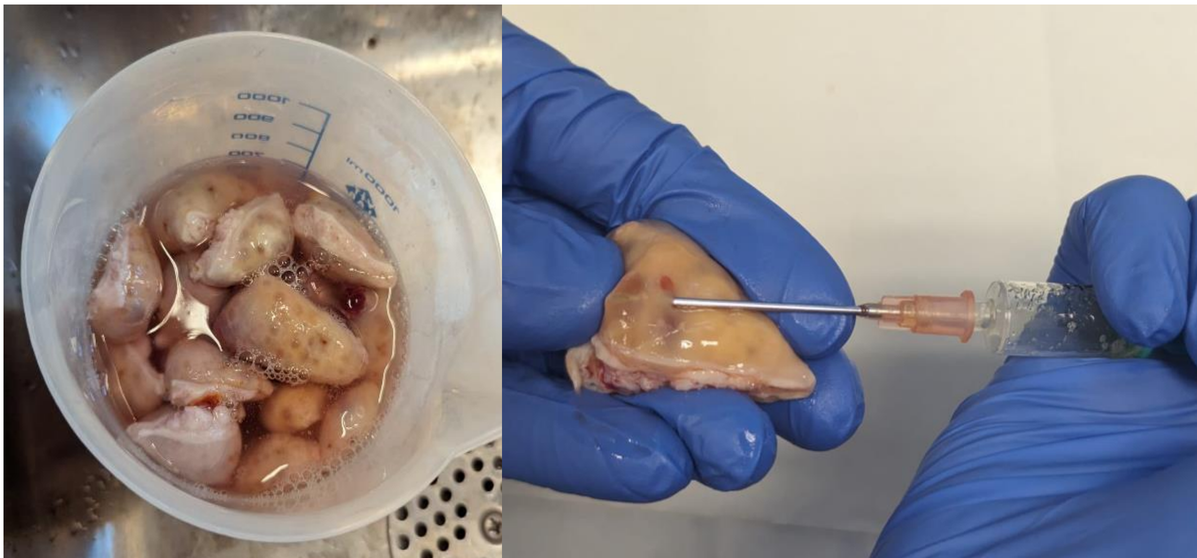

Figure 4. Aspiration and selection of oocytes*. Ovaries were washed in solution three times (left) to drain blood. Immature oocytes were collected by aspiration using a syringe and needle (right). Images taken from personal procedures.*

2.4 *In vitro* maturation of bovine cumulus oocyte complexes

The oocytes were collected by pipette and visualised with a Leica MS5 stereo microscope (Ortomedic AS, Lysaker, Norway), avoiding oocytes with broken/irregular cumulus cells after visualisation. Washed COCs were placed into a 4-well NUCLON (Thermo Fisher Scientific, Waltham, MA, USA) dish containing maturation media and divided equally to prevent overcrowding, no more than 30 COCs per well. The cells were incubated for 24 hours at 38.8 °C with 6% CO_2 in a humidified environment for 21 hours. After this, the COCs were observed

microscopically and the majority of the oocytes were matured, assuming they were at the metaphase II stage.

2.5 CPA exposure and parthenogenesis

The matured oocytes were removed from the incubator after 20 hours and exposed to cryoprotectants for set times. The oocytes were exposed to each respective CPA for 10 mins at room temperature (RT), at varying concentrations. The CPAs were made with Base B in 1ml Eppendorf tubes and stored at 35 °C in the incubator before use. Replicates were performed on the CPAs as seen in table 1. Thereafter, they were placed back into the incubator for 1 hour.

Table 1. Controls and cryoprotectants used for oocyte stress test. *Six control treatments were administered, one non-treated sample and five treated with selected cryoprotectants but without in vitro fertilisation. 17 cryoprotectant treatments were used, with four concentrations varying from 10%, 15%, 20% and mixtures of cryoprotectants at 7.5%.*

Control			
• Non – treated sample	• DMSO 10% - No Fertilisation	• Propylene Glycol 10% - No fertilisation	• Glycerol 10% - No fertilisation
• Ethylene Glycol 7.5% + Propylene Glycol 7.5% - No fertilisation	• Ethylene Glycol 20% - No Fertilisation		
Treatments			
• DMSO 10%	• DMSO 15%	• DMSO 20%	
• DMSO 7.5% + Ethylene Glycol 7.5%	• DMSO 7.5% + Propylene Glycol 7.5%	• DMSO 7.5% + Methanol 7.5%	
• Ethylene Glycol 7.5% + Methanol 7.5%	• Ethylene Glycol 7.5% + Propylene Glycol 7.5%	• Ethylene Glycol 7.5% + Glycerol 7.5%	

• Ethylene Glycol 10%	• Ethylene Glycol 20%	• Propylene Glycol 10%	• Propylene Glycol 20%
• Glycerol 10%	• Glycerol 20%	• Methanol 10%	• Methanol 20%

2.6 Preperation of semen and *in vitro* fertilisation

Droplets of BO- IVF (100 µl) were prepared onto a culture dish. The matured oocytes were transferred into the IVF droplets from the IVM media. IVF droplets (60 µl) with oocytes were added into 400 µl of IVF media prepared in a 4-well plate and placed back into the incubator. Two straws of cryopreserved semen from a single Norwegian Red bull were obtained from nitrogen tanks and thawed at 37 °C for 1 min and collected into an Eppendorf. The semen was transferred into a falcon tube with BO-Semenprep and centrifuged at 330 x g for 5 mins. The supernatant was removed, and the remaining pellet was mixed more with a second semen prep solution and centrifuged again. The prepared semen (20 µl) was diluted with 60 µl PBS. The diluted sperm (3 µl) was placed onto a pre-warmed microscope slide and analysed for motility and concentration using CASA (Microoptics, Barcelona, Spain). This data was recorded for total concentration to be calculated according to sample size of oocytes in wells.

With the sperm and oocytes prepared, sperm was calculated for a final concentration of two million sperm/ml and 40 µl was added to each well. The dish with matured COCs and sperm were incubated at 38.8 °C with 6% CO_2 in a humidified environment for 18 hours.

2.7 *In vitro* culture

After incubating the fertilised oocytes, the early-stage embryos were moved into a falcon tube with 4 ml Biowash. These were divided into two tubes, which were vortexed vigorously against each other to denude the zygotes of cumulus cells and debris. These cells were then washed three times to remove debris into groups of no less than 20 embryos. Cells were washed in BO- IVC and placed in an IVC droplet before being moved into a well with 500 µl IVC covered by 330 µl immersion oil in a 4- well plate. The samples were placed in the incubator at 38.8 °C with 6% CO_2, 6% O_2, and 88% N_2, under humid air. The cleavage rate was recorded on day two, while the blastocyst rate was recorded on day eight.

2.8 Cryopreservation

Compact morulae were selected and placed into a single well on day five, as samples used for cryopreservation. LN_2 was prepared in a nitrogen bucket with a lid, and labelled cryo-tubes were prepared. Morulae were selected (~10-12) and exposed in 500 µl equilibrium media for 8 min in 5% medias, and 4 mins in 3.75% media, respectively. Thereafter, these samples were exposed to 500 µl vitrification media with 200 µl 0.5% sucrose for 30 seconds. The exposed morulae were dropped into metal box with LN_2 using a micropipette and 2 µl of media. Once all the samples were vitrified for each respective CPA, these were collected using acclimatised forceps into cryo-tubes and stored in LN_2 tanks, topped with cotton to prevent floating of the tubes into the tanks.

Table 2. Solutions for equilibrium and vitrification. *Four CPAs were used for equilibrium and vitrification: glycerol, polyethylene glycol, DMSO + ethylene glycol, ethylene glycol + propylene glycol.*

CPA	**Equilibrium Media (%)**	**Vitrification Media (%)**
Glycerol	5%	10%
Propylene glycol	5%	10%
DMSO + ethylene Glycol	3.75% + 3.75%	7.5% + 7.5%
Ethylene glycol + propylene glycol	3.75% + 3.75%	7.5% + 7.5%

2.9 Post-thaw process

Following cryopreservation, the samples had to be recovered in a post-thaw process. A water-bath was prepared, set at 37 °C. Three sucrose concentrations (0.5%, 0.2% and 0.1%) were prepared with Base B media, to be used in the thawing process. The cryopreserved samples were retrieved from samples and held in a bucket with nitrogen to prevent external heating. Tubes were placed in the water bath for thawing, one at a time for 5-6 seconds. Thawed samples were transferred into wells prepared with 0.5%, and droplets of 0.2% and 0.1% sucrose solutions for 30 seconds each, respectively. Following this, the morulae were pipetted into prepared IVC droplets and consequentially moved to IVC media plates for incubation at 38.8 °C with 6% CO_2 and 7% O_2. Thereafter, the morulae were monitored, and blastocyst rate was collected on days 2 and 3 after vitrification.

Table 3. Post- thaw media. *Three media were used for post- thaw process, with concentrations 0.5%, 0.2% and 0.1%.*

Chemicals	Warming Media I	Warming Media II	Warming Media III
Base B	10 ml	10 ml	10 ml
Sucrose	1.78g (0.5%)	0.6g (0.2%)	0.3g (0.1%)

2.10 Staining

On day eight, wherein blastocyst rate had been observed, blastocysts were collected for staining. Blastocysts were taken using a micropipette, placed into PBS for 10 minutes for washing. These were transferred into PFA droplets immersed in oil for 30 mins for fixation. Lastly, the blastocysts were moved into droplets of 1:1000 Hoechst 33342 (H-33342, B2261, Sigma) and placed in a dark drawer for 10 mins. Samples were then fixed onto microscope slides with 3 µl mounting media (Dako, Glostrup, Denmark) and a circular coverslip was placed to enclose the sample onto the slide. These slides were placed in a dark drawer for 24 hours.

The samples were visualised using a Leica DMi8 fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany) with the LAS X programme (Leica Microsystems,

Wetzlar, Germany). The slides were exposed to UV light for Hoechst 33342 stained blastocysts at 20x magnification. The samples were set to be visualised as blue via the LAS X programme, with Hoechst 33342 evaluated with a 420 – 480 nm excitation laser and a 410 – 480 emission filter. The blastocysts were captured on the LAS X programme.

2.11 Statistical analysis

Due to different treatments conducted on the samples, the results of this experiment are descriptive, thus, statistical analysis was not performed on the developmental results.

3. Results

3.1 Cryoprotectant treatments on oocytes and development assessment

A total of 886 oocytes were produced over the course of four weeks (two replicates), with 178 in control groups and 708 exposed to varying concentrations and combinations of CPAs.

Table 4. ***CPA treatments and cleaved bovine oocytes counted by microscope.*** *Sample size is data accumulated from two replicates. Three control treatments and 17 varying CPA treatments were administered. Cleavage rate was measured as sum of cleavage divided by total number of oocytes. Oocyte development was measured at day 2 as not cleaved, two-cell stage, three-cell stage, four-cell stage, and a total cleavage rate (%) calculated.*

Treatment	Total Oocyte	Not cleaved	2 - cell	3 - cell	4 - cell +	Cleavage rate
Control - No treatment	102	20	17	30	35	80.39%
Control - Methanol 20% No IVF	34	34	0	0	0	0
Control - Ethylene glycol 10% - No IVF	42	39	2	0	1	7.14%
Ethylene glycol 10%	37	13	4	15	5	64.86%
Ethylene glycol 20%	35	11	5	17	2	68.57%
Propylene glycol 10%	60	11	14	21	14	81.67%
Propylene glycol 20%	38	8	6	16	7	76.32%
Glycerol 10%	58	13	17	20	8	77.59%
Glycerol 20%	40	14	8	14	3	62.5%
Methanol 10%	39	18	8	12	1	53.85%
Methanol 20%	33	19	3	11	0	42.42%
DMSO 10%	56	18	10	13	3	46.43%
DMSO 20%	34	15	9	6	4	55.88%
DMSO 7.5% + ethylene glycol 7.5%	40	8	8	5	19	80.0%
DMSO 7.5% + glycerol 7.5%	42	16	8	9	9	61.9%
DMSO 7.5% + propylene glycol 7.5%	35	9	9	8	9	74.29%
DMSO 7.5% + methanol 7.5%	37	15	7	3	12	59.46%
Ethylene glycol 7.5% + methanol 7.5%	38	21	5	11	1	44.74%
Ethylene glycol 7.5% + propylene glycol 7.5%	52	14	10	15	13	73.08%
Ethylene glycol 7.5% + glycerol 7.5%	34	8	9	11	6	76.47%

Cleavage rate was measured on day two following IVC, and two CPA treatments showed the highest cleavage rates, being PG 10% with 81.67% and DMSO 7.5% + EG 7.5% with 80% cleavage rate. Furthermore, these CPAs exhibited the greatest number of cells cleaved at the four-cell stage. On the other hand, parthenogenesis was observed for unfertilised EG 10%, wherein 7.14% of the oocytes were cleaved to both two-cell stage and four-cell stage, compared to unfertilised MeOH 20% which did not undergo parthenogenesis. MeOH 20% showed the lowest cleavage rate at 42.42%, wherein it was the only CPA with no 4-cell stage cleavage, similar to its non-fertilised control counterpart. Furthermore, the CPAs that worked in combination with MeOH gave comparatively low cleavage rate. DMSO 7.5% + MeOH 7.5% and EG 7.5% + MeOH 7.5% had much lower rate of cleavage compared to other mixtures, being 59.46% and 44.74%, respectively. Regarding EG 7.5% + MeOH 7.5%, it also had the highest number of non-cleaved cells with 21 oocytes.

3.2 Cryoprotectant treatment development assessment for blastocyst rate

These results were obtained day eight, after exposure to CPAs measuring blastocyst rate. This was measured on day eight, wherein differing growth states were monitored, by registering numbers of young, normal, expanded, expanding, hatched and hatching blastocysts.

***Table 5. CPA treatments and blastocyst states counted via microscopy**. Sample size is combined from two replicates. three control treatments and 17 CPA treatments were accounted for, total oocytes, blastocyst states: young, normal, expanded, hatched & hatching assessed on day eight. Blastocyst rate (%) was calculated with total number of sums of all developed blastocysts divided by total number of oocytes.*

Treatment	Total Oocyte	Young	Normal	Expanded	Hatched	Hatching	Blastocyst Rate
Control - No treatment	102	2	12	11	20	3	47.1%
Control - Methanol 20% No IVF	34	0	0	0	0	0	0%
Control - Ethylene glycol 10% - No IVF	42	0	0	0	0	0	0%
Ethylene glycol 10%	37	0	2	0	6	0	21.6%
Ethylene glycol 20%	35	1	5	3	4	0	37.1%
Propylene glycol 10%	60	0	4	6	6	1	28.3%
Propylene glycol 20%	38	0	2	5	4	4	39.5%
Glycerol 10%	58	0	6	9	6	2	39.7%
Glycerol 20%	40	0	5	3	2	3	32.5%
Methanol 10%	39	1	0	1	4	2	20.5%
Methanol 20%	33	0	0	1	2	0	9.1%
DMSO 10%	56	1	7	9	3	2	39.3%
DMSO 20%	34	0	6	3	2	0	32.4%
DMSO 7.5% + ethylene glycol 7.5%	40	3	3	5	4	0	37.5%
DMSO 7.5% + glycerol 7.5%	42	0	0	3	1	4	19.0%
DMSO 7.5% + propylene glycol 7.5%	35	0	3	2	2	0	19.4%
DMSO 7.5% + methanol 7.5%	37	1	3	1	1	0	16.2%
Ethylene glycol 7.5% + methanol 7.5%	38	0	1	3	0	0	10.3%
Ethylene glycol 7.5% + propylene glycol 7.5%	52	0	5	7	4	5	40.4%
Ethylene glycol 7.5% + glycerol 7.5%	34	1	4	2	1	0	22.9%

The two CPAs which showed high blastocyst rate were EG 7.5% + GLY 7.5% and GLY 10%, with 40.4% and 39.7%, respectively (Table 5). Noticeably, unfertilised control EG 10% did not exhibit further growth into blastocysts despite its high cleavage rate (Table 4). MeOH 20% exhibited the lowest blastocyst rate of 9.1%, alongside EG 7.5% + MeOH 7.5% at 10.3%. These two CPAs followed the same trend of producing the lowest number of divisions, as seen in both cleavage and blastocyst rate (Table 4 and 5).

3.3 Parthenogenesis test

Parthenogenesis was observed among the CPAs which provided the highest blastocyst results from prior experiments. Amongst all the CPAs, all the unfertilised samples did not have any development from oocytes into blastocysts of any form, inferring that there was no parthenogenesis initiated by the CPAs tested.

Table 6. *Measure for parthenogenesis one single experiment.* *Five treatments without in vitro fertilisation were performed. Recorded blastocyst states: young, normal, expanded, hatched and hatching assessed on day 8. Blastocyst rate (%) was calculated with total number of sums of all developed blastocysts divided by total number of oocytes.*

Treatment	Total Oocytes	Young	Normal	Expanded	Hatched	Hatching	Blastocyst Rate %
DMSO 10% - No Fertilisation	20	0	0	0	0	0	0 %
Propylene Glycol 10% - No Fertilisation	20	0	0	0	0	0	0 %
Glycerol 10% - No Fertilisation	19	0	0	0	0	0	0 %
Ethylene Glycol 7.5% + Propylene Glycol 7.5% - No Fertilisation	19	0	0	0	0	0	0 %
Ethylene Glycol 20% - No Fertilisation	21	0	0	0	0	0	0 %

3.4 CPA treatment on compact morulae

The four best performing CPAs from previous experiment (Table 4 and 5) were selected and embryos at the compact morulae stage were exposed to these. The blastocyst rates were measured on day eight post IVF. PG 7.5% + EG 7.5% exhibited the highest blastocyst rate at 92.9% and was the only CPA to exhibit hatching embryos despite the lower number of samples. On the other hand, DMSO 7.5% + EG 7.5% exhibited the lowest blastocyst rate at 40.7%. Furthermore, both this CPA and GLY 10% did not give embryos that did develop further than expanded blastocysts, with no hatched or hatching embryos.

Table 7. CPA treatment on compact morulae and developmental assessment by evaluation of blastocysts obtained *Four treatments: propylene glycol 10%, glycerol 10%, DMSO 7.5% + ethylene glycol 7.5% and propylene glycol 7.5% + ethylene glycol 7.5% treatments and the morulae development into blastocyst stages – young, normal, expanded, hatched & hatching. Blastocyst rate (%) was calculated with total number of sums of all developed blastocysts divided by total number of oocytes.*

Treatment	Morula	Young	Normal	Expanded	Hatched	Hatching	Blastocyst Rate %
Propylene Glycol 10%	30	1	5	8	8	0	73.3%
Glycerol 10%	37	1	10	17	0	0	75.7%
DMSO 7.5% + Ethylene Glycol 7.5%	27	3	3	5	0	0	40.7%
Propylene Glycol 7.5% + Ethylene Glycol 7.5%	28	4	6	6	8	2	92.9%

3.5 Post – thaw assessment

Samples were frozen at the compact morulae stage, and post-thaw, the embryos were allowed to grow into blastocysts and measured for development on day eight post IVF. The morulae did not develop further into blastocysts. There was an increase in granularity seen in all the thawed morulae, and thinner ZP was observed amongst all the thawed morulae (Figure 4). Furthermore, there was an increase in water content shown by the increased distance between the ZP and inner cell mass. There were no observable differences amongst all different thawed morulae.

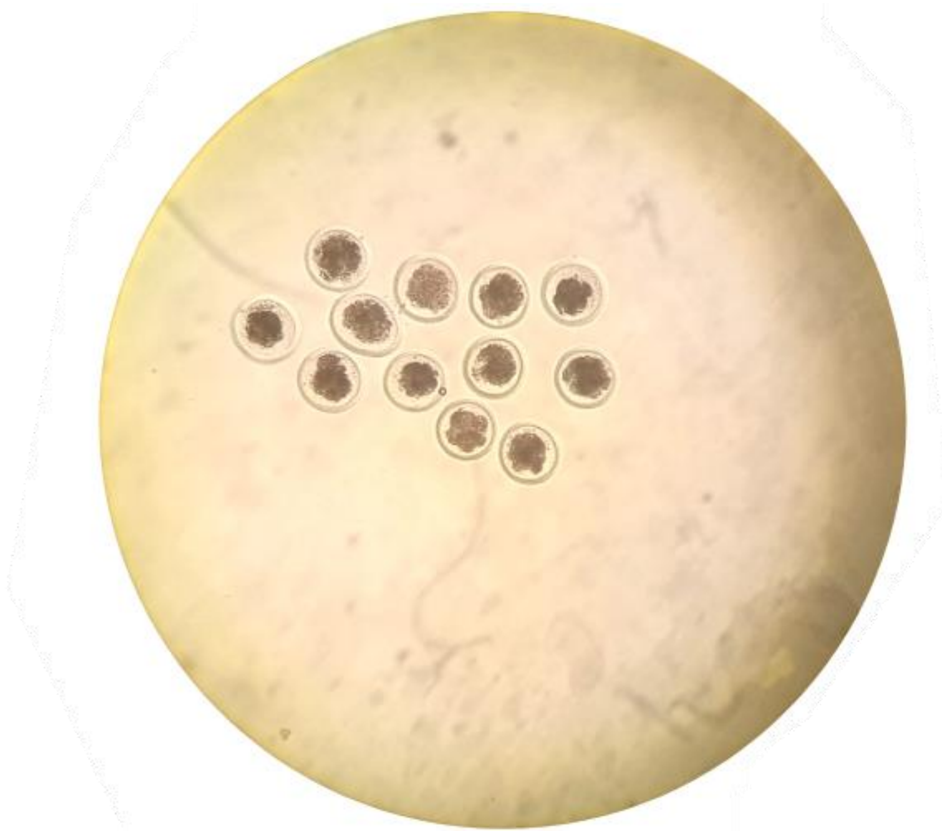

Figure 4. Morulae post-thaw that did not survive *Morulae did not survive after thawing and showed darkness and granularity of the inner cell mass. Image taken from personal procedures via stereomicroscope (10x magnification).*

3.6 Morphological assessment of pre and post - thaw embryos

EG 7.5% + PG 7.5 pre thaw showed circular blastomeres present in the morulae (Figure 5). However, following freezing, the post-thaw blastomeres changed in form, presenting fragmented blastomeres in irregular shapes.

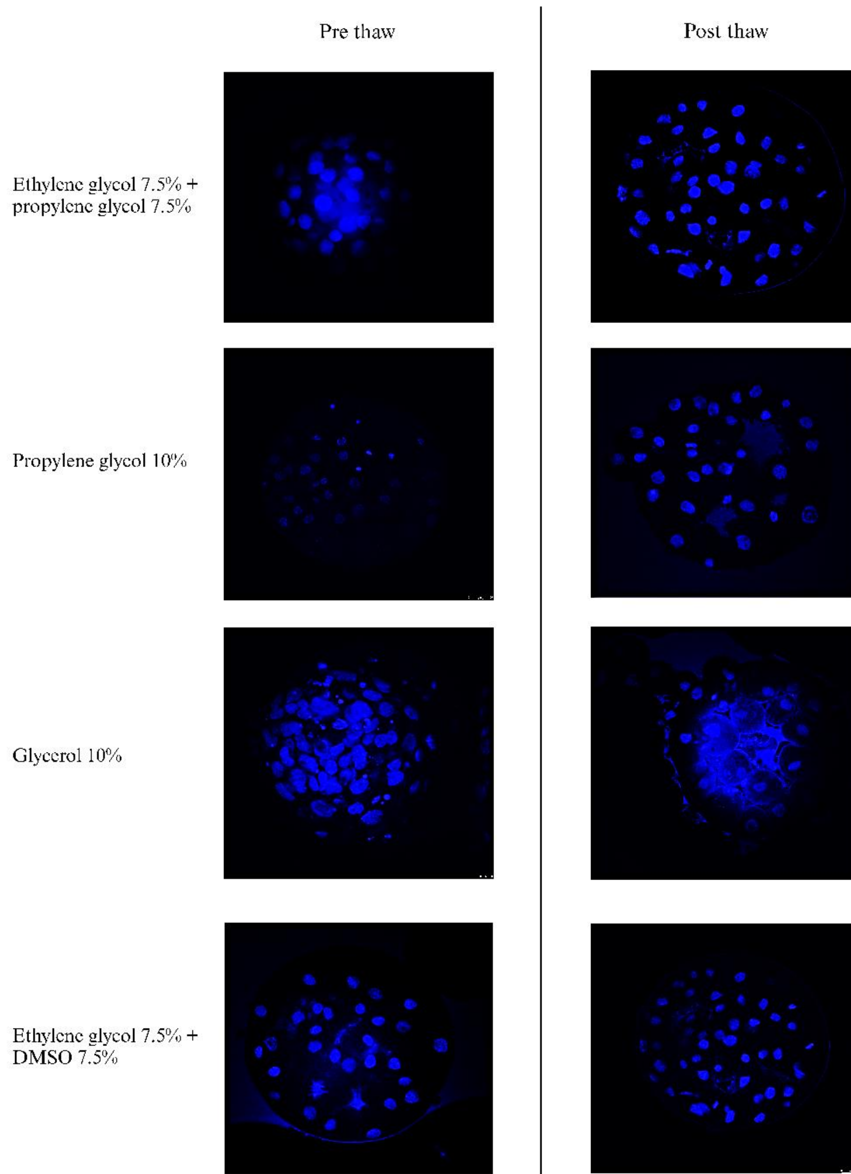

Figure 5. Hoechst 33342 stained blastomeres of morulae imaged by confocal microscopy. *4 treatments; ethylene glycol 7.5% + propylene glycol 7.5%, propylene glycol 10%, glycerol 10%, ethylene glycol 7.5% + DMSO 7.5% were imaged. Pre thaw was exposed to vitrification media but not frozen. Post- thaw underwent vitrification and thawed. All images are scaled at 75μm.*

There was also an increased granularity of blastomeres and distance between blastomeres in the cell, showing reduced compaction. PG 10% pre thaw has a visually low number of blastomeres compared to its post-thaw counterpart, which may be due to the quality of the blastocyst pre-thaw. However, post-thaw had vacuoles present in the perivitelline space and a rupture on the top left of the cell, indicating there has been damage to the ZP. Glycerol 10% pre-thaw was similar to a normal blastocyst having compact blastomeres and several numbers of them. Post-thaw showed rupture of the ZP and spacing between blastomeres. EG 7.5% + DMSO 7.5% pre- and post-thaw show similarities with vacuoles and dispersion of blastomeres throughout the morulae. However, post-thaw showed the same shapes of blastomeres as EG + PG, with reduced number of circular blastomeres present and irregular shapes. The post-thaw morulae showed various degrees of cryoinjury.

4. Discussion

The outcomes of this research have provided insight into the differences between the CPA exposures to bovine oocytes and embryos and their developmental competence. However, the results should be interpreted with caution due to the limitations of the current research regarding methodology. This chapter provides a reflection on the research process. The limitations and potential consequences of the design are discussed, as well as the implication for the interpretation of the results. The chapter ends with several recommendations for future research.

In bovine species, a consecutive success rate of survivability of oocytes following vitrification is difficult to achieve, as oocytes hold a unique structure and have a highly sensitive nature to cooling (Garcia Martínez et al., 2021). However, we know there is a notable correlation between an increase in cryoprotectant concentration and a decrease in survivability of oocytes due to the toxic nature of cryoprotectants. Furthermore, it is important to avoid excessive exposure times and concentration due to the intrinsic toxic nature of CPAs to cells. Thus, there is a need to reduce exposure times to reduce toxic effects and osmotic stress (Lai et al., 2015). As such, there is a need to promote cryosurvival, while increasing developmental competency, morphology, and cell health.

The goal of the first experiment was to identify suitable concentrations following a 10 min exposure time for all the CPA treatments. EG, DMSO and GLY are standard CPAs used industrially and amongst many researchers to improve cryopreservation, showing positive results aligning with their controls (Jaiswal & Vagga, 2023; Marques et al., 2018; Pegg, 2002). As expected, our results showed that the exposed oocytes and embryos underwent morphologically observable changes by reduction of size upon exposure to CPAs, suggesting successful changes in the osmolality of the cell and penetration of intracellular CPAs. This was followed by swelling of the oocyte to its original volume. The relationships of shrinkage to swelling response of the oocytes is linked to temperature, where higher temperatures reduce the time needed for the oocytes to return to their initial cell volumes (Wang et al., 2010). This provides sufficient evidence of penetration of the CPAs into the oocytes, suggesting there are elements of protection provided by the CPAs (Garcia Martínez et al., 2021).

However, studies have demonstrated that cryoinjuries are dependent on the size and shape of the cell and ability of the cell membranes permeability (Hwang & Hochi, 2014), where oocytes

are more likely to undergo damages as it has a large size and a large amount of water (Sciorio et al., 2023). During exposure of CPAs to oocytes, the largest damaging factors would be osmotic stress. Following this observation, the aforementioned PG, EG and DMSO singular and mixtures used in this study showed the highest cleavage and blastocyst rates in this current study, with positive performance amongst those tested. This was more evident with DMSO at the blastocyst stages. In this study, osmotic stress was not measured, however there were not detrimental effects on the developmental competence of oocytes to embryos following CPA exposure regarding osmotic stress. As previously mentioned, the CPAs sufficiently penetrated both the bovine oocytes and embryos upon CPA exposure, seen subjectively via microscopy by the changes in size before and after CPA exposure. In future iterations of this study, osmotic stress could be analysed using real time computer analysis of volumetric changes following CPA exposure (Tu et al., 2022).

Of the CPAs used in this study, EG has the lowest molecular weight (62.07) compared to GLY (92.01), DMSO (78.13) and PG (76.1) thus allowing for it to rapidly diffuse into the cells (Bautista & Kanagawa, 1998). This, however, cannot be stated as the sole reason for the effectiveness of these CPAs. Permeability of the CPAs occur through both facilitated diffusion by AQP3,7 as well as simple diffusion. It has been indicated that GLY and other CPAs utilise predominantly AQP3 and AQP7 in both oocytes and embryos, with DMSO utilising simple diffusion more so (Jin et al., 2011).

One of the CPAs used in this study was methanol. This is a toxic alcohol, where incidentally EG is also considered as such (Ashurst et al., 2023). There are currently to our knowledge no studies on MeOH effects on bovine oocytes and embryos, but it has been applied in equine(Bass et al., 2004) and rodent (Andrews et al., 1993) embryo models. Methanol has been indicated to have detrimental effects on development, causing severe central nervous system (CNS) defects and inducing apoptosis in many cells(Nekoukar et al., 2021). In the current study, cellular damages were not measured; however, day two developmental assessment of MeOH indicates it was a contributor to low levels of cleavage, suggesting it has an effect on the developmental potential of bovine embryos. This was also observed in zebrafish embryos suggesting MeOH to have an inhibitory function on embryonic hatching and morphological deformities (Fu et al., 2021).

In the second experiment, we aimed to observe any parthenogenic activation caused by the CPAs that showed the highest cleavage rate. Parthenogenesis was observed in control

treatment with unfertilised EG 10% when measuring cleavage rate. However, development ceased at the early embryonic stages when measuring blastocyst rate.

As such, this may be parthenogenetically activated by chemical or external stimuli. Several studies have hypothesised oocyte activation to be caused by sperm, where activation is caused by increases in intracellular calcium mediated by inositol triphosphate (IP_3) (Marques et al., 2018; Ross et al., 2008). Therefore, cells which have undergone parthenogenesis, chemically/externally stimulated parthenogenetic cells and fertilised cells could be imaged. The changes in intracellular calcium concentration could be measured as a means to observe calcium mediated responses compared to oocytes that undergo parthenogenesis naturally. However, it cannot be concluded that CPAs induced parthenogenesis due to the low sample size and may have been a naturally occurring attribute of the oocyte that was selected.

Several studies on vitrification of bovine embryos have shown a need for careful selection of CPAs, exposure time and concentration to reduce toxic effects on the embryos. Caamaño *et. al* (2015) successfully froze blastocysts utilising two vitrification solutions of 7.5% EG + 7.5% DMSO and 16.5% EG + 16.5% DMSO in a two step-process followed by droplets into LN_2. These blastocysts yield post-thaw survival resulted in similar outcomes to their slow freezing counterparts. In this current study, blastocysts were exposed to both similar concentrations in a two-step process prior to vitrification.

However, in this current study, the results following post-thaw and subsequent development of the blastocysts did not yield successful results amongst the four CPAs used. All the blastocysts died; however, the cause of death is not clear. A potential cause for this might be the low concentration of cryoprotectants used, wherein of the four CPAs we used, a half concentration of 5% and 10% and 3.25% and 7.5% were used in a two- step process vitrification, respectively. In the current study, the blastocysts were added into equilibrium and vitrification at specific intervals, and visual changes seen by the shrinkage of the blastocyst. After thawing, there was an observable reforming of the blastocyst, returning to its original circular form. This contrasts with the observations by Caamaño *et. al* (2015) following an increased number of both equilibration and post-thaw steps when using a 16- step equilibration process where no visual changes to the blastocyst were observed. These findings imply that injuries caused by osmotic stress may be reduced by incorporating more equilibration steps, potentially enhancing cell survival.

Potential cause of cell death following warming, may be a result of inadequate concentration measured for preparation of the CPAs and sucrose. The varying volumes used in combination may have resulted in dilution of both CPAs and sucrose, leading to a much lower intended concentration than initially presented. As such, future experimentation should utilise changes to the methodology.

DNA damage is a factor to consider for the dead thawed samples, following vitrification. This leads to apoptosis of embryonic cells, and eventual cell death. Vitrification has shown to up-regulate genes involved in stress response and down-regulate genes involved in lipid metabolism and cell adhesion which leads to impaired implantation in the uterus (Gupta et al., 2017). Furthermore, a study by Gutierrez-Castillo et al., (2021) studied the development of vitrified thawed IVP blastocysts after seven days in the uterus. The embryos showed rapid cellular proliferation, especially genes related to DNA repair, suggesting vitrified embryos with high rates of proliferation may result in DNA damage, and subsequent cell death due to pathways functioning to fix the toxic effects. Therefore, this could have been a factor to consider for cell death in the current study, where a comet assay could have been applied to see the levels of DNA integrity between the different vitrified embryos. Furthermore, an RT-PCR could have been used to analyse selective genes related to cold, stress and apoptotic functions to elucidate their activity post-thaw and the consequences of said activity (Lin & Tsai, 2012).

It is known that during the thawing process, cells rehydrate, and the CPAs are removed via osmosis. However, physiochemical stress may arise affecting the spindle, cytoskeleton, zona pellucida and mitochondria (Angel-Velez et al., 2023). Furthermore, it is unknown how these changes may cause alterations in cattle. On the other hand, it has been studied in human oocytes, where changes in the pronucleus and nucleolar had a delayed developmental rate following vitrification (Angel-Velez et al., 2023). As such, this could be measured by time-lapse imaging to see changes that vitrification may cause on the subcellular level.

Assessment of embryo morphology is a key element in assessing the viability of embryos. However, embryo evaluation is a challenging and subjective procedure (Yu et al., 2020). Following vitrification, two samples showed rupture of the cell membrane, the ZP (Figure 5). This rupture is known as the bleb formation, characterised by a bulge or blister-like formation on the cell membrane (Fackler & Grosse, 2008). This arises as a result of decoupling of the cytoskeleton from the plasma membrane, caused by increase in intracellular pressure or local

rupturing of actin filaments. Cellular fragments observed post-thaw in the perivitelline space are typically seen as a sign of low embryo quality (Chi et al., 2011; Van Soom et al., 2003). However, it is not a shown to not have any negative impacts on pregnancy rates. Furthermore, vacuole damage in the perivitelline space was observed among two post thaw-samples, seen as rips within the morulae. This suggests that ice crystallisation formation which should theoretically not be possible with vitrification but may have occurred due to a lower concentration of CPA, thus providing little to no protection for the morulae. As a result, the cryoinjuries to the cell were detrimental to the survivability and developmental competence of the morulae. To further identify elements detrimental to the survivability of the morulae, the use of a TUNEL-labelling and cleavage caspase-3 immunostaining for apoptotic activity would provide vital information of the embryo's viability and morphology (Chi et al., 2011; Najafzadeh et al., 2021).

A lot of the damage previously mentioned cannot be observed following stereomicroscopic visualisation. As such, many studies incorporate the use of transmission electron microscopy and confocal microscopy following staining of select organelles. In a study conducted by Vajta *et al.* (1997) potential damages using transmission electron microscopy compared to light microscopy were analysed. Light microscopy was utilised for developmental assessment in our study. Others have shown that potential damages were not visible following light microscopy, while electron microscopy showed shrinkage of mitochondria, rupturing of cells and disassembly of cell adhesion structures which were fixed at 24 hours, compared to severe damages noted at 4 hours after warming (Vajta et al., 1997) . DNA fragmentation was observed in our study by confocal microscopy and Hoechst staining of blastomeres. This elucidated damage arisen as it was not visible by use of light microscopy, which only provided a few morphological details of the zona pellucida and state of the inner cell mass encompassing the blastomeres.

Thus, for future experimentation, we should consider either increasing concentration of CPAs used or to increase number of steps when equilibrating and post-thaw processes to increase the chances of survivability in the development of blastocysts. However, this may be detrimental to the cell survival rate due to the increasing concentration of CPAs.

5. Conclusion

Singular and combination of commonly used CPAs showed both high cleavage and blastocyst rates. Following vitrification however, it did not yield living blastocysts. This may be due to the low CPA concentration, osmotic damage working on the DNA to induce further damage, cytoskeletal damages, and alteration to the ZP. Hoechst staining showed morphological changes induced by the vitrification, indicating damage to the perivitelline space, ZP and blastomeres which were fragmented.

In future studies to ensure survival and developmental competence after vitrification, a higher concentration of CPAs and extra steps in the post-thaw procedure should be utilised. Furthermore, a TUNEL-assay would aid in providing data on the blastomeres state (living/dead) following vitrification.

6. References

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