Optimized vitrification, cell cycle compatibility and volume of cytoplasts for bovine embryonic cloning

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science (Research)
in Molecular and Cellular Biology

at

The University of Waikato

by

Shantaya Rose Randall

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2022

Abstract

Cell transfer cloning is a promising method for multiplying elite alleles in a population. Together with gene editing, it can increase the genetic gain of livestock and support the adaptation of cattle to climate change. Embryos with superior genetics, including edited genes, can be expanded *in vitro*, and each single embryonic donor cell can be used in cell transfer to produce new embryos with a copy of those genes. Enucleated oocytes in metaphase II (MII cytoplasts) serve as recipient cells for the embryonic donors, supporting their reprogramming and development. Currently, the rate of healthy calves that can be produced from cloning with somatic cell donors is about 5% and the technology is associated with placental, fetal and neonatal abnormalities, referred to as the cloning syndrome. This is largely due to incomplete epigenetic reprogramming in somatic cells. The genome of a pluripotent embryo-derived donor is postulated to require less reprogramming to reach an embryonic state than a somatic donor, which could lead to improved embryo formation, increased numbers of healthy offspring and reduced animal welfare problems. These donors are also hypothesised to be more amenable to DNA editing. While the conditions for cytoplasts that receive somatic donors are well-researched, cytoplast conditions for embryo-derived donors remain to be optimised. The primary aim of this research project was to further the knowledge on bovine cell transfer methodology by trialling different treatments of cytoplasts that receive embryo-derived donors.

Blastocysts were produced through *in vitro* fertilization and plated to generate embryo-derived outgrowths as a source of donors for cloning. The zona pellucida was removed with assisted hatching techniques before plating. The rate of embryo-derived outgrowth formation after natural hatching without assistance was 80%. This was raised to 88% when mechanical zona pellucida dissection was performed on blastocysts before plating, while enzymatically removing the zona pellucida lowered the production of primary embryo outgrowths to 36%. Donor outgrowths were arrested into mitosis by incubation in 500 nM nocodazole overnight before fusion with cytoplasts and activation with ionomycin and cycloheximide. Such chemically activated reconstructs had a 22% pseudo-polar body extrusion rate and blastocyst development was 7%. This was lower than blastocyst development in reconstructs without nocodazole-synchronization and with ionomycin and 6-dimethylaminopurine

activation (13%). Non-synchronized donors were fused to cytoplasts that had received various treatments: aging and cooling, volume increases, and vitrification. Reconstructs were activated with ionomycin and 6-dimethylaminopurine. Embryos constructed with cytoplasts that had been aged and cooled to alter their MII state had lowered blastocyst development (4%) compared to embryos constructed with cytoplasts that were not aged and cooled (15%). Increasing the volume of a MII cytoplast with embryo-derived donors did not significantly affect total blastocyst development (6%) compared to in control cytoplasts without altered volume (11%). Vitrified oocytes had a high survival rate after thawing (97%). Artificially activated zona-free vitrified oocytes were able to support development to the blastocyst stage, although this was at a lower rate (3%) than artificially activated fresh oocytes (22%). Vitrified cytoplasts had a high survival rate after thawing (92%) and were able to produce a blastocyst after cell transfer with somatic donors (2%). However, this blastocyst development occurred at a lower rate than with fresh cytoplasts and somatic donors (8%). The ability of vitrified cytoplasts to support the development of the embryo-derived donors to the blastocyst stage after cell transfer could not be proven.

Before proceeding to *in vivo* cloning trials, confirmation of blastocyst ploidy, and continued refinement of vitrification protocols, through trialling different reagents and concentrations, is required. Further data on the use of vitrified cytoplasts in double ECT is required to elucidate the role of reprogramming factors in cytoplasts on embryo-derived donors. Embryo transfer of the produced blastocysts will be the final measure of the effect of the cytoplast conditions trialled on overall cloning efficiency. Successful embryo transfer would accomplish the ultimate research aim of this project: to establish that bovine embryonic pluripotent stem cells can be successfully reprogrammed during cloning to produce blastocysts and then live offspring.

Acknowledgements

I would like to start by acknowledging my thesis supervisor at AgResearch, Dr Björn Oback. I'm grateful to you for giving me the opportunity to take on this project, and for being so generous with the time you spent helping me. I feel lucky to have had someone so knowledgeable and passionate about molecular biology guiding me. I would also like to thank all the members of the AgResearch animal biotech team for patiently teaching me skills and showing me the ropes. A massive thank you Jingwei, for putting aside countless hours in your already busy schedule to help enucleate, and for sharing your vitrified embryos. Thank you, Blaise, for offering advice and for finding the time to help me with fusion. Thank you, Fan Li, for making all the media. Thank you, Dave, for grading. And to Brigid, Goetz, Sally, Robin, Jess, and Natalie for creating a welcoming and enthusiastic work environment. Thank you to AgResearch for accommodating me, and for the financial support.

Thank you to my university supervisor Dr Linda Peters for your support and your advice on editing this thesis. Thank you to all my classmates at the University of Waikato, especially Dan Cossey, for your motivation and solidarity throughout times of stress. And to my fellow Masters student and partner in crime, Shaan Wilson, I couldn't have done it without you. Thank you for all the extra help with CTs, encouragement, and friendship.

Finally, I am grateful to have been blessed with friends and family who always cheered me on and kept my spirits up. A special thanks to the Randalls; Judi, Mike, Jayden, and Josh, and to Mika Stowers, for all the love.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

0C/1C/2C/4C	DNA quantity of cell
0N/1N/2N	Chromosomal quantity of cell
AC	Alternating current
AOS	Abnormal offspring syndrome
BEF2	Male bovine embryonic fibroblasts
bEPSC	Bovine embryonic pluripotent stem cell
BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
CDK	Cyclin-dependent kinases
CHX	Cycloheximide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR Ribonucleic acid
CT	Cell transfer
DC	Direct current
DMAP	6-dimethylaminopurine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECT	Embryonic cell transfer
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene glycol
ET	Embryo transfer
ePSC	embryonic pluripotent stem cell
FBS	Fetal bovine serum
gRNA	Guide ribonucleic acid
G_0	Non-proliferative phase

G_1	Primary growth phase
G_2	Secondary growth phase
HDR	Homologous directed repair
ICM	Inner cell mass
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
M	Mitosis
MII	Metaphase II
mOsm	milli-osmoles per kilogram
MPF	Maturation promoting factor
mRNA	messenger RNA
n	Number of technical replicates
N	Number of biological replicates
NANOG	Homeobox protein NANOG
NHEJ	Non-homologous end joining
Nocodazole	Methyl N-[6-(thiophene-2-carbonyl)-1H-benzimidazol-2-yl] carbamate
NPPB	No pseudo-polar body
OCT4	Octamer-binding transcription factor ¾
OTX2	Orthodenticle homeobox 2
P	Statistical P-value
PPB	Pseudo-polar body
PBS	Phosphate buffered saline
PC2	Physical containment level 2
PG	Parthenote
PMEL	Premelanosome protein

PRLR	Prolactin
PVA	Polyvinyl alcohol
S	DNA synthesis
SCT	Somatic cell transfer
SEM	Standard error of the mean
SOX2	Sex determining Region Y-Box 2
TM	Tight morula
WT	Wild type
ZF	Zona pellucida free
ZI	Zona pellucida intact
ZP	Zona pellucida

Chapter 1
Introduction

Climate change and global population growth present issues for the agricultural industry that necessitate biotechnological solutions (Karavolias et al., 2021; Wankar et al., 2021). Climate change and the coinciding increase in global temperature has rapidly been worsening and will continue to accelerate under current societal practises. Greenhouse gas emissions have already raised the average temperature of the planet by 1°C since 1850. Temperatures are expected to rise another 0.3-4.8°C by end of twenty-first century. This trend is not sustainable as it causes detrimental changes to the biological landscape that life is acclimatised to surviving in. There is an inability of populations to adequately adapt at the pace of these accelerated environmental changes. Heat stress in domestic populations is one of the significant consequences of the changing environment. New Zealand (NZ) dairy cattle have begun to experience heat stress for ≈20% of their lactation days (Laible et al., 2021). Heat stress impacts feed intake, reduces weight gain, decreases pregnancy rates, decreases general wellbeing, and increases mortality (Karavolias et al., 2021; Wankar et al., 2021). The reduction of milk yield caused by heat stress can range from 14-39% (Wankar et al., 2021). It is predicted that milk yield will decline by a further 1.4 kg/day annually up to 2050.

The effect of heat stress on dairy productivity is problematic due to the regional economical reliance and global nutritional reliance on agriculture. It is predicted that the world population will reach 9.6 billion people by 2050 and the requirement for livestock products is predicted to double in that same time frame (Nations, 2012) (Wankar et al., 2021). Agricultural output must be maintained not only to feed the population but to sustain NZ's economy. Cattle were responsible for 26% of NZ's agricultural revenue in 2020, with dairy exports producing $17.6 billion of that revenue (Zealand, 2021). Increasing agricultural productivity to meet the growing population needs and upholding the contribution to the NZ economy is vital. However, there is the conundrum that increasing domestic cattle populations to meet more these needs would further augment climate change, which in turn decreases productivity further. Agriculture already places a strain on the environment with NZ dairy cattle producing 90.5-98.4 kgs of methane per head annually, making up 17.8% of NZ's carbon

emissions (Environment, 2022). Solutions that address the need for efficient agricultural output while simultaneously avoiding furthering environmental impacts are required. These solutions lie in biotechnology.

DNA editing has been developed as a solution to both support the adaptation of cattle to climate change and increase the productivity of current populations, so larger populations are not required (Karavolias et al., 2021). The presence of edits in a population can then be amplified through embryo-derived cell cloning (Dinnyes et al., 2006). Cloning can increase the number of organisms with elite genomes as it removes the element of chance that arises in natural mating (Mueller & Van Eenennaam, 2022). Even with selective breeding programs, mating will not guarantee all beneficial traits will be inherited by offspring due to the independent assortment of alleles during meiosis. Meanwhile cloning allows a copy of one selected elite genome and the ensured inheritance of beneficial traits (Dinnyes et al., 2006; Mclean et al., 2020). This is especially important when DNA editing, such as for countering heat stress, has been incorporated into the parent genome. Surrogates for cloned embryos will not need to have elite genomes, as they have will not have any genetic contribution to the embryo, meaning more cows will meet motherhood criteria than in selective mating.

The current cloning technique with somatic cell donors has a low live birth rate. Animals that do survive to term are often found to have major health problems due to incomplete reprogramming (Heyman et al., 2002). The use of embryonic pluripotent stem cell (ePSC) donors is hypothesised to improve embryo formation and healthy birth rates after cloning. (Oback & Wells, 2002; Oback & Wells, 2007). ePSCs can give rise to any cell type of embryonic lineage and proliferate indefinitely (Zakrzewski et al., 2019). Their genome is postulated to require less reprogramming to reach an embryonic state than a somatic donor (Oback & Wells, 2002; Oback & Wells, 2007). Clones with ePSC donors are postulated to have reduced health problems (Heyman et al., 2002). ePSCs are also advantageous as they are more amenable to DNA editing (Mclean et al., 2020). However, even with ePSC donors the birth rate from current cloning methods is still too low to be commercially viable (Oback & Wells, 2002; Oback & Wells, 2007).

The goal of this thesis is to close the knowledge gaps in the methodology of bovine embryo-derived cloning by trialling different treatments of oocytes that receive

embryo-derived donors. The ultimate research aim is to establish that ePSCs that can be successfully edited and reprogramed during cloning to produce ePSC-derived blastocysts and then live offspring. The purpose is to accelerate animal breeding, especially breeding of cattle adapted to climate change.

Chapter 2

Literature Review

2.1 Blastocyst production and development

2.1.1 Natural fertilisation and blastocyst development

In vivo blastocysts are developed from an embryo created through the fertilisation of an oocyte with sperm. The DNA content (C) and chromosome numbers (N) of an oocyte changes throughout the meiotic cell cycle and during fertilisation (MacLennan et al., 2015). Before fertilisation oocytes are diploid (2N/2C) with the normal amount of DNA content and a paternal and maternal set of chromosomes. They are in the interphase stage of the cell cycle in which the cell grows, replicates its DNA to become 2N/4C, and prepares for mitosis (figure 2.1.1). The oocyte then enters metaphase I and the chromosomes align on either side of the equatorial plate. It then proceeds to anaphase I which equally divides the chromosomes into two sets. One of these sets is expelled in a polar body leaving an oocyte with a haploid chromosome number (1N/2C). A polar body is a cytoplasmic body that encloses and expels excess DNA from an oocyte (Mogessie et al., 2018). The polar body remains between the cell membrane and the protective layer, the zona pellucida (ZP). This haploid oocyte enters metaphase II (MII) and is arrested in this stage until fertilisation. The arrest in metaphase is encouraged by high levels of M-phase promoting factor (MPF). Upon sperm binding to the oocyte membrane, sperm-borne factors induce oocyte activation through calcium oscillations and MPF breakdown (Leese & Brison, 2015; Sanders & Jones, 2018). Activation causes the ZP to harden, preventing extra sperm from binding and preserving the diploid state of the zygote. The cyclin B and CDK1 subunits of MPF separate, inactivating MPF, and inducing the release from MII phase and the progression of embryo developmental. Anaphase II occurs, segregating the oocyte chromosomes again and releasing a second polar body to leave a 1N/1C oocyte. With the 1N/1C sperm entering the oocyte, it becomes a diploid zygote (2N/2C) (MacLennan et al., 2015).

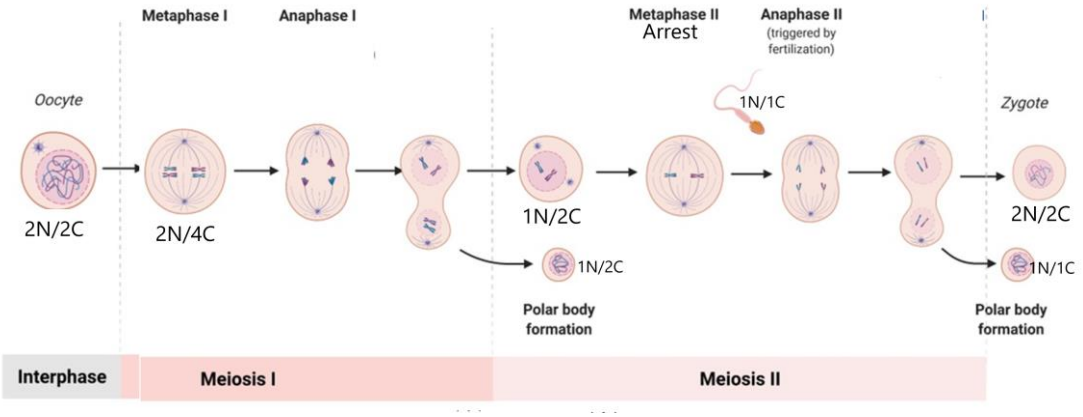


Figure 2.1.1 A schematic representation of meiosis I and II in an oocyte. (Wasielak-Politowska & Kordowitzki, 2022) modified.

The diploid zygote can then begin embryo development and go through a series of cleavage events to form a blastocyst (figure 2.1.2) (Oback & McLean, 2018; Toyooka, 2020; Q. Wei et al., 2017). The first cleavage event produces a two-cell embryo. The individual cells in the embryo are blastomeres. The blastomeres cleave to four cells and then eight cells. They continue to cleave until they form a tight morula (TM) which is a compact cluster of blastomeres tightly bound. The blastomeres in a TM have the maximum surface area of contact between each other while also having desmosomes and gap junctions in-between them. For bovine zygotes, the process to cleave beyond an eight-cell embryo after fertilisation takes approximately five days (Lindner & Wright Jr, 1983). By days six to seven, an early blastocyst can form. The outside of the blastocyst consists of trophoblast cells which will give rise to extraembryonic tissue (Oback & McLean, 2018; Toyooka, 2020; Q. Wei et al., 2017). The inside of the blastocyst consists of the inner cell mass cells (ICM) and a blastocoele cavity. Seven to nine days after fertilisation the early blastocyst can develop to a late blastocyst (Lindner & Wright Jr, 1983). The blastocoele expands, and the ICM cells differentiate into epiblast and hypoblast cells (Oback & McLean, 2018; Toyooka, 2020; Q. Wei et al., 2017). Epiblast cells give rise to the embryo proper. Hypoblast cells give rise to the embryonic yolk sak. Hatching occurs which breakdowns the ZP. Then the blastocyst is ready to implant into the uterine wall.

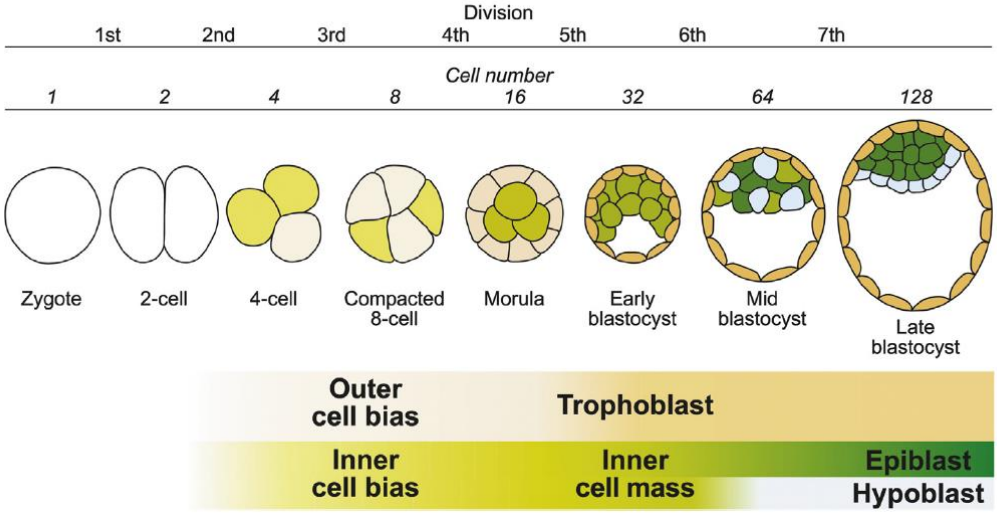


Figure 2.1.2 A schematic representation of bovine preimplantation embryo development. (Oback & McLean, 2018).

2.1.2 Assisted reproductive technology

The scientific field of embryology began in the early 19th century when the stages of embryo development were first observed under the microscope and recorded (Needham & Hughes, 2015). Understanding of embryology has rapidly become more refined and advanced since the early 19th century and has been used to introduce assisted reproductive technologies (ART) into agriculture (Ferré et al., 2020; Mueller & Van Eenennaam, 2022). ART is the use of embryology knowledge to artificially manipulate eggs, sperm, or embryos to produce blastocysts and cause pregnancy. Currently, the types of ART used on NZ dairy cattle include artificial insemination, multiple ovulation embryo transfer, and *in vitro* procedure (IVP). Cell transfer (CT) technology is another promising ART process, but it is currently unviable for commercial wide-scale cattle production. ART has become a preferred practise over natural livestock mating in bovine agriculture. It is used in NZ to produce 70-85% of dairy cattle (LIC, 2021). ART has gained popularity in agriculture because it can be used to increase the genetic gain of a population by increasing the number of offspring from elite parents with desirable phenotypes (Ferré et al., 2020; Mueller & Van Eenennaam, 2022). For example, IVP can produce up to 70 calves per elite dam annually (Harland et al., 2018). The types of desired phenotypes that increase the genetic gain or a herd are ones that reduce environmental footprint, increase suitability

to certain environments, increase milk quality, increase fertility, reduce disease, and improve welfare (Mueller & Van Eenennaam, 2022). ART is also an advantage because genetic editing can be incorporated into the process, embryos or sperm can be frozen for future use (Ferré et al., 2020), and semen can be sexed so that approximately 95% of calves can be expected to be female.

IVP is a popular ART because it allows strong control over the genetic gain of the offspring. Genetic gain is influenced by IVP because while the embryos are *in vitro* there is the opportunity for genetic screening biopsies (de Sousa et al., 2017) and genetic editing (Yum et al., 2018). Another benefit of IVP is the ability to reduce generational intervals by up to a year because oocytes can be collected at any time, including before sexual maturity (Mueller & Van Eenennaam, 2022). The IVP process (figure 2.1.3) begins with the aspiration of immature oocytes from 2-8 mm diameter follicles on a fresh ovary (Ferré et al., 2020). Oocytes of high-quality are selected for *in vitro* maturation. Oocytes of high-quality are ones that are a light even colour and are surrounded by many cumulus cells. They are matured in hypoxic conditions to mimic *in vivo* conditions (Hatırnaz et al., 2018). Fertilisation with elite sperm and subsequent incubation and blastocyst development also occurs *in vitro*. Blastocysts are then graded. Grade 1-2 blastocysts are considered high-quality and are selected based on morphological criteria (Stringfellow et al., 2007). Grade 1-2 blastocysts must have a spherical and symmetrical embryo mass of over 50% of all cellular material within the ZP. Over 15% of blastomeres must be uniform in size, not individual, and not extruded. The ZP surface should be intact and not be flat or concaved. The TE, blastocoele and ICM should also be identifiable. Blastocysts that do not meet these morphological requirements are considered low quality grade 3 blastocysts. Blastocysts can then be injected into a surrogate for embryo transfer (ET), cryopreserved, or cultured *in vitro* to form outgrowths. Using Grade 1-2 blastocyst produced by IVP for ET is preferrable over grade 3 blastocysts because they develop *in vivo* with more cells, with a lower percentage of apoptotic cells, and at a 19% higher rate (Pomar et al., 2005). The rate of pregnancy after ET is lower for blastocysts produced by IVP than natural fertilisation by 46%. Although blastocyst and pregnancy rates are lowered during IVP the benefits make it a commercially superior procedure over natural mating.

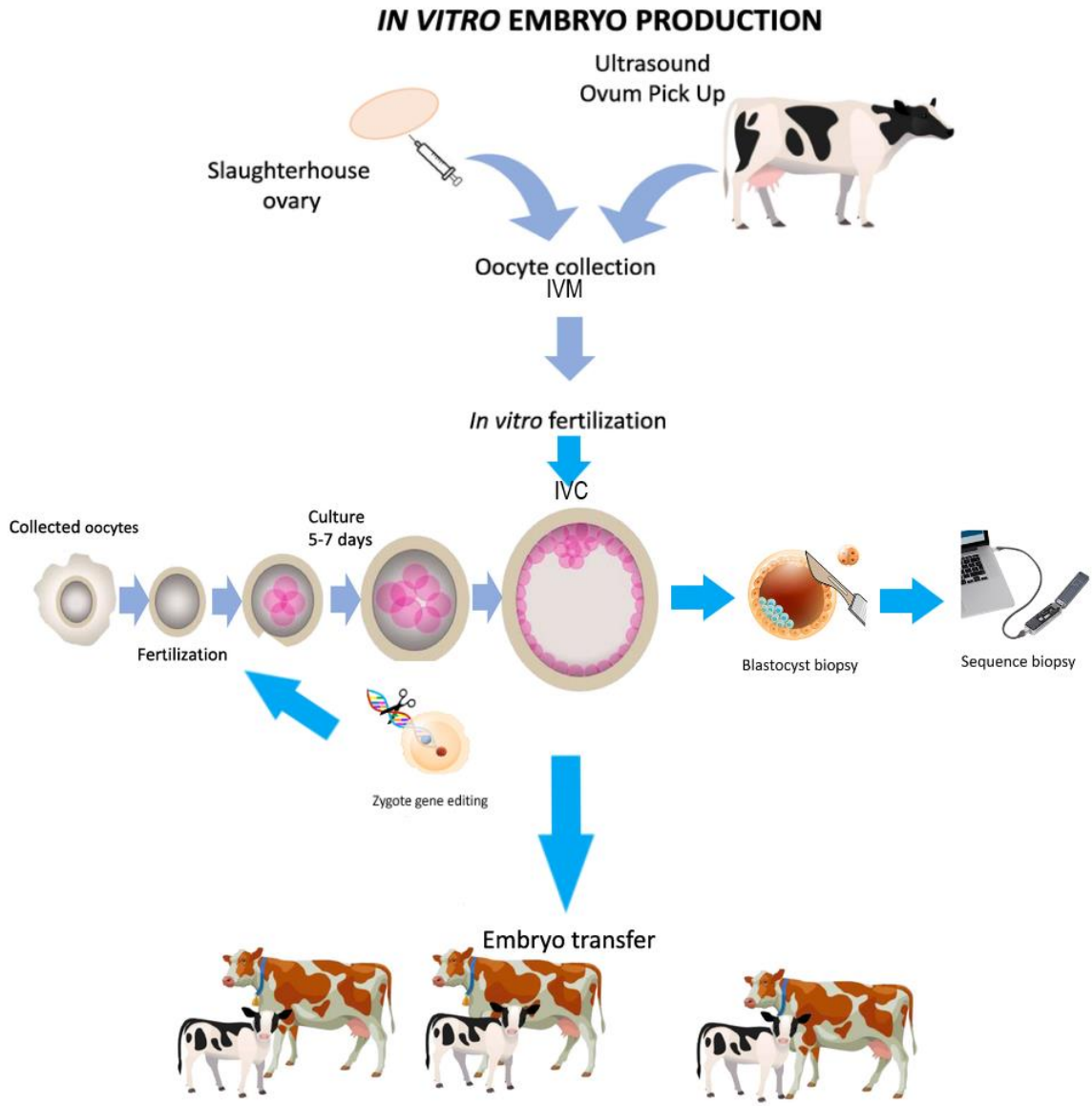


Figure 2.1.3 The IVP process. (McFarlane et al., 2019; Seneda et al., 2021) modified.

2.1.3 Deriving pluripotent stem cells from blastocysts

A bovine blastocyst can be plated and kept in culture after seven to eight days of development. The culture contains media for growth and substrate for attachment. Once the blastocysts attach, they grow into embryo-derived outgrowths consisting of many pluripotent like stem cells (Zhao et al., 2021). Specific substrates in the growth media can maintain the pluripotency of these outgrowths. Culturing is done with blastocysts of genetic value, to augment the number of these valuable cells. Individual cells from these outgrowths can then be used as donors for embryonic-derived cell transfer (ECT).

An ePSC can arise from the ICM of preimplantation blastocysts and can differentiate into any of the three primary germ layers in an organism (endoderm, ectoderm, mesoderm), as well as germ cells (Zakrzewski et al., 2019). These germ layers can differentiate into all the cells in an adult. There are four main functional tests and one main molecular test to determine whether cells are ePSCs (De Los Angeles et al., 2015). The molecular ePSC test is for whether the cells can maintain essential pluripotent protein markers over many passaging events. These markers are octamer-binding transcription factor 4 (Oct4), Sex Determining Region Y-Box 2 (Sox2), and Nanog. All three genes must be expressed at once for pluripotency to be maintained. Their presence is tested for with histological staining or transcriptomics. Epigenetic and metabolic profiles can also be analysed. Molecular tests are less stringent than functional tests. The first and least stringent functional test is the embryoid body assay (Pettinato et al., 2015). Embryoid bodies are three dimensional aggregates of ePSCs that can differentiate into the three germ layers. The events of their *in vitro* differentiation mimic the *in vivo* process. The embryoid bodies can undergo a histological or molecular examination to confirm the presence of each germ layer. The second functional test is whether an ePSC can produce a teratoma when injected into an immune compromised host animal (Gropp et al., 2012). A teratoma is a benign tumour containing cells from all three germ layers. This assay has limitations as teratomas are not generated from single cells so give an assessment of the whole population. They are subject to false positives as incompletely reprogramed cells can produce teratoma-like masses. A third ePSC test is for the ability to form germline chimeras (De Los Angeles et al., 2015). A single suspected ePSC is introduced into a pre-implantation host embryo and evaluated for whether it can support normal development and form all somatic cells and germline cells. This is typically measured by evaluating the somatic cells and germline cells of the chimera for a target gene that was in the ePSC. The fourth, and most stringent functional test is for whether the ePSC can develop into a live offspring after tetraploid completion. An ePSC is introduced into a tetraploid (4N) host which consists of 2x two-cell stage embryos electrofused together. The tetraploid will not develop without an ePSC donor as they can only form the extraembryonic tissue. This tetraploid extra-embryonic tissue can support the development of the ePSC into a normal embryo and live offspring deriving entirely from the ePSC.

In vitro bovine embryonic pluripotent stem cells (bEPSCs) have only recently been established (Bogliotti, Wu, Vilarino, Okamura, Soto, Zhong, Sakurai, Sampaio, Suzuki, & Izpisua Belmonte, 2018; Kinoshita et al., 2021; Pillai et al., 2021; Xiang et al., 2021; Zhao et al., 2021). Bogliotti et al. (2018) cultured bEPSCs that were able to be used as donor cells in CT and supported blastocyst development. However, these were cells in a primed state. They displayed high levels of primed transcriptional and epigenetic features such as an accumulation of histone methylations and high expression levels of *OTX2* which plays a role in the development of optic structures. These markers show a commitment to differentiation. A naïve state is preferrable over a primed state as there is no bias towards a specific cell lineage (De Los Angeles et al., 2015). Zhao et al. (2021) established bEPSCs that have high levels of pluripotency gene expression and can remain genetically stable in long-term culture. They can pass the embryoid bodies test, the teratoma test, and the somatic chimera test. Tetraploid testing was not done and has not been completed in any bEPSCs. Zhao et al. (2021) also demonstrated that the bEPSCs had the capacity to undergo gene editing which makes them promising candidates for CT donors.

2.1.4 Blastocyst hatching

Before plating blastocysts, the ZP can be removed. The ZP is a highly specialized extracellular glycoprotein coat around oocytes (Mousavi et al., 2022). Its purpose is to prevent polyspermy, to protect the oocyte and embryo, and to prevent the separation of blastomeres. Blastocysts hatch from the ZP *in vitro* when there is increased blastocoelic pressure, actin-based trophectodermal projections and trypsin-like proteases (Massip & Mulnard, 1980; Niimura et al., 2010; Seshagiri et al., 2009). There is variation between species in the type of the protease that acts on the ZP, with urokinase-type plasminogen activator being recognised as the one that acts on cattle (Negrón-Pérez & Hansen, 2017). Hatching is important before implantation or plating because hatching exposes adhesion factors which are needed for attachment to the endometrium, or *in vitro* support. The mRNA for Integrin α5 and β1, which are proteins that facilitate adherence to gelatin support during plating, is upregulated by complete zona removal (Ueno et al., 2016). Attachment to support is required for an outgrowth to form. A study by Ueno et al. (2016) demonstrated that complete ZP removal does not affect human blastocyst viability and can improve the rate of *in vitro* blastocyst adhesion and outgrowth formation.

Blastocysts may have an inability to hatch *in vitro* if the structure of the zona is changed by environmental stresses such as an increase in the temperature or pH of the culture medium (Hur et al., 2011). There may also be an inability to hatch if the mother has advanced age (Gabrielsen et al., 2000), if the zona is too thick or hard (Lyu et al., 2005), or the embryo was cryopreserved (Bianchi et al., 2014; Massip & Mulnard, 1980). ZP hardening may be induced by peroxidase-catalysed oxidation that occurs due to a change in the external environment (De Felici & Siracusa, 1982). It can also be caused by cortical granule release at the inappropriate time (Bianchi et al., 2014). Cortical granules in oocytes contain molecules which alter the environment of the ZP including peroxidases. During fertilization, the cortical granules undergo exocytosis and the molecules it stored cause a hardening of the inner face of the ZP. Release of these granules before or after activation, which is associated with causes such as cryopreservation, cause hardening of the ZP. In such cases assisted hatching can be performed.

Two common methods of assisted hatching are mechanical hatching or pronase hatching (Alteri et al., 2018). Pronase is a commercial mixture of proteases including chymotrypsin, trypsin, carboxypeptidase, and aminopeptidase, together with phosphatases (Sigma-Aldrich, 2019). It acts as a proteolytic agent with a broad specificity that can digest many types of proteins down into individual amino acids (Narahashi, 1970; Sigma-Aldrich, 2019). Pronase can facilitate blastocyst attachment by removing the ZP and facilitate the formation of outgrowths by acting on tight junctions between trophoblast cells so the cells can spread more readily (Konwinski et al., 1978). Pronase hatching blastocysts has been found to improve *in vitro* murine development (Konwinski et al., 1978) and pregnancy rates in a variety of species including cattle (Dolgushina et al., 2018; Taniyama et al., 2014). This infers that pronase hatching does not compromise the integrity of the blastocyst and increases the access of binding receptors. Mechanical hatching can be performed by using a fine pipette to peel the zona off or controlled zona dissection. Controlled zona dissection is when the embryo is held in position by with a holding pipette and a microneedle pierces the ZP (Balaban et al., 2002). It is speculated that pronase hatching produces better implantation and pregnancy rates than mechanical hatching (Alteri et al., 2018). Konwinski et al. (1978) observed that *in vitro* murine blastocyst development was lower by 12% after mechanical hatching compared to pronase hatching.

While some literature supports the use of assisted hatching, some literature dismisses its proposed benefits. (Hagemann et al., 2010; Hurst et al., 1998; Mahadevan et al., 1998; Medicine & Technology, 2014). There is a gap in the literature about outgrowth or *in vitro* development rates after assisted hatching, especially bovine ones, as assisted hatching research has been focused on the medical benefits for humans and pregnancy rates (Alteri et al., 2018). Bovine outgrowths on gelatin will be produced in this study but past *in vitro* studies have tested adhesion and outgrowth formation on fibronectin, or no support. There is also a low quantity of studies that explored the effect of pronase hatching on early or late-stage blastocysts rather than day three embryos.

2.2 Cell Transfer Cloning

Cloning is the creation of genetically identical progeny from an original (Dinnyes et al., 2006). The purpose of cloning is to create many copies of an embryo with desirable genetics, to amplify the quantity of elite offspring. One method of cloning is splitting embryos that are early in development into two to four blastomeres (Willadsen & Polge, 1981). This generates twins, triplets, or quadruplets. Another method is TE swapping, whereby one inserts a donor ICM into a host TE (Papaioannou, 1982). The resulting offspring has cells that come exclusively from the donor. In an agricultural context, CT is preferable to splitting embryos and TE swapping because a larger number of clones can be obtained. CT is the method of removing or destroying nuclear DNA from a recipient cell and then replacing it with a donor cell (Dinnyes et al., 2006). The cytoplasm of the recipient cell supports growth and development as instructed by the donor DNA. This new reconstruct can develop into blastocysts *in vitro* and then undergo ET to develop into full animals *in vivo*. Nuclear transfer (NT) is a similar method in which only the nucleus or nuclear DNA from a donor is transferred to the recipient cell rather than the whole donor cell. The difference between NT and CT success is unlikely to be critical. CT offers the advantage over NT of being less labour-intensive as it removes the donor nuclear extraction step. CT is preferrable over other ART methods as it can create a genetic copy of just one elite parent, guaranteeing the elite and potentially edited genetics are passed to multiple offspring (Mueller & Van Eenennaam, 2022). Other ART methods and natural fertilisation cannot guarantee the offspring will have the elite genetics of a parent. However, CT has a significantly lower development to term and birth rate than other ARTs. Although progress has been made

between the first successful CT and modern CTs, the percentage of live healthy births has not been raised to commercially viable levels.

2.2.1 History of cell transfer cloning

NT was first performed on the North American leopard frog *(Rana pipiens)* in the 1950s to test the hypothesis that cell differentiation was irreversible (King & Briggs, 1956). Embryonic cell nuclei were used as donors and proved that cells can be reprogramed to a totipotent embryonic state. Then first successful CT with somatic donors was performed in 1997 by Wilmut et al. (1997). "Dolly" the sheep was the first cloned mammal from a somatic cell and opened the door to research cloning for agriculture. The first successful bovine CT was by Cibelli et al. (1998). After Dolly, interest and research into cloning grew, and many potential applications were investigated. One application of cloning is the re-establishment or expansion of threatened populations (Czernik et al., 2019). Cloning for species preservation was proposed for the endangered white rhinoceros (Saragusty et al., 2016) and used by Wells et al. (1998) to preserve the last surviving cow of the Enderby Island cattle breed. Another application is the production of transgenic animals for medical applications. For example, CRISPR/Cas9 gene editing was used alongside somatic cell cloning, to improve the success of generating pig hearts for xenotransplantation (Boulet et al., 2022; Cooper et al., 2019; Kotz, 2022). A total of nine genetic modifications were introduced into pigs through this technique and were responsible for hearts transplants that survived in their hosts for extended periods of time. Cloning can also be used to study epigenetic reprogramming since the reprogramming of somatic factors is required to achieve pluripotency in somatic cloning (Dinnyes et al., 2006; Fisher & Fisher, 2011). The cloning application most relevant to cattle and agriculture is to increase genomes with high genetic merit, and to multiply high value livestock. An example was the use of somatic CT (SCT) alongside genetic engineering by Brophy et al. (2003) to produce cows that overexpress casein proteins.

2.2.2 Cell transfer methodology

2.2.2.1 Enucleation of oocytes

The nuclear DNA must be removed from an *in vitro* matured oocyte by enucleation to create the recipient cell (Dinnyes et al., 2006) (figure 2.2.1). The recipient cell is called

the cytoplast as it consists purely of cytoplasm. Enucleation can be performed on zona-intact (ZI) or zona-free (ZF) oocytes (Oback et al., 2003). There is no difference in the development rates between ZI and ZF CT, but the ZF method is faster, easier to perform and more readily replicated. Once the ZP is removed with pronase, the oocyte is temporarily stained with Hoechst dye. The dye allows DNA to be located under a UV light. Hoechst dye and UV light can damage the oocyte so the time of exposure to both is minimised. Once the DNA is located, it can be aspirated using a finely pulled pipette. The enucleation is technically difficult to perform and requires an experienced operator. There can be variation in the enucleation competence between operators. If the membrane is compromised in this step or if too much cytoplasm is removed alongside the DNA, the oocyte is no longer viable or loses reprogramming potential. Other methods of removing the DNA of recipient cells include manually bisecting the oocytes and discarding the halves that contain the maternal chromosomes (Vajta et al., 2001) or chemically assisted enucleation (Tani et al., 2006). Needle aspiration is the most accurate, least invasive type of enucleation and minimises the loss of cytoplasm.

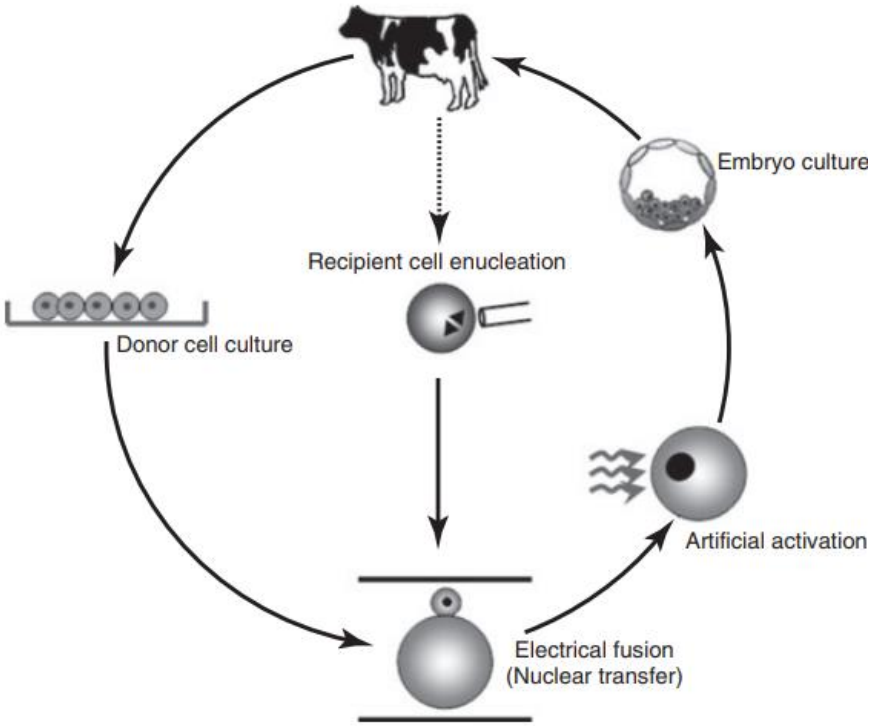


Figure 2.2.1 The main stages of CT. (Dinnyes et al., 2006). Oocytes are collected from ovaries and matured *in vitro*. They are then enucleated to form cytoplasts. Donor cells are disaggregated from blastocyst outgrowths or somatic culture and adhered to the cytoplast to make a couplet. The couplet undergoes electrofusion to form a reconstruct. Artificial activation

of the reconstruct causes release from MII. The reconstruct develops in culture to form a blastocyst for ET or donor cell outgrowths.

2.2.2.2 Donor cell transfer

After enucleation, cytoplasts are stuck to donor cells to produce a couplet (Dinnyes et al., 2006). There are three types of donor cells, each at different developmental stages and with different CT efficiencies. There are embryonic cells which are the least developed and require the least reprogramming. Embryonic cells include blastomeres and embryonic-derived cells from blastocyst outgrowths. Blastomere donors can produce a cloning efficiency which is high enough for commercial use but the number of blastomeres that can be obtained from one embryo is too low to multiply the genotype sufficiently. Primordial or differentiated germ cells can also be used as donors. The third donor type is somatic cells which are the most developed and differentiated. The healthy birth rate from a CT decrease as the developmental stage of the donor cell increases. CT with somatic cells has a lower efficiency than with embryonic donors as they are less differentiated (Hiiragi & Solter, 2005). Oback and Wells (2002) noted that blastocyst development after a CT is lower in embryonic donors compared to somatic donors but the development to term after ET is higher in the embryonic donors. Induced pluripotent somatic cells have better cloning efficiency than adult somatic stem cells and close to embryonic stem cells (Kou et al., 2010). When primordial germ cells are used, if it is before imprinting erasure, they have similar cloning efficiencies to somatic cells (Lee et al., 2002). Zhao et al. (2021) discovered that cloned embryo efficiencies using bEPSC donors (21% blastocyst development) were comparable to that of using fibroblasts as the donors (17% blastocyst development). Embryonic cells are the main donors used in this study. Somatic cells are also used in a minor experiment.

Donor cell colonies must be disaggregated into single cells enzymatically and mechanically before use in a CT. Trypsin can be used for somatic cells (Oback et al., 2003). Disaggregation media containing pronase can be used for ePSCs (Neil & Zimmermann, 1993). Pronase treatment has been shown to increase fusion rates. This is potentially because the pronase removes protein surface charges which increases adhesion between the donor and the cytoplast. Mechanical pipetting up and down further disaggregates the cells. The cells are then adhered to cytoplasts with lectin

(Booth et al., 2001). Lectin is a carbohydrate binding protein that encourages cell-cell adhesion. The lectin sticking step must be less than an hour to avoid toxic effects.

Couplets can then be fused to produce reconstructs. Couplets are placed in fusion buffer with an osmolarity at approximately 165 – 200 mOsm. Osmolarity is a measure of the concentration of particles in a solution. The osmolarity of a typical mammalian cell resides around 280 mOsm (Neil & Zimmermann, 1993). Using a buffer with a lower osmolarity than the cell causes swelling in the couplet and causes cytoskeletal changes. This can increase membrane permeability (Oback et al., 2003) which increases fusion rates. Once the couplet is in the fusion buffer it is aligned parallel to the current's direction with an alternating current (AC). Two direct current (DC) pulses are used to permeabilise membranes of both cells, facilitating fusion. The donor cell DNA is now transferred into the cytoplast.

There are alternatives to electrical fusion including intracellular nuclear injection (Goto et al., 1997) or inactivated Sendai-virus fusion (Song et al., 2011). Nuclear injection is less common because it is labour-intensive and requires high training and experience. It is technically complicated especially when using larger donor cells. In inactivated Sendai-virus fusion, the plasma membranes of the cytoplast and donor cell are brought into close proximity and fused with catalytic viral fusion proteins (Song et al., 2011). The virus method avoids the spontaneous oocyte activation which can occur during electrofusion and improves blastocyst development rates and quality.

2.3.2.3 Reconstruct activation to complete meiosis

Reconstructs need to be artificially activated to mimic the activation that occurs during natural fertilization (Fernandes et al, 2014). As discussed in Section 2.1.1., during normal mammal fertilization a soluble sperm factor triggers calcium oscillations which causes the separation of the subunits of MPF. This inactivates MPF which allows the resumption of meiosis. Without these soluble sperm factors in CT, electrical or chemical artificial activation is used to increase the intracellular calcium. In artificial activation calcium is increased in a single long release instead of the series of pulses that occur *in vivo*. This difference is not significant to development. Electrical activation uses electric field pulses to generate pores in the reconstructs membrane (Cevik et al., 2009). External calcium in the buffer can enter through these pores. Chemical activation also uses the formation of pores for the entrance of extracellular

calcium from the media. Chemical activation can also stimulate the release of calcium from internal deposits (Fernandes et al., 2014; Méo et al., 2007). The ionophore ionomycin is typically used as the chemical activator in CT.

After artificial activation, treatment with a MPF inhibitor is needed. Treatments of 6-dimethylaminopurin (DMAP), cycloheximide (CHX), anisomycin or roscovitine, can prevent the reformation of cyclin B and CDK1 into MPF, preventing the resumption of MII arrest (Fernandes et al., 2014; Méo et al., 2007). DMAP and roscovitine are reversible kinase inhibitors, which include CDK1 as a target (Liu & Yang, 1999; Mermillod et al., 2000). They prevent the phosphorylation of CDK1 preventing its complete activation after re-joining with cyclin B. CHX is protein synthesis inhibitor that can prevent the synthesis of cyclin B (Liu & Yang, 1999). Anisomycin is also a protein synthesis inhibitor that can prevent the synthesis of CDK (Grollman, 1967). A previous student at AgResearch determined that incubation in DMAP produced higher blastocyst development rates than other chemical activation methods (Appleby, 2015).

While ZI reconstructs are cultured in groups, ZF reconstructs must be separated into single group culture (Goovaerts et al., 2010). This is to prevent the exposed membranes from sticking to one another. Single group culture prevents reconstructs being exposed to growth factors excreted by other reconstructs. However, there is no significant difference in development of live calves to term between single and group culture (Oback et al., 2003).

2.2.2.4 Parthenogenotes

A parthenogenote (PG) is a diploid embryo formed from the modified meiosis of an unfertilized haploid oocyte (Engelstädter, 2017; Neaves & Baumann, 2011). During sexual reproduction, the haploid polar bodies degenerate, and the egg cell fuses with sperm to form a diploid zygote. During parthenogenesis, the haploid pseudo-polar body does not degenerate and fuses with a haploid egg cell to form a diploid zygote with only maternal genetic contribution (van der Kooi & Schwander, 2015). PGs can form during asexual reproduction in various species including insects (Liu et al., 2018), reptiles (Kinney et al., 2013), fish (Fields et al, 2015), and amphibians (Neaves & Baumann, 2011). Parthenogenesis cannot occur in mammals because mammals require paternal genetic contribution to account for genetic imprinting (Barlow & Bartolomei, 2014; Hore et al., 2007). Mammals have epigenetic imprinting on specific

genes, which causes an allele to be expressed or inactivated depending on whether the genetic region is of maternal or paternal inheritance. A mammal created by parthenogenesis has only maternal chromosomes. So, where there are inactivated imprinted regions of DNA from maternal contribution, there is no activated imprinted complement of DNA from paternal contribution to balance this, leading to developmental abnormalities.

Parthenogenesis can occur *in vitro* when an oocyte is artificially activated (De et al., 2012; Fernandes et al., 2014; Méo et al., 2007). After activation, the oocyte does not complete meiosis and a second polar body is not extruded, maintaining the diploid state. *In vitro* PGs can then be used as controls for artificial activation efficiency and media quality during CT. Normal PG development indicates successful activation by removing variation caused by donor cell introduction, fusion, and enucleation.

2.3 Cell transfer cloning efficiency

Despite large amounts of research on CT techniques since the first cloned mammals in 1997, there has been little improvement in blastocyst development efficiency and cloning efficiency. Blastocyst development efficiency is the percentage of reconstructs that develop to blastocysts by day seven or eight, out of the number of reconstructs that went into culture. Blastocyst developmental efficiency varies between literature. Literature has reported that ≈33-64% blastocyst development efficiency can be achieved with somatic donors and similar methods to this thesis (Booth et al., 2001; Oback et al., 2003). Bogliotti et al. (2018) reported that ePSCs in CT had a blastocyst development efficiency of ≈10-20%. Cloning efficiency is the percentage of embryos that produce healthy live offspring after ET. Only approximately 5% of somatic bovine CT embryos develop to term and even fewer reach adulthood (Dinnyes et al., 2006). Cloned embryos that do lead to live births often produce offspring with abnormal offspring syndrome (AOS). Medical problems associated with cloned animals include abnormalities in the extraembryonic tissues, obesity, immunodeficiency, respiratory issues, and premature death (Ogura 2013). All these symptoms are welfare concerns.

Further refinement of CT methodology is required for CT to reach its full potential. Factors that need to be optimised include number of cytoplasts used, donor cell type, the removal of epigenetic factors, activation procedures, the correction of polyploidy,

cell cycle synchronization and *in vitro* culture conditions (Akagi et al., 2014; De et al., 2012).

2.3.1 Epigenetic reprogramming

One reason that clones often have medical problems is that the epigenetic reprogramming of the embryo is incomplete which prevents full totipotency (Matoba & Zhang, 2018). Totipotency is the ability of a cell to form all three germ layers and the extraembryonic tissue. Epigenetic alterations are reversible chemical modifications that change the way that DNA is expressed without changing the DNA sequence. Epigenetic attachments include methylation, acetylation, and ubiquination. The attachments can be to the DNA itself or to the amino acid tails of histones that the DNA is wrapped around. The epigenetic attachments change the chromatin structure and accessibility of transcription factors. Epigenetic markers are different between totipotent cells and cells at various stages of differentiation. Epigenetic markers are usually removed by fertilized zygotes to "reprogram" the zygote to totipotency and change which genes can be accessed by transcription factors and expressed. Activation of gene transcription, especially to pluripotency genes, is crucial during early embryo development.

The difference in epigenomes of naturally produced and IVF embryos compared to CT embryos has become an obstacle in the CT process. Bovine CT embryos have higher levels of global DNA and histone methylation, and lower levels of histone acetylation (Dinnyes et al., 2006). Hypermethylation and hypoacetylation decrease access of the chromatin to transcription factors. During CT, the cytoplast carries out reprogramming of the donor nucleus, which includes events such as the replacement of donor cell histones with oocyte stored histones and the removal of DNA modifications (Nashun et al., 2011). If the cell cycle of the donor and cytoplast is compatible, then the donor epigenome can be reprogrammed to increase chromatin accessibility. However epigenetic markers may still be present as some regions of the genome are resistant to reprogramming, resulting in differences in transcriptomes between IVF embryos and cloned embryos (Matoba & Zhang, 2018). This hinders cloning efficiency because it limits the ability of cloned embryos to return to a totipotent state and change the gene expression patterns to differ from a typical embryo. Incomplete epigenetic

reprogramming can cause the proliferation of the donor cell type instead of the formation of a complete organism with all cell types.

Reprogramming must begin before the first mitotic event in the reconstruct. Somatic cells require more reprogramming than embryonic cells as they are more differentiated. To be more differentiated they have more genes transcriptionally blocked with epigenetic alterations. Therefore, somatic cells may require more time to be reprogrammed by the cytoplast. Incomplete epigenetic reprogramming occurs more often in SCT than ECT, causing lower cloning efficiency. Heyman et al. (2002) observed that death between Day 90 of gestation and calving was 43.7% for adult somatic clones, 33.3% for fetal somatic clones, and 4.3% for embryonic clones.

Epigenetic alteration treatments can be used in cloned bovine embryos. This includes treatment with deacetylase inhibitors which have been shown to improve blastocyst development (Akagi et al., 2011; Ding et al., 2008). DNA methylation inhibitor treatments have been trialled but have not improved *in vitro* development of the bovine embryos (Ding et al., 2008). A major epigenetic alteration noted for its resistance to reprogramming during CT is the histone methylation of H3K9me3. This marker is present in CT embryos but not in IVF embryos. This methylation promotes heterochromatin formation which decreases the activation of gene transcription (Czernik et al., 2019). Treatment with histone demethylase can temporarily reduce levels of H3K9me3 in somatic and embryonic donors and improve *in vitro* development after cloning (Antony et al., 2013; J. Wei et al., 2017). No treatment has completely corrected epigenetic reprogramming during CT so far.

2.2.4 Donor cell cycle

Low cloning efficiency can occur due to cell cycle incompatibility (Dinnyes et al., 2006; Du et al., 2002). Cell cycle incompatibility between donor cells and cytoplasts can cause polyploidy or aneuploidy in the resulting embryo. Polyploidy is an abnormally high amount of complete chromosome sets and aneuploidy is an abnormally low amount of complete sets of chromosomes. Polyploidy or aneuploidy occurs in up to 20% of CT blastocysts (Bureau et al., 2003).

The stage of interphase that the donor cell is at during fusion is important to embryo development (Dinnyes et al., 2006). Interphase consists of three stages: growth 1 (G1),

DNA synthesis (S), and growth 2 (G2) (figure 2.3.2) (Yang, 2018). After interphase, mitosis is completed (M). During G1 cells increase their size by transcribing RNA, translating proteins for growth, and copying their organelles. Cyclins and cyclin dependent kinases are required as a checkpoint to cause the progression from G1 to S. Without cyclin signals to progress to the next stage, cells will stay quiescent in the G0-phase. G1/G0 cells have diploid DNA content and chromosome numbers (2N/2C). During S-phase DNA synthesis occurs and the chromosomes are replicated. In S-phase the donor cell progresses from 2N/2C to 2N/4C. A further increase in size and the production of spindle proteins in preparation for division occurs in G2. Another cyclin checkpoint is required in G2 to progress to M or the cells remain quiescent. During G2 the donor cell has 2N/4C. In M-phase, the chromosomes line up and the mitotic spindles separate them into two nuclei. The cell then divides into two daughter cells. If chromosome segregation was correct the daughter cells are identical with 2N/2C each.

During CT, oocytes are left in IVM media for 18-20 hours before removal for enucleation preparation. At this stage, the cytoplasts are in metaphase two (MII) (Dinnyes et al., 2006). During fusion, the donor cells need to be at a stage in the cell cycle that allows correct chromosome segregation with the MII cytoplasts to avoid polyploidy or aneuploidy. Compatible donor cell stages with MII cytoplasts are G1/G0 or G2/M.

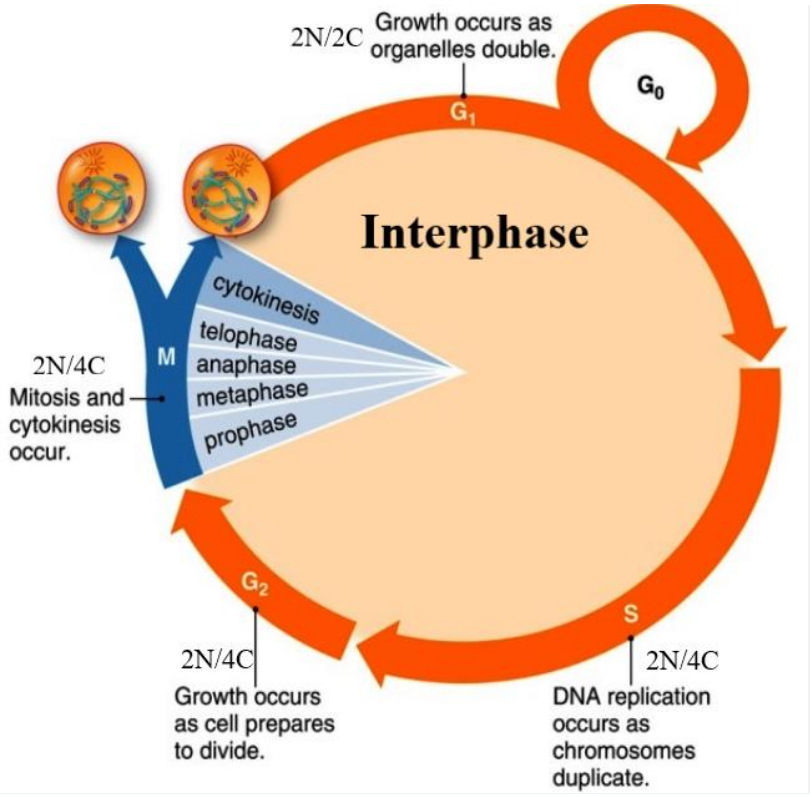


Figure 2.3.1 Stages of interphase in the cell cycle. (James E. Ferrell, 2020).

2.3.2 Serum starvation for donors in G1/G0 phase

Donor cells in the G1/G0 stage are compatible with MII oocytes as they have 2N/2C (Dinnyes et al., 2006). The chromosomes have not yet replicated which allows the reconstruct to have normal ploidy. The high cyclin B/Cdk1 environment in MII cytoplasts stimulates the degradation of the nuclear membrane, and the condensation of chromosomes, allowing their reprogramming. Somatic donor cell colonies can be synchronized into the desired G1/G0 stage for CT with serum starvation (Zakhartchenko et al., 1999). Serum starvation is the process of culturing cells in growth media with a low serum percentage (Sun et al., 2008). In the presence of low serum percentage, the cells enter a quiescent stage and remain in G0 until after fusion. For reconstructs made with donor cells in G1/G0, an activation method which does not allow the expulsion of a pseudo-polar body is required to avoid aneuploidy. DMAP activation can be used as it is an actin depolymerizing drug that prevents the cytokinesis that leads to pseudo-polar body expulsion (Suvá et al., 2019). G1 is shorter in ePSCs than in somatic cells which means fewer embryonic donors are compatible with MII cytoplasts at the time of CT fusion (Bogliotti et al. (2018). Embryonic donors

are vulnerable to cell death during serum starvation so cannot be treated to lengthen G1/G0.

2.3.3 Cytoplast aging for donors in S-phase

Donors in S-phase are incompatible with MII recipient oocytes. The high MPF environment of the MII oocytes would cause the degradation of the nuclear envelope, chromosome condensation and mitotic spindle formation. As the chromosomes are only partially formed during the S-phase they would fragment without the envelope. The DNA cannot undergo normal replication and reconstructs cannot form blastocysts. This is an issue during ECT as bEPSCs donor outgrowths have shown a high proportion of cells in S-phase (27%), more so than somatic cell colony (15%) (Bogliotti et al, 2018).

To be compatible with those donors in S-phase, recipient cytoplasts with a low MPF environment can be used (Dinnyes et al., 2006; Du et al., 2002). This will allow replication to be completed in the S-phase donors before the nuclear membrane breakdown and condensation occurs. A low MPF environment can be created in cytoplasts with aging followed by cooling (Bordignon & Smith, 1998; Fulka Jr et al., 1998; Gall et al., 1996). MPF activity declines with advancing cytoplast age (Fulka Jr et al., 1998). Aging alone cannot reduce MPF levels and H1 kinase activity, which is indicative of the cytoplast being mitotic, but levels drop when aging is combined with cooling (Bordignon & Smith, 1998; Fulka Jr et al., 1998; Gall et al., 1996). Aging and cooling cause an interphase like stage in recipient cells where no chromatin remodelling is done.

The interphase environment of a recipient cytoplasts created by aging and cooling is also compatible with donor cells in G2/M-phase as these cells have completed DNA replication and already have high MPF which dissembles their nuclear membrane for chromosome reprogramming. Donor cells in the G1/G0-phase are not compatible with aged cytoplasts as they need a high MPF environment to degrade the nuclear membrane for chromatin remodelling.

2.3.4 Nocodazole treatment for donors in G2/M

Donor cells in G2/M have abnormal ploidy as they are 2N/4C (Dinnyes et al., 2006). The extrusion of a pseudo-polar body containing the excess sister chromatids returns them to 2N/2C. Pseudo-polar body extrusion from a reconstruct should be checked after activation as it may not occur if sister chromatids did not align properly on the spindle, resulting in a polyploid embryo. Embryonic donors can be synchronized into G2/M with nocodazole treatment (Yabuuchi et al., 2004). Nocodazole treatment halts the cell cycle during mitosis by interfering with the microtubule spindles that separate chromosomes (Tanaka et al., 1995). After activation, reconstructs made with nocodazole-treated donors must be incubated in a media that allows pseudo-polar body extrusion such as protein synthesis inhibitor CHX (Suvá et al., 2019). However, the disadvantage of using CHX over DMAP is that DMAP produces higher blastocyst development rates than other chemical activation methods when using embryonic donors (Appleby, 2015). Embryonic donor cells in M-phase have a higher rate of development into blastocysts than donors in interphase (Yabuuchi et al., 2004).

2.3.5 Double cytoplasts

Increasing the cytoplast volume has been observed to affect the fusion rates, cleavage rates and blastocyst development of a CT run (Appleby et al., 2022; Delaney et al., 2007; Green et al., 2016; Peura et al., 1998). Once the couplet is fused, a second cytoplast can be stuck to the reconstruct and electrofused. Increasing the cytoplast volume can affect CT results because it increases the ability of the cytoplast to reprogram a donor cell (Sayaka et al., 2008). Cytoplasmic factors remove epigenetic modifications in donor cells. *In vivo,* the reprogramming factors of a single oocyte cytoplasm are sufficient for a gamete nucleus. This same volume may not be sufficient for a somatic cell nucleus or ePSC nucleus. The oocyte cytoplasm also contains maternal transcripts, proteins, mRNA, and molecular precursors which are needed for the major burst of transcription during early embryonic development (Liu et al., 2018; Peura et al., 1998). The early embryonic genome activation in bovine embryos occurs at the 8-16 cell stage and before that bovine embryonic development may rely on these maternal factors. Increased maternal transcriptional factors and reprogramming factors in the cytoplasm could increase development. Especially since some of the cytoplasm

and the maternal factors it contains can get removed during enucleation. The exact maternal factors in the cytoplast that affect development have not yet been identified.

Delaney et al. (2007) found that *in vitro* development after bovine SCT was significantly improved in double cytoplasts (58%) compared to controls (43%). Development of grade 1-2 blastocysts was higher in double cytoplasts (41%) compared to controls (3%). The onset of DNA replication was more advanced, and the blastocysts had greater numbers of nuclei. However, pregnancy rates did not differ significantly. There are some discrepancies in the literature around the effects of doubling cytoplasm volume on SCT (Sayaka et al., 2008; Trounson et al., 1998), and a lack of literature around the effect of doubling cytoplast volume during ECT. As embryonic and somatic donors require different levels of reprogramming factors the effect of cytoplast volume on the two donors are likely to differ. One piece of literature on the use of embryonic donors with double cytoplasts has only recently been published. Appleby et al. (2022) used nocodazole-treated bePSCs with double cytoplasts and established that double cytoplasts caused a significant increase in total blastocyst development from 9 ± 4% in single cytoplasts to 18 ± 5% in double cytoplasts. High-quality development also improved from 0% in single cytoplasts to 9 ± 3% in double. However, there was no difference in initial pregnancy establishment with double cytoplasts.

2.3.6 Vitrification of cytoplasts

Vitrification solidifies living cells without forming ice crystals (Chian et al., 2004). Ice crystal formation often accompanies other freezing methods and can damage the nuclear content of the cell. Oocytes and cytoplasts can be preserved through vitrification. The ability to use vitrified recipient cells would increase the efficiency of a CT run (Atabay et al., 2004; Dinnyés et al., 2000; Hou et al., 2005). CT is a day-long procedure, and the enucleation stage takes approximately one hour per 100 cytoplasts for an experienced operator. This time can be further increased during double cytoplast CT when twice the number of cytoplasts is required. CT runs are also limited by the availability of ovaries on the previous day and the timing of aspiration and IVM. Removing the IVM and enucleation steps and replacing them with a cytoplast thawing step would reduce the labour-intensiveness and duration of a CT run. It would remove the limitations of resource availability and timing (Hou et al., 2005). Viable

vitrification of oocytes is also important for preservation of mammalian oocytes from valuable or rare animals. It is therefore of interest whether vitrified bovine cytoplasts and oocytes have a sufficient survival rate and developmental potential for use in CT.

Studies have found that vitrified bovine cytoplasts could have a high survival rate after thawing up to 97% (Booth et al., 1999) and oocytes can have a survival rate up to 77-86% (Dinnyés et al., 2000), 86–90% (Atabay et al., 2004) and 87-98% (Chian et al., 2004). In contrast, some studies have observed that vitrification causes cryoinjury in the meiotic spindle which hinders survival and use of vitrified oocytes (Chen et al., 2003; Girka et al., 2022). Booth et al. (1999) observed that fusion rates were not affected by vitrification but there was a decrease in the cleavage and blastocyst rates per fusion after using vitrified bovine cytoplasts in ECT with blastomere donors. There was a higher rate of lysis indicating incomplete membrane recovery. Despite the increased lysis rate one blastocyst produced from a vitrified cytoplast was transferred to a recipient and produced twins, showing that vitrified cytoplasts can be used for successful CT. Peura et al. (1999) found that fusion, cleavage and blastocyst development was lower with vitrified cytoplasts than fresh in ECT with blastomere donors. Park et al. (2015) saw no change in fusion, cleavage or blastocyst development during bovine SCT with ZI pre-activated cytoplasts. Atabay et al. (2004); Dinnyés et al. (2000) recorded that the fusion, cleavage and blastocyst development rates during CT with vitrified oocytes were not significantly different to fresh oocyte controls after using vitrified oocytes in bovine SCT. Although Atabay et al. (2004) did note that the blastocysts produced from vitrified oocytes had a decreased mean cell number compared to non-vitrified controls.

The vitrification/thawing process has been further refined and improved. Survival rates of bovine oocytes is largely influenced by the protocol and embryologist (Saragusty & Arav, 2011), so the specific vitrification/thawing methods in this thesis may yield different results to the literature. The vitrification methods used on cytoplasts and oocytes by Booth et al. (1999); Hou et al. (2005); Peura et al. (1999) differed from the vitrification methods at AgResearch in terms of solution composition, concentrations, and the time of exposure to the vitrification media. The concentration of dimethyl sulfoxide (DMSO) and ethylene glycol (EG) cryoprotectants and the time of exposure to them is important because cryoprotectants affect the extent of dehydration in the cells (Chian et al., 2004). There is a strong correlation between cryoprotectant

concentration and the cooling-warming rates during vitrification. Hou et al. (2005) discovered that different concentrations of the same medias could cause variation in oocyte survival from 77% to 98%. The type of cryoprotectant used can affect the developmental competence of oocytes. Sucrose can act as an osmotic buffer and its extracellular concentration affects the level of osmotic pressure on the bovine oocyte (Hou et al., 2005; Otoi et al., 1995). This affects the degree of dehydration of the oocytes and the level of influx of cryoprotectants. The high concentration of extracellular sugar increases the osmotic pressure of the solution surrounding the oocytes, rapidly. Furthermore, Otoi et al. (1995) discovered that different concentrations of sucrose in vitrification media affect the developmental competence of bovine oocytes. During thawing, the osmotic buffer of the sucrose can prevent excessive osmotic swelling in the oocyte that could damage it (Hou et al., 2005). Ficoll can transform from the liquid phase into the semisolid and solid phase. The incorporation of ficoll can prevent the formation of ice crystals which would damage the oocytes.

Previous studies do not elucidate whether vitrified bovine cytoplasts can be used in double cytoplasts, when activation of cytoplasts is carried out after thawing instead of before vitrification, or to support reprogramming of bePSC donors.

2.4 Genetic Editing

DNA editing technology can accelerate genetic gain by increasing desirable variants in a herd with more efficacy and more rapidity than breeding with existing natural variation (Mclean et al., 2020; Mueller & Van Eenennaam, 2022; Tait-Burkard et al., 2018). DNA editing allows the control of the combinations of desirable variants without passenger mutations from linkage disequilibrium. Combinations of desirable mutations, and which harmful mutations undergo linkage disequilibrium with these, cannot be controlled during mating. Gene editing also allows the introduction of variants that do not naturally exist within the breeding population. Several methods of DNA editing can be carried out during CT.

A common method of gene editing used alongside CT is Clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein nine (Cas9) (Cong et al., 2013; Jinek et al., 2012). CRISPR and Cas9 are a part of the

bacterial immune system and act on bacteriophage DNA. CRISPR/Cas9 can be adapted for use on DNA other than viral DNA. Cas9 has a nuclease domain that makes double stranded breaks in foreign DNA. In bacterial defence, crRNA is complementary to the target sequence and guides the Cas9 to the location of the desired break. The equivalent of crRNA in editing is gRNA. Once a break is made it can then be repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR), altering the DNA sequence. NHEJ makes random changes to the target DNA sequence by randomly inserting or deleting base pairs before ligating the ends of the break. HDR makes specific changes to the target sequence by using an exogenous DNA template with homologous arms that are complementary around the cut site. HDR is preferrable over NHEJ, because NHEJ is error prone, unpredictable, and does not allow specific edits.

The editors can be introduced to a cell through physical methods (microinjection, lipofection, electroporation) or by using viruses. Microinjection is used for editing target genes in embryos between the one-cell to eight-cell stage. Editing reagents, such as Cas9 and gRNAs in solution, are injected into the cell cytoplasm or pronuclei using a fine needle (Qin et al., 2015). Once the editors act on the target sequence of the cell, all the subsequent cell divisions will include the modified allele. The disadvantage of microinjection is that it requires extensive training, is low in throughput, and requires expensive machinery (Hakim et al., 2021). Electroporation transfection can be performed on donor cells, reconstructs, or early-stage embryos. The cells to be edited are put into in a buffered solution containing the editors with or without a vector. Cas9 mRNA (Hashimoto & Takemoto, 2015) or expressed Cas9 protein (Namula et al., 2019) can be used. A pulse of high-voltage electricity is sent through the solution, creating pores in the membrane through which the vectors or reagents can enter. Electroporation is less labour-intensive and uses cheaper machinery than microinjection. Electroporation is faster as it allows the treatment of multiple embryos, cytoplasts, or donors at once while injection can only be performed on one cell at a time (Kaneko & Nakagawa, 2020). Electroporation also appears to be less invasive than microinjection as electroporated murine zygotes have a 29% higher live birth rate (Qin et al., 2015). However, microinjection of editors resulted in a 35% higher live birth rate with edits. Variables to consider when performing electroporation are the cell type, pulse number, pulse interval, pulse duration, voltage, medium and reagent concentration (Hashimoto & Takemoto, 2015) (Namula et al., 2019). These variables

affect editing rate, transfection rate, mutation rate, blastocyst formation, cleavage rates and embryonic developmental rates (Hashimoto & Takemoto, 2015; Namula et al., 2019; Wei J, 2018). Other gene delivery methods that have been explored but had lower transfection efficiencies in murine oocytes, zygotes and embryos are liposomes, cationic polymers, and viruses (Hakim et al., 2021).

Genetic editors can be introduced to the donor cells (Eghbalsaied et al., 2020; Hyder et al., 2020), or to a zygote (Wei J, 2018). The stage of the introduction of genetic editors determines whether mosaicism occurs (Hennig et al., 2020; Mclean et al., 2020). Mosaic organisms have two or more sets of cells with different allele combinations. Mosaicism can occur if editors are introduced into a one-cell embryo, but the editing occurs after cleavage. After cleavage, the editors will only be in one or some of the divided cells. Only cells that are derived from the successfully edited cell will have the edited sequence. This problem does not occur when introducing edits into ePSC donor cells for ECT because editing occurs at the earliest point in embryogenesis, so all cells of the developing embryo have that same edit. However, when attempting to transfect a whole colony of donor ePSCs with editors it is difficult to guarantee each individual donor is edited. After editors have been applied to a whole colony, individual cells in the colony need to be isolated by methods such as mitotic shake-off, fluorescent sorting, or drug selection and seeded into new clonal line colonies from the single cells (Mclean et al., 2020). All cells in each clonal line will have identical genetic content and these colonies can be checked for the correct edits. Creating clonal lines would require the cells to have high clonogenicity and proliferation potential to amplify the genotype for characterisation and to create enough donors for CT.

CT can be used to increase the number of transferable bovine blastocysts with desirable traits that increase milk production, increase reproductive success, improve animal welfare, and improve sustainability. There has been a large focus on traits that improve dairy cattle welfare. An example of research for cattle welfare is the introduction of the Pc polled allele so dehorning of cattle can be bypassed (Carlson et al., 2016). The allele was introduced into bovine embryo fibroblasts that were cloned with SCT to produce two live polled calves. Cattle have been edited in combination with SCT to produce milk that express the antibiotic lysostaphin which can prevent infection by *S. aureus* (Tait-Burkard et al., 2018). Genes involved in thermotolerance

can be introduced into a cattle population to mitigate heat stress as climate change progresses. This includes edits in the protein gene (*PMEL*) which is a affects bovine coat colour (Laible et al., 2021). A three base pair deletion creates a causative allele for the semi-dominant colour dilution phenotype. This variant is present in Galloway and Highland cattle populations and causes a lighter coat colour that has low absorption of solar radiation. This was introduced with CRISPR/Cas9 of somatic donors and CT into Holstein Friesian dairy cattle. Cattle with homozygote edits had a less absorptive grey and white coat as opposed to their typical black and white coat. Another gene that can be edited for thermotolerance is prolactin receptor (*PRLR*) which affects hair length, hair follicle density, and sweat excretion rate phenotypes (Rodriguez-Villamil et al., 2021). A single base deletion in *PRLR* can cause a dominant slick allele which is characterised by lower hair follicle density, shorter hair, and increased rates of sweating. This variant exists in Senepol cattle populations. It was introduced by Rodriguez-Villamil et al. (2021) into Angus cattle with CRISPR/Cas9 and IVF produced zygotes.

2.5 Research aims and objectives

The aim of this research project was to improve the current bovine ECT methodology, especially the methodology of cytoplast treatment. The goal of this project was to address the low blastocyst output of current methodologies. This research will also increase the knowledge around CT cloning and cytoplast reprograming ability. These findings will contribute to the climate smart-cattle breeding project. The aim of the climate-smart cattle project is to facilitate the multiplication of elite genetics for thermotolerance in NZ dairy cattle populations using ECT.

The research aims were divided into four research objectives.

- Determine the optimal hatching methodology for generating bovine blastocyst outgrowths.
- Investigate the effect of nocodazole cell synchronization on polar body extrusion rates and bovine ECT.
- Optimise methodologies surrounding cytoplast treatment in bovine ECT.
- Investigate the potential of vitrified oocytes and cytoplasts for use in CT.

Chapter 3
Materials and Methods

All research was exempt from animal ethics approval as the study did not proceed past late-stage blastocysts. Research that used genetically modified organisms was performed under the New Zealand (NZ) Hazardous Substances and New Organisms Act 1996. All research was performed under the NZ Biosecurity Act 1993.

3.1 Materials

Appendix I lists the equipment, reagent suppliers, and the in-house recipes used. In-house solutions were made with Milli-Q (MQ) water (15-18 MΩ·cm). Solutions made with non-sterile constituents were sterilised with a 0.2 µm Supor® Hydrophilic polyethersulfone membrane filter (Supor®, Pall laboratories, USA). Glassware and metal apparatuses were heat sterilised at 200°C for two hours in between uses. Heat sensitive materials including aspiration needles were autoclaved at 120°C for 15 minutes before use. The sterility of the lab surfaces was maintained with regular 70% ethanol washes.

All cell culture work was performed in the physical containment level two (PC2) tissue culture laboratory at AgResearch Ltd, Ruakura, Hamilton, New Zealand. All culture work was conducted in a sterile laminar flow hood (Hera guard, Heraeus, Germany). Plates were held in a 38°C 5% CO_2 incubator (Series II water-jacketed CO_2 incubator, Therma Forma, Thermo Scientific, USA).

3.2 *In vitro* production of blastocysts (IVP)

All IVP plates were prepared in a sterile laminar flow hood (Model CF, Gelman Sciences, Australia). Drops were made in petri dishes with automatic electronic pipettors (eLINE and Proline, Sartorius Biohit, Finland). Drops were made in the layouts displayed in Appendix II. Drops were overlaid with mineral oil using a pipette aid and 10 ml serological pipette to deter evaporation and maintain sterility of the drop solution. Plates were gassed in a 38°C 5% CO_2 incubator. Plates were placed on a 36-38°C warm stage while experimental work was carried out before return to gassed

conditions. Washes were done in 35 mm Petri dishes with 3 ml of solution. IVP sheets (Appendix III) were used for records.

Modular incubator chambers (QNA International Pty Ltd., Australia) were prepared with a small amount (~2 cm) of sterile MQ water in the bottom for humidity. A 3 cm indicator dish with 3 mls of B199 + 10% FBS was placed inside the chamber. The seal of the chamber was greased with silicon grease and the clamp was engaged. The chamber was placed in a 38°C Contherm incubator and 5% CO_2, 7% O_2, 88% N_2 gas was released into the chamber for five minutes. After five minutes the gas is sealed inside by airtight clamping, and the chamber was placed in a 38°C dry (non-gassed) incubator. If the modular incubator chamber was correctly gassed the indicator dish was salmon pink or straw coloured after two hours. If the indicator dish was red or purple, then the gassed leaked out and there was a problem with the modular incubator chamber.

3.2.1 *In vitro* maturation (IVM)

One IVM plate was made per 120 oocytes (~30 ovaries). Drops of IVM media, 12 x 40 µl, were made in a 60 mm Petri dish. The IVM plates, aspiration media, H199 + 10% FBS, and B199 + 10% FBS were warmed in an incubator for at least two hours before use. The B199 had the lid loosened to equilibrate to a suitable pH.

Forty ovaries were used per run. They were collected from the Morrinsville, Auckland, or Greenlea abattoirs, NZ in a 29-30°C thermos containing 0.9% saline. Next, 2 ml of warmed aspiration medium was poured into a 15 ml conical tube. One tube was prepared per 15 ovaries transferred and then each tube placed into a 30-35°C test tube warmer. The lid was taken off the conical tube and replaced with a bung with a thick 18-gauge aspiration needle and blunt needle. The end of the blunt needle was made higher than the aspiration needle end to ensure the aspirated oocytes would not go up the aspiration line. The blunt needle was connected to the aspiration pump. Ovaries were strained from the thermos by hand, ensuring most of the fluid was removed and then placed into a wide mouth thermos. The ovaries were rinsed with warm saline twice until no blood remained in the wide mouth thermos. The ovaries were then covered with warm saline. An ovary was taken from the wide-mouth thermos and had the excess saline dabbed off with a paper towel. The aspiration pump (IVF Ultra Quiet VMAR-5100, Cook veterinary products, Switzerland) was turned on with the foot

pedal engaged to 45-50 mm Hg. The clear, yellow follicles between 3-10 mm in diameter were aspirated with the aspiration needle.

The contents of the aspiration tubes were allowed to settle to the bottom. The sediment was collected with a 2 ml syringe attached to a sterile Pasteur pipette and released into a 90 mm Petri dish with aspiration medium covering the base. A single 90 mm dish is used per two tubes. A grid template was placed underneath the dish. While viewing under a stereo microscope (SMZ-2B, Nikon, Japan), the grid was followed, and oocytes were transferred with a pipettor to a H199+10% FBS wash plate. Only oocytes that were light in colour, had even cytoplasm, and were surrounded by unexpanded cumulus cells were selected (Appendix IV). Denuded or spotty oocytes were ignored. The good quality oocytes were washed in H199 + 10% FBS twice. As much debris as possible was left behind. The oocytes were then washed in B199 + 10% FBS. Each IVM drop received 10 µl with 8-12 oocytes. Oocytes were matured in the incubator for 20-22 hours before use in IVF.

3.2.2 Day 0 – *In vitro* fertilisation (IVF)

One IVF plate was made per 60 oocytes. Twelve 30 µl drops of IVF media were made in a 60 mm Petri dish. The drops were overlaid with 8 ml of oil. Approximately 10 ml IVF media, 10 ml HSOF and the IVF plates were warmed in an incubator for at least two hours before use. The IVF media had the lid loosened to equilibrate to a suitable pH. 1 ml of HSOF was kept at room temperature.

A BoviPure® density gradient was created in a centrifuge tube (Falcon) for sperm preparation. 1 ml of 80% BoviPure solution was carefully overlayed with 1 ml of 40% BoviPure solution. Overlaying was done with a syringe and glass Pasteur pipette, running the BoviPure gently down the side of the centrifuge tube. An interface was created between the two BoviPure solutions. One gradient was prepared for one to two semen straws. One straw was used per 200 oocytes. A vitrified bull semen straw (SRB Monowai Debonair) was retrieved and thawed in air for five to ten seconds. It was then immediately placed in the 30-35°C tap water bath for 30 seconds. The straw was dried with a tissue in the laminar flow and the ends were wiped with 70% ethanol. The straw was held in the middle to avoid contamination. Sterile scissors were used to cut open the straw and the contents of the straw were run along the inside of the centrifuge tube, overlaying the BoviPure gradient. The gradient and sperm were centrifuged (Primo

centrifuge, Heraeus Biofuge, Germany) at 300 g for 20-30 minutes. While the gradient was centrifuged the oocytes were prepared.

Oocyte preparation was done 20-22 hours after the oocytes were put into IVM media. The oocytes were removed from the IVM drops and washed through warmed HSOF twice. The cumulus oocyte complexes were pipetted up and down to loosen, but not remove, the cumulus. They were then washed in equilibrated IVF media. Five oocytes in 10 µl were then transferred to each IVF drop. The IVF dish was returned to the incubator until the sperm dilution was ready.

The centrifuged sperm pellet was immediately removed with a glass Pasteur pipette preloaded with a small amount of room temperature HSOF. The removal was immediate to prevent sperm swimming up. The pellet was placed in a fresh 15 ml centrifuge tube. The remaining room temperature HSOF was added drop wise to avoid dilution shock while the tube was flicked gently. The sperm mix was centrifuged at 300 g for five minutes. The supernatant was removed immediately once centrifuging was complete. 200 µl of equilibrated IVF media was added dropwise while the tube was flicked gently. 10 µl of the sperm preparation was added to 190 µl of water in a glass tube for sperm counting. The glass tube was moved in a figure eight motion. The remaining volume (A) of the sperm preparation was measured using a syringe and glass Pasteur pipette. The sperm preparation was kept in a warm dark place during sperm concentration calculation.

Sperm counting (B) was performed with a haemocytometer (Bürker Counting Chamber, Neubauer, Weber, UK) and a thick cover glass (Appendix V). The haemocytometer grid contained 25 large squares, each with 16 smaller squares. The volume of each small square was 0.00025 mm^3. The two shoulders of the haemocytometer were moistened, and the thick cover glass was placed on top with light pressure. 10 µl of the sperm dilution was pipetted at an angle along the edge of the cover glass and haemocytometer, covering the grid. Both sides of the chamber were filled. The sperm was allowed to sit for two to three minutes to settle. The haemocytometer grids were viewed under 400X on a phase contrast light microscope. The sperm heads within the 25 large squares were counted (Appendix V). The sperm on the top and left perimeter of each large square were not included in the count. The

count from both grids was used to calculate the average of sperm count (B); Equation 3.3 was then used to calculate the dilution.

Equation 3.3 : Calculation of sperm dilution to 1.5 million per ml.

$$\frac{\text{Volume of sperm measured (A) x Average of sperm count (B)}}{37.5}$$

$$= \text{Total IVF media volume (C)}$$

$$\text{Total IVF media volume (C)} - \text{Volume of sperm measured (A)}$$

$$= \text{IVF media volume to be added (D)}$$

The calculated IVF media volume (D) was added to the sperm preparation (A) to create the final diluted sperm sample. 10 µl of diluted sperm sample was added to each IVF drop containing oocytes to make a final concentration of 1 million/ml. The drops were checked for sperm motility before the plates were returned to the incubator for 18-24 hours.

3.2.3 Day 1 – *In vitro* culture (IVC)

18-24 hours after IVF was completed, IVC was performed. IVC plates were prepared at least two hours in advance. 2 x 40 µl wash drops and 6 x 20 µl drops of early SOF (ESOF) were made in a 35 mm Petri dish. The drops were overlaid with 3 ml of oil. One plate per 60 zygotes was made. The plates were equilibrated in a modular incubator for at least two hours before use. 10 ml HSOF was warmed in an incubator for two hours.

The zygotes were removed from the IVF plates washed through warmed HSOF. The zygotes were pipetted up and down to loosen cumulus cells. The cumulus cells were striped in a micro test tube (Eppendorf) with hyaluronidase (1 mg/ml) in HSOF+BSA. The micro test tube was vortexed for three minutes at 2000 rpm on a minishaker (SM1 minishaker IKA®, Germany). The test tube was centrifuged (Spectrafuge mini C1301, Labnet International INC., USA) for ten seconds. The pellet was removed and placed into a second HSOF wash dish. The remaining supernatant was searched.

A pulled Pasteur pipette attached to a mouth pipette was used to pick up zygotes and transfer them to the central wash drops of the IVC plates. The Pasteur pipette was pulled by heating the neck over a Bunsen burner flame until the glass was softened. The glass was quickly removed from the flame while simultaneously being pulled outwards to lengthen and constrict the glass. The neck was snapped, and the tip was softened in the flame. Mouth pipettes were used to minimise media transfer and for control over a small group. 8-12 zygotes were moved to each 20 µl drop. The IVC plates were returned to the modular incubator chamber and the chamber was regassed before return to the dry incubator.

3.2.4 Day 5 – Culture medium change (Changeover)

Five days after IVF, the embryos were moved from the ESOF media into late SOF (LSOF) media. Mineral oil in a flask and the LSOF media had their lids loosened and were gassed for five minutes at 2 psi. At the end of the five minutes the lids were sealed, and the media and oil were left to equilibrate for an hour. After the hour, changeover plates were made with 2 x 40 µl wash drops and 6 x 20 µl drops of LSOF in a 35 mm Petri dish. The drops were overlaid with 3 ml of oil. One LSOF plate per ESOF was made. The plates were equilibrated in a modular incubator for at least two hours before use. Embryos were analysed for lysis, cleavage beyond the one-cell stage, cleavage beyond the eight-cell stage, and the formation of tight morulae. This was recorded on the IVP record sheet. Embryos that had cleaved to or above the eight-cell stage were transferred through both wash drops in the LSOF plate with a pulled Pasteur pipette at the end of a mouth pipette. 8-12 embryos were moved to each 20 µl drop. The IVC plates were returned to the modular incubator chamber and the chamber was regassed before return to the dry incubator.

3.2.5 Day 8 – Grading embryo development

Embryo development and grade was scored seven days and eight days after IVF. An experienced grader viewed the embryos under a microscope and recorded the number of tight morulae and the number of blastocysts. Each was assigned a grade between 1-3 following a universal guide (Appendix VI). Grades 1-2 were considered high-quality and were used for hatching experiments or outgrowth generation for ECT donors. Grading was completed by the same operators each time to avoid human bias and inconsistency.

3.3 Cell transfer cloning

To produce embryos with the same genetic makeup as an elite outgrowth, CT was done in a sterile laminar flow hood (Model CF, Gelman Sciences, Australia). Drops were made in Petri dishes using the in the layouts displayed in Appendix III with automatic electronic pipettors (eLINE and Proline, Sartorius Biohit, Finland). Drops were overlaid with mineral oil using a pipette aid and 10 ml serological pipette to deter evaporation and maintain sterility of the drop solution. Plates were gassed in a 38°C 5% CO_2 incubator. All live-cell work was done on 36-38°C warm stages. Washes were done in 35 mm Petri dishes with 3 ml of solution. Pulled Pasteur mouth pipettes were used throughout CT unless otherwise stated. CT data was documented on record sheets (Appendix VII) and fusion sheets (Appendix VIII).

3.3.1 CT solution and plate preparation

To reduce the length of the run, some CT solutions and plates were made the afternoon prior to CT (Table 3.1). These were stored in a 4°C fridge overnight. Solutions and plates that required fresh reagents were made on the morning of ECT (Table 3.5.1.2). All solutions were prepared in universal tubes or 50 ml flasks. Plates were gassed in an incubator or a modulator chamber for at least two hours before use according to their location listing in Table 3.5.1.1 and Table 3.5.1.1. On the morning of CT fusion buffer was left on a bench to warm to room temperature, stage plates were placed on the stage to warm, incubator plates were placed in the incubator for equilibrating, and ECT solutions were placed in the dry incubator to warm.

Table 3.3-1. Plates prepared the day prior to CT

Plate Name	Solution	Drop Configuration	Number of Plates	Location
Oocyte	H199 +3 mg/ml BSA	12 x 30 µl	1	Warm stage
Cytoplast	H199 +3 mg/ml BSA	12 x 30 µl	1	Warm stage
Couplet	H199 +3 mg/ml BSA	12 x 30 µl	1 per treatment	Warm stage
Couplet + nocodazole	H199 +3 mg/ml BSA 500 nM nocodazole	12 x 30 µl	1 per treatment	Warm stage
Post Fusion	H199 +3 mg/ml BSA	12 x 30 µl	1 per treatment	Warm stage
ESOF – Ca	ESOF - Ca + 10% FBS	12 x 30 µl	1 per treatment	Incubator
ESOF	ESOF	3 x 40 µl, 32 x 5 µl	1 per treatment + 1 for PGs	Modular incubator chamber
Embryo hold	Embryo hold	1 x 40 µl	1	15˚C water bath
Embryonic donor	H199 +3 mg/ml BSA + 0.1% Y-27632	6 x 40 µl	1 per donor group	Warm stage
Embryonic donor + nocodazole	H199 +3mg/ml BSA + 0.1% Y-27632 + 500 nM nocodazole	6 x 40 µl	1 per donor group	Warm stage
Somatic donor	H199 + 0.5% FBS + 0.1% Y-27632	6 x 40 µl	1 per donor group	Warm stage

Table 3.3-2 Plates prepared the morning of cell transfer

Plate Name	Solution	Drop Configuration	Number of Plates	Location
Lectin	1980 µl H199 + 3 mg/ml BSA + 20 µl Lectin	12 x 30 µl	1 per treatment	Warm stage
Lectin + nocodazole	1980 µl H199 + 3 mg/ml BSA + 20 µl Lectin + 500 nM nocodazole	12 x 30 µl	1 per treatment	Warm stage
Stain	995 µl H199 + 3 mg/ml BSA + 5 µl Hoechst	12 x 30 µl	1	Warm stage
Pronase	H199 + 0.5% pronase	2 x 50 µl	1	Warm stage
Embryonic Disaggregation	Disaggregation media + 0.1% CB + 0.1% Y-27632	100 µl	1	Tissue culture bench
Embryonic Disaggregation + nocodazole	1 ml Disaggregation media + 500 nM nocodazole	100 µl	1	Tissue culture bench
Somatic disaggregation	H199 + 0.5% FBS	100 µl	1	Incubator
DMAP	1992 µl ESOF + 8 µl DMAP	3 x 40 µl, 40 x 5 µl	1 per treatment + 1 for PGs	Incubator
CHX	2 µl CHX in 1 ml ESOF	3 x 40 µl, 32 x 5 µl	1 per treatment	Incubator

3.3.2 Oocyte preparation

Oocytes were aspirated and added to IVM plates using the same method described in Section 3.2.1. Oocytes were matured in IVM media for 18-20 hours before use in CT. This was to allow the oocyte genome to mature and arrest at MII, and to ensure donor reprogramming factors and cycle resumption factors were present in the cytoplasm.

After maturation was complete oocytes were collected from the IVM culture with a mechanical pipette and washed in a 35 mm dish of H199 + 3 mg/ml BSA. The oocytes were pipetted up and down to loosen cumulus cells. The cumulus cells were striped by placing the oocytes into a micro test tube (Eppendorf) with hyaluronidase (1 mg/ml) in H199 + BSA. The micro test tube was vortexed for three minutes at 2000 rpm on a minishaker (SM1 minishaker IKA®, Germany). The test tube was centrifuged (Spectrafuge mini C1301, Labnet International INC., USA) for ten seconds. The pellet was removed and placed into a second H199 + 3 mg/ml BSA wash dish. The remaining supernatant was searched for oocytes. Each oocyte was rotated with a mouth pipette to search for a polar body in between the membrane and ZP. This indicated that the oocyte was in MII. Oocytes with no polar body were set aside with the zona still intact in a labelled drop in the oocyte dish. They were used as positive control PGs. A pronase plate was prepared without mineral oil overlay. Oocytes with polar bodies were transferred to the pronase. The oocytes were watched under the microscope until the zona loosened and grew faint. They were immediately washed through H199 + 3 mg/ml BSA twice to avoid the pronase digesting the oocyte membrane. They were then moved into the oocyte dish, 30 per drop, and allowed sufficient time to recover from the pronase.

3.3.3 Enucleation of oocytes

3.3.3.1 Enucleation tool preparation

Before the day of CT, a capillary puller (P-87, Sutter Instruments, USA) was set to heat = 320, pull = 150, velocity = 250, and time = 0. Borosilicat glass capillaries (GC100T-15 thin wall, Harvard Apparatus Ltd, UK) were inserted into the puller and carefully clamped at the ends. One capillary was pulled to make an enucleation needle and one to make a separation pipette. Capillaries were stored in a dust-free environment. Handling of the capillaries was done at the ends to avoid contaminating the area that would become the needle points.

A microforge (MP-9, Narishige, Japan) set to 10X magnification was used to break the enucleation needle at ~3 units (~ 32 µm). Clean perpendicular breaks were introduced by lowering the needle onto the glass bead and then turning off the heat quickly. A 30º bend was introduced at ~10 units (80 µm). The bend was made by positioning the capillary about 10 units above the glass bead and heating the filament.

The capillary was slowly moved to the right as the shaft dropped to get a smooth curvature without narrowing the inside diameter.

The microforge was used to break the separation pipette at ~12-18 units (~ 100-150 µm). The tip was closed and rounded by holding the tip near the heated glass bead. A 30º bend was introduced at ~25 units (200 µm).

Tools could be reused in later CTs after being rinsed in trypsin to remove debris and prevent clogging.

3.3.3.2 Enucleation

Oocytes were moved to the stain plate for ten minutes. This was adequate time for the stain to bind to DNA and inadequate time for the cytotoxic effects. 30 oocytes were stained at a time as 30 oocytes could be enucleated before the stain faded. Stained oocytes were enucleated in multiple drops of H199 + 10% FBS in a 90 mm Petri dish lid. Enucleation was performed with a micromanipulation microscope (MO-188, Nikon Narishige, Japan). The enucleation pipette and separation needle were affixed to three-axis oil hydraulic hanging joystick micromanipulators with a 0.2 mL Gilmont® micrometer syringe (Cole-Parmer Instruments, USA). The separation pipette was placed alongside the edge of an oocyte to steady it during enucleation. A UV light was turned on at the lowest level to fluoresce the stain in the nuclear contents of the oocyte. The oocyte was rotated until the DNA was located and accessible. The enucleation needle was lined up with the metaphase plate and gently inserted. The DNA was pulled out with as little cytoplasm as possible to leave a cytoplast. The UV light was on for the minimum period possible to prevent any UV damage. The cytoplast was pinched off the enucleation needle using the separation needle and moved to the ‘Cytoplast’ plate.

3.3.4 Aging cytoplasts

When completing experiments with aged cytoplasts, after enucleation a group of cytoplasts were set aside and labelled in the ‘Cytoplast’ plate. 35 hours after IVM these cytoplasts were transferred into the embryo hold plate. The plate was then left in a 15°C water bath for 11 hours. The cytoplasts were removed from embryo hold and

washed in H199 + 3 mg/ml BSA. Then they underwent attachment with fresh donors and resumed CT.

3.3.5 Donor cell preparation

Donor cell preparation was carried out in a sterile laminar flow hood (Hera guard, Heraeus, Germany) and plates were held in a 38°C 5% CO_2 incubator (Series II water-jacketed CO_2 incubator, Therma Forma, Thermo Scientific, USA). Either embryo-derived donors or somatic fibroblasts were used. On the day of CT, the colonies were lifted and disaggregated into single cells. This was done by one CT operator while another was enucleating. Mechanical pipettes were used in tissue culture.

3.3.5.1 Embryo-derived donors

Outgrowths with ePSCs were cultured for one to two weeks leading up to CT following Section 3.3.10. Large healthy outgrowths were chosen. Before lifting the embryonic outgrowths, the growth media was removed from the well and the well was PBS washed. 50 µl of disaggregation medium was added. The plate was returned to the 5% CO_2 38°C incubator for two to five minutes or until the outgrowth had lifted. Lifting of the outgrowth was confirmed with a phase-contrast microscope. A 200 µl pipette was used to transfer the cells to the embryonic disaggregation plate for transporting to the embryology lab. The colony was washed in H199 + 3 mg/ml BLSA before being moved into the first drop in the embryonic donor plate. The total time of the colony in disaggregation media was kept below ten minutes. A finely pulled pipette with a small diameter (≈ 100 µm) was used to mouth pipette the outgrowth up and down to fragment it. As the fragments were moved between drops in the donor plate, subsequently smaller diameter pipettes (≈ 70, 50, and 30 µm) were used to create smaller fragments until there were single donor cells.

When nocodazole synchronization was used, 500 nM of nocodazole was added to the complete growth media 18-20 hours before the donors were lifted. This synchronized the donors in mitosis. The disaggregation media, disaggregation plate, embryonic donor plate, lectin plate and couplet plate also had nocodazole to maintain the synchronization of the donors until fusion.

3.3.5.2 Somatic donors

Cryovials (Cryotube ™ Vials, Thermo Scientific, Denmark) of frozen male bovine embryonic fibroblasts (BEF2) were removed from liquid nitrogen storage and immediately thawed in a 38°C water bath. The cells were added to a 15 ml centrifuge tube containing 5 ml of prewarmed fibroblast growth media. The tube was centrifuged (Biofage Primo Centrifuge, Heraeus, Germany) at 1000 rpm for four minutes. The supernatant was aspirated off (Air Cadet, Thermo Scientific, USA) and the pellet was resuspended in another 5 mls of fibroblast media. The suspension was moved to a 60 mm tissue culture dish. Fibroblasts were incubated at 5% CO_2 and 38°C. Four hours after plating they were PBS washed three times. They were incubated in serum starvation media for five to six days before CT. This synchronized the fibroblasts in G1/G0. On the day of CT, fibroblasts were PBS washed and incubated in trypsin for four minutes. Lifting was confirmed with a phase-contrast microscope. The trypsin was inactivated with 5 mls of H199 + 0.5% FBS. The cells were centrifuged, and the supernatant was aspirated off. The cells were resuspended in 500 µl of H199 + 0.5% FBS. These single donors were spread amongst the drops of the somatic donor plate.

3.3.6 Cytoplast and donor cell adhesion

Cytoplasts were moved to the lectin plate for adhesion to donor cells. Lectin caused carbohydrate binding between the cytoplasts and donors. Five cytoplasts were placed in each drop and spread out to prevent them from sticking to each other. Five to ten donor cells were placed in each lectin drop and spread out to prevent sticking to other donors or couplets. Each cytoplast was rolled with the mouth pipette over a single donor cell to create a couplet. The couplet was gently pressed to maximise the surface area of contact between the cytoplast and donor. The couplet was left in the lectin for five minutes, and then pipetted up and down to ensure the cells were securely stuck. Couplets were spread throughout the couplet plate to prevent sticking to other cells and to prevent any toxicity from the lectin.

3.3.7 Fusion

A parallel plate fusion chamber was prepared in-house. The electrodes of an electro cell manipulator (BTX ECM 200, Biotechnologies and Experimental Research Inc, USA) were clamped to the fusion chamber. The chamber was flooded with

hypo-osmolar fusion buffer with calcium (164 – 207 mOsm). A pipette was used to remove any bubbles between the electrodes. The electro cell manipulator was set to 25 V AC current amplitude, 600 V DC pulse amplitude, two pulses, and 10 µsec pulse duration. Couplets were washed in a 35 mm Petri dish of fusion buffer to equilibrate to the hypo-osmolarity. Couplets were checked again for adherence and returned to lectin if they had separated to be restuck. The mouth pipette was washed to prevent any oil interfering with the current. Five to ten couplets were transferred into the 3 mm gap between the electrodes. The AC current was used to automatically align the couplets. Any couplets that were not aligned perpendicular to the fusion plates were removed or manually rotated to be perpendicular to electrodes. The DC pulses were initiated to cause fusion. Couplets were washed in the 35 mm petri dish of fusion buffer before being moved to the post fusion plate. After 30 minutes the couplets were scored as fused, lysed, or non-fused. Non-fused couplets that were still adhered could undergo a second round of fusion. Fused reconstructs were washed through HSOF – Ca + 10% FBS. They were then washed through the first two rows of the ESOF – Ca + 10% FBS plate. Fused reconstructs were placed in each drop of the bottom row of ESOF – Ca + 10% FBS plate and kept in the incubator for three hours until activation.

3.3.8 Double cytoplast fusion

To increase the volume of reprogramming factors in a reconstruct, there was the option of fusing a second cytoplast. Once a reconstruct was scored as fused, it could be moved to an unused lectin plate. A second cytoplast could be rolled onto the reconstruct. The fusion steps were repeated with the electro cell manipulator was set to 25 V AC current amplitude, 450 V DC pulse amplitude, two pulses, and 10 µsec pulse duration. Fused double cytoplast reconstructs were kept in separate ESOF – Ca + 10% FBS plates. Activation was done three hours after at the halfway point between the time of fusion of the single cytoplasts and the time of fusion of the double cytoplasts.

3.3.9 Activation of reconstructs

Reconstructs were activated to cause the release from MII arrest and the progression of embryo development. Each group of reconstructs was activated separately and kept in separate plates. Reconstructs were washed in a 35 mm of HSOF + 1 mg/ml BSA for one minute. They were transferred to a 35 mm dish of freshly made ionomycin solution for 4 minutes 30 seconds. The reconstructs were then transferred to a 35 mm dish of

HSOF + 30 mg/ml BSA for at least three minutes. HSOF + 1 mg/ml BSA and HSOF + 30 mg/ml BSA were prepared before the day of CT. The ionomycin was thawed and added to the HSOF + 1 mg/ml BSA at 1 µl/mL right before activation. The reconstructs were put through the three wash drops in the DMAP plate, and then placed in one 5 ul drop each. They were left in the incubator for four hours. Ionomycin/DMAP activation was also done with the PGs. When using reconstructs made with nocodazole-treated donors, CHX was used instead of DMAP to allow polar body extrusion. CHX and DMAP are inhibitors of phosphorylation or protein synthesis and prevent cytoplasmic factors from inhibiting the resumption of mitosis.

3.3.10 Embryo culture

Four hours after activation, the embryos were removed from the DMAP or CHX plates and washed through a 35 mm petri dish of warmed HSOF twice. The embryos were transferred through the three large wash drops of the ESOF plates. Each embryo was then move to a 5 µl drop. The ESOF plates were returned to the modular incubator chamber and the chamber was regassed before return to the dry incubator. Five days after CT, the changeover method described in Section 3.2.4 was followed. The LSOF drops were single culture instead of group culture. Single culture plates had 3 x 40 µl and 32 x 5 µl drops in a 60 mm petri dish, overlaid with oil. Seven days after CT, the embryos and PGs were graded as described in Section 3.2.5. On day eight blastocysts were vitrified for use in ETs or plated to produce more donors.

3.4 Generation of embryo-derived outgrowth culture

All cells in the outgrowths formed from plated blastocysts are termed embryo-derived cells. Testing for pluripotency was limited so they cannot be confidently termed ePSCs.

3.4.1 Culture plate preparation

Complete growth media was prepared monthly for embryo-derived outgrowth culture. Fresh activin A (Prospec Bio, Israel) was added at 20 ng/mL to the growth media every three days. Fresh fibroblast growth factor two (Sigma Aldrich, USA) was added at 20 ng/mL to the growth media each day of use. The wells of a flat-bottom 96-well plate (Nunc, Thermo Scientific, NZ) were flooded with ≈50 µL of filtered (Acrodisc

32 mm syringe filter with 0.2 µm membrane, Supor® Pall medical, UK) gelatin (0.1%). The gelatin was left to set for at least 20 minutes at room temperature before removal with a pipette. This facilitates adhesion of the outgrowth to the well. 100 µl of PBS was pipetted into each well on the perimeter of the dish to counter evaporation of the complete media. At least 20 minutes before 100 µl of the complete media was pipetted into wells inside the perimeter. One well was prepared per blastocyst. The plate was held in a 5% CO_2 incubator at 38°C.

3.4.2 Blastocyst plating

Embryo-derived outgrowths were produced from hatched bovine blastocysts. Plating of fresh blastocysts was performed eight days after IVP, or CT. Vitrified blastocysts were plated two hours after thawing. Blastocysts were checked for a ZP. If they were hatched and no ZP was visible they were plated as they were. If the ZP remained it was removed either mechanically or enzymatically. Plating was then done under the laminar flow with a bent pulled pipette and mouth pipettor. The bend was created by heating the neck of the Pasteur pipette over a Bunsen burner and pulling the softened glass at a ≈120° angle. The angle allowed for use of the pipette directly under the microscope lens. Each blastocyst was mouth pipetted into the bottom centre of a prepared well. The plate was returned to the incubator every three minutes and allowed to equilibrate again for five minutes. The completed plate was left in the incubator.

3.4.2.1 Mechanical hatching

To mechanically remove a biopsied ZP, a Pasteur pipette was pulled to have a diameter slightly smaller than the ZP. The blastocyst was mouth pipetted through the fine pipette until the ZP was peeled off. If the zona was not biopsied, a cut was made with 0.5 x 25 mm gauge needle (Neolus needle, Terumo, Europe NV). The zona was pinned in position by one needle and gently nicked with another needle. The embryo could then be removed with the mouth pipette.

3.4.2.2 Enzyme hatching

To enzymatically remove the ZP the blastocysts were placed in a drop of pronase in a 35 mm Petri dish. They were watched under a microscope until the ZP had expanded

in size and was fainter in appearance. The embryos were then washed in the LSOF, or embryo hold medium.

3.4.3 Embryo-derived cell culture

Half-media changes were done every two to three days. Fresh complete growth media was aliquoted and equilibrated in the incubator for 20 minutes. 50 µl of old media was gently pipetted from the top of each well. 50 µl of fresh pre-gassed media was then gently pipetted into each well.

3.5 Vitrification and thawing

3.5.1 Vitrification of embryos and oocytes

A CVM cryobath with a CVM vitrification block (Cyrologic, Australia) was half filled with liquid nitrogen and left for ten minutes. When the liquid nitrogen had stopped bubbling around the vitrification block, the block was adequately cooled. The cryobath was then filled with liquid nitrogen to a level just below the top surface of the block. The level of liquid nitrogen was checked and topped up throughout the vitrification procedure. Fibreplug sleeves (Cyrologic, Australia) were placed in the slots of the block to cool while cells were vitrified. Fibreplugs were prelabelled with the date, type of cell, hook number and number of cytoplasts/oocytes or embryos. A four-well plate with vitrification solutions (table 3.6.1) was prepared and left on a warm stage for ten minutes. The 4-well plate was remade every 30-40 minutes.

Table 3.5-1 Solution concentrations in a four-well vitrification plate for bovine embryos and oocytes

Well	Base media (Embryo hold – BSA + 20% FBS) (µl)	Sucrose Solution with Ficoll (Base media + 1M sucrose + 0.01g/mL Ficoll 70) (µl)	DMSO (µl)	EG (µl)
1	1000	0	0	0
2	1000	0	0	0

3	850	0	75	75
4	0	700	150	150

1 ml of MQ water was placed between the wells for humidity. Oocytes prepared as described in Section 3.3.1 can be vitrified ZP free (ZF) or with the ZP intact (ZI). For ZF oocytes the zona was removed with pronase as described in Section 3.4.2. Blastocysts from CT or IVP could be vitrified. The oocytes/embryos were transferred between wells with a thick pulled Pasteur pipette and mouth pipetter. They were washed in well one and well two for a minute. Then they were moved to well three for three minutes. While the oocytes/embryos were in well three, 2 x 20 µl drops of the solution from well four were pipetted on the inverted lid of the four-well plate. The second drop could be made slightly smaller than 20 µl to increase the ease of picking up cells. The oocytes/embryos were transferred from well three to the first 20 µl drop in as small a volume as possible. Using a vitrification micro-pipette (0.1-3 µl mLine, Sartorius Biohit, Finland) set to 2.5 µl, solution from the second drop was aspirated and released directly over the first drop to cause the cells to sink. Then all the oocytes/embryos were picked up in 2.5 µl. and transferred onto the hook of the fibreplug. The hook was held against the surface of the cooling block until the droplet vitrified. The time between exposure to well four solution and placement onto the cooling block was recorded. The goal was to keep the time in well four solution as short as possible, with one minute being the maximum time. The droplet was checked for white cloudiness. White cloudiness was caused by water slowly freezing within the cells which meant the vitrification was not successful. The fibreplug was slotted inside a cooled sleeve in the vitrification block. Liquid nitrogen was poured into a small polystyrene box until half full. The sleeved fibreplugs were quickly transferred from the cyroblock to the polystyrene box with forceps. A goblet was retrieved from the liquid nitrogen storage tank and placed into the box. With forceps, the sleeved fibreplugs were slid into the goblet while always remaining submerged in the liquid nitrogen. The goblet was checked for air bubbles. The empty spaces in the goblet are filled with cotton wool. The goblet was quickly placed back into a cannister in the storage tank and cotton wool was placed in the empty spaces in the canister to prevent the goblet floating.

3.5.2 Thawing of embryos and oocytes

When embryos were being thawed, a 60 mm petri dish with 6 x 20 μL drops of embryo hold under oil was prepared. When oocytes were being thawed a 60 mm petri dish with 6 x 20 μL drops of H199 + 10% FBS under oil was prepared. Post-thawing plates were warmed on a 36-38°C stage during the thawing process.

A four-well plate with thawing solutions was prepared and left on a warm stage for ten minutes. The concentration and placement of the solutions in the wells varies between embryos (table 3.4) and oocytes/cytoplasts (table 3.5). The four-well thawing plate was remade every 30-40 minutes. 1 ml of MQ water was placed between the wells for humidity. Liquid nitrogen was poured into a small polystyrene box (1/2 full). The desired fibreplugs were retrieved from the liquid nitrogen storage tank with forceps, and immediately placed in polystyrene box. In one quick movement, a single fibreplug was removed from the liquid nitrogen with forceps, pulled out of plastic sleeve and the hook was dipped into the first well. The vitrified oocyte or embryo thawed into the solution immediately. Each embryo or oocyte was then moved between sequential wells with a mouth pipette following the timings described in tables 3.4 and 3.5. After well four the embryos were transferred to the embryo hold plate. After well four the oocytes were transferred to the H199 plate. After two hours they were evaluated for survival. Survival was measured by intact membrane integrity. Surviving oocytes could then undergo parthenogenetic activation following the methods in Sections 3.3.9 and 3.3.10. Surviving cytoplasts could be used to stick to donors in CT as described in Section 3.3.6. Surviving embryos could be plated as described in Section 3.4.2 to produce CT donors.

Table 3.5-2 Solution concentrations in a four-well thawing plate for bovine embryos

Well	Base media (Embryo hold – BSA + 20% FBS) (μl)	Sucrose Solution (Base media + 1 M sucrose) (μl)	Time in well (min)
1	500	500	2.0
2	730	270	3.0

3	840	160	3.0
4	1000	0	2.5

Table 3.5-3 Solution concentrations in a four-well thawing plate for bovine oocytes

Well	Base media (Embryo hold – BSA + 20% FBS) (µl)	Sucrose Solution (Base media + 1 M sucrose) (µl)	Time in well (min)
1	0	1000	1
2	500	500	3
3	750	250	3
4	1000	0	5

3.6 Statistical analysis

Microsoft Excel® (version 2205) was used for data analysis. Fusion was calculated as a percentage out of couplets stuck excluding couplets where lysis occurred in the donor or cytoplast. Lysis was calculated as a percentage out of reconstructs into IVC. Embryo development was calculated as a percentage out of reconstructs in IVC. Error bars were determined with standard error of the mean (SEM). Error bars are absent in the results of preliminary studies which had only a single replicate. At least three replicates were done, if possible. Significance was calculated with Fisher's exact test of independence in 2 x 2 tables. Fisher's exact test is a statistical analysis used to determine if there are non-random associations between two variables in an experiment with a small number of observations. The null hypothesis is that the variables are equal because they are independent with no association. Fisher's exact test of independence was appropriate for objectives 2-4 as the percentage of blastocyst development was low. Fisher's exact test of independence was appropriate for objective 1 as the sample size for each hatching method was small. Significance was

given as a *P* value. A *P* value less than 0.05 was arbitrarily defined as significant and a *P* value less than 0.01 as highly significant.

Chapter 4

Results

4.1 Objective 1: Determining the optimal hatching methodology for generating bovine blastocyst outgrowths

4.1.1 IVP blastocyst generation

IVP runs (replicates *n*=7) were performed to generate blastocysts for the hatching experiments or plated to create donor outgrowths for ECT. Five days after IVF was completed, fertilized oocytes that went into IVC **(***N*=944**)** and had cleaved beyond the one-cell stage were counted and determined to be 88 ± 1% (Figure 4.1.1). Seven-eight days after IVF was completed, fertilized oocytes that had developed into grade 1-3 blastocysts were counted and determined to be 36 ± 5%. Grade 1-2 blastocysts were counted and determined to be 14 ± 3%. Grade 1-2 blastocysts were considered high-quality and were used for future experiments.

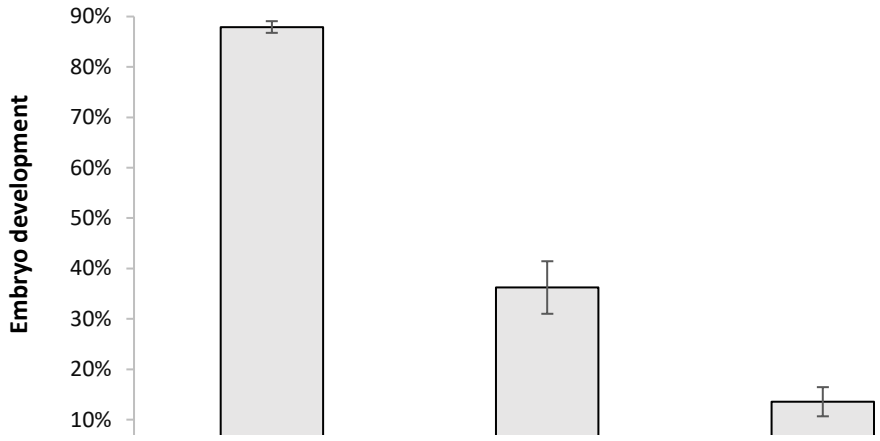
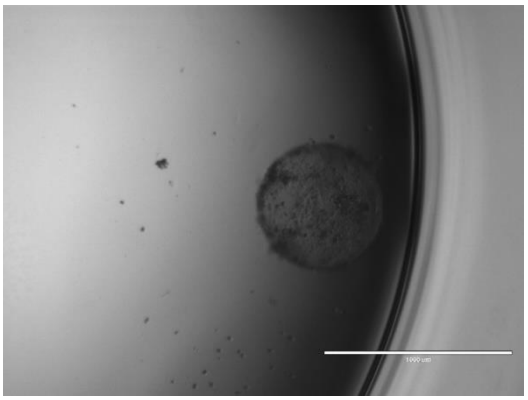


Figure 4.1.1 IVP development. Cleavage was calculated on day 5 out of the number of fertilized oocytes that went into IVC. Blastocysts development was calculated on day 7 or 8 out of the number of fertilized oocytes that went into IVC. Grade B1-3 is the total number of blastocysts and grade B1-2 includes the high-quality blastocysts. Error bars=SEM, fertilized oocytes in IVC *N*=944, Replicates *n*=7.

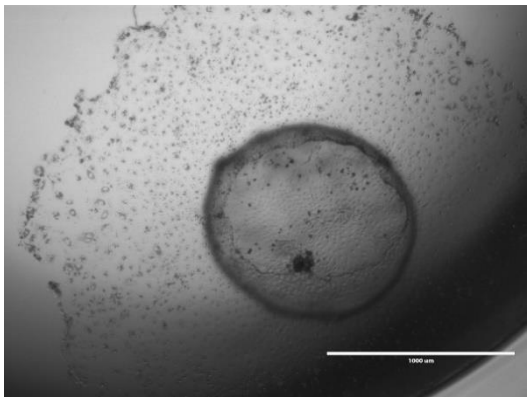
4.1.2 Blastocyst hatching methods to generate outgrowths

Healthy embryo-derived outgrowths are required as a source of donors in ECT. Four techniques of blastocysts hatching were tested to determine the optimal method of creating these required outgrowths. Eight days after IVF was performed, high-quality wild-type (WT) blastocysts (B1-2) were selected to trial these techniques (*n*=7). Blastocysts that had hatched naturally were plated without further treatment to act as the positive control (*N*=20). A group of non-hatched blastocysts were selected and plated with the ZP still intact to act as the negative control (*N*=15). The remaining non-hatched blastocysts were divided into two treatment groups; mechanically hatched (*N*=16) and pronase hatched (*N*=11). The outgrowth efficiency (the number of blastocysts that formed outgrowths) of each technique was recorded for two to seven days after plating (Figure 4.1.2). Any additional outgrowths that formed after day seven were added to the 7+ period, but any outgrowths that died after days 7+ were not subtracted from the days 7+ period. The techniques that lead to outgrowths by days 2-3, in order of highest to lowest efficiency, were natural hatching (45 ± 19%), mechanical hatching (31 ± 20%), and non-hatching (7 ± 15%) (Figure 4.1.3). Pronase hatching did not lead outgrowths on days 2-3. Natural hatching had an outgrowth efficiency that was significantly higher than the pronase hatching (*P*=0.017) and non-hatching (*P*=0.029) by days 2-3. The differences in outgrowth efficiencies between other techniques were insignificant. The order of highest to lowest outgrowth efficiency on days 4-5 was mechanical hatching (88 ± 13%), natural hatching (80 ± 16%), non-hatching (67 ± 18%) and then pronase hatching (18 ± 15%). The outgrowth efficiency of pronase hatched blastocysts was significantly lower than the naturally hatched (*P*=0.001), mechanically hatched (*P*=0.0027), and non-hatched (*P*=0.037). The differences in outgrowth efficiencies between the other methods were insignificant. The order of highest to lowest outgrowth efficiency on day 7 was, mechanical hatching (75 ± 17%), natural hatching (55 ± 19%), non-hatching (47 ± 20%) and then pronase hatching (36 ± 19%). The differences in outgrowth efficiencies between all the methods were insignificant by day 7.

A)



B)



C)

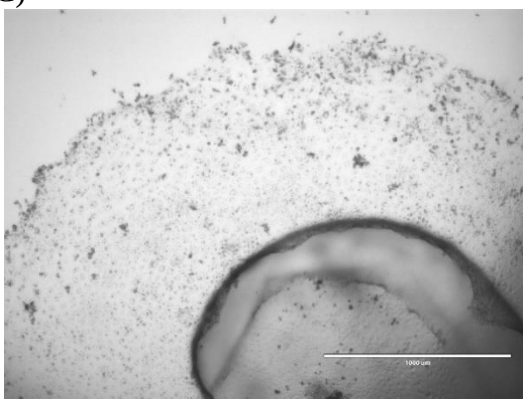


Figure 4.1.2 A positive control outgrowth from the hatching experiments on day 3 (A), day5 (B) and day 7 (C). A was not recorded as an outgrowth. B and C were recorded as outgrowths.

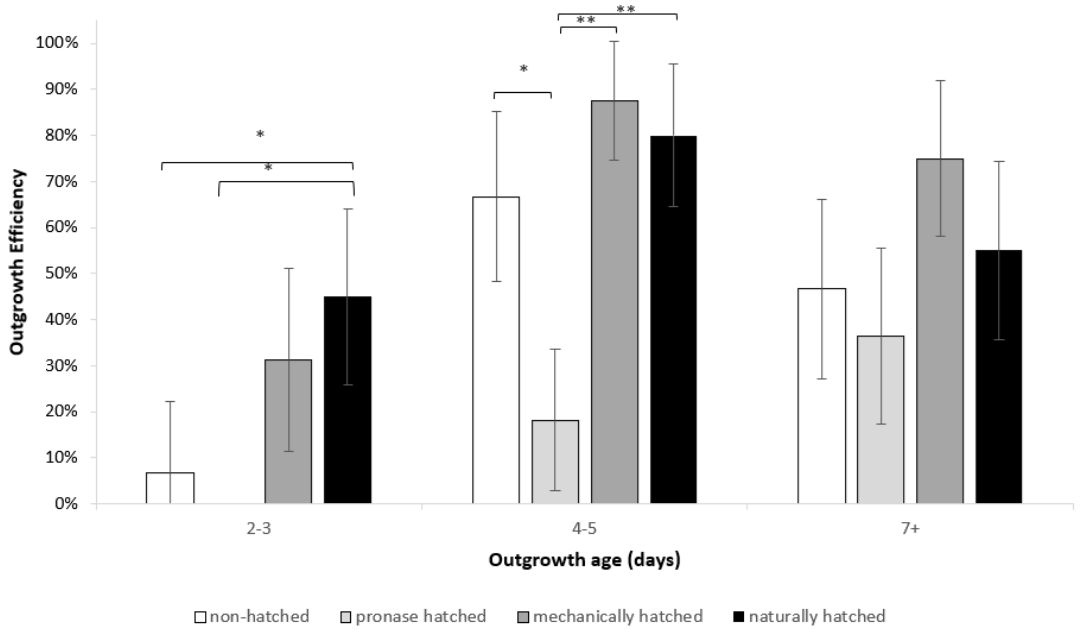


Figure 4.1.3 The effect of different blastocyst hatching techniques on embryo-derived outgrowth efficiency. WT blastocysts were non-hatched, pronase hatched, mechanically hatched, or naturally hatched. Outgrowth age refers to number of days since blastocyst plating. Outgrowth efficiency is the proportion of blastocysts plated that form an outgrowth. Error bars=SEM. Total blastocysts plated; non-hatched *N*=15, pronase hatched *N*=11, mechanically hatched *N*=16, and naturally hatched *N*=20. Replicates *n*=7. Significance * = $P < 0.05$, ** = $P < 0.01$.

4.2 Objective 2: Investigating the effect of nocodazole cell synchronization on polar body extrusion rates and on bovine ECT

4.2.1 Polar body extrusion

After the outgrowth efficiency was optimised by testing the different hatching techniques, this optimised procedure was used to generate embryo-derived donors for ECT. Embryo-derived donors used in ECT throughout this thesis were developed from blastocysts that were mechanically hatched or naturally hatched. Embryo-derived donor cells were synchronized into mitosis with 500 nM of nocodazole for 18-20 hours to promote the compatibility of the donors with cytoplasts in MII. Nocodazole incubation was expected to increase the proportion of bovine embryo-derived donors arrested in mitosis to approximately 49% up from 6% (Appleby et al., 2022). Putative mitotic donors were selected, based on their relatively larger size, and fused with MII cytoplasts.

To analyse the effect of nocodazole and CHX treatment on embryo development, ECT with nocodazole-synchronized donors was performed (*n*=3). As a control reconstructs were formed with non-synchronized embryonic donors and then were incubated in DMAP after activation. Non-treated donors were likely a mix of cells at different cycle stages. Embryonic donors were pronase-treated, disaggregated, attached to cytoplasts, and fused. Successful fusion was recorded an hour after exposure to DC pulses and was quantified out of couplets stuck, excluding couplets where lysis had occurred in the cytoplast or donor. Fusion between MII cytoplasts and embryonic donors was the same regardless of whether the donor was treated with nocodazole (*N*=126; 82 ± 2%), or not (*N*=121; 82 ± 4%, *P*=1). (Figure 4.2.1).

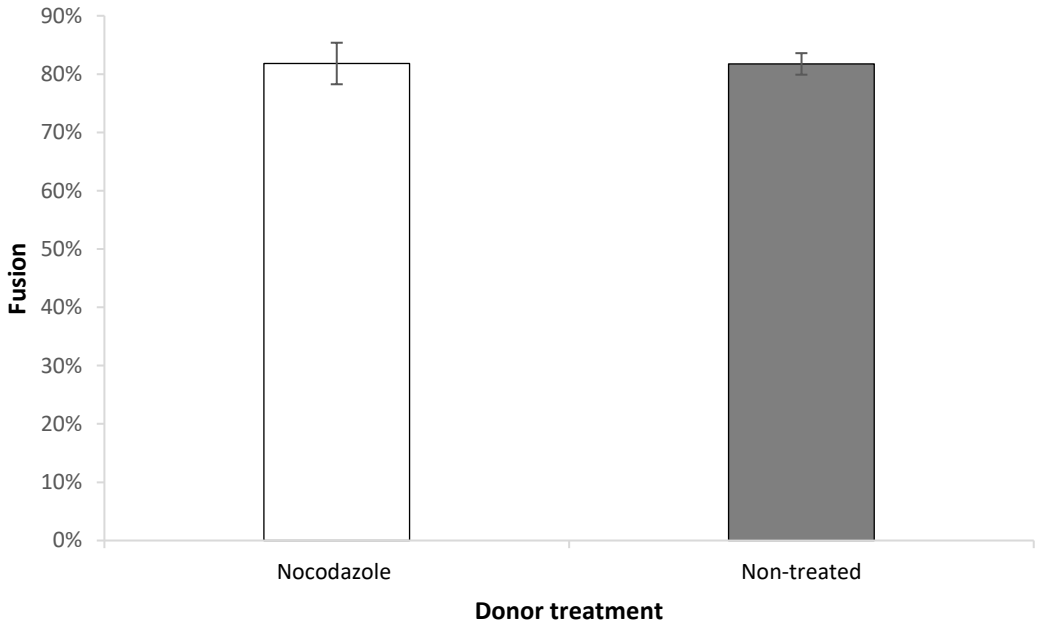


Figure 4.2.1 Fusion of CT couplets with embryonic donors that were treated with 500 nM nocodazole or non-treated. Fusion was calculated out of number of couplets that successfully fused out of couplets stuck excluding couplets where lysis in the cytoplast or donor occurred. Error bars=SEM. Significance * = *P*<0.05, ** = *P*<0.01. Total number of couplets; nocodazole-treated *N*=121, non-treated *N*=126. Replicates *n*=3.

Reconstructs were activated with ionomycin/CHX to allow pseudo-polar body extrusion for the return to a diploid state (*N*=94, *n*=3). Four hours after incubation in CHX, embryos were separated into those that formed pseudo-polar bodies (PPB) or no pseudo-polar bodies (NPB) in between the ZP and plasma membrane (Figure 4.2.2). The proportion of reconstructs that did not form pseudo-polar bodies (78 ± 5%) was

significantly higher than the amount of reconstructs that did form pseudo-polar bodies (22 ± 4%, $P=10^{-14}$).

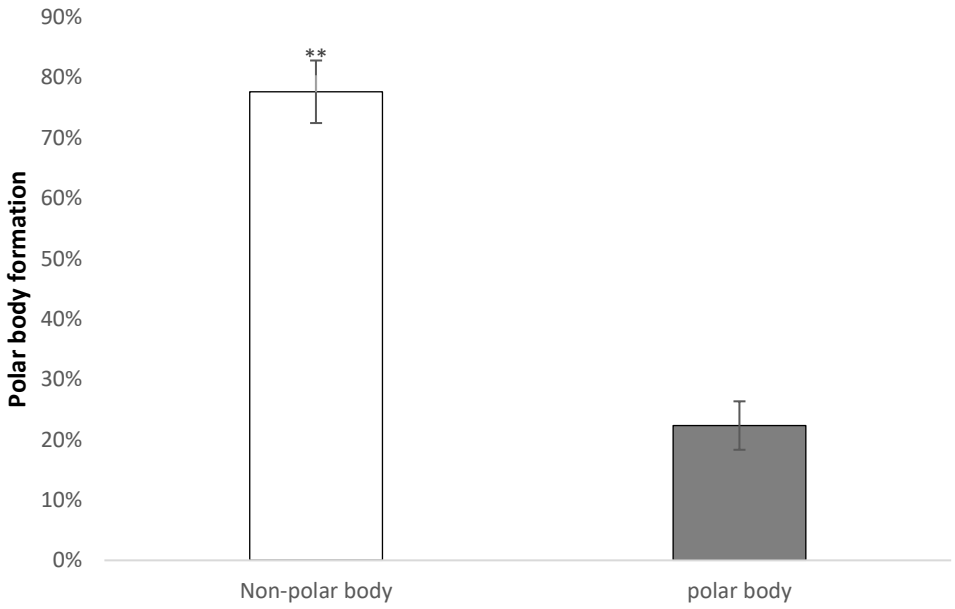


Figure 4.2.2 Polar body formation in CT reconstructs with embryonic donors treated with 500 nM nocodazole and activated with CHX. Four hours after CHX activation, embryos were separated into those that formed pseudo-polar bodies or no pseudo-polar bodies. * = $P<0.05$, ** = $P<0.01$. Total number of reconstructs treated with nocodazole $N=94$. Replicates $n=3$

.

4.2.2 Development of ECT embryos with synchronized and non-synchronized donors

Reconstructs with non-synchronized donors were incubated in DMAP, which inhibited pseudo-polar body formation. Reconstructs with nocodazole-treated donors that formed PPBs ($N=21$), reconstructs with nocodazole-treated donors that had NPPB extrusion ($N=73$) and non-treated reconstructs ($N=97$) were kept in ESOF culture for 5 days. On day 5, lysis in the embryos reconstructed with the different donor treatments was recorded (figure 4.2.3). Lysis on day 5 occurred in the non-treated group (11 ± 6%) and the nocodazole-treated PPB group (5 ± 4%) but not in the nocodazole-treated NPPB group (0 ± 0%). The difference in lysis between the embryos with non-treated donors and embryos with nocodazole-treated donors is only significant when those embryos did not have polar bodies ($P=0.0032$). The difference in lysis between the nocodazole-treated NPPB and PPB embryos was not significant.

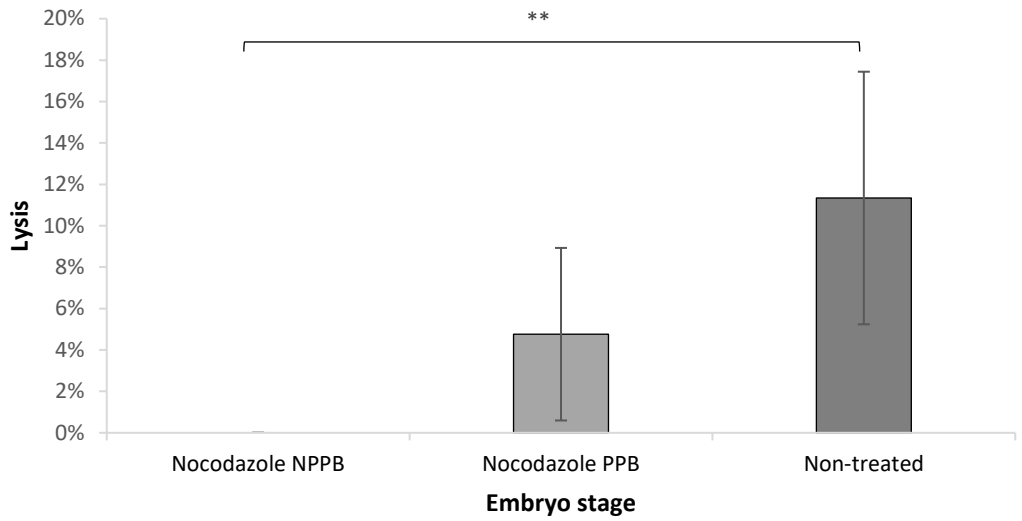


Figure 4.2.3 Lysis of ECT embryos with donors treated or not treated with 500nM nocodazole. After CHX activation, treated embryos were separated into those that formed PPB or NPPB. After DMAP activation, non-treated embryos were NPPB. Lysis was calculated on day 5 out of number of reconstructs that went into IVC. Error bars = SEM. Significance * = $P<0.05$, ** = $P<0.01$. Total number of reconstructs into IVC; nocodazole NPPB $N=73$, nocodazole PPB $N=21$, non-treated $N=97$. Replicates $n=3$.

There was no significant difference in day 5 cleavage or day 7 blastocyst development between embryos with non-treated donors and embryos with nocodazole-treated donors with or without pseudo-polar body formation (P>0.05) (figure 4.2.4). Cleavage was the same after nocodazole treatment with NPPB (68 ± 3%), nocodazole treatment with PPB (67 ± 14) and no treatment (67 ± 4). Total blastocyst development was highest after no treatment (13 ± 3%), and then nocodazole treatment with NPPB (7 ± 1%). No blastocysts developed after nocodazole treatment with PPB (0 ± 0). High grade blastocysts developed from the no treatment group (4 ± 3%), and the NPPB nocodazole treatment group (1 ± 2%).

Data from three repeat experiments was used for the final figures and calculations. A 4th repeat was completed, which showed that it was possible to produce blastocysts from the nocodazole PPB group. However, this run was not included due to an issue with culture medium evaporation that specifically affected the DMAP group. One grade 3 blastocyst (25%) was produced from the nocodazole PPB group in the 4th repeat which was not significantly different from B1-3 development in the NPPB

group (6%). No embryos from the PPB or NPPB treated group developed into grade 1-2 blastocysts in the 4th repeat.

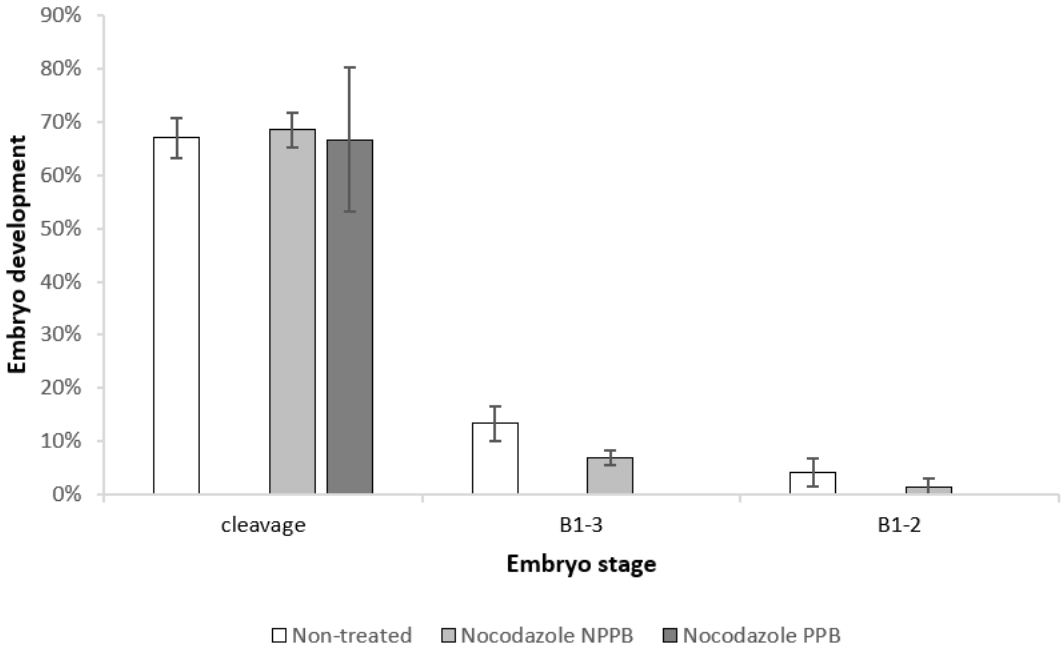


Figure 4.2.4 Development of ECT embryos with donors treated or not treated with 500nM nocodazole. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Development in blastocysts grade 1-3 or grade 1-2 were calculated on day 7 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$. Total number of reconstructs into IVC; nocodazole NPPB $N=73$, nocodazole PPB $N=21$, non-treated $N=97$. Replicates $n=3$.

4.3 Objective 3: Optimise cytoplast treatment for bovine ECT

4.3.1 Cytoplast age

To create a cytoplast environment low in MPF for compatibility with donor cells in S-phase, cytoplast aging and cooling was trialled ($n=3$). After enucleation, cytoplasts were split into an aged and non-aged group. The non-aged group was attached and fused to embryonic donors sequential to enucleation ($N=121$). The remaining cytoplasts were aged in embryo hold medium in a 15°C water bath overnight (9.5 hours), prior to being attached and fused to embryo-derived donors ($N=110$). Successful fusion was recorded an hour after exposure to DC pulses and was quantified

out of couplets stuck, excluding couplets where lysis had occurred in the cytoplast or donor. There was no significant difference in fusion between aged cytoplasts (83 ± 3%) and non-aged cytoplasts (91 ± 4%, *P*>0.05) (figure 4.3.1).

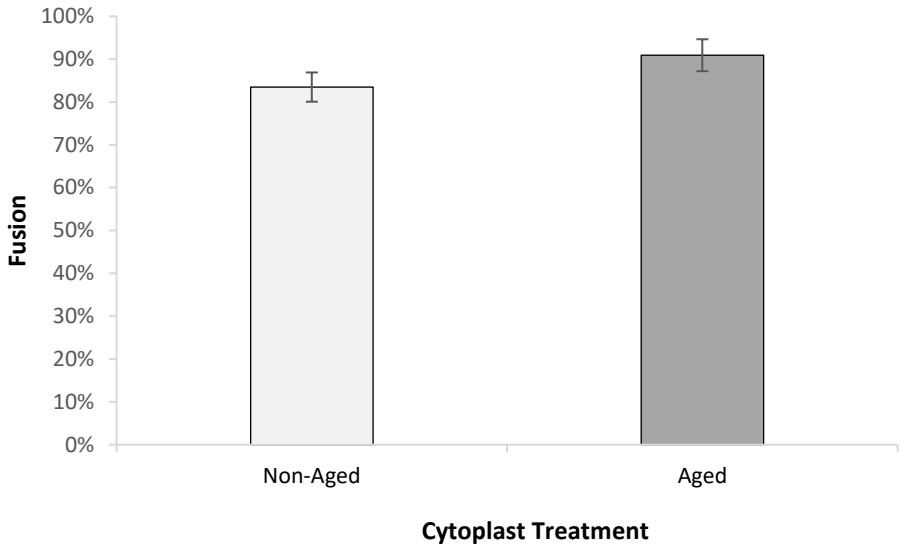


Figure 4.3.1 Fusion of ECT couplets after non-aged and aged treatment. Successful fusion was recorded an hour after exposure to DC pulses and was out of couplets stuck excluding couplets where lysis occurred in the cytoplast or donor. Error bars=SEM. Significance * = *P*<0.05, ** = *P*<0.01 Total number of couplets; Non-aged cytoplasts *N*=121, Aged cytoplasts *N*=110. Replicates *n*=3.

Three hours after fusion, reconstructs underwent ionomycin/DMAP activation before being transferred to IVC. Lysis on day 5 was significantly higher after aged treatment (*N*=85; 28 ± 6%) compared to non-aged treatment (*N*=88; 8 ± 3%, *P*=0.00085) (Figure 4.3.2). A trend was seen of higher development in embryos constructed with non-aged cytoplasts compared to embryos constructed with aged cytoplasts (Figure 4.3.3). There was higher cleavage (66 ± 9% and 61 ± 6%, respectively, *P*>0.05), significantly higher total blastocyst development (15 ± 4% and 4 ± 3%, respectively. *P*=0.019) and higher B1-2 quality blastocyst development (2 ± 2% and 0 ± 0%, respectively, *P*>0.05) after non-aged treatment compared to aged treatment. No high-quality blastocysts developed from embryos constructed with aged cytoplasts while two developed from embryos constructed with non-aged cytoplasts. After the experiments with aged cytoplasts, the remaining CT experiments were completed with non-aged cytoplasts.

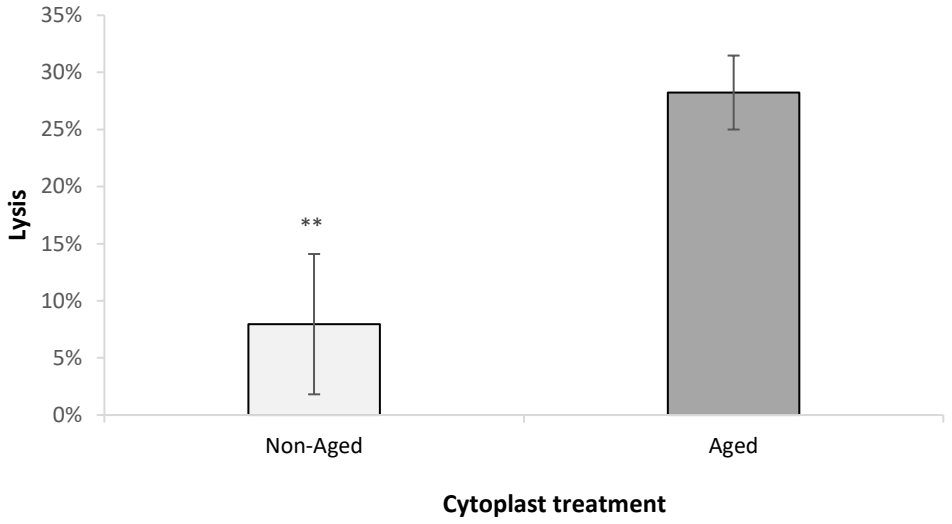


Figure 4.3.2 Day 5 lysis of ECT reconstructs after non-aged and aged treatment of the cytoplasts. Lysis was calculated on day 5 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of couplets; Non-aged cytoplasts N=88, Aged cytoplasts N=85. Replicates n=3.

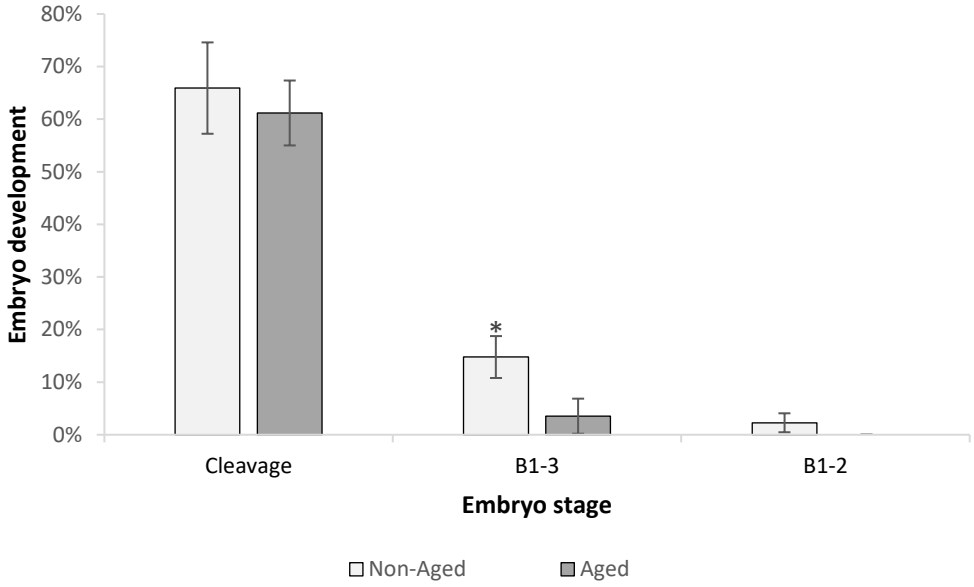


Figure 4.3.3 Development of ECT reconstructs after non-aged and aged treatment of the cytoplasts. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Grade 1-3 and grade 1-2 blastocyst development was calculated on day 7 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of couplets; Non-aged cytoplasts N=88, Aged cytoplasts N=85. Replicates n=3.

4.3.2 Cytoplast volume

Double cytoplasts were trialled to examine the effect of increasing cytoplasmic volume on the ability to reprogram embryonic donors and the ability to support development (*n*=5). Approximately one hour after the first fusion round (*N*=405), successfully fused cytoplasts were divided into two groups: single cytoplasts and double cytoplasts. The double cytoplast group had a second cytoplast attached to the fused reconstruct and then underwent a second round of fusion (*N*=119). Successful fusion was recorded for the double cytoplast group approximately one hour after the second round of fusion. Fusion was significantly higher between a reconstruct and cytoplast (double cytoplasts) than a donor and cytoplast (single cytoplasts) (100 ± 0% and 75 ± 5%, respectively. $P=10^{-13}$) (Figure 4.3.4).

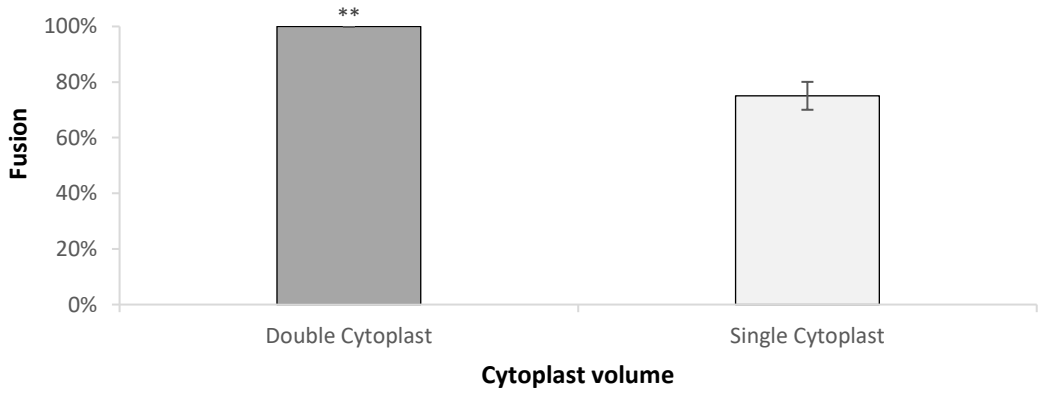


Figure 4.3.4 Fusion of ECT couplets with double and single cytoplasts. Successful fusion was recorded an hour after exposure to DC pulses and was out of couplets stuck excluding couplets where lysis in the cytoplast or donor occurred. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of couplets; Single cytoplasts *N*=405, Double cytoplasts *N*=119. Replicates *n*=5.

DMAP/ionomycin activation was completed three hours after the point in-between fusion of the single cytoplasts and fusion of the double cytoplasts. Reconstructs made from single cytoplasts (*N*=147) and double cytoplasts (*N*=110) were kept in *in vitro* culture (*n*=5). There was no significant difference in lysis of embryos on day 5 between the single cytoplast group (13 ± 8%) and the double cytoplast (10 ± 14%. $P>0.05$) (figure 4.3.5).

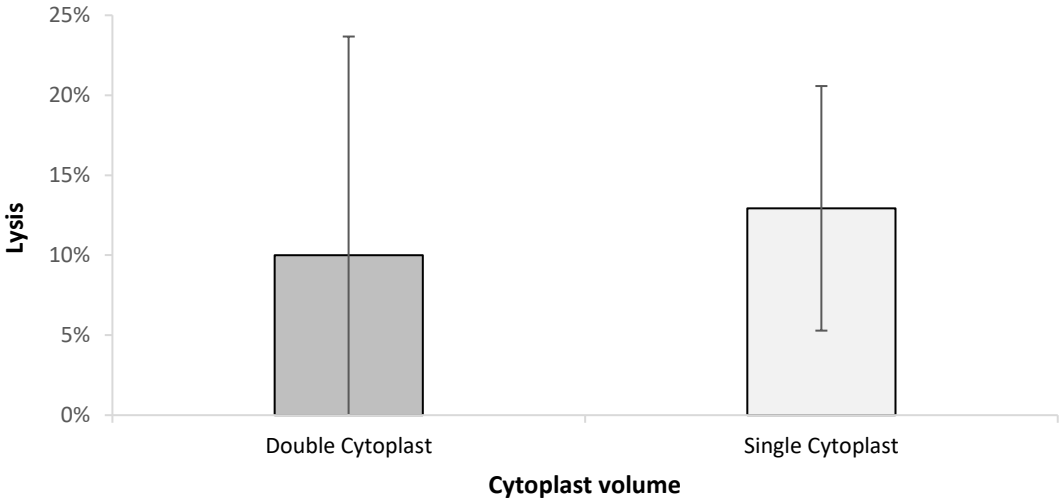


Figure 4.3.5 Day 5 lysis of ECT reconstructs with double and single cytoplasts. Lysis was calculated on day 5 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$. Total number of day 5 reconstructs; Double cytoplasts $N=110$, single cytoplasts $N=147$. Replicates $n=5$.

Although cleavage on day 5 was significantly higher in double cytoplasts than in single cytoplasts (87 ± 7% and 67 ± 9%, respectively. $P=0.00018$), there were no differences in development on day 7 (Figure 4.3.6). While day 5 data included five replicates, the day 7 data had a replicate excluded due to precipitate in the LSOF lysing the embryos ($n=4$, double cytoplasts $N=87$, single cytoplasts $N=111$). On day 7, there was no significant difference in higher total blastocyst development (11 ± 4% and 6 ± 2%, respectively. $P>0.05$) and or high-quality blastocysts development (6 ± 3% and 5 ± 1%, respectively. $P>0.05$) between the single cytoplast group and the double cytoplast group. As double cytoplasts offered no advantage, the remaining experiments were completed with single cytoplasts.

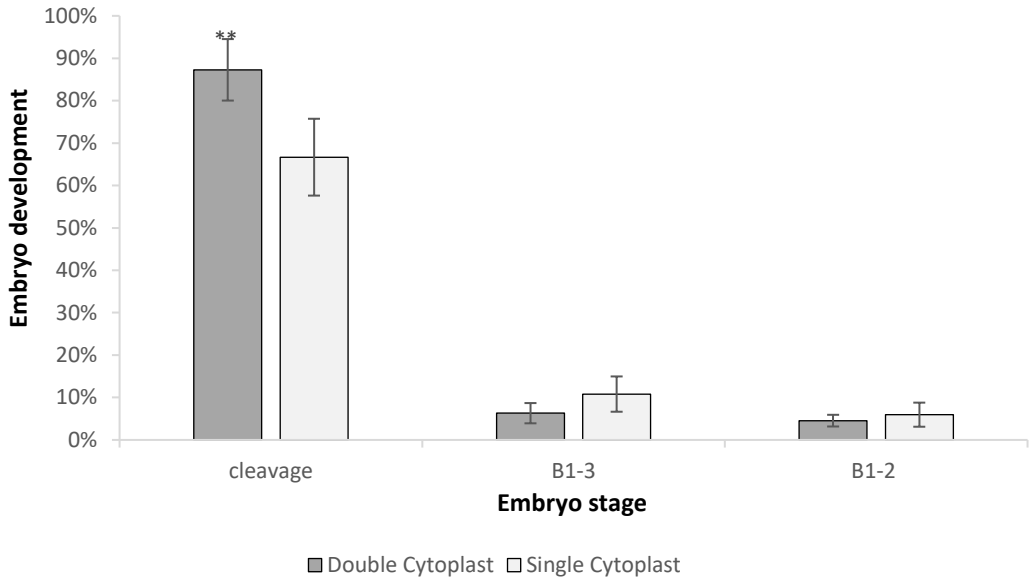


Figure 4.3.6 Development of ECT reconstructs with double and single cytoplasts. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Total number of day 5 reconstructs; Double cytoplasts *N*=110, single cytoplasts *N*=147. Replicates *n*=5. Grade 1-3 and grade 1-2 development in blastocysts was calculated on day 7 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$. Total number of day 7 reconstructs; Double cytoplasts *N*=87, single cytoplasts *N*=111. Replicates *n*=4.

4.4 Objective 4: Potential of vitrified bovine oocytes and cytoplasts for use in CT

4.4.1 Survival after thawing

To analyse whether the vitrification protocol compromised the physical integrity of oocytes or cytoplasts, survival after thawing was tested (*n*=8). Oocytes were vitrified with the ZP intact (ZI) (*N*=208) or ZP-free (ZF) (*N*=231). The ZP in the ZI group remained intact during the thawing process and during PG development. Cytoplasts had the ZP removed for enucleation, so all cytoplasts were vitrified in a ZF state. After thawing, oocytes and cytoplasts were kept in H199 + 10% FBS for two hours and then survival out of the number thawed was recorded (Figure 4.4.1).

The rate of survival of cytoplasts was dependant on the enucleation operator and their level of experience. The less experienced operator produced cytoplasts (*N*=38, *n*=2) with a strong significant decrease in survival compared to the more experienced operator (*N*=307, *n*=8), (45 ± 16%, and 92 ± 3% respectively, $P=10^{-11}$). Due to this difference, only the cytoplasts enucleated by the experienced operator were included in the final survival data displayed. Only the cytoplasts enucleated by the experienced operator were used for CT experiments.

Survival was highest in the ZI oocyte group (99 ± 1%), then the ZF oocyte group (96 ± 2%), and then lowest in the cytoplast group (92 ± 3%). ZI oocytes had significantly increased survival over cytoplasts (*P* =0.00022). The differences between ZF oocytes and the other two groups was insignificant. In an overall comparison between cytoplasts and oocytes regardless of their zona state (*N*=439, *n*=8), the oocytes had significantly higher survival than the cytoplasts (97 ± 2%, *P*=0.00084.). Oocytes that survived were used in artificial activation experiments to produce PGs and cytoplasts that survived were used in vitrified CT experiments.

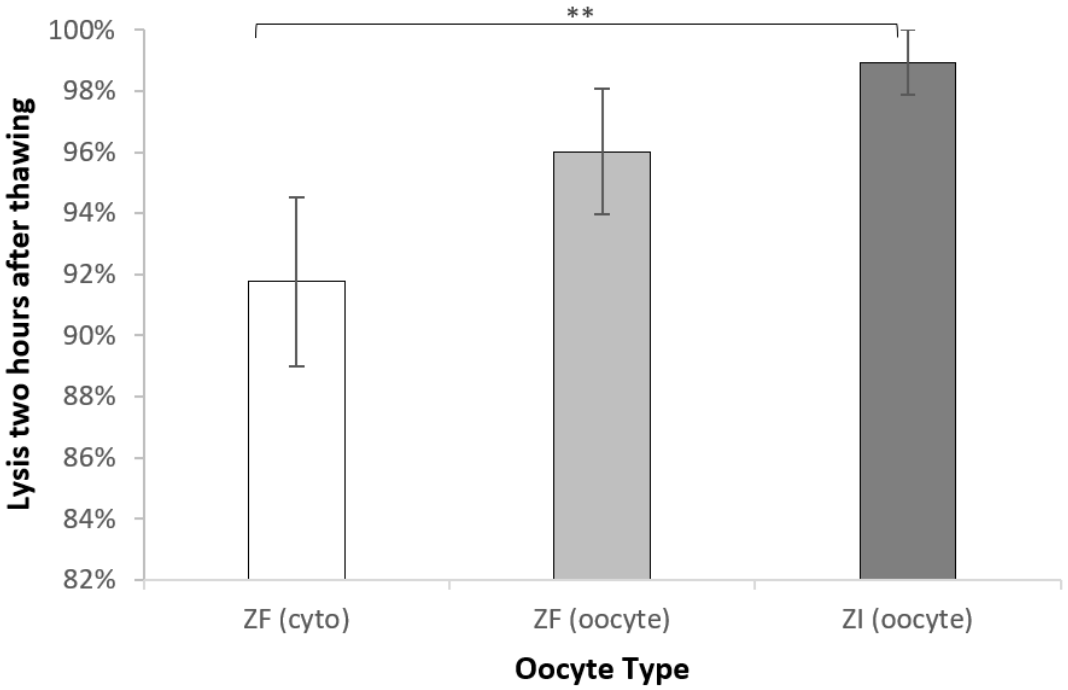


Figure 4.4.1 Survival of oocytes and cytoplasts two hours after thawing. Survival was calculated out of the number thawed. ZF=ZP-free. ZI=ZP-intact. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number thawed: cytoplasts N=307, ZF oocytes N=231, ZI oocytes N=208. Replicates n=8.

4.4.2 Artificial activation of thawed oocytes

To analyse whether the vitrification protocol compromised the developmental factors in oocytes, PG development was tested (n=3). Vitrified ZI oocytes (N=78) and vitrified ZF oocytes (N=82) were thawed. The survival rates two hours after thawing fell into the ranges described in Section 4.4.1 with no difference between ZI (95 ± 2%) and ZF (100 ± 0%, $P<0.05$). Lysed oocytes were removed from the experiment at this stage and not included in day 5 lysis calculations or IVC sample size. Vitrified oocytes (ZI N=70, ZF N=75) and fresh oocytes (ZI N=101, ZF N=68) underwent ionomycin activation and incubation in DMAP for four hours. The oocytes were then placed in ESOF for five days before lysis was recorded (Figure 4.4.2). The vitrified groups had higher lysis than each fresh group. Lysis was significantly higher in vitrified ZF oocytes (33 ± 9%) than in fresh ZF oocytes (6 ± 6%, P=0.0044) and in fresh ZI oocytes (11 ± 2%, P=0.035). Lysis was significantly higher in vitrified ZI oocytes (26 ± 5%) than in fresh ZF oocytes ($P=10^{-05}$) and in fresh ZI oocytes (P=0.00021). The presence of a ZP did not cause a significant difference in lysis in either the vitrified oocytes or the fresh oocytes.

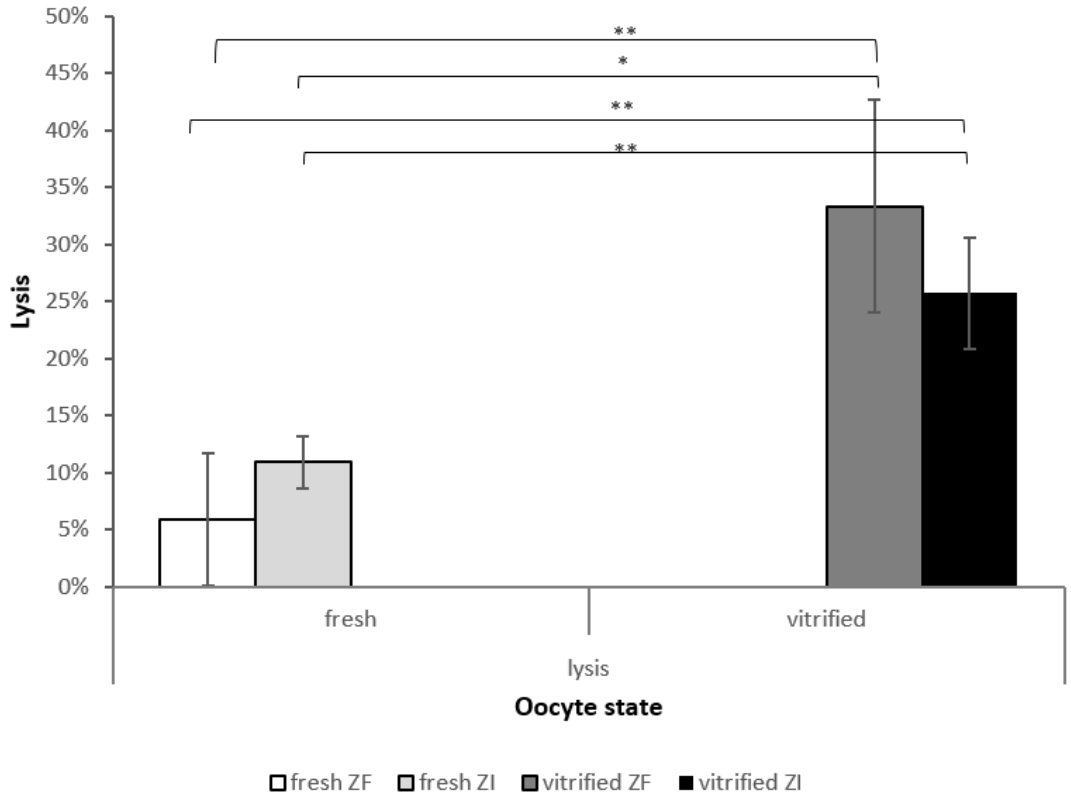


Figure 4.4.2 Lysis in fresh and thawed oocytes with and without ZPs, five days after PG activation. The number of lysed oocytes out of the number of oocytes into IVC was recorded. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of oocytes into IVC: fresh ZF $N=68$, fresh ZI $N=101$, vitrified ZF $N=75$, and vitrified ZI $N=70$. Replicates $n=3$.

The presence of a ZP did not cause a significant difference in cleavage on day 5, or blastocyst development on day 7 in either the vitrified oocytes or the fresh oocytes (Figure 4.4.3). Cleavage and blastocyst development was higher in fresh groups than vitrified groups. Cleavage was higher in fresh ZF (57 ± 19%) and ZI oocytes (61 ± 16%) than in vitrified ZF (48 ± 9%.) and ZI oocytes (49 ± 21%). However, this difference was not statistically significant ($P>0.05$). Total blastocyst development was significantly higher in fresh ZF PGs (22 ± 11%) than in vitrified ZF PGs (3 ± 3%, $P=0.00059$) and vitrified ZI PGs (0 ± 0%, $P=10^{-05}$). Total blastocyst development was significantly higher in fresh ZI PGs (17 ± 8%) than in vitrified ZF PGs ($P=0.0036$) and in vitrified ZI PGs (P=0.00014). High-quality blastocyst development was significantly higher in fresh ZF PGs (10 ± 5%) than in vitrified ZF PGs (1 ± 1%, $P=0.045$) and in vitrified ZI PGs (0 ± 0%, $P=0.012$). High-quality blastocyst development was not significantly higher in fresh ZI PGs (5 ± 3%) than in vitrified ZF and ZI PGs.

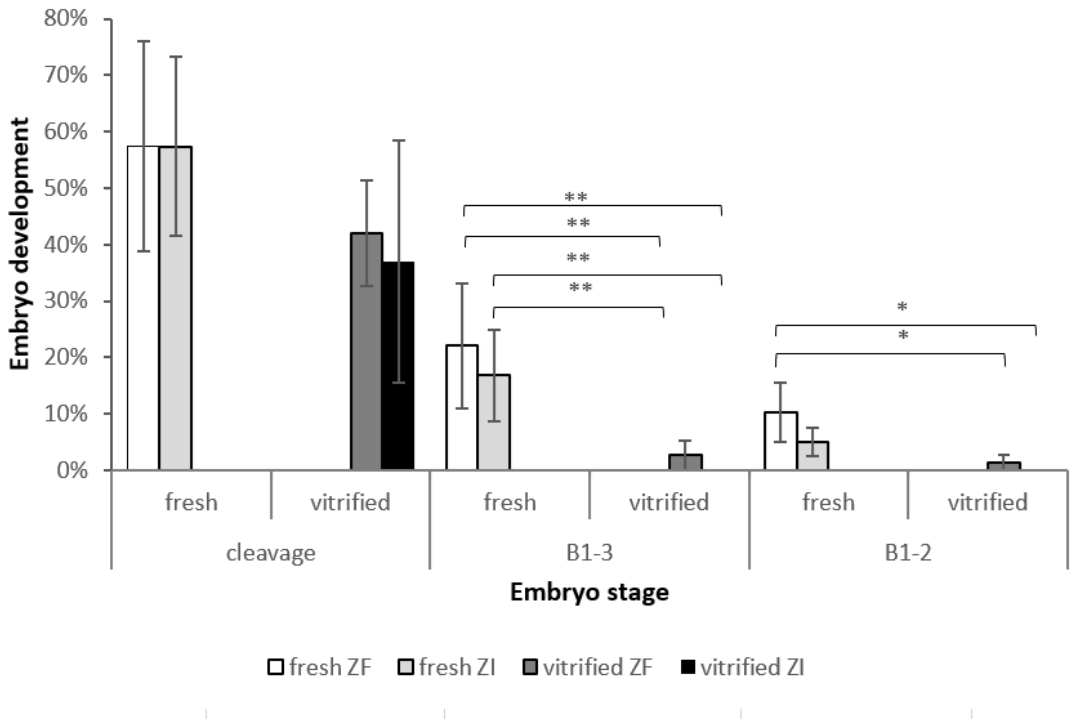


Figure 4.4.3 Development in fresh and thawed oocytes with and without ZPs after artificial activation. 5 days after activation cleaved embryos out of number of oocytes into IVC was recorded. 7 days after activation grade 1-3 and grade 1-2 blastocysts out of the number of oocytes into IVC was recorded. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of oocytes into IVC: fresh ZF N= 68, fresh ZI N=101, vitrified ZF N=75, and vitrified ZI N=70. Replicates n=3.

4.4.3 CT with vitrified cytoplasts

4.4.3.1 Development of single thawed cytoplasts in SCT

To analyse whether the vitrification protocol compromised reprogramming factors in cytoplasts, their ability to reprogram somatic donors in CT was tested (n=3). Vitrified ZF oocytes (N=113) were thawed. The survival rate two hours after thawing (88 ± 3%) fell into the cytoplast range described in Section 4.4.1 (92 ± 3%). Lysed oocytes were removed from the experiment at this stage and not included in day 5 lysis calculations. Two hours after thawing, vitrified cytoplasts were attached and fused to serum starved somatic donors (N=85). Fresh cytoplasts were attached and fused to serum starved somatic donors immediately after enucleation (N=165). Fusion of somatic donors with fresh cytoplasts (93 ± 4%) was not significantly different to fusion with vitrified cytoplasts (85 ± 1%, $P>0.05$) (Figure 4.4.4).

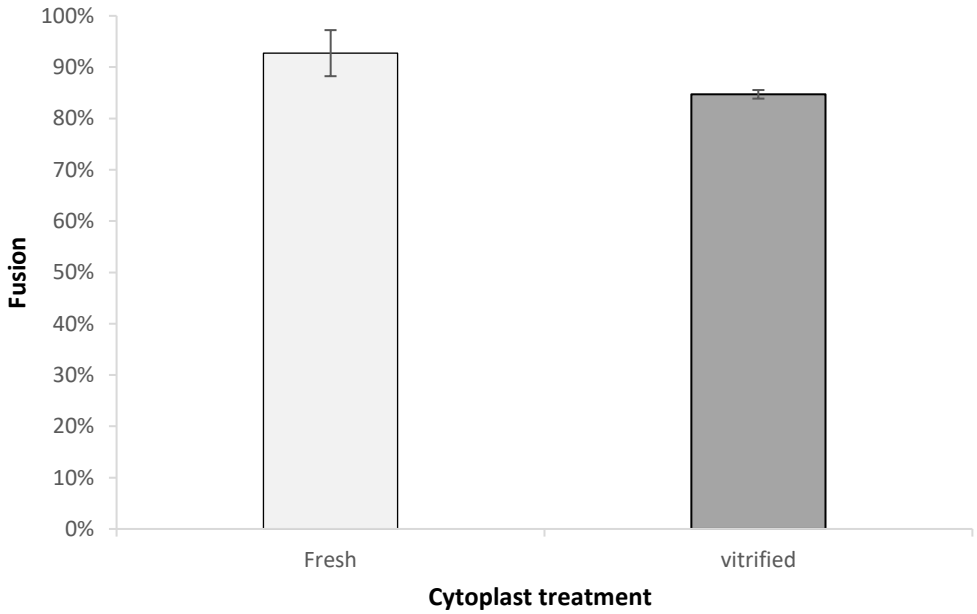


Figure 4.4.4 Fusion of somatic donors with fresh and vitrified/thawed cytoplasts. Successful fusion was recorded an hour after exposure to DC pulses and was out of couplets stuck excluding couplets where lysis occurred in the cytoplast or donor. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of couplets; Fresh cytoplasts $N=165$, vitrified cytoplasts $N=85$. Replicates $n=3$.

Lysis and cleavage were recorded 5 days after SCT out of the number of embryos that went into ESOF that were constructed with the vitrified cytoplasts ($N=65$) or fresh cytoplasts ($N=99$). Embryos produced from vitrified cytoplasts had significantly higher lysis (42 ± 2%) than embryos produced with fresh cytoplasts (15 ± 5%, $P=0.00034$) (Figure 4.4.5).

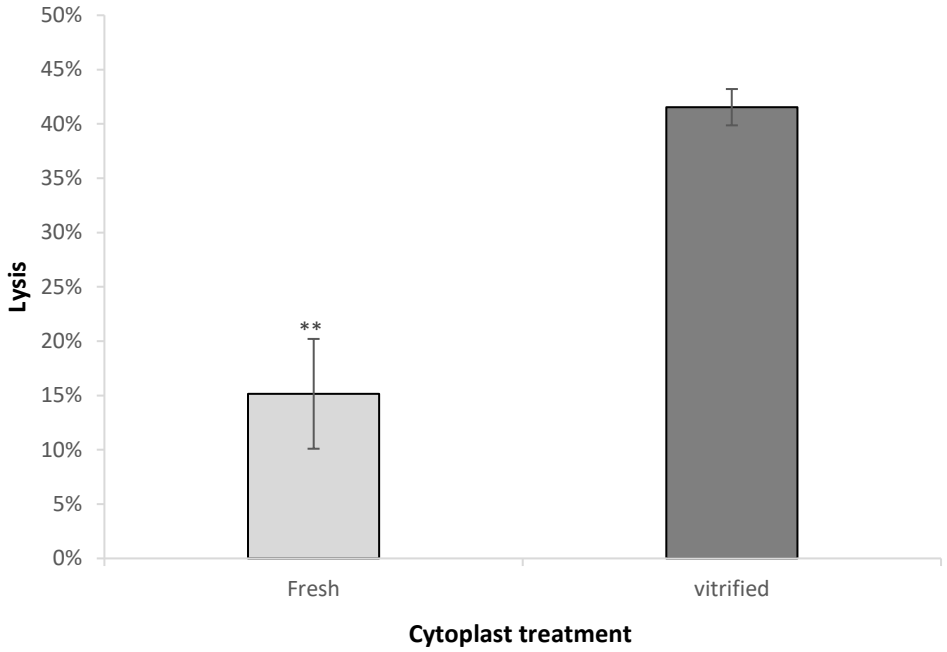


Figure 4.4.5 Day 5 lysis of SCT embryos constructed with fresh or vitrified/thawed cytoplasts and somatic donors. 5 days after SCT the lysed embryos out of the number of reconstructs into IVC was recorded. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of reconstructs into IVC: fresh cytoplasts $N=99$, vitrified cytoplasts $N=65$. Replicates n=3.

Development was higher in embryos produced with fresh cytoplasts than with vitrified cytoplasts (Figure 4.4.6). Day 5 cleavage was significantly higher in embryos produced with fresh cytoplasts (84 ± 10%) than with vitrified cytoplasts (45 ± 5%. $P=10^{-7}$). Total blastocyst development was higher in embryos produced with fresh cytoplasts (8 ± 2%) than with vitrified cytoplasts (2 ± 1%) but this was not statistically significant ($P>0.05$). High-quality blastocyst development was higher in embryos produced with fresh cytoplasts (5 ± 2%) than with vitrified cytoplasts (0 ± 0%) but this was also not statistically significant ($P>0.05$).

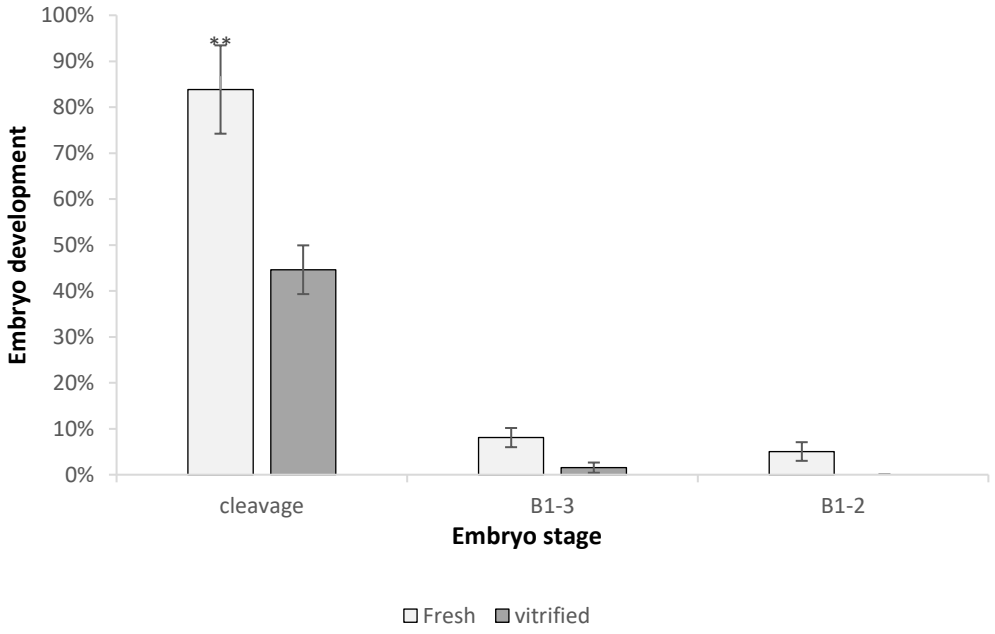


Figure 4.4.6 Development of SCT embryos constructed with fresh or vitrified/thawed cytoplasts and somatic donors. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Grade 1-3 and grade 1-2 development in blastocysts was calculated on day 7 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = P<0.05, ** = *P*<0.01 Total number of reconstructs into IVC: fresh cytoplasts *N*=99, vitrified cytoplasts *N*=65. Replicates *n*=3.

4.4.3.2 Development of single thawed cytoplasts in ECT

Vitrified cytoplasts were used in ECT to analyse whether the vitrification protocol compromised the reprogramming factors required for embryonic donors (*n*=5). Vitrified ZF oocytes (*N*=197) were thawed. The survival rate two hours after thawing (91 ± 3%) fell into the cytoplast range described in Section 4.4.1 (92 ± 3%). Lysed oocytes were removed from the experiment at this stage and not included in day 5 lysis calculations. Two hours after thawing, vitrified cytoplasts were attached and fused to embryonic donors (*N*=176). Fresh cytoplasts were attached and fused to embryonic donors immediately after enucleation (*N*=239). Fusion of embryonic donors with fresh cytoplasts (86 ± 3%) was significantly higher than with vitrified cytoplasts (72 ± 4%, *P*=0.001) (Figure 4.4.7).

The fusion of fresh cytoplasts was not different with an embryonic donor or somatic donor (86 ± 3% and 93 ± 4% respectively. *P*>0.05). This means that donor cell type alone did not influence fusion and any differences in fusion can be attributed to the

vitrification process. The fusion of vitrified cytoplasts with somatic donors (85 ± 1%) in section 4.4.3.1, was significantly higher than with embryo-derived donors (72 ± 4%. *P*=0.0005) in section 4.4.3.2. The vitrification process alone did not influence somatic fusion, but it did affect fusion when using embryonic donors.

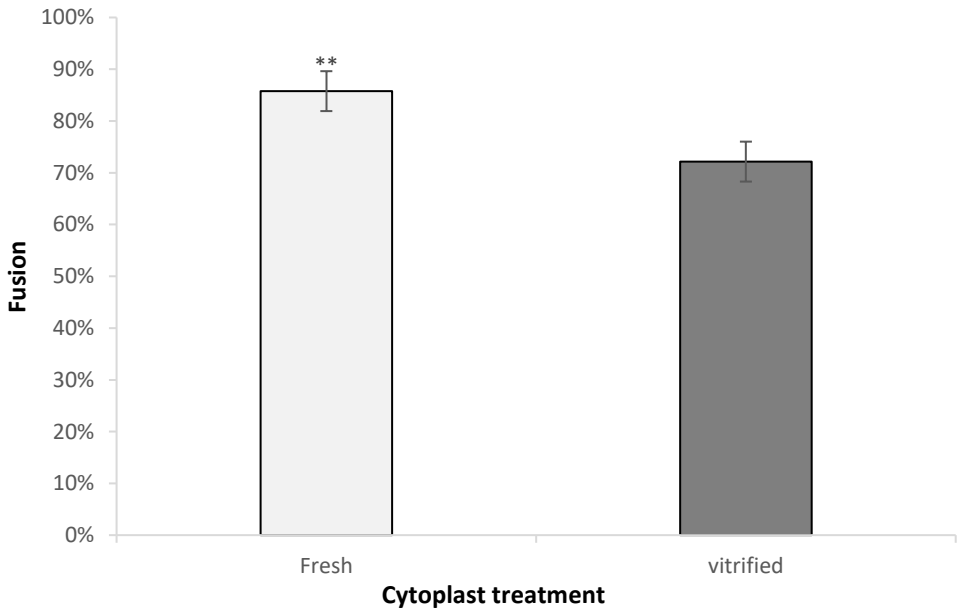


Figure 4.4.7 Fusion of embryonic donors with fresh and vitrified/thawed cytoplasts. Successful fusion was recorded an hour after exposure to DC pulses and was out of couplets stuck excluding couplets where lysis occurred in the cytoplast or donor. Error bars=SEM. Significance * = *P*<0.05, ** = *P*<0.01 Total number of couplets; Fresh cytoplasts *N*=239, vitrified cytoplasts *N*=176. Replicates *n*=5.

The reconstructs were then placed in ESOF for 5 days before lysis and cleavage were recorded (vitrified cytoplasts in IVC *N*=110, fresh cytoplasts in IVC *N*=148). Embryos produced from vitrified cytoplasts had significantly higher lysis (50 ± 10%) than embryos produced with fresh cytoplasts (14 ± 5%, $P=10^{-10}$) (Figure 4.4.8).

There was no significant difference in lysis between CT with somatic (Section 4.4.3.1) or embryonic donors (Section 4.4.3.2) with fresh cytoplasts indicating the donor cell type alone was not a major factor in lysis (15 ± 5% and 14 ± 5% respectively. *P*>0.05). There was no significant difference in lysis between CT with somatic (Section 4.4.3.1) or embryonic donors (Section 4.4.3.2) with vitrified cytoplasts indicating the combination of cell type and vitrification was not the cause for lysis (42 ± 2% and 50 ± 10% respectively. *P*>0.05). In both types of CT the lysis was higher with vitrified cytoplasts indicating it was the vitrification process alone causing the increase in lysis.

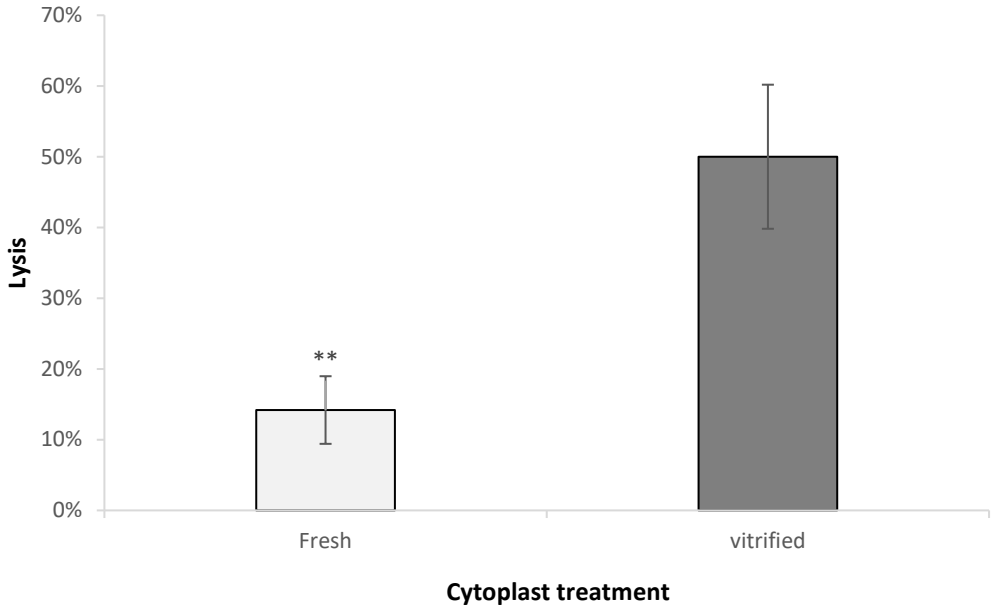


Figure 4.4.8 Day 5 lysis of ECT embryos constructed with fresh and vitrified/thawed cytoplasts. 5 days after activation lysed embryos out of number of reconstructs into IVC was recorded. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of reconstructs into IVC: fresh cytoplasts $N=148$, vitrified cytoplasts $N=110$. Replicates $n=5$.

Blastocysts developed from embryos produced with fresh cytoplasts but no blastocysts were produced from vitrified cytoplasts (Figure 4.4.9). There was no significant difference in day 5 cleavage between embryos produced with fresh cytoplasts (57 ± 11%) and with vitrified cytoplasts (49 ± 10%, $P>0.05$). Total blastocyst development was higher in in embryos produced with fresh cytoplasts (3 ± 5%) than with vitrified cytoplasts (0 ± 0%, $P>0.05$). High-quality blastocyst development was higher in in embryos produced with fresh cytoplasts (2 ± 3%) than with vitrified cytoplasts (0 ± 0%, $P>0.05$). However, the differences in blastocyst development were not found to be statistically significant.

There was no significant difference in cleavage or blastocyst development between CT with somatic (Section 4.4.3.1) or embryonic donors (Section 4.4.3.2) with fresh cytoplasts indicating the cell type alone was not a major factor in development. There was no significant difference in cleavage or blastocyst development between CT with somatic (Section 4.4.3.1) or embryonic donors (Section 4.4.3.2) with vitrified cytoplasts indicating the combination of cell type and vitrification did not affect development. Any difference in development between cytoplast types was caused by the vitrification process alone regardless of cell type.

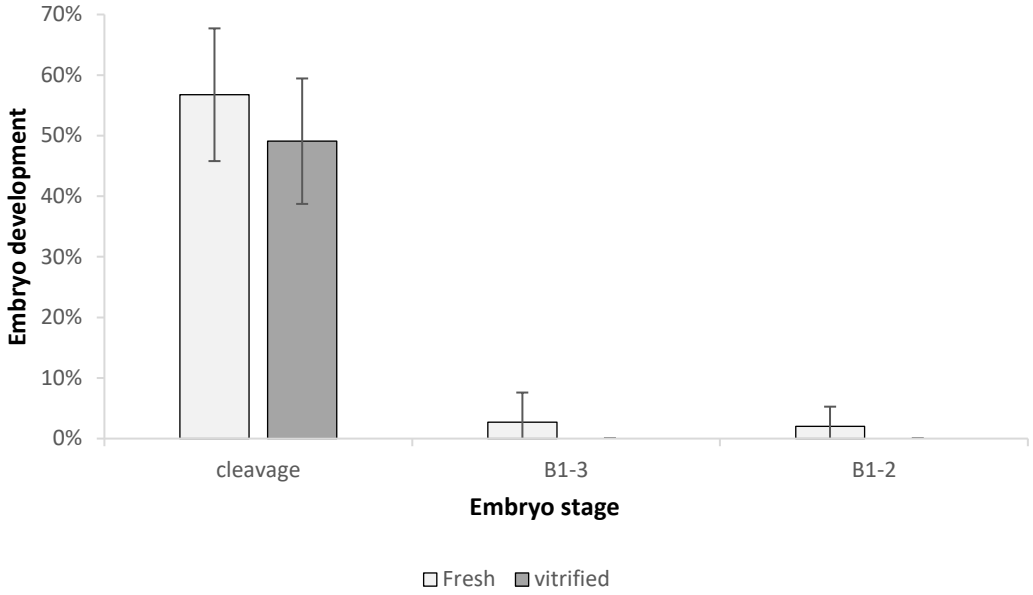


Figure 4.4.9 Development of ECT embryos constructed with fresh and thawed cytoplasts. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Grade 1-3 and grade 1-2 development in blastocysts was calculated on day 7 out of number of reconstructs that went into IVC. Error bars = SEM. Significance * = $P<0.05$, ** = $P<0.01$. Total number of reconstructs into IVC: fresh cytoplasts $N=148$, vitrified cytoplasts $N=110$. Replicates $n=5$.

4.4.3.3 Development of double thawed cytoplasts in CT with somatic donors

It is known that using fresh-fresh double cytoplasts in SCT improves development (Delaney et al., 2007; Green et al., 2016). The production of a blastocyst in 4.4.3.1 showed that vitrified cytoplasts contain sufficient intact factors to support reprogramming and development in SCT. So, it is plausible that sufficient cytoplasmic factors endure after vitrification to aid development when used in conjunction with a fresh cytoplast in SCT. To test this hypothesis a preliminary trial with fresh-vitrified double cytoplasts SCT was trialled ($n=1$). Reconstructs were produced with a fresh cytoplast and serum starved somatic donor. After successful fusion, reconstructs were fused to a second fresh cytoplasts ($N=13$) or to a vitrified/thawed cytoplast ($N=13$). There was no difference in the fusion of fresh-fresh double reconstructs (100%) and the fusion of fresh-vitrified double reconstructs (100%, $P=1$) (Figure 4.4.10).

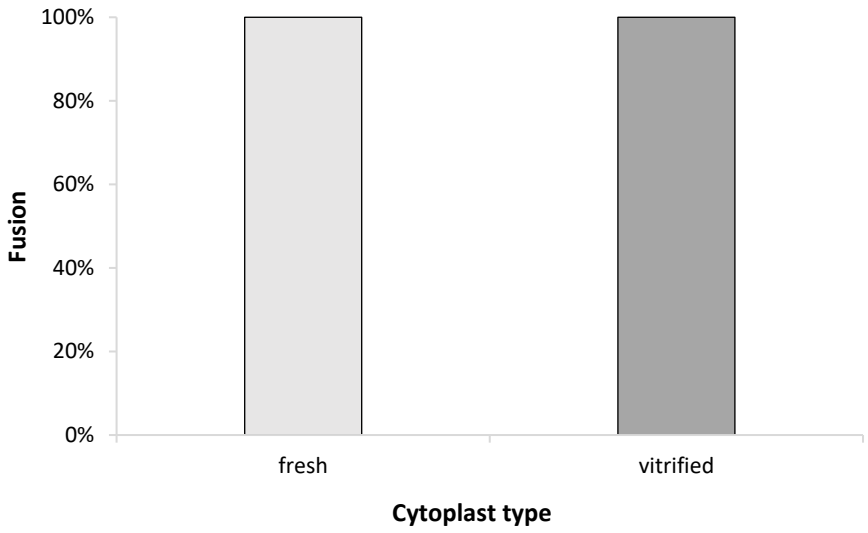


Figure 4.4.10 Fusion of somatic reconstructs with a second fresh or vitrified cytoplast. Successful fusion was recorded an hour after exposure to DC pulses and was out of couplets stuck excluding couplets where lysis occurred in the cytoplast or donor. Fresh refers to fresh-fresh couplets. Vitrified refers to fresh-vitrified couplets. Significance * = $P<0.05$, ** = $P<0.01$ Total number of couplets; Fresh-fresh cytoplasts $N=13$, fresh-vitrified cytoplasts $N=13$. Replicates $n=1$.

The reconstructs were then placed in ESOF for 5 days before lysis and cleavage were recorded. There was no difference in lysis between embryos produced from fresh-fresh cytoplasts ($N=9$; 11%) and embryos produced with fresh-vitrified cytoplasts ($N=10$; 10%. $P=1$) (Figure 4.4.11).

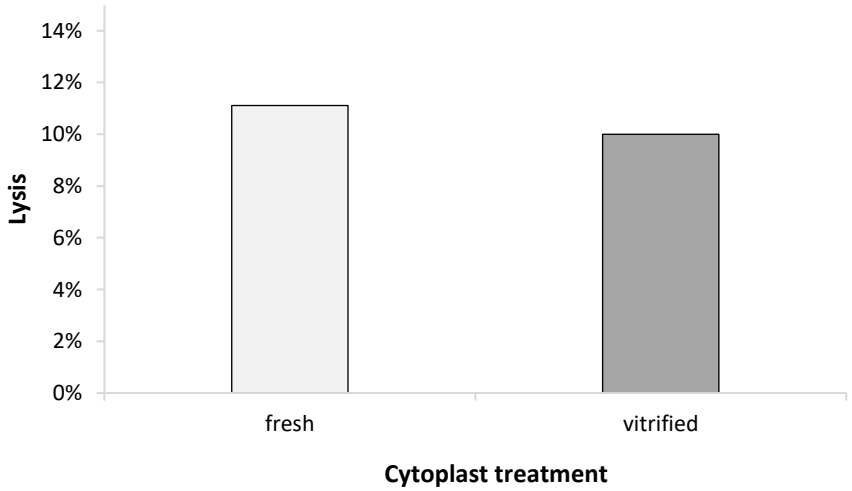


Figure 4.4.11 Day 5 lysis of somatic double CT embryos constructed with fresh-fresh or fresh-vitrified cytoplasts. 5 days after activation lysed embryos out of number of reconstructs into IVC was recorded. Fresh refers to fresh-fresh reconstructs. Vitrified refers to fresh-

vitrified reconstructs. Significance * = $P<0.05$, ** = $P<0.01$ Total number of reconstructs into IVC: fresh-fresh cytoplasts $N=9$, fresh-vitrified cytoplasts $N=10$. Replicates $n=1$.

Blastocysts developed from embryos produced with fresh-fresh cytoplasts, but no blastocysts were produced from fresh-vitrified cytoplasts (Figure 4.4.12). There was no significant difference in day 5 cleavage between embryos produced with fresh-fresh cytoplasts (89%) and with fresh-vitrified cytoplasts (90%, $P=1$). Total blastocyst development was higher in in embryos produced with fresh-fresh cytoplasts (11%) than with fresh-vitrified cytoplasts (0%, $P>0.05$). No high-quality blastocyst developed from embryos produced with fresh-fresh cytoplasts (0) or with fresh-vitrified cytoplasts (0%, $P=1$). However, the differences in blastocyst development were not found to be statistically significant due to small samples sizes and a low number of repeats.

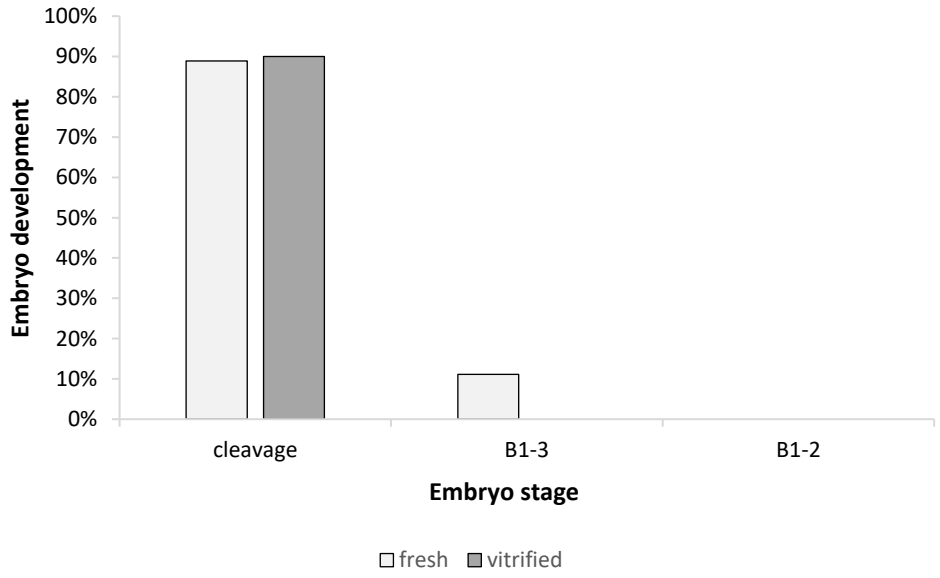


Figure 4.4.12 Development of somatic double CT embryos constructed with fresh-fresh or fresh-vitrified cytoplasts Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Grade 1-3 and grade 1-2 development in blastocysts was calculated on day 7 out of number of reconstructs that went into IVC. Fresh refers to fresh-fresh couplets. Vitrified refers to fresh-vitrified couplets. Significance * = $P<0.05$, ** = $P<0.01$. Total number of reconstructs into IVC: fresh-fresh cytoplasts $N=9$, vitrified-fresh cytoplasts $N=10$. Replicates $n=1$.

.

4.4.4 Summary of development of vitrified cytoplasts and oocytes

The effect of vitrification alone on development was evaluated in Section 4.4.2 and Section 4.4.3. Next, whether the interaction of vitrification and enucleation had any effect on development was analysed. The CT data with vitrified cytoplasts with somatic donors and embryo-derived donors (Section 4.4.3.1 and Section 4.4.3.2) was pooled (*n*=8). The pooled vitrified data was compared data on the artificial activation of vitrified ZF oocytes (Section 4.4.2) (*n*=3).

The survival rate two hours after thawing was not different between vitrified ZF oocytes used for artificial activation (*N*=82; 95 ± 2%) in Section 4.6.2 and vitrified cytoplasts used for CT in Section 4.4.3.1 and 4.4.3.2 (*N*=310; 90 ± 2%). Lysed oocytes were removed from the experiment at this stage and not included in day 5 lysis calculations.

Lysis on day 5 was not different between vitrified ZF oocytes used for artificial activation (*N*=75; 33 ± 9%) in Section 4.4.2 and vitrified cytoplasts used for CT in Section 4.4.3.1 and 4.4.3.2 (*N*=175; 47 ± 7%) (figure 4.4.13).

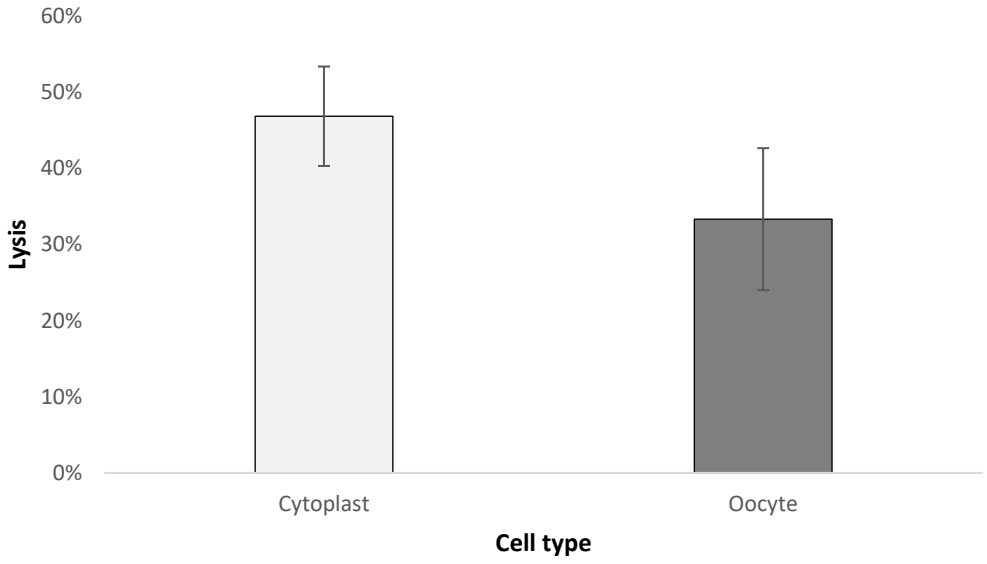


Figure 4.4.13 Day 5 lysis of CT embryos constructed with thawed cytoplasts and artificially activated embryos constructed with ZF thawed oocytes. 5 days after CT the lysed embryos out of the number of reconstructs into IVC was recorded. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of reconstructs into IVC: vitrified

cytoplasts *N*=175, vitrified ZF oocytes *N*=75. Replicates for CT with vitrified cytoplasts *n*=8, replicates for artificial activation with vitrified ZF oocytes *n*=3.

Cleavage on day 5 was not different between vitrified ZF oocytes used for artificial activation (48 ± 9%) in Section 4.4.2 and vitrified cytoplasts used for CT in Section 4.4.3.1 and 4.4.3.2 (47 ± 6%) (figure 4.4.14). Total blastocyst development on day 7 was not different between vitrified ZF oocytes used for artificial activation (3 ± 3%) in Section 4.6.2 and vitrified cytoplasts used for CT in Section 4.6.4.1 and 4.6.4.2 (1 ± .5%). High-quality blastocyst development on day 7 was not different between vitrified ZF oocytes used for artificial activation (1 ± 1%) in Section 4.4.2 and vitrified cytoplasts used for CT in Section 4.6.4.1 and 4.6.4.2 (0 ± 0%).

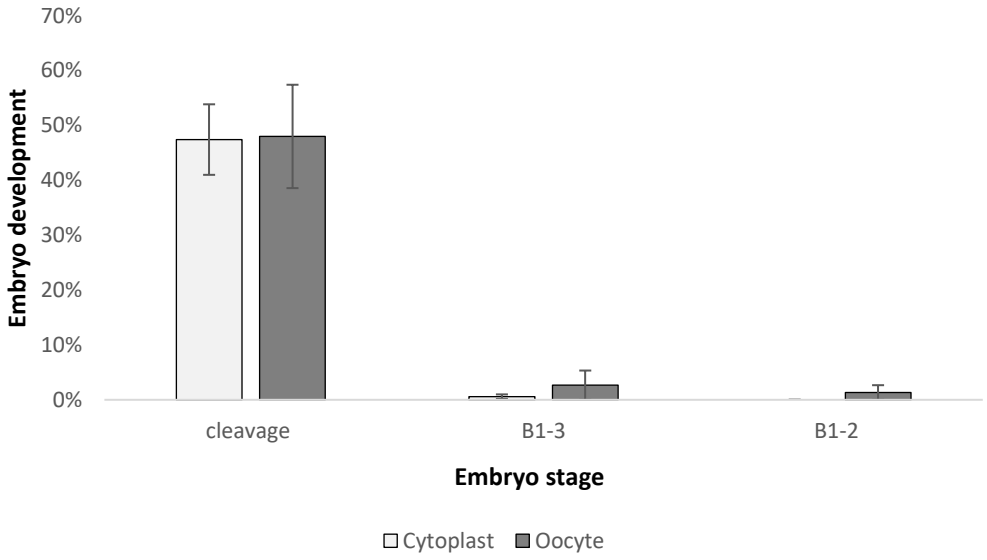


Figure 4.4.14 Development of CT embryos constructed with thawed cytoplasts and artificially activated embryos constructed with ZF thawed oocytes. Cleavage was calculated on day 5 out of number of reconstructs that went into IVC. Grade 1-3 and grade 1-2 development in blastocysts was calculated on day 7 out of number of reconstructs that went into IVC. Error bars=SEM. Significance * = $P<0.05$, ** = $P<0.01$ Total number of reconstructs into IVC: vitrified cytoplasts *N*=175, vitrified ZF oocytes *N*=75. Replicates for CT with vitrified cytoplasts *n*=8, replicates for artificial activation with vitrified ZF oocytes *n*=3.

Chapter 5
Discussion

The main goal of this thesis was to improve blastocyst development in bovine ECT by optimising the methodology around the generation of embryo-derived donor outgrowths, cytoplast conditions, and cell synchronization. This will contribute towards the ultimate research aim of being able to multiple genetically elite bovine embryos for accelerated genetic gain in cattle populations. Accelerated genetic gain has benefits for animal welfare, the environment, the economy, and has biomedical applications. A major motivation for improving ECT efficiency is so it can be used to introduce thermotolerance alleles into cattle populations with the intention of mitigating the effects of climate change. There were four main objectives completed as part of this thesis. Firstly, the effects of four different hatching techniques on bovine blastocysts were compared and the optimal technique for generating embryo-derived outgrowths was determined. Secondly, the effect of nocodazole cell synchronization in combination with CHX activation in bovine ECT was compared to standard activation with no synchronization. Thirdly, the optimal methodology surrounding cytoplast age and volume in bovine ECT was determined. Finally, the potential of vitrified bovine oocytes and cytoplasts to be used in CT was assessed.

5.1 Objective 1: Determining the optimal hatching methodology for generating bovine blastocyst outgrowths

5.1.1 IVP blastocyst generation

Late-stage blastocysts were required to test the different hatching techniques and to produce donor outgrowths for ECT. Late-stage blastocysts were produced with IVP. The standard range of embryo development in commercial bovine IVP in 2021 was 60-80% cleavage and 30-40% blastocyst development (Crowe et al., 2021; Nogueira et al., 2021). Cleavage and blastocyst development from IVP in this study was on the higher end of those standard ranges. A standard range of high-quality blastocyst development is difficult to deduce as grade is based on a visual judgement and there may be inconsistency between studies.

5.1.2 Blastocyst hatching methods

Embryo-derived outgrowths from bovine blastocysts are required as a source of donors for ECT. The ability to produce usable outgrowths with persisting pluripotency was only established in 2018 (Bogliotti et al, 2018). Consequently, the methodology of creating bovine outgrowths varies between research groups and still requires further study. One factor that influences the production of these outgrowths, which still requires investigation, is the treatment of the ZP before plating.

Four hatching techniques were tested to study the importance of hatching, the effect of assisted hatching compared to natural hatching, and the optimal assisted hatching method. The two non-assisted techniques were natural hatching as the positive control, and non-hatching as the negative control. The two assisted techniques were mechanical hatching and pronase hatching. Mechanical hatching involved slicing through the ZP with a needle and pipetting the blastocyst out. The pronase digested the proteins in the ZP down into individual amino acids (Narahashi, 1970) and acted on tight junctions between trophoblast cells so the cells could spread more readily (Konwinski et al., 1978). Pronase mimics the urokinase-type plasminogen activator that is secreted from bovine TE to act on the ZP during natural hatching (Negrón-Pérez & Hansen, 2017). The trypsin-like protease is the main requirement for natural hatching along with blastocoelic pressure and actin-based trophectodermal projections (Massip & Mulnard, 1980; Niimura et al., 2010; Seshagiri et al., 2009). The number of outgrowths formed in each hatching group was monitored over 7 days. Many outgrowths that died between the 4-5 and 7+ period did so due to over confluency because passaging was not done during these experiments. This made the data on days 7+ outgrowth potentially unreliable and a poor marker of healthy outgrowth efficiency. Outgrowth efficiencies on days 4-5 were most important as these are the ideal ECT donors. On days 2-3, the outgrowth would be too small to produce enough donors for CT. Wilson (2021) observed that older outgrowths such as the days 7+ outgrowths may be less suitable for CT than younger outgrowths. Older outgrowths produce larger donor cells which is indicative of cells potentially being in S-phase or G2/M-phase and being multinucleated. This leads to a higher death and a lowered rate of development during ECT. If the purpose of these outgrowths was to sustain cells with valuable genetic editing, instead of creating embryo-derived donors for CT, establishing outgrowths

with persisting pluripotency and survival over a longer duration would be prioritised and the 7+ period would be more relevant.

The importance of hatching before plating was not as great as predicted. Outgrowths formed from the non-hatched negative control in all periods, reaching 67% on days 4-5. Unexpectedly, this lies within the range of hatched bovine outgrowth efficiencies reported by other studies. Verma et al. (2013) reported a 59-85% bovine outgrowth efficiency and Bogliotti et al. (2018) reported a 52-100% bovine outgrowth efficiency. However, it is difficult to directly compare outgrowth efficiencies between different studies because there is large variation in culture media composition and embryo sources. Also, initial outgrowth efficiency is not the focus of bEPSC studies and so it is often underreported. Another unexpected finding from the negative control group was that it had a higher outgrowth efficiency than the pronase hatched group in every period. Pronase hatching in this thesis only reached 36% outgrowth efficiency, indicating the pronase may have damaged the blastocyst. This is contradictory to Konwinski et al. (1978) who demonstrated that using pronase on non-hatched murine blastocysts allowed them to develop *in vitro* at 88-90% while the non-treated group developed at 52-56%. The difference between non-hatched and pronase hatched in this thesis was only significant on days 4-5. Many of the non-hatched negative control outgrowths were observed to die from becoming too confluent on days 7+ which may be a reason why the difference from the pronase group is no longer statistically significant on days 7+. The outgrowth efficiency from the negative control remained lower than the positive control and mechanically hatched group in all periods, but the difference was only significant from the positive control on days 2-3 and not days 4-5. A potential reason the negative control had a higher outgrowth efficiency than expected was that the blastocysts did not remain non-hatched during the 7 days. Some blastocysts were able to naturally hatch after being transferred to a plating well, and some of these were then able to form outgrowths. Hatching is essential for adhesion and outgrowth formation (Ueno et al., 2016), but these results show that if natural hatching has not occurred by day 8, then assisted hatching may not be necessary before plating. Placing a non-hatched blastocyst into a plating well without treatment may yield more outgrowths than opting to perform assisted hatching with pronase.

Assisted hatching did not improve outgrowth efficiency over natural hatching regardless of whether the assisted technique was pronase or mechanical. Naturally

hatched blastocysts reached an outgrowth efficiency of 80% which fits in the range of naturally hatched bovine outgrowth efficiencies reported in literature (Verma et al., 2013; Bogliotti et al., 2018). The positive control group had a higher outgrowth efficiency than the pronase hatched group in every period. This difference was statistically significant on days days 4-5. The low outgrowth efficiency from pronase hatching compared to natural hatching is contradictory to some literature. A study by Dolgushina et al. (2018) concluded that pronase hatching human IVF blastocysts lead to a significantly higher pregnancy rate, inferring that pronase hatching does not compromise the integrity of the blastocyst and increases the access of binding receptors. Taniyama et al. (2014) also found that pronase treatment on bovine embryos had positive effects on pregnancy rate. This contradiction between the literature and this thesis could indicate that pronase hatching can improve blastocyst attachment to the endometrium but not to *in vitro* gelatin support.

The outgrowth efficiency of naturally hatched blastocysts was higher than that of mechanically hatched blastocysts on days 2-3, but lower on days 4-7+. Mechanical hatching produced the highest outgrowth efficiency of all the techniques at 88%, the higher end of the literature range (Verma et al., 2013; Bogliotti et al., 2018). However, the advantage of mechanical hatching over natural hatching was not statistically significant in any of the periods. A potential reason that mechanical hatching could have an advantage over natural hatching is that it bypasses the strong contractions that sometimes occur during natural hatching (Alteri et al., 2018). These strong contractions have a negative influence on hatching compared to weak contractions as they cause elevated energy expenditure (Niimura, 2003). Contractions in bovine blastocysts are observed to precede or follow hatching (Massip & Mulnard, 1980). They are not a requirement for hatching to succeed and have even been observed to hinder bovine hatching in some cases. Overall, there was no significant evidence that assisted hatching could have an advantage over natural hatching. This aligns with literature that dismisses there being any advantages of assisted hatching (Hagemann et al., 2010; Hurst et al., 1998; Mahadevan et al., 1998; Medicine & Technology, 2014).

Mechanical hatching was the optimal assisted hatching method which did not match expectations given by the literature. Konwinski et al. (1978) discovered that mechanical hatching lowered *in vitro* murine blastocyst development to 12% while pronase hatching lead them to developing at 88-90%. Alteri et al (2018) speculated

that mechanical hatching could cause squeezing or injury to the blastocyst. They discussed how pronase may have an advantage over mechanical hatching as it thins the resilient inner zona layer while sometimes mechanical hatching only removes the outer layer. Bovine blastocysts have a more readily digestible outer layer and a more compact inner layer that is harder to penetrate (Alteri et al., 2018; Habermann et al., 2011; Vanroose et al., 2000). Between the two layers there is zonal glycan segregation, and a difference in thickness with the inner zona being thicker. Pores are present in the outer layer which decrease in size towards the inner layer, where pores are absent. The method of mechanical hatching used in this thesis was controlled zona dissection which may have penetrated the inner layer as well, explaining the increased outgrowth efficiency. Controlled zona dissection is when the embryo is held in position by with a holding pipette and a microneedle pierces the ZP (Balaban et al., 2002). Controlled zona dissection can penetrate the inner ZP to further facilitate adherence (Lyu et al., 2005; Stein et al., 1995).

Other types of assisted hatching that were not included in this thesis are acid drilling and laser hatching (Alteri et al., 2018). Acid drilling is a chemically assisted hatching method in which Tyrode acid is blown over the external surface of a ZP with a pipette. After the acid has created a hole in the membrane it is washed off. Laser hatching is when a diode laser is attached to an inverted microscope. The laser positioning, focusing, and aligning is computer controlled. A precise hole or point of thinning is created. Literature does not show that acid Tyrode hatching or laser hatching can significantly improve *in vivo* implantation rates over pronase hatching or mechanical hatching (Bordignon & Smith, 1998; Feng et al., 2009). However, alternative assisted hatching methods may positively affect *in vitro* bovine attachment efficiency as Cohen and Feldberg (1991) observed that acid drilling produced significantly more murine outgrowths than mechanical hatching. Laser may have the advantage of being a more consistent and reliable method for hatching for outgrowths as it is easier to focus on a target area. A controlled and precise hole can be created, and there is less variation between operators (Alteri et al., 2018). Compared to pronase or mechanical hatching, laser has the disadvantages of being more expensive, having potential thermal effects and needing specialized equipment.

Another method of blastocyst preparation that can be performed before plating is the removal of trophoblast cells as well as the zona. Outgrowths can be generated from an

ICM isolated with immunosurgery, instead of the whole blastocyst (Bogliotti, Wu, Vilarino, Okamura, Soto, Zhong, Sakurai, Sampaio, Suzuki, Belmonte, et al., 2018; Van Stekelenburg-Hamers et al., 1995). Immunosurgery removes trophoblast cells with anti-bovine antibodies. The antibodies bind to the external trophoblast cells, while the internal ICM is protected by the trophoblast cells and their zonular tight junctions. A reagent that induces lysis can then bind to the antibodies. This method allows an increase in epiblast and hypoblast cells which are pluripotent while avoiding the increase of trophoblast cells which downregulate pluripotency genes over time (Chambers & Tomlinson, 2009; Maruotti et al., 2012). Immunosurgery was not used in this thesis as it is time-consuming process and comes with the small risk of damaging the ICM during the immunological reaction, especially if there are any tears in the trophectoderm (Khan, 2021). Immunosurgery is also not necessary for sufficient outgrowth efficiencies as Bogliotti et al. (2018) reported bEPSCs outgrowths generated with either ICMs or whole blastocyst had low differences in efficiencies. Wolf et al. (2011) found that the ESC-like outgrowth efficiency of porcine blastocysts was not significantly different between whole blastocysts and immunosurgery ICMs. They noted that whole blastocysts produced a high rate of solitary outgrowths where ESC-like cells grew as a clean homogeneous cell population while immunosurgery increased the number of surrounding, flat cells. Immunosurgery may be necessary for sufficient production of cloning compatible donors as Hada et al. (2022) reported that murine trophoblast-derived stem cells have additional epigenetic barriers to reprogramming and resulted in lowered cloning efficiencies when used as donors. Exclusion of trophoblast cells through immunosurgery may produce outgrowths with more donors that are compatible with reprogramming in embryo-derived CT.

5.1.3 Future recommendations

A future recommendation is to address the observation that outgrowths that died from over confluency between days 4-5 and days 7+, which distorted the data on days 7+. Thus, passaging of confluent outgrowths should be carried out in the future to compare the length of survival of outgrowths from each hatching technique. This would also give information on the rate at which outgrowths grow after each hatching technique. The cause of death and time of death of outgrowths should be recorded to properly analyse the day 7+ data.

The sample sizes were also low and largely varied between each technique. This may have been the reason for high p-values. Repeats with larger sample sizes should also be conducted in the future.

The duration of blastocyst exposure to pronase is subjective to the operator. If left for too long the pronase may have started digesting the trophoblast epithelium of the blastocyst. This could have removed proteins on that domain that were important for matrix attachment which would have lowered the survival and affected the outgrowth efficiency. Thus, it is recommended to repeat these experiments but by another operator to determine if this is a concern. These experiments could be repeated with standardised parameters for more consistency to minimise operator effects such as timing the length of exposure to pronase and processing a set number of blastocysts at a time.

Finally, in the future using blastocysts produced by means other than IVM may improve outgrowth efficiency. Bogliotti et al. (2018) discovered that embryos were sourced from IVM had decreased outgrowth efficiency compared to embryos sourced from ovum pick up IVF and SCT.

5.2 Objective 2: Investigating the effect of nocodazole cell synchronization on polar body extrusion rates and on bovine ECT

After embryo-derived donor outgrowths were established, the compatibility of these donors with the CT cloning process was investigated. The compatibility of the cell cycle stage in the donor and in the cytoplast affects the viability of the blastocyst (Dinnyes et al., 2006; Du et al., 2002). At the time of disaggregation and fusion to the recipient cytoplasts, the donor cells are at unknown and varied stages of the cell cycle. The proportion of bEPSC donors in each cell phase was unknown but is likely to be similar to the proportions displayed in table 5.1 as estimated by Bogliotti et al. (2018); Wilson (2021). Wilson (2021) also estimates that the total of cells in G1, G2 and M phase to be ≈28%. Wilson (2021) is likely to be the most similar to donors in this thesis as the bEPSC outgrowths were cultured in the exact same conditions as this study.

Table 5.2 The proportion of bEPSC in embryo-derived outgrowths in each cell cycle according to literature

Study	S-phase %	G0-phase %	G2/M-phase %
Wilson (2021)	≈49	≈23	-
Bogliotti et al. (2018)	27	45	23

The cytoplasts at the time of fusion are in MII which means they have a high MPF environment (Dinnyes et al., 2006; Du et al., 2002). This environment stimulates the degradation of the nuclear envelope in donor cells, the condensation of chromosomes, and mitotic spindle formation to facilitate mitosis. Reprogramming of the donor DNA also occurs. Reconstructs can be activated with ionomycin three hours post fusion to re-establish the nuclear envelope and resume mitosis (Alberio et al., 2000). Not all stages of the cell cycle in the donors are compatible with this cytoplast environment so not all reconstructs can successfully develop to embryos. Donor cells in G1/G0 and G2/M are compatible but donors in S-phase are not. Synchronization of embryo-derived donors into the G0/G1-phase is challenging while synchronization to G2/M is achievable (Yabuuchi et al., 2004).

Embryo-derived donor cells were synchronized into G2/M with nocodazole treatment to improve the number of donors that were compatible with the MII cytoplasts during ECT. Nocodazole treatment halted the cell cycle during mitosis by interfering with the microtubule spindles that separate chromosomes (Tanaka et al., 1995). In G2/M the replication of the DNA has been completed so the cells are ready for the nuclear membrane to be dissembled and for the chromosomes to be reprogramed (Dinnyes et al., 2006). Nocodazole incubation for 21 hours has been reported to six-fold increase the proportion of bESCs arrested in mitosis to half the colony (Appleby et al., 2022). Other literature reported that bovine blastomeres treated with nocodazole had an increase in mitotic cells compared to blastomeres not treated. The increase was reported to be as high as 6-fold (Tanaka et al., 1995), and even 9-fold (Techakumphu, 1993). Following activation, the 2N/4C reconstructs with nocodazole synchronized donors were treated with CHX to allow the extrusion of a PPB to return them to 2N/2C

(Suvá et al., 2019). The development of the reconstructs with nocodazole synchronized donors was compared to the development of reconstructs with non-synchronized donors which varied in cell cycle stage.

The non-synchronized donor cells in the G1/G0 stage were compatible with the MII cytoplasts as the DNA had not yet replicated, so was not affected by the degradation of the nuclear membrane (Dinnyes et al., 2006; Du et al., 2002). Those donors had 2N/2C which meant the reconstruct had normal ploidy. Non-synchronized donors were incubated in DMAP after activation to prevent the expulsion of a PPB so that aneuploidy was avoided (Suvá et al., 2019).

The non-synchronized donors in S-phase were incompatible with MII recipient oocytes (Dinnyes et al., 2006; Du et al., 2002). As the donor chromosomes were only partially replicated during the S-phase, condensation formed chromosomes with gaps that could not undergo normal replication. This prevented the formation of blastocysts.

5.2.1 Polar body extrusion after nocodazole synchronization and ionomycin/CHX activation

Embryo-derived donors were synchronized into mitosis with 500 nM of nocodazole for 18-20 hours before being fused to MII cytoplasts. The reconstructs underwent ionomycin activation before incubation in CHX to allow pseudo-polar body extrusion for the correction of ploidy. Four hours after incubation in CHX, embryos were separated into those that formed pseudo-polar bodies (PPB) or had no pseudo-polar bodies (NPPB) in between the ZP and plasma membrane. There was a significant increase in the amount of reconstructs that had NPPB compared to those that has PPBs. The amount of reconstructs that developed PPBs was low. As a result, the amount of reconstructs with correct ploidy after nocodazole treatment may be low and viability of the blastocysts produced from nocodazole-treated donors may be low. Appleby (2015) demonstrated that nocodazole-treated blastocysts that were reconstructed with bESC donors and had extruded a PPB had 60 chromosomes, whereas those that were NPPB had about 120 chromosomes. 60 chromosomes is the normal ploidy for a bovine blastocyst (Khatun et al., 2011; Udroiu & Sgura, 2017). Ideta et al. (2005); Tani et al. (2001) observed that NPPB blastocysts formed using mitotic somatic donors and CHX incubation had a 75-100% tetraploid rate while those that were PPB were diploid. As the proportion of donors used in mitosis was unknown, it is also possible that some

blastocysts produced from nocodazole-treated donors may be viable. If a blastocyst in CHX that was NPPB was not synchronized correctly and had a donor that was in G1- or S-phase, the blastocyst would be diploid and useable for CT. Appleby et al. (2022) established that half of 500 nM overnight nocodazole-treated bEPSCs are in mitosis. If this is correct than half of NNPB blastocysts may be diploid. The expulsion rate in this thesis was lower than in CT with mitotic bovine blastomeres and ethanol/CHX activation by Alberio et al. (2000) who reported a 50% PPB expulsion rate. The PPB expulsion rate in this thesis was closer to Ideta et al. (2005); Tani et al. (2001) who saw a 30% PPB expulsion rate after using mitotic somatic donors and CHX incubation.

A plausible explanation for the low extrusion rate could be that the chromosomes did not organise and segregate correctly which hindered the formation of a PPB (Alberio et al., 2000). The interval between fusion and activation is important for the correct organisation of the chromosomes in reconstructs. If reconstructs are activated prematurely, incorrect segregation could obstruct PPB formation. Alberio et al. (2000) found that two hours after fusion with a bovine M-phase blastomere, half of the reconstructs showed a premature condensed chromatin arrangement that may not segregate normally. Condensed chromosomes not arranged on the equatorial plate were observed for three hours after fusion. DNA synthesis was observed at a significantly higher rate in embryos that did not extrude a PPB-like structure than in those that extruded a PPB showing that the extrusion of a PPB after improper chromatin organisation may be occurring. Proper organisation of the metaphase plates occurred between three to seven hours post fusion. The interval between fusion and activation in this thesis was three hours from the midpoint of fusion for each treatment group. When fusion of a second treatment group occurred an hour after fusion of the first, the first treatment group may be activated slightly less than three hours after fusion, meaning chromosomes would not segregate properly.

Electrofusion can be associated with developmental defects in CT embryos because it can cause premature activation by stimulating an increase in intracellular Ca^{2+} (Mitalipov et al., 2007; Song et al., 2011). The fusion buffer used in this study contained Ca^{2+} which could enter the reconstructs through fusion pores. Premature activation could cause a decrease in MPF which would lead to the reformation of the nuclear envelope before proper nuclear remodelling. This could lead to cell cycle arrest, decreases in blastocyst quality and blastomere apoptosis. As the interval

between fusion and activation has been shown to be important to development (Alberio et al., 2000), premature electrofusion activation could be an issue.

Another plausible reason for the low extrusion rate was that the interval between activation and PPB searching meant some PPBs were not observed. Appleby (2015) performed ECT with mitotic embryo-derived cells and produced a PPB reconstruct that developed to a blastocyst. In this reconstruct, the PPB expulsion was observed six hours after incubation in CHX. Alberio et al (2000) also recorded their PPB expulsion rate up to five hours after activation and incubation in CHX to allow sufficient time for nuclear remodelling after activation.

5.2.2 Development of embryos with nocodazole synchronized donors

To analyse whether mitotic synchronization had any positive effects on embryo development, ECT was performed with synchronized donors and CHX incubation alongside ECT with non-synchronized donors and DMAP incubation. It was presumed that the non-synchronized donors that developed to blastocysts were in G1/G0 or G2/M phase because S-phase donors are not compatible with MII cytoplasts (Dinnyes et al., 2006; Du et al., 2002). As the S-phase was likely to be the dominant phase in the outgrowth (Wilson, 2021), it was expected that approximately half of reconstructs would not develop (Table 5.1). It is likely that the proportion of donors in M, G1 and G2 phase was ≈28%, and the proportion of donors in G1 was ≈23% meaning it was reasonable to expect that development would have been low from reconstructs formed that were not successfully synchronized into mitosis and extruded a PPB.

Day 5 lysis was not observed in the nocodazole-treated NPPB group, but it was observed in the nocodazole-treated PPB group. There was a strongly significant increase in lysis in the embryos that received non-synchronized donors than in the embryos that received synchronized donors and were NPPB. This may affirm that the non-synchronized group had a higher proportion of donors in S-phase. The difference between the PPB embryos and both other embryo groups was not statistically significant, but this was likely due to the low sample size of reconstructs that extruded PPBs.

There was no difference in cleavage or blastocyst development between embryos with mitotic synchronization or without mitotic synchronization, regardless of whether the reconstruct formed a PPB. No high-quality blastocysts could be produced from the treated PPB group. Again, the lack of significance seen in differences between the PPB group and other groups is likely due to its small sample size (n=21) as only 22% of the treated group extruded PPBs. These experiments showed that mitotic synchronized embryo-derived donors can produce blastocysts in CT with and without PPBs but they may not improve blastocyst development compared to non-synchronized donors. This does not necessarily mean that synchronization would not improve bovine cloning overall, as the effects of ploidy on pregnancy and birth rate may be more prevalent than on blastocyst development. Chromosomal abnormalities often cause pregnancy failure and a decrease in the fertility of cattle (Dzitsiuk & Tipilo, 2019).

Low development could have been observed in the nocodazole treated group because the concentration and length of incubation of nocodazole was detrimental. The microtubule-depolymerising action of nocodazole can become permanently detrimental to the centrosome structure if it occurs at heightened concentrations or over extended periods (Jordan et al., 1992). If this occurs the centrosome cannot reform after removal from nocodazole. The reconstruct is reliant on this donor centrosome as the maternal centrosome in the cytoplast was removed during enucleation. Too low a concentration or too low a period of exposure does not block the function of the microtubles so an optimal time and concentration must be used. This thesis followed the methodology of Appleby et al. (2022) who established that treatment of bovine embryo-derived donors with 500 nM nocodazole for 21 hours produces a high level of mitotic arrest. There are alternative cytoskeletal inhibitors that can be used for mitotic synchronization such as colcemid, colchicine, benomyl and vinblastine (Alberio et al., 2000; Tanaka et al., 1995). Nocodazole is the preferable mitotic synchronizer as it has been observed to be more reversible than benomyl, colcemid, colchicine or vinblastine.

A bigger sample size for the PPB treated group may have led to stronger differences between groups and led to conclusions similar to those in literature. Other literature has observed that mitotic synchronization in bovine CT can cause a significant decrease to blastocyst development. Techakumphu (1993) found that when nocodazole-treated bovine blastomeres were used as donors in ECT with MII cytoplasts their development halved compared to controls. Alberio et al. (2000)

discovered that they could not produce blastocysts during bovine ECT with mitotic synchronized blastomere donors. They concluded that under the conditions tested M-phase donor cells could not properly remodel and support normal embryonic development. They suspected that this was due to improper chromatin remodelling of mitotic nuclei after CT, which caused chromosomal abnormalities. Ideta et al. (2005); Tani et al. (2001) discovered that blastocyst development was not different between PPB and NPPB reconstructs formed with mitotic somatic donors and CHX incubation. Other literature has also confirmed that the MPF inhibitor used after activation in bovine CT can cause a significant change to blastocyst development. Akagi et al. (2020); Bhak et al. (2006) found that the blastocyst formation rate after CHX incubation was significantly higher than after DMAP incubation in bovine SCT without mitotic synchronization. Bhak et al. (2006) observed that CHX produced a significantly higher number of diploid blastocysts than DMAP and that half of the DMAP blastocysts had abnormal ploidy. In contrast, Appleby (2015) discovered that DMAP incubation led to almost double total and high-quality blastocyst development than CHX incubation during bovine ECT with mitotic synchronized donors in both groups. Blastocyst development was higher in both the CHX (14%) and DMAP group (24%) in Appleby's study than reported in similar experiments this thesis. However, double the total blastocyst development after DMAP activation compared to CHX activation was also seen in this thesis when only the CHX group received nocodazole synchronization. The similar improvements seen may indicate that the non-synchronized group in this thesis performed better because of the DMAP and not because they weren't nocodazole treated. Appleby (2015) found that after DMAP in CT with non-synchronized embryo-derived donors, somatic donors, and PGs there was a greater incidence of aneuploidy and polyploidy. So, despite the higher blastocyst development after DMAP, the chromosomal abnormalities may still make CHX the more appropriate option.

It will be of interest to see whether the synchronized NPPB blastocysts can produce healthy calves as they are expected to be tetraploid, which was reinforced by karyotyping by Appleby (2015). However, they only karyotyped one NPPB blastocyst from a mitotic bEPSC donor. Without confirming the polyploidy in this study with karyotyping, it is possible the NPPBs do not have 120 chromosomes as expected. Parmenter (1940) showed that PGs that extruded a second PPB could achieve diploidy by delaying the cell cycle to enable an extra round of DNA replication.

Endoreduplication, by which a cell undergoes multiple S-phases without an subsequent cytokinesis has been observed in bovine embryos and oocytes (Hanada et al., 2005). It is possible the reverse could occur, and that a delay in DNA replication before cell division could occur. The embryos may have a way to regulate their DNA content and correct polyploidy. To my knowledge there are no published examples of this proposed mechanism and no live chromatin image analysis was undertaken to prove this[1]. Appleby (2015) proposed that this was possible as she identified DMAP activated CT reconstructs with mitotic donors that had spreads with 60 chromosomes when they would have expected 120 because of the DMAP preventing PPB extrusion.

5.2.3 Future recommendations

The proportion of donor cells in each cell cycle phase had to be estimated from previous literature. The estimations provided by Wilson (2021) are likely to be fairly accurate as the outgrowths were grown with the same media conditions, were from the same source and were cultured in the same laboratory. To ensure further accuracy in the future, it is recommended that Hoechst stain is used to identify condensed nuclei on the remaining disaggregated synchronized and non-synchronized cells to identify the proportion in mitosis and interphase.

Karyotype analysis should also be conducted on the blastocysts constructed from nocodazole-treated donors to confirm their ploidy. Karyotype examinations can reveal the number and structure of chromosomes (Khatun et al., 2011; Udroiu & Sgura, 2017). A karyotype of a normal diploid bovine cell would reveal 29 pairs of acrocentric autosomes and two metacentric sex chromosomes. If the assumption that the nocodazole-treated donors were synchronized to M-phase is true, karyotyping would reveal higher rates of diploid blastocysts constructed with synchronized donors than with non-synchronized donors. This finding would encourage the use of nocodazole in ECT when CHX is being utilized. If the assumption that treated reconstructs with PPBs are diploid is true, karyotyping would reveal higher rates of diploid blastocysts in the treated PPB group and higher rates of tetraploid blastocysts in the NPPB group.

[1] As of 1st November 2022.

This finding would allow the appropriate selection of blastocysts for ET and the NPPB blastocysts would be discarded.

5.3 Objective 3: Optimise cytoplast treatment for bovine ECT

5.3.1 Cytoplast age

The proportion of donors in S-phase used in the aging experiments is unknown but is likely to be half as shown by staining of outgrowths cultured in the same conditions as in this thesis (Wilson, 2021). This means approximately half of the reconstructs made with MII cytoplasts were likely to be unviable. To be compatible with the large population of donors in S-phase, recipient cytoplasts with a low MPF environment can be used (Dinnyes et al., 2006; Du et al., 2002). A cytoplast with this environment exists in an interphase-like stage and will not perform nuclear breakdown and chromatin remodelling on the donor while in this state. Replication can be completed in the S-phase donors before the nuclear membrane breakdown and chromosome condensation.

Aging followed by cooling is one technique used to lower MPF in cytoplasts (Bordignon & Smith, 1998; Fulka Jr et al., 1998; Gall et al., 1996). Advancing cytoplast age causes a decline in MPF activity (Fulka Jr et al., 1998). MPF activity plateaus at 20-24 hours after maturation, holding the bovine oocyte in MII (Wu et al., 1997). MPF remains high until 30 hours of maturation where it decreases until basal levels at 44-48 hours. To ensure MPF lowers to a point where the cytoplast no longer induces nuclear envelope breakdown in donors, aging for at least 44 hours is required. The aging must be accompanied by cooling as high levels of MPF were detected in cytoplasts that were aged at room temperature, but low levels were detected in cytoplasts aged at 10°C (Gall et al., 1996). H1 kinase activity, which is indicative of the oocyte being meiotic, was lower in cytoplasts that were aged and cooled to 12°C compared to in cytoplasts that were aged but not cooled (Bordignon & Smith, 1998). Temperature reduction may be crucial as it has been shown to stimulate repeated calcium oscillations which could induce activation and the release from MII in some mammals. Enucleation was also required before cooling and aging to create an S-phase compatible environment. Enucleation alone did not affect MPF, as MPF remained high

in enucleated cytoplasts with aging and cooling. Lee and Campbell (2006) also established that the enucleation of ovine oocytes did not affect MPF activity. Li et al. (2014) observed that a large fraction of cyclin B, a subunit of MPF, accumulated around the spindle which gets removed during enucleation. They found that MPF activity in oocytes was decreased following porcine enucleation, but this decrease was not statistically significant as a fraction of MPF was cytoplasmic and not removed. However, in combination with aging and cooling, enucleation can lower MPF (Gall et al., 1996). High MPF levels were detected in aged, cooled, non-enucleated oocytes but basal MPF levels were detected in aged, cooled, enucleated oocytes.

The cytoplast environment after aging and cooling was also compatible with donor cells in G2/M-phase as these cells had completed replication and could degrade their nuclear membrane for chromatin remodeling without a high MPF environment (Dinnyes et al., 2006; Du et al., 2002). Donor cells in the G1/G0-phase were not compatible as they needed a high MPF environment to degrade their nuclear membrane for chromatin remodeling. The proportion of donors in G1/G0 was likely to be ≈23% so the total proportion of cells predicted to be compatible with aged and cooled cytoplasts was up to ≈77% (Wilson, 2021).

Fusion was higher for aged cytoplasts than non-aged cytoplasts, although this was not statistically significant. Muggleton-Harris and Hayflick (1976) discovered that aged cytoplasts had higher fusion rates with somatic donors and Sendai viruses than the non-aged cytoplasts and speculated that this could be due to a change in the membrane with age that facilitates easier fusion with other cellular membranes. This varied from a study by Liu et al. (2000) that noticed that the fusion rates of donor cells with aged cytoplasts enucleated at the TII stage were lower than those with non-aged cytoplasts enucleated at the MII stage, potentially due to the membrane becoming more fragile and vulnerable to electrofusion with age.

Lysis on day 5 was significantly higher after aged treatment. This suggests the aging process may have compromised cell integrity which could contribute to lower blastocyst development.

Both cleavage and blastocyst generation rates were higher in the non-aged group than in the aged group. This difference was significant for total blastocyst development but

not significant for high-quality blastocyst development. This result could suggest that the aging process did not sufficiently reduce the levels of MPF or that the proportion of donors in G1/G0 was higher than expected. The results in this thesis did not correlate with literature which found that aging cytoplasts followed by cooling in bovine CT appears beneficial (Heyman et al., 1994; Misica-Turner et al., 2007) or was not different to controls (Shiga et al., 1999). The lowered blastocyst development of aged cytoplasts in this thesis was supported by other literature, which observed that oocyte aging could decrease viability after CT as prolonged culture may have affected some crucial cytoplasmic constituents required for normal development (Bordignon & Smith, 1998).

Gall et al. (1996) established that enucleation must accompany aging and cooling to cause a drop in MPF levels. However, using non-enucleated oocytes for aging is more common and has been reported to have an advantage for blastocyst development (Bordignon & Smith, 1998; Liu et al., 2000). Yang (1991), on the other hand, noted that using aged oocytes instead of cytoplasts may be an issue because aging can lead to alterations in the cytoskeleton that cause inward migration of the metaphase plate which may reduce the rate of enucleation of the aged oocytes and affect CT efficiency.

An alternative method to create a low MPF environment in recipient cytoplasts is chemical pre-activation. Chemical pre-activation involves activating cytoplasts with ionomycin before fusion and putting them into a presumed G1/S-phase (Du et al., 2002). Then the cytoplast is kept in an inhibitor of protein phosphorylation or synthesis until fusion is completed. Stice et al. (1994) discovered that using preactivated cytoplasts in bovine CT is beneficial when using blastomeres as donors. Du et al. (2002) observed that pre-activating bovine cytoplasts improved blastocyst development by 25% when using blastomere donors. Kurosaka et al. (2002) discovered that pre-activating bovine cytoplasts improved blastocyst development by 16% when using somatic donors in S-phase. Bordignon and Smith (1998) observed that blastocysts constructed bovine blastomeres had higher development when using preactivated-aged oocytes compared to using aged-cooled cytoplasts.

5.3.2 Cytoplast volume

Increasing the volume of the cytoplast during bovine CT has been observed to affect the fusion, cleavage and blastocyst development of reconstructs with somatic donors (Delaney et al., 2007; Green et al., 2016), blastomere donors (Peura et al., 1998) and embryo-derived donors (Appleby et al., 2022). A larger cytoplast has an increased number of maternal factors which reprogram the donor DNA (Sayaka et al., 2008). The increase in maternal transcripts, proteins, mRNA, and molecular precursors in the reconstruct may better facilitate the major burst of transcription required during early embryonic development (Liu et al., 2018; Peura et al., 1998).

There is a lack of literature around the effects of increased cytoplasmic volume on bovine ECT. Appleby et al. (2022) used double cytoplasts with bEPSC donors but these were synchronized into mitosis with nocodazole treatment, which does not establish whether double cytoplasts can support the development and reprogramming of donors at different cycle stages, including the G1/G0-phase, which is also compatible with MII cytoplasts. To investigate the effect of cytoplast volume on fusion and embryonic development in bovine ECT, reconstructs with non-synchronized embryo-derived donors and double cytoplasts were made. The double cytoplast group had another cytoplast attached to the fused reconstruct and underwent a second round of fusion. Fusion was significantly higher between a reconstruct and cytoplast (double cytoplasts) than a donor and cytoplast (single cytoplasts), which is likely due to the difference in cell sizes. A large difference in cell size can lower electrofusion efficiency (Stice & Keefer, 1993). The difference in size and surface contact area between a cytoplast and an embryo-derived donor is much greater than between a cytoplast and a reconstruct. Trounson et al. (1998) ascribed the low fusion rates of their fibroblasts (15–20 µm diameter), and cytoplasts (100 µm diameter) to the disparity in the size of the two cells. They found that fusion with a fibroblast was improved as the size of the cytoplast was decreased. This is further validated by all the double cytoplasts in this thesis, with minimal size difference between the reconstruct and second cytoplast, having 100% fusion. The difference in membrane composition can also affect fusion efficiency. The heterotypic fusion that occurs between different cell types can have lower efficiency than homotypic fusion between two identical cell types due to differences in the number and type of cell specific proteins (Liu et al., 2022).

There was no difference in lysis on day 5 showing that a larger cell volume is not detrimental to an embryo's growth up to day 5. Although cleavage on day 5 was significantly higher in embryos with double cytoplasts than embryos with single cytoplasts, there was no significant difference in development on day 7. This change in significance could be due to the day 5 data having an extra replicate and greater sample size than the day 7 data. Another possibility is that the larger diameter may have impaired the cell machinery of cell division, countering the positive effects of the extra cytoplasmic factors (Sayaka et al., 2008).

It is difficult to make direct comparisons to literature about cytoplast volume in CT when most studies do not use embryo-derived donors or ePSCs. Different types of donor cells may require different volumes of cytoplasmic factors for complete reprogramming and to adequately support development. There is also dissonance in the conclusions that literature makes about the effect of double cytoplasts in SCT. Some literature supports the trend seen in this thesis such as Trounson et al. (1998), who saw no improvement in blastocyst development in bovine SCT and Sayaka et al. (2008) who also observed no difference in blastocyst development with double cytoplasts in murine SCT. Meanwhile other literature saw contradictory trends in SCT which lead them to conclude double cytoplasts improve *in vitro* total bovine blastocyst development by 15% (Delaney et al., 2007) and high-quality ovine blastocyst development 9% (Green et al., 2016). Peura et al. (1998) observed no differences in fusion or cleavage rates between bovine double cytoplast SCT and single cytoplast SCT with blastomere donors but saw significantly increased blastocyst development. Embryo-derived donors may have required more reprogramming than blastomeres and therefore the increase in reprogramming factors was sufficient for blastomeres but not embryo-derived donors. It is unlikely that the embryo-derived donors required more reprogramming factors than somatic cells, so the explanation does not account for discrepancies with SCT literature. There is also discrepancy between this thesis and double cytoplast embryo-derived literature. Appleby et al. (2022) used nocodazole-treated bePSCs donors and discovered that double cytoplasts caused blastocyst development twice as high as the blastocyst development seen in single cytoplasts. In this thesis, the double cytoplast development only reached a third of the double cytoplast blastocyst development seen by Appleby et al. (2022), The single cytoplasts development was similar to at Appleby et al. (2022) meaning the difference in cell synchronisation cannot explain why double cytoplasts did not improve

development in this thesis. The high-quality development in double cytoplasts seen by Appleby et al. (2022) was similar to this thesis. For Appleby et al. (2022) this was an improvement from high-quality blastocyst development in single cytoplasts but there was no difference in this thesis. These disparities prompt the need for further data on double cytoplasts in ECT. The reason some literature sees improvement in cloning from use of double cytoplasts and the mechanism behind this is still unclear. The mechanism behind improvements in reprogramming potential and developmental potential may be the increase of reprogramming activators or the dilution of reprogramming inhibitors.

While no effect on blastocyst development was seen from double cytoplasts, a difference in overall cloning efficiency may be seen after ET. While Appleby et al. (2022); Delaney et al. (2007) saw an improvement in bovine blastocyst development, double cytoplasts did not improve initial pregnancy establishment. While Sayaka et al. (2008) saw no difference in murine blastocyst development with double cytoplasts they saw a significant decrease in the survival of cloned embryos to term. Blastocyst development efficiency does not reflect or necessarily correlate to overall cloning efficiency. Sayaka et al. (2008) attributed the difference between their blastocyst efficiency and overall cloning efficiency to the greater size of the embryos impairing the machinery of cell division which can produce abnormal karyotypes. Abnormal karyotypes do not inhibit preimplantation development but can cause fatality during foetal development so double cytoplast embryos may develop to blastocysts but not to term.

5.3.3 Future recommendations

Gall et al. (1996) observed that aged cytoplasts had high levels of MPF unless the temperature was lowered to 10°C for four hours after 44 hours of aging. Bordignon and Smith (1998) found that H1 kinase activity was lowered after 43 hours of aging and then another three hours of cooling at 12°C. In this thesis, cooling was done at 15°C for 11 hours after 35 hours of aging which is higher than the temperatures tested by those studies. It is even possible that the temperature was slightly higher than 15°C as the thermometers in the water bath had measurements that varied after the cytoplasts were aged which could suggest the water bath was faulty. Therefore, aging in this thesis may not have been cold enough to adequately cause an interphase like state in

the cytoplasts. Testing for levels of MPF after cooling/aging at 15°C could be done in the future to confirm the cytoplasts had reached an interphase state.

5.4 Objective 4: Investigate the potential of vitrified bovine oocytes and cytoplasts for use in CT

Large quantities of cytoplasts are required for a CT run. The preparation of oocytes on the morning of CT takes approximately two hours and then enucleation takes an hour per 100 oocytes. The ability to use vitrified recipient cells would bypass the preparation and enucleation stage, replacing it with a shorter thawing protocol. Thawing cytoplasts on the morning of CT takes approximately one hour per 80 oocytes. It removes the requirement for fresh oocytes to be available the day prior to CT. Therefore, viable vitrified cytoplasts can increase the efficacy of an CT run (Atabay et al., 2004; Dinnyés et al., 2000; Hou et al., 2005).

5.4.1 Survival after thawing

The first phase of determining the viability of vitrified bovine oocytes was testing their ability to survive the vitrification and thawing process.

The level of experience of the enucleation operator had a strong influence on the survival of cytoplasts. Cytoplasts enucleated by the less experienced operator had a significantly lower survival rate than cytoplasts enucleated by the more experienced operator. Enucleation is technically difficult, and the membrane can become compromised or too much cytoplasm can get removed if it is performed incorrectly (Dinneys et al, 2006). This can lower the survival rate, viability, and reprogramming potential of the oocyte. Reducing the bovine cytoplast volume can lower blastocyst development and decrease blastocyst cell numbers (Zakhartchenko et al., 1997) indicating that removal of too much cytoplasm by an inexperienced enucleation operator would decrease viability. The post-vitrification survival rates of cytoplasts from the less experienced operator were excluded as they would drastically skew the final calculations.

The survival rate of bovine cytoplasts in this thesis was only slightly lower than Booth et al. (1999); Peura et al. (1999) who achieved around 98% survival. The bovine ZI

oocyte survival rate in this thesis was very close to complete survival which exceeded the rates in literature which put it at 79-98% (Atabay et al., 2004; Chian et al., 2004; Dinnyés et al., 2000). To my knowledge, literature did not describe survival of ZF bovine oocytes[2]. The oocyte survival rate in this study is higher than stated in the previous studies because the vitrification/thawing process has been refined and improved over time. The survival rates of bovine oocytes is largely influenced by the protocol and embryologist (Saragusty & Arav, 2011). Some potential reasons that a few cells lysed after vitrification and thawing include mitochondrial damage, changes to the plasma membrane, cytoskeletal damage, cracking because of temperature differences between the outer layers and the core, and spindle depolymerization (Booth et al., 1999; Dinnyés et al., 2000; Saragusty & Arav, 2011). Or if the vitrification process fails ice crystals may have caused cellular damage.

Overall, regardless of whether the oocytes were ZF or ZI, the oocytes had a significantly higher survival rate two hours after thawing than the cytoplasts. This prompted the question of whether enucleation causes a degree of fragility to the membrane that leaves it susceptible to lysis when exposed to vitrification. However, when the effect of interaction of vitrification and enucleation on development was evaluated, it was revealed that there was no difference in lysis on day 5, cleavage or blastocyst development between artificially activated ZF vitrified oocytes and CT reconstructs with vitrified cytoplasts. It cannot be confirmed that the interaction of vitrification and enucleation was the reason for low development from vitrified cytoplasts. Although, high-quality blastocysts could be developed from the vitrified ZF oocytes, but not cytoplasts. Further investigation into the interaction of enucleation and vitrification is required.

When the state of the zona was taken into consideration, survival two hours after thawing only significantly exceeded cytoplasts when the oocyte was ZI but not ZF. ZI oocytes showed almost complete survival. This suggests that the ZP provides protection during the vitrification process. Oocytes have high cytoplasmic lipid content that increases chilling sensitivity and less submembranes actin filaments and microtubules which makes their membrane less robust than embryos (Saragusty & Arav, 2011). Therefore, the additional protection of the membrane may be important

[2] As of 1st November 2022.

to oocytes and cytoplasts during vitrification. ZP proteins are involved in localized actin remodelling and the organisation and stabilization of membrane (Fernandes et al., 2010). The ZP can act as a barrier to movement of water and cryoprotectants into and out of the oocyte (Saragusty & Arav, 2011). Choi et al. (2015) found that ZI murine oocytes had a post-vitrification survival rate that was seven-fold higher than ZF murine oocytes. They discovered that ZI murine oocytes had an elastic modulus that is more than 85 times higher than that of ZF oocytes. Elastic modulus is a measure of resistance to non-permanent deformation. To my knowledge there is no literature which directly compares the survival rates of vitrified bovine oocytes with and without ZPs. The higher survival in the ZI oocytes indicates that they may have more intact cytoplasmic components. Using vitrified ZI oocytes and enucleating them after thawing may have yielded higher CT development than using vitrified/thawed cytoplasts. Using vitrified oocytes instead of using vitrified cytoplasts in CT would lengthen the duration of CT as it requires the enucleation step. Using vitrified oocytes instead of fresh oocytes would shorten the duration of CT as it would eliminate the need for IVM and the oocyte preparation steps, such as cumulus stripping.

The survival rates of cytoplasts and oocytes after vitrification and thawing was high and sufficient to proceed to the next test of their potential for CT.

5.4.2 Artificial activation of thawed oocytes

After establishing that oocytes can survive the vitrification/thawing process, thawed oocytes underwent ionomycin/DMAP activation to assess whether the nuclear or cytoplasmic components essential for normal embryo development were degraded during the vitrification/thawing process. Parthenogenesis can occur *in vitro* when a non-enucleated oocyte is artificially activated to produce a diploid embryo with only maternal genetic material (De et al., 2012; Fernandes et al., 2014; Méo et al., 2007). Artificial activation can be used as a preliminary study before CT to assess whether an oocyte is capable of supporting development.

Lysis was significantly higher in vitrified oocytes after activation than in fresh oocytes. Fresh oocytes had significantly higher total blastocyst development and significantly higher grade 1-2 blastocyst development than vitrified oocytes. Blastocyst development was taken from the total number of oocytes into IVC and did not exclude oocytes that lysed by day 5. The low blastocyst development of the vitrified oocytes if

a reflection of their low rate of survival to day 5. So, while vitrified oocytes can still support development to the blastocyst stage, the vitrification process lowers this developmental potential. Dinnyés et al. (2000); Hou et al. (2005) also found that artificial activation with vitrified bovine ZI oocytes produced embryos with lower cleavage and blastocyst development than with fresh oocytes. However, when O_2 incubation levels were increased from 5%, like in this thesis, to 20% the difference in blastocyst development disappeared. Authors have no included speculation as to the reason for this phenomenon. The exact reason why the vitrification/thawing process negatively affected oocyte developmental competence cannot be confirmed, but it is likely to be because of the effect of vitrification on the temperature-sensitive meiotic spindle. Some studies have observed that vitrification causes cryoinjury in the meiotic spindle which hinders survival and the use of vitrified oocytes (Atabay et al., 2004; Booth et al., 2001; Dinnyés et al., 2000; Girka et al., 2022; Saragusty & Arav, 2011). This spindle makes bovine oocytes more difficult to vitrify than bovine embryos. The meiotic spindle is required for the proper separation of chromosomes, completion of meiosis, and normal embryo development (Chen et al., 2003). The heterodimers of the microtubules in the spindle can dissociate in the rapidly decreasing temperatures of vitrification, compromising the spindle. During warming the spindle repolymerizes but it may not be the correct conformation. The alternative reason is that maternal developmental factors, especially those required for early embryonic activation, were damaged during the vitrification/thawing process. Vitrified cytoplasts, which had the meiotic spindle removed, also displayed lowered developmental competence which may suggest that a damaged meiotic spindle was not a detrimental issue in oocytes, only damaged developmental factors. Although if the developmental factors were intact, reprogramming factors may have been a causative problem in cytoplasts while the meiotic spindle was a causative problem in oocytes.

Although it has been suggested that the ZP can provide protection from lysis during the vitrification/thawing process, it does not affect lysis after artificial activation for fresh or vitrified oocytes. There was no significant difference in cleavage or blastocyst development between oocytes with or without a zona, regardless of whether they were vitrified or not ($P>0.05$). To my knowledge, there are no reports in previous studies

that the ZP impacts development after artificial activation[3]. Booth et al. (2001) found that the artificial activation of bovine ZI oocytes produced embryos with significantly lower lysis than with ZF oocytes. However, they saw no significant difference in blastocyst development. Even though the difference in blastocyst development in this thesis was not statistically significant, we can conclude that ZF vitrified oocytes can develop to blastocysts while we cannot make that conclusion with ZI vitrified oocytes. If oocytes are vitrified with an intact zona to increase thawing survival, the zona should then be removed prior to CT. This will not hinder the current CT protocol as CT in this thesis is performed with a zona free system to make enucleation easier and fusion rates higher (Booth et al., 2001; Oback et al., 2003).

The rate of cleavage and blastocyst development in our ZI fresh control PGs was lower than the range in the literature. Both Dinnyés et al. (2000) and Hou et al. (2005) found that ZI fresh PGs had about 1.3-fold higher cleavage and about two-fold higher blastocyst development. After activation, the rate of cleavage in the ZI vitrified oocytes was within the reported range but blastocyst development was nil, much lower than reported. Both Dinnyés et al. (2000) and Hou et al. (2005) found that ZI vitrified PGs had the same cleavage at 50% but approximately 20% higher blastocyst development. Overall Dinnyés et al. (2000); Hou et al. (2005) observed a reduction in both cleavage and blastocyst development in artificially activated vitrified oocytes compared to fresh oocytes. A reduction was also observed in this thesis but at a lower baseline indicating that the artificial activation system in this thesis was suboptimal. The variation in development between this thesis and the literature is likely to be caused by differences in the composition, concentrations, and the time of exposure of the vitrification media. The concentration of cryoprotectants and the time of exposure to cryoprotectants is important because they affect the extent of dehydration in the cells (Chian et al., 2004). Cryoprotectant concentration affects cooling-warming rates during vitrification. Hou et al. (2005) found that increasing the concentrations of cryoprotectants increased oocyte survival by 21%. The type of cryoprotectant used can affect the developmental competence of oocytes. Chian et al. (2004) found that development after IVF with vitrified oocytes was higher when using propylene glycol instead of DMSO. DMSO can cause spindle polymerisation with increased risk for polyploidy. Propylene glycol

[3] As of 1st November 2022.

is less toxic and does not cause spindle polymerisation. Dinnyés et al. (2000) opted to exclude DMSO during the vitrification of ZI oocytes and produced blastocysts at higher rate after artificial activation than was displayed in by oocytes in this thesis which were vitrified with DMSO in the media. This could further suggest that the presence of DMSO does damage cells.

Ovary collections alternated between seasons and the ages of the mothers. There are seasonal changes in the membrane phospholipid composition and cholesterol levels in bovine oocytes (Buschiazzo et al., 2017). There is lower cholesterol levels in bovine oocytes in winter and in summer compared to autumn and spring which lowers the membrane fluidity. Lowered fluidity can lower the tolerance of the oocytes to cryopreservation. Murine vitrified oocytes have been observed to have decreasing survival, cleavage rate and blastocyst development rate with maternal age (Yan et al., 2010).

The development of two blastocysts, including a high-quality one, from the artificial activation of ZF oocytes proved that the oocytes are capable of supporting development after the vitrification thawing process.

5.4.3 SCT with thawed cytoplasts

CT with vitrified ZF cytoplasts was performed to test whether the reprogramming factors in the cytoplasm were also affected by the vitrification/thawing process. Fusion of serum starved somatic donors with fresh cytoplasts was not different than with vitrified cytoplasts. Park et al. (2015) also found no difference between fresh and vitrified cytoplasts in bovine SCT when using ZI cytoplasts. Embryos produced from vitrified cytoplasts had significantly higher lysis than embryos produced with fresh cytoplasts. Day 5 cleavage was significantly higher in embryos produced with fresh cytoplasts than with vitrified cytoplasts. There was a four-fold difference in total blastocyst development (B1-3) between embryos produced with fresh cytoplasts than with vitrified cytoplasts. This difference was not statistically different, but this is likely due to low sample numbers. The reason that a significant change was seen in cleavage but not in blastocyst development may be due to culture difficulties between days 5-7. One of the three repeats had reconstructs that all cleavage by day 5 but then lysed by day 7 in both treatment groups lowering blastocyst development rates. Lysis between days 5-7 also occurred in all PG controls indicating that the lysis may have been caused

by a media or oil issue. High-quality blastocysts were produced from the fresh cytoplasts, but no high-quality blastocysts could be produced with vitrified cytoplasts, indicating development and/or reprogramming may be hindered to some degree by the vitrification of cytoplasts. Park et al. (2015) also found that blastocyst production was significantly lowered when using vitrified ZI cytoplasts in bovine SCT compared to fresh. However, when the cytoplasts were activated before vitrification, the blastocyst development was no longer significantly different from fresh cytoplasts.

Studies that completed SCT with vitrified ZI oocytes, which were enucleated after thawing, found that the fusion, cleavage, and blastocyst development rates were not significantly different to those from fresh oocyte controls (Atabay et al., 2004; Dinnyés et al., 2000). Hou et al. (2005), on the other hand, found that using ZI vitrified oocytes for bovine SCT produced significantly lower fusion, cleavage, and blastocyst development than fresh oocytes ET with a blastocyst from a vitrified oocyte did achieve one live birth which shows that vitrified oocytes are capable of reprogramming and supporting development to term. In all the SCT studies, vitrified oocytes lead to blastocyst development in the 20% range, higher than has been shown in this thesis (Atabay et al., 2004; Dinnyés et al., 2000; Hou et al., 2005). This could suggest that either the removal of the ZP makes the cell more vunerable to vitrification damage or that the culture conditions in this thesis are suboptimal. There is a need for ECT experiments which use thawed ZI oocytes and then denudes them for ZF CT. There is also a need for improving baseline blastocyst development produced by the methods in this thesis.

The production of a blastocyst showed that vitrified/thawed cytoplasts can maintain their reprogramming ability to a level that is sufficient for a somatic donor, although at lowered rates compared to fresh cytoplasts. The ability to produce a high-quality blastocyst for ET has not yet been proven with the current AgResearch vitrification/thawing methods. Total blastocyst development occurred at very low levels, so protocols would need significant improvement before they can be used for routine CT.

5.4.4 ECT with thawed cytoplasts

It was proven that it is possible for vitrified cytoplasts to maintain the ability to reprogram somatic donors and support development to a blastocyst stage. The next

step was to test whether vitrified cytoplasts could support the reprogramming and development of embryonic donors. To my knowledge, no studies on the ability of vitrified recipient cells to support the reprogramming and development of ePSCs have been completed[4]. Bovine ECT with vitrified cytoplasts paired with blastomere donors has been performed by Booth et al. (1999); Peura et al. (1999). These studies varied in embryonic donor type, vitrification, and CT protocols compared to this thesis.

While no difference with somatic donors was seen, fusion of embryonic donors with fresh cytoplasts was significantly higher than with vitrified cytoplasts. The fusion of fresh cytoplasts was not significantly different with embryonic donors in Section 4.4.3.1 or somatic donors in Section 4.4.3.2, meaning the smaller size of the embryonic donor cell size was not influencing fusion. The fusion of a somatic donors with vitrified cytoplasts was significantly higher than with embryonic donors. The difference between donor types was not a factor in fresh cytoplast fusion, and vitrification did not influence somatic fusion. Thus, the difference in fusion between vitrified and fresh cytoplasts was enhanced when using embryonic donors. While a difference in fusion was seen between vitrified cytoplasts and fresh cytoplasts when the embryonic donor was an ePSC, Booth et al. (1999); Peura et al. (1999) did not observe a difference when the donor was a blastomere. The fusion rates with vitrified cytoplasts in this thesis for both donor types aligned with the range in Booth et al. (1999) but the fusion rate with embryonic donors was slightly less than in Peura et al. (1999).

Embryos produced from vitrified cytoplasts had significantly higher lysis than embryos produced with fresh cytoplasts. There was no significant difference in lysis between somatic or embryonic donors with vitrified cytoplasts. In both types of CT, it appears the lysis is from the vitrification compromising cytoplast membrane integrity. Booth et al. (1999) also found that there was a higher rate of lysis with blastomere donors indicating that incomplete membrane recovery occurs in vitrified cytoplasts.

ECT development was not significantly different between embryos produced with fresh vs vitrified cytoplasts, but a larger sample size is needed. Two of the five repeats had reconstructs that all reached the cleavage stage but then lysed in both treatment

[4] As of 1st November 2022.

groups lowering blastocyst development rates. Lysis between days 5-7 also occurred in all PG controls indicating that the lysis may have been caused by a LSOF media or oil issue. Differences between cleavage and blastocyst development using vitrified cytoplasts were not significantly different between using somatic or embryonic donors. Both Booth et al. (1999); Peura et al. (1999) found that the use of vitrified cytoplasts reduced cleavage and blastocyst development after CT with blastomere donors, especially if cytoplasts were activated after vitrification and before fusion. Peura et al. (1999) found that reconstructs from blastomeres and vitrified cytoplasts produced blastocysts at 4-9% rate and Booth et al. (1999) found that reconstructs from blastomeres and vitrified cytoplasts could produce blastocysts at a 7% rate, showing that vitrified cytoplasts can support to development of some embryonic donor cell types. Booth et al. (1999) show that vitrified cytoplasts were able to support development to term as they produced homozygotic twins from a blastocyst formed with a vitrified cytoplast and a blastomere donor. However, the twins died, and it is unknown whether the death was related to the twin pregnancy or the CT.

The reason vitrified cytoplasts were able to produce blastocysts in the literature but not in this thesis may be due to the difference between ePSCs and blastomeres or due to differences in methodology. The concentration of DMSO and EG cryoprotectants and the time of exposure to them is crucial because cryoprotectant affect the extent of dehydration in the cells (Chian et al., 2004). Booth et al. (1999); Peura et al. (1999) used higher concentrations of DMSO and EG during vitrification than were used in this thesis, which could be a reason they saw blastocyst development from ECT while we did not. The presence and concentration of sucrose was important because it acted as an osmotic buffer and its extracellular concentration affects the level of osmotic pressure on the bovine oocyte (Hou et al., 2005; Otoi et al., 1995). This affects the degree of dehydration of the oocytes and the level of influx of cryoprotectants. Furthermore, Otoi et al. (1995) found that different concentrations of sucrose in vitrification media affected the developmental competence of bovine oocytes. During thawing, the osmotic buffer of the sucrose can prevent excessive osmotic swelling in the oocyte that could damage it (Hou et al., 2005). Sucrose was included in the vitrification and thawing solutions in this thesis. Booth et al. (1999); Peura et al. (1999) used sucrose at lower concentrations or excluded it from their vitrification and thawing solutions. The difference in degrees of osmotic buffer may have been a reason for the difference in development between this thesis and the literature. The cytoplasts used

by Booth et al. (1999); Peura et al. (1999) were activated before vitrification or activated after vitrification and before fusion which may add additional variation to the development. Activating cytoplasts before vitrification has been shown to lower the levels of reductive oxidation stress close to the level in fresh cytoplasts while the level remains heightened in vitrified cytoplasts that were not pre-activated (Park et al., 2015). The transcriptome of activated then vitrified cytoplasts is closer to fresh cytoplasts than non-activated vitrified cytoplasts.

Blastocysts, including high-quality ones were produced from embryonic donors with fresh cytoplasts but no blastocysts were produced using vitrified cytoplasts. Although vitrified cytoplasts were capable of supporting reprogramming and development in somatic donors, we cannot conclude that they can support embryonic donors. The sample size was higher for ECT with vitrified cytoplasts than SCT indicating that the quantity of ECT reconstructs was not the reason no blastocysts were produced.

5.4.5 Double SCT with thawed cytoplasts

The maternal factors in a second fresh cytoplast which contribute to the improvement in blastocyst development in double SCT are unknown (Delaney et al., 2007; Green et al., 2016). The production of one blastocyst in Section 4.4.3.1 showed that vitrified cytoplasts can contain sufficient intact factors to produce blastocysts from SCT in principle. So, it is plausible that sufficient cytoplasmic factors endure after vitrification to aid development when used in conjunction with a fresh cytoplast in SCT. Or the additional vitrified cytoplast may dilute anti-reprogramming factors in the reconstruct. There was no difference in the fusion of fresh-fresh double reconstructs and the fusion of fresh-vitrified double reconstructs as both displayed full fusion. There was also no difference in lysis, cleavage or blastocyst development between embryos produced from fresh-fresh cytoplasts and embryos produced with fresh-vitrified cytoplasts. So far, it cannot be shown that blastocysts can be produced from fresh-vitrified cytoplasts. It cannot yet be proven that sufficient maternal factors are preserved in a bovine vitrified cytoplast to contribute to the development and reprogramming of a somatic reconstruct. The experiment to test this hypothesis had only one repeat and an extremely low sample size for each group. Considering that the average control SCT blastocyst development in this thesis was 8% (data not shown), a sample size of 9-10 was not enough to expect blastocyst formation. The low sample size and low number

of repeats was due to the limitation of thesis time constraints, covid lockdowns, and covid resource constraints. The results of this experiment are only preliminary and unreliable. At least two more repeats are required to draw any conclusions from the data.

5.4.6 Future recommendations

In the future, trailing vitrified oocytes instead of cytoplasts for the CT experiments should be considered. The higher survival rates in this thesis and the higher CT development rates in the literature suggests that ZI oocytes maintain reprogramming and developmental ability better than cytoplasts.

In the future, reagents can be added before or after the vitrification of bovine oocytes to minimize meiotic spindle damage (Girka et al., 2022; Hwang et al., 2013). A microtubule recovery agent can be added to post-thawing media to aid meiotic spindle repolymerization and recovery. Y-27632 can inhibit rho-associated coiled-coil kinase activity in vitrified bovine oocytes (Hwang et al., 2013). Inhibiting these proteins prevents cytoplasmic division and apoptosis leading to increased developmental competence. Girka et al. (2022) found that the proportion of bovine oocytes with normal microtubule distribution and chromosome arrangement could be increased with Y-27632 after thawing. They also found that treatment with microtubule stabilizing agents, such as cysteine or paclitaxel, before vitrification can prevent microtubule depolymerization. Cysteine encourages glutathione accumulation to protect against reactive oxygen species for recovery.

Additional repeats are also required of the double cytoplast vitrified SCT in order to obtain conclusive data.

Finally, a molecular analysis of biochemical properties that are intact and damaged before and after vitrification in oocytes and cytoplasts may reveal the reason that both cell types displayed lowered developmental potential.

5.5 General discussion and future recommendations

This study demonstrated that there was a broad range in blastocyst development between the 19 different ECT experiments, independent of the variation introduced by different treatments. The total blastocyst development in PG controls varied from 0-41% between runs and high-quality blastocyst development varied from 0-18%. The total blastocyst development in non-treated ECT controls varied from 0-25% and high-quality blastocyst development varied from 0-17%. As the conditions for controls were the same throughout experiments it would be expected that the development rates should be similar. The large range may be explained by the weekly variation in ovary quality. Ovary collections alternated between different abattoirs which caused the lengths of incubation in saline to differ by a couple of hours. Collections also varied in the different ages of mothers and the season. Summer and winter ovine oocytes can vary in the number of 2–8 mm follicles per ovary, and the cleavage and blastocyst rate following chemical activation (Zeron et al., 2001).

The average production of blastocysts from ECT with all treatments in this study was ≈ 7% out of reconstructs that went into IVC. The average production of blastocysts from ECT with standard protocol and no treatment was ≈ 9% out of reconstructs that went into IVC. Blastocyst production was lower than Bogliotti et al. (2018) who used bEPSCs as CT donors and established a blastocyst development rate of 10-20% and Zhao et al. (2021) who used bEPSCS in CT to establish a blastocyst development rate of 21%. Other studies have found that of CT embryos derived from an ES cell nucleus 10–30% reach the morula/blastocyst stage *in vitro* (Oback & Wells, 2002). Cloning with bovine cultured ICM donors has also been able to generate blastocysts at a rate of 15% (Sims & First, 1994). The lowered average rate of blastocyst development in this study compared to the literature can be explained by the variation between runs. Some runs had zero development, bringing the averages down. The maximum blastocyst development from non-treated controls achieved in a CT run in this thesis was 25%, which exceeds rates in other literature with bEPSCs, meaning embryo-derived cloning protocols in this thesis can improve blastocyst development with further refinement and consistency.

As no ETs were completed with blastocysts during this thesis, their ability to produce live offspring is unknown. Although we can conclude whether a treatment has

beneficial effects on bovine blastocyst development, no conclusions on the effect on overall cloning efficiency can be made. A treatment that improves blastocyst development may not necessarily improve pregnancy and healthy birth rates. For example, the TM/blastocyst development of CT with cumulus or fibroblast nuclei (55%) is higher than with an ES cell nucleus (10–30%) while blastocysts that reach adulthood is 5–15-fold higher from ES cell nuclei than cumulus or fibroblast nuclei (Oback & Wells, 2002). High-quality blastocysts should undergo ET into recipient animals in order to calculate overall cloning efficiency. Cloning efficiency with blastocysts from double, vitrified, aged, or synchronized treatments can be compared to the cloning efficiencies of non-treated ECT blastocysts. The cloning efficiency of the ECT blastocysts can be compared to the cloning efficiency of SCT blastocysts for insight into the value of the embryo-derived donor cells.

To advance the findings of this study, testing the cells of the embryo-derived outgrowths for pluripotency is required to confidently confirm the cells are ePSCs. Testing should begin with molecular assessment of pluripotency. This could be done through either reverse transcription quantitative real-time PCR or histological staining for pluripotency markers such as *SOX2, OCT4* and *NANOG* can be done (De Los Angeles et al., 2015). Testing alkaline phosphatase levels which are downregulated during lineage differentiation can also illuminate the degree of pluripotency. The level of DNA methylation can be used to determine whether the cells are in a ground state (low levels of methylation) or primed state (high levels of methylation). bEPSCs and fibroblasts have comparable levels of DNA methylation which can be revealed through bisulfite sequencing (Zhao et al., 2021). Molecular tests can suggest pluripotency, but then functional assays are needed to confirm the developmental potential of the ePSCs (De Los Angeles et al., 2015). The next steps of testing for pluripotency would be functional assessments such as an embryoid body assay, teratoma formation or tetraploid formation (De Los Angeles et al., 2015; Pettinato et al., 2015). Finally, a germline chimera can be formed (De Los Angeles et al., 2015). A single suspected ePSC with can be introduced to a preimplantated host embryo and evaluated for whether it can support normal development and form all somatic cells and germline cells. This is typically measured by evaluating the somatic cells and germline cells of the chimera for expression of a target gene such as *tdTomato* that was in the ePSC (Zhao et al., 2021). It is important to determine pluripotency in the embryo-derived

donor cells as reducing the reprogramming required is the main motivation for using embryonic donors over somatic donors in CT.

Further steps to encourage the reprogramming of donor cells is to include treatments for epigenetic alterations. It has been observed that blastocysts from CT have incomplete reprogramming of the epigenome (Ding et al., 2008). The demethylation of some DNA regions which occurs after normal fertilization, is absent after CT fusion. As a result, CT embryos have hypermethylation which promotes heterochromatin. This limits the access of transcription factors which decreases the activation of gene transcription (Czernik et al., 2019). Treatments for the inhibition of DNA methylation transfer during replication have been trialled, such as 5-aza-dC, which improved *in vitro* development of the bovine embryos (Ding et al., 2008). Increasing acetylation loosens the chromatin configuration to allow access to the transcription factors (Czernik et al., 2019). Treatment with deacetylase inhibitors such as Trichostatin A and scriptaid have been trialled and shown to increase acetylation and improve blastocyst development after CT (Satoshi Akagi et al., 2011; Ding et al., 2008). One main epigenetic alteration noted for its resistance to reprogramming during CT is the histone methylation of H3K9me3 (Antony et al., 2013). This marker is present in CT embryos but not IVF embryos. Overexpression of the demethylase Kdm4b in donor cells has been shown to decrease H3K9me3 and H3K936me3 levels in bovine embryos (Meng et al., 2020). Kdm4E and Kdm4D used in cattle SCT can decrease methylation of H3K9me3 and improve blastocyst development (Liu et al., 2018). Incorporation of epigenetic reprogramming treatments such as KDM4D/E isoforms into ECT should be trialled to analyse its effect on embryonic donor methylation and to increase developmental potential of embryos. Especially when using trophoblast donor cells which have high levels of H3K9me3 accumulation and are highly resistant to genomic reprogramming during cell transfer (Hada et al., 2022). When Hada et al. (2022) removed H3K9me3 with Kdm4D mRNA the genome was reprogrammed and the trophoblast cells produced live offspring from ECT. This process could be crucial as the embryo-derived donor cells used by the outgrowths in this thesis are predominantly trophoblastic.

5.6 Conclusion

To conclude, suitable treatments of the ZP to generate embryo-derived outgrowths from blastocysts were established. Blastocysts were produced through *in vitro* fertilization at 82% efficiency and used for embryo-derived donor outgrowths. These outgrowths were formed at a maximum efficiency of 88% on days 4-5 of growth, which was achieved through mechanical ZP dissection. It was established that pronase treating the ZP lowered the production of embryo-derived donor outgrowths to 36%. Blastocyst production from ECT was achieved using cells from the embryo-derived outgrowths. Arresting the embryo-derived outgrowths into mitosis with incubation in 500nM nocodazole overnight did not improve blastocyst development with MII cytoplasts and ionomycin/CHX activation. Arresting the embryo-derived outgrowths into mitosis lead to a 22% PPB extrusion rate in reconstructs. A lowered level of blastocyst development was seen in the reconstructs with extrusions. A series of treatments on the bovine MII cytoplasts before they received embryo-derived donors without mitotic synchronization, and subsequent ionomycin/DMAP activation, were trialled. The first treatment was aging and cooling the cytoplasts to alter their MII state. Aging and cooling increased the lysis of reconstructs and decreased the blastocyst development of embryos to 4%. Aged and cooled cytoplasts were not observed to lead to high-quality blastocysts. Next, increasing the volume of a MII cytoplast was trialled and was not observed to have any effect on embryonic development with embryo-derived donors. The effect of vitrification into the reprogramming and developmental potential of oocytes and cytoplasts was investigated. Oocytes were successfully vitrified and thawed with a survival rate of 97%. These vitrified oocytes supported PG development to the blastocyst stage but at a lower rate than fresh oocytes did. Cytoplasts were successfully vitrified and thawed with a survival rate of 92%. These vitrified cytoplasts supported development to the blastocyst stage after CT with somatic donors but at a lower rate than fresh cytoplasts did. The ability of vitrified cytoplasts to support the development of the embryo-derived donors to the blastocyst stage after CT could not be proven.

References

Akagi, S., Matsukawa, K., Mizutani, E., Fukunari, K., Kaneda, M., Watanabe, S., & Takahashi, S. (2011, Feb). Treatment with a histone deacetylase inhibitor after nuclear transfer improves the preimplantation development of cloned bovine embryos. *J Reprod Dev, 57*(1), 120-126. https://doi.org/10.1262/jrd.10-058a

Akagi, S., Matsukawa, K., & Takahashi, S. (2014). Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *Journal of Reproduction and Development, 60*(5), 329-335. https://doi.org/https://doi.org/10.1262/jrd.2014-057

Akagi, S., Tamura, S., & Matsukawa, K. (2020). Timing of the first cleavage and in vitro developmental potential of bovine somatic cell nuclear transfer embryos activated by different protocols. *Cellular reprogramming, 22*(1), 36-42. https://doi.org/https://doi.org/10.1089/cell.2019.0074

Alberio, R., Motlik, J., Stojkovic, M., Wolf, E., & Zakhartchenko, V. (2000). Behavior of M‐phase synchronized blastomeres after nuclear transfer in cattle. *Molecular Reproduction and Development: Incorporating Gamete Research, 57*(1), 37-47. https://doi.org/10.1002/1098-2795(200009)57:1

Alteri, A., Vigano, P., Maizar, A. A., Jovine, L., Giacomini, E., & Rubino, P. (2018). Revisiting embryo assisted hatching approaches: a systematic review of the current protocols. *Journal of assisted reproduction and genetics, 35*(3), 367-391. https://doi.org/https://doi.org/10.1007/s10815-018-1118-4

Antony, J., Oback, F., Chamley, L. W., Oback, B., & Laible, G. (2013). Transient JMJD2B-mediated reduction of H3K9me3 levels improves reprogramming of embryonic stem cells into cloned embryos. *Molecular and cellular biology, 33*(5), 974-983. https://doi.org/https://doi.org/10.1128/MCB.01014-12

Appleby, S. J. (2015). *Using putative bovine embryonic stem cell-like cells for nuclear transfer cloning to determine their functional potential* [Masters, University of Waikato]. Hamilton, New Zealand. https://hdl.handle.net/10289/11477

Appleby, S. J., Turner, P. M., Turner, F. C., Dhali, A., MacLean, Z. L., & Oback, B. (2022). Serial embryonic cloning improves in vitro but not in vivo development from mitotic pluripotent cells in cattle. *Frontiers in genetics*, 1957. https://doi.org/https://doi.org/10.3389/fgene.2022.933534

Atabay, E., Takahashi, Y., Katagiri, S., Nagano, M., Koga, A., & Kanai, Y. (2004). Vitrification of bovine oocytes and its application to intergeneric somatic cell nucleus transfer. *Theriogenology, 61*(1), 15-23. https://doi.org/https://doi.org/10.1016/S0093-691X(03)00179-1

Balaban, B., Urman, B., Alatas, C., Mercan, R., Mumcu, A., & Isiklar, A. (2002). A comparison of four different techniques of assisted hatching. *Human Reproduction, 17*(5), 1239-1243. https://doi.org/https://doi.org/10.1093/humrep/17.5.1239

Barlow, D. P., & Bartolomei, M. S. (2014). Genomic imprinting in mammals. *Cold Spring Harbor perspectives in biology, 6*(2), a018382. https://doi.org/https://doi.org/10.1101/cshperspect.a018382

Bhak, J.-S., Lee, S.-L., Ock, S.-A., Choe, S.-Y., & Rho, G.-J. (2006). Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. *Animal Reproduction Science*, *92*(1-2), 37-49. https://doi.org/https://doi.org/10.1016/j.anireprosci.2005.04.016

Bianchi, V., Macchiarelli, G., Borini, A., Lappi, M., Cecconi, S., Miglietta, S., Familiari, G., & Nottola, S. A. (2014). Fine morphological assessment of quality of human mature oocytes after slow freezing or vitrification with a closed device: a comparative analysis. *Reproductive Biology and Endocrinology*, *12*(1), 1-13. https://doi.org/https://doi.org/10.1186/1477-7827-12-110

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., Belmonte, J. C. I., & Ross, P. J. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proceedings of the National Academy of Sciences*, *115*(9), 2090-2095. https://doi.org/doi:10.1073/pnas.1716161115

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., & Izpisua Belmonte, J. C. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proceedings of the National Academy of Sciences*, *115*(9), 2090-2095. https://doi.org/https://doi.org/10.1073/pnas.1716161115

Booth, P., Tan, S., Reipurth, R., Holm, P., & Callesen, H. (2001). Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. *Cloning & Stem Cells*, *3*(3), 139-150. https://doi.org/https://doi.org/10.1089/153623001753205098

Booth, P., Vajta, G., Høj, A., Holm, P., Jacobsen, H., Greve, T., & Callesen, H. (1999). Full-term development of nuclear transfer calves produced from open-pulled straw (OPS) vitrified cytoplasts: work in progress. *Theriogenology*, *51*(5), 999-1006. https://doi.org/https://doi.org/10.1016/S0093-691X(99)00045-X

Bordignon, V., & Smith, L. C. (1998). Telophase enucleation: an improved method to prepare recipient cytoplasts for use in bovine nuclear transfer. *Molecular Reproduction and Development: Incorporating Gamete Research*, *49*(1), 29-36. https://doi.org/https://doi.org/10.1002/(SICI)1098-2795(199801)49:1<29::AID-MRD4>3.0.CO;2-Q

Boulet, J., Cunningham, J. W., & Mehra, M. R. (2022). Cardiac xenotransplantation: challenges, evolution, and advances. *Basic to Translational Science*, *7*(7), 716-729. https://doi.org/https://doi.org/10.1016/j.jacbts.2022.05.003

Brophy, B., Smolenski, G., Wheeler, T., Wells, D., L'Huillier, P., & Laible, G. (2003). Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein. *Nature biotechnology*, *21*(2), 157-162. https://doi.org/https://doi.org/10.1038/nbt783

Bureau, W. S., Bordignon, V., Leveillee, C., Smith, L., & King, W. (2003). Assessment of chromosomal abnormalities in bovine nuclear transfer embryos and in their donor cells. *Cloning & Stem Cells*, *5*(2), 123-132. https://doi.org/https://doi.org/10.1089/153623003322234722

Buschiazzo, J., Ríos, G. L., Canizo, J. R., Antollini, S. S., & Alberio, R. H. (2017). Free cholesterol and cholesterol esters in bovine oocytes: Implications in survival and membrane raft organization after cryopreservation. *PloS one*, *12*(7), e0180451. https://doi.org/https://doi.org/10.1371/journal.pone.0180451

Carlson, D. F., Lancto, C. A., Zang, B., Kim, E.-S., Walton, M., Oldeschulte, D., Seabury, C., Sonstegard, T. S., & Fahrenkrug, S. C. (2016). Production of hornless dairy cattle from genome-edited cell lines. *Nature biotechnology*, *34*(5), 479-481. https://doi.org/https://doi.org/10.1038/nbt.3560

Cevik, M., Taş, A., Akkoc, T., Bağiş, H., & Arat, S. (2009). A comparative study of parthenogenetic activation and in vitro fertilization of in vitro matured bovine oocytes. *Turkish Journal of Veterinary & Animal Sciences*, *33*(5), 393-399. https://doi.org/https://doi.org/10.3906/vet-0803-5

Chambers, I., & Tomlinson, S. R. (2009). The transcriptional foundation of pluripotency. https://doi.org/https://doi.org/10.1242/dev.024398

Chen, S., Lien, Y., Chao, K., Ho, H.-N., Yang, Y., & Lee, T. (2003). Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a review article. *Molecular and Cellular Endocrinology*, *202*(1-2), 101-107. https://doi.org/https://doi.org/10.1016/S0303-7207(03)00070-4

Chian, R.-C., Kuwayama, M., Tan, L., Tan, J., Kato, O., & Nagai, T. (2004). High survival rate of bovine oocytes matured in vitro following vitrification. *Journal of Reproduction and Development*, *50*(6), 685-696. https://doi.org/https://doi.org/10.1262/jrd.50.685

Choi, J. K., Yue, T., Huang, H., Zhao, G., Zhang, M., & He, X. (2015). The crucial role of zona pellucida in cryopreservation of oocytes by vitrification. *Cryobiology*, *71*(2), 350-355. https://doi.org/https://doi.org/10.1016/j.cryobiol.2015.08.012

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de León, F. A., & Robl, J. M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science*, *280*(5367), 1256-1258. https://doi.org/https://DOI:10.1126/science.280.5367.1256

Cohen, J., & Feldberg, D. (1991). Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. *Molecular Reproduction and Development*, *30*(1), 70-78. https://doi.org/https://doi.org/10.1002/mrd.1080300110

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., & Marraffini, L. A. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, *339*(6121), 819-823. https://doi.org//doi/abs/10.1126/science.1231143

Cooper, D. K., Hara, H., Iwase, H., Yamamoto, T., Li, Q., Ezzelarab, M., Federzoni, E., Dandro, A., & Ayares, D. (2019). Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation*, *26*(4), e12516. https://doi.org/https://doi.org/10.1111/xen.12516

Crowe, A. D., Lonergan, P., & Butler, S. T. (2021). Invited review: Use of assisted reproduction techniques to accelerate genetic gain and increase value of beef production in dairy herds. *Journal of Dairy Science*, *104*(12), 12189-12206. https://doi.org/https://doi.org/10.3168/jds.2021-20281

Czernik, M., Anzalone, D. A., Palazzese, L., Oikawa, M., & Loi, P. (2019). Somatic cell nuclear transfer: Failures, successes and the challenges ahead. *International Journal*

of Developmental Biology, *63*(3-4-5), 123-130. https://doi.org/https://doi:10.1387/ijdb.180324mc

De, A. K., Malakar, D., Jena, M. K., Dutta, R., Garg, S., & Akshey, Y. S. (2012). Zona-free and with-zona parthenogenetic embryo production in goat (Capra hircus)—effect of activation methods, culture systems and culture media. *Livestock Science*, *143*(1), 35-42. https://doi.org/https://doi.org/10.1016/j.livsci.2011.08.012

De Felici, M., & Siracusa, G. (1982). "Spontaneous" hardening of the zona pellucida of mouse oocytes during in vitro culture. *Gamete Research*, *6*(2), 107-113. https://doi.org/https://doi.org/10.1002/mrd.1120060203

De Los Angeles, A., Ferrari, F., Xi, R., Fujiwara, Y., Benvenisty, N., Deng, H., Hochedlinger, K., Jaenisch, R., Lee, S., & Leitch, H. G. (2015). Hallmarks of pluripotency. *Nature*, *525*(7570), 469-478. https://doi.org/https://doi.org/10.1038/nature15515

de Sousa, R. V., da Silva Cardoso, C. R., Butzke, G., Dode, M. A. N., Rumpf, R., & Franco, M. M. (2017). Biopsy of bovine embryos produced in vivo and in vitro does not affect pregnancy rates. *Theriogenology*, *90*, 25-31. https://doi.org/https://doi.org/10.1016/j.theriogenology.2016.11.003

Delaney, T., Oback, B., & Wells, D. (2007). Doubling cytoplast volume increases embryo development following somatic cell nuclear transfer in cattle. 4th Annual Conference of the Asian Reproductive Biotechnology Society,

Ding, X., Wang, Y., Zhang, D., Guo, Z., & Zhang, Y. (2008). Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2′ -deoxycytidine and trichostatin A. *Theriogenology*, *70*(4), 622-630. https://doi.org/https://doi.org/10.1016/j.theriogenology.2008.04.042

Dinnyes, A., Cindy Tian, X., & Oback, B. (2006). Nuclear transfer for cloning animals. *Reviews in Cell Biology and Molecular Medicine*. https://doi.org/https://doi.org/10.1002/3527600906.mcb.200400139.pub2

Dinnyés, A., Dai, Y., Jiang, S., & Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. *Biology of reproduction*, *63*(2), 513-518. https://doi.org/https://doi.org/10.1095/biolreprod63.2.513

Dolgushina, N., Ibragimova, E., Romanov, A. Y., Makarova, N., Dovgan, A., Syrkasheva, A., & Kalinina, E. (2018). Role of pronase hatching in enhancing the effectiveness of assisted reproductive technology programs. *Akusherstvo i ginekologiya= Obstetrics and Gynecology*, *3*, 70-74. https://doi.org/https://doi.org/10.18565/aig.2018.3.70-74

Du, F., Sung, L. Y., Tian, X. C., & Yang, X. (2002). Differential cytoplast requirement for embryonic and somatic cell nuclear transfer in cattle. *Molecular Reproduction and Development: Incorporating Gamete Research*, *63*(2), 183-191. https://doi.org/https://doi.org/10.1002/mrd.10172

Dzitsiuk, V., & Tipilo, H. (2019). Chromosomal anomalies in dairy cattle as reasons of impaired fertility. *Agricultural Science and Practice*, *6*(1), 60-66. https://doi.org/https://doi.org/10.15407/agrisp6.01.060

Eghbalsaied, S., Hyder, I., & Kues, W. A. (2020). A versatile bulk electrotransfection protocol for murine embryonic fibroblasts and iPS cells. *Scientific reports*, *10*(1), 1-10. https://doi.org/https://doi.org/10.1038/s41598-020-70258-w

Engelstädter, J. (2017). Asexual but not clonal: evolutionary processes in automictic populations. *Genetics*, *206*(2), 993-1009. https://doi.org/https://doi.org/10.1534/genetics.116.196873

Environment, M. f. t. (2022). New Zealand's Greenhouse Gas Inventory 1990–2020. *Ministry for the Environment*, *1*, 149-182.

Feng, H. L., Hershlag, A., Scholl, G. M., & Cohen, M. A. (2009). A retroprospective study comparing three different assisted hatching techniques. *Fertility and sterility*, *91*(4), 1323-1325. https://doi.org/https://doi.org/10.1016/j.fertnstert.2008.02.133

Fernandes, C. B., Devito, L. G., Martins, L. R., Blanco, I. D. P., de Lima Neto, J. F., Tsuribe, P. M., Gonçalves, C. G. P., & da Cruz Landim-Alvarenga, F. (2014). Artificial activation of bovine and equine oocytes with cycloheximide, roscovitine, strontium, or 6-dimethylaminopurine in low or high calcium concentrations. *Zygote*, *22*(3), 387-394. https://doi.org/https://doi.org/10.1017/S0967199412000627

Fernandes, I., Chanut-Delalande, H., Ferrer, P., Latapie, Y., Waltzer, L., Affolter, M., Payre, F., & Plaza, S. (2010). Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. *Developmental cell*, *18*(1), 64-76. https://doi.org/https://doi.org/10.1016/j.devcel.2009.11.009

Ferré, L., Kjelland, M., Strøbech, L., Hyttel, P., Mermillod, P., & Ross, P. (2020). Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animal*, *14*(5), 991-1004. https://doi.org/https://doi.org/10.1017/S1751731119002775

Fisher, C. L., & Fisher, A. G. (2011). Chromatin states in pluripotent, differentiated, and reprogrammed cells. *Current opinion in genetics & development*, *21*(2), 140-146. https://doi.org/https://doi.org/10.1016/j.gde.2011.01.015

Fulka Jr, J., First, N. L., Loi, P., & Moor, R. M. (1998). Cloning by somatic cell nuclear transfer. *Bioessays*, *20*(10), 847-851. https://doi.org/https://doi.org/10.1002/(SICI)1521-1878(199810)20:10<847::AID-BIES10>3.0.CO;2-F

Gabrielsen, A., Bhatnager, P. R., Petersen, K., & Lindenberg, S. (2000). Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following in vitro fertilization treatment. *Journal of assisted reproduction and genetics*, *17*(6), 323-328. https://doi.org/https://doi.org/10.1023/A:1009453011321

Gall, L., Dedieu, T., Chesne, P., Ruffini, S., Sevellec, C., Peynot, N., Renard, J. P., & Heyman, Y. (1996). Bovine embryo cloning: characterization of the recipient cytoplasts by phosphorylation patterns and kinase activities. *Development, growth & differentiation*, *38*(5), 517-525. https://doi.org/https://doi.org/10.1046/j.1440-169X.1996.t01-4-00008.x

Girka, E., Gatenby, L., Gutierrez, E. J., & Bondioli, K. R. (2022). The effects of microtubule stabilizing and recovery agents on vitrified bovine oocytes. *Theriogenology*, *182*, 9-16. https://doi.org/https://doi.org/10.1016/j.theriogenology.2022.01.031

Goovaerts, I., Leroy, J., Jorssen, E., & Bols, P. (2010). Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture. *Theriogenology*, *74*(9), 1509-1520. https://doi.org/https://doi.org/10.1016/j.theriogenology.2010.06.022

Goto, K., Tanaka, M., Ookutsu, S., & NAKANISHI, Y. (1997). Production of a nuclear transferred calf by the intracytoplasmic injection of donor cells. *Journal of Reproduction and Development*, *43*(3), 257-260. https://doi.org/ https://doi.org/10.1262/jrd.43.257

Green, A., Oback, F., Oliver, J., Popovic, L., McGowan, L., Appleby, S., Meng, F., Hyndman, D., Carson, D., & Wells, D. (2016). 28 DOUBLING OOCYTE CYTOPLASM VOLUME INCREASES BLASTOCYST QUALITY FOLLOWING INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER IN ARGALI SHEEP (OVIS AMMON). *Reproduction, Fertility and Development*, *28*(2), 144-144. https://doi.org/https://doi.org/10.1071/RDv28n2Ab28

Grollman, A. P. (1967). Inhibitors of protein biosynthesis: II. Mode of action of anisomycin. *Journal of Biological Chemistry*, *242*(13), 3226-3233. https://doi.org/ttps://doi.org/10.1016/S0021-9258(18)95953-3

Gropp, M., Shilo, V., Vainer, G., Gov, M., Gil, Y., Khaner, H., Matzrafi, L., Idelson, M., Kopolovic, J., Zak, N. B., & Reubinoff, B. E. (2012). Standardization of the teratoma assay for analysis of pluripotency of human ES cells and biosafety of their differentiated progeny. *PloS one*, *7*(9), e45532. https://doi.org/10.1371/journal.pone.0045532

Habermann, F. A., André, S., Kaltner, H., Kübler, D., Sinowatz, F., & Gabius, H.-J. (2011). Galectins as tools for glycan mapping in histology: comparison of their binding profiles to the bovine zona pellucida by confocal laser scanning microscopy. *Histochemistry and cell biology*, *135*(6), 539-552. https://doi.org/https://doi.org/10.1007/s00418-011-0814-2

Hada, M., Miura, H., Tanigawa, A., Matoba, S., Inoue, K., Ogonuki, N., Hirose, M., Watanabe, N., Nakato, R., & Fujiki, K. (2022). Highly rigid H3. 1/H3. 2–H3K9me3 domains set a barrier for cell fate reprogramming in trophoblast stem cells. *Genes & development*, *36*(1-2), 84-102. https://doi.org/https://doi.org/10.1101/gad.348782.121

Hagemann, A. R., Lanzendorf, S. E., Jungheim, E. S., Chang, A. S., Ratts, V. S., & Odem, R. R. (2010). A prospective, randomized, double-blinded study of assisted hatching in women younger than 38 years undergoing in vitro fertilization. *Fertility and sterility*, *93*(2), 586-591. https://doi.org/https://doi.org/10.1016/j.fertnstert.2009.01.116

Hakim, B. A., Tyagi, V., Agnihotri, S. K., Nath, A., Agrawal, A. K., Jain, A., Singh, D., Konwar, R., & Sachdev, M. (2021). Electroporation of mouse follicles, oocytes and embryos without manipulating zona pellucida. *Journal of Developmental Biology*, *9*(2), 13. https://doi.org/https://doi.org/10.3390/jdb9020013

Hanada, H., Takeda, K., Tagami, T., Nirasawa, K., Akagi, S., Adachi, N., Takahashi, S., Izaike, Y., Iwamoto, M., & Fuchimoto, D. I. (2005). Chromosomal instability in the cattle clones derived by somatic cell nuclear ‐ transfer. *Molecular Reproduction and Development: Incorporating Gamete Research*, *71*(1), 36-44. https://doi.org/https://doi.org/10.1002/mrd.20283

Harland, C., Durkin, K., Artesi, M., Karim, L., Cambisano, N., Deckers, M., Tamma, N., Mullaart, E., Coppieters, W., & Charlier, C. (2018). Rate of de novo mutation in dairy

cattle and potential impact of reproductive technologies. *Proc World Congr Genet Appl to Livest Prod*.

Hashimoto, M., & Takemoto, T. (2015). Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. *Scientific reports*, *5*(1), 1-8. https://doi.org/https://doi.org/10.1038/srep11315

Hatırnaz, Ş., Ata, B., Hatırnaz, E. S., Dahan, M. H., Tannus, S., Tan, J., & Tan, S. L. (2018). Oocyte in vitro maturation: A sytematic review. *Turkish journal of obstetrics and gynecology*, *15*(2), 112. https://doi.org/https://doi.org/10.4274/tjod.23911

Hennig, S. L., Owen, J. R., Lin, J. C., Young, A. E., Ross, P. J., Van Eenennaam, A. L., & Murray, J. D. (2020). Evaluation of mutation rates, mosaicism and off target mutations when injecting Cas9 mRNA or protein for genome editing of bovine embryos. *Scientific reports*, *10*(1), 1-9. https://doi.org/https://doi.org/10.1038/s41598-020-78264-8

Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous, S., Vignon, X., & Renard, J. P. (2002). Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biology of reproduction*, *66*(1), 6-13. https://doi.org/https://doi.org/10.1095/biolreprod66.1.6

Heyman, Y., Chesné, P., Lebourhis, D., Peynot, N., & Renard, J. P. (1994). Developmental ability of bovine embryos after nuclear transfer based on the nuclear source: in vivo versus in vitro. *Theriogenology*, *42*(4), 695-702. https://doi.org/https://doi.org/10.1016/0093-691X(94)90386-W

Hiiragi, T., & Solter, D. (2005). Reprogramming is essential in nuclear transfer. *Molecular Reproduction and Development: Incorporating Gamete Research*, *70*(4), 417-421. https://doi.org/https://doi.org/10.1002/mrd.20126

Hore, T. A., Rapkins, R. W., & Graves, J. A. M. (2007). Construction and evolution of imprinted loci in mammals. *Trends in Genetics*, *23*(9), 440-448. https://doi.org/https://doi.org/10.1016/j.tig.2007.07.003

Hou, Y.-P., Dai, Y.-P., Zhu, S.-E., Zhu, H.-B., Wu, T.-Y., Gong, G.-C., Wang, H.-P., Wang, L.-L., Liu, Y., & Li, R. (2005). Bovine oocytes vitrified by the open pulled straw method and used for somatic cell cloning supported development to term. *Theriogenology*, *64*(6), 1381-1391. https://doi.org/https://doi.org/10.1016/j.theriogenology.2005.03.012

Hur, Y. S., Park, J. H., Ryu, E. K., Yoon, H. J., Hur, C. Y., Lee, W. D., & Lim, J. H. (2011). Effect of artificial shrinkage on clinical outcome in fresh blastocyst transfer cycles. *Clinical and experimental reproductive medicine*, *38*(2), 87. https://doi.org/https://doi.org/10.5653/cerm.2011.38.2.87

Hurst, B. S., Tucker, K. E., Awoniyi, C. A., & Schlaff, W. D. (1998). Assisted hatching does not enhance IVF success in good-prognosis patients. *Journal of assisted reproduction and genetics*, *15*(2), 62-64. https://doi.org/https://doi.org/10.1007/BF02766826

Hwang, I.-S., Hara, H., Chung, H.-J., Hirabayashi, M., & Hochi, S. (2013). Rescue of vitrified-warmed bovine oocytes with rho-associated coiled-coil kinase inhibitor. *Biology of reproduction*, *89*(2), 26, 21-26. https://doi.org/https://doi.org/10.1095/biolreprod.113.109769

Hyder, I., Eghbalsaied, S., & Kues, W. A. (2020). Systematic optimization of square-wave electroporation conditions for bovine primary fibroblasts. *BMC molecular and cell biology*, *21*(1), 1-8. https://doi.org/https://doi.org/10.1186/s12860-020-00254-5

Ideta, A., Urakawa, M., Aoyagi, Y., & Saeki, K. (2005). Early morphological nuclear events and developmental capacity of embryos reconstructed with fetal fibroblasts at the M or G1 phase after intracytoplasmic nuclear injection in cattle. *Journal of Reproduction and Development*, *51*(2), 187-194. https://doi.org/https://doi.org/10.1262/jrd.16030

James E. Ferrell, J. (2020, 5th September 2022). Cell cycle. *AccessScience*. https://doi.org/https://doi.org/10.1036/1097-8542.116150

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816-821. https://doi.org/https://doi.org/10.1126/science.1225829

Jordan, M. A., Thrower, D., & Wilson, L. (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *Journal of cell science*, *102*(3), 401-416. https://doi.org/https://doi.org/10.1242/jcs.102.3.401

Kaneko, T., & Nakagawa, Y. (2020). Genome editing of rodents by electroporation of CRISPR/Cas9 into frozen-warmed pronuclear-stage embryos. *Cryobiology*, *92*, 231-234. https://doi.org/https://doi.org/10.1016/j.cryobiol.2020.01.016

Karavolias, N. G., Horner, W., Abugu, M. N., & Evanega, S. N. (2021). Application of gene editing for climate change in agriculture. *Frontiers in Sustainable Food Systems*, *5*, 685801. https://doi.org/https://doi.org/10.3389/fsufs.2021.685801

Khan, H. A. (2021). Isolation and Characterization of Stem Cells. In *Advances in Application of Stem Cells: From Bench to Clinics* (pp. 51-71). Springer. https://doi.org/https://doi.org/10.1007/978-3-030-78101-9_3

Khatun, M., Arifuzzaman, M., & Ashraf, A. (2011). Karyotype for identification of genetic abnormalities in cattle. *Asian Journal of Animal and Veterinary Advances*, *6*(2), 117-125. https://doi.org/https://doi.org/10.3923/ajava.2011.117.125

King, T. J., & Briggs, R. (1956). Serial transplantation of embryonic nuclei. Cold Spring Harbor symposia on quantitative biology,

Kinney, M. E., Wack, R. F., Grahn, R. A., & Lyons, L. (2013). Parthenogenesis in a Brazilian Rainbow Boa (E picrates cenchria cenchria). *Zoo Biology*, *32*(2), 172-176. https://doi.org/https://doi.org/10.1002/zoo.21050

Kinoshita, M., Kobayashi, T., Planells, B., Klisch, D., Spindlow, D., Masaki, H., Bornelöv, S., Stirparo, G. G., Matsunari, H., & Uchikura, A. (2021). Pluripotent stem cells related to embryonic disc exhibit common self-renewal requirements in diverse livestock species. *Development*, *148*(23), dev199901. https://doi.org/https://doi.org/10.1242/dev.199901

Konwinski, M., Solter, D., & Koprowski, H. (1978). Effect of removal of the zona pellucida on subsequent development of mouse blastocysts in vitro. *Reproduction*, *54*(1), 137-143. https://doi.org/https://doi.org/10.1530/jrf.0.0540137

Kou, Z., Kang, L., Yuan, Y., Tao, Y., Zhang, Y., Wu, T., He, J., Wang, J., Liu, Z., & Gao, S. (2010). Mice cloned from induced pluripotent stem cells (iPSCs). *Biology of reproduction*, *83*(2), 238-243. https://doi.org/https://doi.org/10.1095/biolreprod67.2.643

Kurosaka, S., Nagao, Y., Minami, N., Yamada, M., & Imai, H. (2002). Dependence of DNA synthesis and in vitro development of bovine nuclear transfer embryos on the stage of the cell cycle of donor cells and recipient cytoplasts. *Biology of reproduction*, *67*(2), 643-647. https://doi.org/https://doi.org/10.1095/biolreprod67.2.643

Laible, G., Cole, S.-A., Brophy, B., Wei, J., Leath, S., Jivanji, S., Littlejohn, M., & Wells, D. (2021). Holstein Friesian dairy cattle edited for diluted coat color as a potential adaptation to climate change. *BMC genomics*, *22*(1), 1-12. https://doi.org/https://doi.org/10.1186/s12864-021-08175-z

Lee, J.-H., & Campbell, K. H. (2006). Effects of enucleation and caffeine on maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities in ovine oocytes used as recipient cytoplasts for nuclear transfer. *Biology of reproduction*, *74*(4), 691-698. https://doi.org/https://doi.org/10.1095/biolreprod.105.043885

Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A., & Ishino, F. (2002). Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. https://doi.org/https://doi.org/10.1242/dev.129.8.1807

Leese, H. J., & Brison, D. R. (2015). *Cell signaling during mammalian early embryo development* (Vol. 843). Springer. https://doi.org/https://doi.org/10.1007.978-1-4939-2480-6

Li, S., Kang, J.-D., Jin, J.-X., Hong, Y., Zhu, H.-Y., Jin, L., Gao, Q.-S., Yan, C.-G., Cui, C.-D., & Li, W.-X. (2014). Effect of demecolcine-assisted enucleation on the MPF level and cyclin B1 distribution in porcine oocytes. *PloS one*, *9*(3), e91483. https://doi.org/https://doi.org/10.1371/journal.pone.0091483

Lindner, G. M., & Wright Jr, R. W. (1983). Bovine embryo morphology and evaluation. *Theriogenology*, *20*(4), 407-416. https://doi.org/https://doi.org/10.1016/0093-691X(83)90201-7

Liu, J.-L., Wang, M.-K., Sun, Q.-Y., Xu, Z., & Chen, D.-Y. (2000). Effect of telophase enucleation on bovine somatic nuclear transfer. *Theriogenology*, *54*(6), 989-998. https://doi.org/https://doi.org/10.1016/S0093-691X(00)00407-6

Liu, L., & Yang, X. (1999). Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activated bovine oocytes. *Biology of reproduction*, *61*(1), 1-7. https://doi.org/https://doi.org/10.1095/biolreprod61.1.1

Liu, X., Wang, Y., Gao, Y., Su, J., Zhang, J., Xing, X., Zhou, C., Yao, K., An, Q., & Zhang, Y. (2018). H3K9 demethylase KDM4E is an epigenetic regulator for bovine embryonic development and a defective factor for nuclear reprogramming. *Development*, *145*(4), dev158261. https://doi.org/https://doi.org/10.1242/dev.158261

Liu, X., Zhang, W., Farooq, U., Rong, N., Shi, J., Pang, N., Xu, L., Niu, L., & Meng, L. (2022). Rapid cell pairing and fusion based on oscillating bubbles within an acoustofluidic

device. *Lab on a Chip*, *22*(5), 921-927. https://doi.org/https://doi.org/10.1039/d1lc01074c

Lyu, Q., Wu, L., Li, Y., Pan, Q., Liu, D., Xia, K., Liang, D., Cai, F., Long, Z., & Dai, H. (2005). An improved mechanical technique for assisted hatching. *Human Reproduction*, *20*(6), 1619-1623. https://doi.org/https://doi.org/10.1093/humrep/deh809

MacLennan, M., Crichton, J. H., Playfoot, C. J., & Adams, I. R. (2015). Oocyte development, meiosis and aneuploidy. Seminars in cell & developmental biology,

Mahadevan, M., Miller, M., Maris, M., & Moutos, D. (1998). Assisted hatching of embryos by micromanipulation for human in vitro fertilization: UAMS experience. *The Journal of the Arkansas Medical Society*, *94*(12), 529-531.

Maruotti, J., Muñoz, M., Degrelle, S. A., Gómez, E., Louet, C., Monforte, C. D., De Longchamp, P. H., Brochard, V., Hue, I., & Caamaño, J. N. (2012). Efficient derivation of bovine embryonic stem cells needs more than active core pluripotency factors. *Molecular Reproduction and Development*, *79*(7), 461-477. https://doi.org/https://doi.org/10.1002/mrd.22051

Massip, A., & Mulnard, J. (1980). Time-lapse cinematographic analysis of hatching of normal and frozen—thawed cow blastocysts. *Reproduction*, *58*(2), 475-478. https://doi.org/https://doi.org/10.1530/jrf.0.0580475

Matoba, S., & Zhang, Y. (2018). Somatic cell nuclear transfer reprogramming: mechanisms and applications. *Cell Stem Cell*, *23*(4), 471-485. https://doi.org/https://doi.org/10.1016/j.stem.2018.06.018

McFarlane, G. R., Salvesen, H. A., Sternberg, A., & Lillico, S. G. (2019). On-farm livestock genome editing using cutting edge reproductive technologies. *Frontiers in Sustainable Food Systems*, *3*, 106. https://doi.org/https://doi.org/10.3389/fsufs.2019.00106

Mclean, Z., Oback, B., & Laible, G. (2020). Embryo-mediated genome editing for accelerated genetic improvement of livestock. *Front. Agric. Sci. Eng*, *7*(148), 10.15302. https://doi.org/https://doi.org/10.15302/J-FASE-2019305

Medicine, P. C. o. t. A. S. f. R., & Technology, P. C. o. t. S. f. A. R. (2014). Role of assisted hatching in in vitro fertilization: a guideline. *Fertility and sterility*, *102*(2), 348-351. https://doi.org/https://doi.org/10.1016/j.fertnstert.2014.05.034

Méo, S., Yamazaki, W., Ferreira, C., Perecin, F., Saraiva, N., Leal, C., & Garcia, J. (2007). Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. *Zygote*, *15*(4), 295-306. https://doi.org/https://doi.org/10.1017/S0967199407004285

Mermillod, P., Tomanek, M., Marchal, R., & Meijer, L. (2000). High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. *Molecular Reproduction and Development*, *55*(1), 89-95. https://doi.org/https://doi.org/10.1002/(SICI)1098-2795(200001)55:1<89::AID-MRD12>3.0.CO;2-M

Misica-Turner, P. M., Oback, F. C., Eichenlaub, M., Wells, D. N., & Oback, B. (2007). Aggregating embryonic but not somatic nuclear transfer embryos increases cloning efficiency in cattle. *Biology of reproduction*, *76*(2), 268-278. https://doi.org/https://doi.org/10.1095/biolreprod.106.050922

Mitalipov, S., Zhou, Q., Byrne, J., Ji, W., Norgren, R. B., & Wolf, D. (2007). Reprogramming following somatic cell nuclear transfer in primates is dependent upon nuclear remodeling. *Human Reproduction*, *22*(8), 2232-2242. https://doi.org/https://doi.org/10.1093/humrep/dem136

Mogessie, B., Scheffler, K., & Schuh, M. (2018). Assembly and positioning of the oocyte meiotic spindle. *Annual review of cell and developmental biology*, 381-403. https://doi.org/https://doi.org/10.1146/annurev-cellbio-100616-060553

Mousavi, F. S., Ahmadi, E., Shirazi, A., Shams‐Esfandabadi, N., & Nazari, H. (2022). The effect of chemical treatment of the sheep embryo zona pellucida on the ability of blastocysts to hatch after vitrification and warming. *Veterinary Medicine and Science*, *8*(1), 405-410. https://doi.org/https://doi.org/10.1002/vms3.632

Mueller, M. L., & Van Eenennaam, A. L. (2022). Synergistic power of genomic selection, assisted reproductive technologies, and gene editing to drive genetic improvement of cattle. *CABI Agriculture and Bioscience*, *3*(1), 1-29. https://doi.org/https://doi.org/10.1186/s43170-022-00080-z

Muggleton-Harris, A. L., & Hayflick, L. (1976). Cellular aging studied by the reconstruction of replicating cells from nuclei and cytoplasms isolated from normal human diploid cells. *Experimental Cell Research*, *103*(2), 321-330. https://doi.org/https://doi.org/10.1016/0014-4827(76)90269-X

Namula, Z., Wittayarat, M., Hirata, M., Hirano, T., Nguyen, N. T., Le, Q. A., Fahrudin, M., Tanihara, F., & Otoi, T. (2019). Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes. *In Vitro Cellular & Developmental Biology-Animal*, *55*(8), 598-603. https://doi.org/https://doi.org/10.1007/s11626-019-00385-w

Narahashi, Y. (1970). Proteolytic enzymes. *Methods Enzymol*, *19*, 651. https://doi.org/https://doi.org/10.1016/0076-6879(70)19052-5

Nashun, B., Akiyama, T., Suzuki, M. G., & Aoki, F. (2011). Dramatic replacement of histone variants during genome remodeling in nuclear-transferred embryos. *Epigenetics*, *6*(12), 1489-1497. https://doi.org/https://doi.org/10.4161/epi.6.12.18206

Neaves, W. B., & Baumann, P. (2011). Unisexual reproduction among vertebrates. *Trends in Genetics*, *27*(3), 81-88. https://doi.org/https://doi.org/10.1016/j.tig.2010.12.002

Needham, J., & Hughes, A. (2015). *A history of embryology*. Cambridge University Press. https://doi.org/https://doi.org/10.1086/347231

Negrón-Pérez, V. M., & Hansen, P. J. (2017). The bovine embryo hatches from the zona pellucida through either the embryonic or abembryonic pole. *Journal of assisted reproduction and genetics*, *34*(6), 725-731. https://doi.org/https://doi.org/10.1007/s10815-017-0933-3

Neil, G. A., & Zimmermann, U. (1993). [14] Electrofusion. *Methods in enzymology*, *220*, 174-196. https://doi.org/https://doi.org/10.1016/0076-6879(93)20082-E

Niimura, S. (2003). Time-lapse videomicrographic analyses of contractions in mouse blastocysts. *Journal of Reproduction and Development*, *49*(6), 413-423. https://doi.org/https://doi.org/10.1262/jrd.49.413

Niimura, S., Ogata, T., Okimura, A., Sato, T., Uchiyama, Y., Seta, T., Nakagawa, H., Nakagawa, K., & Tamura, Y. (2010). Time-lapse videomicrographic observations of blastocyst hatching in cattle. *Journal of Reproduction and Development*, 1008250299-1008250299. https://doi.org/https://doi.org/10.1262/jrd.10-069h

Nogueira, B. G. R., de Souza, L. F. A., Puelker, R. Z., Giometti, I. C., Firetti, S. M. G., Dias, T. S. d. S. B., & Castilho, C. (2021). Factors affecting the in vitro production of bovine embryos in a commercial program. *Research, Society and Development, 10*(2), e16110212264-e16110212264. https://doi.org/https://doi.org/10.33448/rsd-v10i2.12264

Oback, B., & McLean, Z. (2018). Inner Cell Mass Development. https://doi.org/http://dx.doi.org/10.1016/B978-0-12-801238-3.64479-3

Oback, B., & Wells, D. (2002). Donor cells for nuclear cloning: many are called, but few are chosen. *Cloning & Stem Cells*, *4*(2), 147-168. https://doi.org/https://doi.org/10.1089/153623002320253328

Oback, B., & Wells, D. (2007). Donor cell differentiation, reprogramming, and cloning efficiency: elusive or illusive correlation? *Molecular Reproduction and Development*, *74*(5), 646-654. https://doi.org/https://doi.org/10.1002/mrd.20654

Oback, B., Wiersema, A., Gaynor, P., Laible, G., Tucker, F., Oliver, J., Miller, A., Troskie, H., Wilson, K., & Forsyth, J. (2003). Cloned cattle derived from a novel zona-free embryo reconstruction system. *Cloning & Stem Cells*, *5*(1), 3-12. https://doi.org/https://doi.org/10.1089/153623003321512111

Otoi, T., Yamamoto, K., Koyama, N., & Suzuki, T. (1995). In vitro fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. *Cryobiology*, *32*(5), 455-460. https://doi.org/https://doi.org/10.1006/cryo.1995.1045

Papaioannou, V. E. (1982). Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts. https://doi.org/https://doi.org/10.1242/dev.68.1.199

Park, M. J., Lee, S. E., Kim, E. Y., Lee, J. B., Jeong, C. J., & Park, S. P. (2015). Effective oocyte vitrification and survival techniques for bovine somatic cell nuclear transfer. *Cellular Reprogramming (Formerly" Cloning and Stem Cells"), 17*(3), 199-210. https://doi.org/https://doi.org/10.1089/cell.2014.0072

Parmenter, C. L. (1940). Chromosome numbers in Rana fusca parthenogenetically developed from eggs with known polar body and cleavage histories. *Journal of Morphology*, *66*(2), 241-260. https://doi.org/https://doi.org/10.1002/jmor.1050660204

Pettinato, G., Wen, X., & Zhang, N. (2015). Engineering strategies for the formation of embryoid bodies from human pluripotent stem cells. *Stem cells and development*, *24*(14), 1595-1609. https://doi.org/https://doi.org/10.1089/scd.2014.0427

Peura, T., Lewis, I., & Trounson, A. O. (1998). The effect of recipient oocyte volume on nuclear transfer in cattle. *Molecular Reproduction and Development: Incorporating Gamete Research, 50*(2), 185-191. https://doi.org/https://doi.org/10.1002/(SICI)1098-2795(199806)50:2<185::AID-MRD9>3.0.CO;2-G

Peura, T., Vajta, G., Lane, M., Boekel, K., & Trounson, A. (1999). Vitrification of bovine cytoplasts for nuclear transfer. *Theriogenology*, *51*(1), 211. https://doi.org/https://doi.org/10.1016/s0093-691x(99)91770-3

Pillai, V. V., Koganti, P. P., Kei, T. G., Gurung, S., Butler, W. R., & Selvaraj, V. (2021). Efficient induction and sustenance of pluripotent stem cells from bovine somatic cells. *Biology open*, *10*(10), bio058756. https://doi.org/https://doi.org/10.1242/bio.058756

Pomar, F. R., Teerds, K., Kidson, A., Colenbrander, B., Tharasanit, T., Aguilar, B., & Roelen, B. (2005). Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. *Theriogenology*, *63*(8), 2254-2268. https://doi.org/https://doi.org/10.1016/j.theriogenology.2004.10.015

Qin, W., Dion, S. L., Kutny, P. M., Zhang, Y., Cheng, A. W., Jillette, N. L., Malhotra, A., Geurts, A. M., Chen, Y.-G., & Wang, H. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics*, *200*(2), 423-430. https://doi.org/https://doi.org/10.1534/genetics.115.176594

Rodriguez-Villamil, P., Ongaratto, F., Bostrom, J., Larson, S., & Sonstegard, T. (2021). 13 Generation of SLICK beef cattle by embryo microinjection: A case report. *Reproduction, Fertility and Development*, *33*(2), 114-114. https://doi.org/https://doi.org/10.1071/RDv33n2Ab13

Sanders, J. R., & Jones, K. T. (2018). Regulation of the meiotic divisions of mammalian oocytes and eggs. *Biochemical Society Transactions*, *46*(4), 797-806. https://doi.org/https://doi.org/10.1042/BST20170493

Saragusty, J., & Arav, A. (2011). Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, *141*(1), 1. https://doi.org/https://doi.org/10.1530/REP-10-0236

Saragusty, J., Diecke, S., Drukker, M., Durrant, B., Friedrich Ben‐Nun, I., Galli, C., Göritz, F., Hayashi, K., Hermes, R., & Holtze, S. (2016). Rewinding the process of mammalian extinction. *Zoo Biology*, *35*(4), 280-292. https://doi.org/https://doi.org/10.1002/zoo.21284

Sayaka, W., Satoshi, K., Van Thuan, N., Hiroshi, O., Takafusa, H., Eiji, M., Thuy, B. H., Masashi, M., & Teruhiko, W. (2008). Effect of volume of oocyte cytoplasm on embryo development after parthenogenetic activation, intracytoplasmic sperm injection, or somatic cell nuclear transfer. *Zygote*, *16*(3), 211-222. https://doi.org/https://doi.org/10.1017/S0967199408004620

Seneda, M. M., Delchiaro, S. B., Zangirolamo, A. F., Alfieri, A. A., & Morotti, F. (2021). Folliculogenesis, Fertility and Biotechnology in Dairy Cattle. https://doi.org/https://doi.org/10.5772/intechopen.101243

Seshagiri, P. B., Roy, S. S., Sireesha, G., & Rao, R. P. (2009). Cellular and molecular regulation of mammalian blastocyst hatching. *Journal of reproductive immunology*, *83*(1-2), 79-84. https://doi.org/https://doi.org/10.1016/j.jri.2009.06.264

Shiga, K., Fujita, T., Hirose, K., Sasae, Y., & Nagai, T. (1999). Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese black bulls. *Theriogenology*, *52*(3), 527-535. https://doi.org/https://doi.org/10.1016/S0093-691X(99)00149-1

Sigma-Aldrich. (2019). Pronase. *Sigma Aldrich Product Documents*. www.sigmaaldrich.com

Sims, M., & First, N. (1994). Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proceedings of the National Academy of Sciences, 91*(13), 6143-6147. https://doi.org/https://doi.org/10.1073/pnas.91.13.6143

Song, B.-S., Kim, J.-S., Yoon, S.-B., Lee, K.-S., Koo, D.-B., Lee, D.-S., Choo, Y.-K., Huh, J.-W., Lee, S.-R., & Kim, S.-U. (2011). Inactivated Sendai-virus-mediated fusion improves early development of cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated apoptosis. *Reproduction, Fertility and Development, 23*(6), 826-836. https://doi.org/https://doi.org/10.1071/RD10194

Stein, A., Rufas, O., Amit, S., Avrech, O., Pinkas, H., Ovadia, J., & Fisch, B. (1995). Assisted hatching by partial zona dissection of human pre-embryos in patients with recurrent implantation failure after in vitro fertilization. *Fertility and sterility, 63*(4), 838-841. https://doi.org/https://doi.org/10.1016/S0015-0282(16)57490-1

Stice, S. L., & Keefer, C. L. (1993). Multiple generational bovine embryo cloning. *Biology of reproduction, 48*(4), 715-719. https://doi.org/https://doi.org/10.1095/biolreprod48.4.715

Stice, S. L., Keefer, C. L., & Matthews, L. (1994). Bovine nuclear transfer embryos: oocyte activation prior to blastomere fusion. *Molecular Reproduction and Development, 38*(1), 61-68. https://doi.org/https://doi.org/10.1002/mrd.1080380111

Stringfellow, D. A., Seidel, S. M., & International Embryo Transfer Society. (2007). *Manual of the International Embryo Transfer Society a procedural guide and general information for the use of embryo transfer technology, emphasizing sanitary procedures*. International Embryo Transfer Society.

Sun, X., Wang, S., Zhang, Y., Wang, H., Wang, L., Ying, L., Li, R., & Li, N. (2008). Cell-cycle synchronization of fibroblasts derived from transgenic cloned cattle ear skin: effects of serum starvation, roscovitine and contact inhibition. *Zygote, 16*(2), 111-116. https://doi.org/https://doi.org/10.1017/S0967199407004522

Suvá, M., Canel, N. G., & Salamone, D. F. (2019). Effect of single and combined treatments with MPF or MAPK inhibitors on parthenogenetic haploid activation of bovine oocytes. *Reproductive Biology, 19*(4), 386-393. https://doi.org/https://doi.org/10.1016/j.repbio.2019.09.001

Tait-Burkard, C., Doeschl-Wilson, A., McGrew, M. J., Archibald, A. L., Sang, H. M., Houston, R. D., Whitelaw, C. B., & Watson, M. (2018). Livestock 2.0–genome editing for fitter, healthier, and more productive farmed animals. *Genome biology, 19*(1), 1-11. https://doi.org/https://doi.org/10.1186/s13059-018-1583-1

Tanaka, H., TAKAHASHI, Y., HISHINUMA, M., KANAGAWA, H., & KARIYA, T. (1995). Influence of nocodazole on the development of donor blastomeres from 16-cell stage bovine embryos in nuclear transfer. *Japanese Journal of Veterinary Research, 43*(1), 1-14. https://doi.org/https://doi.org/10.14943/jjvr.43.1.1

Tani, T., Kato, Y., & Tsunoda, Y. (2001). Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biology of reproduction, 64*(1), 324-330. https://doi.org/https://doi.org/10.1095/biolreprod64.1.324

Tani, T., Shimada, H., Kato, Y., & Tsunoda, Y. (2006). Demecolcine-assisted enucleation for bovine cloning. *Cloning and stem cells*, *8*(1), 61-66. https://doi.org/https://doi.org/10.1089/clo.2006.8.61

Taniyama, A., Nishino, Y., Inoue, T., Taura, Y., Takagi, M., Otoi, T., Sato, M., & Kubota, C. (2014). Effects of chemical zona pellucida thinning with pronase on the development of bovine embryos. *Journal of the Japan Veterinary Medical Association*, *67*(11), 833-838. https://doi.org/https://doi.org/10.12935/jvma.67.833

Techakumphu, M. (1993). Viability of bovine blastomeres after metaphase arrest with nocodazole. *Theriogenology*, *39*, 328 abstr. https://doi.org/https://doi.org/10.1016/0093-691x(93)90183-6

Toyooka, Y. (2020). Trophoblast lineage specification in the mammalian preimplantation embryo. *Reproductive Medicine and Biology*, *19*(3), 209-221. https://doi.org/https://doi.org/10.1002/rmb2.12333

Trounson, A., Lacham-Kaplan, O., Diamente, M., & Gougoulidis, T. (1998). Reprogramming cattle somatic cells by isolated nuclear injection. *Reproduction, Fertility and Development*, *10*(8), 645-650. https://doi.org/https://doi.org/10.1071/RD98095

Udroiu, I., & Sgura, A. (2017). Cytogenetic tests for animal production: state of the art and perspectives. *Animal genetics*, *48*(5), 505-515. https://doi.org/https://doi.org/10.1111/age.12581

Ueno, S., Ezoe, K., Yabuuchi, A., Uchiyama, K., Okimura, T., Okuno, T., Kobayashi, T., & Kato, K. (2016). Complete zona pellucida removal from vitrified-warmed human blastocysts facilitates earlier in-vitro attachment and outgrowth. *Reproductive BioMedicine Online*, *33*(2), 140-148. https://doi.org/https://doi.org/10.1016/j.rbmo.2016.05.007

Vajta, G., Lewis, I. M., Hyttel, P., Thouas, G. A., & Trounson, A. O. (2001). Somatic cell cloning without micromanipulators. *Cloning*, *3*(2), 89-95. https://doi.org/https://doi.org/10.1089/15204550152475590

van der Kooi, C. J., & Schwander, T. (2015). Parthenogenesis: birth of a new lineage or reproductive accident? *Current Biology*, *25*(15), R659-R661. https://doi.org/https://doi.org/10.1016/j.cub.2015.06.055

Van Stekelenburg‐Hamers, A. E., Van Achterberg, T. A., Rebel, H. G., Fléchon, J. E., Campbell, K. H., Weima, S. M., & Mummery, C. L. (1995). Isolation and characterization of permanent cell lines from inner cell mass cells of bovine blastocysts. *Molecular Reproduction and Development*, *40*(4), 444-454. https://doi.org/https://doi.org/10.1002/mrd.1080400408

Vanroose, G., Nauwynck, H., Soom, A. V., Ysebaert, M.-T., Charlier, G., Oostveldt, P. V., & de Kruif, A. (2000). Structural aspects of the zona pellucida of in vitro-produced bovine embryos: a scanning electron and confocal laser scanning microscopic study. *Biology of reproduction*, *62*(2), 463-469. https://doi.org/https://doi.org/10.1095/biolreprod62.2.463

Verma, V., Huang, B., Kallingappa, P. K., & Oback, B. (2013). Dual kinase inhibition promotes pluripotency in finite bovine embryonic cell lines. *Stem cells and development*, *22*(11), 1728-1742. https://doi.org/https://doi.org/10.1089/scd.2012.0481

Wankar, A. K., Rindhe, S. N., & Doijad, N. S. (2021). Heat stress in dairy animals and current milk production trends, economics, and future perspectives: the global scenario. *Tropical Animal Health and Production, 53*(1), 1-14. https://doi.org/https://doi.org/10.1007/s11250-020-02541-x

Wasielak-Politowska, M., & Kordowitzki, P. (2022). Chromosome Segregation in the Oocyte: What Goes Wrong during Aging. *International Journal of Molecular Sciences, 23*(5), 2880. https://doi.org/https://doi.org/10.3390/ijms23052880

Wei, J., Antony, J., Meng, F., MacLean, P., Rhind, R., Laible, G., & Oback, B. (2017). KDM4B-mediated reduction of H3K9me3 and H3K36me3 levels improves somatic cell reprogramming into pluripotency. *Scientific reports, 7*(1), 1-14. https://doi.org/https://doi.org/10.1038/s41598-017-06569-2

Wei J, G. P., Cole S, Brophy B, Oback B, Laible G. (2018). Developing the laboratory conditions for bovine embryos genome editing by electroporation. *The World Congress on Genetics Applied to Livestock Production, 11*, 1118.

Wei, Q., Zhong, L., Zhang, S., Mu, H., Xiang, J., Yue, L., Dai, Y., & Han, J. (2017). Bovine lineage specification revealed by single-cell gene expression analysis from zygote to blastocyst. *Biology of reproduction, 97*(1), 5-17. https://doi.org/https://doi.org/10.1093/biolre/iox071

Wells, D. N., Misica, P. M., Tervit, H. R., & Vivanco, W. H. (1998). Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reproduction, Fertility and Development, 10*(4), 369-378. https://doi.org/https://doi.org/10.1071/R98109

Willadsen, S., & Polge, C. (1981). Attempts to produce monozygotic quadruplets in cattle by blastomere separation. *The Veterinary Record, 108*(10), 211-213. https://doi.org/https://doi.org/10.1136/vr.108.10.211

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., & Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature, 385*(6619), 810-813. https://doi.org/https://doi.org/10.1089/clo.2006.0002

Wilson, S. H. (2021). *Improving bovine donor cell culture conditions for embryo-derived cell transfer cloning* The University of Waikato].

Wolf, X., Rasmussen, M., Schauser, K., Jensen, A., Schmidt, M., & Hyttel, P. (2011). OCT4 expression in outgrowth colonies derived from porcine inner cell masses and epiblasts. *Reproduction in domestic animals, 46*(3), 385-392. https://doi.org/https://doi.org/10.1111/j.1439-0531.2010.01675.x

Wu, B., Ignotz, G., Bruce Currie, W., & Yang, X. (1997). Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. *Biology of reproduction, 56*(1), 253-259. https://doi.org/https://doi.org/10.1095/biolreprod56.1.253

Xiang, J., Wang, H., Zhang, Y., Wang, J., Liu, F., Han, X., Lu, Z., Li, C., Li, Z., & Gao, Y. (2021). LCDM medium supports the derivation of bovine extended pluripotent stem cells with embryonic and extraembryonic potency in bovine–mouse chimeras from iPSCs and bovine fetal fibroblasts. *The FEBS Journal, 288*(14), 4394-4411. https://doi.org/https://doi.org/10.1111/febs.15744

Yabuuchi, A., Yasuda, Y., Kato, Y., & Tsunoda, Y. (2004). Effects of nuclear transfer procedures on ES cell cloning efficiency in the mouse. *Journal of Reproduction and Development*, *50*(2), 263-268. https://doi.org/https://doi.org/10.1262/jrd.50.263

Yan, J., Suzuki, J., Yu, X., Kan, F. W., Qiao, J., & Chian, R.-C. (2010). Cryo-survival, fertilization and early embryonic development of vitrified oocytes derived from mice of different reproductive age. *Journal of assisted reproduction and genetics*, *27*(11), 605-611. https://doi.org/https://doi.org/10.1007/s10815-010-9450-3

Yang, V. W. (2018). The cell cycle. *Physiology of the Gastrointestinal Tract*, 197-219. https://doi.org/https://doi.org/10.1016/B978-0-12-809954-4.00008-6

Yang, X. (1991). Age-dependent activation, enucleation and nuclear transfer of bovine oocytes matured in vitro and in vivo. *Biol Reprod*, *44*, 141.

Yum, S.-Y., Youn, K.-Y., Choi, W.-J., & Jang, G. (2018). Development of genome engineering technologies in cattle: from random to specific. *Journal of animal science and biotechnology*, *9*(1), 1-9. https://doi.org/https://doi.org/10.1186/s40104-018-0232-6

Zakhartchenko, V., Durcova-Hills, G., Stojkovic, M., Schernthaner, W., Prelle, K., Steinborn, R., Müller, M., Brem, G., & Wolf, E. (1999). Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *Reproduction*, *115*(2), 325-331. https://doi.org/https://doi.org/10.1530/jrf.0.1150325

Zakhartchenko, V., Stojkovic, M., Palma, G., Wolf, E., & Brem, G. (1997). Enucleation of bovine oocytes with minimal cytoplasmic volume: effect on development of nuclear transfer embryos. *Theriogenology*, *1*(47), 238. https://doi.org/https://doi.org/10.1016/s0093-691x(97)82365-5

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: past, present, and future. *Stem cell research & therapy*, *10*(1), 1-22. https://doi.org/https://doi.org/10.1186/s13287-019-1165-5

Zealand, B. a. L. N. (2021). Compendium of New Zealand farm facts 2021. *New Zealand: Beef and Lamb*, *45*.

Zeron, Y., Ocheretny, A., Kedar, O., Borochov, A., Sklan, D., & Arav, A. (2001). Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction*, *121*(3), 447-454. https://doi.org/https://doi.org/10.1530/rep.0.1210447

Zhao, L., Gao, X., Zheng, Y., Wang, Z., Zhao, G., Ren, J., Zhang, J., Wu, J., Wu, B., & Chen, Y. (2021). Establishment of bovine expanded potential stem cells. *Proceedings of the National Academy of Sciences*, *118*(15), e2018505118. https://doi.org/https://doi.org/10.1073/pnas.2018505118

Appendices

Appendix I. Materials

Aspiration media
H199 medium with 2% (w/v) FBS (Gibco, Thermo fisher, USA) and 925 IU/ml Heparin (CP Pharmaceuticals, UK). Stored at 4°C.

B199
Bicarbonate-buffered medium M199 with 0.2 mM pyruvate, 25 mM sodium bicarbonate, and 0.086 mM kanamycin monosulfate. Stored at 4°C.

Bovine Serum Albumin (BSA)
Fatty-acid-free bovine albumin. Sigma Aldrich (USA). Stored at 4°C.

BoviPure™ 40%
200 µl BoviPure™ with 300 µl BoviDilute. Nidacon (Sweden). Stored at room temperature.

BoviPure™ 80%
400 µl BoviPure™ with 100 µl BoviDilute. Nicadon (Sweden). Stored at room temperature.

CHX
5mg/ml Cycloheximide in DMSO. Sigma Aldrich (USA). Stored at -20°C.

Complete growth media
pEPSC base media with 3 mM CHIR99021 (41368, Stemgent), 0.3 mM WH-4-023 (R&D systems, USA), 2.5 mM XAV939 (R&D systems, USA), 65 µg/mL ascorbic acid (Sigma Aldrich, USA), 20 ng/mL activin A (Prospec Bio, Israel), 10 ng/mL IL-6, 10 ng/mL Y27632 (Reagents Direct, USA), 10% Knockout Serum Replacement, 20 ng/mL fibroblast growth factor two (Sigma Aldrich, USA). Stored at 4°C.

Dimethyl sulfoxide (DMSO)
Dimethyl sulfoxide, Sigma Aldrich (USA). Stored at room temperature.

Disaggregation media
HSOF – Ca – BSA with 0.1 mg/ml PVA, 0.02% EDTA, and 5.0 mg/ml pronase. Supplemented with 0.1% Cyotochalasin B and 0.1% Y27632 the day prior to CT. Stored at 4°C for up to 2 weeks.

DMAP
74.62 mg/mL 6-dimethylaminopurine in DMSO. Sigma Aldrich (USA). Stored at -20°C.

EG
Ethylene glycol, Sigma Aldrich (USA). Stored at room temperature.

Embryo Hold
107.8 mM sodium chloride, 3.99 mM potassium chloride, 1.20 mM monopotassium phosphate, 0.33 mM sodium pyruvate, 0.49 mM magnesium chloride hexahydrate, 38.61 g/L MOPS sodium salt, 25 mM sodium bicarbonate, 1.71 mM calcium chloride dihydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 0.081 g/L Non-Essential Amino Acid, 1 mM Gluta-Max, 0.22 g/L BM Essential Amino Acid, 17.37 g/L MOPS free acid. Stored at 4°C.

ESOF
107.8 mM sodium chloride, 0.33mM sodium pyruvate, 7.15 mM potassium chloride, 1.71 mM calcium chloride dihydrate, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.15 mM glucose, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 0.081 g/L Non-Essential Amino Acid, 1 mM Gluta-Max, and 8 mg/ml BSA. Stored at 4°C.

ESOF – Ca
107.8 mM sodium chloride, 0.33mM sodium pyruvate, 7.15 mM potassium chloride, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.15 mM glucose, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 0.081 g/L Non-Essential Amino Acid, 1 mM Gluta-Max, and 8 mg/ml BSA. Stored at 4°C.

Ethanol
70% ethanol in Milli-Q water. Fisher Chemicals (USA). Stored at room temperature.

Fetal calf serum (FBS)
Fetal bovine serum, Gibco, Thermo Fisher, (USA). Stored at 4°C.

Fibroblast growth media
DMEM/F-12 with 10% FBS. Gibco, Thermo fisher, USA

Ficoll
Ficoll® PM 70, Sigma Aldrich (Sweden). Stored at room temperature.

Fusion buffer (205)

36.4 g/L Mannitol, 7.7 mg/L calcium chloride, 24.9 mg/L magnesium chloride, 2 ml/L Hepes, and 0.5 g/L BSA. Stored at 4°C.

Gelatin
0.1% gelatin powder dissolved in Milli-Q water. Sigma Aldrich (USA). Stored at room temperature.

H199
Hepes-buffered M119 with 15 mM Hepes, 0.086 mM kanamycin monosulfate, and 5 mM sodium bicarbonate. Stored at 4°C.

Hoechst
bisBenzimide H 33342 trihydrochloride in Milli-Q water. Sigma Aldrich (USA). Stored at 4°C.

HSOF
107.8 mM sodium chloride, 5 mM sodium bicarbonate, 7.15 mM potassium chloride, 0.3 mM monopotassium phosphate, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM calcium chloride dihydrate, and 3 mg/mL BSA. Stored at 4°C.

Hyaluronidase
0.1% Bovine Testicular Hyaluronidase in HSOF or H199, Sigma Aldrich (USA). Stored at -20°C.

Ionomycin
1 mg Ionomycin salt in 268 µL DMSO. Sigma Aldrich (USA). Stored at -20°C.

IVF media
107.8 mM sodium chloride, 0.33 mM sodium pyruvate, 7.10 mM potassium chloride, 0.3 mM monopotassium phosphate, 25 mM sodium bicarbonate, 3.32 mM sodium lactate, 1.71 mM calcium chloride dihydrate, 8 mg/ml BSA, with 0.04 mM kanamycin sulfate, and supplemented on the day of use with 10 µg/ml heparin, 1 mM pyruvate, 20 µM penicillamine and 10 µM hypotaurine. Stored at 4°C.

Lectin
2 mg/mL Phytohaemagglutinin (PHA) in H199 + 3 mg/mL BSA solution. Sigma Aldrich (USA). Stored at -20°C.

LSOF
107.8 mM sodium chloride, 0.33 mM sodium pyruvate, 1.20 mM monopotassium phosphate, 25 mM sodium bicarbonate, 1.71 mM calcium chloride dihydrate, 0.50 mM magnesium chloride hexahydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 1 mM DNP (2-, 4-dinitrophenol), 1 mM Gluta-Max, 0.22 g/L

BM Essential Amino Acid, 0.081 g/L Non-Essential Amino Acid, and 8 mg/ml BSA. Stored at 4°C.

Maturation media
10 µg/mL ovine follicle-stimulating hormone in B199, OVAGEN; ICP Bio (NZ), 1 µg/mL ovine luteinizing hormone, and 1 µg/mL 17-β-estradiol. Supplemented on day of use with 0.1 mM cysteamine (2- Mercaptoethylamine). Stored at 4°C.

Milli-Q water
Produced in Millipore Milli-Q plus unit, Bio Lab, (USA). Stored at room temperature.

Mineral Oil
M5310, Sigma Aldrich (USA). Stored at room temperature or 38°C in 5% CO_2.

Nocodazole
1mg/ml nocodazole in DMSO. Sigma Aldrich (USA). Stored at -20°C.

pEPSC base media
DMEM/F-12 (Gibco, Thermo Fisher, NZ) with 5 µl/mL 100X N2 (Gibco, Thermo Fisher, NZ), 10 µl/mL B27 (Gibco, Thermo Fisher, NZ), 10 µl/mL Penicillin-Streptomycin (Gibco, Thermo Fisher, NZ), 10 µl/mL Non-Essential Amino Acid, and 0.1 mM beta-mercaptoethanol (Sigma Aldrich, USA). Stored at 4°C.

Phosphate buffered saline (PBS)
8.4 mM disodium hydrogen orthophosphate 2-hydrate, 150 mM sodium chloride, and 1.8 mM sodium dihydrogen orthophosphate 1-hydrate dissolved in Milli-Q water, pH 7.4. Stored at room temperature

Pronase
0.5% Protease in H199. Sigma Aldrich (USA). Stored at -20°C.

Saline
0.9% sodium chloride in Milli-Q water. Sigma Aldrich (USA). Stored at 32°C.

Serum starvation media
DMEM/F-12 with 0.5% Fetal bovine serum. Gibco, Thermo Fisher, (USA).

Sucrose
D-Sucrose, Sigma Aldrich (USA). Stored at room temperature.

Trypsin.
0.25% Trypsin in PBS, Gibco, Thermo Fisher (NZ). Stored at 4°C.

Y27632
10 ng/mL Y27632 in DMSO (Reagents Direct, USA). Stored at -20°C.

Appendix II. IVP record sheet

Experiment :

Run Number :

IVM	IVF	IVC	C/O
Date/time : Wednesday, 18 May 2022	Thursday, 19 May 2022	Friday, 20 May 2022	Tuesday, 24 May 2022
Source/collector:	No. oocytes :	No. oocytes :	No. oocytes :
No. ovaries : No. oocytes : Quality :	Bull :	Treatment : E SOF	Treatment : L SOF
Treatment :	IVF treatm :		deg 1 cell <8 >8
notes :	notes :	notes :	notes :

End of Culture Date : Friday, 27 May 2022

Total	1-cells	Total	1-cells	Total	1-cells

Appendix III. Plate layouts

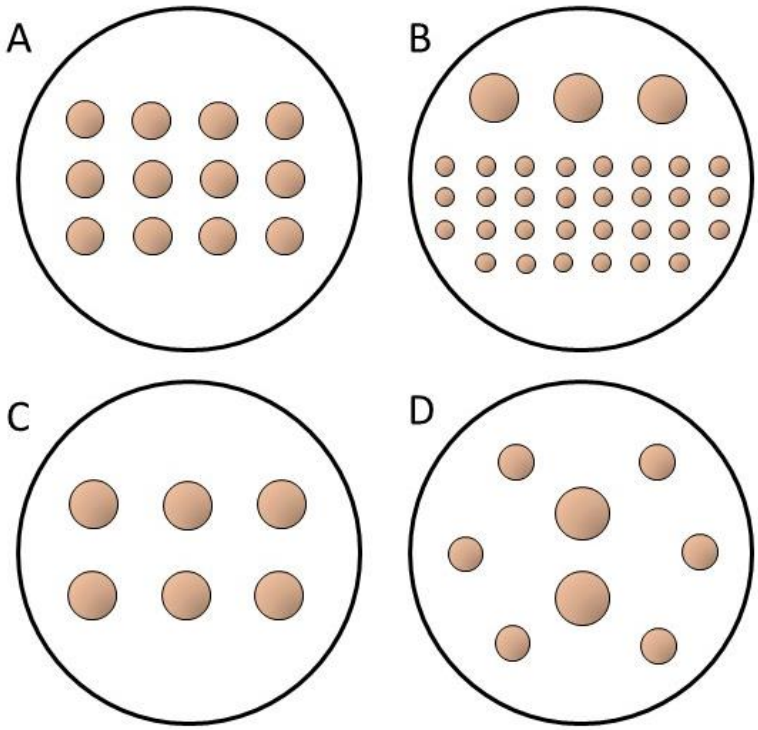


Figure 5.6.1 Petri dish plate layouts. A) IVP and CT oocyte preparation plates. 12 x 30 µl or 12 x 40 µl drops in a 60 mm. B) Single embryo culture in a 60 mm. Top row 3 x 40 µl wash drops, lower rows 30-40 x 5 µl drops. C) Disaggregation-related plates. 6 x 40 µl drops in a 60 mm. D) Group embryo culture. 2 x 40 µl central wash drops surrounded by 6 x 20 µl culture drops in a 35 mm. A-C) Overlaid with 8 mls of mineral oil. D) Overlaid with 3 mls of mineral oil.

Appendix IV. Bovine oocyte grading

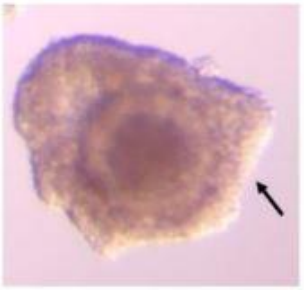
I. Cumulus cell morphology

Cat. 1) oocyte is surrounded by **> 5 layers of compact granulosa cells**

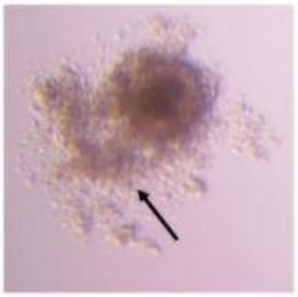
Cat. 2) oocyte is surrounded by **> 5 layers of less compact granulosa cells**

Cat. 3) oocyte is surrounded by **> 5 layers of expanded granulosa cells**

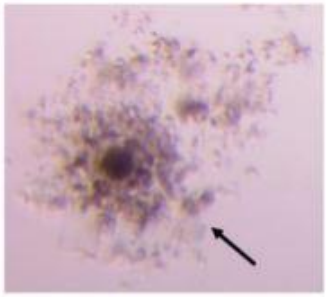
Cat. 4) oocyte is surrounded by **≤ 5 layers of granulosa cells and/or not completely surrounding the oocyte**



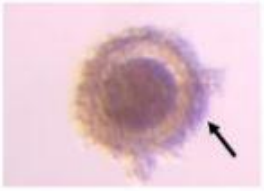
Cat. 1) Cumulus consists of more than 5 layers. **Granulosa cells are compact and dense.**



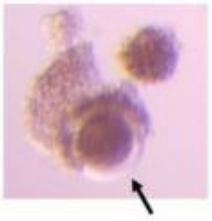
Cat. 2) Cumulus consists of more than 5 layers. **Granulosa cells are less compact and start to expand.**



Cat. 3) Cumulus consists of more than 5 layers. **Granulosa cells are expanded**



Cat. 4) Cumulus consists of **less than 5 layers.**



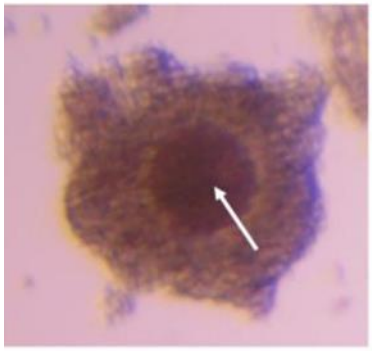
Cat. 4) Granulosa cells are **not completely surrounding the oocyte.**

II. Ooplasm morphology

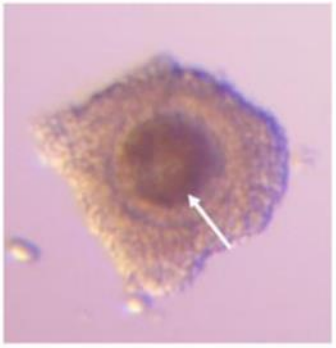
Cat. 1) Ooplasm is **homogeneously dark**

Cat. 2) Ooplasm is **dark and slightly granular** (acceptable granularity)

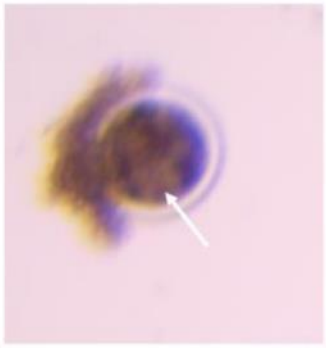
Cat. 3) Ooplasm is **heterogeneous** (mix of dark and pale areas, the granularity is non-acceptable).



Cat. 1) Ooplasm is **homogeneously dark**



Cat. 2) Ooplasm is **dark and slightly granular**



Cat. 3) Ooplasm is a **heterogeneous** mix of dark and pale areas

Grade	Description
1	Many layers of tight cumulus surrounding the oocyte. Cytoplasm is homogeneous with fine granulation in a light even greyish colour.
2	Cumulus layers are less compact and fewer. The ooplasm is slightly coarser, still even in coloration.
3	Corona only, zona pellucida may be exposed. The edge of the cytoplasm can be darker, uneven in colour.
4	Naked Oocyte with little to no cumulus cells. Cytoplasm is still ok, but usually uneven, can be lightly spotted with moderate size granules or with a darker zone around the periphery
5	Expanded cumulus with degenerated cells as satellites, the cytoplasm looks very pale with a darker edge, very pigmented dark black, brown, or dark clusters (clumping of organelles), and indicates a progression towards degeneration.
6	Degenerate. Oocyte cytoplasm is degenerate/absent/lysed. This grade also includes atretic, expanded oocytes, and any empty zonas

Figure 5.6.2 Grading of bovine oocytes for IVM based on cumulus and ooplasm morphologies. In house.

Appendix V. Haemocytometer counting

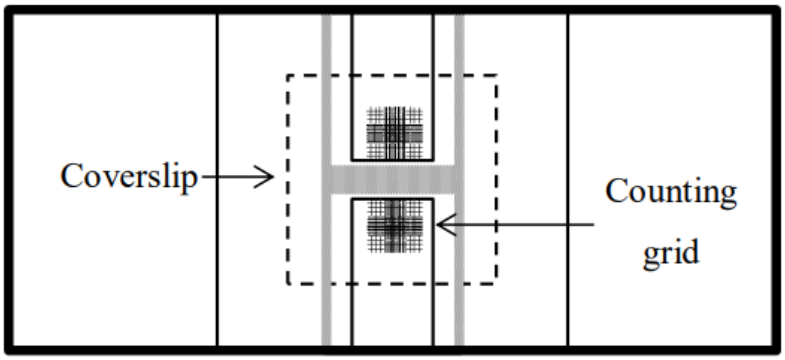


Figure 5.6.3 Aerial view of Haemocytometer.

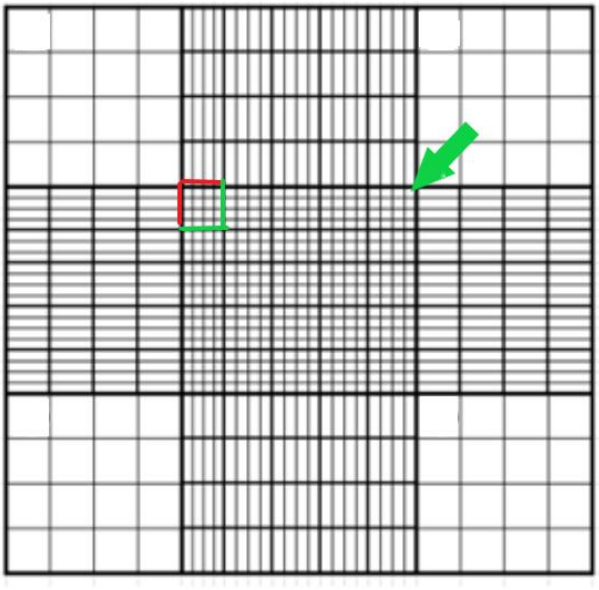


Figure 5.6.4 Counting grid on haemocytometer. The central square with triple lines

Appendix VI. Bovine embryo development and grading

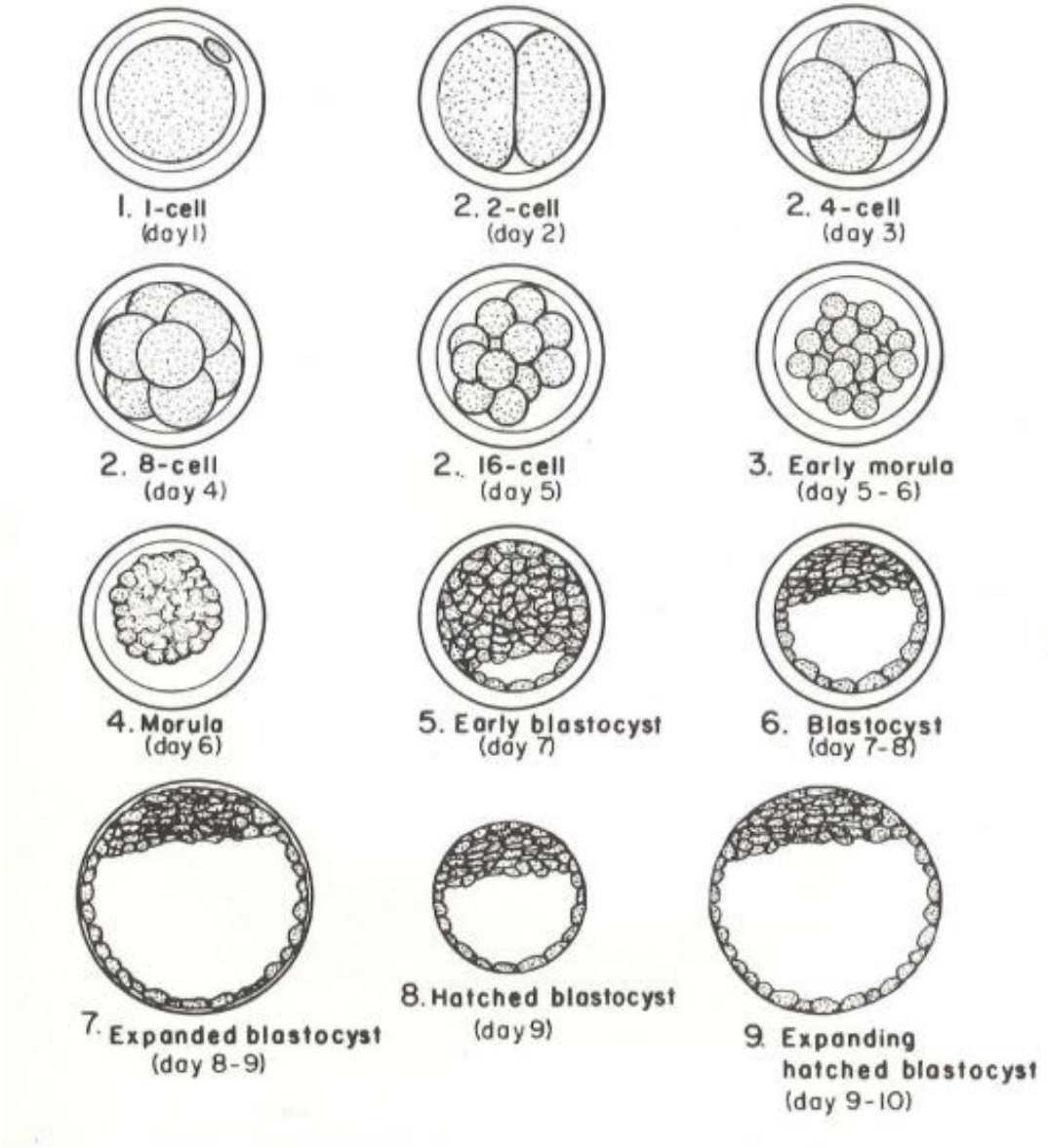
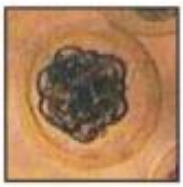


Figure 5.6.5 Developmental stages of a bovine embryo. Development starting from IVC (day 1) to an expanded hatched blastocyst. Image from international embryo grading guide (Stringfellow et al., 2007).

BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

Cycle day: 6
Stage Code: 3
Quality Code:1
Comments: a

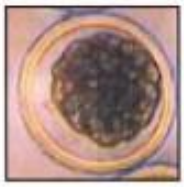


Cycle day: 6.5
Stage Code: 3
Quality Code:1
Comments: a

Cycle day: 6.5
Stage Code: 3
Quality Code: 2
Comments: a, b

Cycle day: 6.5
Stage Code: 3
Quality Code: 2
Comments: a, b

Cycle day: 6.5
Stage Code: 4
Quality Code: 1
Comments: b,c,d



Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 4
Quality Code:1
Comments: d

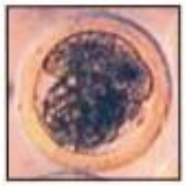
Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments: d

Cycleday: 7
Stage Code: 4
Quality Code: 1
Comments: d

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b



Cycle day: 7
Stage Code: 4
Quality Code:2
Comments: b, e

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

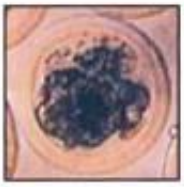
Comments:

a. If this embryo is collected on day 7 or later, the stage is not consistent with the expected stage of development and, therefore, should be lowered one quality code.
b. Large cells that were extruded from the embryo mass prior to the 16-cell stage easily make up more than 15% of the total cellular material through stage 5 embryos.
c. Large individual blastomeres indicate compaction is not complete and is an early stage 4.
d. Single or small extruded blastomeres comprise less than 15% of the total cellular material and the embryo is consistent with the expected stage of development.
e. Sperm on zona pellucida.

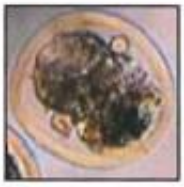
BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

Cycle day: 7
Stage Code: 4
Quality Code:2
Comments:

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments:f,g



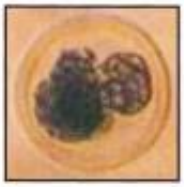
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g



Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments:f, g



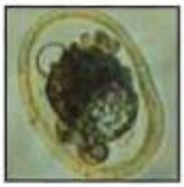
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g, h

Cycle day: 7
Stage Code: 5
Quality Code:l
Comments:

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments: d

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments: d, i



Cycle day: 7
Stage Code: 5
Quality Code: 2
Comments: e

Cycle day: 7
Stage Code: 5
Quality Code: 2
Comments:

Cycle day: 7
Stage Code: 5
Quality Code:2
Comments:

Cycle day: 7
Stage Code: 5
Quality Code: 3
Comments: g

Comments:

d. Single or small blastomeres comprise less than 15% of the total cellular material and the embryo is consistent with the expected stage of development.
e. Sperm on zona pellucida.
f. Embryos with many extruded cells or debris must be carefully rolled over to determine the presence and quality of any viable embryo mass.
g. Quality code 3 embryos have an embryo mass that is less than 50% of all cellular material within the zona pellucida.
h. This embryo has a nice but very small mass. If the embryo mass is less than 25% of all cellular material, it should be given quality code 4 (non-viable).
i. Irregular shape is a common variation in blastocoel development.

Figure 5.6.6 Examples of bovine embryos at various stages and qualities. Images of embryos with their grade and reasoning for the grade. Grading determined by international embryo grading guide (Stringfellow et al., 2007).

Appendix VII. Cell transfer record sheet

Experiment : | **Run Number :**

IVM 19/05/2022	
Time :	
Person(s) :	
No. ovaries :	
No. oocytes :	
Mother age :	
Abattoir:	
Approx. collection time :	
No. with CL:	
Comments:	

Cloning solutions

Solution	Fresh ?	Date Thawed	Date made	Batch person
H199 - FCS				
HSOF				
HSOF-BSA				
HSOF-Ca-BSA				
ESOF-Ca-BSA				
SOF				
FCS				
BSA				
Scriptaid				
Lectin				
6-DMAP				
Ionomycin				
Hyaluronidase				
Pronase				
FB				

NT 20/05/2022	
Time out IVM :	
Oocyte breakdown	
Homo +PB:	
Homo -PB:	
Hetero:	
Dead:	
Enucleator(s):	
Culture system:	

24hr C/O 21/05/2022

Group	Lysed	1-c	2-c	3-c	>3c	Frag

D5 C/O 25/05/2022

Group	Lysed	1-c	>1-c	Frag	TM	B

Experiment comments:

End of Culture Date : 26/05/2022 Grader:

	Total		1-cell	Total		1-cell	Total		1-cell

Appendix VIII. Fusion record sheet

FBA Zona-Free

Experiment:_____

Date:_____

Cell information:_____

Cell cycle synchronisation: _____

Drop	Plate	SC/ DC	No. Cytos	Time Fused	Cytoplast lysed PF	Donor lysed PF	No. Fused	No. not fused	Comments

Fusion Parameters

	Parameter 1	Parameter 2	Parameter 3
Amplitude			
µsec			
PF AC time			

Fusion rate

Group	No. fused	Total	Percent
Total			

Group	No. into ESOF –Ca	Time into ESOF – Ca	No. for Act.	Time HSOF + 1mg/ml	Time Iono.	No. into DMAP	Time into DMAP	No. into IVC	Time into IVC
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		: