LIPID QUANTIFICATION AND CRYOPRESERVATION OF IN VITRO PRODUCED JERSEY CATTLE EMBRYOS

A Thesis presented to the Faculty of California Polytechnic State University, San Luis Obispo

In Partial Fulfillment of the Requirements for the Degree Master of Science in Agriculture

by Katherine A. Rhodes-Long

August 2020
COMMITTEE MEMBERSHIP

TITLE: Lipid quantification and cryopreservation of in vitro produced Jersey cattle embryos

AUTHOR: Katherine A. Rhodes-Long

DATE SUBMITTED: August 2020

COMMITTEE CHAIR: Fernando Campos-Chillón, M.S., D.V.M., Ph.D., D.A.C.T., Professor of Animal Science

COMMITTEE MEMBER: Joy Altermatt, D.V.M., M.S., D.A.C.T., Associate Professor of Animal Science

COMMITTEE MEMBER: Moises Barceló-Fimbres, D.V.M., M.S., Ph.D., Research Veterinarian, Swinging Udders Veterinary Services
ABSTRACT

Lipid quantification and cryopreservation of in vitro produced Jersey cattle embryos

Katherine A. Rhodes-Long

Cryopreservation of in vivo derived Jersey bovine embryos have resulted in a 10% lower pregnancy rate compared to other dairy breeds. Poor embryo survival after cryopreservation has been partially attributed to the high lipid content of Jersey embryos. In vitro-produced (IVP) bovine embryos have darker cytoplasm than their in vivo-derived counterparts because of higher lipid accumulation. High lipid accumulation is associated with impaired embryo quality. Forskolin is an adenylate cyclase activator that regulates cAMP levels in cells and has been shown to induce lipolysis in IVP embryos. L-carnitine is required for transport of fatty acids from the intermembrane space of the mitochondria into the mitochondrial matrix to support the process of \( \beta \)-oxidation, and enhances ATP production. We hypothesized that the lipid content of in vivo-produced and IVP Jersey embryos is higher than respective Holstein embryos and that forskolin + L-carnitine would reduce lipid content of IVP embryos and vitrification with embryo collapse would improve the cryosurvival of Jersey IVP embryos. The objectives of this experiment were (1) to analyze lipid content of in vivo and IVP Jersey and Holstein cattle embryos, (2) to evaluate the effect of forskolin + L-carnitine added to IVP culture media, and (3) evaluate Jersey IVP survival rates after three cryopreservation procedures. The factorial experimental design for objectives one and two used two breeds (Holstein and Jersey) and three embryo production methods (in vivo, IVP, and IVP + forsk/L-C). In vivo produced embryos (n = 27 blastocysts) were collected from superstimulated donors by routine procedures 7.5 days after AI. IVP embryos (n = 259 blastocysts) were produced by standard procedures; briefly, oocytes were aspirated from 2- to 8-mm follicles from slaughterhouse ovaries and matured for 24 h in SMM medium (BoviPro, MOFA Global, Verona, WI, USA). Matured oocytes were fertilized using semen from two different bulls for each breed, and embryos were cultured in BBH7 medium (BoviPro, MOFA Global) alone or with the addition of 1.5mM L-carnitine during maturation and embryo culture with forskolin (10 \( \mu \)M) added at Day 5 of culture at 38.5°C in 5% \( \text{O}_2 \), 5% \( \text{CO}_2 \), and 90% \( \text{N}_2 \). The lipid content of embryos was quantified by staining Day 7 blastocysts with 1 \( \mu \)g mL\(^{-1} \) Nile red dye (580–596 nm), after which a digital photograph of the equatorial part of the embryo was taken at 40\( \times \), and fluorescence intensity (FI) was measured with Image Pro...
software. Data was analyzed by ANOVA, and means were compared using Tukey’s HSD. For the third objective, grade 1 Jersey IVP blastocysts (n=356) were divided into six treatments using a 2x3 factorial design comparing intact (IB) vs collapsed blastocoele (CB) and three cryopreservation methods: slow freezing (SF) vs vitrification using open pulled straws (OPS) or cryotop (CT). Slow freezing embryos were equilibrated in 0.7 M glycerol and 0.1 M galactose in holding media for 10 min, held for 10 min at -6°C, seeded after 5 min, decreased to -32 °C at 0.5 °C /min, held at -32°C for 5 min, and finally plunged into liquid nitrogen. Vitrified embryos were equilibrated in 1.5 M ethylene glycol (EG) for 5 min, exposed to 7 M EG + 0.6 M galactose for 30 s while loaded into OPS or placed onto CT, then immediately plunged into liquid nitrogen. SF embryos were thawed in air for 10 s and placed in a water bath at 37°C for 45 s. Vitrified embryos were warmed directly into holding medium at 37°C supplemented with 1.0 M, 0.5 M and 0.25 M galactose for 3 minutes each. Subsequently, embryos were cultured in BBH7 and re-expansion rates were assessed at 24 and 48h post warming and data was evaluated by GLIMX. For objective 1, Jersey and Holstein IVP embryos had higher lipid content than Holstein in vivo-produced embryos (P < 0.05), but were not different than Jersey in vivo-derived embryos (P > 0.1). Forskolin + L-carnitine lowered the lipid content (P < 0.05) of both IVP Jersey and Holstein embryos and was not different (P > 0.1) than in vivo-produced embryos. For experiment 2, re-expansion rates were higher for CT, than OPS, and SF (85 vs. 66 vs. 72% ± 0.4, respectively; p<0.05). Main effect means for re-expansion were higher for CB than IB (79 vs 68% ± 0.3; p<0.05). In conclusion, IVP embryos have higher lipid accumulation over Holstein in vivo embryos. Addition of forskolin and L-carnitine to embryo culture media has the potential to lower embryo lipid accumulation and possibly improve embryo viability and cryotolerance of IVP embryos. The CT method and collapsing the blastocoele prior to cryopreservation resulted in higher blastocyst survival rate. Further studies including transfer of embryos to recipients are necessary to corroborate these results.

Keywords: Jersey Cattle, IVP, Lipids, Cryopreservation, Embryos
ACKNOWLEDGMENTS

Firstly, I would like to thank my thesis advisor, Dr. Fernando Campos-Chillón, for giving me this opportunity and not giving up on me. He constantly challenged me to not only be a better researcher, but also a better person. He was always available to answer my countless questions and continues to be a valuable mentor. I cannot thank him enough for the encouragement and support.

Secondly, I would like to express my gratitude to Dr. Moises Barceló-Fimbres for his valuable and constructive suggestions during the planning and development of this research work. His willingness to give his time so generously is very much appreciated. I would also like to thank Dr. Joy Altermatt for her patient guidance, enthusiastic encouragement and useful critiques. I very much enjoyed my time working with her and assisting her with her work in bovine and equine reproduction. A big thank you to Dr. Daniel Peterson who saw potential in me and allowed me to sit in on his graduate seminars. He inspired me to pursue research and take the next step in my academic career.

Lastly, I wish to extend my special thanks to my lab mates and student assistants who spent long, tedious hours in the Cal Poly Embryology Laboratory making all of our projects a success. A special shout out to my closest friends, Alexander DeCarli and Samra Wali, for making my time at Cal Poly memorable. Thank you to my husband, Vishnu Jyothindran, for his love, support, and encouragement to push me to follow through on the writing of this thesis.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. LITERATURE REVIEW</td>
<td>2</td>
</tr>
<tr>
<td>2.1 Differences Between In Vivo and In Vitro Produced (IVP) Embryos</td>
<td>2</td>
</tr>
<tr>
<td>2.2 Lipids and Metabolic Regulators</td>
<td>2</td>
</tr>
<tr>
<td>2.3 Lipid Quantification</td>
<td>6</td>
</tr>
<tr>
<td>2.4 Cryopreservation</td>
<td>7</td>
</tr>
<tr>
<td>2.5 Objectives</td>
<td>10</td>
</tr>
<tr>
<td>3. MATERIALS AND METHODS</td>
<td>12</td>
</tr>
<tr>
<td>3.1 Embryo Production</td>
<td>12</td>
</tr>
<tr>
<td>3.1.1 In vivo embryo production</td>
<td>12</td>
</tr>
<tr>
<td>3.1.2 IVP embryo production</td>
<td>13</td>
</tr>
<tr>
<td>3.2 Nile Red Staining and Lipid Quantification</td>
<td>14</td>
</tr>
<tr>
<td>3.3 Cryopreservation Experimental Design</td>
<td>14</td>
</tr>
<tr>
<td>3.4 Blastocoele Collapse</td>
<td>15</td>
</tr>
<tr>
<td>3.5 Cryopreservation</td>
<td>16</td>
</tr>
<tr>
<td>4. RESULTS</td>
<td>17</td>
</tr>
<tr>
<td>4.1 Lipid Quantification</td>
<td>17</td>
</tr>
<tr>
<td>4.2 Cryopreservation</td>
<td>18</td>
</tr>
<tr>
<td>5. DISCUSSION</td>
<td>19</td>
</tr>
<tr>
<td>6. CONCLUSION</td>
<td>22</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>45</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fluorescent intensity of in vivo and IVP produced Jersey and Holstein embryos</td>
<td>17</td>
</tr>
<tr>
<td>2. Re-expansion of embryos after cryopreservation</td>
<td>18</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mechanism of L-carnitine. L-carnitine facilitates movement of fatty acids into the mitochondria by converting them into fatty acyl-carnitine. The fatty acyl-carnitine then undergoes β-oxidation and is then converted to ATP (adapted from Dunning and Robker 2012)</td>
<td>5</td>
</tr>
<tr>
<td>2. Mechanism of Forskolin. Forskolin stimulates the adenylate cyclase pathway to initiate lipolysis by activating the PKA pathway. The PKA pathway breaks triglyceride-rich lipid droplets down to fatty acids that are shuttled into the mitochondria by L-carnitine (adapted from Sudano et al. 2013).</td>
<td>6</td>
</tr>
<tr>
<td>3. Graphic representation of the difference between conventional slow freezing and vitrification. Slow freezing method has a slow and rapid cooling phase to allow for proper ice seeding in the media surrounding the embryo where vitrification has only rapid cooling and no ice formation (Kasai &amp; Mukaida, 2004).</td>
<td>8</td>
</tr>
<tr>
<td>4. Superovulation and in vivo flush timeline: Visual representation of superovulation timeline from insertion of progesterone CIDR to non-surgical flush of day 7 in vivo embryos</td>
<td>13</td>
</tr>
<tr>
<td>5. In vitro production timeline</td>
<td>14</td>
</tr>
<tr>
<td>6. Cryopreservation experimental design</td>
<td>15</td>
</tr>
<tr>
<td>7. Visual representation of the set up for blastocoele collapse</td>
<td>15</td>
</tr>
<tr>
<td>8. Visualization of Nile Red fluorescent staining</td>
<td>17</td>
</tr>
</tbody>
</table>
Chapter 1
INTRODUCTION

Reproductive technologies such as in-vitro fertilization (IVF), embryo transfer, and embryo cryopreservation are invaluable ‘tools’ available for dairy and beef cattle breeds to achieve specific breeding and herd genomic goals. California is the leading dairy producing state (NASS & USDA, 2019) with 40.4 billion pounds of milk, generating $6.37 billion cash receipts from milk production (CDFA 2019) and over 189,000 jobs (2018 Impact Report-California Dairy Pressroom). While Holstein cattle are a popular dairy breed partially due to their higher milk production per cow, 29.1kg daily milk yield compared to 20.9kg per day for Jersey (Capper & Cady, 2012), the Jersey breed is becoming in higher demand as a primary breeds or as cross-breeds in dairies. This is due in part to the inherent higher fat content in their milk, which is ideal for producing milk, yogurts, and cheese, as well as their ability to maximize their feed efficiency (Kristensen et al., 2015). With genomic selection for superior individuals becoming more readily available, there is a need to efficiently propagate these valuable genetics within and between herds of cattle across the world. One method of propagating and shipping these valuable genetics is through cryopreservation of in-vivo and IVF produced Jersey cattle embryos. However, the cryosurvival of this particular breed tends to be lower and results in a lower pregnancy rates compared to other breeds of cattle such as the Holstein (Steel & Hasler, 2004). It is thought that Jersey cattle embryos have a higher lipid content than their Holstein counterparts and could account for the differences in cryosurvival and subsequent pregnancy rates between the two breeds (Baldoceda et al., 2016; Steel & Hasler, 2004).
2.1 Differences Between In Vivo and In Vitro Produced (IVP) Embryos

In vitro fertilization (IVF) in the cattle industry is becoming increasingly popular, with one of the major advantages being that it can be performed on pregnant cattle without harming the fetus (Hansen, 2006). IVP embryos are much easier to obtain and produce in large quantities over embryo flushing. Embryos from good quality heifers and cows can be continuously produced while allowing them to remain in the milking herd. However, in-vitro derived embryos clear differences compared to their in vivo counterparts despite the advances being made to embryonic culture systems (Lonergan et al., 2003). IVP embryos often have very electron dense cytoplasm, have weaker intracellular bonds that result in looser blastomere formation, and are buoyant in density (Baldocena et al., 2015). They tend to show gene overexpression compared to in vivo embryos and have a higher apoptotic rate (Fair et al., 2001; Rizos et al., 2003). IVP embryos have a higher activity of the glycolytic pathway and consequent inhibition of the oxidative phosphorylation pathway, which impairs embryo development because too little energetic substrate is partitioned to the pentose phosphate pathway in favor of lipid accumulation and increased lipid synthesis precursors (Sutton-McDowall et al., 2010). This is known as the crab tree effect (Gardner et al., 2000). This metabolic difference can attribute to the higher lipid accumulation of the IVP embryos as compared to their in vivo counterparts. They also show an altered metabolism where there is an increase in lactate production and increase in oxidation of energy substrates, which could be a stress response to the in vitro culture system (Khurana & Niemann, 2000). These differences in morphological quality and metabolic systems impact pregnancy rates after transfer of the fresh or frozen IVP embryos (Hasler et al., 1995).

2.2 Lipids and Metabolic Regulators

Lipids can be broken up into four general classes: triglycerides, free fatty acids, cholesterol, and phospholipids. Phospholipids are the structural units of cell membranes, where their composition determines the physiochemical properties and functionality of the membrane including permeability, fluidity, and thermal phase behavior (Edidin, 2003; Stubbs & Smith, 1984). The phospholipid composition in cell
membranes can vary between species as well as embryo origin (IVP vs in vivo) (Ferguson & Leese, 2006; Ferguson & Leese, 1999; McEvoy et al., 2000). Cholesterol and phospholipids interact in the cell membrane and the level and ratio of cholesterol can change the cell membrane’s fluidity (Edidin, 2003; Horvath & Seidel, 2006). Triglycerides are mainly stored in intracellular lipid droplets and are the most abundant lipid in the cytoplasm of mammalian cells. They are important source of energy for oocytes and embryos (Ferguson & Leese, 2006)

Lipids present in bovine embryos seem to play a significant role on energy storage and cell structure. These lipids can also modify the physiological properties and metabolic functions of biological membranes (Ferguson & Leese, 2006). Bovine embryos metabolize glycogen, proteins, and lipids from the single-celled zygote to well past the blastocyst stage (Ferguson & Leese, 1999). While there may be structural components, lipids seem primarily to provide a large potential energy reserve for the developing embryos.

Jersey cattle embryos are more naturally lipid-rich than other Bos Taurus breeds, and this is a suggested reason for their lower tolerance to cryopreservation, due in part to lipids causing an interference of water movement out of the cells and stability of the plasma membrane (Frers et al., 2008). Similarly, due to artificial culture conditions as well as an altered metabolism, in-vitro produced bovine embryos have a higher lipid content than their in vivo derived counterparts and this has been associated with impaired embryo quality and reduced cryotolerance (Abe et al., 2002; Ferguson & Leese, 1999; Rizos et al., 2003).

Mechanical dilapidation is performed in porcine oocytes and embryos via centrifugation and physically removing the lipid via aspiration with a micromanipulation system (Nagashima et al., 1994). Mechanical dilapidation showed an increase cryotolerance (Nagashima et al., 1994, Fu et al., 2011; Men, et al., 2006; Nagashima et al., 1999) and even resulted in live births of piglets (Hiruma et al., 2006). However, embryos showed decreased developmental competence following dilapidation with altered energy metabolism and increased ROS stress (Wang et al., 2015). Apart from the reduced developmental capacity, the process is labor-intensive and time-consuming which would prevent it from becoming commercially available.

Chemical dilapidation is a viable option of reducing intracellular lipids of IVP embryos and potentially increases embryonic survival post thaw by reducing the chance of mechanical collisions between lipid droplets within the cells (Fu et al., 2011). The addition of phenazine ethosulfate (PES) showed intracellular
l lipid decrease (Barceló-Fimbres & Seidel, 2007) but there were toxicity concerns. In a 2008 study done by Barceló-Fimbres and Seidel, the effects of caffeine, epinephrine and forskolin on embryonic lipid reduction were evaluated. Caffeine was shown to be detrimental in high doses, as seen by the reduced number of blastocysts, and epinephrine was not as effective in reducing intracellular lipids as forskolin. L-carnitine had been shown to promote lipid utilization and increase cryosurvival in both oocytes and embryos in several species (Abdelrazik et al., 2009; Dunning et al., 2011; Held-Hoelker et al., 2017; Yazaki et al., 2013). Other compounds, such as vitamin K2, focused on increasing mitochondrial efficiency to increase lipid utilization (Baldoceda et al., 2013).

L-carnitine, a small hydrophilic molecule synthesized from lysine and methionine (Montjean et al., 2012; Wu et al., 2011), has been shown to play a major and beneficial role in enhancing lipid metabolism in both oocytes and embryos of several species (Chankitisakul et al., 2013; Dunning et al., 2011). It exists in two stereoisomers: active L-carnitine and inactive D-carnitine. L-carnitine is required for transport of fatty acids from the intermembrane space of the mitochondria into the mitochondrial matrix [Figure 1] to support the process of β-oxidation (Phongnimitr et al., 2013; You et al., 2012), and enhances ATP production which helps decrease the overall lipid content of the oocyte or embryo. L-carnitine has also been shown to increase nuclear maturation and meiotic competence in the MII stage oocytes by improving mitochondrial activity, relocating mitochondria in the cytoplasm (You et al., 2012), improving spindle microtubule assembly, and chromosome alignment (Moawad et al., 2013). L-carnitine acts as an antioxidant by protecting and stabilizing mitochondrial membranes and DNA against degradation by reactive oxygen species (ROS) (Wu et al., 2011; Yazaki et al., 2013). It protects the cells from apoptosis, reduces cytoskeletal damage and protects developing embryos against oxidative stress (Dunning & Robker, 2012; Phongnimitr et al., 2013). L-carnitine shows dual effects of regulating both lipid levels and accumulation of ROS, thus improving blastocyst development and cryotolerance (Baldoceda et al., 2013).
Forskolin, a diterpene derived from the roots of the Indian Coleus plant, *Coleus forskohlii* (Seamon, 1981), has been reported to decrease the levels of lipids in porcine and bovine embryos (Fu et al., 2011; Men et al., 2006; Sanches et al., 2013). Forskolin works to re-sensitize cell receptors by activating adenylyl cyclase, which in turn raises cAMP and activates both the protein kinase A (PKA; regulates glycogen, sugar, and lipid metabolism) and Epac (exchange protein directly activated by cAMP) pathways which influence glycogen breakdown (Seamon 1981; Men et al. 2006; Meneghel et al. 2017) [Figure 2].
Figure 2 Mechanism of Forskolin. Forskolin stimulates the adenylate cyclase pathway to initiate lipolysis by activating the PKA pathway. The PKA pathway breaks triglyceride-rich lipid droplets down to fatty acids that are shuttled into the mitochondria by L-carnitine (adapted from Sudano et al. 2013).

The potential benefits of the addition of L-carnitine and Forskolin to the in-vitro production should lead to a quality blastocyst with a higher cryotolerance, thus allowing Jersey embryos to be more effectively cryopreserved and valuable genetics to be shared.

2.3 Lipid Quantification

Lipids in embryos can be quantified in several ways. Previous studies used gas chromatography (Kim et al., 2001; McEvoy et al., 2000) or assay kits (Ferguson & Leese, 1999). These methods often utilize costly equipment and require large pools of embryos or oocytes. An alternative method used for visualizing and semi-quantifying lipids is Sudan Black B staining (Abe et al., 2002; Barceló-Fimbres & Seidel, 2007; Subramaniam & Chaubal, 1990). Sudan Black B selectively stains intracellular neutral lipids, i.e. triglycerides, a dark blue-black color (Subramaniam & Chaubal, 1990). This method allows for a visual
representation of the distribution of lipids in the cell as well as relative size of the lipid droplets. This method this easy to implement however, is very time consuming and is subject to observer’s bias. Nile red, 9-diethylamino-5H- benzo[c]phenoxazin-5-one, is a lipophilic fluorescent dye that target lipids, primarily triglycerides (Barceló-Fimbres & Seidel, 2008; Bonilla & Hansen, 2005; Genicot et al., 2005; Greenspan & Fowler, 1985). When Nile red binds to neutral lipid droplets present in mammalian oocytes and embryos, it emits fluorescence and allows the user to analyze the embryos using an imaging program. This gives you the fluorescent intensity for each embryo on a whole, rather than counting individual lipid droplets. In a 2011 study to validate the use of Nile Red, Barceló-Fimbres and Seidel compared the lipid content of bovine oocytes and embryos using both Sudan Black B and Nile Red fluorescent staining (Barceló-Fimbres & Seidel, 2011). They compared arbitrary fluorescent units (AFU) obtained from Nile red fluorescence and an imaging software to the relative size of lipid droplets seen with Sudan Black B stain with a regression analysis. They found Nile red to be a comparable alternative to Sudan Black B staining.

2.4 Cryopreservation

Cryopreservation of cattle embryos is a way to spread and preserve valuable genetics and the transfer of frozen embryos have been gaining in popularity. Data reported by the International Embryo Transfer Society (IETS) showed an shift from transferring fresh embryos to in-vitro produced frozen embryos in North America from 19% in 2013 to just under 60% in 2019 (IETS, 2019). The first reported success in cryopreservation of mammalian embryos was by Whittingham et al. in 1972 with mouse embryos. Whittingham’s research with different cryoprotectants, cooling rates, and warming rates lead to the development of conventional slow-freezing which is still the most widely used method of cryopreserving embryos in the livestock industry (Hasler, 2014). It involves the simultaneous gradual dehydration of the embryo in a cryoprotectant along with a gradual cooling of the embryo and its surrounding media before being plunged into liquid nitrogen (Kasai & Mukaida, 2004; Whittingham et al., 1995). Equilibrating the embryos or oocytes in a permeating cryoprotectant such as glycerol or ethylene glycol causes extracellular water to crystalize which results in a high osmotic gradient (Saragusty & Arav, 2011). This in turn will draw out intracellular water, allowing the intracellular matrix to vitrify preventing intracellular freezing which
causes membrane damage (Mazur, 1963). This method does not require extensive training, however the cooling system could be costly and the process time consuming.

Figure 3 Graphic representation of the difference between conventional slow freezing and vitrification. Slow freezing method has a slow and rapid cooling phase to allow for proper ice seeding in the media surrounding the embryo where vitrification has only rapid cooling and no ice formation (Kasai & Mukaida, 2004).
Vitrification is a rapid physiological phase change from a fluid to a glass-like solid which virtually eliminates the potential of lethal ice crystal formation (Saragusty & Arav, 2011; Vajta, Holm, & Kuwayama, 1998) which helps increase embryo survival. Cellular dehydration during the short equilibration steps prior to freezing allows both the intracellular space and the extracellular solution to vitrify at the same time, reducing the chance of osmotic damage and ice crystal formation that can damage cell membranes (Saragusty & Arav, 2011; Sudano et al., 2013). The combination of high cryoprotectant concentrations, extreme elevation in solution viscosity, and small volumes causes a rapid phase change in the liquid surrounding the embryo from a liquid, to a glass-like solid (Kasai & Mukaida, 2004). This virtually eliminates damaging ice crystal formation and increases the embryo survivability. Vitrification requires only a microscope and a Styrofoam container to plunge the vitrification device into but it does require training that is more extensive.

Kaidi et al. 2001 compared the effects of conventional slow freezing and vitrification on the morphology and metabolism of in vitro-produced bovine blastocysts. Similar survival and hatching rates were observed as well as a decreased cell number after warming/thawing. The total number of trophoderm cells were lower in embryos frozen conventionally versus the vitrified group after 72 hrs of culture, as well as a decrease in glucose, pyruvate, and oxygen uptake compared to the vitrified group. The conventionally frozen group also had an increase in glycolytic activity, indicating a stress response to this procedure.

Do et al. 2018 compared pregnancy rates of vitrified and non-vitrified in-vitro produced bovine blastocysts and found no statistical difference between the two groups. The pregnancy rates were 41.3% and 40% on day 30 for the fresh transfer group and vitrified group respectively. They followed up with pregnancy checks on days 30 and 60 and observed a pregnancy rate of 37.6% and 40.0% for the fresh and vitrified groups respectively. Pereira et al. 2019 reported similar findings with in-vitro produced Nellore (Bos indicus) embryos where overall pregnancy rates were not statistically significant between the fresh and vitrified groups (47% fresh vs 40% vitrified). Pereira further noted that there was no statistical difference between the fresh and vitrified groups in terms of day 60 pregnancy loss (7.69% fresh and 6% vitrified).

In vitro produced embryos have been shown to have a lower tolerance to cryopreservation procedures compared to their in vivo counterparts (Fair et al., 2001; Hasler et al., 1995). Most of these observations have been associated with higher embryonic lipid content (H. Abe et al., 2002; Seidel, 2006). Increased
intracellular lipid stores lead to larger cell volume which may cause osmotic issues and a lower buoyant density (Ferguson & Leese, 1999). Larger cell volume means a low surface to volume ratio, limiting the efficiency of the movement of water and cryoprotectant across the cellular membrane (Pereira & Marques, 2008).

It has also been thought that fully expanded blastocysts are more sensitive to vitrification. The large volume of fluid in the large expanded blastocoele cavity could impair the rapid equilibration of the embryo in cryoprotectant and leads to intra-blastocoelic ice crystal formation (Feng et al., 2010; Iwayama, Hochi, & Yamashita, 2011). Collapsing the blastocoele cavity prior to vitrification has been shown to improve pregnancy rates following warming in humans, equine, bovine, and porcine studies (Choi et al., 2011; Iwayama et al., 2011; Kovačič et al., 2018; Lin et al., 2008; Min et al., 2014). Blastocoele collapse, also referred to as forced or artificial blastocoele collapse, can be done either mechanically using a needle inserted into the blastocoele cavity and aspirating the fluid (Chen et al., 2005) or by laser pulse between 2 trophectoderm cells (Mukaida et al., 2006).

2.5 Objectives

The purpose of this thesis was to determine if Jersey cattle embryos did have higher lipid content compared to their Holstein counterparts, determine if the addition of metabolic regulators L-carnitine and forskolin could reduce the lipid content of IVP embryos, and compare different methods of cryopreservation to determine the optimal protocol for Jersey IVP embryos. The first objective was to produce in vivo and in vitro bovine embryos from Jersey and Holstein cows, then measure and compare the lipid content from each group of embryos. We anticipated that the lipid content in Jersey embryos would be higher than their respective Holstein counterparts and IVP embryos would have a higher lipid content than in vivo produced embryos. The second objective was to determine if the addition of metabolic agents, L-carnitine and forskolin, decreased lipid content of in vitro Jersey and Holstein embryos. We expected that the addition of L-carnitine and forskolin would reduce the lipid content of the IVP embryos to resemble that of in vivo embryos for both breeds. The third objective was to test two methods of vitrification and one method of slow cryopreservation on in vitro produced Jersey cattle embryos from which the blastocoele fluid had or had not
been removed, and to evaluate their post warming survival. We expected vitrification with blastocoele collapse to have higher re-expansion rate post warming over slow frozen embryos or vitrified embryos without collapsing the blastocoel.
Chapter 3
MATERIALS AND METHODS

3.1 Embryo Production

3.1.1 In vivo embryo production

In vivo produced embryos (n=27) were collected by standard procedures 7 days after A.I. Lactating Jersey cows at the Cal Poly Dairy were stimulated to induce superovulation according to the MoFA Pluset protocol. A controlled Internal Drug Release device, CIDR (EAZI-BREED™ CIDR® Cattle insert, Zoetis USA) containing progesterone was inserted into the vagina on stimulation day zero. On stimulation day two, 86µg/mL GnRH (Fertagyl; Intervet Inc, Summit, NJ) was administered intramuscularly in the neck or rump. Stimulated cows received 8 decreasing doses FSH (Pluset Flex H; MoFA, Verona, WI) for a total of 700 IUs or 575 IUs FSH for cows or heifers respectively on days four to eight via intramuscular shots to the rump. Prostaglandin (250mg/mL, i.m.) Estrumate; Intervet Inc, Summit, NJ) were administered along with the 6th and 7th FSH injections. The cows were monitored for signs of heat and artificially inseminated with frozen-thawed semen collected from either a Jersey or Holstein bull twice on day 9, approximately 12 hours apart. A non-surgical embryo flush was performed on day 17 of the stimulation cycle to collect day 8 in vivo embryos.

Embryos were non-surgically retrieved by standard procedures (Seidel 1991). Briefly, superovulated cows were restrained in the stocks and their tail tied loosely up. The cows were rectally palpated and a sterilized two-way Foley catheter connected to an embryo collection cup was inserted into vagina, through the cervix, and into the uterus. The bulb of the catheter was inflated just past the cervix with a saline solution to ensure the catheter would not slip out during the embryo collection. A uterine body flush with a continuous flow method was performed using approx. 1-2 liters of flush solution total into the uterus at a slow, steady rate and the flush solution emptied through an embryo collection cup with a mesh filter. The uterus was rectally manipulated to encourage complete emptying of the flush fluid from the uterus and maximize embryos collected. The embryo collection cup was then returned to the dairy embryology laboratory and the mesh filter rinsed to prevent embryos from becoming stuck to it and lost. Embryos were visualized with a stereomicroscope at 40x and picked up using a pipette. Recovered embryos were immersed in a pH-stable
holding solution and were brought to the main embryology laboratory in building 10 to be stained with Nile Red.

![Figure 4](image)

**Figure 4 Superovulation and in vivo flush timeline:** Visual representation of superovulation timeline from insertion of progesterone CIDR to non-surgical flush of day 7 in vivo embryos.

### 3.1.2 IVP embryo production

Slaughterhouse derived ovaries were collected from Central Valley Meat Co. in Hanford, CA and transported at room temperature in saline solution to the Embryology Laboratory at Cal Poly. Ovaries were then rinsed thoroughly in saline then oocytes were aspirated from 2 to 8 mm follicles. Recovered oocytes were then matured for 24h in (TCM)-199 (Sigma, St Louis, MO, USA) supplemented with 0.2 mM Na-pyruvate, 15 ng mL⁻¹ ovine follicle stimulating hormone (oFSH) [NIDDK-oFSH-20], 50 ng mL⁻¹ human recombinant epidermal growth factor (hrEGF) (Sigma), 10% fetal calf serum (FCS), and 1.5mM L-carnitine in an incubator at 38.5°C and 5% CO₂ in air. Sperm from two different bulls for each breed were processed through a 40/80 Sperm-Talp Percoll (Sigma P1644) gradient and matured oocytes were co-incubated with the sperm for 16 hours. Presumptive zygotes were vortexed then washed in holding media to remove degenerating cumulus and excess sperm. Nile Red: Subsequent embryos were cultured in BBH7 medium (BoviPro, MOFA Global, WI, USA) supplemented with 1.5mM L-carnitine at 38.5°C in 5% O₂, 5% CO₂, and Embryos were then cultured until day 7 with the forskolin group having 10µM forskolin supplementation added at day 5, while the control group had no additives.

Embryos for the cryopreservation project were produced as described above. Embryos were cultured in BBH7 medium (BoviPro, MOFA Global, WI, USA) 1.5mM L-carnitine at 38.5°C in 5% O₂, 5% CO₂, and
90% N\textsubscript{2} for 6 days with 10µM forskolin supplementation added at day 5 then shipped overnight to Colorado State University in a shipping incubator at 38.5°C (Figure 5). Embryos were kept in an incubator until processing.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{in_vitro_production_timeline}
\caption{In vitro production timeline}
\end{figure}

### 3.2 Nile Red Staining and Lipid Quantification

Day 7 blastocysts were removed from culture and either fixed in a 10% formalin in a modified phosphate-buffered-solution (mPBS) + 0.01% PVA solution at pH 7.4 or 100% ethanol for the negative control overnight (minimum of 16 hours) in a dark box at room temperature. The respective groups were then washed in a PBS + 0.01% PVA solution (mPBS) to remove excess fixative before being added to a solution of 1 µg/mL Nile red dye (580-596nm) in mPBS for 30 minutes in the dark at room temperature. Embryos were then washed once more through mPBS to remove excess Nile Red dye before being mounted on a slide in 5µl glycerol with a maximum of 10 embryos per drop. The mounted embryos were immediately viewed on an epifluorescent confocal microscope with a FITC filter and a digital photograph of the equatorial section of the embryo was taken at 40x. Fluorescence intensity (FI) was then measured with Image Pro software from 0 to 255 shades for each pixel (0=no lipids, 255=highest lipid accumulation (Barceló-Fimbres & Seidel, 2011). Comparisons within and between groups were evaluated by ANOVA.

### 3.3 Cryopreservation Experimental Design

This experiment randomly divided grade 1 blastocysts (n=359) produced at the Cal Poly Embryology Lab into 6 treatments (replicated 13 times) in a 3x2 factorial design (3 cryopreservation methods: vitrification using cryotop or Open Pulled Straw (OPS) and a modified slow freezing protocol; 2 embryo manipulations: intact and collapsed blastocoele) (Figure 6).
3.4 Blastocoele Collapse

The embryos for the collapsed blastocoele group were randomly chosen and collapsed by removing the blastocoele fluid. Removal of fluid from the blastocoele cavity was performed with a Narishige micromanipulation system. Embryos were stabilized with the inner cell mass (ICM) at 6 or 12 o’clock with a holding pipette to prevent ICM disruption while a handmade aspiration pipette approximately 10 μm in diameter was inserted between two trophectoderm cells into the center of the blastocoele. Once the needle was inside the blastocoele, approximately 75% of the fluid was removed using slow, steady suction and these collapsed embryos were immediately vitrified or slow frozen to prevent re-expansion of the blastocoele cavity.

Figure 6 Cryopreservation experimental design

Figure 7 Visual representation of the set up for blastocoele collapse
3.5 Cryopreservation

Slow Frozen embryos were equilibrated in 0.7 M glycerol and 0.1 M galactose in holding media (Syngro; Bioniche Animal Health, Athens, GA) for 10 minutes during which time they were loaded into 0.25 mL straws. A controlled rate freezer was used for the following protocol: Hold for 10 min at -6°C, decrease in a controlled rate to -32°C at -0.5°C/min, and hold at -32°C for 5 minutes before being plunged into liquid nitrogen. Straws were seeded 5 minutes into the process with forceps pre-cooled in LN₂ to initiate the ice crystallization process. Embryos were stored in a LN₂ tank until thawing. Slow frozen embryos were thawed in air for 10 seconds then placed in a water bath at 37°C for 45 seconds before being expelled into a petri dish then placed in culture.

Vitrified embryos were equilibrated in 1.5 M ethylene glycol (EG) in holding media (500mL) for 5 minutes, followed by a brief exposure to 7 M EG + 0.6 M galactose in holding media for 30 seconds during which time embryos were either loaded by capillary action into Open Pulled Straws (OPS) or placed onto cryotops with a pipette holding approximately 1μL of medium with the embryo. The OPS or cryotop containing the embryo were immediately plunged into liquid nitrogen. Embryos were stored in a LN₂ tank until thawing. Vitrified embryos were warmed by removing the OPS or cryotop from the liquid nitrogen and submerging the end of the respective device containing the embryo directly into holding medium at 37°C supplemented with 1.0 M galactose for 3 minutes, then through 0.5 M and 0.25 M galactose for 3 minutes each.

Binomial data (expansion or not) and qualitative data (embryo grades) were evaluated by ANOVA using GLM procedures of SAS and means was compared using Tukey’s honestly significant difference (hsd) test.
4.1 Lipid Quantification

Jersey and Holstein IVP embryos had higher lipid content than Holstein *in vivo*-produced embryos \((P < 0.05)\), but were not different than Jersey *in vivo*-derived embryos \((P > 0.1)\). L-C+Forsk lowered the lipid content \((P < 0.05)\) of both IVP Jersey and Holstein embryos and was not different \((P > 0.1)\) than *in vivo*-produced embryos.

**Table 1** Fluorescent intensity of *in vivo* and IVP produced Jersey and Holstein embryos

<table>
<thead>
<tr>
<th>Embryo Production Methods</th>
<th>Breed</th>
<th>In vivo (n=27)</th>
<th>IVP (n=60)</th>
<th>IVP+L-C+Forsk (n=199)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holstein (FI)</td>
<td>43.2 ± 3.3 a</td>
<td>55.7 ± 2.7 b</td>
<td>44.5 ± 1.6 a</td>
</tr>
<tr>
<td></td>
<td>Jersey (FI)</td>
<td>49.2 ± 7.9 ab</td>
<td>56.9 ± 3.1 b</td>
<td>46.1 ± 1.6 a</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Values within columns and rows with different subscripts differ \((P<0.05)\).

**Figure 8** Visualization of Nile Red fluorescent staining
4.2 Cryopreservation

A generalized linear mixed model fit by the adaptive Gaussian Hermite approximation was used to analyze the raw data (Analyzed by Dr. Jeffrey Sklar at the Cal Poly Statistics Department). The formula is as follows:

\[ Re.\expansion_Y \sim Method + Stage + Treatment + 1/Replicate \]

\( Re.\expansion_Y \sim Method + Stage + Treatment + (1 | Replicate) \), with Replicate being a random effect. An odds ratios and 95% confidence intervals for the true odds ratios was calculated. Important results from Table 3: The estimated odds ratio .38 can be interpreted as the following: The odds of re-expansion for an embryo frozen under Method OPS are predicted to be \((1-0.38)100 = 62\%\) lower than the odds of re-expansion for an embryo frozen under Method Cryotop (adjusting for Stage and Treatment). The estimated odds ratio .42 can be interpreted as the following: The odds of re-expansion for an embryo frozen under Method SF are predicted to be \((1-0.42)100 = 58\%\) lower than the odds of re-expansion for an embryo frozen under Method Cryotop (adjusting for Stage and Treatment). The estimated odds ratio .58 can be interpreted as the following: The odds of re-expansion for an embryo treated with the control are predicted to be \((1-0.58)100 = 58\%\) lower than the odds of re-expansion for an embryo treated with the collapsed technique (adjusting for Stage and Treatment) (Table 2). Stage was not significant.

Table 2 Re-expansion of embryos after cryopreservation

<table>
<thead>
<tr>
<th>Method</th>
<th>Treatment</th>
<th>n=</th>
<th>Exp (%)</th>
<th>Hatched (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryotop</td>
<td>Collapsed</td>
<td>51</td>
<td>88.24</td>
<td>a 52.94</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>48</td>
<td>81.25</td>
<td>31.25</td>
</tr>
<tr>
<td>OPS</td>
<td>Collapsed</td>
<td>55</td>
<td>83.64</td>
<td>b 47.27</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>52</td>
<td>48.08</td>
<td>11.54</td>
</tr>
<tr>
<td>SF</td>
<td>Collapsed</td>
<td>81</td>
<td>70.37</td>
<td>c 50.62</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>72</td>
<td>75.00</td>
<td>22.22</td>
</tr>
</tbody>
</table>

a,b,c Values with different letters are significantly different (p=0.0067)
In the present study, the supplementation of L-carnitine on days 0 and 2 along with forskolin on day 5 did have a statistically significant impact on lipid accumulation in the IVP L-C+Forsk group compared to the IVP control group. The IVP L-C+Forsk group was not statistically different than the in vivo group, which shows the addition of the L-carnitine and forskolin lead to a decrease in lipid content comparable to an in vivo produced embryo of their respective breed.

We expected the Jersey and Holstein IVP embryos to have higher lipid content over both the Jersey and Holstein in vivo produced groups, however the data indicated that the in vitro groups were significantly different than the in vivo Holstein (P < 0.05) but not the in vivo Jersey group (P > 0.1). We also expected the Jersey groups to have a higher lipid content than the respective Holstein groups. The data did suggest the Jersey groups did have a numerically higher lipid content but was not significantly different from their Holstein counterparts. The results comparing the in vivo embryos and the in vitro embryos may have been skewed due to the small sample size of the in vivo group (n=27). A follow up study with a comparable number of vivo embryos for both breeds to get a more accurate picture of lipid composition is necessary.

The quantification of lipids has allowed us to gain insight into the lipid composition of embryos for many species (H. Abe et al., 2002; Barceló-Fimbres, 2007; Meneghel et al., 2017); however, few direct comparisons between Jersey cattle embryos and Holstein or other breeds of cattle exist. Steele and Hasler found that frozen Jersey embryos were resulting in a lower pregnancy rate compared to frozen Holstein embryos and were among the first to suggest that there might be a difference in lipid composition between the breeds (Steel & Hasler, 2004). Studies that do comparisons of Jersey embryo lipid content to Holsteins or other cattle breeds also corroborates with our findings that Jersey embryos do have a higher lipid content. Weathers found IVP Jersey embryos had a higher lipid content over IVP embryos generated from beef cattle (Weathers, 2008; Weathers & Prien, 2014). This difference was detected through a modified specific gravity technique combined with electron microscopy. Baldoceda, et al. compared the lipid content of Jersey and Holstein embryos, along with mitochondrial activity and differential expression of genes associated with lipid metabolism. They found that Jersey embryos displayed a darker cytoplasm and an increased lipid
accumulation, as well as lower mitochondrial activity, compared to Holstein cattle embryos (Baldoceda et al., 2016).

We expected the collapsed embryo groups to have a better survival rate following cryopreservation across all freezing groups due to the preemptive removal of blastocoele fluid that could slow the movement of cryoprotectant into the embryos. Expanded blastocysts may have decreased cryotolerance due to the inability to dehydrate fully and inadequate permeation of cryoprotectants leading to subsequent ice crystal formation in the blastocoele cavity (Iwayama et al., 2011). Collapsing embryos prior to vitrification is a common practice used in human IVF and has been used with success for porcine and equine embryos (Choi et al., 2011; Lin et al., 2008; Min et al., 2014; Mukaida et al., 2006).

The vitrification-collapsed groups did show a higher survival rate compared to the un-collapsed group, but the un-collapsed group performed better in the slow freezing method. The slow freezing method has a longer equilibration time which allows the embryos to naturally collapse due to osmotic shrinkage which would negate the benefits of artificial collapsing (Duke et al., 2004).

The vitrification group using cryotop had the highest re-expansion rate post warming over the other two groups. We expected one of the vitrification methods to perform the best due to the reduction of damaging ice crystal formation that would disrupt the membranes of the embryos and cause cellular death. One potential difference between the cryotop and OPS methods is a large surface area of the media droplet containing the embryo has direct contact with the LN$_2$ when using the cryotop vs the smaller surface area exposed by the OPS. This could have had an impact on the cooling rate of the media and embryos and subsequent embryo survival. Liu et al. compared OPS and cryotop for the vitrification of porcine embryos and found that the cryotop method was superior (Liu et al., 2008). Cryotop was also found to be superior to OPS for vitrifying bovine embryos (Morató et al., 2008).

Future studies are needed to investigate whether cryotop vitrification with artificial blastocoele collapsing can be a viable alternative to conventional slow freezing in terms of usability in the field. Warming vitrified embryos takes additional technical skill as well as a portable laboratory set-up. The benefits of vitrifying embryos on a device such as a cryotop can be overlooked, if it is not a practical procedure to carry out in the field.
The mechanisms and driving forces behind excess lipid accumulation in IVP embryos versus in vivo produced counterparts is still not fully understood. If light is shone on those processes, then we can more finely tune the maturations and culture media to better suit the embryo and bring their quality up to more closely resemble the in vivo conditions. IVP mitochondria is often altered in its structure, lower in number, and show decreased efficiency which could compromise the embryo’s lipid metabolism (H. Abe et al., 2002; Crosier et al., 2001). One way to potentially improve lipid metabolism is through improving mitochondrial function with the addition of compounds such as vitamin K2 (Baldoceda et al., 2013; Vos et al., 2012), resveratrol (T. Abe et al., 2017), and Co-enzyme Q (Bergamini et al., 2012). Additional studies could also take a closer look in the benefits that the addition of forskolin and L-carnitine have directly on the mitochondria and its functioning.

The best indicator of the success of our lipid-reduced, vitrified embryos is to obtain live births. A continuation of this project could look at the live birth characteristics of jersey cattle born from IVP embryos that were collapsed, vitrified on a cryotop then transferred.
Bovine IVP embryos of both Jersey and Holsteins had a higher lipid content than Holstein in vivo embryos. A larger sample of Jersey in vivo embryos is needed to have a more accurate picture of the average lipid composition vs Holstein embryos. The addition of L-carnitine and forskolin was shown to decrease the lipid content in both the Jersey and Holstein IVP groups. The cryotop method of vitrification had the highest rate of blastocyst re-expansion compared to the OPS and slow freezing groups while the blastocyst collapse was the most effective in the vitrification groups. Further studies are needed using Jersey IVP embryos produced with the addition of metabolic regulators looking at the live pregnancy rates following cryopreservation.
REFERENCES


Bonilla, L., & Hansen, P. J. (2005). Determining Lipid Content in Embryos using Nile Red Fluorescence, (Figure 1), 1–3.


