EFFECTS OF POLYDATIN ON IN VITRO BOVINE EMBRYO DEVELOPMENTAL COMPETENCE, METABOLISM, AND CRYOPRESERVATION

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ABSTRACT

Effects of Polydatin on In Vitro Bovine Embryo Developmental Competence, Metabolism and Cryopreservation

Corie Marie Owen

Bovine in vitro produced embryos suffer from poor developmental competence and altered metabolism which hinders their cryotolerance. Overall, the goals of this thesis were to improve oocyte and embryo culture with the antioxidant polydatin and to optimize slow freezing procedures. This thesis was designed as three experiments, and in each experiment, oocytes were aspirated from abattoir ovaries, matured for 23h, fertilized with semen from 1 of 3 bulls, and cultured in synthetic oviductal (SOF) based medium (SCF1) in 38.5 °C in 5% O_2, 5% CO_2 and 90% N_2. Stage 7 blastocysts were stained with Nile Red for lipid content or Mitotracker Red CMX-Rosamine for mitochondrial activity or Cell Rox Green for reactive oxygen species. Ten images per embryo were acquired using confocal microscopy at 5-µM step size and detected fluorescence by IMAGE PRO software. Embryos were also slow frozen in ethylene glycol and analyzed for post-thaw re-expansion after 24 and 48 hours. Experiment one was a 2x2x2 factorial design to test two culture media (SCF1 and a conventional SOF media), the use of a sucrose dehydration prior to slow freezing (2 min in 0 or 0.6 M sucrose), and the length of equilibration in ethylene glycol prior to slow freezing (10 or 20 min). We determined that embryos cultured in SCF1 had increased blastocyst rate, mitochondrial activity, and cryotolerance and decreased lipid accumulation ($p<0.01$). Additionally, stage 7 embryos equilibrated for 10 minutes had increased post-thaw apoptosis ($p<0.01$) and tended to have lower post-thaw re-expansion compared to blastocysts equilibrated for 20 min ($p<0.1$). In experiment two, effects of the antioxidant polydatin on all stages of embryo production were evaluated. Adding polydatin to maturation media improved blastocyst rate and post thaw re-expansion compared to adding it to all stages of production($p<0.05$); furthermore, these embryos had increased mitochondrial polarity and decreased lipid content compared to the control embryos ($p<0.05$). In experiment three a 2x2 factorial was designed to test the effects of polydatin on maturation medium, blastocyst rate, mitochondrial polarity, lipid content, ROS level, and cryotolerance. In addition, the effects of in straw rehydration coupled with antioxidants on post-thaw re-expansion were evaluated. Overall, oocytes matured with polydatin had increased mitochondrial activity ($p<0.05$) and tended to have higher blastocyst rate and lower ROS levels ($p<0.1$), but there was no difference in lipids or cryotolerance. Also, no difference was found in post-thaw survival when using in-straw rehydration. Overall, the results of our studies show that SCF1 improved embryonic development, and that polydatin had modest improvements on embryonic metabolism. Additionally, using a 20 min equilibration in ethylene glycol prior to slow freezing improved cryopreservation. Future studies should include a pregnancy study to validate the results seen in vitro.

Keywords: bovine, in vitro produced embryos, metabolism, polydatin, cryotolerance

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Chapter 1

INTRODUCTION

In vitro-produced (IVP) embryos have large potential in the dairy industry. Recent advances in assisted reproductive technologies, specifically ovum pick up and embryo transfer, have made IVP embryos easier to produce. By super-ovulating a genetically superior female and collecting oocytes, producing embryos, and transferring them into recipient animals, we can produce more offspring from a higher producing animal in one reproductive cycle. In 2015 alone, over 40,000 ovum pick-ups were performed in the United States (Moore & Hasler, 2017).

Unfortunately, bovine IVP embryos differ from *in vivo* derived (IVD) embryos and suffer from poor development, cryotolerance, and hindered metabolisms. Only 30-40% of retrieved oocytes develop to the blastocyst stage, or the stage at which embryos can be frozen of transferred. Of this, only 50% of transferred embryos will result in a pregnancy, resulting in only a 15-20% success rate compared to a 50% pregnancy rate for frozen IVD embryos (Moore & Hasler, 2017; Reader, Stanton, & Juengel, 2017).

One major factor for the decreased development is the poor metabolism of IVP bovine embryos. IVP embryo mitochondria have a hooded, immature appearance that are unable to produce enough ATP for the developing embryo (Reader et al., 2017). Additionally, lipid droplets are more abundant and have a different composition than intracellular lipids in IVD embryos (Reader et al., 2017). Although the exact impact the increased lipids have is unknown, they are associated with poor cryotolerance (Seidel, 2006; Sudano et al., 2016). Jersey cattle produce embryos with higher lipid content, and in turn exhibit lower cryotolerance than other breeds (Baldoceda et al., 2016). The poor metabolism and increased lipid content could explain the lower cryotolerance observed in IVP embryos.

The cause of the developmental problems are still unknown, but are likely due to improper culture conditions (M. Takahashi, 2012). Oocytes are cultured in hyperoxic conditions which leads to increased production of reactive oxygen species (ROS) (Kitagawa, Suzuki, Yoneda, & Watanabe, 2004). However, culturing oocytes at lower oxygen tension (5%) does not improve

blastocyst rates and in one study increased ROS production, suggesting that other factors influence the high ROS levels in IVP embryos (Pereira et al., 2010; N. A. S. Rocha-Frigoni, Leão, Nogueira, Accorsi, & Mingoti, 2015). ROS are found at higher levels in IVP embryos than IVD embryos and disrupt normal cellular functions, mutate DNA, and decrease mitochondrial function (Bain, Madan, & Betts, 2011; R. Li, Jia, & Trush, 2016).

The disconnect between the follicular and *in vitro* maturation conditions decreases the quality of the oocyte and leads to the poor development of IVP embryos. Oocytes that are matured *in vivo,* collected, fertilized, and cultured *in vitro* develop to the blastocyst stage 70% of the time, further suggesting the developmental problems may stem from the oocyte (Wrenzycki & Stinshoff, 2013). Improving oocyte maturation *in vitro* could help alleviate developmental problems seen in bovine IVP embryos and improve their efficiency in the industry.

The purpose of this study was to

1. Improve embryonic metabolism, developmental competence, and cryopreservation by using the antioxidant polydatin
2. Optimize slow-freezing procedures to improve post-thaw survival

Chapter 2

LITERATURE REVIEW

2.1 Oocyte Maturation and Physiology

2.1.1 Oocyte maturation

One of the most common procedures in cattle assisted reproduction (ART) is the use of *in vitro* oocyte maturation. Immature oocytes can be retrieved from abattoir ovaries or via ovum pick up from live animals and matured *in vitro* prior to fertilization. During maturation, the oocyte must progress from prophase I to metaphase II to allow fertilization to occur (Lonergan & Fair, 2016). The process of maturation consists of nuclear, cytoplasmic, and epigenetic events that progress the oocyte to a stage where fertilization is possible.

2.1.1.1 Nuclear Maturation

Nuclear maturation is the progression of the oocyte through meiosis to metaphase II. Immature oocytes are arrested at prophase I and must resume meiosis prior to fertilization. The meiotic arrest is maintained via a complex signal cascade which depends on maturation promoting factor (MPF) (Adhikari & Liu, 2014). Oocytes maintained within follicles are retained at prophase I by low activity of maturation MPF. MPF is a complex of cyclin dependent kinase (CDK1) and the regulatory subunit cyclin B (Adhikari & Liu, 2014; Han & Conti, 2006). CDK1 is regulated by cyclic adenosine 3’5’-monophosphate (cAMP) which is formed by adenylyl cyclase from ATP. There are two models for how cAMP is able to enter the oocyte, though the exact mechanism is still unknown (Adhikari & Liu, 2014; Dekel N., 1988)

The first mechanism of cAMP production is within the somatic cells, also known as cumulus cells, surrounding the oocyte. The cAMP is then able to enter the oocyte via gap junctions between the cumulus cells and the oocytes themselves (Dekel N., 1988). These gap junctions are composed of proteins, most notably connexin 43 (Cx43) (Kidder & Mhawi, 2002). In rat ovarian follicles, the LH surge phosphorylates and decreases expression of Cx43, which disrupts the gap junctions and

decreases communication between the cells (Norris et al., 2008). With no communication between cumulus cells and oocytes, oocytes will resume meiosis.

Another mechanism of cAMP production is through the oocyte itself. Denuded mouse oocytes cultured with forskolin, which is an agonist of adenylyl cyclase and prevents formation of cAMP, have increased levels of cAMP (Schultz, Montgomery, & Belanoff, 1983). Thus, the oocyte must be able to produce cAMP on its own. cAMP within the oocyte is presumed to be mediated by G-protein-coupled receptors (GPCR) on the oocyte plasma membrane (Mehlmann et al., 2004). Gs protein is stimulated by GPR3 on the oocyte and produces adenylyl cyclase which then initiates the production of cAMP (Mehlmann et al., 2004). By producing cAMP in both the oocyte and the cumulus cells, cAMP is kept at high levels in the oocyte.

The high levels of cAMP must be maintained for the oocyte to remain arrested. Cyclic guanosine monophosphate (cGMP) helps maintain these levels throughout the oocyte by inhibiting phosphodiesterase 3A (PDE3A). PDE3A hydrolyzes cAMP and degrades it to 5'AMP which allows meiosis to resume (Norris et al., 2009). cGMP is produced in cumulus cells and enters the oocyte through gap junctions. Therefore, once the LH surge occurs and gap junctions break, the cGMP will no longer enter the cell and meiosis will resume (Norris et al., 2009). cGMP is required to maintain high levels of cAMP, which activates other proteins through a signal cascade to prevent meiotic resumption.

cAMP begins the signal cascade by activating protein kinase A (PKA). PKA changes the function of Wee1B kinase and Cdc25 phosphatase to phosphorylate CDK1 (Han & Conti, 2006). PKA directly phosphorylates Wee1B, which inhibits CDK1 (Han, Chen, Paronetto, & Conti, 2005). Inversely, Cdc25 phosphatase reverses the inhibition by phosphorylating CDK1. PKA phosphorylates Cdc25 to inactivate the protein and maintain meiotic arrest (Rudolph, 2007). High levels of PKA activity keeps CDK1 inactive through the activity of Wee1B kinase and Cdc25 to maintain meiotic arrest (Adhikari & Liu, 2014). CDK1 is bound to cyclin B to form the MPF complex. The oocyte itself maintains high levels of cyclin B; however, if it is not continuously degraded by anaphase promoting complex, meiosis will spontaneously resume (Mao, Lou, Lou, Wang, & Jin,

2014; Reis, Chang, Levasseur, & Jones, 2006). The LH surge activates MPF and meiosis is resumed.

Nuclear maturation is key for the resumption of meiosis following fertilization. Meiotic arrest must be maintained by high levels of cAMP until the LH surge, at which time cAMP levels drop and meiosis may resume. cAMP is produced within both the oocyte and the cumulus cells, though the exact mechanism for cAMP production is unknown. Nuclear maturation is a key aspect of oocyte maturation and is integral to the developmental competence of the oocyte.

2.1.1.2 Cytoplasmic Maturation

While the oocyte undergoes nuclear maturation, the organelles reorganize, cytoplasmic RNA is transcribed, and proteins are synthesized in a process known as cytoplasmic maturation. Multiple studies have elucidated the process of nuclear maturation, but cytoplasmic maturation is less understood (Ferreira et al., 2009; Mao et al., 2014). During cytoplasmic maturation, organelles must reorganize to best support the cell. Specifically, mitochondria, Golgi apparatus, endoplasmic reticulum (ER), and cortical granules play an important role during this redistribution (Ferreira et al., 2009; FitzHarris, Marangos, & Carroll, 2007; Mao et al., 2014; Paola, Bello, & Michaut, 2015; Stojkovic et al., 2001).

Mitochondria produce ATP which is heavily relied upon for energy during the maturation process. In immature oocytes, mitochondria are typically clustered around the germinal vesicle (Jimenez, Laporte, Duvezin-Caubet, Courtout, & Sagot, 2014). As the cell progresses through meiosis, the organelles become more evenly distributed throughout the cell and cluster in areas where ATP is most needed (Dumollard, Duchen, & Sardet, 2006; Jimenez et al., 2014; Nagano, Katagiri, & Takahashi, 2006; Stojkovic et al., 2001). The ATP concentration increases as the mitochondria move to peripheral locations and the oocyte matures (Stojkovic et al., 2001). One important use for the increased ATP concentration is spindle formation and chromosome segregation. High levels of ATP are required for the formation of spindle by acting through motor proteins (Heald, 2000; X. Zhang, Wu, Lu, Guo, & Ma, 2006). The spindle is produced through

dynamic instability, meaning one side is continuously degraded while the other end is built through ATP powered motor proteins. (Heald, 2000). Without ATP, the motor proteins cannot build the spindle and the chromosomes will not segregate, which explains why oocytes with higher levels of ATP have increased fertilization and blastocyst rates (Nagano et al., 2006; Stojkovic et al., 2001; X. Zhang et al., 2006). Mitochondria have an important role within the developing oocyte and embryo, and their energy production is critical for development.

The Golgi apparatus is important for intracellular trafficking, protein modification and delivery (Mao et al., 2014). In immature mouse oocytes, the Golgi is dispersed throughout the cytoplasm, though it is more concentrated at the interior of the cell (Moreno, Schatten, & Ramalho-Santos, 2002). As the oocyte matures, the Golgi fragments and disperses throughout the oocyte, though the significance of this is still unknown (Mao et al., 2014).

The ER also redistributes itself from the start of maturation to metaphase II. At the germinal vesicle stage (GV) the ER is located evenly throughout the oocyte; however, once the oocyte reaches metaphase II, the ER clusters in the oocyte cortex (FitzHarris et al., 2007). The changes in the ER are likely responsible for the calcium influx required for fertilization. Ca^{2+} is released by the ER in response to 1,4,5-triphophate (IP3). The Ca^{2+} is critical to block polyspermy by depolarizing the membrane and preventing further sperm entry into the cell (Machaca, 2004). The redistribution of the ER brings them closer to the membrane, thus enhancing the polyspermy block and preventing polyspermy.

The final organelle of interest is the cortical granules, whose function is not fully understood. Cortical granules are derived from the Golgi during maturation and move to the oocyte cortex by metaphase II. These cortical granules release their contents at fertilization; however, they must be close to the edges of the oocyte by metaphase II to bind to the plasma membrane and release their contents into the perivitelline space (Paola et al., 2015). Sperm binding to the plasma membrane initiates the cortical reaction (Ducibella, 1996). The binding initiates a G-coupled protein receptor cascade which activates the inositol phosphate (PIP_2) pathway. IP_3 binds to the ER, causing a calcium release which signals the granules to dock to the plasma membrane via SNARE

proteins. (Ducibella, 1996; Liu, 2011). Once the cortical granules dock, they exocytose their contents into the perivitelline space (Hosoe & Shioya, 1997; Mao et al., 2014). The contents of the granules include proteins, enzymes, and proteases that change the structure of the zona pellucida and block more sperm from entering through the perivitelline space (Haley & Wessel, 2004). Immature oocytes are unable to exocytose cortical granules, and therefore unable to block polyspermy (W. Wang, Hosoe, Li, & Shioya, 1997). The cortical reaction is essential to oocyte maturation and marks the end of oocyte maturation and the beginning of embryonic development after fertilization.

Overall, cytoplasmic maturation is an important aspect of oocyte maturation that is not yet fully understood. Many different organelles such as the mitochondria, ER, Golgi apparatus, and cortical granules all play important roles during the maturation of the oocyte, though much more research is needed to fully elucidate these processes.

2.1.1.3 Epigenetic Maturation

Throughout oocyte maturation, specific genes must be reprogrammed through epigenetic regulation to ensure normal development. Multiple studies have suggested that *in vitro* matured oocytes experience a different pattern of gene regulation than *in vivo* matured oocytes, which can lead to abnormal phenotypes and poor development (El Hajj & Haaf, 2013; Horsthemke & Ludwig, 2005). In mammals, imprinting is required in female and male gametes to prevent expression of genes from both parents (Hanna & Kelsey, 2014). Imprinting requires methylation of specific genes or region of DNA to silence them and prevent their transcription (Hanna & Kelsey, 2014). If imprinting does not occur, genes will be transcribed from both maternal and paternal DNA and can lead to developmental abnormalities and even fetal death (Bartolomei, 2009). The methylation of specific genes in the oocyte is completed by the end of oocyte maturation and is imperative for normal development of the offspring (El Hajj & Haaf, 2013).

Oocytes matured *in vivo* and *in vitro* have very different methylation patterns (Heinzmann et al., 2011). IVP blastocysts differ greatly from blastocysts that were matured and fertilized *in vivo*

and cultured *in vitro*. Interestingly, the methylation pattern of *in vivo* derived 4 and 16 cell embryos are more similar to blastocysts matured and fertilized *in vivo* than IVP embryos (Salilew-Wondim et al., 2015). Most of the differentially methylated genes are correlated to ion transport, regulation of cell morphogenesis, metabolism, and cytoskeleton regulation (Salilew-Wondim et al., 2015). These incorrect methylation patterns can impact the embryo or the developing offspring. Specifically, large offspring syndrome has been associated with incorrect methylation of the IGF2R gene (Heinzmann et al., 2011). The culture conditions impact the methylation status of oocytes, leading to disorders in the oocyte, embryo, or offspring.

Epigenetic maturation plays a key role in the development of the offspring. Incorrect gene methylation impacts development and leads to major problems in the offspring. IVP embryos suffer from an altered methylation pattern which may contribute to the poor development observed in these embryos.

2.1.4 Oocyte Energy Production

2.1.4.1 Production of Reactive Oxygen Species

ROS are oxygen containing reactive species that may or may not have unpaired electrons. ROS with unpaired electrons are referred to as free radicals and those without unpaired electrons are referred to as non-radical ROS (R. Li et al., 2016). There are many types of ROS, but common ones include superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), or lipid hydroperoxides (LOOH) (R. Li et al., 2016). They are produced by the donation of an extra electron to an O_2 molecule, which then can produce highly stable H_2O_2, which can cross cell membranes, or becomes unstable OH^- in the presence of irons (R. Li et al., 2016). OH^- causes oxidative damage by disrupting normal redox reactions, mutating DNA, and causing lipid peroxidation (M. Takahashi, 2012).

The electron transport chain within the mitochondria produces ROS under normal physiological conditions and is the main source of ROS. Specifically, ROS production occurs in the electron accepting domains of complex I and III (Orrenius, Gogvadze, & Zhivotovsky, 2007).

Oocytes contain nearly 100x more mitochondria than somatic cells, which correlates with higher ROS production (Carling, Cree, & Chinnery, 2011; Seki & Mazur, 2012). Oocytes matured *in vitro* have lower glycolysis levels which requires them to rely on oxidative metabolism to produce ATP and increases ROS production (Wrenzycki & Stinshoff, 2013). Additionally, oocytes cultured *in vitro* are subjected to high level of oxygen tension (20% compared to the 3-13% common in the follicular environment) which increases ROS production since more oxygen is available to accept electrons in the electron transport chain (Velez-Pardo, Morales, Del, & Olivera-Angel, 2007). The ROS mutate mitochondrial DNA, which leads to poorly functioning mitochondria that produce even higher levels of ROS, further exacerbating the oxidative stress (Orrenius et al., 2007). Mitochondria produce a majority of ROS within cells and represent a major source of oxidative stress.

Another common source of ROS is from the ER. The ER is responsible for protein folding, and oxidizing conditions are required for the formation of the proper bonds (Tu & Weissman, 2004). Specifically, disulfide bonds must be produced by electron transport which uses O_2 as the final electron acceptor and produces ROS (Tu & Weissman, 2002). Additionally, the production of disulfide bonds requires the oxidation of glutathione (GSH) (K. Zhang & Kaufman, 2008). GSH is one of the most important nonenzymatic antioxidants and plays a key role in neutralizing ROS (M. Takahashi, 2012). Thus, depleting the cell's source of GSH could prevent the cell from being able to properly protect itself from oxidative damage. When excess ROS are produced during the protein folding process, calcium leaks from the ER and accumulates in the inner matrix of the mitochondria and depolarizes the mitochondrial membrane (Görlach, Klappa, & Kietzmann, 2006; K. Zhang & Kaufman, 2008). The membrane depolarization disrupts the electron transport chain and increases production of ROS, which in turn causes more calcium release from the ER, and leads to a cycle of increasing ROS production (Görlach et al., 2006). Additionally, the change in calcium homeostasis often disrupts the protein-folding process in the ER. This stresses the ER and induces the unfolded protein response which causes the ER to refold proteins and which produces higher levels of ROS (K. Zhang & Kaufman, 2008). Overall, ER stress produces more ROS and activates inflammatory responses and apoptosis (Malhotra & Kaufman, 2007).

2.1.4.2 Enzymatic and Non-Enzymatic antioxidants

Naturally produced antioxidants within the female tract and the embryos themselves help protect offspring from ROS. These antioxidants can be enzymatic or non-enzymatic in nature and assist in neutralizing ROS to decrease the stress placed on the embryos and gametes.

The mitochondria produces an antioxidant system that helps eliminate some of the excess ROS produced within the electron transport chain (Figure 1) (Starkov, 2008). Superoxide dismutase (SOD) converts O_2^- to H_2O_2 to prevent the more detrimental OH^- from being produced (Combelles, Holick, Paolella, Walker, & Wu, 2010). Glutathione peroxidase (GPx) catalyzes the reaction of H_2O_2 to H_2O within the mitochondria by reducing H_2O_2. In return, reduced glutathione (GSH) changes to its oxidized state (GSSG). High levels of GSH are indicative of low levels of oxidative stress, since GPx does not need to function at high levels (El-Wahsh, Fuller, Davidson, & Rolles, 2003; Pasqualotto et al., 2009). Catalase (Cat) also functions to reduce H_2O_2 to H_2O; however, it functions in the cytoplasm rather than within the mitochondria (M. Takahashi, 2012).

The oocyte undergoes maturation within the follicle, and follicular fluid contains antioxidants that help protect the maturing oocyte from oxidative stress (Combelles et al., 2010). SOD is produced within follicles and granulosa cells and may help combat formation of ROS (Combelles et al., 2010). Interestingly, Cat activity, SOD activity, and GPx activity is highest in small follicles, but total antioxidant capacity is highest in large follicles (Combelles et al., 2010; Gupta et al., 2011). The antioxidants produced within the follicle may help combat ROS in the maturing oocyte and improve the development. In human patients, oocytes collected from follicles with higher levels of SOD and Cat have increased fertilization rates (Pasqualotto et al., 2009). Although the exact role of the antioxidants within the follicle is not known, they clearly impact the maturing oocyte and could have an impact on the developing embryo.

After ovulation, the oocyte is exposed to enzymes within the female tract to help eliminate excess levels of ROS. Specifically, SOD, GPx, and Cat are expressed within the oviducts of the female and play an important role in eliminating ROS within the tract that could impact the developing offspring (Pasqualotto et al., 2009). GSH is produced within the embryos themselves

and by the oviductal environment, further suggesting the importance of the female tract in regulating the oxidative stress of developing embryos (M. Takahashi, 2012). These enzymatic and non-enzymatic antioxidants produced within the female tract help protect the embryo from oxidative stress.

These antioxidants produced within the follicle and female tract likely play a role in the development of the oocyte and embryo, though more research is needed to determine the exact mechanisms. Interestingly, supplementing the maturation media with SOD does not improve blastocyst rates in bovids (Ali, Bilodeau, & Sirard, 2003). The disconnect between antioxidants *in vitro* and *in vivo* could cause some of the metabolic problems seen in IVP embryos (Figure 1).

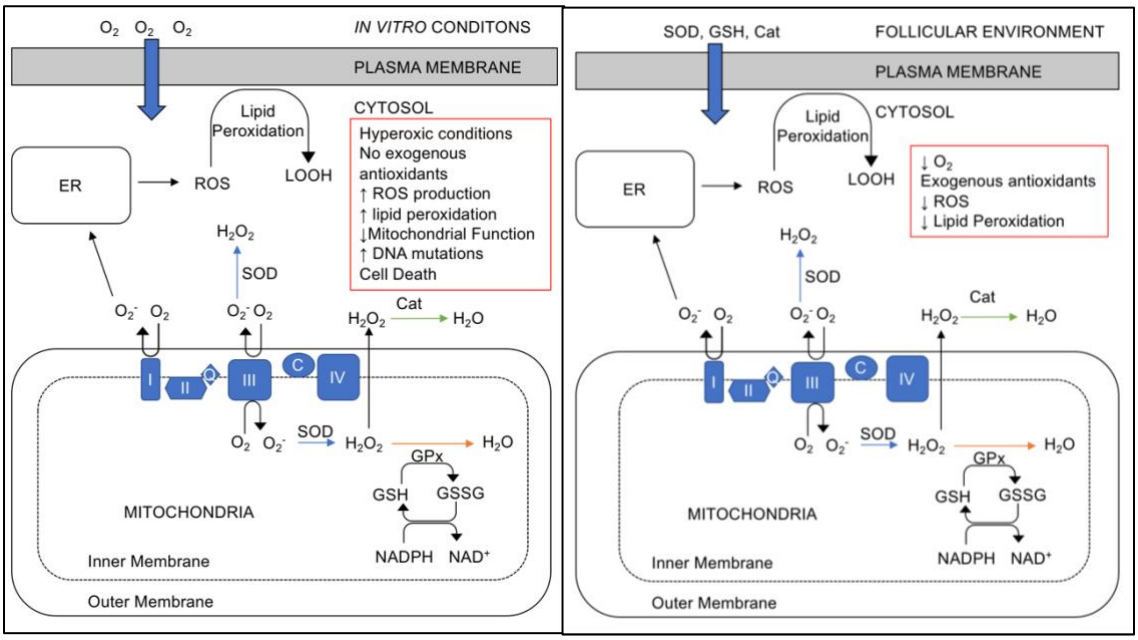


Figure 1. Production of ROS and antioxidant defenses in follicular environment and *in vitro* conditions.

2.2 Antioxidants

Between the lower oxygen tension and female produced antioxidants, oxidative stress in IVD embryos is lower than IVP embryos. Embryos produced *in vitro* have higher levels of ROS, as well as lower levels of antioxidant enzymes GSH, SOD, and Cat which impairs development (Kitagawa

et al., 2004; R. Li et al., 2016; M. Takahashi, 2012). One way to improve IVP embryos is supplementing their development with antioxidants to improve metabolism and decrease oxidative stress.

2.2.1 Antioxidant use in IVM

Oocyte maturation is the most important determining factor in embryonic development, and thus improving the IVM conditions has been an important area of research. Since many problems stem from high levels of ROS in oocytes, antioxidants may decrease the levels *in vitro* (Ali et al., 2003; Sovernigo et al., 2017). Many different antioxidants have been studied in bovine oocyte maturation to improve oocyte metabolism and improve developmental competence (Córdova, Morató, Izquierdo, Paramio, & Mogas, 2010; Tian et al., 2014; F. Wang, Tian, Zhang, et al., 2014).

One major antioxidant studied *in vitro* is melatonin. Melatonin is a naturally occurring free radical scavenger that can upregulate GSH and SOD (Rodriguez et al., 2004; Tian et al., 2014). When added to bovine maturation media, 1 nM melatonin improves cleavage rates and subsequent blastocyst rate and cell number. (Tian et al., 2014). Another study showed that 1 nM melatonin improved the number of bovine oocytes that resumed meiosis and lowered ROS levels (Rodrigues-Cunha et al., 2016). Overall, melatonin improves maturation when used in bovine IVM.

L-ascorbic acid is another free radical scavenger used commonly in IVP embryos. L-ascorbic acid is a naturally occurring antioxidant which combats oxidative stress by scavenging free radicals (Córdova et al., 2010). However, when bovine oocyte maturation medium is supplemented with 100 μg/mL L-ascorbic acid, the subsequent blastocyst rate is lower than the control (Córdova et al., 2010). Additionally, 50 μg/mL L-ascorbic acid has no effect on cleavage or blastocyst rate when added to porcine maturation media (Nohalez et al., 2018). Although L-ascorbic acid is a well-documented antioxidant, it has not yet shown benefits in IVM.

Another well studied antioxidant is resveratrol, which is a naturally occurring polyphenolic compound found in wine (Alcaín, Minor, Villalba, & Cabo, 2010). When added during oocyte maturation at 1 μM, resveratrol improves cumulus cell expansion, polar body formation, GSH

levels, and hatched blastocyst rate, and reduces ROS (F. Wang, Tian, Zhang, et al., 2014). The improved development is likely caused by the upregulation sirtuin-1 (SIRT1) gene which is important for metabolism, mitochondrial biogenesis, and decreasing oxidative stress (Alcaín et al., 2010; F. Wang, Tian, Zhang, et al., 2014).

2.2.2 Antioxidant use in IVF

Antioxidant use in IVF is less studied than IVM and has produced contradictory results (Ali et al., 2003; Cheuquemán et al., 2015; Pang et al., 2016). ROS levels are quite high in sperm due to the high levels of ATP produced by mitochondria (Sanocka & Kurpisz, 2004). ROS are required by the sperm to undergo capacitation; however, *in vivo* the excess ROS are naturally dissipated throughout the female tract (O'Flaherty, Beorlegui, & Beconi, 1999). *In vitro,* these ROS are not eliminated and are maintained in the fertilization media (Nohalez et al., 2018; O'Flaherty et al., 1999). However, results on antioxidant use in IVF are inconsistent. Using L-ascorbic acid in porcine fertilization has no effect on blastocyst rate (Nohalez et al., 2018). Using melatonin in bovine IVF has contradictory reports, as it has led to significantly lower and higher blastocyst rates in different studies (Cheuquemán et al., 2015; Pang et al., 2016). Other bovine studies have reported that high levels of the antioxidant cysteine or the antioxidant enzymes SOD or catalase lead to decreased blastocyst rate when added to the fertilization media. Furthermore, lower levels have no significant effect on the blastocyst rate (Ali et al., 2003). Overall, results using antioxidants in this step of embryo culture have contradictory results and are less studied than other stages, though some beneficial reports warrant further exploration into how antioxidants impact this stage of culture (Pang et al., 2016).

2.2.3 Antioxidant use in IVC

Embryo culture is the last step in embryo production and another potential place to eliminate ROS. IVC is typically completed at lower oxygen tension (5%) which decreases ROS production; however, the increased ROS production from the previous two steps could lead to impaired mitochondrial function which will produce more ROS throughout IVC (Ali et al., 2003). In

addition, embryos are typically maintained in the same culture drop for up to 8 days, leading to increased buildup of ROS with no means for elimination (Ali et al., 2003).

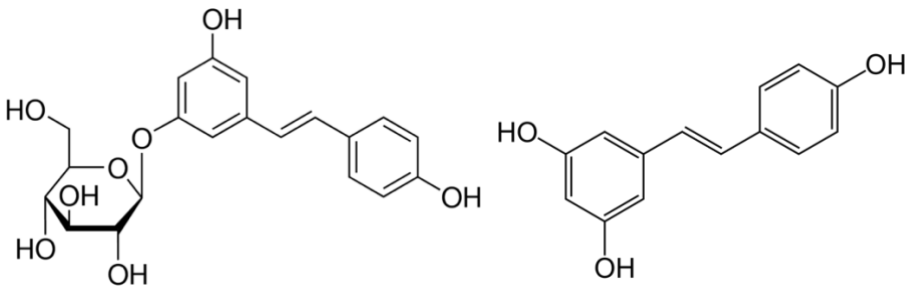
Melatonin has been used in culture systems for its antioxidant potential. When added to culture medium at 100 nM, melatonin improves blastocyst rate, post-warming survival following vitrification, and makes gene expression more similar to IVD embryos (F. Wang, Tian, Zhou, et al., 2014). Additionally, blastocyst rate increases when 1 μM melatonin is added to murine culture medium (Ishizuka, Kuribayashi, Murai, Amemiya, & Itoh, 2000). Overall, melatonin exhibits beneficial effects when added to mammalian embryo culture.

Another commonly studied antioxidant is beta-mercaptoethanol (BME) which can upregulate intracellular scavengers such as GSH (Geshi, Yonai, Sakaguchi, & Nagai, 1998). When added at 50 μM to porcine embryo culture, BME has no effect on embryo development (Hosseini et al., 2009). However, when 10 μM was added to bovine culture medium, the blastocyst rate increased (Geshi et al., 1998). Another study suggested 100μM BME increases GSH production when added to bovine embryo culture, as well as decreasing the proportion of apoptotic cells; however, there was no beneficial effects when added at 50 μM (Feugang et al., 2004). BME is one of the most studied antioxidants and has strong potential to improve IVC.

Resveratrol is also often added to culture systems due to its ability to upregulate SIRT1 activity. One study suggested that adding resveratrol to bovine culture medium at too high of concentration (5 or 10 μM) greatly decreases blastocyst rate (Salzano et al., 2014). However, when added at .5 μM, blastocyst rate and cryotolerance were enhanced (Salzano et al., 2014). Similarly, a study showed that when .5 μM resveratrol was added to culture medium, mitochondrial activity increased and lipid content decreased, as well as improving development, cryotolerance, and expression of SOD and GPx. (Abe et al., 2017). Resveratrol is a dose dependent antioxidant with the best results at low concentrations; however, when added at lower concentrations, resveratrol can improve IVC of bovine embryos. Overall, there is enough evidence to suggest adding antioxidants to the embryo culture medium has the ability to improve development by scavenging free radicals and upregulating production of intracellular antioxidants.

2.2.4 Polydatin

Polydatin is a naturally occurring antioxidant and a precursor of resveratrol. Polydatin is found in red wine at concentrations seven times higher than resveratrol, suggesting it is the most commonly occurring form of the antioxidant (Romero-Pérez, Ibern-Gómez, Lamuela-Raventós, & de La Torre-Boronat, 1999). Polydatin has a similar chemical structure as resveratrol, except the hydroxyl group on the third carbon is replaced with a glucoside group (Ravagnan et al., 2013) (Figure 2).



Polydatin Resveratrol

Figure 2. Chemical structure of polydatin and resveratrol.

The substitution of the hydroxyl group for the glucoside group leads to conformational changes that causes differences in biological properties. The glucoside group leads to a twisted conformation in the molecule that is not seen in resveratrol (Regev-Shoshani, Shoseyov, Bilkis, & Kerem, 2003). The twist causes steric inhibition which may prevent enzymes from oxidizing the molecule and increases the molecule's stability in solution (Ravagnan et al., 2013; Regev-Shoshani et al., 2003). The increased stability may increase its efficiency in embryo culture, thus making it an attractive addition to the embryo production media.

Additionally, the glucoside group makes polydatin more bioavailable to cells. Resveratrol can only enter cells via passive diffusion or endocytosis on lipid rafts (Delmas & Lin, 2011). Polydatin can be taken up by cells via the same mechanisms; however, it also can be actively transported by sodium-dependent glucose transporters expressed in bovine oocytes (Augustin et

al., 2001; Du, Peng, & Zhang, 2013). Since polydatin can be taken up by cells via both passive and active transport, its bioavailability is higher than resveratrol which could increase its antioxidant capacity. (Lanzilli et al., 2012).

One important antioxidant mechanism is free radical scavenging, or the ability to neutralize the unpaired electron in ROS. Multiple studies have suggested polydatin has higher free radical scavenging capabilities than resveratrol *in vitro* (Fabris, Momo, Ravagnan, & Stevanato, 2008; Su et al., 2013). The difference in scavenging properties is thought to be due to the conformational change (Fabris et al., 2008). The hydroxyl group on C-4 is the most reactive group on resveratrol and polydatin and is responsible for the majority of the free radical scavenging (Regev-Shoshani et al., 2003). When this hydroxyl group donates an electron to ROS, resveratrol may form a dimer that inhibits it's antioxidant capabilities (Fabris et al., 2008; Regev-Shoshani et al., 2003). Meanwhile, the glucoside group on polydatin contributes to steric hindrance which prevents polydatin from dimer formation so it is more available to scavenge ROS (Fabris et al., 2008; Regev-Shoshani et al., 2003). The formation of the resveratrol dimer is supported by spectrophotometric data showing a secondary molecule in resveratrol that is not present in polydatin, as well as the faster consumption rate of resveratrol *in vitro* (Fabris et al., 2008).

Another important antioxidant mechanism is the inhibition of lipid peroxidation. Lipid peroxidation occurs when lipids, most commonly polyunsaturated fatty acids, are oxidized by ROS (Gaschler & Stockwell, 2017). The oxidation of membrane lipids greatly alters ion gradients, membrane fluidity and permeability, and causes ferroptosis (Gaschler & Stockwell, 2017; Yang & Stockwell, 2016). Ferroptosis is a form of regulated cell death that is characterized by high levels of iron and accumulation of lipid peroxides (Yang & Stockwell, 2016). Additionally, intracellular and membrane lipid peroxidation creates lipid hydroperoxides which act similarly to ROS and disrupt cellular processes, leading to damage and eventually cell death (Ayala, Muñoz, & Argüelles, 2014). Lipid peroxidation may be a key cause of cellular death in frozen embryos, as embryos that are slow frozen in the presence of a lipid peroxidation inhibitor are more likely to survive freezing (Tarín & Trounson, 1993). Polydatin has protective effects against lipid peroxidation, and is more effective than vitamin E or resveratrol in preventing lipid peroxidation (Fabris et al., 2008). The protective

effect is delayed, which suggests polydatin may help prevent against lipid peroxidation much longer after initial exposure (Fabris et al., 2008).

The final method that allows polydatin to inhibit oxidative stress is by the upregulation of the SIRT1 pathway. The sirtuin family of proteins are nicotinamide adenosine dinucleotide $^{+}$ (NAD^{+}) dependent protein deacetylases. SIRT1 deacetylates histones and non-histone proteins by transferring acetyl groups to NAD^{+} which changes gene expression (Chung et al., 2010; Du et al., 2013).. Polydatin upregulates the SIRT1 gene in many cells including embryonic cells, oocytes, kidney, cardiovascular, and epithelial cells (Chen & Lan, 2017; Huang et al., 2015; Khan et al., 2017; Ma, Gong, Mo, & Wu, 2016). Specifically, polydatin upregulates SIRT1 in bovine *in vitro* matured oocytes (Khan et al., 2017). When added at 1 μM, polydatin increases SIRT1 expression, and decreases expression of inflammation genes *Nfkb*, *COX2*, *iNOS*, and *BAX*. Additionally, embryonic quality is improved by increasing the number of cells in blastocysts and decreasing apoptotic cells and ROS levels (Khan et al., 2017). In addition to upregulation of SIRT1, polydatin increases the affinity of SIRT1 for NAD^{+} in vitro, suggesting that polydatin influences the SIRT1 pathway both by increasing expression but also by enhancing the efficiency of the enzyme (Su et al., 2013). Overall, polydatin greatly enhances SIRT1 activity which improves metabolic function.

Polydatin is similar to resveratrol in its ability to protect cells from oxidative stress. By limiting scavenging free radicals, limiting lipid peroxidation, and upregulating the SIRT1 pathway, polydatin has many mechanisms to combat ROS (Figure 3). However, polydatin is more stable and more bioavailable to cells than resveratrol, which suggests polydatin may be a better choice for embryonic culture.

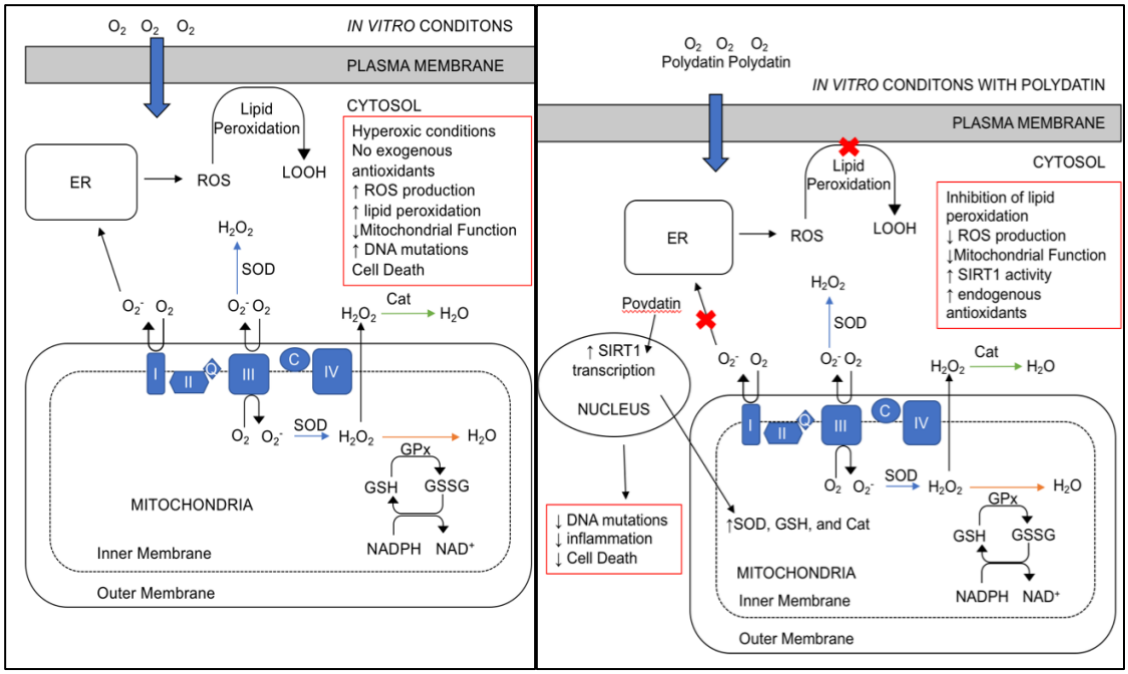


Figure 3. Proposed mechanism of polydatin on *in vitro* ROS metabolism

2.3 Cryopreservation

Cryopreservation is an essential part of an embryo transfer program, with nearly 70% of cattle embryos frozen rather than transferred immediately (Moore & Hasler, 2017). The long-term storage of bovine embryos allows for optimization of protocol timing, as producers can prepare the exact number of recipient animals that they need rather than estimating a number for fresh embryos. Additionally, embryos can be shipped across the world to increase genetic variation amongst herds. Finally, cryopreservation allows unique genetic material from high producing animals to be stored after death to retain their genetics for future generations.

2.3.1 Cryopreservation Methods

Cryopreservation is the process of cooling embryos for long term storage. Cryopreserved embryos can be stored in liquid nitrogen until recipient cows are prepared which optimizes the timing of transfers. There are two commonly used methods for cryopreserving embryos; slow-freezing and vitrification.

2.3.1.1 Slow Freezing

Slow freezing involves slowly cooling embryos to low temperatures before freezing them at -196°C. Throughout this process, water is turned into ice which damages cells. Specifically, intracellular ice formation is extremely detrimental to cells and is the most important factor for survival (Inaba et al., 2016). For successful slow freezing, water must be removed from the cells (Chaytor et al., 2012; Santis & Coticchio, 2011). Once water is outside the cells, extracellular ice crystal formation creates an osmotic gradient that further draws more water from the cells to complete the dehydration process (Santis & Coticchio, 2011; Saragusty & Arav, 2011). Dehydration must occur to prevent formation of intracellular ice; however, it can be lethal to the cells and lead to the solute effect which causes irreparable damage to the cell membranes (Chaytor et al., 2012). Using cryoprotectants can help manage the dehydration rate

Cryoprotectants can either be permeating or extracellular. Permeating cryoprotectants enter the cell and replace the water (Chaytor et al., 2012). These molecules are not able to enter the cell as quickly as water is drained, leading to a volume reduction within cells that may decrease viability (Saragusty & Arav, 2011). Additionally, these cryoprotectants can be toxic to cells which affects survival (Chaytor et al., 2012). Meanwhile, extracellular cryoprotectants are not able to cross the membrane, and thus dehydrate the cells via osmotic equilibrium (Santis & Coticchio, 2011). A combination of permeating and extracellular cryoprotectants increases the post-thaw survival rate (Chaytor et al., 2012).

After embryos are exposed to cryoprotectants, they must be cooled to the desired temperature. Water will remain in liquid form below the melting point in a process known as

undercooling (Morris & Acton, 2013). This undercooling leads to the entire sample forming an ice crystal matrix that can damage cells during thawing (Fuller & Paynter, 2004). When ice forms at lower temperatures, it will remain ice until higher temperatures when thawed which increases damages to the cells (Morris & Acton, 2013). To prevent the undercooling, embryos are frozen in straws and seeded at -6.3°C to help initiate the ice crystal formation process at a relatively high temperature (Acker & McGann, 2003). Seeding helps begin ice crystal formation and helps improve survival rates of frozen embryos.

Following seeding, embryos are cooled at a controlled rate until they reach -196°C, at which point they can be stored in liquid nitrogen tanks (Acker & McGann, 2003). The cooling rate must be carefully controlled; if it is too slow the cells will die from excessive dehydration, and if it is too fast, intracellular ice will form (Fuller & Paynter, 2004). Embryos can be maintained in liquid nitrogen tanks until they can be transferred into synchronized recipient cows. Embryos that are slow frozen in 1.5 M ethylene glycol (EG) can be directly transferred into the cow, meaning the thawed contents of the straw can be expelled directly into the uterus of the female. The direct transfer of embryos makes slow freezing an attractive option for producers due to the ease of transfer in the field (Voelkel & Hu, 1992). Slow freezing in EG is the industry standard due to standardized procedures and is commonly used in practice worldwide (Inaba et al., 2016).

2.3.1.2 Vitrification

Vitrification is the process of rapidly cooling cells with a very low volume to avoid any ice crystal formation, thus decreasing the risk of cellular damage. By rapidly cooling the cells, water is transformed from liquid to glass and ice crystal formation is bypassed (Seki & Mazur, 2012). The success of vitrification is reliant upon three main properties shown in the equation below: the cooling and warming rate, the viscosity of the medium, and the volume of the medium (Arav, 2014).

$$Probability\ of\ vitrification = \frac{cooling\ and\ warming\ rate\ \times viscosity}{volume}$$

The first important aspect of vitrification is the cooling and warming rates of the procedure. The high cooling rate is typically achieved by cooling the cells to 0°C before plunging the sample directly into liquid nitrogen (Yavin & Arav, 2007). This creates a high cooling rate that increases survival rates of embryos by 37% (Arav, 2014). Recently, the warming rate has found to also be important for survival. Mouse embryos warmed at a rate of 2000°C/min had a survival rate of 76%, whereas embryos warmed at a rate of 1450°C/min only had a 26% survival rate (Seki & Mazur, 2012). Thus, both rapid cooling and warming of vitrified embryos is required for success. The next important aspect of survival of vitrification is the viscosity of the medium the cells are suspended within. Higher levels of cryoprotectants within the medium enhance the glass transition and make the medium more viscous (Arav, 2014). The final determinant for successful vitrification is the volume of the medium. Smaller volumes allow for better heat transfer which creates faster cooling rates and increases the chance of survival. (Arav, 2014).

Vitrification has shown promise in cryobiology due to the high success rates; however, there are some practical considerations which make it less preferred in the cattle industry (Mandawala, Harvey, Roy, & Fowler, 2016). Vitrification occurs in either open, where the embryos are in direct contact with the liquid nitrogen, or closed systems which have a barrier that prevents contact. (Mandawala et al., 2016). A study in human embryo vitrification suggested that open vitrification is more reliable and successful (De Munck, Santos-Ribeiro, Stoop, Van de Velde, & Verheyen, 2016). Unfortunately, open vitrification presents a contamination risk since pathogens in the liquid nitrogen can transfer into the sample (Mandawala et al., 2016; Tedder et al., 1995). Although there is no clinical evidence of contaminated embryos, hepatitis has transferred into bone marrow samples from contaminated liquid nitrogen (De Munck et al., 2016; Tedder et al., 1995). The risk of contamination makes an open vitrification system not preferred in ART, and the less reliable closed system is the current standard (Mandawala et al., 2016).

Another problem with vitrification is that embryo warming requires a trained embryologist to move embryos through a series of steps (Mandawala et al., 2016). This must be performed on a farm, which increases labor and the risk of pathogenic contamination due to the conditions (Dochi

et al., 1998). Overall, vitrification presents great promise in the industry due to increased survival and decreased damage, but further work is done to improve the feasibility of implementing this technique into the cattle industry (Inaba et al., 2016).

2.3.2 Cryopreservation in Bovine Embryos

Slow freezing is currently preferred over vitrification within the cattle industry due to the ease of direct transfer (Inaba et al., 2016). Direct transfer allows a technician to place the embryo directly into the recipient without need for a long thawing process, which eliminates the need for an embryologist or microscope in the field and makes slow freezing the more convenient procedure. Unfortunately, IVP embryo cryotolerance is low due to apoptosis and membrane damage which leads to poor survival (Inaba et al., 2016; Seidel, 2006).

Cellular volume is changed drastically during freezing and thawing as water enters and exits cells. This change in cell volume leads to osmotic stress (Seidel, 2006). IVP bovine embryos typically have a more rigid membrane due to higher lipid content, which renders them less able to undergo the volume change (Seidel, 2006). In response to osmotic stress, cells activate genes related to cell rescue and heat shock proteins once thawed. These genes typically include energy generation and increase in ROS production (Odani, Komatsu, Oka, & Iwahashi, 2003). ROS are also produced upon thawing when oxygen is reintroduced back into the cells, leading to a burst of production (El-Wahsh et al., 2003). These two mechanisms coupled together could overwhelm the cells with high levels of ROS.

ROS impact the freezing process by inducing lipid peroxidation. Lipid peroxidation occurs when ROS scavenge electrons from lipids and create unstable lipid hydroperoxides (Gaschler & Stockwell, 2017). Polyunsaturated fatty acids in phospholipids are especially susceptible to degredation which destabilizes the membrane (Orrenius et al., 2007). These unstable membranes allow cryoprotectants and other enzymes and undesirable metabolites to enter the cell at toxic level (Seki & Mazur, 2012). The influx of extracellular molecules within the cells leads to cellular damage and often death (Fuller & Paynter, 2004). Using lipid peroxidation inhibitors during freezing

improves the success rate of cryopreservation, further demonstrating the importance lipid peroxidation on the ability of the cell to survive freezing (Tarín & Trounson, 1993).

In addition to altering the membrane stability, lipid peroxidation also creates harmful oxygen radicals that further damage the cells (Gaschler & Stockwell, 2017). Lipid peroxidation produces more ROS which impact mitochondrial metabolism. Impaired mitochondria trigger apoptosis and lead to cell death (Orrenius et al., 2007). Since bovine IVP embryos have higher levels of intracellular lipids than IVD embryos, there are potentially more peroxidation byproducts that negatively impact the cell (Seidel, 2006; Sudano et al., 2016). This could potentially explain the decreased cryotolerance seen in IVP embryos compared to fresh embryos and why lipid heavy Jersey embryos do not survive freezing as often as Holstein embryos (Baldoceda et al., 2016; Seidel, 2006; Sudano et al., 2016). Furthermore, high levels of ROS also lead to increased DNA fragmentation via apoptosis which decreases post-thaw survival (Inaba et al., 2016).

Slow freezing bovine blastocysts mainly effects the trophectoderm cell membranes (Inaba et al., 2016). The trophectoderm is required to pump Na+ into the blastocoel cavity to cause expansion. Damaged trophectoderm cells may be unable to produce the electrochemical gradient required to pump fluid into the blastocoel cavity (Inaba et al., 2016; Watson & Barcroft, 2001). Thus, the embryos don't survive the freezing process because they are unable to reform a blastocoel cavity and do not re-expand (Inaba et al., 2016). Re-expansion indicates few trophectoderm cell losses and is the best determinant of an embryos ability to survive once transferred into a recipient cow or human (Hasler et al., 1995; Inaba et al., 2016; Shu et al., 2009). Additionally, in ruminants, the trophectoderm is responsible for interferon Tau (INFT) secretion, which causes pregnancy recognition in the female (Spencer & Hansen, 2015). Inadequate INFT production by the trophectoderm is most likely a major part of pregnancy loss in cattle (Arosh et al., 2004). Thus, if the trophectoderm cells are lost or damaged at high levels, they will be unable to produce enough INFT to initiate pregnancy recognition once transferred into the dam.

2.3.2.1 Possible solutions to cryopreservation in bovine embryos

There are two methods to improve cryotolerance of bovine IVP embryos: changing the culture conditions to produce less lipid heavy embryos or optimizing the slow freezing conditions. Previous research in our lab has focused on modifying the culture conditions to produce less lipid heavy embryos (Rhodes-Long, Campos-Chillòn, Barceló-Fimbres, & Altermatt, 2016; Roberts, Campos-Chillòn, Barceló-Fimbres, & Altermatt, 2016). This review will focus more on optimizing the slow freezing technique and utilizing antioxidants in the freezing medium to improve post-thaw survival.

2.3.2.1.1 Optimizing Techniques

One potential way to improve embryo slow-freezing survival is to optimize the actual slow freezing procedure. The standard slow freezing procedure involves embryos being equilibrated in 1.5 M EG for 10 minutes prior to being loaded into straws and cooled slowly (Hasler et al., 1995; Inaba et al., 2016). Alterations to this procedure could help improve the dehydration and osmotic stress of the embryos and increase their post thaw viability.

One potential mechanism to improve slow freezing success rates is by forced blastocoel collapse (Feng et al., 2010; Min et al., 2014). Bovine embryos slow frozen after forced blastocoel collapse had increased re-expansion and decreased apoptotic cells post-thaw compared to embryos slow frozen without the forced collapse (Min et al., 2014). Unfortunately, the re-expansion rates were still lower than vitrified or fresh embryos and apoptosis was still higher (Min et al., 2014). Though forced collapse of the blastocoel cavity shows promise, the success rates still do not match survival of vitrified embryos.

A recent technique using different concentrations of EG within the straw has presented as another possible way to improve cryopreservation (Sanches et al., 2016). One main problem with cryopreservation is the water exiting and entering the cells too quickly which puts osmotic stress on the cells and leads to damage (Seidel, 2006). Osmotic stress can be eliminated by lowering the concentration of the cryoprotectant the embryo is exposed to once thawed. By surrounding the

embryo with 0.75M EG (opposed to the 1.5M EG the embryos are frozen in) and mixing the columns prior to transfer, the lower concentration of EG may slow the rate at which water reenters the cell and increased conception rates (Sanches et al., 2016). Unfortunately, this method was not tested against a standard slow freezing technique so no comparison is possible, though it is noted that the pregnancy rates were still lower than IVD embryos frozen in the same manner (Sanches et al., 2016).

2.3.2.1.2 Antioxidant use in freezing medium

Introducing antioxidants to the freezing medium may alleviate oxidative stress placed on the embryos throughout the freezing process. Decreasing ROS can lead to less lipid peroxidation and DNA fragmentation that could improve post-thaw survival (Tatone, Emidio, Vento, Ciriminna, & Artini, 2010).

Studies using antioxidants in freezing medium of embryos is limited (Lane, Maybach, & Gardner, 2002). Ascorbate significantly improves the ability of mouse blastocysts to survive slow freezing when added directly to the freezing media (Lane et al., 2002). The enhanced cryotolerance was coupled with lower levels of hydrogen peroxide, though no other ROS were tested. Additionally, there was decreased leakage of extracellular enzymes into the cells, suggesting that ascorbate decreased membrane damage (Lane et al., 2002).

Despite few studies in embryos, the use of antioxidants in sperm cryopreservation are widely studied (Agarwal, Durairajanayagam, & du Plessis, 2014; Donnelly, McClure, & Lewis, 1999; Kobayashi, Miyazaki, Natori, & Nozawa, 1991; Said & Agarwal, 2012). Sperm are very susceptible oxidative stress and lipid peroxidation, which makes them likely to be damaged by the cryopreservation process (Said & Agarwal, 2012). L-ascorbic acid, vitamin E, SOD, and catalase have all significantly improved cryotolerance in human spermatozoa by increasing post thaw-motility (Donnelly et al., 1999; Kobayashi et al., 1991; Said & Agarwal, 2012). Supplementing with SOD specifically decreased lipid peroxidation levels in the spermatozoa (Kobayashi et al., 1991). Additionally, human sperm frozen with ascorbate and catalase have lower levels of ROS and higher

post-thaw motility (Z. Li, Lin, Liu, Xiao, & Liu, 2010). Adding antioxidants directly to the freezing medium could improve post-thaw survival by helping decrease oxidative stress embryos encounter during the freezing process.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

All materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

3.2 Embryo Production

3.2.1 Ovary transport and oocyte collection

Bovine ovaries were collected from a slaughterhouse (Cargill Meat Solutions Inc., Fresno, CA), cooled to 22-25°C, and transported back to the lab within 4 hours of harvest in 0.15M saline. Cumulus oocyte complexes were aspirated via vacuum pump aspiration using an 18-gauge needle from 2-8 mm follicles. Oocytes with even cytoplasm and at least three even layers of cumulus cells were washed through ovum pick-up media (MOFA Global, Verona, WI) twice; then, once through oocyte washing medium (MOFA Global, Verona, WI). Finally, COCs were then washed once through maturation medium (TCM-199, 10% FCS, 4.03 mM pyruvate, 25 μg/mL gentamicin, 5 IU FSH, and 1 μL/mL L-carnitine) and cultured in maturation media for 22-24h at 38.5°C and 5% CO_2 in air.

3.2.2 Sperm Preparation and In Vitro Fertilization

Frozen semen from fertility proven bulls was thawed in 37-35°C water for 30-45 s. Semen was gently expelled onto a Sperm Separation gradient (MOFA Global, Verona, WI). Semen was centrifuged at 600 xg for 12 minutes. Following centrifugation, the pellet was removed and pipetted gently into 1 mL of pre-equilibrated fertilization medium (FCDM with 2 μg/mL heparin) (Mendes, Burns, De La Torre-Sanchez, & Seidel, 2003). The sperm were then centrifuged again at 600 xg for 4 minutes. The supernatant was removed, and sperm concentration was determined using a hemocytometer. The sperm concentration was adjusted to $10x10^6$ sperm/mL in FCDM. Oocytes

were moved to FCDM medium overlain with oil. Following preparation, sperm was added at a concentration of $1x10^6$ sperm/mL, and the oocytes and sperm were co-incubated for 16-24 h at 38.5°C in 5% CO_2 in air.

3.2.3 Embryo Culture

Following co-incubation, zygotes were vortexed for two minutes in oocyte wash with gentamicin. Presumptive zygotes were washed 6 times through oocyte wash with gentamicin and once through embryo wash medium (MOFA Global, Verona, WI), then placed into pre-equilibrated culture drops of synthetic oviductal fluid medium for conventional freezing medium (SCF1) (Owen, Barceló-Fimbres, Altermatt, & Campos-Chillòn, 2017). SCF1 is an in house made culture media with a conventional synthetic oviductal fluid medium (SOF) base, supplemented with 5% conditioned medium and 1 μL/mL L-carnitine. On day one, presumptive zygotes were supplemented with 5 mM vitamin K_2, and on day five with 10 μM forskolin. Presumptive zygotes were cultured for 7-8 days in 5% O_2 and 5% CO_2 and 90% N_2 at 38.5°C. Blastocyst rate was assessed on day 7 and 8 post fertilization.

3.3 Embryo Assessments

3.3.1 Mitochondrial Polarity Assessment

Stage 7 blastocysts were washed three times through PBS-PVP, then stained for mitochondrial polarity using 300 nM Mitotracker Red CMX-Rosamine (Thermo Fischer, Waltham, MA) for 50 minutes in culture conditions. Blastocysts were washed 3 more times through PBS-PVP, then fixed in 4% paraformaldehyde for 3-7 days away from light at 4°C. Following fixation, blastocysts were washed 3 times through PBS-PVP, counter-stained with 1 μg/mL DAPI for 15 minutes in the dark. Blastocysts were then washed 3 more times in PBS-PVP and transferred to the confocal microscope. Twenty images were taken at 40x magnification with a 5 μm step size. Images were analyzed by determining the mean fluorescence of the embryo and subtracting the

background to calculate the corrected mean total fluorescence in 10 representative images in IMAGE J software (NIH).

3.3.2 Lipid Content

Stage 7 blastocysts were washed three times through PBS-PVP, then fixed for 3-7 days in 3.9% paraformaldehyde. Following fixation, blastocysts were washed three times in PBS-PVP, and counter-stained with DAPI for 15 minutes in the dark. Embryos were washed once again through PBS-PVP; then, stained with 1 μg/mL Nile Red for 30 minutes. Finally, embryos were washed 3 times in PBS-PVP then placed in PBS-PVP and transferred to the confocal microscope. Blastocysts were imaged at 40x using and 20 images were taken at a 5 μm step size. Images were analyzed by determining the mean fluorescence of the embryo and subtracting the background to calculate the corrected mean total fluorescence in 10 representative images in IMAGE J software.

3.3.3 Reactive Oxygen Species Assessment

Stage 7 blastocysts were washed 3 times in Syngro Holding Medium (Vetoquinol, Canada) and stained with 5 μM Cell Rox Green (Thermo Fischer, Waltham MA) for 30 minutes in culture conditions. After staining, blastocysts were washed 3 more times in Syngro Holding Medium and fixed in 10% formalin for 30 minutes. Blastocysts were then washed 3 times in Syngro Holding Medium and counter-stained with DAPI in the dark for 15 minutes, and finally placed in Syngro Holding Medium and transported to the confocal microscope within 4 hours of staining. Blastocysts were imaged at 40x. Twenty images were taken at a 5 μm step size. Images were analyzed by determining the mean fluorescence of the embryo and subtracting the background to calculate the corrected mean total fluorescence in 10 representative images in IMAGE J software.

3.4 Embryo Slow Freezing and Post-Thaw Assessment

Stage 7 or higher blastocysts were slow frozen following an equilibration in 1.5 M EG with 0.5 M sucrose for 10 minutes. Blastocysts were loaded into 0.25cc straws and then placed into the cryochamber (Chrysalysis C2346S, Napa Valley CA) at -6°C. Embryos were held for one minute at -6°C prior to seeding, then held for another 9 minutes before they were cooled at a rate of -0.6°C per minute to a final temperature of -33°C before being plunged in liquid nitrogen. Straws were stored in liquid nitrogen until thawing.

Straws were thawed in air for 10 sec followed by 30 sec in 32-35 °C water. Straw contents were expelled, and blastocysts were washed 10 times in oocyte wash with oocyte wash medium before being placed into pre-equilibrated culture drops. Re-expansion was assessed at 24 and 48h.

3.4.1 TUNEL Assay

Re-expanded blastocysts were assessed for apoptosis using a TUNEL assay. Therefore, they were washed three times in PBS-PVP, and fixed in 3.7% paraformaldehyde for 3-7 days. Following fixation, blastocysts were washed three times in PBS-PVP, and permeabilized in 0.5% Triton X for 30 minutes at room temperature. Blastocysts were washed three more times in PBS-PVP; then, stained with TUNEL in the dark at 38.5°C and 85% humidity. Blastocysts were washed 3 more times in PBS-PVP, then counter-stained with 1 μg/mL DAPI for 15 minutes in the dark. Finally, they were washed three more times through PBS-PVP, then transferred to the confocal microscope in PBS-PVP and imaged at 40x with 20 images per embryo were taken at a 5 μM step size. Ten images per embryo were analyzed by counting all cells and all apoptotic cells and calculating the percent of dead cells.

3.5 Statistical Analysis

Statistical analysis was performed in SAS (SAS Institute, Cary, NC, USA). Blastocyst rate and re-expansion data were analyzed for normality using the Shapiro Wilk test, arcsine transformed

as appropriate, and analyzed via ANOVA with LSD to separate means. Replicates and bulls were included in the model to account for weekly variations in ovary batch as well as a bull effect. Mitotracker, Nile Red, and, ROS analysis data were analyzed for normality using the Shapiro Wilk test, log transformed as appropriate, and analyzed via ANOVA with LSD to separate means.

Chapter 4

EXPERIMENTS

4.1 Introduction

IVP embryos can potentially increase economic gain and production efficiency in the dairy industry (Moore & Hasler, 2017). Unfortunately, these embryos are different from their IVD counterparts in morphology, gene expression, development, metabolism, and cryotolerance (Barceló-Fimbres & Seidel, 2007; Heinzmann et al., 2011; Inaba et al., 2016; Nagano et al., 2006). The poor development and cryotolerance of IVP embryos hinders success rates when transferred into recipient cows and limits widespread use of the technology (Moore & Hasler, 2017).

IVP embryos suffer from increased intracellular lipid content, dysfunctional mitochondria, and high levels of ROS that lead to poor development and cryotolerance (Abe et al., 2017). Although culture conditions have improved, most media are still unable to adequately mimic *in vivo* conditions (Seidel, 2006). Furthermore, developing oocytes and embryos are subjected to high levels of microscopic light, hyperoxic conditions, and improper culture substrates (Barceló-Fimbres & Seidel, 2007; Kitagawa et al., 2004; Korhonen et al., 2009). Embryos are cultured in IVC media for up to 8 days, and thus improving culture conditions could play an important role in improving embryonic metabolism and cryotolerance (Lonergan & Fair, 2016; Rizos, Ward, Duffy, Boland, & Lonergan, 2002).

One potential cause of the decreased embryo cryotolerance is intracellular lipid accumulation due to improper culture conditions (Seidel, 2006). Synthetic oviductal fluid (SOF) is a commonly used culture media that was formulated based on the components of ewe oviductal fluid (Tervit, Whittingham, & Rowson, 1972). Over time the formulation has changed slightly, most notably adding amino acids and bovine serum albumin to improve development (Gandhi, Lane, Gardner, & Krisher, 2000). Studies using SOF have reported a 30% blastocyst rate but poor cryotolerance (D. Rizos, Ward, Boland, & Lonergan, 2001; Shirazi, Nazari, Ahmadi, Heidari, & Shams-Esfandabadi, 2009). Embryos cultured in SOF are more lipid heavy than IVD embryos, and although the exact mechanism of lipid accumulation is unknown, two potential sources are the use

of fetal calf serum and glucose in culture (De La Torre-Sanchez, Gardner, Preis, Gibbons, & Seidel, 2006; Seidel, 2006). Fetal calf serum contains globulins, growth factors, and proteins that support embryo growth; however, it has been correlated with increased lipid accumulation and decreased cryotolerance (Barceló-Fimbres & Seidel, 2007). Glucose is used as an energy source in culture media although it is not utilized by embryos until the compaction stage (Barceló-Fimbres & Seidel, 2007). Improper glucose utilization leads to increased pentose phosphate pathway activity and ROS production which impairs mitochondrial activity and increases lipid accumulation (Barceló-Fimbres & Seidel, 2007; Kimura, Spate, Green, & Roberts, 2005). However, glucose is an important component for synthesis of nucleotide precursors, and thus is necessary for developing embryos and should not be omitted from the culture media (Vajta, Rienzi, Cobo, & Yovich, 2010).

Lipid accumulation is correlated with decreases in cryotolerance, as breeds with higher milk fat such as Jersey's tend to have more lipid heavy embryos and are less likely to survive freezing (Baldoceda et al., 2016). Lipids most likely impact cryopreservation through lipid peroxidation and membrane stability (Tarín & Trounson, 1993). In contrast, lipid accumulation may be a symptom of poor development and more of a marker for poor quality than the cause of low cryotolerance (Sudano et al., 2011). In either case, decreasing lipid content may improve cryotolerance by reducing lipid peroxidation or improving overall embryo quality. Our lab has developed a novel culture media called SCF1 which is an SOF base supplemented with metabolic regulators l-carnitine, Vitamin K_2, forskolin, and conditioned medium from Vero cells that potentially will improve embryonic development and enhance cryotolerance (Owen et al., 2017; Roberts et al., 2016).

While our lab has investigated regulation of mitochondrial activity and lipid accumulation, ROS production has not been studied. ROS are highly reactive oxygen containing compounds such as H_2O_2, OH^-, and O_2^- (R. Li et al., 2016). They are naturally produced within cells during oxidative phosphorylation and are not harmful at low levels (Starkov, 2008). However, when ROS levels are too high, they can disrupt normal cellular reactions, mutate DNA, cause poor mitochondrial activity,

lipid peroxidation, and potentially cell death (Kitagawa et al., 2004; R. Li et al., 2016; Tatone et al., 2010).

ROS are produced at all stages of production within IVP embryos. Oocytes are typically matured in 20% O_2 compared to the 3-13% O_2 they would naturally be exposed to within the follicle (Ali et al., 2003). Higher levels of O_2 increases oxidative phosphorylation, thus increasing the ROS production (M. Takahashi, 2012). Additionally, the follicle produces antioxidants SOD, Cat, and GPx that aid the maturing oocyte in preventing ROS accumulation (Combelles et al., 2010). Adding antioxidants such as melatonin, L-ascorbic acid, and resveratrol to oocyte maturation medium has beneficial effects on subsequent embryonic development (Córdova et al., 2010; Rodrigues-Cunha et al., 2016; F. Wang, Tian, Zhang, et al., 2014).

The effects of antioxidants on IVF are less studied. ROS are required during sperm capacitation, thus lowering ROS levels too far could have negative impacts (O'Flaherty et al., 1999). However, oocytes and sperm are co-cultured at 20% O_2 which is higher than the oxygen tension in the female tract which could increase ROS production (Cheuquemán et al., 2015). Additionally, the sperm contribute ROS that would be dispersed in the female tract *in vivo*, but are maintained within the fertilization medium *in vitro* (Nohalez et al., 2018). Adding melatonin to IVF media has contradictory reports; in one study, blastocyst rate improved, while in another it decreased (Cheuquemán et al., 2015; Pang et al., 2016). Further studies are warranted to investigate the effects of antioxidants in fertilization media.

Embryos are cultured in media for up to 8 days after fertilization, leading to accumulation of ROS (Salzano et al., 2014). Adding melatonin, BME, or resveratrol to culture increases blastocyst rate in bovine embryos (Feugang et al., 2004; Salzano et al., 2014; F. Wang, Tian, Zhou, et al., 2014). Resveratrol specifically is a good candidate to add to bovine culture conditions due to its ability to decrease lipid levels and increase mitochondrial activity and cryotolerance (Abe et al., 2017). Unfortunately, resveratrol is unstable and easily oxidized which makes it unable to last in embryo culture (Regev-Shoshani et al., 2003). Polydatin, a glucoside precursor of resveratrol, may

be a better option for embryo culture since it is not oxidized as easily and has higher bioavailability since it can be transported into cells via glucose transporters (Lanzilli et al., 2012). Additionally, polydatin limits lipid peroxidation and upregulates the SIRT1 pathway which improves overall embryonic metabolism (Fabris et al., 2008; Huang et al., 2015). A previous study by Khan et al., (2017) showed that adding polydatin to bovine oocyte maturation, blastocyst rate and SIRT1 increased, while expression of inflammatory genes such as *NFkb* and *BAX* decreased.

While polydatin has promising effects on oocyte maturation, its effects have not been studied on all stages of embryo production. A previous study in porcine added L-ascorbic acid to all stages of production and found no effects in any treatment group; however, L-ascorbic acid is a scavenger and does not have the metabolic impacts polydatin has (Nohalez et al., 2018). Overall, the objective of this study was to determine the effects of polydatin added to IVM, IVF, IVC on blastocyst rate, mitochondrial activity, lipid levels, and cryotolerance. We hypothesized that adding polydatin to all stages of culture would increase blastocyst rate, mitochondrial activity, and cryotolerance while lowering lipid levels.

While culture conditions may impact cryotolerance, optimizing the procedure may also have beneficial effects. During slow freezing, ice crystal formation must be limited to prevent organelle and membrane damage and apoptosis (Inaba et al., 2016; L. Li, Zhang, Zhao, Xia, & Wang, 2012). Cattle embryos are typically slow frozen in 1.5 M EG to allow direct transfer into a recipient cow upon thawing (Voelkel & Hu, 1992). The concentrations of EG are low to prevent toxicity to the cells; however, they may be too low to fully dehydrate the cells (L. Li et al., 2012). Expanded blastocysts require more dehydration than non-expanded blastocysts due to the fluid filled blastocoel cavity, and a longer equilibration time may help improve survival rates (L. Li et al., 2012). Additionally, nonpermeable cryoprotectants such as sucrose can help enhance the cryotolerance by further dehydrating cells (Tominaga, Iwaki, & Hochi, 2007). Sugars such as sucrose draw more water outside the cells and bind to polar heads within the membrane to stabilize the cells against changes in volume (Palasz, Thundathil, De La Fuente, & Mapletoft, 2000). Optimizing the slow freezing procedure using different equilibration times and extracellular

cryoprotectants to dehydrate cells potentially reduce intracellular ice crystal formation and improve cryotolerance of *in vitro* produced embryos.

When embryos are thawed, oxygen influx and increased production of heat shock proteins leads to a burst of ROS production (El-Wahsh et al., 2003; Odani et al., 2003). The ROS burst increases lipid peroxidation, DNA mutations, and leads to apoptosis which decreases post-thaw survival (Tatone et al., 2010). One potential mechanism to alleviate the oxidative stress is using antioxidants in the freezing medium (Lane et al., 2002). The antioxidants in the freezing medium can prevent the sudden onset of high levels of ROS, potentially improving post thaw survival rates (Higginbotham et al., 2017; Lane et al., 2002). Previous research in our lab showed improved re-expansion rates post-thawing when l-ascorbic acid was added to EG; however, the use of another antioxidant such as polydatin has not been studied (Higginbotham et al., 2017). In addition to oxidative stress, slow frozen embryos encounter osmotic stress due to water reentering the cells too quickly (Seidel, 2006). The quick water movement causes blastomeres to swell and potentially burst which results in cell death and embryonic loss (Sanches et al., 2016). A recent study by Sanches et al., (2016) investigated using a rehydration column with a lower concentration of EG to help prevent water from reentering the cells too quickly. Using this technique, they produced similar conception rates of 40.19% for slow frozen or 35.89% for vitrified bovine blastocysts which is in contrast to many studies showing that vitrification produced higher conception rates (Inaba et al., 2016; L. Li et al., 2012; Sanches et al., 2016; Saragusty & Arav, 2011). Sucrose could improve post-thaw survival by further slowing the rehydration and limiting osmotic stress (Coticchio et al., 2006; Palasz et al., 2000; Tominaga et al., 2007). Adding sucrose to the rehydration column could potentially help eliminate post-thaw osmotic stress and improve survival rates.

The objectives of experiment 1 were to:

1) Validate the ability of SCF1 to improve embryonic development, metabolism, and cryotolerance

2) Optimize the slow freezing protocol that had the least negative impact on *in vitro* produced bovine embryos

We hypothesized that:

1) Embryos cultured in SCF1 would have increased blastocyst rate, mitochondrial activity, cryotolerance, and decreased lipid levels and post-thaw apoptosis
2) Embryos dehydrated in sucrose and allowed a longer equilibration time would have increased cryotolerance and decreased post-thaw apoptosis

The objectives of experiment 2 were to determine the effects of polydatin added to IVM, IVF, IVC on blastocyst rate, mitochondrial activity, lipid levels, and cryotolerance. We hypothesized that adding polydatin to all stages of culture would increase blastocyst rate, mitochondrial activity, and cryotolerance while lowering lipid levels.

The objectives of experiment 3 were to:

1) Determine the effects of polydatin on blastocyst rate, lipid levels, mitochondrial activity, ROS levels, and cryotolerance
2) Determine the effects of in straw rehydration coupled with L-ascorbic acid and polydatin on cryotolerance

We hypothesized that:

1) Oocytes matured in a media with polydatin would have increased blastocyst rate, mitochondrial activity, and cryotolerance, and lower levels of lipids and ROS than the control oocytes
2) Blastocysts frozen with antioxidants and rehydrated in-straw would have increased cryotolerance compared to control cryopreserved embryos

4.2 Experimental Design

This thesis is separated into three separate experiments. In the first experiment, we utilized a 2x2x2 factorial. The factors were culture media (SCF1 vs. SOF), sucrose dehydration (0 vs. 0.6M), and equilibration time of embryos to EG (10 vs. 20 min). The experiment was replicated 9 times with 3127 oocytes and semen from one of four fertility proven bulls. In the second experiment, embryos were produced with polydatin added to all three steps of production and all possible combinations, which produced 8 possible treatments (Table 1). The experiment was replicated 7 times with 3320 oocytes. The final experiment was designed as a 2x2 factorial design. The first factor was the presence of 0 or 1 μM polydatin in the maturation medium. The second factor was the use of a rehydration column containing 0.75 M EG and 0.6 M sucrose with 1 mM l-ascorbic acid and 1 μM polydatin in both the rehydration and embryo columns or using only 1.5 M EG and 0.5 M sucrose throughout the entire straw. This experiment was replicated 12 times with 3318 oocytes placed into culture.

Table 1. Treatment groups for polydatin experiment. Embryos were produced with 1μM polydatin added to either maturation (IVM), fertilization (IVF), or culture (IVC), and all possible combinations and assigned a treatment group number 1-8. A "+" in the table represents the addition of polydatin, while a "–" indicates no polydatin was added.

	Polydatin		
	IVM	**IVF**	**IVC**
1	**-**	**-**	**-**
2	**+**	**-**	**-**
3	**-**	**+**	**-**
4	**-**	**-**	**+**
5	**+**	**+**	**-**
6	**-**	**+**	**+**
7	**+**	**-**	**+**
8	**+**	**+**	**+**

4.3 Materials and Methods

4.3.1 Embryo Production

Embryos were produced following the procedures listed in chapter 2 with the following modifications:

1) Only Holstein ovaries were collected from the abattoir for experiment 1
2) Presumptive zygotes were randomly assigned to either SOF or SCF1 in experiment 1
3) Embryos cultured in SCF1 were supplemented with Vitamin K_2 on day 3 instead of day 5 in experiment 1
4) COC's were washed once through OPU and twice through oocyte wash medium to eliminate excess heparin carry over from the OPU in experiments 2 and 3
5) 0 or 1 μM polydatin was added to the maturation medium in experiment 2 and 3
6) 0 or 1 μM polydatin was added to the fertilization medium in experiment 2
7) 0 or 1 μM polydatin was added to the culture medium in experiment 2

4.3.2 Blastocyst Rate, Mitochondrial Activity, Lipid Content, and Post-Thaw Survival Assessment

Embryos were assessed for blastocyst rate on days 7 and 8. Embryos were staged and graded according to IETS guidelines (Mapletoft, 2013). Mitochondrial activity, lipid content, and ROS levels were assessed following the procedures listed in chapter 2 with the following modification in experiment 2 and 3:

1) Fluorescence was analyzed per cell to further refine the process. In each slice where fluorescence was measured, the total number of cells was manually counted. Then, the fluorescence per slice was divided by the number of cells to get an average fluorescence/cell.

Stage 7 grade 1 blastocysts were slow-frozen following a 2 min dehydration in either 0 or 0.6 M sucrose followed by equilibration in 1.5 M EG and 0.5 M sucrose for 10 or 20 min in the first experiment. In the subsequent experiments, stage 7 blastocysts were slow frozen following a 20

min equilibration in EG which was determined to be the best treatment with the following modification:

1) 1 mM L-ascorbic acid was added to the EG based on a previous experiment in our lab that showed increased post-thaw re-expansion (Higginbotham, Owen, Barceló-Fimbres, & Campos-Chillòn, 2017)
2) EG was supplemented with 0 or 1 mM l-ascorbic acid and 0 or 1 μM polydatin in experiment 3
3) Rehydration columns were comprised of 1.5 M EG and 0.5 M sucrose or 0.75 M EG and 0.6 M sucrose with 1 mM l-ascorbic acid and 1 μM polydatin in experiment 3

Re-expansion and post-thaw apoptosis were assessed following procedures listed in chapter 2.

4.3.3 Statistical Analysis

Statistics were performed for the first experiment as described in chapter two except:

1) Post-thaw survival rate was arcsine transformed for normality and analyzed by ANOVA with means separated by Tukey's HSD. Differences $p<0.05$ were considered significant
2) TUNEL was analyzed by ANOVA with means separated by Tukey's HSD with differences $p<0.05$ considered significant

In the second experiment, blastocyst rate and re-expansion rate data were analyzed following the procedures listed in chapter 2. Mitochondrial activity and lipid levels were log transformed and analyzed in JMP using an ANOVA with Tukey's HSD to separate means, with $p<0.05$ considered significant. Data from the third experiment were analyzed following the procedure described in chapter 2.

4.4 Results

4.4.1 Experiment 1

Embryos cultured in SCF1 had a higher blastocyst rate (35.0± 1.7) than embryos cultured in SOF medium (22.44± 2.9, $p<0.01$, Figure 4).

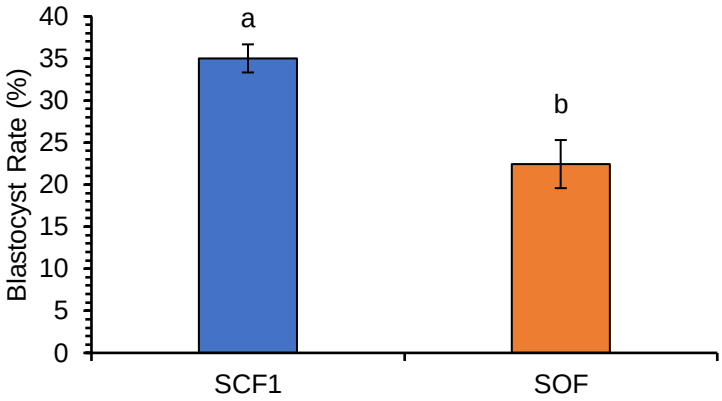


Figure 4. Blastocyst rate of embryos cultured in SCF1 or SOF (Mean±SEM). Embryos were cultured in either SCF1 (n=1781) or SOF (n=1391) for up to 8 days, then blastocyst rate was visually assessed. SCF1 produced a higher blastocyst rate than SOF ($^{a,b}p<0.01$).

Stage 7 embryos grade 1 embryos were stained for lipid content or mitochondrial polarity with Nile Red or Mitotracker, respectively. SCF1 embryos had lower lipid levels and higher levels of mitochondrial activity than embryos cultured in SOF ($p<0.01$, Figure 5).

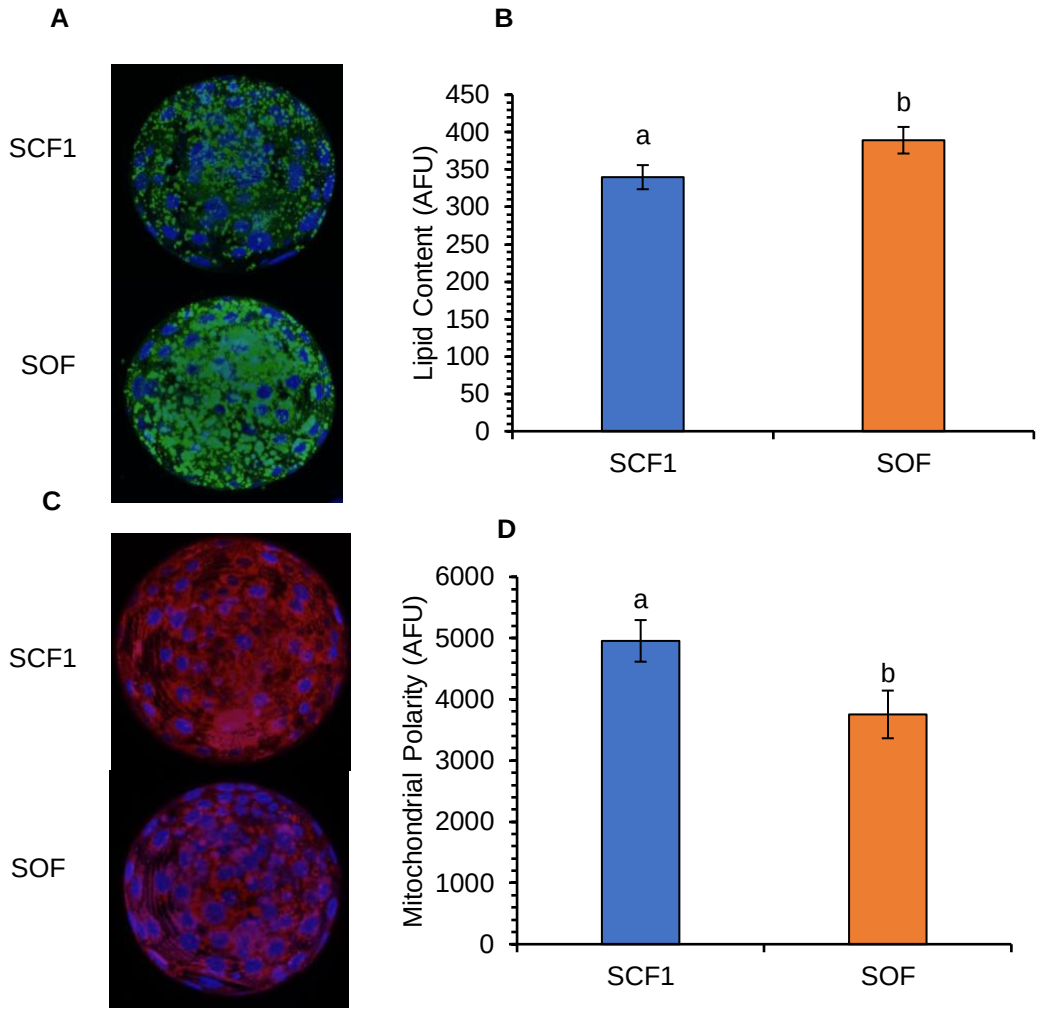


Figure 5. Lipid content and mitochondrial activity and of embryos cultured in SCF1 or SOF (Mean±SEM). Stage 7 embryos were stained with Nile Red (A, n=16 per treatment) or Mitotracker Red CMX-Rosamine (C, n=17 per treatment) and imaged via confocal microscopy. Fluorescence was analyzed in IMAGEJ software and quantified (C, D). Embryos cultured in SCF1 had lower lipid levels and higher mitochondrial activity than embryos cultured in SOF ($^{a,b}p<0.01$).

Stage 7 grade 1 embryos were slow frozen after a 2 min dehydration in 0 or 0.6M sucrose and a 10 or 20 min equilibration in EG. No significant interactions were observed; therefore, only main effects were analyzed. Embryos that were cultured in SCF1 had a higher re-expansion rate (82.3 ± 2.7%) than embryos cultured in SOF (49.6 ± 4.5%, $p<0.01$). There was no difference between dehydration in 0 or 0.6M sucrose ($p>0.1$). Finally, embryos equilibrated for 20 min

(69.8±3.9%) tended to have a higher re-expansion rate than embryos equilibrated for 10 min (62.1± 4.7%, p<0.1, Figure 6).

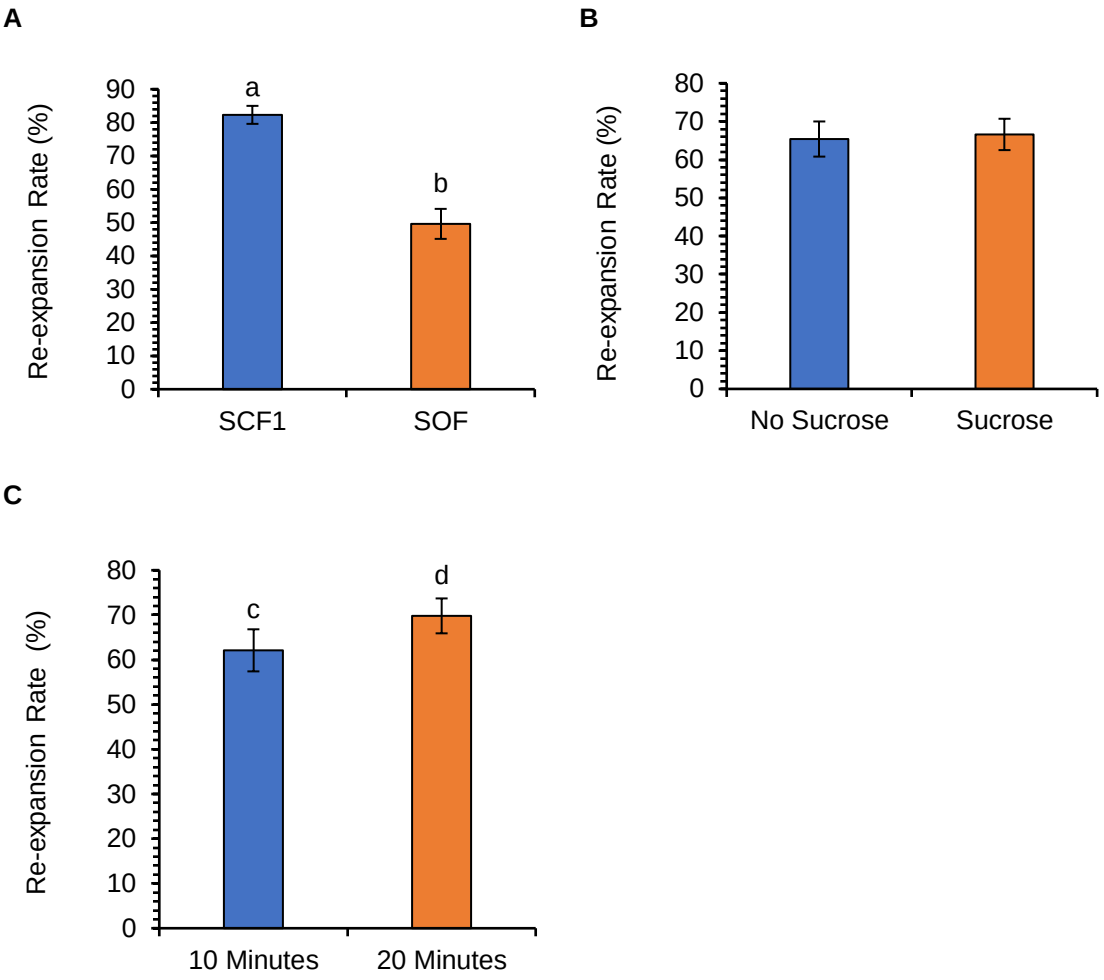


Figure 6. Main effects of culture media (A), sucrose rehydration (B), and equilibration time in EG (C) on the re-expansion rate of slow frozen embryos (Mean±SEM). Embryos cultured in SCF1 (n=358) had a higher re-expansion rate than embryos cultured in SOF (n=198, $^{a,b}p<0.01$). There was no difference between embryos dehydrated prior to equilibration in 0 (n=277) or 0.6M sucrose (n=279). Embryos equilibrated for 20 min in EG (n=270) tended to have a higher re-expansion rate than embryos equilibrated for 10 min (n=286) prior to slow freezing ($^{c, d}$ p<0.1).

Re-expanded blastocysts were stained with TUNEL to analyze apoptosis. No significant interactions were observed so only main effects were analyzed. Embryos cultured in SOF had a

higher percent of apoptotic cells than embryos cultured in SCF1 (31.6±2.0 vs. 20.4±2.6, $p<0.01$). In addition, embryos equilibrated for 20 minutes had lower percent of apoptotic cells than embryos equilibrated for 10 minutes (21.4±2.0 vs. 30.6±2.5, $p<0.01$). Sucrose dehydration had no difference on apoptosis in re-expanded blastocysts ($p>0.1$, Figure 7).

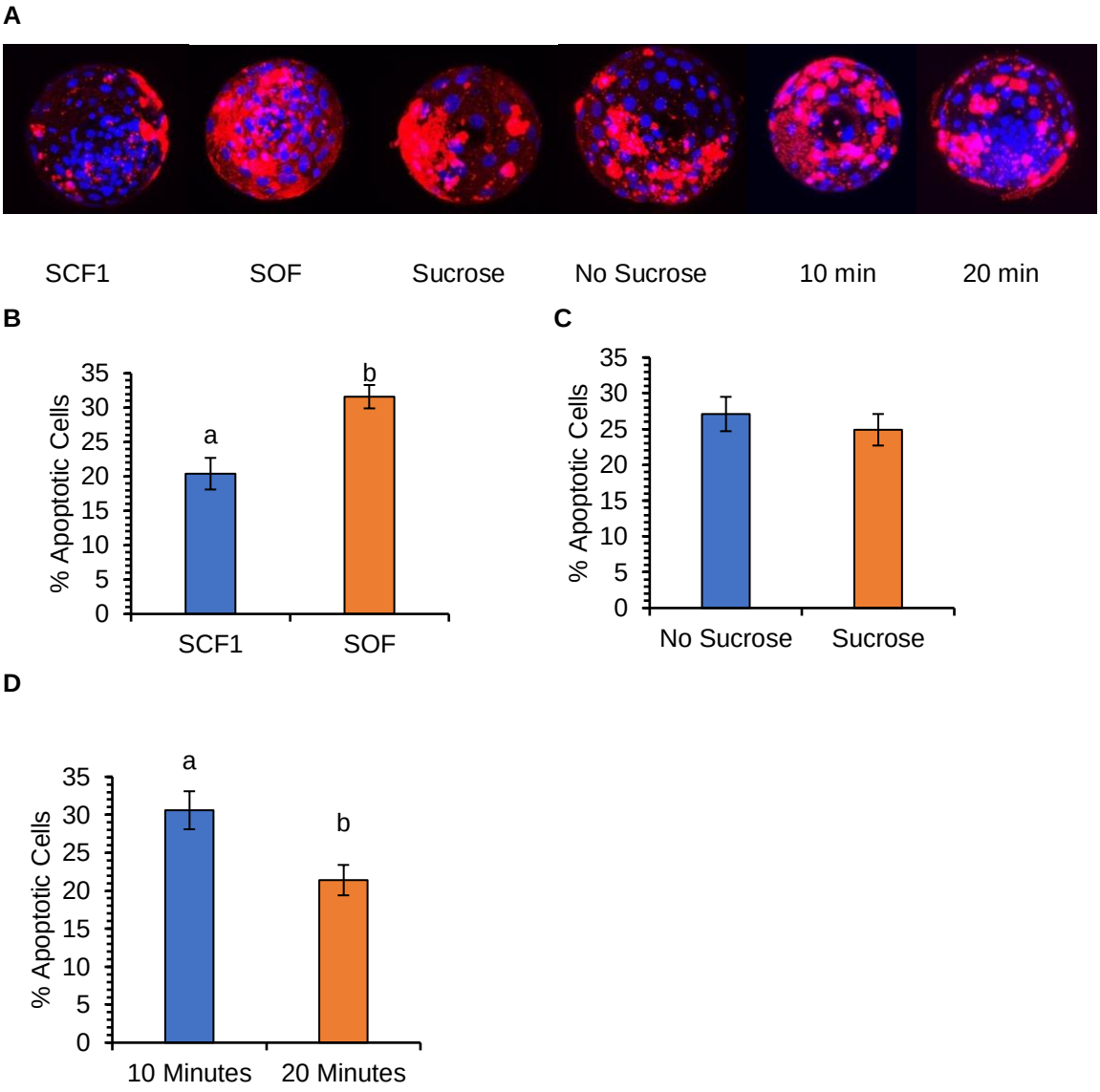


Figure 7. Main effects of culture media, sucrose rehydration, and equilibration time in EG on the percent of apoptotic cells of slow frozen IVP embryos (Mean±SEM). Nuclei are stained blue with DAPI and fragmented DNA is stained red with TUNEL, and the overlapping pink nuclei representing apoptotic cells (A). Apoptotic cells were counted as a percent of total cells. Embryos cultured in either SCF1 (n=85) had a lower percent of apoptotic cells rate than embryos cultured in SOF (n=63, B). There was no difference between embryos that were dehydrated prior to equilibration in in 0 (n=72) or 0.6M sucrose (n=76, C). Embryos equilibrated for 20 min (n=72) had a lower percent of apoptotic cells than embryos equilibrated for 10 min (n=76) prior to slow freezing (D) (^{a,b} $p<0.01$).

4.4.2 Experiment 2

Adding polydatin to the maturation media only (Treatment 2) increased the blastocyst rate compared to when it was added to IVM, IVF, and IVC (Treatment 8, $p<0.01$). There were no differences in any other treatment group (Figure 8).

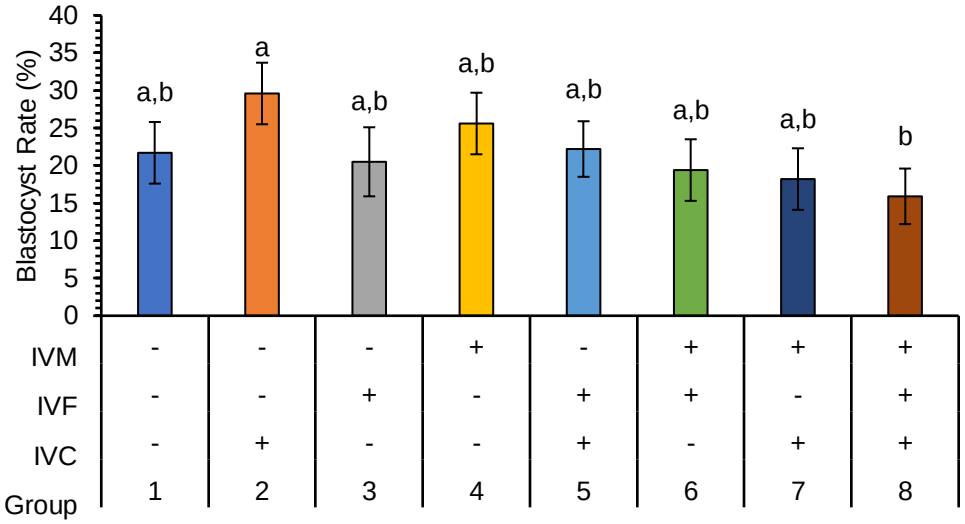


Figure 8. Blastocyst rate of bovine embryos cultured with polydatin added to different stages of production (Mean±SEM). 1 μM polydatin was added to IVM, IVF, or IVC and all possible combinations (Table 1, n= 398, 342, 460, 385, 292, 383, 582 oocytes per culture per Treatment 1-8, respectively). The blastocyst rate was higher when oocytes were matured with polydatin (Treatment 2) than when polydatin was added to all three stages of production (Treatment 8, $p<0.01$).

When polydatin was added to only maturation media, developed blastocysts had higher mitochondrial activity then when added to no stages of production (Treatment 1), when added only to IVF (Treatment 3), when added to only culture (Treatment 4), when added to IVF and IVC (Treatment 6) or when added to IVM and IVC (Treatment 7, $p<0.05$). Additionally, when added to all 3 stages of production (Treatment 8), mitochondrial activity was higher then Treatments 1, 3, 4,

6, and 7 ($p<0.05$, Figure 9). Treatment 2 also had the lowest amount of lipids compared to all other treatments ($p<0.05$, Figure 9).

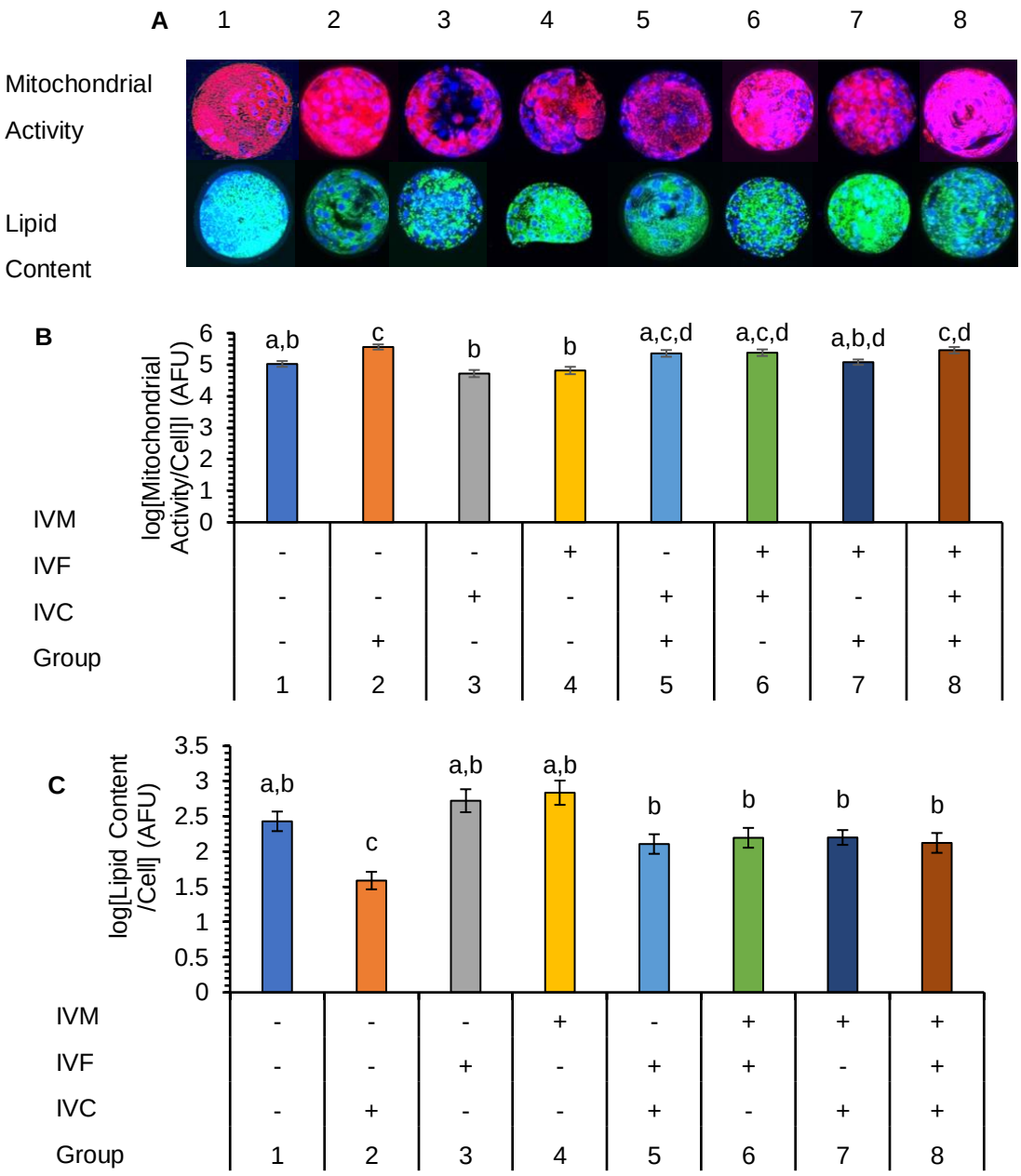


Figure 9. Mitochondrial activity and lipid levels of stage 7 embryos produced with polydatin added to various stages of production (A). Mitochondrial activity (n=5, 5, 8, 7, 5, 4, 7, or 8 embryos stained per Treatment 1-8, respectively) and lipid content (n=4, 4, 7, 5, 4, 3, 3, or 4 embryos stained per Treatment 1-8, respectively) were analyzed by determining the corrected mean total fluorescence/cell (B, C, Mean±SEM). Data were log transformed for normality ($^{a,b,c,d}p<0.05$).

Adding polydatin only to maturation medium (Treatment 2) increased cryotolerance compared to when it was added to all production stages (Treatment 8, $p<0.05$). There were no other differences in cryotolerance ($p>0.1$, Figure 10).

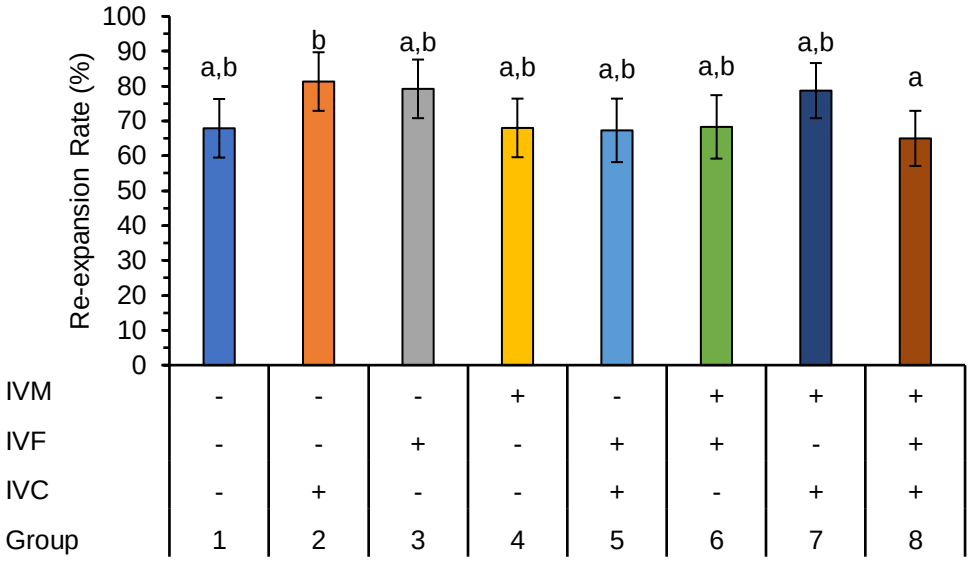


Figure 10. Re-expansion rates of embryos produced with polydatin added to various stages of production (Mean±SEM). Embryos were slow frozen, then thawed and re-expansion was assessed at 24 and 48 h (n=40, 49, 47, 91, 60, 48, or 50 frozen embryos per Treatment 1-8, respectively). Adding polydatin to only maturation medium (Treatment 2) produced a higher re-expansion rate then when added to all stages of production (Treatment 8, $p<0.05$).

4.4.3 Experiment 3

Oocytes matured in polydatin tended to have an increased blastocyst rate (16.01±1.00 vs 14.37±1.03, respectively, $p=0.0665$, Figure 11).

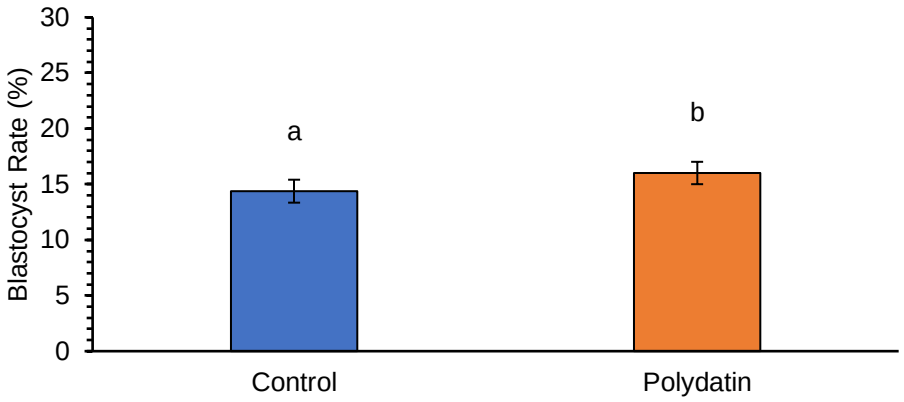


Figure 11. Effect of polydatin in maturation media on blastocyst rate (Mean±SEM).. Oocytes were matured with either 0 (n=1734) or 1 μM (n=1584) polydatin. Blastocyst rate was assessed on day 7 and 8. Blastocyst rate tended to be higher in oocytes matured with polydatin ($^{a,b}p<0.1$).

Stage 7 embryos were analyzed for ROS level, lipid content, or mitochondrial activity and the fluorescence was quantified (Figure 12). Oocytes matured with polydatin produced blastocysts that tended to have less ROS ($p=0.0994$) and had higher mitochondrial activity than the control blastocysts ($p<0.05$). There was no difference in lipid content between the two treatments ($p>0.1$).

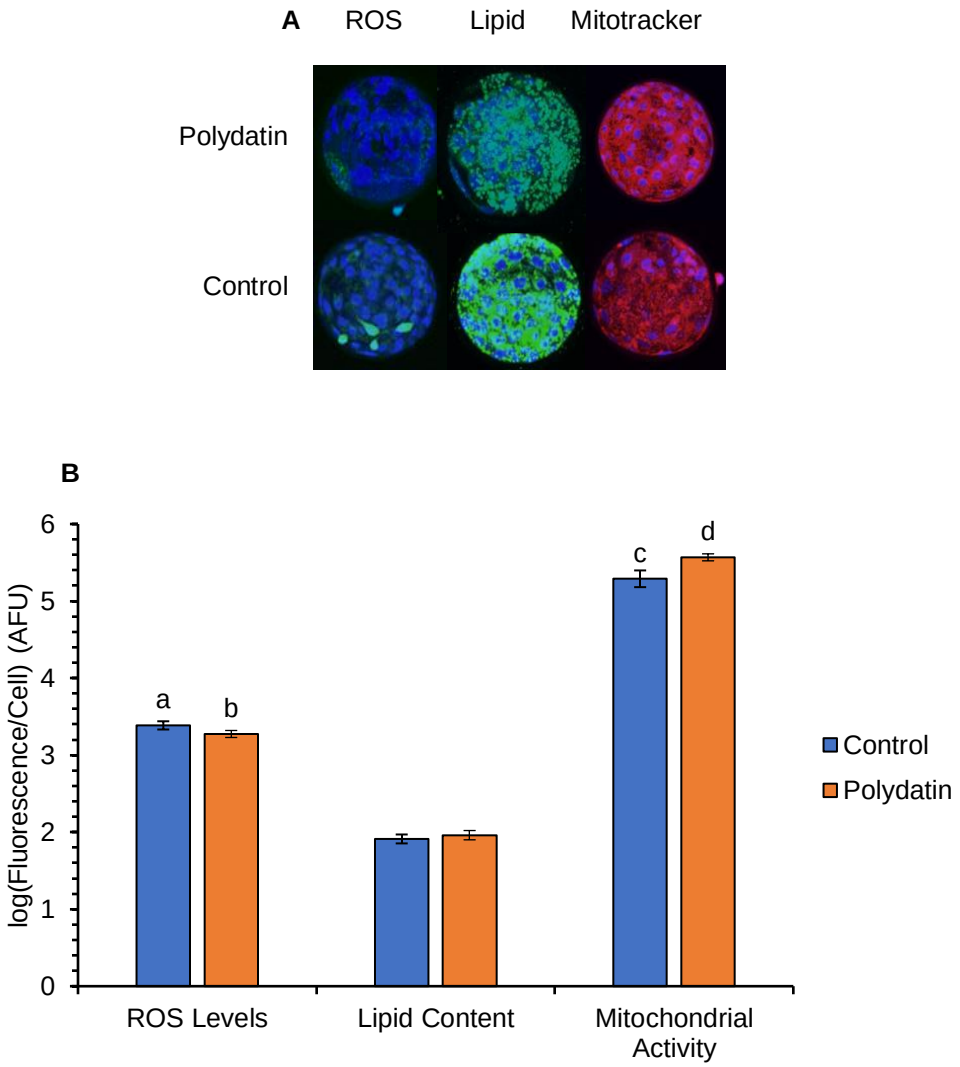


Figure 12. The effect of polydatin on ROS levels, lipid content, and mitochondrial activity on bovine IVP embryos (Mean±SEM). Embryos were stained with Cell Rox Green (n=14 for control and 13 for polydatin treatment, left images), Nile Red (n=9 for both treatments, middle images), or Mitotracker Red CMX-Rosamine (n=9 for control and 12 for polydatin treatments, right images) (A). All stains were counterstained with DAPI, then analyzed for corrected mean fluorescence per cell in IMAGEJ (B). Oocytes matured with polydatin tended to have less ROS (^{a,b} $p<0.1$) and higher mitochondrial activity than control embryos (^{c,d} $p<0.05$) There was no difference in lipid content ($p>0.05$).

There was no significant interaction between the maturation media and the use of in-straw rehydration, therefore, only main effects are shown. There were no significant differences between

all four treatment groups, though overall polydatin in the maturation medium and an in-straw rehydration produced the highest re-expansion rate numerically (Figure 13).

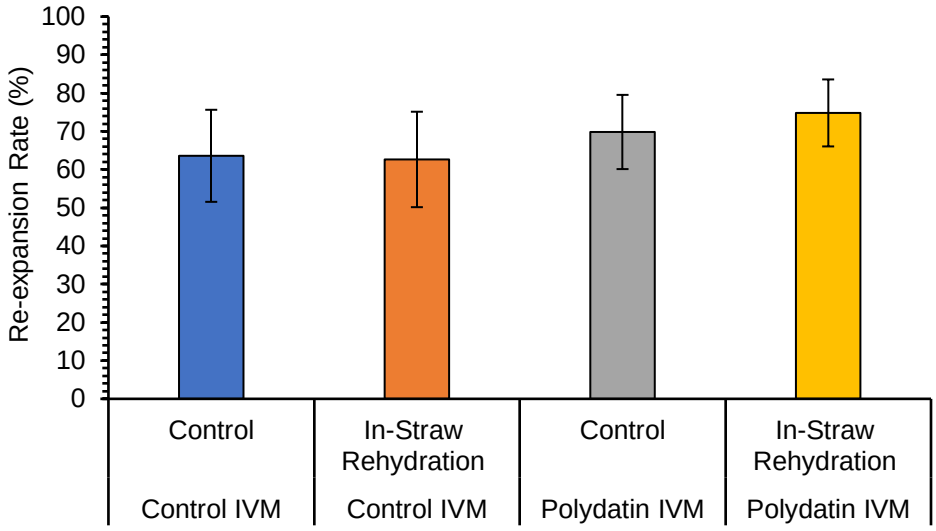


Figure 13. Differences in post-thaw survival between oocytes matured with 0 or 1 μM polydatin and rehydrated in the straw (Mean±SEM). Oocytes were matured in a control or polydatin treated maturation medium, then developed stage 7 blastocysts were slow frozen in a medium containing a control (n=52 for control oocytes and 51 for polydatin treated oocytes) or an in-straw rehydration column coupled with 1 mM L-ascorbic acid and 1 μM polydatin (n=58 for control oocytes and polydatin treated oocytes). There was no difference between the treatment groups ($p>0.05$).

4.5 Discussion

Embryonic culture impacts the metabolism, gene expression, and cryotolerance of bovine embryos and needs improvement to better mimic the uterine environment (Kocyigit, 2016). SOF is commonly for bovine embryo culture, though the embryos produced have decreased cryotolerance and increased lipids (Shirazi et al., 2009). SCF1 is SOF FCS free and supplemented with l-carnitine, conditioned medium from Vero cells, vitamin K_2 and forskolin to improve development. L-carnitine helps transport fatty acids into mitochondria to enhance β-oxidation to reduce lipid levels (Longo, Frigeni, & Pasquali, 2016). When added to embryo culture medium, l-carnitine improves

blastocyst rate and cryotolerance and lowers lipid levels which is in agreement with our results (T. Takahashi et al., 2013). The conditioned medium secretes growth factors and cytokines into the medium which support the embryo (Maeda, 1996). Previous studies have shown that conditioned medium improves blastocyst rate of IVP bovine embryos when added at day 0 of culture (Barceló-Fimbres, Gouze, Mtango, & Koppang, 2015; Maeda, 1996). Vitamin K_2 acts as an electron carrier in the mitochondria which helps improve function and increase ATP output (Baldoceda-Baldeon, Gagné, Vigneault, Blondin, & Robert, 2014). When added to IVC, embryos have higher blastocyst rate and mitochondrial activity (Baldoceda-Baldeon et al., 2014). Finally, forskolin is an activator of cAMP, and significantly reduces the amount of lipids found in porcine embryos; however, does not improve blastocyst rate in bovine embryos (Paschoal et al., 2012). When all metabolic regulators were used in combination, the novel SCF1 medium improved embryonic developmental competence and metabolism of bovine embryos produced *in vitro.* A previous study in our lab compared every possible combination of these metabolic regulators and also found low lipid levels, high mitochondrial activity, blastocyst rate, and re-expansion rate in SCF1 compared to other combinations of forskolin, vitamin K, and conditioned medium (Roberts et al., 2016).

Embryos cultured in SCF1 had decreased post-thaw apoptosis and increased post-thaw re-expansion rate. The enhanced cryotolerance is likely due to the reduced lipid content which improves post thaw survival (Seidel, 2006; T. Takahashi et al., 2013). Lipid reduction is often associated with post-thaw survival; however, it is not necessarily the cause of increased cryotolerance and is more likely a marker of overall embryo stress (T. Takahashi et al., 2013). In early apoptosis, oxidative stress inhibits ß-oxidation of lipids within the mitochondria, leading to increased lipid droplet accumulation (Boren & Brindle, 2012). Thus, the embryos cultured in SCF1 may have less lipid accumulation due to decreased stress, indicating that they may be more competent than embryos cultured in SOF (Boren & Brindle, 2012; T. Takahashi et al., 2013). Since cryotolerance is often a marker of overall embryo quality, the embryos cultured in SCF1 may be of higher quality due to the less stressful culture conditions which lead to the improved cryotolerance and decreased post-thaw apoptosis observed (T. Takahashi et al., 2013).

In addition to studying the effects of SCF1, we determined the impact of a sucrose dehydration and different equilibration times on cryotolerance and post-thaw apoptosis. Sucrose is a macromolecule often used in cryopreservation to regulate the osmolarity by preventing water from entering or exiting the cell too quickly (Palasz et al., 2000). Sucrose is unable to cross the membrane bilayer, and instead slowly draws water out from within the cell to prevent ice crystal formation (Gómez-Fernández, Gómez-Izquierdo, Tomás, Mocé, & de Mercado, 2012). We did not observe any changes in post-thaw apoptosis or re-expansion when embryos were dehydrated in 0 or 0.6M sucrose. A relatively low concentration of sucrose was used in this study and our freezing medium was already supplemented with 0.5 M. Perhaps the sucrose within the freezing medium was enough to dehydrate the embryos regardless of the 2 min dehydration prior to equilibration. An additional effect of sucrose is stabilizing the membrane by entering the phospholipid bilayer and hydrogen bonding with the polar heads (Aisen, Medina, & Venturino, 2002; Pollock et al., 2016). Stabilizing the membrane can prevent abrupt changes in cell volume and limit osmotic shock. Studies in sperm cryopreservation have suggested that different sugars have better cryoprotective effects with different species due to the different lipid makeup of their membranes (Aisen et al., 2002; Fernández-Santos et al., 2007; Gómez-Fernández et al., 2012; Sarıözkan et al., 2012). Raffinose, a trisaccharide composed of a glucose, fructose, and galactose subunit have improved post-thaw viability of bull sperm compared to sucrose, and may be able to bind more efficiently with the plasma membranes in bovids (Tuncer et al., 2011).

In addition to a sucrose dehydration, the effects of an increased equilibration in EG were evaluated. EG replaces water within cells to decrease damaging ice crystal formation (Saragusty & Arav, 2011). Cryoprotectants are toxic to cells, and it is advised to limit the embryos exposure; however, EG has low toxicitiy and thus embryos may be able to survive a longer exposure time (Kaidi et al., 2001). Allowing embryos to equilibrate in EG for 20 minutes led to decreased post-thaw apoptosis and a tendency to have higher post-thaw survival. The exact trigger of apoptosis during slow freezing is unknown, though two likely mechanisms are intracellular ice crystal formation leading to cellular damage and resulting in stress that leads to apoptosis, or the lysis of

membranes due to changes in volume that releases enzymes and lead to apoptosis (L. Li et al., 2012).

Stage 7 blastocysts are typically around 200 microns in diameter, compared to approximately 170 microns for nonexpanded blastocysts (Mori, Otoi, & Suzuki, 2002). The increase in diameter is due to the increase in size of the blastocoel cavity as more fluid enters, and a longer equilibration time may be necessary to remove the fluid and prevent ice crystal formation (L. Li et al., 2012). Expanded blastocysts have a higher apoptotic index than nonexpanded blastocysts post cryopreservation, suggesting that fluid within the cavitity must be removed prior to slow freezing (Kader et al., 2009). Allowing embryos to equilibrate for 20 min may have removed more fluid from the blatocoel cavity, thus reducing ice crystal formation and limiting apoptosis. Despite differences in apoptotic status, Kader et al., (2009) did not report a change in post-thaw survival between expanded and non-expanded blastocysts. It is important to note however, that the Kader study utilized mouse embryos that had been previously frozen and thus may have had different effects than our study. Nevertheless, his study does suggest that other factors may impact post-thaw survival that could explain why we did not observe differences in post thaw re-expansion, despite seeing increased apoptosis in the embryos equilibrated for 10 min in EG. One potential cause is changes in metabolism in thawed embryos that limit their survival (Kaidi et al., 2001). Slow frozen bovine embryos have decreased glucose and pyruvate uptake compared to vitrified and control embryos (Kaidi et al., 2001). The decreased glucose uptake is likely due to disturbed membrane transport systems in trophoblast cells which decreases expression of GLUT1 (Inaba et al., 2016; Kaidi et al., 2001; Uechi, Tsutsumi, Morita, & Taketani, 1997). Decreasing energy consumption leads to decreased ATP production which decreases embryonic growth by preventing spindle formation, leading to decreased mitosis and halted development (Heald, 2000; X. Zhang et al., 2006). Additionally, decreased ATP production could limit the Na^+/K^+ pumps that pump sodium into the blastocoel cavitiy (Inaba et al., 2016). By limiting the action of the pumps, the embryos may not be able to reexpand and thus would not survive the slow freezing process. In rat embryos, glucose concentration, however not pyruvate concentration, limiting glucose uptake directly limited the formation of the blastocoel cavity (Brison & Leese, 1994). Thus, the inability of the embryos to re-

expand following slow freezing may be due to energy uptake in addition to apoptosis. This could explain why we observed a decrease in apoptosis but only a tendency for increased re-expansion in embryos slow frozen after a 20 min equilbration.

Following the results of experiment 1, the goal of the subsequent experiments were to alleviate oxidative stress by utilizing the antioxidant polydatin in embryo culture. Adding polydatin to maturation media increased blastocyst rate and cryotolerance compared to when it was added to all stages of production; however, there was no difference with the control (Treatment 1). The decreased development and cryotolerance in embryos produced with polydatin in all culture stages (Treatment 8) compared to only adding polydatin to maturation media (Treatment 2) could be due to the dose dependent nature of the antioxidant. Previous studies suggested that resveratrol supplementation at a concentration greater than 1 μM decreased development and led to higher levels of apoptosis in bovine and parthogenic porcine embryos (Lee, Wang, Chaille, & Machaty, 2010; Salzano et al., 2014). Additionally, in human cancer cells increased exposure to resveratrol led to pro apoptotic and anti-proliferative effects *in vitro* (Hsieh & Wu, 1999; Kuwajerwala et al., 2002; Lin et al., 2008). Cancer cells exposed to higher levels of resveratrol inhibit the G_1\S phase transition during mitosis which decreases proliferation (Hsieh & Wu, 1999). High resveratrol exposure also increases Cox2 expression which stimulates the p53 gene, which is responsible for increasing ATP production to repair DNA (Lin et al., 2008). If the stress is too severe; however, p53 will inhibit repair mechanisms and initiate apoptosis (Lin et al., 2008). Apoptotic cells have increased mitochondrial membrane potential due to p53 activity (Boren & Brindle, 2012). The increased mitochondrial activity increases ROS, and specifically hydrogen peroxide interacts with thiols on enzymes responsible for fatty acid oxidation which increases lipid accumulation (Boren & Brindle, 2012; Korge & Weiss, 2006). This agrees with our results, which showed increased mitochondrial activity and lipid content in embryos treated with polydatin at all stages of production. Thus, it is likely that the embryos from Treatment 8 had a high apoptotic index and poor quality due to toxic effects from polydatin.

Interestingly, we observed that adding polydatin to only maturation media did not improve blastocyst rate or cryotolerance compared to the control. This is in contrast to previous reports

that suggested polydatin improved blastocyst rate when added to bovine maturation media; however, one study suggested that adding resveratrol to maturation media does not enhance blastocyst rate (Khan et al., 2017; Takeo et al., 2014). Takeo et al., (2014) determined that the addition of resveratrol lead to more normal fertilization outcomes but did not change the overall blastocyst rate. They also determined that the embryos quality was improved due to increased mitochondrial activity, copy number, and ATP levels (Takeo et al., 2014). Similarly, our study suggested embryos from Treatment 2 were higher quality due to increased mitochondrial activity and lower lipid levels, though there were no differences in blastocyst rate and cryotolerance. However, in the Takeo study (2014) they added resveratrol at a concentration of 20 μM which should be toxic according to other studies (Lee et al., 2010; Salzano et al., 2014). The contradicting results with both polydatin and resveratrol suggest that other factors play a key role in how the antioxidants are able to impact IVM, and future studies will need to elucidate the mechanism by which polydatin and resveratrol improve embryo quality.

In our final experiment, we chose to further evaluate the impact of polydatin on oocyte maturation. Overall, there were modest, yet significant improvements to embryonic development and metabolism when polydatin was added to the maturation medium, and there was a tendency for a higher blastocyst rate. We observed significantly higher mitochondrial activity, which is a marker for embryonic quality (Baldoceda-Baldeon et al., 2014). Increased mitochondrial activity is associated with increased ATP levels and lower levels of lipids, often due to increased β-oxidation (Baldoceda-Baldeon et al., 2014; Ferguson & Leese, 2006; Nagano et al., 2006). Impaired mitochondrial function can also decrease birth weight and lead to problems for the developing offspring (Wakefield, Lane, & Mitchell, 2011).

The increased mitochondrial activity may also explain why polydatin only tended to decrease the ROS levels. Previous studies have reported that adding polydatin or resveratrol to maturation significantly lowered ROS levels; however, neither study investigated the impact of ROS on the mitochondria (Khan et al., 2017; F. Wang, Tian, Zhang, et al., 2014). ROS are a natural byproduct of oxidative phosphorylation, and increased mitochondrial activity is directly correlated with increased ROS production (Orrenius et al., 2007; M. Takahashi, 2012). By upregulating the

SIRT1 pathway, polydatin increases production of antioxidant genes such as SOD and Cat which can counteract some of the stress an embryo encounters; however, the enhanced mitochondrial function also likely produced more ROS within the embryos (Khan et al., 2017; Orrenius et al., 2007). Therefore, the reduction in ROS may not have been significant due to the increased mitochondrial activity observed in our study.

Although polydatin increased mitochondrial activity, it is unknown if this is due to enhancing the function of the mitochondria or increasing the number of mitochondria. Polydatin upregulates the SIRT1 gene which increases mitochondrial biogenesis (Coticchio et al., 2006; Palasz, Thundathil, De La Fuente, & Mapletoft, 2000; Tominaga, Iwaki, & Hochi, 2007). Therefore, it is possible that the mitochondria in oocytes treated with polydatin are still functioning poorly, but there is a higher number leading to the increased fluorescence. Increasing mitochondrial number does not impact ATP levels; however, increasing mitochondrial function has a large effect on ATP levels (Ge et al., 2012). If the increase in mitochondrial fluorescence seen in this experiment was due to increased numbers of mitochondria, it may not actually correspond to improved embryonic function or ATP levels. Reports using resveratrol have reported conflicting data on mitochondrial copy number in bovine embryos (Abe et al., 2017; Takeo et al., 2014). In one study by Abe et al., (2017), mitochondrial membrane potential was enhanced but copy number was not different when resveratrol was added; however, in the Takeo et al., study (2014), they reported increased mitochondrial membrane potential as well as increased copy number. Differences between these studies suggest that other factors influence how polydatin and resveratrol impact the mitochondria and overall embryo, and more research should be done to further understand the antioxidants.

There was no difference in lipid content between the polydatin treated embryos and the control embryos, which contradicted our results from the previous experiment. One possible explanation for this discrepancy could be due to the breed differences. Different breeds of cattle respond differently to metabolic regulators (Baldoceda, Gagné, Ferreira, & Robert, 2015; Baldoceda et al., 2016). For example, Jersey cattle embryos exposed to l-carnitine have less lipid reduction than Holstein embryos (Baldoceda et al., 2015). Since the breed of ovaries in the present study was unknown, it is possible that the breeds mainly used in this study had less response to

polydatin than the breeds in the previous study, leading to the different results. In the previous experiment, we only had a small sample size of 4 embryos stained in Treatment 2 due to time constraints, and these embryos came from only 3 replicates. Since we do not know the breed makeup of the ovaries obtained each week, it is possible that these embryos may have been mainly comprised of one breed that responded differently to the polydatin. In this study, the embryos stained were produced from 6 different replicates and may be more representative of the entire experiment. Future studies should examine the effects of polydatin on lipid content in one specific breed to determine how polydatin impacts embryonic lipid metabolism.

Similar to our results in experiment 2, blastocysts produced from oocytes matured in polydatin did not have increased cryotolerance compared to the control. Previous studies have suggested similar findings, and show that decreasing ROS levels with antioxidant supplementation during maturation does not impact cryotolerance (N. A. S. Rocha-Frigoni et al., 2015; N. A. S. Rocha-Frigoni, Leão, Nogueira, Accorsi, & Mingoti, 2014). Although adding antioxidants to IVM can improve embryonic quality, it may not be able to overcome all stressors the embryo faces throughout production which leads to the decreased cryotolerance (N. A. S. Rocha-Frigoni et al., 2014). The half-life of polydatin is only 4 hours and once removed from IVM medium, it is not in cells for an extended period of time (Du et al., 2013). After fertilization, presumptive zygotes are exposed to decreased temperatures and UV light while their cumulus cells are removed which may lead to increased ROS production (Cagnone & Sirard, 2016). Unfortunately, the embryos are unable to transcribe endogenous antioxidants until the activation of the embryonic genome which leaves the embryos unprotected and susceptible to oxidative stress which compromises embryo quality (Cagnone & Sirard, 2016; M. Takahashi, 2012).

Finally, we determined the impact of using in-straw rehydration coupled with the antioxidants l-ascorbic acid and polydatin on cryotolerance. In the original study that developed in-straw rehydration columns, the authors did not determine re-expansion rates *in vitro* or compare the novel technique to a more traditional slow freezing technique (Sanches et al., 2016). Thus, although our technique did not improve re-expansion *in vitro* does not mean that it would not impact conception rates. Pregnancy rates from our novel freezing technique should be assessed to

determine if there could be increased success after transferring these embryos. Sanches et al., (2016) did not examine the effects of antioxidants in the freezing media; however, previous research in our lab has suggested that adding L-ascorbic acid to EG improved post-thaw survival rates (Higginbotham et al., 2017; Sanches et al., 2016). We did not test polydatin on its own to determine if it improved post-thaw survival compared to the l-ascorbic acid. However, polydatin enters cells via GLUT1 transporters which are downregulated in trophoblast cells in thawed embryos (Uechi et al., 1997). Thus, it is possible that the addition of polydatin may not enhance post-thaw survival and should be evaluated on its own to determine its effects on survival. Numerically, oocytes matured with polydatin and slow frozen using in-straw rehydration coupled with antioxidants had the highest cryotolerance. The total number of embryos frozen in this study was relatively low, with only about 50 embryos frozen per treatment. This sample size may not have been large enough to determine differences between the treatments, and the study should be repeated with larger numbers.

Overall, oocytes matured with polydatin had some indications of being more metabolically competent that oocytes matured without the antioxidant. Though the results were modest, they did indicate that polydatin may enhance embryonic development. Additionally, the use of in straw rehydration produced the numerically highest post-thaw survival rates. Future studies should include a pregnancy study to determine if the post-thaw survival rates could have an impact *in vivo*.

Chapter 5

Conclusion

Bovine IVP embryos are developmentally and metabolically abnormal compared to IVD embryos and work needs to be done to improve the *in vitro* culture process. Overall, the focus of this thesis was to improve bovine embryonic culture conditions by determining the effects of our novel culture media SCF1, using the antioxidant polydatin, and optimizing slow freezing protocols. We determined that SCF1 produced embryos with higher developmental competence and cryotolerance, improved mitochondrial activity and decreased lipid content. Additionally, adding polydatin to oocyte maturation media had the biggest impact on embryonic development and produced embryos with higher mitochondrial activity compared to the control. Finally, we discovered that embryos equilibrated for 20 minutes prior to slow freezing had the best post-thaw survival, and that using an in-straw rehydration coupled with antioxidants produced the numerically highest survival rate. Future studies should validate these observations with pregnancy data in order to confirm the results seen *in vitro*.

REFERENCES

Abe, T., Kawahara-Miki, R., Hara, T., Noguchi, T., Hayashi, T., Shirasuna, K., … Iwata, H. (2017). Modification of mitochondrial function, cytoplasmic lipid content and cryosensitivity of bovine embryos by resveratrol. *The Journal of Reproduction and Development*, *63*(5), 455–461. https://doi.org/10.1262/jrd.2016-182

Acker, J. P., & McGann, L. E. (2003). Protective effect of intracellular ice during freezing? *Cryobiology*, *46*(2), 197–202.

Adhikari, D., & Liu, K. (2014). The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Molecular and Cellular Endocrinology*, *382*(1), 480–487. https://doi.org/10.1016/j.mce.2013.07.027

Agarwal, A., Durairajanayagam, D., & du Plessis, S. S. (2014). Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reproductive Biology and Endocrinology : RB&E*, *12*. https://doi.org/10.1186/1477-7827-12-112

Aisen, E. G., Medina, V. H., & Venturino, A. (2002). Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology*, *57*(7), 1801–1808. https://doi.org/10.1016/S0093-691X(02)00653-2

Alcaín, F. J., Minor, R. K., Villalba, J. M., & Cabo, R. de. (2010). Small Molecule Modulators of Sirtuin Activity. In *The Future of Aging* (pp. 331–356). Springer, Dordrecht. https://doi.org/10.1007/978-90-481-3999-6_10

Ali, A. A., Bilodeau, J. F., & Sirard, M. A. (2003). Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology*, *59*(3–4), 939–949.

Arav, A. (2014). Cryopreservation of oocytes and embryos. *Theriogenology*, *81*(1), 96–102. https://doi.org/10.1016/j.theriogenology.2013.09.011

Arosh, J. A., Banu, S. K., Kimmins, S., Chapdelaine, P., MacLaren, L. A., & Fortier, M. A. (2004). Effect of Interferon-τ on Prostaglandin Biosynthesis, Transport, and Signaling at the Time of Maternal Recognition of Pregnancy in Cattle: Evidence of Polycrine Actions of

Prostaglandin E2. *Endocrinology*, *145*(11), 5280–5293. https://doi.org/10.1210/en.2004-0587

Augustin, R., Pocar, P., Navarrete-Santos, A., Wrenzycki, C., Gandolfi, F., Niemann, H., & Fischer, B. (2001). Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. *Molecular Reproduction and Development*, *60*(3), 370–376. https://doi.org/10.1002/mrd.1099

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal [Research article]. https://doi.org/10.1155/2014/360438

Bain, N. T., Madan, P., & Betts, D. H. (2011). The early embryo response to intracellular reactive oxygen species is developmentally regulated. *Reproduction, Fertility and Development*, *23*(4), 561–575. https://doi.org/10.1071/RD10148

Baldoceda, L., Gagné, D., Ferreira, C. R., & Robert, C. (2015). Genetic influence on the reduction in bovine embryo lipid content by l-carnitine. *Reproduction, Fertility, and Development*. https://doi.org/10.1071/RD14215

Baldoceda, L., Gilbert, I., Gagn�, D., Vigneault, C., Blondin, P., Ferreira, C. R., & Robert, C. (2016). Breed-specific factors influence embryonic lipid composition: comparison between Jersey and Holstein. *Reproduction, Fertility and Development*, *28*(8), 1185. https://doi.org/10.1071/RD14211

Baldoceda-Baldeon, L. M., Gagné, D., Vigneault, C., Blondin, P., & Robert, C. (2014). Improvement of bovine in vitro embryo production by vitamin K2 supplementation. *Reproduction*, *148*(5), 489–497. https://doi.org/10.1530/REP-14-0324

Barceló-Fimbres, M., Gouze, J. N., Mtango, N. R., & Koppang, R. (2015). 127 Vero Cells Conditioned Medium Improves Embryonic Development During in Vitro Culture of Bovine Embryos. *Reproduction, Fertility and Development*, *27*(1), 155–155. https://doi.org/10.1071/RDv27n1Ab127

Barceló-Fimbres, M., & Seidel, G. E. (2007). Effects of either glucose or fructose and metabolic regulators on bovine embryo development and lipid accumulation in vitro. *Molecular Reproduction and Development*, *74*(11), 1406–1418. https://doi.org/10.1002/mrd.20700

Barceló-Fimbres, M., & Seidel, G. E. (2007). Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during in vitro culture of bovine embryos on embryonic development after cryopreservation. *Molecular Reproduction and Development*, *74*(11), 1395–1405. https://doi.org/10.1002/mrd.20699

Bartolomei, M. S. (2009). Genomic imprinting: employing and avoiding epigenetic processes. *Genes & Development*, *23*(18), 2124–2133. https://doi.org/10.1101/gad.1841409

Boren, J., & Brindle, K. M. (2012). Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation. *Cell Death and Differentiation*, *19*(9), 1561–1570. https://doi.org/10.1038/cdd.2012.34

Brison, D. R., & Leese, H. J. (1994). The role of exogenous energy substrates in blastocoele fluid accumulation in the rat. *Zygote (Cambridge, England)*, *2*(1), 69–77.

Cagnone, G., & Sirard, M.-A. (2016). The embryonic stress response to in vitro culture: insight from genomic analysis. *Reproduction*, *152*(6), R247–R261. https://doi.org/10.1530/REP-16-0391

Carling, P. J., Cree, L. M., & Chinnery, P. F. (2011). The implications of mitochondrial DNA copy number regulation during embryogenesis. *Mitochondrion*, *11*(5), 686–692. https://doi.org/10.1016/j.mito.2011.05.004

Chaytor, J. L., Tokarew, J. M., Wu, L. K., Leclère, M., Tam, R. Y., Capicciotti, C. J., … Ben, R. N. (2012). Inhibiting ice recrystallization and optimization of cell viability after cryopreservation. *Glycobiology*, *22*(1), 123–133. https://doi.org/10.1093/glycob/cwr115

Chen, L., & Lan, Z. (2017). Polydatin attenuates potassium oxonate-induced hyperuricemia and kidney inflammation by inhibiting NF-κB/NLRP3 inflammasome activation via the AMPK/SIRT1 pathway. *Food & Function*, *8*(5), 1785–1792. https://doi.org/10.1039/c6fo01561a

Cheuquemán, C., Arias, M. E., Risopatrón, J., Felmer, R., Álvarez, J., Mogas, T., & Sánchez, R. (2015). Supplementation of IVF medium with melatonin: effect on sperm functionality and in vitro produced bovine embryos. *Andrologia*, *47*(6), 604–615. https://doi.org/10.1111/and.12308

Chung, S., Yao, H., Caito, S., Hwang, J., Arunachalam, G., & Rahman, I. (2010). Regulation of SIRT1 in cellular functions: role of polyphenols. *Archives of Biochemistry and Biophysics*, *501*(1), 79–90. https://doi.org/10.1016/j.abb.2010.05.003

Combelles, C. M. H., Holick, E. A., Paolella, L. J., Walker, D. C., & Wu, Q. (2010). Profiling of SOD isoenzymes in compartments of the developing bovine antral follicle. *Reproduction (Cambridge, England)*, *139*(5), 871–881. https://doi.org/10.1530/REP-09-0390

Córdova, B., Morató, R., Izquierdo, D., Paramio, T., & Mogas, T. (2010). Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the in vitro maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. *Theriogenology*, *74*(8), 1341–1348. https://doi.org/10.1016/j.theriogenology.2010.06.003

Coticchio, G., De Santis, L., Rossi, G., Borini, A., Albertini, D., Scaravelli, G., … Cecconi, S. (2006). Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Human Reproduction*, *21*(7), 1771–1776. https://doi.org/10.1093/humrep/del073

De La Torre-Sanchez, J. F., Gardner, D. K., Preis, K., Gibbons, J., & Seidel, G. E. (2006). Metabolic regulation of in vitro-produced bovine embryos. II. Effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. *Reproduction, Fertility, and Development*, *18*(5), 597–607.

De Munck, N., Santos-Ribeiro, S., Stoop, D., Van de Velde, H., & Verheyen, G. (2016). Open versus closed oocyte vitrification in an oocyte donation programme: a prospective randomized sibling oocyte study. *Human Reproduction*, dev321. https://doi.org/10.1093/humrep/dev321

Dekel N. (1988). Regulation of Oocyte Maturation. *Annals of the New York Academy of Sciences*, *541*(1), 211–216. https://doi.org/10.1111/j.1749-6632.1988.tb22258.x

Delmas, D., & Lin, H.-Y. (2011). Role of membrane dynamics processes and exogenous molecules in cellular resveratrol uptake: consequences in bioavailability and activities. *Molecular Nutrition & Food Research*, *55*(8), 1142–1153. https://doi.org/10.1002/mnfr.201100065

Dochi, O., Yamamoto, Y., Saga, H., Yoshiba, N., Kano, N., Maeda, J., … Inohae, S. (1998). Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. *Theriogenology*, *49*(5), 1051–1058.

Donnelly, E. T., McClure, N., & Lewis, S. E. (1999). The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, *14*(5), 505–512.

Du, Q.-H., Peng, C., & Zhang, H. (2013). Polydatin: a review of pharmacology and pharmacokinetics. *Pharmaceutical Biology*, *51*(11), 1347–1354. https://doi.org/10.3109/13880209.2013.792849

Ducibella, T. (1996). The cortical reaction and development of activation competence in mammalian oocytes. *Human Reproduction Update*, *2*(1), 29–42. https://doi.org/10.1093/humupd/2.1.29

Dumollard, R., Duchen, M., & Sardet, C. (2006). Calcium signals and mitochondria at fertilisation. *Seminars in Cell & Developmental Biology*, *17*(2), 314–323. https://doi.org/10.1016/j.semcdb.2006.02.009

El Hajj, N., & Haaf, T. (2013). Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. *Fertility and Sterility*, *99*(3), 632–641. https://doi.org/10.1016/j.fertnstert.2012.12.044

El-Wahsh, M., Fuller, B., Davidson, B., & Rolles, K. (2003). Hepatic cold hypoxia and oxidative stress: implications for ICAM-1 expression and modulation by glutathione during experimental isolated liver preservation. *Cryobiology*, *47*(2), 165–173.

Fabris, S., Momo, F., Ravagnan, G., & Stevanato, R. (2008). Antioxidant properties of resveratrol and piceid on lipid peroxidation in micelles and monolamellar liposomes. *Biophysical Chemistry*, *135*(1), 76–83. https://doi.org/10.1016/j.bpc.2008.03.005

Feng, G., Zhang, B., Shu, J., Zhou, H., Zhou, L., Gan, X., & Liu, Y. (2010). [Effects of artificial shrinkage of blastocoeles before vitrification on pregnancy outcome]. *Zhonghua Fu Chan Ke Za Zhi*, *45*(11), 838–842.

Ferguson, E. M., & Leese, H. J. (2006). A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Molecular Reproduction and Development*, *73*(9), 1195–1201. https://doi.org/10.1002/mrd.20494

Fernández-Santos, M. R., Martínez-Pastor, F., García-Macías, V., Esteso, M. C., Soler, A. J., de Paz, P., … Garde, J. J. (2007). Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa. *Theriogenology*, *67*(4), 738–753. https://doi.org/10.1016/j.theriogenology.2006.10.005

Ferreira, E. M., Vireque, A. A., Adona, P. R., Meirelles, F. V., Ferriani, R. A., & Navarro, P. A. A. S. (2009). Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*, *71*(5), 836–848. https://doi.org/10.1016/j.theriogenology.2008.10.023

Feugang, J.-M., de Roover, R., Moens, A., Léonard, S., Dessy, F., & Donnay, I. (2004). Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology*, *61*(1), 71–90.

FitzHarris, G., Marangos, P., & Carroll, J. (2007). Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein. *Developmental Biology*, *305*(1), 133–144. https://doi.org/10.1016/j.ydbio.2007.02.006

Fuller, B., & Paynter, S. (2004). Fundamentals of cryobiology in reproductive medicine. *Reproductive BioMedicine Online*, *9*(6), 680–691. https://doi.org/10.1016/S1472-6483(10)61780-4

Gandhi, A. P., Lane, M., Gardner, D. K., & Krisher, R. L. (2000). A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Human Reproduction*, *15*(2), 395–401. https://doi.org/10.1093/humrep/15.2.395

Gaschler, M. M., & Stockwell, B. R. (2017). Lipid peroxidation in cell death. *Biochemical and Biophysical Research Communications*, *482*(3), 419–425. https://doi.org/10.1016/j.bbrc.2016.10.086

Ge, H., Tollner, T. L., Hu, Z., Dai, M., Li, X., Guan, H., … Dong, Q. (2012). The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. *Molecular Reproduction and Development*, *79*(6), 392–401. https://doi.org/10.1002/mrd.22042

Geshi, M., Yonai, M., Sakaguchi, M., & Nagai, T. (1998). Improvement of in vitro co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with 13-mercaptoethanol, 8.

Gómez-Fernández, J., Gómez-Izquierdo, E., Tomás, C., Mocé, E., & de Mercado, E. (2012). Effect of different monosaccharides and disaccharides on boar sperm quality after cryopreservation. *Animal Reproduction Science*, *133*(1), 109–116. https://doi.org/10.1016/j.anireprosci.2012.06.010

Görlach, A., Klappa, P., & Kietzmann, T. (2006). The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. *Antioxidants & Redox Signaling*, *8*(9–10), 1391–1418. https://doi.org/10.1089/ars.2006.8.1391

Gupta, S., Choi, A., Yu, H. Y., Czerniak, S. M., Holick, E. A., Paolella, L. J., … Combelles, C. M. H. (2011). Fluctuations in total antioxidant capacity, catalase activity, and hydrogen peroxide levels of follicular fluid during bovine folliculogenesis. *Reproduction, Fertility, and Development*, *23*(5), 673–680. https://doi.org/10.1071/RD10270

Haley, S. A., & Wessel, G. M. (2004). Regulated Proteolysis by Cortical Granule Serine Protease 1 at Fertilization. *Molecular Biology of the Cell*, *15*(5), 2084–2092. https://doi.org/10.1091/mbc.e03-11-0843

Han, S. J., Chen, R., Paronetto, M. P., & Conti, M. (2005). Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse. *Current Biology : CB*, *15*(18), 1670–1676. https://doi.org/10.1016/j.cub.2005.07.056

Hanna, C. W., & Kelsey, G. (2014). The specification of imprints in mammals. *Heredity*, *113*(2), 176–183. https://doi.org/10.1038/hdy.2014.54

Hasler, J. F., Henderson, W. B., Hurtgen, P. J., Jin, Z. Q., McCauley, A. D., Mower, S. A., … Trimmer, S. A. (1995). Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*, *43*(1), 141–152. https://doi.org/10.1016/0093-691X(94)00020-U

Heald, R. (2000). Motor Function in the Mitotic Spindle Minireview. *Cell*, *102*(4), 399–402. https://doi.org/10.1016/S0092-8674(00)00044-1

Heinzmann, J., Hansmann, T., Herrmann, D., Wrenzycki, C., Zechner, U., Haaf, T., & Niemann, H. (2011). Epigenetic profile of developmentally important genes in bovine oocytes. *Molecular Reproduction and Development*, *78*(3), 188–201. https://doi.org/10.1002/mrd.21281

Higginbotham, A. R., Owen, C. M., Barceló-Fimbres, M., & Campos-Chillòn, L. F. (2017). 213 Effect of Novel Sof Medium and L-Ascorbic Acid During Cryopreservation of in Vitro-Produced Jersey Cattle Embryos. *Reproduction, Fertility and Development*, *29*(1), 215–216. https://doi.org/10.1071/RDv29n1Ab213

Horsthemke, B., & Ludwig, M. (2005). Assisted reproduction: the epigenetic perspective. *Human Reproduction Update*, *11*(5), 473–482. https://doi.org/10.1093/humupd/dmi022

Hosoe, M., & Shioya, Y. (1997). Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote*, *5*(4), 371–376. https://doi.org/10.1017/S0967199400003956

Hosseini, S. M., Forouzanfar, M., Hajian, M., Asgari, V., Abedi, P., Hosseini, L., … Nasr-Esfahani, M. H. (2009). Antioxidant supplementation of culture medium during embryo development

and/or after vitrification-warming; which is the most important? *Journal of Assisted Reproduction and Genetics*, *26*(6), 355–364. https://doi.org/10.1007/s10815-009-9317-7

Hsieh, T., & Wu, J. M. (1999). Differential Effects on Growth, Cell Cycle Arrest, and Induction of Apoptosis by Resveratrol in Human Prostate Cancer Cell Lines. *Experimental Cell Research*, *249*(1), 109–115. https://doi.org/10.1006/excr.1999.4471

Huang, K., Chen, C., Hao, J., Huang, J., Wang, S., Liu, P., & Huang, H. (2015). Polydatin promotes Nrf2-ARE anti-oxidative pathway through activating Sirt1 to resist AGEs-induced upregulation of fibronetin and transforming growth factor-β1 in rat glomerular messangial cells. *Molecular and Cellular Endocrinology*, *399*, 178–189. https://doi.org/10.1016/j.mce.2014.08.014

Inaba, Y., Miyashita, S., Somfai, T., Geshi, M., Matoba, S., Dochi, O., & Nagai, T. (2016). Cryopreservation method affects DNA fragmentation in trophectoderm and the speed of re-expansion in bovine blastocysts. *Cryobiology*, *72*(2), 86–92. https://doi.org/10.1016/j.cryobiol.2016.03.006

Ishizuka, B., Kuribayashi, Y., Murai, K., Amemiya, A., & Itoh, M. T. (2000). The effect of melatonin on in vitro fertilization and embryo development in mice. *Journal of Pineal Research*, *28*(1), 48–51. https://doi.org/10.1034/j.1600-079x.2000.280107.x

Jimenez, L., Laporte, D., Duvezin-Caubet, S., Courtout, F., & Sagot, I. (2014). Mitochondrial ATP synthases cluster as discrete domains that reorganize with the cellular demand for oxidative phosphorylation. *J Cell Sci*, *127*(4), 719–726. https://doi.org/10.1242/jcs.137141

Han, S. J., & Conti, M. (2006). *New Pathways from PKA to the Cdc2/cyclin B Complex in Oocytes: Wee1B as a Potential PKA Substrate* (Vol. 5). https://doi.org/10.4161/cc.5.3.2395

Kader, A., Agarwal, A., Abdelrazik, H., Sharma, R. K., Ahmady, A., & Falcone, T. (2009). Evaluation of post-thaw DNA integrity of mouse blastocysts after ultrarapid and slow freezing. *Fertility and Sterility*, *91*(5, Supplement), 2087–2094. https://doi.org/10.1016/j.fertnstert.2008.04.049

Kaidi, S., Bernard, S., Lambert, P., Massip, A., Dessy, F., & Donnay, I. (2001). Effect of Conventional Controlled-Rate Freezing and Vitrification on Morphology and Metabolism of Bovine Blastocysts Produced In Vitro. *Biology of Reproduction*, *65*(4), 1127–1134. https://doi.org/10.1095/biolreprod65.4.1127

Khan, I., Kim, S. W., Lee, K.-L., Song, S.-H., Mesalam, A., Chowdhury, M. M. R., … Kong, I.-K. (2017). Polydatin improves the developmental competence of bovine embryos in vitro via induction of sirtuin 1 (Sirt1). *Reproduction, Fertility and Development*, *29*(10), 2011–2020. https://doi.org/10.1071/RD16302

Kidder, G. M., & Mhawi, A. A. (2002). Gap junctions and ovarian folliculogenesis. *Reproduction (Cambridge, England)*, *123*(5), 613–620.

Kimura, K., Spate, L. D., Green, M. P., & Roberts, R. M. (2005). Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. *Molecular Reproduction and Development*, *72*(2), 201–207. https://doi.org/10.1002/mrd.20342

Kitagawa, Y., Suzuki, K., Yoneda, A., & Watanabe, T. (2004). Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology*, *62*(7), 1186–1197. https://doi.org/10.1016/j.theriogenology.2004.01.011

Kobayashi, T., Miyazaki, T., Natori, M., & Nozawa, S. (1991). Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Human Reproduction (Oxford, England)*, *6*(7), 987–991.

Kocyigit, A. (2016). A Review of In Vitro Culture Systems in Bovine Reproductive Biotechnologies, *01*(01). Retrieved from https://www.elynsgroup.com/journal/article/a-review-of-in-vitro-culture-systems-in-bovine-reproductive-biotechnologies

Korge, P., & Weiss, J. N. (2006). Redox regulation of endogenous substrate oxidation by cardiac mitochondria. *American Journal of Physiology. Heart and Circulatory Physiology*, *291*(3), H1436-1445. https://doi.org/10.1152/ajpheart.01292.2005

Korhonen, K., Sjövall, S., Viitanen, J., Ketoja, E., Makarevich, A., & Peippo, J. (2009). Viability of bovine embryos following exposure to the green filtered or wider bandwidth light during in vitro embryo production. *Human Reproduction (Oxford, England)*, *24*(2), 308–314. https://doi.org/10.1093/humrep/den432

Kuwajerwala, N., Cifuentes, E., Gautam, S., Menon, M., Barrack, E. R., & Reddy, G. P. V. (2002). Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis. *Cancer Research*, *62*(9), 2488–2492.

Lane, M., Maybach, J. M., & Gardner, D. K. (2002). Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Human Reproduction*, *17*(10), 2686–2693. https://doi.org/10.1093/humrep/17.10.2686

Lanzilli, G., Cottarelli, A., Nicotera, G., Guida, S., Ravagnan, G., & Fuggetta, M. P. (2012). Anti-inflammatory Effect of Resveratrol and Polydatin by In Vitro IL-17 Modulation. *Inflammation*, *35*(1), 240–248. https://doi.org/10.1007/s10753-011-9310-z

Lee, K., Wang, C., Chaille, J. M., & Machaty, Z. (2010). Effect of Resveratrol on the Development of Porcine Embryos Produced In Vitro. *Journal of Reproduction and Development*, *56*(3), 330–335. https://doi.org/10.1262/jrd.09-174K

Li, L., Zhang, X., Zhao, L., Xia, X., & Wang, W. (2012). Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing. *Molecular Reproduction and Development*, *79*(3), 229–236. https://doi.org/10.1002/mrd.22018

Li, R., Jia, Z., & Trush, M. A. (2016). Defining ROS in Biology and Medicine. *Reactive Oxygen Species (Apex, N.C.)*, *1*(1), 9–21. https://doi.org/10.20455/ros.2016.803

Li, Z., Lin, Q., Liu, R., Xiao, W., & Liu, W. (2010). Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. *Journal of Andrology*, *31*(5), 437–444. https://doi.org/10.2164/jandrol.109.007849

Lin, H.-Y., Sun, M., Tang, H.-Y., Simone, T. M., Wu, Y.-H., Grandis, J. R., … Davis, F. B. (2008). Resveratrol causes COX-2- and p53-dependent apoptosis in head and neck squamous cell cancer cells. *Journal of Cellular Biochemistry*, *104*(6), 2131–2142. https://doi.org/10.1002/jcb.21772

Liu, M. (2011). The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology*, *9*, 149. https://doi.org/10.1186/1477-7827-9-149

Lonergan, P., & Fair, T. (2016). Maturation of Oocytes in Vitro. *Annual Review of Animal Biosciences*, *4*, 255–268. https://doi.org/10.1146/annurev-animal-022114-110822

Longo, N., Frigeni, M., & Pasquali, M. (2016). Carnitine Transport and Fatty Acid Oxidation. *Biochimica et Biophysica Acta*, *1863*(10), 2422–2435. https://doi.org/10.1016/j.bbamcr.2016.01.023

Ma, Y., Gong, X., Mo, Y., & Wu, S. (2016). Polydatin inhibits the oxidative stress-induced proliferation of vascular smooth muscle cells by activating the eNOS/SIRT1 pathway. *International Journal of Molecular Medicine*, *37*(6), 1652–1660. https://doi.org/10.3892/ijmm.2016.2554

Machaca, K. (2004). Increased sensitivity and clustering of elementary Ca2+ release events during oocyte maturation. *Developmental Biology*, *275*(1), 170–182. https://doi.org/10.1016/j.ydbio.2004.08.004

Maeda, J. (1996). In vitro development of bovine embryos in conditioned media from bovine granulosa cells and vero cells cultured in exogenous protein- and amino acid-free chemically defined human tubal fluid medium. *Biology of Reproduction*, *54*(4), 930–936. https://doi.org/10.1095/biolreprod54.4.930

Malhotra, J. D., & Kaufman, R. J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants & Redox Signaling*, *9*(12), 2277–2293. https://doi.org/10.1089/ars.2007.1782

Mandawala, A. A., Harvey, S. C., Roy, T. K., & Fowler, K. E. (2016). Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology*, *86*(7), 1637–1644. https://doi.org/10.1016/j.theriogenology.2016.07.018

Mao, L., Lou, H., Lou, Y., Wang, N., & Jin, F. (2014). Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reproductive BioMedicine Online*, *28*(3), 284–299. https://doi.org/10.1016/j.rbmo.2013.10.016

Mapletoft, R. (2013). BOVINE EMBRYO TRANSFER, 5.

Mehlmann, L. M., Saeki, Y., Tanaka, S., Brennan, T. J., Evsikov, A. V., Pendola, F. L., … Jaffe, L. A. (2004). The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science (New York, N.Y.)*, *306*(5703), 1947–1950. https://doi.org/10.1126/science.1103974

Mendes, J. O. B., Burns, P. D., De La Torre-Sanchez, J. F., & Seidel, G. E. (2003). Effect of heparin on cleavage rates and embryo production with four bovine sperm preparation protocols. *Theriogenology*, *60*(2), 331–340. https://doi.org/10.1016/S0093-691X(03)00029-3

Min, S.-H., Kim, J.-W., Lee, Y.-H., Park, S.-Y., Jeong, P.-S., Yeon, J.-Y., … Koo, D.-B. (2014). Forced Collapse of the Blastocoel Cavity Improves Developmental Potential in Cryopreserved Bovine Blastocysts by Slow-Rate Freezing and Vitrification. *Reproduction in Domestic Animals*, *49*(4), 684–692. https://doi.org/10.1111/rda.12354

Moore, S. G., & Hasler, J. F. (2017). A 100-Year Review: Reproductive technologies in dairy science. *Journal of Dairy Science*, *100*(12), 10314–10331. https://doi.org/10.3168/jds.2017-13138

Moreno, R. D., Schatten, G., & Ramalho-Santos, J. (2002). Golgi apparatus dynamics during mouse oocyte in vitro maturation: effect of the membrane trafficking inhibitor brefeldin A. *Biology of Reproduction*, *66*(5), 1259–1266.

Mori, M., Otoi, T., & Suzuki, T. (2002). Correlation between the cell number and diameter in bovine embryos produced in vitro. *Reproduction in Domestic Animals = Zuchthygiene*, *37*(3), 181–184.

Morris, G. J., & Acton, E. (2013). Controlled ice nucleation in cryopreservation--a review. *Cryobiology*, *66*(2), 85–92. https://doi.org/10.1016/j.cryobiol.2012.11.007

Nagano, M., Katagiri, S., & Takahashi, Y. (2006). ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. *Zygote*, *14*(4), 299–304. https://doi.org/10.1017/S0967199406003807

Nohalez, A., Martinez, C. A., Parrilla, I., Roca, J., Gil, M. A., Rodriguez-Martinez, H., … Cuello, C. (2018). Exogenous ascorbic acid enhances vitrification survival of porcine in vitro-

developed blastocysts but fails to improve the in vitro embryo production outcomes. *Theriogenology*, *113*, 113–119. https://doi.org/10.1016/j.theriogenology.2018.02.014

Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., … Jaffe, L. A. (2008). Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development (Cambridge, England)*, *135*(19), 3229–3238. https://doi.org/10.1242/dev.025494

Norris, R. P., Ratzan, W. J., Freudzon, M., Mehlmann, L. M., Krall, J., Movsesian, M. A., … Jaffe, L. A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development (Cambridge, England)*, *136*(11), 1869–1878. https://doi.org/10.1242/dev.035238

Odani, M., Komatsu, Y., Oka, S., & Iwahashi, H. (2003). Screening of genes that respond to cryopreservation stress using yeast DNA microarray. *Cryobiology*, *47*(2), 155–164.

O'Flaherty, C. M., Beorlegui, N. B., & Beconi, M. T. (1999). Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. *Theriogenology*, *52*(2), 289–301. https://doi.org/10.1016/S0093-691X(99)00129-6

Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2007). Mitochondrial oxidative stress: implications for cell death. *Annual Review of Pharmacology and Toxicology*, *47*, 143–183. https://doi.org/10.1146/annurev.pharmtox.47.120505.105122

Owen, C. M., Barceló-Fimbres, M., Altermatt, J. L., & Campos-Chillòn, L. F. (2017). 45 Survival of Holstein in Vitro-Produced Embryos Cultured in Novel Synthetic Oviductal Fluid Media (scf1) and Dehydrated Prior to Cryopreservation. *Reproduction, Fertility and Development*, *29*(1), 129–130. https://doi.org/10.1071/RDv29n1Ab45

Palasz, A. T., Thundathil, J., De La Fuente, J., & Mapletoft, R. J. (2000). Effect of Reduced Concentrations of Glycerol and Various Macromolecules on the Cryopreservation of Mouse and Cattle Embryos. *Cryobiology*, *41*(1), 35–42. https://doi.org/10.1006/cryo.2000.2262

Pang, Y.-W., Sun, Y.-Q., Jiang, X.-L., Huang, Z.-Q., Zhao, S.-J., Du, W.-H., … Zhu, H.-B. (2016). Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. *Molecular Reproduction and Development*, *83*(11), 993–1002. https://doi.org/10.1002/mrd.22742

Paola, M. de, Bello, O. D., & Michaut, M. A. (2015). Cortical Granule Exocytosis Is Mediated by Alpha-SNAP and N-Ethilmaleimide Sensitive Factor in Mouse Oocytes. *PLOS ONE*, *10*(8), e0135679. https://doi.org/10.1371/journal.pone.0135679

Paschoal, D., Sudano, M., Guastali, M., Maziero, R., Crocomo, L., Carlos Ona Magalhaes, L., … Landim-Alvarenga, F. (2012). Forskolin effect on the cryosurvival of in vitro-produced bovine embryos in the presence or absence of fetal calf serum. *Zygote (Cambridge, England)*, *22*, 1–12. https://doi.org/10.1017/S0967199412000354

Pasqualotto, E. B., Lara, L. V., Salvador, M., Sobreiro, B. P., Jr, E. B., & Pasqualotto, F. F. (2009). The role of enzymatic antioxidants detected in the follicular fluid and semen of infertile couples undergoing assisted reproduction. *Human Fertility*, *12*(3), 166–171. https://doi.org/10.1080/14647270903207941

Pereira, M. M., Machado, M. A., Costa, F. Q., Serapiao, R. V., Viana, J. H. M., & Camargo, L. S. A. (2010). Effect of oxygen tension and serum during IVM on developmental competence of bovine oocytes. *Reproduction, Fertility, and Development*, *22*(7), 1074–1082. https://doi.org/10.1071/RD10007

Pollock, K., Yu, G., Moller-Trane, R., Koran, M., Dosa, P. I., McKenna, D. H., & Hubel, A. (2016). Combinations of Osmolytes, Including Monosaccharides, Disaccharides, and Sugar Alcohols Act in Concert During Cryopreservation to Improve Mesenchymal Stromal Cell Survival. *Tissue Engineering. Part C, Methods*, *22*(11), 999–1008. https://doi.org/10.1089/ten.tec.2016.0284

Ravagnan, G., Filippis, A. D., Cartenì, M., Maria, S. D., Cozza, V., Petrazzuolo, M., … Donnarumma, G. (2013). Polydatin, A Natural Precursor of Resveratrol, Induces β-Defensin Production and Reduces Inflammatory Response. *Inflammation*, *36*(1), 26–34. https://doi.org/10.1007/s10753-012-9516-8

Reader, K. L., Stanton, J.-A. L., & Juengel, J. L. (2017). The Role of Oocyte Organelles in Determining Developmental Competence. *Biology*, *6*(3). https://doi.org/10.3390/biology6030035

Regev-Shoshani, G., Shoseyov, O., Bilkis, I., & Kerem, Z. (2003). Glycosylation of resveratrol protects it from enzymic oxidation. *The Biochemical Journal*, *374*(Pt 1), 157–163. https://doi.org/10.1042/BJ20030141

Reis, A., Chang, H.-Y., Levasseur, M., & Jones, K. (2006). APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division. *Nature Cell Biology*, *8*, 539–540. https://doi.org/10.1038/ncb1406

Rhodes-Long, K., Campos-Chillòn, L. F., Barceló-Fimbres, M., & Altermatt, J. L. (2016). 90 Lipid Content of in Vivo- and in Vitro-Produced Jersey and Holstein Cattle Embryos and the Effect of Forskolin on Embryo Lipid Reduction. *Reproduction, Fertility and Development*, *28*, 174. https://doi.org/10.1071/RDv28n2Ab90

Rizos, D., Ward, F., Boland, M. P., & Lonergan, P. (2001). Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology*, *56*(1), 1–16. https://doi.org/10.1016/S0093-691X(01)00538-6

Rizos, Dimitrios, Ward, F., Duffy, P., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*, *61*(2), 234–248. https://doi.org/10.1002/mrd.1153

Roberts, M., Campos-Chillòn, L. F., Barceló-Fimbres, M., & Altermatt, J. L. (2016). Effect of Media, Metabolic Regulators, and Stage of Development on Lipid Content and Mitochondrial Polarity of in Vitro-Produced Holstein Embryos. *Reproduction, Fertility and Development*, *28*(2), 174–174. https://doi.org/10.1071/RDv28n2Ab89

Rocha-Frigoni, N. A. S., Leão, B. C. da S., Nogueira, É., Accorsi, M. F., & Mingoti, G. Z. (2015). Effects of gaseous atmosphere and antioxidants on the development and cryotolerance of bovine embryos at different periods of in vitro culture. *Zygote (Cambridge, England)*, *23*(2), 159–168. https://doi.org/10.1017/S0967199413000361

Rocha-Frigoni, N. A. S., Leão, B. C. S., Nogueira, É., Accorsi, M. F., & Mingoti, G. Z. (2014). Reduced levels of intracellular reactive oxygen species and apoptotic status are not correlated with increases in cryotolerance of bovine embryos produced in vitro in the presence of antioxidants. *Reproduction, Fertility, and Development*, *26*(6), 797–805. https://doi.org/10.1071/RD12354

Rodrigues-Cunha, M. C., Mesquita, L. G., Bressan, F., Collado, M. del, Balieiro, J. C. C., Schwarz, K. R. L., … Leal, C. L. V. (2016). Effects of melatonin during IVM in defined medium on oocyte meiosis, oxidative stress, and subsequent embryo development. *Theriogenology*, *86*(7), 1685–1694. https://doi.org/10.1016/j.theriogenology.2016.05.026

Rodriguez, C., Mayo, J. C., Sainz, R. M., Antolín, I., Herrera, F., Martín, V., & Reiter, R. J. (2004). Regulation of antioxidant enzymes: a significant role for melatonin. *Journal of Pineal Research*, *36*(1), 1–9.

Romero-Pérez, A. I., Ibern-Gómez, M., Lamuela-Raventós, R. M., & de La Torre-Boronat, M. C. (1999). Piceid, the major resveratrol derivative in grape juices. *Journal of Agricultural and Food Chemistry*, *47*(4), 1533–1536.

Rudolph, J. (2007). Cdc25 phosphatases: structure, specificity, and mechanism. *Biochemistry*, *46*(12), 3595–3604. https://doi.org/10.1021/bi700026j

Said, T., & Agarwal, A. (2012). Antioxidants in Sperm Cryopreservation. In S. J. Parekattil & A. Agarwal (Eds.), *Male Infertility* (pp. 431–437). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4614-3335-4_41

Salilew-Wondim, D., Fournier, E., Hoelker, M., Saeed-Zidane, M., Tholen, E., Looft, C., … Tesfaye, D. (2015). Genome-Wide DNA Methylation Patterns of Bovine Blastocysts Developed In Vivo from Embryos Completed Different Stages of Development In Vitro. *PLoS ONE*, *10*(11). https://doi.org/10.1371/journal.pone.0140467

Salzano, A., Albero, G., Zullo, G., Neglia, G., Abdel-Wahab, A., Bifulco, G., … Gasparrini, B. (2014). Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos. *Animal Reproduction Science*, *151*(3–4), 91–96. https://doi.org/10.1016/j.anireprosci.2014.09.018

Sanches, B. V., Lunardelli, P. A., Tannura, J. H., Cardoso, B. L., Colombo Pereira, M. H., Gaitkoski, D., … Seneda, M. M. (2016). A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, *85*(6), 1147–1151. https://doi.org/10.1016/j.theriogenology.2015.11.029

Sanocka, D., & Kurpisz, M. (2004). Reactive oxygen species and sperm cells. *Reproductive Biology and Endocrinology : RB&E*, *2*, 12. https://doi.org/10.1186/1477-7827-2-12

Santis, L. D., & Coticchio, G. (2011). Theoretical and experimental basis of slow freezing. *Reproductive BioMedicine Online*, *22*(2), 125–132. https://doi.org/10.1016/j.rbmo.2010.10.012

Saragusty, J., & Arav, A. (2011). Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction (Cambridge, England)*, *141*(1), 1–19. https://doi.org/10.1530/REP-10-0236

Sarıözkan, S., Bucak, M. N., Canturk, F., Özdamar, S., Yay, A., Tuncer, P. B., … Caner, Y. (2012). The effects of different sugars on motility, morphology and DNA damage during the liquid storage of rat epididymal sperm at 4°C. *Cryobiology*, *65*(2), 93–97. https://doi.org/10.1016/j.cryobiol.2012.05.007

Schultz, R. M., Montgomery, R. R., & Belanoff, J. R. (1983). Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Developmental Biology*, *97*(2), 264–273. https://doi.org/10.1016/0012-1606(83)90085-4

Seidel, G. E. (2006). Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology*, *65*(1), 228–235. https://doi.org/10.1016/j.theriogenology.2005.09.025

Seki, S., & Mazur, P. (2012). Ultra-Rapid Warming Yields High Survival of Mouse Oocytes Cooled to −196°C in Dilutions of a Standard Vitrification Solution. *PLOS ONE*, *7*(4), e36058. https://doi.org/10.1371/journal.pone.0036058

Shirazi, A., Nazari, H., Ahmadi, E., Heidari, B., & Shams-Esfandabadi, N. (2009). Effect of culture system on survival rate of vitrified bovine embryos produced in vitro. *Cryobiology*, *59*(3), 285–290. https://doi.org/10.1016/j.cryobiol.2009.08.003

Shu, Y., Watt, J., Gebhardt, J., Dasig, J., Appling, J., & Behr, B. (2009). The value of fast blastocoele re-expansion in the selection of a viable thawed blastocyst for transfer. *Fertility and Sterility*, *91*(2), 401–406. https://doi.org/10.1016/j.fertnstert.2007.11.083

Sovernigo, T. C., Adona, P. R., Monzani, P. S., Guemra, S., Barros, F. D. A., Lopes, F. G., & Leal, C. L. V. (2017). Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reproduction in Domestic Animals*, *52*(4), 561–569. https://doi.org/10.1111/rda.12946

Spencer, T. E., & Hansen, T. R. (2015). Implantation and Establishment of Pregnancy in Ruminants. In *Regulation of Implantation and Establishment of Pregnancy in Mammals* (pp. 105–135). Springer, Cham. https://doi.org/10.1007/978-3-319-15856-3_7

Starkov, A. A. (2008). The Role of Mitochondria in Reactive Oxygen Species Metabolism and Signaling. *Annals of the New York Academy of Sciences*, *1147*, 37–52. https://doi.org/10.1196/annals.1427.015

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P. B., & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biology of Reproduction*, *64*(3), 904–909.

Su, D., Cheng, Y., Liu, M., Liu, D., Cui, H., Zhang, B., … Mei, Q. (2013). Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0054505

Sudano, M. J., Paschoal, D. M., Rascado, T. da S., Magalhães, L. C. O., Crocomo, L. F., Sudano, M. J., & Landim-Alvarenga, F. da C. (2011). Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology*, *75*(7), 1211–1220. https://doi.org/10.1016/j.theriogenology.2010.11.033

Sudano, M. J., Rascado, T. D. S., Tata, A., Belaz, K. R. A., Santos, V. G., Valente, R. S., … Landim-Alvarenga, F. D. C. (2016). Lipidome signatures in early bovine embryo

development. *Theriogenology*, *86*(2), 472-484.e1. https://doi.org/10.1016/j.theriogenology.2016.03.025

Takahashi, M. (2012). Oxidative stress and redox regulation on in vitro development of mammalian embryos. *The Journal of Reproduction and Development*, *58*(1), 1–9.

Takahashi, T., Inaba, Y., Somfai, T., Kaneda, M., Geshi, M., Nagai, T., & Manabe, N. (2013). Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. *Reproduction, Fertility, and Development*, *25*(4), 589–599. https://doi.org/10.1071/RD11262

Takeo, S., Sato, D., Kimura, K., Monji, Y., Kuwayama, T., Kawahara-Miki, R., & Iwata, H. (2014). Resveratrol Improves the Mitochondrial Function and Fertilization Outcome of Bovine Oocytes. *The Journal of Reproduction and Development*, *60*(2), 92–99. https://doi.org/10.1262/jrd.2013-102

Tarín, J. J., & Trounson, A. O. (1993). Effects of stimulation or inhibition of lipid peroxidation on freezing-thawing of mouse embryos. *Biology of Reproduction*, *49*(6), 1362–1368.

Tatone, C., Emidio, G. D., Vento, M., Ciriminna, R., & Artini, P. G. (2010). Cryopreservation and oxidative stress in reproductive cells. *Gynecological Endocrinology*, *26*(8), 563–567. https://doi.org/10.3109/09513591003686395

Tedder, R. S., Zuckerman, M. A., Goldstone, A. H., Hawkins, A. E., Fielding, A., Briggs, E. M., … Patterson, K. G. (1995). Hepatitis B transmission from contaminated cryopreservation tank. *Lancet (London, England)*, *346*(8968), 137–140.

Tervit, H. R., Whittingham, D. G., & Rowson, L. E. A. (1972). Successful Culture in Vitro of Sheep and Cattle Ova. *Journal of Reproduction and Fertility*, *30*(3), 493–497. https://doi.org/10.1530/jrf.0.0300493

Tian, X., Wang, F., He, C., Zhang, L., Tan, D., Reiter, R. J., … Liu, G. (2014). Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. *Journal of Pineal Research*, *57*(3), 239–247. https://doi.org/10.1111/jpi.12163

Tominaga, K., Iwaki, F., & Hochi, S. (2007). Conventional freezing of in vitro-produced and biopsied bovine blastocysts in the presence of a low concentration of glycerol and sucrose. *The Journal of Reproduction and Development*, *53*(2), 443–447.

Tu, B. P., & Weissman, J. S. (2002). The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Molecular Cell*, *10*(5), 983–994.

Tu, B. P., & Weissman, J. S. (2004). Oxidative protein folding in eukaryotes: mechanisms and consequences. *The Journal of Cell Biology*, *164*(3), 341–346. https://doi.org/10.1083/jcb.200311055

Tuncer, P. B., Sarıözkan, S., Bucak, M. N., Ulutaş, P. A., Akalın, P. P., Büyükleblebici, S., & Canturk, F. (2011). Effect of glutamine and sugars after bull spermatozoa cryopreservation. *Theriogenology*, *75*(8), 1459–1465. https://doi.org/10.1016/j.theriogenology.2010.12.006

Uechi, H., Tsutsumi, O., Morita, Y., & Taketani, Y. (1997). Cryopreservation of mouse embryos affects later embryonic development possibly through reduced expression of the glucose transporter GLUT1. *Molecular Reproduction and Development*, *48*(4), 496–500. https://doi.org/10.1002/(SICI)1098-2795(199712)48:4<496::AID-MRD10>3.0.CO;2-S

Vajta, G., Rienzi, L., Cobo, A., & Yovich, J. (2010). Embryo culture: can we perform better than nature? *Reproductive BioMedicine Online*, *20*(4), 453–469. https://doi.org/10.1016/j.rbmo.2009.12.018

Velez-Pardo, C., Morales, A. T., Del, M. R., & Olivera-Angel, M. (2007). Endogenously generated hydrogen peroxide induces apoptosis via mitochondrial damage independent of NF-kappaB and p53 activation in bovine embryos. *Theriogenology*, *67*(7), 1285–1296. https://doi.org/10.1016/j.theriogenology.2007.01.018

Voelkel, S. A., & Hu, Y. X. (1992). Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen-thawed embryos to recipient females. *Theriogenology*, *37*(3), 687–697.

Wakefield, S. L., Lane, M., & Mitchell, M. (2011). Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biology of Reproduction*, *84*(3), 572–580. https://doi.org/10.1095/biolreprod.110.087262

Wang, F., Tian, X., Zhang, L., He, C., Ji, P., Li, Y., … Liu, G. (2014). Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertility and Sterility*, *101*(2), 577–586. https://doi.org/10.1016/j.fertnstert.2013.10.041

Wang, F., Tian, X., Zhou, Y., Tan, D., Zhu, S., Dai, Y., & Liu, G. (2014). Melatonin Improves the Quality of In Vitro Produced (IVP) Bovine Embryos: Implications for Blastocyst Development, Cryotolerance, and Modifications of Relevant Gene Expression. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093641

Wang, W., Hosoe, M., Li, R., & Shioya, Y. (1997). Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. *Development, Growth & Differentiation*, *39*(5), 607–615. https://doi.org/10.1046/j.1440-169X.1997.t01-4-00008.x

Watson, A. J., & Barcroft, L. C. (2001). Regulation of blastocyst formation. *Frontiers in Bioscience: A Journal and Virtual Library*, *6*, D708-730.

Wrenzycki, C., & Stinshoff, H. (2013). Maturation environment and impact on subsequent developmental competence of bovine oocytes. *Reproduction in Domestic Animals = Zuchthygiene*, *48 Suppl 1*, 38–43. https://doi.org/10.1111/rda.12204

Yang, W. S., & Stockwell, B. R. (2016). Ferroptosis: Death by Lipid Peroxidation. *Trends in Cell Biology*, *26*(3), 165–176. https://doi.org/10.1016/j.tcb.2015.10.014

Yavin, S., & Arav, A. (2007). Measurement of essential physical properties of vitrification solutions. *Theriogenology*, *67*(1), 81–89. https://doi.org/10.1016/j.theriogenology.2006.09.029

Zhang, K., & Kaufman, R. J. (2008). From endoplasmic-reticulum stress to the inflammatory response. *Nature*, *454*(7203), 455–462. https://doi.org/10.1038/nature07203

Zhang, X., Wu, X. Q., Lu, S., Guo, Y. L., & Ma, X. (2006). Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles. *Cell Research*, *16*(10), 841–850. https://doi.org/10.1038/sj.cr.7310095