DISSERTATION

INVESTIGATION OF POTENTIAL ADVANCEMENTS IN BOVINE ASSISTED REPRODUCTIVE TECHNOLOGIES

Submitted by

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ABSTRACT

INVESTIGATION OF POTENTIAL ADVANCEMENTS IN BOVINE ASSISTED REPRODUCTIVE TECHNOLOGIES

The assisted reproductive technologies (ART) area of research within bovine encompasses a diverse group of technologies including artificial insemination, cryopreservation of gametes and embryos, in vitro fertilization, induction of multiple ovulations, sex determination of sperm and embryos, and more. These techniques have been studied thoroughly within the last forty years, yet there is a plethora of knowledge to still be discovered on a molecular basis extending to longitudinal effects of ART interference generationally. One large sector of ART research in the bovine industry is to gain information that will aide in the advancement of current protocols and practices to increase productivity and cost effectiveness. This includes improving cryopreservation of gametes, creating higher in vitro embryo production yield, reducing costs, and developing easier practices for technicians with hopes of providing a more efficient system, from both a labor and fiscal standpoint. The following dissertation contributes specifically to this research sector by providing novel information regarding cryopreservation and capacitation timing of bovine spermatozoa treated with cholesterol prior to cryopreservation, embryo production rates when such spermatozoa are used within an in vitro fertilization (IVF) system, and the use and metabolomic components of spent media from bovine embryo culture.

The first chapter of this dissertation will provide background information on the complex

sperm capacitation and acrosome reaction process, and methods on how to assess and modify the capacitation process via molecular processes, both pre- and post-cryopreservation. This lays the foundation for the research presented here, which focuses on the loading of bovine spermatozoa with cholesterol prior to cryopreservation, and what effect this treatment has on the cells post-thaw in relation to capacitation timing analyzed using flow cytometry within the second chapter. The first chapter also provides background to the fertilization process and in vitro production of embryos which is examined by using cholesterol-loaded spermatozoa for IVF presented in the third chapter. Additionally, contextual information is explained regarding the type of culture systems within IVF, where embryos are individually cultured or in groups. This information is required for the fourth chapter which includes experimentation with spent culture media and the study of the metabolomic components of media exposed to group embryo culture.

Pre-loading bovine sperm with cholesterol prior to freezing is known to increase cryosurvival, though the timing of capacitation in these sperm has not yet been evaluated. The objective of the study performed in chapter two was to determine if there is a potential delay in capacitation timing in these sperm due to the increased cholesterol content. Flow cytometric evaluation was utilized to assess viability, and stain technology to assess acrosome intactness (Propidium Iodide/FITC-PNA), intracellular calcium levels (Propidium Iodide/FLUO 3-AM) and membrane fluidity (Merocyanine 540/YO-PRO-1). Through this investigation it was determined that treatment of sperm with cholesterol-loaded cyclodextrin (CLC) (2 mg/mL) increased cryosurvival and viability over time, though longer time to capacitate was required compared with non-treated sperm. Treatment with CLC also significantly decreased membrane fluidity in sperm ($P<.05$). by 1 hour, resulting in increased intracellular calcium and increased acrosome reaction, and consequently viability loss by 3 hours. Taken together, these results

determined sperm pre-loaded with cholesterol display significantly improved post-thaw viability but require longer to capacitate which may hinder fertilization capacity and/or require adjustments to timing of in vitro fertilization.but require longer to capacitate which may hinder fertilization capacity and/or require adjustments to timing of in vitro fertilization.

Given the delayed capacitation timing of sperm treated with CLC, it was then investigated if CLC-treated sperm could be successful in IVF to determine if the additional retained cholesterol hindered fertilization ability and consequently, embryo production. The research summarized in chapter three examines the fertilization ability of CLC-treated sperm and subsequent in vitro embryo development by offsetting sperm preparation timing in order to adjust for delayed capacitation due to the increased cholesterol content of CLC-treated sperm. Additionally, treatment with methyl-β-cyclodextrin (MβCD) was utilized as a capacitation inducing agent by causing an efflux of cholesterol from the sperm plasma membranes during the sperm preparation incubation period. Bovine sperm pre-loaded with cholesterol via CLC prior to cryopreservation did not result in significant difference in cleavage or blastocyst formation ($P>0.05$) when sperm preparations were completed 90 minutes prior to co-incubation to adjust for delayed capacitation timing. There was significant decline ($P<0.05$) in embryo cleavage percentage in both control and CLC-treated sperm groups when MβCD was used as a capacitation inducer at 2 mg/ml. The findings presented in chapter three demonstrate that bovine sperm treated with CLC resulted in a nonsignificant difference in embryo production when used in IVF, even when delayed capacitation timing is adjusted for in sperm preparation. This concludes that the fertilization ability of the spermatozoa treated with CLC was not impaired, and that the most important application of CLC-treated sperm is not within in vitro fertilization examination, but rather, within in vivo models utilizing AI synchronization timing adjusted for

delayed capacitation. Here the benefit of improved cryosurvival rates can be best utilized, potentially resulting in the ultimate goal of increased fertility. One of the most widely pursued research avenues to improve embryo development is advancements in culture media and conditions. Many attempts have been made to optimize in vitro production of embryos by component supplementation and recipe adjustment. An additional culture condition with a dramatic impact on bovine preimplantation embryogenesis is whether embryos are cultured individually or in groups, where increased embryo production is observed in group culture conditions. These items together, have created an active area of research which analyzes culture media after exposure to embryo culture to examine the artifacts secreted by embryos including various amino acids, proteins, and other metabolites that are considered to be embryotrophic, or positive signals from companion embryos. Chapter four experiments include culturing embryos both individually and in groups with spent culture media from a group culture donor cycle with embryo production analyzed via cleavage and blastocyst formation with time-lapse imaging. Similar cleavage percentages between control and spent media were observed, however, an increase in blastocyst formation was observed in grouped embryos compared with single embryos. No difference ($P>.05$) was observed in the timing of developmental events in embryos cultured in control or spent media within individual culture. Analysis of the metabolomic profile of spent culture media with gas chromatography-mass spectrometry (GC-MS) was also completed, suggesting potential embryotrophic factors in the spent media. These findings conclude that bovine embryos progress in a more timely and developmentally differentiated degree when cultured within groups, and artifacts left within the spent culture media were not sufficient to result in significant increase in embryo production within spent media culture.

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DEDICATION

I dedicate this work to my best friend and biggest fan, thank you for your never-ending love and support. I couldn't have done it without you. I love you, man.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Prior to a spermatozoon being able to penetrate and fertilize an oocyte, it must undergo capacitation and the acrosome reaction, which requires a set of timed biological and physiological events. These changes begin in vivo when spermatozoa come in contact with seminal plasma at the time of ejaculation within the female reproductive tract. Alternatively, in vitro capacitation changes occur when sperm come in contact with specific media. Due to the complex nature of capacitation, in vitro fertilization protocols must accommodate these processes with appropriate timing of sperm preparation. Such protocols, when implemented correctly, can garner valuable research data allowing IVF to evolve from an experimental procedure to treat infertile animals towards a genomic accelerator for many breeds (Sirard, 2018). These may improve embryo production, and potentially pregnancy rates, which is a main driver behind research in domestic animals due to economic benefits.

The first birth of a live calf resulting from in vitro fertilization (IVF), 'Virgil', was a milestone in the use of assisted reproductive technologies (ART; Brackett et al., 1982), and since the use of ART has substantially increased in the livestock industry (Viana, 2019). Today, bovine in vitro produced (IVP) embryos are used around the world, with an increase in the number of IVP embryos generated (12% over 2019) and an increase in the proportion of those IVP embryos transferred (5% over 2019), suggesting improvement in in vitro embryo production systems (Viana, 2019). This technology plays a central role in bovine production systems by aiding in crossbreeding schemes, genetic selection strategies, and can be integrated to improve pregnancy rates in herds with low fertility and economical use of valuable semen with limited

availability. However, limitations of the technology decreasing the desirability of IVP embryos include increased cost, lack of access to proper facilities and/or technician proficiency, sub-optimal embryonic and fetal development, and potential for abnormal offspring (Hansen, 2006; Hasler, 2014). Most of these technical problems can be overcome through research, which is why it is critical to continually update and modify protocols, increase education and access to advancing technology, and while difficult, perform follow-up monitoring of pregnancy rates and fetal development. In this review, capacitation and in vitro production of embryos will be discussed within the scope of bovine reproduction, to further understand how sperm capacitation timing and modifications to IVF can improve embryo production, pregnancy, and ultimately calving rates.

1.2 Capacitation

1.2.1 *Capacitation Overview*

Capacitation is defined as the biological and physiological changes sperm must undergo to acquire the ability to successfully penetrate and fertilize an oocyte (Chang, 1957; Farooqui, 1983; Parrish et al., 1999). The term 'capacitation' was proposed by Austin in 1952, though the phenomenon of mammalian spermatozoa acquiring their fertilization capacity after a period of time in the female reproductive tract was first described in 1951 in rabbits (Chang, 1950) and rats (Austin, 1951). This concept of capacitation which occurs in vivo can be mimicked in vitro by the use of media containing appropriate pH and compounds (Chang, 1951; Visconti, 2009). Changes begin as soon as sperm is ejaculated and continue for hours in the female tract in vivo or in capacitation medium in vitro. These changes are a series of sequential and parallel processes.

Processes of capacitation include the acrosome reaction, hyperactivation of sperm, and phosphorylation of tyrosine. Only capacitated sperm have the ability to undergo the acrosome reaction and subsequently fertilize an oocyte. Preparation for the acrosome reaction occurs at the sperm head whilst preparation for hyperactivity occurs at the sperm tail (Jin & Yang, 2017). Salicioni et al., (2007) describes capacitation as the culmination of fast and slow events. Fast events include the initiation of sperm motility, which occurs as soon as the sperm are released from the epididymis. Slow events are defined as changes in the motility patterns (e.g. hyperactivation) and the stimulation of a physiological agonist, and the phosphorylation of tyrosine providing sperm the capacity to undergo the acrosome reaction (Salicioni 2007; Visconti 2009).

Sperm capacitation was first noted over 50 years ago, however the molecular basis of the process is still not well understood, as it is regulated by a variety of factors. Briefly, capacitation involves the removal of decapacitation factors, cholesterol loss, activation of cyclic adenosine monophosphate (cAMP) and the protein kinase A (PKA) system, protein tyrosine phosphorylation, and increased intracellular pH, bicarbonate, calcium, and potassium levels (Bailey, 2010; Jin & Yang, 2017). Capacitation is regulated by the activation of many intracellular signaling pathways, involving cAMP, PKA, receptor tyrosine kinases, and non-receptor tyrosine kinases (Naz & Rajesh, 2004). Many molecules have been identified that regulate these pathways, such as calcium, bicarbonate, reactive oxygen species (ROS), γ-aminobutyric acid (GABA), progesterone, estrogen, angiotensin, and cytokines (Jin & Yang, 2017; Naz & Rajesh, 2004).

First, the removal of cholesterol by a cholesterol acceptor from sperm plasma membranes occurs, usually in the form of fatty acid-free (FAF) albumin within medium in vitro. High-

density lipoproteins are present within the oviduct and other fluids in the genital tract in vivo increasing the membrane permeability (Leahy & Gadella, 2015). Then, a calcium (Ca^{2+}) influx occurs through the stimulation of bicarbonate (HCO^{3-}) (Okamura et al., 1985; Salicioni et al., 2007) and membrane channels (Ren & Xia, 2010). This influx activates secondary messenger systems including soluble adenylate cyclase (sAC) to produce cAMP. The increased transmembrane movement of HCO^{3-} into the cell is associated with the increase in intracellular pH during capacitation. (Breitbart, 2002; Hess et al., 2005; Visconti et al., 1995). The secondary messengers that activate sAC also activate the PKA signaling pathway. The PKA system, through the inhibition of protein phosphatases and/or the activation of tyrosine kinases, then allows an increase in protein phosphorylation and stimulation of acrosome exocytosis (Jin & Yang, 2017). This increase in cAMP leads to activation of the PKA signaling system regulating protein tyrosine phosphorylation, which consequently regulates several sperm processes including hyper activated motility and the acrosome reaction within capacitation and zona pellucida (ZP) binding (Grasa et al., 2009; Jin & Yang, 2017).

1.2.2 *Protein Tyrosine Phosphorylation*

Because the terminally differentiated spermatozoa are transcriptionally and translationally inactive, highly compartmentalized, and unable to synthesize new proteins, it can be argued that the reliance on post-translational modification (e.g. protein phosphorylation) is greater than many other cell types as a means of altering their function (Naresh & Atreja 2015; Urner & Sakkas, 2003). Protein phosphorylation regulates capacitation, hyperactivated motility, and the acrosome reaction, which all must take place for spermatozoa to reach and fuse with the oocyte (Naresh & Atreja, 2015; Sati et al., 2014; Urner & Sakkas, 2003).

Increased tyrosine phosphorylation of sperm membrane proteins has shown to be required for induction of mammalian sperm capacitation (Arcelay et al., 2008; Ioki et al., 2016; Visconti et al., 1995-1), and this change has been demonstrated in several species (human; Bailey, 2010; rat; Lewis & Aitken, 2001; mouse; Visconti et al., 1995-2). The major activator of signal transduction pathways appears to be through phosphorylation of tyrosine residues, however, phosphorylation can also occur at serine and threonine residues (Naz & Rajesh, 2004). If tyrosine residues are inhibited, capacitation, acrosome reaction, and fertilization are blocked (Naresh & Atreja, 2015). In addition, serum albumin, HCO^{3-}, and Ca^{2+} are required for tyrosine phosphorylation (Muratori et al., 2011), and without any of these factors tyrosine phosphorylation and capacitation will not occur (Visconti, 1995a).

Proteomic and immunocytochemistry studies in many species have shown that sequences of protein tyrosine phosphorylation differ between locations of spermatozoa when assessed during capacitation and upon binding to the zona pellucida (Roy & Atreja, 2008; Urner et al., 2001). Immunofluorescence studies of human sperm have shown the flagellum to be the primary structure containing localization of tyrosine phosphorylated proteins, particularly on cells involved in zona pellucida binding, oolemma binding, and gamete fusion (Sati et al., 2014). In many species the flagellum is the primary component that undergoes tyrosine phosphorylation, including monkey, hamster, rat, and mouse, however this is not true in boar (Naz and Rajesh 2004). Additionally, the cAMP/PKA-dependent signaling cascade that induces tyrosine phosphorylation may be responsible for hyperactivation of sperm in response to extracellular Ca^{2+} (Harayama et al., 2012; Tateno et al., 2013), specifically within the flagellum (Mariappa et al., 2010; Urner & Sakkas, 2003), which in turn is needed for zona pellucida penetration by spermatozoa. Studies by Sati et al., (2014) suggest that specific tyrosine phosphorylation

residues are present during capacitation and various regions of human sperm when binding to the zona pellucida. Additionally, there is a common regulatory pathway of tyrosine phosphorylation related to sperms' ability to fertilize (Sati et al., 2014). Once capacitated, there is a redistribution of proteins which undergo tyrosine phosphorylation at the neck and acrosome regions of the sperm upon binding to zona pellucida (Naresh & Atreja, 2015; Sati et al., 2014). It has been reported that conformational changes are facilitated by tyrosine phosphorylation of chaperone proteins on the sperm head surface which results in zona recognition (Asquith et al., 2004; Ecroyd et al., 2003; Mitchell et al., 2007; Naresh & Atreja, 2015).

1.2.3 *Hyperactivated Motility*

The first of two characteristics that distinguish capacitated sperm is hyperactivated motility. In most species hyperactivity occurs spontaneously and time-dependently with capacitation, therefore the presence of this characteristic will indicate that capacitation has occurred (Jin & Yang, 2017). Hyperactivity is defined as when regular wave-like beats are replaced with stronger, wider amplitude beats (also known as whiplash beats) of the tail that push the sperm forward in vigorous lurches, which is visualized as a 'star-spin' pattern (Kay & Robertson, 1998). This change in motility pattern was first reported by Yanagimachi in 1969 in epididymal sperm from golden hamsters and has since been studied in many species. Hyperactivation increases swimming efficiency in viscous mucus, assists sperm to detach from the oviductal epithelium within the female tract, and is required for the penetration of the zona pellucida (Loux et al., 2013; Qi et al., 2007; Suarez, 2008).

Hyperactivation appears to involve several precursors including increasing intracellular levels of Ca^{2+}, cAMP, ATP, pH and the activation of the CatSper ion channel. It has been demonstrated that two key factors in the regulation of mammalian sperm motility are Ca^{2+} and

cAMP (Yanagimachi, 1994). cAMP has been suggested to activate motility by enhancing beat frequency, though it does not induce the deep flagellar bending characteristic of hyperactivation (Suarez, 2008). Calcium is the predominate secondary messenger that initiates hyperactivation (Suarez, 2008; Sun et al., 2017) derived from multiple locations within spermatozoa.

In sperm, there are two main sources for calcium ions: (1) a portion that are stored in the calcium pump located in the head of sperm, where a redundant nuclear envelope exists in the same position as the IP3 receptor in the neck region, as well as in (2) the mitochondria of the midpiece (Costello et al., 2009; Quill et al., 2001). While there are voltage-gated Ca^{2+} channels located in the testis that function in other locations (such as the brain and heart), the CatSper ion channel is exclusively expressed in spermatozoa (Sun et al., 2017).

1.2.4 *Acrosome Reaction*

The second characteristic of capacitated sperm is the ability to undergo the acrosome reaction, which is an exocytotic event that involves fusion of a secretory vesicle (the acrosome) with the sperm plasma membrane that is essential for fertilization (Bedford, 2011; Graham, 1996). While it is believed the molecular machinery and mechanisms involved in the acrosome reaction are similar to that of other secretory vesicle reactions, the capacity and morphology of spermatozoa becomes altered drastically unlike other cells.

The first steps of the acrosome reaction are a series of membrane fusion events. Mammalian spermatozoa have some of the same molecules involved in membrane fusion as other exocytotic cells, such as Rab3A, SNAREs, synatotagmins, -SNAP, and NSF (Hirohashi, 2016). These point fusions between the outer acrosomal membrane and the overlying cell membrane release the necessary acrosomal enzymes for the sperm to digest through the outer oocyte cumulus complex (Graham, 1996, Yanagimachi, 1994). These point fusions facilitate the

loss of the acrosome from the anterior head and the release of soluble components from the acrosomal matrix (Kim et al., 2001) that play a role in dispersal of the cumulus matrix. A possible lytic agent of the acrosomal matrix in early studies was believed to be hyaluronidase in the acrosome, however more recent studies have identified glycosylphosphatidylinositol (GPI)-anchored hyaluronidases, SPAM/PH-20 and Hyal5, localized on the surface of the acrosome-intact spermatozoa, the sperm plasma membrane, and the outer acrosome membrane respectively in mice (Kimura, 2009). The exposure of the inner acrosomal membrane and plasma membrane overlying the equatorial segment of the sperm head prepares spermatozoa for fusion with the oolemma by exposing oolemma-binding proteins, and aides in the adhesion and penetration of the ZP (Cuasnicu et al.,2016; Graham, 1996; Okabe, 2016). Once the appropriate membranes are exposed, crucial interactions between sperm and oolemma binding proteins are enabled, primarily by the IZUMO1 protein.

IZUMO1 is an integral membrane protein with an extracellular immunoglobin-like domain and a short cytoplasmic tail that is localized on the acrosome cap area of both the outer and inner acrosomal membrane (Inoue et al., 2005). During the acrosome reaction, IZUMO1 migrates out from the outer (but not inner) acrosomal membrane to the equatorial segment of sperm plasma membrane (Sosnik et al., 2010). This movement of IZUMO1 to the equatorial segment was captured on video in 2012 by Satouh et al., demonstrating that one function of the acrosome reaction is to supply protein(s) from the outer acrosomal membrane to the plasma membrane.

IZUMO1 disbursement is also involved in sperm-egg fusion. Prior to sperm-egg fusion IZUMO1 is mainly localized to the equatorial segment, but it disperses in the egg plasma membrane rapidly when the sperm-egg fusion takes place (Satouh, 2012). The IZUMO1 on the

inner acrosomal membrane does not disperse even after fusion, but is incorporated into the cytoplasm of the egg as an inner acrosomal membrane structure complex. It was also recently determined that JUNO, a GPI-anchored protein expressed on the oocyte surface essential for female fertility, was found to interact with IZUMO1 to facilitate sperm-egg fusion (Bianchi et al., 2014). In the study it was determined that if female mice were lacking JUNO they were infertile, and JUNO-deficient eggs did not fuse with normal sperm (Bianchi et al., 2014). Disruption of IZUMO1 did not affect the ability of the sperm to penetrate the ZP, but fusing ability with the oocyte was completely impaired when JUNO was absent (Inoue et al., 2005). These findings indicate that both the presence of IZUMO1 and JUNO are essential for adequate sperm-egg fusion.

1.3 Assessment and Modifications of Capacitation

1.3.1 *Assessment of Capacitation and the Acrosome Reaction*

Though the rise in intracellular calcium does not encompass all of capacitation, it can be used to indicate whether capacitation in a spermatozoon has been initiated (Handrow et al., 1989; Nolan et al., 1992; Parrish et al., 1999). An increase in intracellular calcium occurs prior to the acrosome reaction, with the acrosome reaction being prevented in media that is void of calcium, and measured by a concentration dependent increase in calcium levels. In bull sperm, the acrosome reaction can be confirmed by utilizing the fluorescent probe Fluo-3 AM and flow cytometry (Nolan et al., 1992). Fluo-3 AM is a membrane permeant probe passively loaded into cells via incubation, which becomes fluorescent in the presence of free Ca^{2+}. However, these authors cautioned the use of this probe because in this application it provides an estimate of calcium in the entire cell rather than specific regions, therefore this methodology may be measuring calcium levels from other cellular components and not specifically the acrosome

(Nolan et al., 1992). Calcium levels have also been evaluated in bull sperm using the fluorescent stain Indo-1/AM and flow cytometry, which is also cell-permeant dye, though this measures intracellular ionized calcium concentration (Bailey & Buhr, 1993; Collin et al., 2000).

While there are many staining methods available to assess sperm capacitation and the acrosome reaction, flow cytometric analysis of lectins conjugated with fluorescent molecules are commonly used due to the short incubation time required, objectivity, and ability to evaluate a large number of cells quickly. A variety of lectins are available for use, with peanut lectin from the peanut plant, *Arachis hyogaea* (FITC-PNA), being the most commonly used as it is very specific for terminal beta-galactose (Molec and Probes). This fluorescent dye binds specifically to the outer acrosomal membrane in spermatozoa. Therefore, these lectins only bind to spermatozoa that have undergone the acrosome reaction. Acrosome-reacted spermatozoa staining positive for FITC-PNA can be visualized with a microscope. Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain, commonly used to detect dead cells in a population since it is not permeant to live cells. When counterstained with PI, data is able to be collected as the percentage of live plasma membrane intact (PI negative) sperm that are acrosome intact (FITC-PNA negative), or acrosome reacted (FITC-PNA positive) (Flesch et al., 1998). Cell populations that are PI positive are non-viable.

Another component that can be used to assess capacitation in spermatozoa is protein tyrosine phosphorylation (PTP). Because changes in PTP have a clear association with capacitation, this has also been studied via flow cytometry to determine if sperm have completed capacitation. Specifically in post-thaw bovine spermatozoa, changes have been observed in protein tyrosine phosphorylation of the sperm flagellar proteins (Bailey et al., 2000) with an increase in these levels relating to their improved tolerance to cryopreservation and fertility

(Harayama et al., 2010). Kumaresan et al., (2012) developed a protocol for staining with the use of fluorescein isothiocynante (FITC)- conjugated monoclonal anti-phosphotyrosine (PTYR) antibody produced in mouse containing antibodies that specifically recognize phosphorylated tyrosine residues, and counterstained PI to indicate viability. This combination allows cytometric monitoring of the percentage of capacitated and plasma membrane intact sperm. These authors noted a linear increase in tyrosine phosphorylation throughout the cryopreservation process between fresh and frozen-thawed semen in boar, with the conclusion that higher levels of tyrosine phosphorylation results in better cryopreservation results (Kumaresan et al., 2012).

An alternative approach to measuring capacitation is the application of flow cytometry using the lipophilic dye, merocyanine-540 (M-540). This dye is usually excluded from the plasma membrane, but has the ability to enter between phospholipids of the exoplasmic leaflet when sperm plasma membrane phospholipid disorder occurs in bicarbonate ion-induced capacitation (Guthrie & Welch, 2005). This phospholipid disorder is also referred to as membrane scrambling. During capacitation, removal of cholesterol from the sperm plasma membrane by albumin occurs, which increases membrane permeability and disorder allowing for Ca^{2+} influx and the capacitation cascade to occur (Jin & Yang, 2017). Increased M-540 fluorescence indicates greater phospholipid disorder (increased membrane fluidity), and therefore can be indicative of capacitation. When counterstained with Yo-Pro-1, a nuclear marker that binds to the DNA of dying cells, populations of viable spermatozoa (low Yo-Pro-1 fluorescence) and low M-540 fluorescence are considered to be non-capacitated, and those with high M-540 fluorescence are considered to be capacitated and viable, or in the process of capacitation (Gadella & Harrison, 2000; Guthrie & Welch, 2005).

1.3.2 *Induction of Capacitation and the Acrosome Reaction*

Multiple chemical compounds have been used to induce capacitation in sperm. One example is Ionomycin, an antibiotic and calcium ionophore used as an artificial activating substance for both oocytes and sperm (Ebner et al., 2015). Ionomycin induces the acrosome reaction by elevating the intracellular Ca^{2+} level, which is essential for capacitation and acrosome reaction. Another Ca^{2+} ionophore commonly used to induce acrosome reaction is A23187 or Calcimycin. It has been shown that mouse spermatozoa treated with A23187 can fertilize oocytes without activation of the cAMP/PKA pathway. This suggests that the cAMP/PKA pathway is upstream of the intracellular Ca^{2+} increase required for acrosome and hyperactivation under normal in vitro conditions (Tateno et al., 2013).

Another compound that has been used to induce capacitation in vitro is dilauroylphosphatidylcholine, or PC-12. Dilauroylphosphatidylcholine (PC-12) liposomes rapidly induce an acrosome reaction in bull sperm (Graham et al., 1986). PC-12 liposomes "spontaneously transfer short-chain phospholipids from vesicles to cells" (Nolan et al., 1992) which causes lipid scrambling in the plasma membrane and creates ion pores in the plasma membrane lipid bilayer increasing permeability to ions (Nolan et al., 1992). This change in membrane physiology allows for fusion between the plasma membrane and the outer leaflet of the acrosome, likely due to PC-12 accumulation in the outer membrane leaflet, which causes a shift in cholesterol from the inner to outer leaflet. This leaves the inner unstable and therefore susceptible to fusion (Nolan et al., 1992). This results in a 'normal' acrosome reaction in vitro.

1.3.3 *Cyclodextrin, Cholesterol, and Cholesterol-Loaded Cyclodextrins*

Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and possess an external hydrophilic face and an internal hydrophobic core (Dodziuk, 2006) that can encapsulate hydrophobic compounds (Mocé et al., 2010). There are three types of

cyclodextrins (α, β, and ϒ). The addition of methyl or hydroxypropyl residues to cyclodextrin increases the ability to dissolve hydrophobic compounds and enhances solubility in water (Yancey et al., 1996). β-cyclodextrin is the most studied and frequently used cyclodextrin due to the molecules' affordability, availability, and wide range of complex-forming capabilities through host-guest interactions.

Recently, cyclodextrins have been used to extract cholesterol from cell membranes**.** These molecules have a high affinity for sterols in vitro and are very efficient in stimulating the efflux of cholesterol from the membrane of sperm (Companyó et al., 2007; Visconti et al., 1999). Because these molecules are capable of removing cholesterol from plasma membranes, they have been used to capacitate and acrosome react sperm from many species, including but not limited to bulls (Aguila et al., 2015; Visconti et al., 1999), mouse (Visconti et al., 1999), ram (Garcia-Alvarez et al., 2015), stallion (Bromfield et al., 2014), and goat (Iborra et al., 2000). In the presence of methyl-β-cyclodextrin (MβCD), Visconti et al., (1999) reported that bull sperm were able to capacitate and acrosome react in media without bovine serum albumin (BSA), and Aguila et al., (2015) reported that sperm incubated with MβCD enhanced the capacitation effects of BSA resulting in improved zona-binding ability. With cyclodextrins being effectively used to modify cholesterol within sperm plasma membranes, it is pertinent to examine the roles of cholesterol in cellular function.

Cholesterol is a hydrophobic molecule that has multiple effects on membranes, including reduction of membrane permeability, stabilization, influence on membrane phase transition, serving as a membrane antioxidant, facilitating morphological membrane characteristics, enabling cell-cell interactions, as well as providing suitable microenvironments for membrane-associated proteins (Crockett, 1998). The cholesterol to phospholipid ratio is an important

determining factor of membrane stability and fluidity at low temperature. Cholesterol maintains the phospholipids in a random, lamellar arrangement as temperature decreases (Amann & Pickett, 1987) and modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids (Watson, 1981). In model membranes, increasing the ratio of cholesterol to phospholipid broadens the phase transition and reduces the membrane leakage and membrane phase separations (Drobnis et al., 1993). Therefore, the treatment of cholesterol prior to cryopreservation could reduce the sensitivity of sperm membranes to cooling damage, thus reducing or eliminating the lateral phase separation of the lipids (Watson, 1981).

In contrast, if cyclodextrins are pre-loaded with cholesterol, they can insert cholesterol into cell membranes (Navratil et al., 2003) due to cyclodextrin's inclusion complexes allowing for hydrophobic compounds, such as cholesterol, to be encapsulated within the molecule. In fact, they even transfer cholesterol more efficiently than phospholipid acceptors (Zidovetzki & Levitan, 2007)**.** Methyl-β-cyclodextrin is most commonly used to pre-load sperm with cholesterol and create a cholesterol-loaded cyclodextrin or CLC (Mocé et al., 2010). Treating sperm with CLC prior to cryopreservation improves cryosurvival rates when a single parameter or both motility and viability are assessed (Amorim et al., 2009; Mocé & Graham, 2006; Moraes et al., 2010; Purdy & Graham 2004a; Purdy et al., 2005). Purdy and Graham (2004a) demonstrated that treatment of bull sperm with CLC prior to freezing increased the sperm cholesterol content 2-3 times, increasing the cholesterol to phospholipid ratio from 0.45 to approximately 0.9. This makes the sperm less susceptible to cold shock as it is known that species with ratios >0.8 are not sensitive to cold shock, and therefore survive freezing more successfully (Mocé & Graham, 2006). Though, even with the increased sperm survivability post-

thaw, it is unclear why treated CLC sperm used for artificial insemination with standard breeding procedures resulted in pregnancy rates similar to control sperm (Graham & Purdy, 2004b).

Cholesterol remains elevated even after thawing, which causes concern for the effect of CLC treatment on sperm functionality. However, promising results seen in ram sperm treated with CLC prior to cryopreservation may be an indication that the impacts may be minimal in vivo. Ram sperm exhibited higher percentages of motile sperm after 3 hours of incubation at 38.5°C (45% motile) than those not treated with CLC (26% motile) in addition to higher cryosurvival rates (48% treated compared to 35% control) immediately after thawing (Mocé et al., 2010). This increased longevity during incubation reflects a greater likelihood of the sperm to survive in the female reproductive tract in vivo, thus giving more time to undergo capacitation and fertilize the oocyte (Fiser et al., 1991).

During cryopreservation, sperm are stressed while undergoing membrane phase transition, and are specifically subjected to osmotic stress as water is removed and cryoprotectants are added to the cells, as well as when the extracellular solution freezes and thaws (Hammerstedt et al., 1990). Spermatozoa respond to these osmotic changes by swelling or shrinking, which can be lethal if they expand or condense past their osmotic limits. Treating bull sperm with CLC broadens this osmotic tolerance limit by broadening the phase transition, regardless of hypertonic or hypotonic conditions (Moraes et al., 2010) which may be another mechanism by which CLC treatment increases sperm cryosurvival. Purdy et al., (2005) reported no changes in membrane fluidity in bull sperm after CLC treatment demonstrating no negative effect of CLC treatment on sperm membrane fluidity.

As previously mentioned, one of the first steps in sperm capacitation is a loss of cholesterol from the plasma membrane, which begins a cascade of events that make the sperm

acrosome react and capable to fertilize. Due to the increased cholesterol content in sperm plasma membranes treated with CLC, it is likely that the capacitation timing of, and the subsequent ability of sperm to acrosome react is altered. In fresh bull sperm, CLC treatment retards capacitation and the timing of the acrosome reaction, however cryopreserved control and CLC-treated sperm underwent capacitation and acrosome reaction within the same time period assessed within 1 hour (Purdy & Graham, 2004b). While this result was not expected, it is proposed there is additional damage to sperm membranes (a result of cryopreservation) that placed the sperm into a cryo-capacitated state that the additional cholesterol does not counteract (Purdy & Graham, 2004b).

1.4 Fertilization

1.4.1 *Acrosome Reaction and the Zona Pellucida*

For several decades it was believed that mammalian sperm acrosomes must be intact to bind to the zona pellucida (ZP) prior to undergoing exocytosis, enabling sperm to penetrate and fertilize the egg (Wassarman et al., 2004). However, more recent studies involving genetically engineered mouse strains that express green fluorescent protein (GFP) in their acrosomes have shown that almost all fertilizing mouse sperm have undergone acrosomal exocytosis prior to penetrating the zona pellucida (Foster & Gerton, 2016; Jin et al., 2011). It can be proposed that both concepts hold some truth, as in eutherian mammals, natural fertilization occurs when sperm ascend to the oviduct ampulla, pass through the cumulus oophorous surrounding the oocyte, loosely bind to the ZP (primary binding), followed by tight binding to the ZP (secondary binding), and penetration through the ZP, culminating in sperm-oocyte membrane fusion (Jin et al., 2011). In this sequence, the acrosome may be intact for primary binding but must be reacted before secondary binding. This concept is in line with B.M. Gadella's review that indicates once

the sperm cell attaches to the ZP (primary binding) it will be triggered to undergo the acrosome reaction which allows for hyperactivated motility to drill through the ZP (secondary ZP binding coinciding with sequential local ZP digestion and rebinding) (Gadella, 2010).

The bovine ZP is composed of three heavily glycosylated proteins, ZPA (homolog of human ZP2), ZPB (homolog of human ZP4/ZP3β) and ZPC (homolog of human ZP3/ZP3α). ZPC/ZP3 and ZPB/ZP4 are predicted to be the primary receptors for sperm binding and induction of acrosomal exocytosis, and ZPA/ZP2 is responsible for secondary sperm-zona binding (Sutovsky, 2018). While many species have similarities within zona proteins, it has recently been shown bovine ZP4 exhibits sperm-binding activity, with the N-terminal domain ZP4 (bZP4 ZP-N1) as the sperm-binding region (Dilimulati et al., 2022). The bZP4 ZP-N1 domain contains three sites identified in sperm binding: site I: from Gln-41 to Pro-46, site II: from Leu-65 to Ser-68 and site III: from Thr-108 to Ile-123. Additionally, sperm bind to the bZP4 ZP-N1 domain in a species-selective manner and N-glycosylation is not required for sperm-binding activity (Dilimulati et al., 2022).

1.4.1 *Oocyte Penetration and Activation*

Mammalian fertilization proceeds in a cascade-like manner with the various steps being inseparable. As previously mentioned, this comprises many processes, including sperm migration through the female reproductive tract in normal fertilization, sperm penetration through the cumulus cell mass, sperm adhesion and binding to the ZP, acrosomal exocytosis, sperm-egg interaction and fusion, and subsequent initiation of embryonic development through oocyte activation (Satouh & Ikawa 2018; Sutovsky, 2018; Wassarman et al., 2004). The cumulus oophorus of the oocyte is involved in growth and maturation, as well as helps maintain meiotic arrest until the oocyte reaches cytoplasmic maturation (Van Soom et al., 2002). Because bovine

oocytes are ovulated at an immature state of metabolic arrest at metaphase II (MII), oocytes must go through oocyte activation, a term given to a set of essential events that take place when sperm and egg unite.

Oocyte activation includes the following defining features; changes in egg cytosolic Ca^{2+} concentrations with repetitive increases and decreases for hours (referred to as Ca^{2+} oscillations), cortical granule exocytosis, resumption and completion of meiosis II, second polar body extrusion, pronuclear formation, and DNA synthesis (Satouh & Ikawa 2019; Xu et al., 1994). While there are many factors contributing to oocyte activation, it is proposed that a sperm-borne oocyte activation factor (SOAF) in the sperm head induces the Ca^{2+} oscillations by increasing 1,4,5-triphosphate (IP_3) from the successful activation of oocytes after intracytoplasmic sperm injection (ICSI) (Saunders et al., 2002). When sperm are treated with IP_3 inhibitors, there is a lack of Ca^{2+} oscillations indicating IP_3 is critical in this mechanism. Currently, the favored SOAF candidate is phospholipase C zeta 1 (PLCZ1) as it has been shown to trigger oocyte Ca^{2+} oscillations equivalent to those caused by sperm entry when PLCZ1 mRNA was injected into metaphase II oocytes (Saunders et al., 2002).

To avoid polyspermy, the oocyte becomes largely refractory to further sperm fusion events to prevent the creation of nonviable polyploid embryos. Once successful zona penetration has occurred, the oocyte implements a delaying strategy to only allow one sperm cell at a time from the perivitelline space to bind and fuse to the oolemma. After the fusion of the oocyte and sperm, immediately the oolemma is depolarized by releasing the contents of the cortical granules of the oocyte into the perivitelline space, blocking further sperm-oocyte fusion. Then the zona pellucida hardens, ceasing additional sperm-oocyte interaction. This essentially blocks more sperm from entering the perivitelline space by the release of enzymes to trigger a faster-acting

block via biochemical changes in the oolemmal membrane (Bianchi 2014; Gadella, 2010). It is also noted that the rapid shedding of JUNO from the egg membrane after fertilization (disappearing within 40 minutes post fertilization) may provide a possible mechanism for the membrane to block polyspermy in mammalian eggs (Bianchi, 2014). The release of zinc into the extracellular space at fertilization, termed zinc sparks, has also recently been noted to contribute to the block of polyspermy through modification of the zona pellucida in bovine (Que et al., 2019).

1.5 In Vitro Production of Bovine Embryos

1.5.1 *Overview*

The complex technology that is in vitro fertilization (IVF) has required decades of experimentation beginning with rodents in the 50's (Austin, 1951; Chang, 1951), though the birth of Louise Brown in 1978 intensified and renewed interest tremendously in large mammal applications. Since the world's first IVF calf born in 1982 (Brackett et al., 1982), embryos are now routinely produced in the laboratory. In fact, according to the global totals reported to International Embryo Technology Society (IETS) in 2020 1,156,422 embryos were produced in vitro, compared to 361,728 in vivo derived embryos (Viana, 2020). This process entails three techniques: maturation of oocytes in vitro (IVM), in vitro fertilization (IVF) of matured oocytes with motile, capacitated sperm, and in vitro culture (IVC) of the presumptive zygotes up to the blastocyst stage. Embryo development is strongly influenced by the integrity of each stage in the process, with each presenting unique challenges.

The goal of in vitro maturation (IVM) is to cause immature oocytes to reach the same stage of development as their in vivo matured counterpart, which requires both the nuclear and cytoplasmic maturation processes. For the developmental ability and fertilization of oocytes,

these two processes seem to be equally important (Eppig et al., 1996). Nuclear maturation, the meiotic resumption process, transforms prophase oocytes to metaphase II (MII) oocytes (Mikkelsen, 2005). Following meiotic resumption, the nuclear membrane dissolves, which is called GV breakdown (GVBD) (Eppig et al., 1996). To initiate the maturation process in vitro, the oocyte must be removed from its follicular environment and depleted of intracellular cAMP stores (Downs & Eppig, 1984). The first successful report of IVM was bovine achieved with cattle oocytes as indicated by polar body extrusion and metaphase plate appearance by pioneer Sir Robert Edwards (1965). Major medium components and incubation conditions described in his report are still used today with slight modification. Factors that affect IVM success include the method of oocyte retrieval, bovine serum and other protein sources supplemented in the media, and hormone supplementation of the IVM media (Abd El-Aziz et al., 2016).

Fertilization in vivo takes place in the oviduct of the female reproductive tract. In vitro fertilization requires that conditions are optimized for two gametes instead of one, unlike IVM. Sperm and oocyte must be provided with appropriate culture conditions favorable for their individual metabolic activity (Xu & King, 1990). Effects on fertilization success include individual bull variability, use of fresh vs frozen semen, sperm separation methods, and additives in media to induce capacitation and acrosome reaction. Individual variation among bulls is one of the most important factors in IVF success, and may be related to the bulls' age, stage of season, and ejaculate sperm quality (Xu & King, 1990). Numerous studies have examined the use of fresh and frozen semen in IVF (Ferré et al., 2020), determining that fresh semen takes longer to capacitate (Parrish, 2014), and membrane integrity (Mahmoud et al., 2013) as well as nuclear DNA fragmentation (Mukhopadhyay et al., 2011), decrease significantly in fresh vs. frozen semen. However, frozen sperm is desirable as it provides the potential to preserve

genetically desirable bulls, and can be utilized to avoid seasonal concerns while not requiring the coordination of oocyte and semen collection. As previously discussed, capacitation is a complex series of biochemical and physiological reactions which can be stimulated chemically within the fertilization media. Treatment of the semen with a medium of High Ionic Strength (HIS) like Brackett and Oliphant (BO) (Bracket, 1982), use of TALP-medium (Agarwal, 2017), supplementation of heparin (which most likely mimics the in vivo mechanism of capacitation) (Parrish et al., 1989), or addition of a calcium ionophore such as A23187 (Fukuda et al., 1985) all artificially induce capacitation in bovine spermatozoa.

Development of in vitro embryo culture has allowed researchers to observe and learn more about embryo development in real time within research applications, as well as provide the necessary tools to non-surgically transfer embryos at a later stage of development. Within recent years, this has become instrumental in the widespread commercial application of in vitro embryo production. The greatest challenge is the dynamic conditions during the first few days of embryonic development within the oviduct, and the transition from pre- to post-genome activation within embryo metabolism (Sirard, 2018).

1.5.2 *Culture of Bovine Embryos In Vitro*

Many research efforts have been made to improve existing culture systems, optimize high quality embryo development, and increase yield of pregnancy rate from viable embryos (dos Santos et. al., 2021). Early attempts at IVC used an egg white medium (Hammond, 1949) or egg-saline medium (Dowling, 1949) supplying protein, nutrients, and growth factors though the results were not promising. Synthetic Oviductal Fluid (SOF) was developed based on the ionic and protein contents of oviductal fluid of sheep (Restall & Wales, 1966) and this type of media was first used for bovine IVC by Tervit and colleagues in 1972. In addition to the use of SOF,

oxygen tension was also decreased to better approximate the conditions of the oviduct (Whitten, 1971). While improvements were being developed, early successes in bovine embryo culture were seen either from 1-2 cell embryos to the 8 cell stage, *or* from the 8 cell stage to the morula/blastocyst stage. A great majority of bovine zygotes stop developing within a few cell cycles after initiating cleavage. This developmental block arises in the bovine embryo at the eight-cell-stage, and is likely correlated with the cytoplasmic quality of the oocyte. Oocytes harbor all mRNAs and proteins needed to reach the fourth or fifth cell cycle, however, embryos that fail to transcribe their own genome consequently fail to further develop (Meirelles et al., 2004). This failure to develop was coined the "8 cell bock". During the same time point as the "block," the embryonic genome activation (EGA) occurs, which is a shift from the embryo's dependence on maternal mRNA to its own embryonic mRNA (Abd El-Aziz et al., 2016). Two main mechanisms are believed to cause this: inability to overcome the chromatin repression and (i) activate transcription of important developmental genes and/or (ii) to react to injuries caused by environment (Betts & King, 2001).

In addition to the application of low oxygen tension, co-culture of embryos with oviductal cells resulted in the first completely in vitro system for bovine embryo production resulting in the birth of 5 healthy calves (Eyestone & First, 1989). Somatic cell co-culture was adopted by many research groups in a variety of species with benefits including increased blastocyst cell count, faster cleavage, higher blastocyst rates, better hatching rates, increased pregnancy outcomes, and most importantly, more live births (Orsi & Reischl, 2007). Drawbacks of co-culture, such as differing media needs, viral transfection possibility, and lack of replicability, led researchers to explore other media options.

Adding Fetal Bovine Serum (FBS) to media was also investigated, showing increased blastocyst formation and faster development with the addition of FBS (Van Langenhonckt et al., 1997), though this also resulted in a trend of heavier birth weights, termed Large Offspring Syndrome (LOS) which is an overgrowth disorder in ruminants. Due to this unfortunate outcome of serum supplementation, modern embryo culture is trending toward cell-free, serum-free conditions. These contain mediums specifically developed with amino acids present and exchanged frequently to avoid ammonia build up, with the determination that embryos must be cultured in groups to simulate a co-culture environment (Abd El-Aziz et al., 2016). However, uncovering the right combination of constituents within media that is able to successfully support embryos through the 8 cell block, as well as aide in the formation of healthy blastocysts has proven a considerable task. Group culture medium additives that have shown some promise include transforming growth factor β and basic fibroblast growth factor (Larson et al., 1992), defined amino acids (Gardner et al., 1994), polyvinyl alcohol, insulin, transferrin, selenium, myo-inositol, and epidermal growth factor (Wang et al., 2012).

One of the most widely pursued research avenues to improve embryo development is advanced culture media and conditions, which can be implemented in a wide range of laboratories (dos Santos et al., 2021; Reed et al., 2011; Woolridge et al., 2019). It is known embryos of high-quality require different substrates at different stages of development. For energy generation early on embryos mainly use pyruvate, lactate and aspartate, and have a limited ability to metabolize glucose. Glutamine and aspartate are also options, though at the cost of energy and consequently cleavage rates (Gardner & Harvey, 2015). Around the time of embryonic genome activation (EGA) (prior to morulae compaction) increased biosynthesis, cell proliferation, and blastocoel expansion increases demand for adenosine triphosphate (ATP)

(Gardner & Harvey, 2015). After compaction, embryos then increase oxygen consumption, mitochondrial activity, and the ability to metabolize glucose and generate energy through the glycolic pathway (Gardner & Harvey, 2015). From here, glucose can be directed to the pentose-phosphate pathway, which generates ribose chains for RNA synthesis and DNA, with NADH which is required for the reduction of the important antioxidant glutathione (Rieger et al., 1992). Essential amino acids are required for the differentiation of the inner cell mass (ICM), while non-essential amino acids and glutamine improves blastocoel formation and hatching (Lane & Gardner, 1996; Lane & Gardner, 2007; Steeves & Gardner 1999).

1.5.3 *Group versus Single Embryo Culture*

A second culture condition that may have a dramatic impact on success is how embryos are cultured: individually or in groups. One practical reason in favor of individual culture is the ability to follow morphological changes and development rates in individual embryos, as this can provide valuable information to track individual embryonic kinetics and development. Another argument made within the human industry to culture embryos individually is to consider that embryos in groups may be exposed to negative effects of dying or delayed embryos cultured in the same drop (Reed et al., 2011). Single embryo culture has become most popular within the human industry due to there being fewer zygotes to culture from each oocyte retrieval in humans compared with bovine practices, as well as the utmost importance in retaining command of DNA identity within embryos (Wydooghe, et al., 2014). Advancement in time-lapse incubation has also made single embryo culture systems more desirable by providing the ability to identify embryo kinetics in real-time, allowing for later analysis of data, and video monitoring tracking embryo development without having to interrupt the culture system for media exchanges or re-plating (Ciray et al., 2012, Sugiumura et al., 2017, Velez et al., 2020).

However in contrast, bovine embryos generally develop to a lesser stage in individual culture, most likely due to their very sensitive nature to suboptimal culture conditions (Goovaerts et al., 2012). Interestingly enough, group culture appears to benefit both mono- and poly-ovulatory species. The advantages of group culture may be a result of the secretion of different substances on the neighboring embryos and on the embryo itself. Paracrine (the effects of one embryo on the other) and autocrine (the effects of the embryo on itself) effects may account for the improved laboratory outcome in group culture (Matsuura, 2014). This secretion of embryotrophic, or positive signals from companion embryos, is thought to be the rationale for better quality embryos (Reed, 2006). The analysis of the embryo secretome in recent years has become an active area of research demonstrating that embryos secrete various amino acids, proteins, and other metabolites (D'souza, 2018; Katz-Jaffe & Schoolcraft, 2006; Krisher et al., 2014; Lindgren et al., 2018). Considering this crosstalk between embryos and the surrounding environment, it is plausible to assume this is why group cultured embryos perform better and potentially leave behind positive factors within the group culture media.

There are also factors that contribute to embryo interactions within group culture outside of the media. These include but are not limited to the ratio of media volume to the number of embryos (embryo density), embryo spacing/contact, and quality of companion embryos (Reed et al., 2011) which also must be considered when determining the optimum culture system. Overall, it is most common practice for bovine embryos to be cultured within groups due to better blastocyst formation results (Wydooghe et al., 2014), however this limits the availability to track individual embryo development in culture.

1.6 Summary

Sperm capacitation is a complex process that exhibits sensitively timed compounding actions. Because of this, capacitation is extremely difficult to study in vivo, therefore extensive research has been completed analyzing the capacitation and acrosome reaction process in vitro. Knowledge gained from better understanding of capacitation timing and processes paired with spermatozoa-oocyte interaction and IVF research can be used to better increase IVP outcomes. Culture conditions, including media composition and number of embryos cultured, greatly impact IVP outcomes within the bovine system and beyond. Future development of enhanced IVP protocols is necessary to further the understanding of functions and limitations of bovine reproduction within laboratories, aiding both research and industrial sectors.

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CHAPTER 2: EFFECT OF CHOLESTEROL-LOADED CYCLODEXTRIN TREATMENT OF BOVINE SPERM ON CAPACITATION TIMING[1][2]

2.1. Introduction

Cryopreservation of bovine sperm facilitates the storage and transport of germplasm for use in artificial insemination (AI) and other advanced reproductive technologies. However, cryopreservation can cause irreversible cell damage, thus hindering functionality. Damage occurs to the plasma membrane during the phase transition, and can be reduced by increasing lipid composition, though this alteration also affects the ability of sperm to capacitate and the acrosome to react (Purdy & Graham 2004a). Treating sperm with CLC prior to cryopreservation increases cryosurvival rates, (Purdy & Graham 2004a; Mocé & Graham 2006), though even with a higher number of viable sperm cells, in vitro embryo production rates and pregnancy rates from artificial insemination with control or CLC-treated sperm were not different (Purdy & Graham 2004b). One potential explanation of this unexpected result is a delay of frozen-thawed CLC-treated sperm to undergo capacitation. Our goal was to investigate the effects of CLC treatment on in vitro capacitation timing.

Cholesterol is a hydrophobic molecule that has multiple effects on membranes, including stabilizing the membrane, reducing membrane permeability, influencing the membrane phase transition, serving as a membrane antioxidant, facilitating morphological membrane characteristics, enabling cell-cell interactions, as well as providing suitable microenvironments for membrane-associated proteins (Crockett 1998). Cholesterol:phospholipid ratio is an

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important determining factor of membrane stability and fluidity at low temperature. Cholesterol maintains phospholipids in a random, lamellar arrangement as temperature decreases (Amann & Pickett 1987) and modulates fluidity of membranes by interacting with the fatty acyl chains of phospholipids (Watson 1981). In model membranes, increasing the cholesterol:phospholipid ratio broadens the phase transition and reduces membrane leakage and membrane phase separations (Drobnis et al., 1993). Therefore, treatment of sperm with cholesterol prior to cryopreservation could reduce sensitivity of sperm membranes to cooling damage, thus reducing or eliminating the lateral phase separation of lipids (Watson 1981).

Cyclodextrins can be used to insert or remove cholesterol from cell membranes. Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and possess an external hydrophilic face and an internal hydrophobic core (Dodziuk 2006) that can encapsulate hydrophobic compounds, such as cholesterol (Mocé et al., 2010). These molecules have a high affinity for sterols in vitro, are very efficient in stimulating efflux of cholesterol from the membrane of sperm (e.g. Companyó et al., 2007; Visconti et al., 1999) and, if pre-loaded with cholesterol, can insert cholesterol into cell membranes (Navratil et al., 2003). The addition of methyl residues to cyclodextrins enhance their solubility in water and ability to dissolve hydrophobic compounds (Yayncey et al., 1996). Therefore methyl-β-cyclodextrin (MβCD) is most commonly used to pre-load sperm with cholesterol, creating a CLC (Mocé et al., 2010). Treating sperm with CLC prior to cryopreservation increases sperm cryosurvival rates (Purdy & Graham 2004a; Mocé & Graham 2006), though even with better survival no evidence has emerged that indicates this result is associated with increased fertility rates in vitro. This lack of evidence may suggest compromised sperm fertilization ability and/or the need to alter the timing of IVF with CLC-treated sperm.

The objective of this study was to investigate the role of CLC on *in vitro* timing of capacitation in frozen-thawed bull sperm. Assessments included viability and acrosomal integrity, intracellular calcium levels, and membrane disorder. Treatment with MβCD at various concentrations, and calcium ionophore, Ionomycin, was completed to determine if there would be an effect on capacitation timing within this sperm population.

2.2 Materials and Methods

2.2.1 *Animal Handling*

Cattle handling and semen collection followed the National Association of Animal Breeders Management Guidelines regarding bull care. Semen was collected offsite, thus IACUC approval wasn't required by CSU. Rocky Mountain Sire Services has good standing with Certified Semen Services, Inc. (a subsidiary of NAAB). The 10 bovine ejaculates used for this study were provided by Select Sires, Inc. (Plain City, OH) and collected by RMSS (Bennett, CO 39°44'23°N 104°31'36°W, Climate Zone 4) from beef cattle breeds housed outside in the same facility. Collections were performed in December during breeding soundness examinations.

2.2.2 *Chemicals, Reagents & Disposables*

All research grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical reagents such as flow cytometry fluorophores were also purchased from Sigma Chemical Co. (St. Louis, MO) and Thermo Scientific (Carlsbad, CA) unless otherwise stated. Disposables were purchased from Life Science Products (Frederick, CO) and VWR International (Radnor, PA).

2.2.3 *Preparation of Cyclodextrin-loaded Cholesterol*

In this study, cyclodextrins were used to modify sperm membranes. The procedures of Purdy and Graham (2004a) were followed. Briefly, 1 g MβCD was dissolved in 2 mL methanol,

and 200mg cholesterol dissolved in 1 mL chloroform. The solution was mixed until clear and the solvents evaporated. The resulting powder was stored at 23°C until use, and hereafter referred to as CLC. A working solution of CLC was prepared by adding 50 mg of cholesterol-loaded cyclodextrin to 1 mL of Tyrode's albumin lactate pyruvate (TALP) (Graham et al., 1986) following the procedures of Purdy and Graham (2004a), and mixed thoroughly to create a CLC concentration of 50 mg/mL (345 mOsm).

2.2.4 *Semen Collection and Cryopreservation*

One ejaculate from ten bulls was collected by artificial vagina (AV) method (Alexander 2014). All ejaculates had a minimum concentration of 1 x 10^9/mL, >70 % normal morphology, and greater than 60% motile sperm. Ejaculates were transported to the lab immediately and then, prior to cryopreservation, each ejaculate was split into 2 aliquots. CLC treatment and cryopreservation of sperm were conducted as described by Moce & Graham (2006). One aliquot served as the control, and the other was treated with 2 mg CLC/120x10^6 sperm. Both aliquots were incubated for 15 min at 22°C,then diluted with 20% egg yolk-Tris diluent to a concentration of 100 x 10^6 sperm/mL and cooled to 5°C over 2 hours. Once cooled, aliquots were diluted 1:1 (vol:vol) with 14% glycerolated egg yolk-Tris diluent (final glycerol concentration 7%), loaded into 0.5 mL straws, frozen in nitrogen vapor 4 cm above the surface of liquid nitrogen for 15 min, and plunged into liquid nitrogen for storage at -196°C until thawing.

2.2.5 *Flow Cytometric Evaluation*

Cryopreserved semen from each of the ten ejaculates was used to evaluate the effect of cyclodextrin concentration and the effect of ionomycin treatment on capacitation timing between control and CLC-treated sperm over 180 min. For both groups (control and CLC-treated sperm),

straws were thawed in a 37°C water bath for 30 seconds, and motile sperm were isolated by washing thawed semen through a 45/90 Percoll® gradient (Parrish et al., 1995) at 800 g for 20 min. Supernatant was removed and sperm washed again in 2 mL TALP by centrifugation for 5 min at 300 g. Washed sperm samples were pooled and diluted with TALP medium to 15 mL total, then separated into 2.5 mL borosilicate glass tubes suitable for analysis by flow cytometry with 2-5 x 10^6 sperm/mL.

Within one sub-sample group, 0.5 mL of washed, diluted sample was treated with MβCD at concentrations of 0, 1 or 2 mg/mL to test the effect of MβCD on capacitation timing by cholesterol efflux. Within a second sub-sample group, 2µL of 0.05 mM ionomycin was added to 0.5 mL of washed, diluted sample to act as a capacitating agent by increasing intracellular calcium levels.

Flow cytometry assessment of sperm was conducted using a BD Accuri ™ C6 flow cytometer (BD Biosciences Systems & Reagents Inc, San Jose, CA) equipped with standard optical filters FL1 533/30 nm, FL2 585/40 nm, FL3 >670 nm, and FL4 675/25 nm operating at 66 µL/min to acquire 10,000 DNA-positive events for each assay performed. Calibration of the instrument was performed with 19 flow cytometry calibration beads prior to use. For all flow cytometric evaluations in this study, a sperm sample killed by snap freezing was utilized as a negative control. A clean and decontamination cycle was run according to manufacturer instruction between each use. All assays below were analyzed with FlowJo software (BD Biosciences Systems & Reagents Inc, San Jose, CA) programmed with gating schemes set in a logarithmic scale.

2.2.6 *Fluorescent Stains*

Fluorescent stains were used to assess capacitation factors of spermatozoa treated with CLC. Stains included propidium iodide (PI) (0.15 mM) and FITC-coupled peanut-agglutinin (FITC-PNA) (0.83 mM) to assess acrosomal integrity and viability, FLUO 3-AM (1 mM in DMSO) and PI to assess intracellular calcium levels, and Merocyanine 540 (M540; 5 mM) with YO-PRO™-1 Iodide (0.1 mM) to assess membrane disorder or scrambling. Flow cytometric data on all samples were collected at 0-, 15-, 30-, 60-, 120-, and 180-minute staining times. Data were collected for all parameters on all ejaculates for both control and CLC treated sperm. Live sperm populations were considered to have compromised acrosome integrity when negative for PI and positive for FITC-PNA (Purdy 2008), high intracellular calcium levels with negative propidium iodide and positive FLOU-3 AM (Nolan et al., 1992), and high plasma membrane disorder when M540 and negative for YO-PRO™-1 Iodide (Guthrie & Welch 2005).

2.2.7 *Statistical Analysis*

All statistical analyses were performed with SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Separate analyses were performed for the percent live sperm, live acrosome-reacted sperm, live sperm with high intracellular calcium levels, and live sperm with high membrane fluidity. Values for these parameters are presented as the mean $\pm$ SEM (Figures 2.1-2.4). Percentage data were transformed (arcsin) prior to analysis. Initially, MβCD levels (0, 1, and 2 mg/mL) were analyzed by ANOVA, at each time point within each treatment (control, control+ionomycin, CLC and CLC+ionomycin) to determine if MβCD administration affected sperm parameters. No differences ($P>0.05$) were determined in any sperm parameter for the different MβCD levels within the 4 sperm treatments at any specific time point. Therefore, data for the 3 MβCD treatments were pooled in the final analyses. A Repeated Measurements Analysis of Variance was used to detect differences temporally within each

treatment. Within each time point, treatment differences were determined using ANOVA and means were separated using Student–Newman–Keuls (SNK).

2.3. Results

From the ten ejaculates used in this split ejaculate study, there are 4 treatment groups (control, control + ionomycin, CLC, and CLC + ionomycin) which were evaluated for acrosomal integrity, intracellular calcium level, and membrane disorder by flow cytometry over time up to 180 min. The treatment of CLC prior to cryopreservation was the main effect, with ionomycin as a secondary treatment being tested. Varied MβCD concentration levels of 0, 1, and 2 mg/mL revealed no difference at any time points (Table 2.1).

Table 2.1 Average percentage of live cells observed between time 0 and 180 min across treatments concentrations in mg/mL. No significant differences were found (P> 0.05). (P<0.05) from their respective mean values at time zero. Mean values within a single time period with different superscripts indicate significant difference between treatments (P<0.05).

	Control			CLC		
Time (min)	**[0]**	**[1]**	**[2]**	**[0]**	**[1]**	**[2]**
0	44.8	44.7	44.9	61.2	61	61.1
15	43.1	42.8	42.5	59.8	59.3	58
30	42	42.3	41.8	58.2	58.1	56.7
60	39.4	39.9	38.6	52.6	54.6	52.7
120	24	25.9	24	33.5	36.9	37.1
180	16.3	17.2	16	22.1	23.4	22.2

	Control + Ionomycin			CLC + Ionomycin		
Time (min)	**[0]**	**[1]**	**[2]**	**[0]**	**[1]**	**[2]**
0	42.8	41.9	41.7	60.4	59.3	58.1
15	38.7	38.5	37.6	56.7	54.8	54.6
30	35.9	35.1	32.6	54.1	48.7	47.6
60	24.5	24.1	21.3	40.1	33.5	30.8
120	14.4	14.3	12.4	15.4	14.5	11.3
180	10.1	10.6	9.2	10.7	9.3	7.7

The percentage of live post-thaw sperm cells treated with CLC prior to freezing exhibited significantly higher viability compared with control sperm at time zero ($P<0.01$), and displayed the highest percent of live cells at all time points between 0-180 min (Figure 2.1). From time 0, significant viability loss was seen at 30 minutes for ionomycin-treated CLC sperm, at 60 min for ionomycin-treated control sperm and CLC sperm, though not until 120 min for control sperm ($P<0.05$). The greatest loss of cell viability (50 percentage points) was seen in the CLC sperm treated with ionomycin though this group displayed the second highest post-thaw viability at time 0. The least amount of viability loss was seen in the control sperm without ionomycin treatment with 28 percentage points lost at 3 hours.

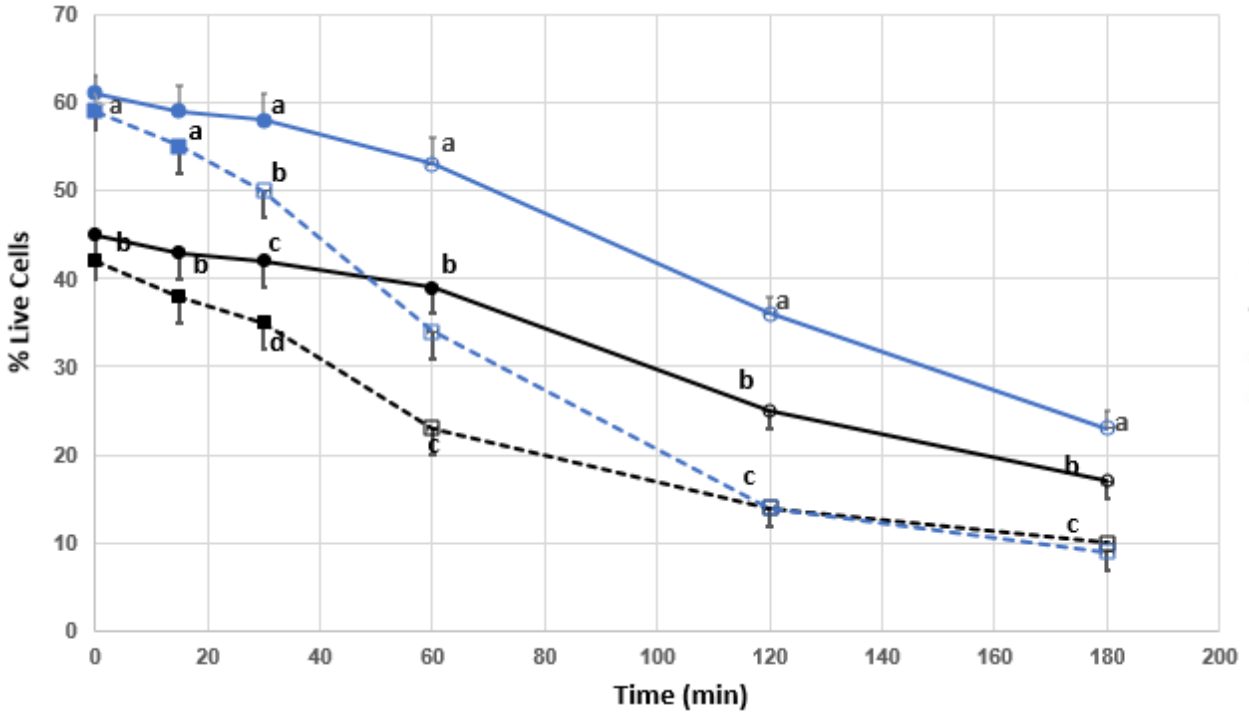

Figure 2.1 Time-dependent post-thaw survival of cryopreserved sperm ± CLC. Percent survival of live bull sperm post-thaw was analyzed as a function of time from 0 to 180 minutes post-thaw. Sperm from 10 bulls were evenly aliquoted by volume and cryopreserved without treatment (Control, black solid lines) or in the presence of 2 mg/mL CLC, (blue lines) prior to freezing. Additionally, both groups were subject to treatment with 0.05 mM ionomycin to induce the acrosome reaction and capacitate sperm. Solid lines (—●—) indicate no treatment and dashed lines (---■---) indicate treatment with ionomycin. Open circles or squares indicate significant difference

(P<0.05) from their respective mean values at time zero. Mean values within a single time period with different superscripts indicate significant difference between treatments (P<0.05).

A significantly higher percentage of live acrosome-reacted sperm was noted in the non-treated CLC group, treated control group, and treated CLC sperm group at 120 min as compared to time 0 for each treatment (*P*<0.05). A significant difference was not seen in control sperm until 180 min (*P*<0.05). CLC sperm displayed the lowest percent of live-acrosome reacted cells at all time points (Figure 2.2). The highest increase in percent of live acrosome- reacted cells was seen in CLC sperm and control sperm treated with ionomycin (12 and 8 percent increase respectively).

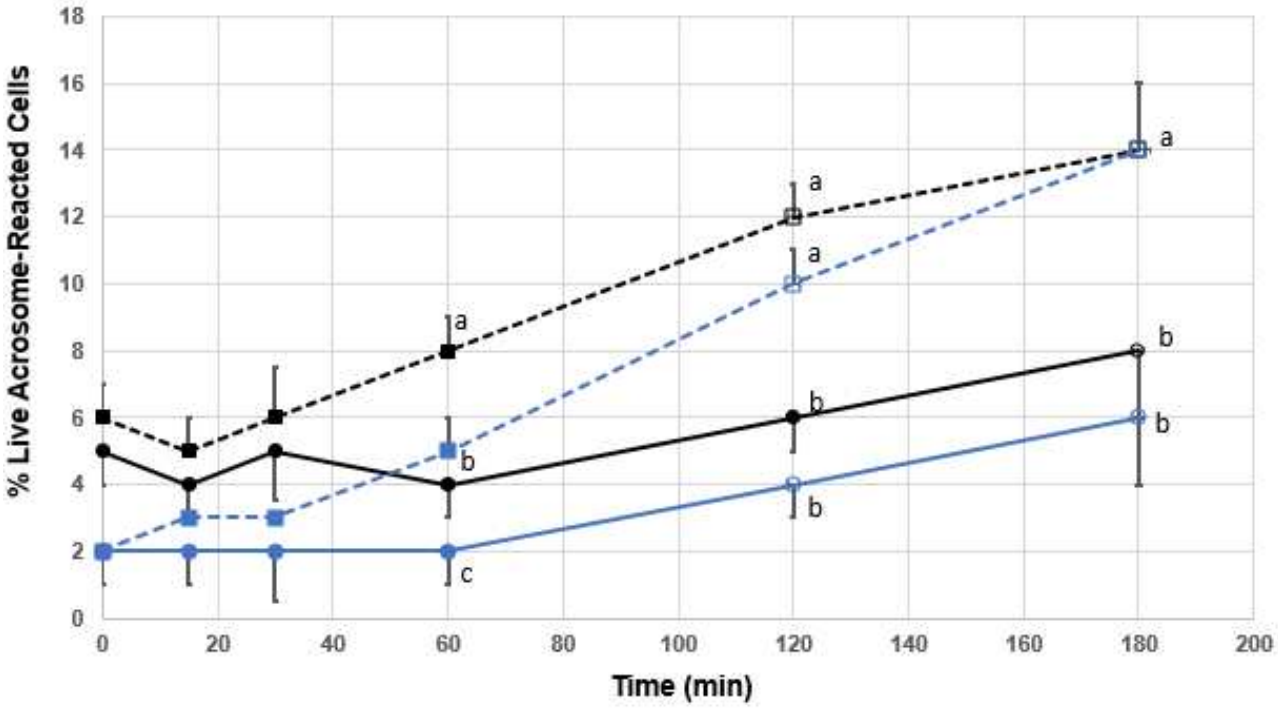

Figure 2.2 Post-thaw survival of live acrosome-reacted bull sperm cells ± CLC and stained with PI/FITC-PNA analyzed as a function of time from 0 to 180 minutes. Sperm from 10 bulls were evenly aliquoted by volume and cryopreserved without treatment (Control, black solid lines) or in the presences of 2 mg/mL CLC (blue lines) prior to freezing. Additionally, both groups were subject to treatment with 0.05 mM ionomycin to induce the acrosome reaction and capacitate sperm. Solid lines (—●—) indicate no treatment and dashed lines (---■---) indicate treatment with ionomycin. Open circles or squares indicate significant difference (P<0.05) from their respective

mean values at time zero. Mean values within a single time period with different superscripts indicate significant difference between treatments (P<0.05).

Ionomycin treatment resulted in sperm intracellular calcium increasing quickly, peaking at 15 and 30 min for control and CLC sperm respectively, with non-treated control sperm peaking at 60 min post-thaw and at 120 min for CLC sperm. Ionomycin treatment revealed a decline in percentage of live cells with high intracellular calcium after peaking at time 30 for both control and CLC sperm, indicating the cells had capacitated. When comparing peak percentage of live cells with high intracellular calcium to the final time point of 3 hours, CLC sperm treated with ionomycin had the greatest drop in percentage points (56) and CLC sperm with no treatment had the least amount of decline at 6 percent. A significant difference was determined in non-treated control and CLC cells at 15 min and 120 min for ionomycin-treated groups ($P<0.05$). These results validate the use of ionomycin to induce capacitation by increasing intracellular calcium levels (Figure 2.3).

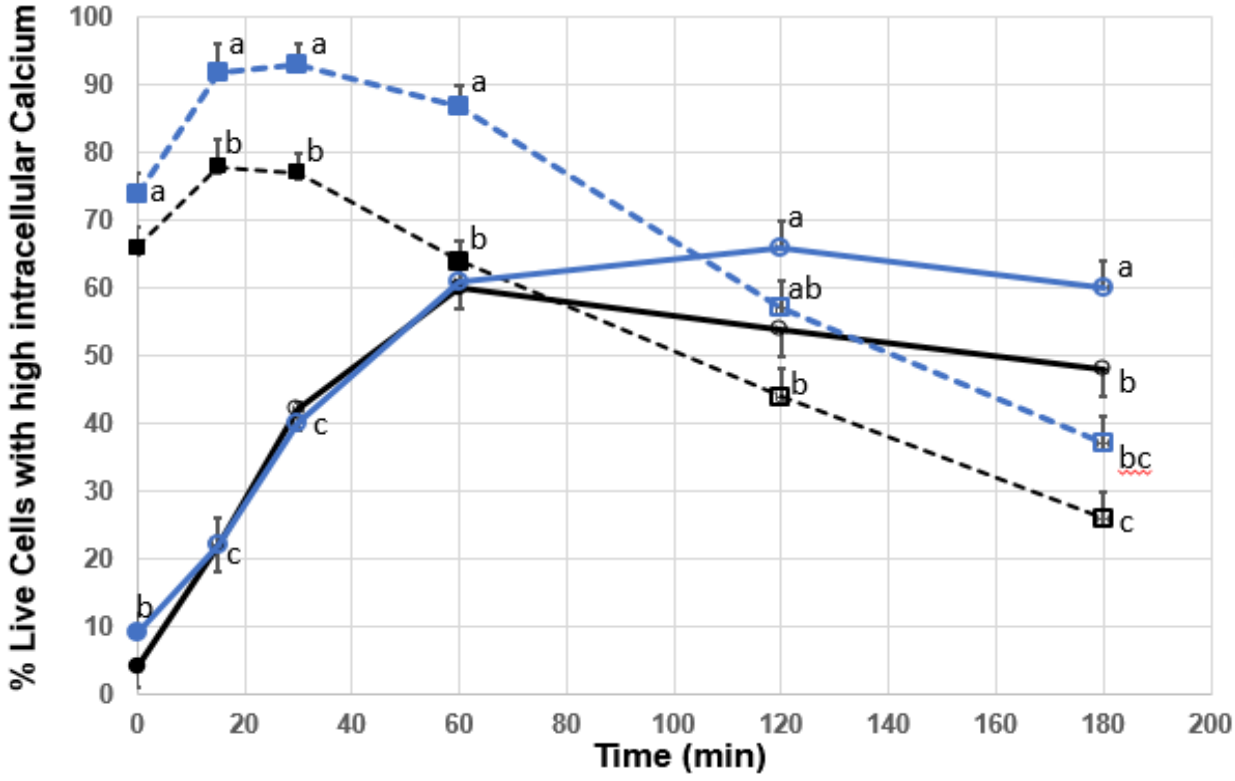

Figure 2.3 Post-thaw survival of sperm cells incubated in the presence of CLC and revealing high intracellular calcium following exposure to staining with PI/FLUO 3-AM analyzed as a function of time from 0 to 180 minutes post-thaw. Sperm from 10 bulls were evenly aliquoted by volume and cryopreserved without treatment (Control, black solid lines) or in the presences of 2 mg/mL CLC (blue lines) prior to freezing. Additionally, both groups were subjected to treatment with 0.05 mM ionomycin to induce the acrosome reaction and capacitate sperm. Solid lines (—●—) indicate no treatment and dashed lines (---■---) indicate treatment with ionomycin. Open circles or squares indicate significant difference (P<0.05) from their respective mean values at time zero. Mean values within a single time period with different superscripts indicate significant difference between treatments (P<0.05).

When analyzing percentage of live cells exhibiting membrane disorder, there was no significant difference in control sperm, and CLC sperm only displayed significant difference at 180 min (*P*<0.05). Ionomycin-treated control and CLC sperm were both different at 120 min from time 0 (*P*<0.05). Non-treated CLC sperm displayed the lowest percentage of live cells exhibiting membrane disorder (Figure 2.4).

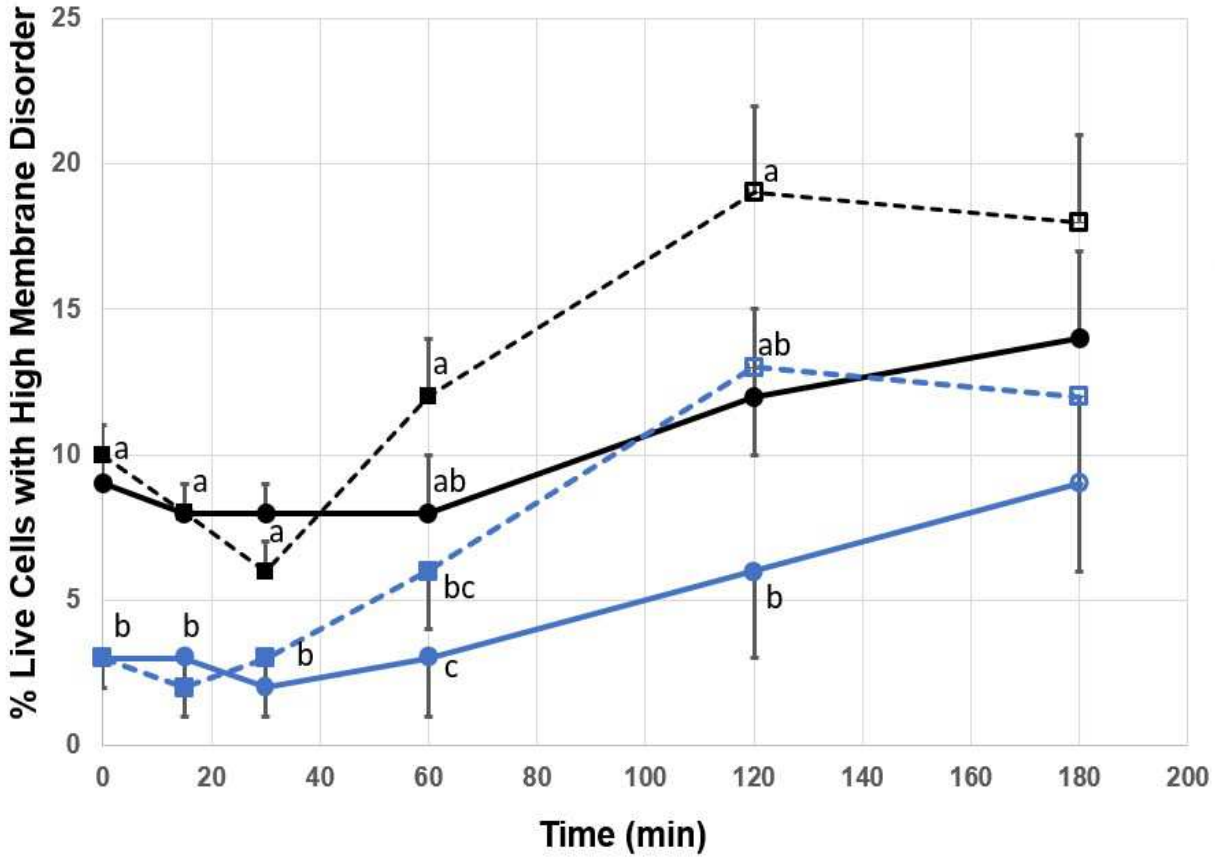

Figure 2.4 Membrane disorder in live post-cryo-preserved sperm cells incubated in CLC. The degree of disorder was revealed by the application of the stain Merocyanine 540/YO-PRO-1 with cells presenting as high fluidity by percent of live bull sperm post-thaw and analyzed as a function of time from 0 to 180 minutes post-thaw. Sperm from 10 bulls were evenly aliquoted by volume and cryopreserved without treatment (Control, black solid lines) or in the presences of 2 mg/mL CLC(blue lines) prior to freezing. Additionally, both groups were subject to treatment with 0.05 mM ionomycin to induce the acrosome reaction and capacitate sperm. Solid lines (—●—) indicate no treatment and dashed lines (---■---) indicate treatment with ionomycin. Open circles or squares indicate significant difference ($P<0.05$) from their respective mean values at time zero. Mean values within a single time period with different superscripts indicate significant difference between treatments ($P<0.05$).

Similar percentage difference between the lowest point to peak was matched between non-treated control and CLC sperm (6 and 7 percent respectively) and between ionomycin treated control and CLC sperm (13 and 11 percent respectively). These similarities indicate that increased cholesterol within the plasma membrane of the sperm cells limited disorder potential, and that when treated with ionomycin displayed similar reaction results across the control and CLC sperm.

2.4. Discussion

Cryopreservation of bovine sperm negatively effects viability, induces synthesis of reactive oxygen species, and causes DNA damage (Gürler et al., 2016). In this and previous studies, treatment of bovine sperm with CLC prior to freezing supports increased post-thaw viability both initially after thaw and over time (Purdy & Graham 2004a; Mocé & Graham 2006). Our results displayed significantly higher percentage of live post-thaw sperm cells treated with CLC prior to freezing compared with control sperm at time zero ($P<0.01$). These improved cryosurvival rates suggest that CLC treated sperm can more effectively withstand the damaging conditions of cryopreservation, and yield otherwise unlikely survivability. Recent studies have indicated increased post thaw viability when CLC is added prior to cryopreservation of bovine sperm packaged for low dose insemination, which is a potential application for the use of CLC to optimize the preservation of valuable genetic donors (Lone et al., 2021).

In previous studies, MβCD improved the capacitation status as assessed by the increase in plasma membrane fluidity, intracellular calcium concentration, induced acrosome reactivity and zona pellucida (ZP)-binding ability (Águila et al., 2015), and increased rates of activation and fertilization (Kato, Shoei, & Nagao 2011) in bovine sperm in vitro. However, our results indicated no significant difference ($P>0.05$) at 0, 1, and 2 mg/mL at all time points. This difference may indicate that the concentration of MβCD used in our present study was not high enough to induce cholesterol efflux in CLC-treated sperm, as in previous studies there was a decrease observed in a dose-dependent manner compared to the control sperm (Águila et al., 2015). High concentrations of MβCD were not used due to indication of DNA integrity being compromised at 5mM (Águila et al., 2015), however, treatment at these levels may be necessary to see an effect on CLC-treated sperm. CLC-treated sperm are estimated to have an increased cholesterol:phospholipid ratio from 0.45 to 0.9 or higher (Purdy & Graham 2004a), therefore, to see an effect of MβCD on these sperm, treatment with up to twice the concentration used in the current study may be required. Further studies assessing MβCD effect at concentrations greater than 2 mg/mL would be required to evaluate cholesterol efflux in CLC-treated sperm, while also assessing potential damage to DNA integrity.

When assessing percent of live acrosome-reacted sperm, the group treated with CLC was associated with the lowest percent of live cells reacted at all time points, supporting the hypothesis that CLC-treated sperm require more time to react acrosomally, and therefore capacitate more slowly compared with control sperm. Previous studies evaluated this reaction over 30 minutes (Purdy & Graham 2004b), however evaluation over a longer 3 hour timeframe allowed for a more in depth assessment. This hypothesis is also supported when assessing percent of live cells exhibiting membrane disorder. Our results reinforce the inference that

adding cholesterol to frozen-thawed sperm cells cultured in the above conditions induces membranes to become more rigid (Purdy et al., 2005), an observation based on the finding of increased membrane stability of CLC- treated sperm. This was true for sperm cells both treated with ionomycin and non-treated CLC sperm, with significant difference seen between most time points ($P<0.05$).

Ionomycin, a potent and selective calcium ionophore., has been used as a research tool to stimulate hyperactivation in bovine sperm. A similar calcium ionophore A23187 has been used to induce sperm capacitation of bovine sperm (Purdy & Graham 2004b), though A23187 acts by incorporating into the sperm plasma membrane and transporting calcium stores across the plasma membrane (Talbot et al., 1976), whereas Ionomycin acts by inducing the release of cytosolic calcium stores (Morgan & Jacob 1994). Our results support the work of Purdy and Graham (2004b) with CLC-treated sperm and control sperm exhibiting similar percentages of cells with high intracellular calcium. Figure 3 displays a sharp increase in the percentage of live cells with increased intracellular Ca^{2+} indicating capacitation was occurring, though after 60 min (con) or 120 min (CLC-treated) there is a steady decline in live cells displaying high intracellular calcium, suggesting that population of cells died once capacitated. Sperm treated with ionomycin displayed the highest increase in percentage of live acrosome-reacted cells over time, also supporting the notion that capacitation has occurred. Loss of motility was also observed in subsamples treated with ionomycin. In total, these results support other reports, in addition to providing insight on the effect of ionomycin treatment on bovine sperm capacitation when accessing acrosome reaction, intracellular Ca2+ levels, and membrane disorder. These results support the hypothesis that the additional cholesterol added to sperm via CLC-treatment does

delay capacitation timing in vitro, as shown from later acrosome reaction, decreased membrane disorder, and effect on intracellular calcium levels.

Cholesterol loading prior to freezing resulted in delayed capacitation timing. This delay may be the cause for the absence of increased fertility in vitro, but further in vitro investigation is required for confirmation of this conclusion. Additionally, further investigation of the molecular mechanisms involved in delayed capacitation of CLC-treated sperm would help determine the cause of the delay and determine why there is no improvement in fertility associated with CLC-treated sperm. Paired with additional study of potential uses for CLC treatment in cryopreservation of low-dose insemination units, CLC treatment could highly benefit the cryopreservation of valuable genetic sires and/or provide higher yield of insemination units per ejaculate.

2.5. Conclusion

In conclusion, treatment of bovine sperm pre-loaded with cholesterol prior to freezing via CLC (2mg/mL) improved cryosurvival and post-thaw viability, initially and over time by significantly decreasing membrane fluidity in sperm. However, CLC-treated sperm exhibit delayed capacitation by 1 hour based on assessment of viability and acrosomal integrity, intracellular calcium levels, and membrane disorder. MβCD treatment (1mg/mL and 2mg/mL) did not impact capacitation, though ionomycin (0.05mM) induced capacitation by 1 hour, resulting in increased intracellular calcium and acrosome reaction. Further investigation of retained cholesterol post-thaw is required, as longer time to capacitate may hinder fertilization capacity and/or require adjustments to IVF timing.

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CHAPTER 3: IN VITRO FERTILIZATION USING CHOLESTEROL-LOADED BOVINE SPERM TREATED WITH METHYL-β-CYCLODEXTRIN

3.1 Introduction

Cholesterol plays an important role in mammalian cell functions and integrity, making it a molecule of choice when trying to alter sperm cryopreservation for use in in vitro fertilization. In sperm, cholesterol is a critical first molecule within the capacitation cascade of which removal from the plasma membranes occurs (Leahy & Gadella, 2015). Additionally, the cholesterol to phospholipid ratio is an important determining factor of membrane stability and fluidity at low temperature. Within spermatozoa plasma membranes, cholesterol maintains the phospholipids in a random, lamellar arrangement as temperature decreases (Amann & Pickett, 1987) and modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids (Watson, 1981). Within mammalian species a wide range of cholesterol to phospholipid ratios exist, with high susceptibility to 'cold shock' (Watson, 1981) being observed in lower ratio species such as boar, bull, ram and stallion, and high resistance seen in rabbit, human, and fowl (Mocé et al., 2010). Given this information, multiple studies have been conducted around the premise of increasing cholesterol content within spermatozoa (Mocé et al., 2010). To achieve this increase, cyclodextrins molecules have been used to modify cholesterol content in spermatozoa plasma membranes (Mocé et al., 2010).

Cyclodextrins are water-soluble cyclic oligosaccharides obtained by the enzymatic degradation of starch (Singh et al., 2002). The glucose subunits (α-D-glucopyranose) connected by type α-1,4 have a hydrophobic center capable of encapsulating hydrophobic compounds (Mocé et al. 2010; Singh et al., 2002). This specific structure allows free cyclodextrin to selectively deplete cholesterol from isolated or intact membranes from a variety of cells,

including both sperm and oocytes (Companyó et al., 2007; Nagao et al., 2010; Visconti et al., 1999). The addition of methyl residues to cyclodextrins enhance their solubility in water and ability to dissolve hydrophobic compounds, with methyl-β-cyclodextrin (MβCD) being the most potent in respect to its affinity for cholesterol binding. MβCD has been used to capacitate bovine sperm, shown to increase the rate of activation and fertilization compared to control (Kato et al., 2011) and improved capacitation status as assessed by increased plasma membrane fluidity, intracellular calcium concentration, acrosome reactivity, and zona pellucida (ZP)-binding ability (Aguila et al., 2015).

Transversely, cyclodextrins can be used as a carrier molecule for the incorporation of cholesterol into plasma membranes (Amorim et al., 2009; Horvath & Seidel 2006; Purdy & Graham 2004a). Due to the binding ability of MβCD to cholesterol, it is most commonly used to pre-load sperm with cholesterol, creating a cholesterol-loaded cyclodextrin, or CLC (Mocé et al., 2010; Yancey et al., 1996). Treating bovine sperm with CLC prior to cryopreservation increases the cholesterol to phospholipid ratio of sperm plasma membranes to 0.9, reducing cold shock sensitivity and resulting in improved sperm cryosurvival rates (Mocé & Graham 2006; Purdy & Graham 2004a). It is suggested that because the CLC-treated sperm retain added cholesterol post-thaw and possess a much higher cholesterol phospholipid ratio, the sperm require additional time to capacitate due to the higher amount of cholesterol required to efflux prior to activation.

Our objective was to test the fertilization ability of CLC-treated sperm and subsequent embryo development within bovine in vitro fertilization (IVF) by offsetting sperm preparation timing in order to adjust for delayed capacitation due to the increased cholesterol content of CLC-treated sperm. Additionally, treatment with MβCD was utilized as a capacitation inducing agent by causing an efflux of cholesterol from the sperm plasma membranes during the sperm

preparation incubation period. Based on these interactions, our goal was to determine if significant differences in embryo production rates would be observed in bovine IVF using CLC-treated sperm with altered post-thaw preparation timing, and treatment of MβCD as a capacitation agent prior to co-incubation with oocytes.

3.2 Materials and Methods

3.2.1 *Animal Handling*

Cattle handling and semen collection followed the National Association of Animal Breeders Management Guidelines regarding bull care. Semen was collected offsite, thus IACUC approval wasn't required by CSU. Rocky Mountain Sire Services has good standing with Certified Semen Services, Inc. (a subsidiary of NAAB). The 10 bovine ejaculates used for this study were provided by Select Sires, Inc. (Plain City, OH) and collected by RMSS (Bennett, CO 39°44'23°N 104°31'36°W, Climate Zone 4) from beef cattle breeds housed outside in the same facility. Collections were performed in December during breeding soundness examinations.

3.2.2 *Chemicals, Reagents, & Disposables*

All research grade chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Disposables were purchased from Life Science Products (Frederick, CO) and VWR International (Radnor, PA).

3.2.3 *Preparation of Cyclodextrin-loaded Cholesterol*

In this study, cyclodextrins were used to modify sperm membranes. The procedures of Purdy and Graham (2004a) were followed. Briefly, 1 g MβCD was dissolved in 2 mL methanol, and 200 mg cholesterol dissolved in 1 mL chloroform. The solution was mixed until clear and the solvents evaporated. The resulting powder was stored at 23°C until use, and hereafter referred to as CLC. A working solution of CLC was prepared by adding 50 mg of cholesterol-

loaded cyclodextrin to 1 mL of Tyrode's albumin lactate pyruvate (TALP) (Graham et al., 1986) following the procedures of Purdy and Graham (2004a), and mixed thoroughly to create a CLC concentration of 50 mg/mL (345 mOsm).

3.2.4 *Semen Collection and Cryopreservation*

One ejaculate from ten bulls was collected by artificial vagina (AV) method (Alexander 2014). All ejaculates had a minimum concentration of 1 x 10^9/mL, >70 % normal morphology, and greater than 60% motile sperm. Ejaculates were transported to the lab immediately and then, prior to cryopreservation, each ejaculate was split into 2 aliquots. CLC treatment and cryopreservation of sperm were conducted as described by Mocé & Graham (2006). One aliquot served as the control, and the other was treated with 2 mg CLC/120x10^6 sperm. Both aliquots were incubated for 15 min at 22°C, then diluted with 20% egg yolk-Tris diluent to a concentration of 100 x 10^6 sperm/mL and cooled to 5°C over 2 hours. Once cooled, aliquots were diluted 1:1 (vol:vol) with 14% glycerolated egg yolk-Tris diluent (final glycerol concentration 7%), loaded into 0.5 mL straws, frozen in nitrogen vapor 4 cm above the surface of liquid nitrogen for 15 min, and plunged into liquid nitrogen for storage at -196°C until thawing.

3.2.5 *Ovary Collection and Oocyte Recovery*

Ovaries were collected from a local abattoir within two hours of slaughter between March and April. Ovaries were transported within two hours of slaughter in an insulated container with approximately 500 ml saline stored at 25-30°C. Cumulus oocyte complexes (COCs) were vacuum aspirated using an 18-gauge needle attached to a vacuum pump set to 50mm Hg, (Pioneer Pro Pump, Life Global Group, LLC, Guliford, CT, USA) from follicles 2-8 mm in size within 3-4 hours of ovary collection. Once collected, COCs were washed in HCDM-

M, a Colorado State University (CSU) chemically defined medium for handling oocytes, several times to remove debris before being placed into 4 well dishes containing 1ml of CSU chemically defined media for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h. COCs with at least 3 visible cumulus layers were included and visibly degenerate COCs were discarded.

3.2.6 *In Vitro Maturation, Fertilization, and Culture*

Selected COCs placed into wells containing IVM were supplemented with 1 µl/ml follicle stimulating hormone, 1 µl/ml luteinizing hormone, 1 µl/ml epidermal growth factor, 10 µl/ml estradiol, and 10 µl/ml cysteamine. Each well contained a maximum of 50 COCs cultured at 38.5°C in 5% CO_2 for 23 ± 1 h.

After 23 ± 1 h maturation, oocytes were transferred in approximately 20µl medium from IVM dishes to 4 well plates pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h containing 430µl of CSU chemically defined medium for in vitro fertilization (FCDM). Semen was selected from three sires proven successful for in vitro fertilization (IVF). Frozen-thawed semen was washed to isolate motile sperm through a 45/90 Percoll® gradient (Parrish et al., 1995) at 800 g for 20 min. The supernatant was removed, washed again in 2 ml HCDM-1, a CSU chemically defined medium for early embryo handling, and centrifuged for 5 min at 300 g. Sperm was resuspended in HCDM-1 with post-thaw progressive motility after washing ranging from 85-95% assessed using light microscopy at 200X magnification, and quantified using a hemocytometer in preparation for the use in IVF. Preparations of each semen treatment were split into one aliquot for non-treatment and one to be treated with 2mg/mL methyl-β-cyclodextrin in HCDM-1 solution. All four treatment groups (Control, CLC, Control + MβCD, and CLC + MβCD) were incubated in 5% CO_2 and air for 90 minutes prior to addition to the

oocytes. Preparation of sperm was completed 90 minutes ahead of the 23 h mark of oocyte maturation to ensure maturation timing was considered for both the spermatozoa and oocytes at time of fertilization. Sperm were added to COCs to a final concentration of 0.5×10^6 sperm/ml, and co-incubated in a final volume of 500 µl FCDM for 18 h at 38.5°C and 5% CO_2 air.

After 18 h of co-incubation, presumptive zygotes were mechanically stripped of cumulus cells using a Vortex-Genie® 2 (Scientific Industries, Inc., Bohemia, NY, USA) after transfer into a 1.5 ml microcentrifuge tube with approximately 60 µl of media. After vortexing at maximum speed for 60 seconds, zygotes were promptly added to HCDM-1 and washed through multiple drops until all debris was removed. Once clean, presumptive zygotes were transferred to 4 well dishes pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h in wells of 500 µl of CSU chemically defined medium for in vitro culture of early embryos (CDM-1) and cultured in groups of 35 ± 5. Embryo cleavage was recorded after 56 h of culture by transferring zygotes into warmed CSU chemically defined medium for embryo handling (HCDM-2) for assessment. Zygotes containing ≥4 blastomeres were transferred into dishes pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h with wells containing 500µl CDM-2, the CSU chemically defined medium for in vitro culture of late embryos, to be cultured for an additional 96 h in 5% CO_2, 5% O_2, 90% N_2 at 38.5°C. The maximum number of embryos per well was 35. Embryos were assessed for blastocyst formation on day 7 (96 h).

3.2.7 *Statistical Analysis*

The percentages of cleaved embryos reaching the blastocyst stage were determined from 4 replicates, each containing (30±5) oocytes/treatment, for semen from 3 bulls. The means and SE for these parameters are presented (Figure 3.1). Percentage data were transformed (arcsin) and treatment differences, bull differences and treatment x bull interactions determined by

ANOVA. Treatment differences were separated using SNK. Additional Chi-square and data variability analyses were performed using Graph-Pad Prism 9.2 (GraphPad Software, La Jolla, CA, USA).

3.3 Results

Between all 4 groups, a total of 1,641 COCs were fertilized, with per treatment group having a mean ± SEM (410±1) with each containing (30±5) oocytes/treatment well. There was no significant difference in cleavage or blastocyst stage formation between control and CLC sperm (P>0.05), indicating there was no difference in embryo production between oocytes fertilized with control or CLC sperm (Figure 3.1).

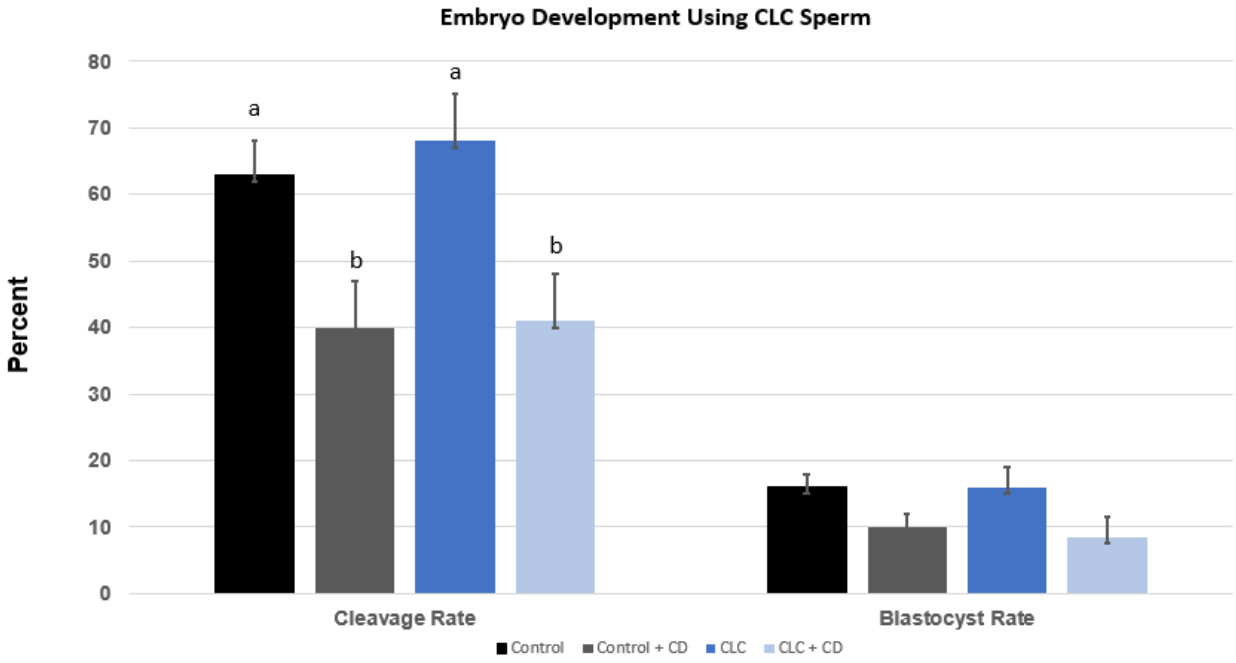

Figure 3.1 Percentages of oocyte cleavage and percentages of oocytes that reached the blastocyst stage fertilized with sperm from 3 bulls cryopreserved without treatment (Control) or treated with cholesterol by 2mg/ml cholesterol-loaded cyclodextrin (CLC) prior to freezing. At thaw sperm was prepared either without further treatment or treated with 2mg/ml of methyl-β-cyclodextrin (CD) to induce sperm capacitation, then added to oocytes in vitro 90 minutes after sperm preparation. Means associated with different superscripts are significant (P<0.05). N=12

Significant decrease in number of embryos cleaved was observed when sperm used for insemination was treated with 2 mg/mL MβCD (*P*<0.05), but there was no significant difference

between groups at blastocyst stage development (P>0.05) (Table 3.1). These results indicate MβCD treatment of sperm at 2mg/mL provided no benefit to embryo production, but rather hindered cleavage percentages.

Table 3.1 Development of in vitro produced (IVP) bovine embryos produced from cumulus oocyte complexes (COC) collected from abattoir ovaries fertilized with control, control treated with methyl-β-cyclodextrin (CD), CLC, or CLC treated with methyl-β-cyclodextrin (CD). Blastocyst development per COC and per cleaved embryo was calculated on number of embryos on Day 7.

End Point	Control	Control +CD	CLC	CLC + CD
Cleaved Embryo (%)	271/411 (65.9%)[a]	162/416 (38.9%)[b]	275/405 (67.9%)[a]	171/409 (41.8%)[b]
Blast per COC (%)	68/411 (16.5%)	34/416 (8.2%)	68/405 (16.8%)	34/409 (8.3%)
Blast per Cleaved (%)	68/271 (25.1%)	24/162 (21.1%)	68/275 (24.7%)	34/171 (19.9%)

[ab] Within rows, superscripts differ to indicate significance (P< 0.05, Chi-square test).

3.4 Discussion and Conclusion

Our study concluded there was no difference in embryonic development by using CLC treated sperm when pre-incubated for 90 minutes prior to fertilization of oocytes. There was no significant difference (P>0.05) between control and CLC sperm in the rates of cleavage and blastocyst stage of formation, which is an indicator that the 90-minute pre-incubation period was sufficient to allow for the CLC-treated spermatozoa to capacitate on a delayed schedule. When treated with MβCD to induce cholesterol efflux, there was a significant decline in embryo development at the cleavage stage (P>0.05) suggesting the cyclodextrin reduced fertilization, most likely due to sperm capacitation occurring outside of the optimum window for oocyte fertilization. Another potential contributing factor of reduced cleavage could be the small amount of MβCD that was transferred within the fertilization media at insemination which may have a negative effect on the oocytes. This could have resulted in cholesterol efflux from the oocytes within the wells, causing decreased fertilization from effected oocyte quality. Further studies on

the secondary effects of MβCD on oocytes from exposure within transferred fertilization medium would need to be completed to determine if such exposure results in oocyte toxicity or other negative effects on the oocyte.

Previous work reported the use of control and CLC-treated sperm in bovine IVF displayed similar percentages of oocytes cleaved and embryos developed to the blastocyst stage when sperm was inseminated after incubation for 60 minutes prior to insemination or immediately after preparation (Purdy & Graham 2004b). These findings support our results using control and CLC-treated sperm with 90-minute incubation prior to insemination, however this also indicates that the 90-minute period was not optimal for sperm capacitation to result in increased embryo production. The 90-minute time period either did not allow enough time for cholesterol to be removed from the plasma membrane of sperm, or allowed too much time to pass resulting in a missed optimum capacitation window to observe improved embryo production. More likely, the additional cholesterol inserted into the plasma membrane by cholesterol-loaded cyclodextrin before freezing is a permanent fixture which may interfere with capacitation timing, however, has a negligible effect on fertilization capability within standard in vitro co-incubation timing.

Aforementioned studies concluded that cholesterol-loading prior to freezing is beneficial to cryosurvival of bovine sperm in both initial post-thaw viability and over duration of time, however these sperm also display delayed capacitation timing (LaVelle et al., 2022). In combination with IVF results, this data can be used to propose that CLC-treated sperm do not have relevance in bovine IVF applications, but could be more effectively utilized in fertility trials involving artificial insemination (AI). When tested in donkey, CLC treated sperm produced no significant difference in fertility (Oliveira et al., 2014), mirroring our in vitro results while other

studies displayed decreased fertility by AI when using CLC treated sperm in cattle (Purdy & Graham 2004b; Sattar et al., 2014), rams (Purdy et al., 2010), boars (Tomás et al., 2013), and horses (Spizziri et al., 2010). However, the implications of our results suggest there is a need to adjust for the delay of capacitation timing by over 90 minutes when using CLC-treated sperm for the benefit of improved cryosurvival, specifically when developing synchronization protocols for AI with hopes of increasing pregnancy and offspring yield.

In summary, when used in in vitro fertilization, bovine sperm pre-loaded with cholesterol via CLC prior to cryopreservation did not result in significant difference in cleavage or blastocyst formation ($P>0.05$) when sperm preparations were completed 90 minutes prior to co-incubation to adjust for delayed capacitation timing of CLC-treated sperm. There was significant decline ($P<0.05$) in embryo cleavage percentage in both control and CLC-treated sperm groups when MβCD was used as a capacitation inducer at 2 mg/ml. Collectively, these findings in combination with previous CLC work, establish that bovine sperm treated with CLC prior to cryopreservation results in improved cryosurvival, but no difference in embryo production when used in IVF even when delayed capacitation timing is adjusted for in sperm preparation. This indicates that the fertilization ability of the spermatozoa treated with CLC was not impaired, and important application of bovine CLC-treated sperm is not within in vitro fertilization examination. Rather, in vivo models utilizing AI synchronization timing adjusted for delayed capacitation could more effectively harness the benefit of improved cryosurvival rates, potentially resulting in the ultimate goal of increased fertility.

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CHAPTER 4: METABOLOMIC PROFILING OF SPENT CULTURE MEDIA AND USE IN INDIVIDUAL AND GROUP BOVINE IVF CULTURE CONDITIONS

4.1 Introduction

Since the birth of "Virgil," the first calf born from an in vitro produced embryo, research efforts have focused on improving existing culture systems, optimizing high-quality embryo development, and increasing the pregnancy rate from viable embryos (Brackett et al., 1982; dos Santos et al., 2021). While new, advanced technologies have emerged, many of these options are fiscally impossible for smaller laboratories to implement, leaving a disparity requiring more affordable, simple techniques to be incorporated into protocols. One of the most widely pursued research avenues to improve embryo development is advanced culture media and conditions that can be implemented in a wide range of laboratories (dos Santos et al., 2021; Reed et al., 2011; Wooldridge et al., 2019). Culture media composition should intend to mimic the environment embryos encounter in vivo within the female reproductive tract, with many attempts made to optimize in vitro culture conditions of embryos by component supplementation and recipe adjustment.

Embryos require different substrates at different stages of development. For energy generation early on, embryos mainly use pyruvate, lactate, and aspartate, and have a limited ability to metabolize glucose. Glutamine and aspartate are also options, though at the cost of energy and, consequently, cleavage rates (Gardner & Harvey, 2015). Around the time of embryonic genome activation (EGA) (prior to morulae compaction) increased biosynthesis, cell proliferation, and blastocoel expansion increases demand for ATP. After compaction, embryos then increase oxygen consumption, mitochondrial activity, and the ability to metabolize glucose and generate energy through the glycolic pathway (Gardner & Harvey, 2015). At this point

glucose can be directed to the pentose-phosphate pathway, which generates ribose chains for RNA synthesis and DNA, with NADH, which is required for the reduction of the important antioxidant glutathione (Rieger et al., 1992). Essential amino acids are required for the differentiation of the inner cell mass (ICM), while non-essential amino acids and glutamine improves blastocoel formation and hatching (Lane & Gardner, 1996; Lane & Gardner, 2007; Steeves & Gardner, 1999). Due to these very specific requirements our objective was to determine what compounds remained in spent culture media after embryos had completed culture by analyzing the metabolomic profile of spent culture media with gas chromatography-mass spectrometry (GC-MS).

A second culture condition that may have a dramatic impact on advances in in vitro bovine preimplantation embryogenesis is whether embryos are cultured individually or in groups. Interestingly, group culture appears to benefit both mono- and poly-ovulatory species (Reed et al., 2011). The primary rationale for this outcome is that group culture exposes embryos to the secretion of embryotrophic, or positive signals from companion embryos, resulting in embryos more likely to proceed developmentally (Reed 2006). The analysis of the embryo secretome in recent years has become an active area of research, and has revealed that embryos secrete various amino acids, proteins, and other metabolites (D'souza et al., 2018; Katz-Jaffe et al., 2006; Krisher et al., 2014; Lindgren et al., 2018). Considering this reception by embryos to such factors in their surrounding environment, it is plausible to assume this is why embryos cultured in groups develop more rapidly, by potentially releasing embryotrophic factors within the group culture media. Given this possibility, we cultured embryos both individually and in groups with spent culture media from a group culture donor cycle. Our goal was to determine if

individual embryos would benefit from culturing in spent media by analyzing embryo production via cleavage and blastocyst formation with time-lapse imaging.

By this means we assessed embryo production rates in response to culture in "spent" media, which is media collected after group-cultured embryo conditions from a donor IVF cycle. Both control and spent media were tested within individual and group culture. This culture protocol, in combination with the GC-MS results, provided additional insight into possible embryotrophic factors that are artifacts in bovine IVF culture media after embryo incubation.

4.2 Materials and Methods

4.2.1 *Ovary Collection and Oocyte Recovery*

Ovaries were collected from a local abattoir within two hours of slaughter between March and April. Ovaries were transported within approximately two hours of slaughter in an insulated container with approximately 500 ml saline stored at 25-30°C. Cumulus oocyte complexes (COCs) were vacuum aspirated using an 18-gauge needle attached to a vacuum pump set to 50mm Hg, (Pioneer Pro Pump, Life Global Group, LLC, Guliford, CT, USA) from follicles 2-8 mm in size within 3-4 hours of ovary collection. Once collected, COCs were washed in HCDM-M, a Colorado State University (CSU) chemically defined media for handling oocytes, several times to remove debris before being placed into 4 well dishes containing 1ml of CSU chemically defined media for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5°C in 5% CO_2 and air for $\geq$ 5 h. COCs with at least 3 visible cumulus layers were included and visibly degenerate COCs were discarded.

4.2.2 *In Vitro Maturation, Fertilization, and Culture for Donor Media*

Selected COCs placed into wells containing IVM were supplemented with 1 µl/ml follicle stimulating hormone, 1 µl/ml luteinizing hormone, 1 µl/ml epidermal growth factor, 10

µl/ml estradiol, and 10 µl/ml cysteamine. Each well contained a maximum group of 50 COCs cultured at 38.5°C in 5% CO_2 for 23 ± 1 h.

After 23 ± 1 h maturation, oocytes were transferred from IVM dishes to 4 well plates pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h containing 430µl of CSU chemically defined medium for in vitro fertilization (FCDM). Semen was selected from three single sires proven successful for in vitro fertilization (IVF). Frozen-thawed semen was washed to isolate motile sperm through a 45/90 Percoll® gradient (Parrish et al., 1995) at 800 g for 20 min. The supernatant was removed, washed again in 2 ml HCDM-1, a CSU chemically defined medium for early embryo handling, and centrifuged for 5 min at 300 g. Sperm was resuspended in HCDM-1 with post-thaw progressive motility after washing ranging from 85-95% assessed using light microscopy at 200X magnification, and quantified using a hemocytometer in preparation for the use in IVF. Sperm were added to COCs to a final concentration of 0.5×10^6 sperm/ml, and co-incubated in a final volume of 500 µl FCDM for 18 h at 38.5°C and 5% CO_2 air.

After 18 h of co-incubation, presumptive zygotes were mechanically stripped of cumulus cells using a Vortex-Genie® 2 (Scientific Industries, Inc., Bohemia, NY, USA) after transfer into a 1.5 ml microcentrifuge tube with approximately 60 µl of media. After vortexing at maximum speed for 60 seconds, zygotes were promptly added to HCDM-1 and washed through multiple drops until all debris was removed. Once clean, presumptive zygotes were transferred to 4 well dishes pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h in wells of 500 µl of CSU chemically defined medium for in vitro culture of early embryos (CDM-1) and cultured in groups of 42-50. Embryo cleavage was recorded after 56 h of culture by transferring zygotes into warmed CSU chemically defined medium for embryo handling (HCDM-2) for assessment. CDM-1 was pooled from all wells into a warmed 5 ml snap-top tube and placed into a 5% CO_2

5% O_2, 90% N_2 at 38.5°C incubator until further use. Zygotes containing ≥4 blastomeres were transferred into dishes pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h with wells containing 500µl CDM-2, the CSU chemically defined medium for in vitro culture of late embryos, to be cultured for an additional 96 h in 5% CO_2, 5% O_2, 90% N_2 at 38.5°C. The maximum number of embryos per well was 35. Embryos were assessed for blastocyst formation on day 7 (96 h). CDM-2 was pooled into a warmed 5 ml snap-top tube and placed into a 5% CO_2, 5% O_2, 90% N_2 at 38.5°C incubator for later use.

4.2.3 *In Vitro Maturation, Fertilization, and Culture for Spent Media Application Individually and in Groups:*

Selected COCs were placed in a 4 well dish containing 1 ml IVM supplemented with 1 µl/ml of follicle-stimulating hormone, luteinizing hormone, and epidermal growth factor, and 10 µl/ml of estradiol and cysteamine. Each well contained a maximum group of 50 COCs for culture at 38.5°C in 5% CO_2 for 23 ± 1 h.

After 23 ± 1 h maturation, oocytes were transferred from IVM dishes to 4 well plates pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h containing 430µl of CSU chemically defined medium for in vitro fertilization (FCDM). Semen was selected from three single sires proven successful for in vitro fertilization (IVF). The same corresponding sire that was used for the donor media was used for the spent media challenge cycle for consistency within replicates. Frozen-thawed semen was washed to isolate motile sperm through a 45/90 Percoll® gradient (Parrish et al., 1995) at 800 g for 20 min. The supernatant was removed, washed again in 2 ml HCDM-1, a CSU chemically defined medium for early embryo handling, and centrifuged for 5 min at 300 g. Sperm was resuspended in HCDM-1 with post-thaw progressive motility after washing ranging from 85-95% assessed using light microscopy at 200X magnification, and

quantified using a hemocytometer in preparation for the use in IVF. Sperm were added to COCs to a final concentration of 0.5 × 106 sperm/ml, and co-incubated in a final volume of 500 µl FCDM for 18 h at 38.5°C and 5% CO_2 air. The culture method remained constant between the donor media cycle and the spent media challenge cycle until the vortex stage due to the lack of incubation capability relative to gas content in the single culture incubation system.

After 18 h of co-incubation, presumptive zygotes were mechanically stripped of cumulus cells using a Vortex-Genie® 2 (Scientific Industries, Inc., Bohemia, NY, USA) after transfer into a 1.5 ml microcentrifuge tube with approximately 60 µl of media. After vortexing at maximum speed for 60 seconds, zygotes were promptly added to HCDM-1 and washed through multiple drops until all debris was removed. Once clean, presumptive zygotes were split into two groups: group culture and single culture. Group culture embryos were cultured in groups of 50 embryos in 4 well dishes pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h with each well containing 500 µl control CDM-1 or wells of 500 µl of spent CDM-1 that was collected from the donor IVF cycle. Both populations of embryos were cultured in the same conditions for 56 h at 38.5°C in 5% CO_2, 5% O_2, 90% N_2. Embryo cleavage was recorded after 56 h of culture by transferring zygotes into warmed HCDM-2 for assessment. Zygotes containing ≥4 blastomeres from the control media wells were transferred into wells containing 500 µl of CDM-2 control equilibrated medium, and zygotes containing ≥4 blastomeres from the spent CDM-1 from the donor IVF cycle were transferred into wells containing 500 µl of spent CDM-2 from the donor IVF cycle. Both treatment groups were subjected to an additional 96 h in 5% CO_2, 5% O_2, 90% N_2 at 38.5°C. The maximum embryo quantity per well was 35 embryos. Embryos were assessed for blastocyst formation on day 7 (96 h).

For the single culture group, the presumptive zygotes were transferred after vortexing into a prepared CultureCoin®, which is the embryo culture dish for use in the MIRI®TL an Esco Medical Time-Lapse incubator. CultureCoins® were prepared according to manufacturer instructions by off-gassing the dishes for 12 h prior to loading the pre-warmed dishes with approximately 25 µl of pre-equilibrated CDM-1 to each well, and promptly adding 2.5-3 ml Ovoil™ (Vitrolife). These dishes have individual culture wells for 14 embryos per dish. Following completion of the preparation of the dishes the presumptive zygotes were loaded into dishes containing either control CDM-1 or spent CDM-1 from the donor IVF cycle. Coins were placed into the MIRI® TL incubator for 56 h at 38.5°C in 5% CO_2, 5% O_2, 90% N_2. Embryo cleavage was recorded after 56 h of culture. Zygotes containing ≥4 blastomeres from the control media dishes were transferred into CultureCoins® prepared with control CDM-2 medium, and zygotes containing ≥4 blastomeres from the spent CDM-1 from the donor IVF cycle were transferred into CultureCoins® prepared with spent CDM-2 from the donor IVF cycle. Loaded CultureCoins® were placed into the MIRI®TL incubator for 96 h at 38.5°C in 5% CO_2, 5% O_2, 90% N_2. Embryos were assessed for blastocyst formation on day 7 (96 h). For visual representation of this timeline, see Figure 4.1. For each replicate, 3 dishes per treatment group were cultured, with a maximum number of 42 embryos per treatment per cycle.

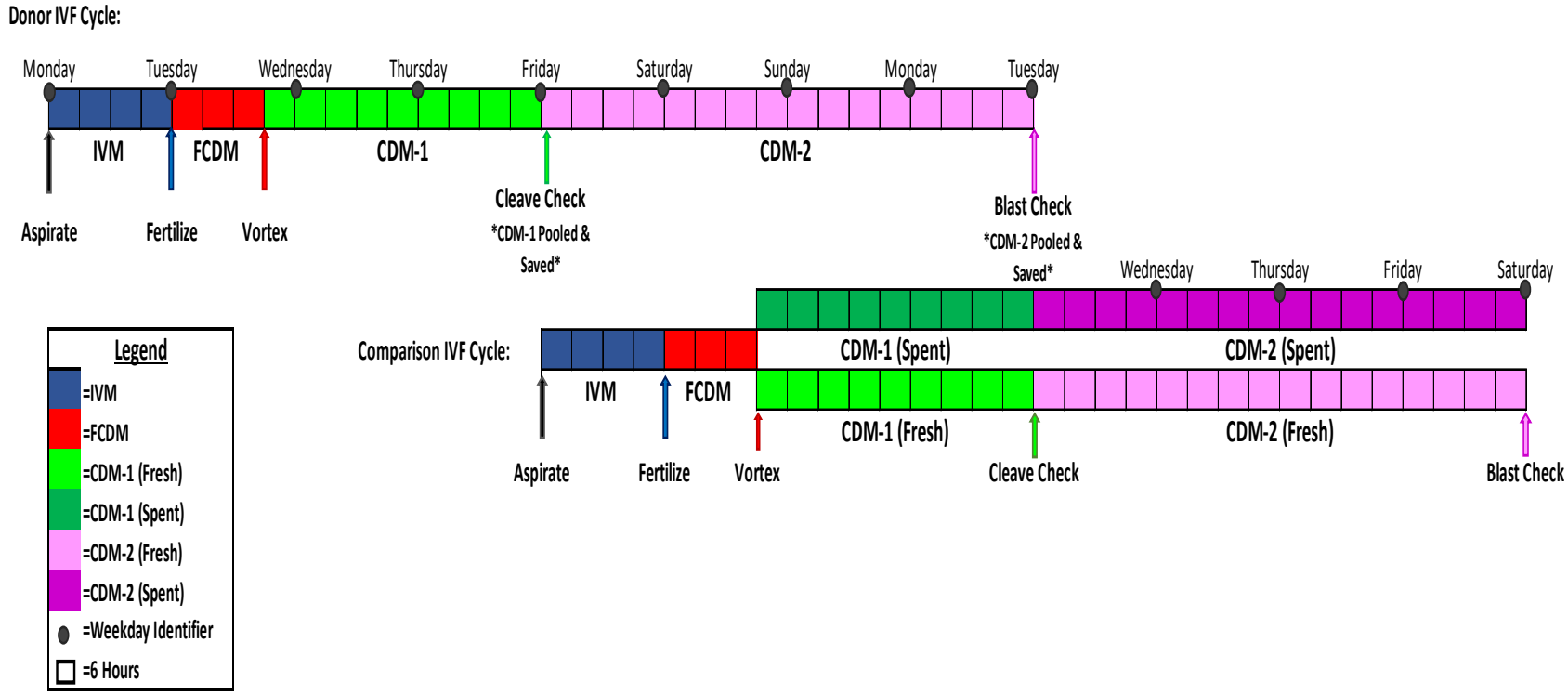

Figure 4.1 The timeline of the donor IVF Cycle and the spent media IVF cycle are displayed. This image is to scale based on time with overlay of cycle timing depicted. Each color represents the type of culture media used at specific time points, with blue indicating IVM or maturation media, red indicating FCDM or fertilization media, green indicating CDM-1 (fresh being light green and spent being dark green) or early culture media from vortex to cleavage check, and CDM-1 (fresh being light purple and spent being dark purple) or late culture media from cleavage to blastocyst formation. Each square represents 6 hours of time, and each day is also indicated.

4.2.4 *Embryo Production Statistical Analysis*

Embryo cleavage and blastocyst production were analyzed with a Fisher's Exact test. P-values of <0.5 were considered significant. All statistics were generated using Graph-Pad Prism 9.2 (GraphPad Software, La Jolla, CA, USA).

4.2.5 *Gas Chromatography-Mass Spectrophotometry (GC-MS)*

Remaining sample volumes from both pooled culture mediums, CDM-1 and CDM-2 from the donor IVF cycles, as well as control samples of CDM-1 and CDM-2 were frozen in a cryovial at 40°C until analysis. Analysis was performed by the Colorado State University Analytical Resources Core Facility (RDI:SCR_021758).

4.2.6 *Sample Preparation*

Samples (100 µl) were mixed with cold 100% methanol (400 µl), vortexed, and incubated at -20°C overnight. Supernatant (200 µl) was recovered following centrifugation at 3000 g and 4°C for 10 min, transferred to a vial containing 1 ml of internal standard solution, and subjected to solvent evaporation under nitrogen until dry. Aliquots of 100 µl supernatant from each sample extract were combined to generate a quality control (QC) sample, which was then split into multiple QCs, each 200 µl. The QCs were mixed with 1 ml of IS and processed as was the experimental samples. The IS solution contained 6.3 µg/ml alanine-13C3, 0.63 µg/ml phenylalanine-13C6, 0.32 µg/ml fumaric acid-13C4, 3.2 µg/ml tryptophan-13C11, 0.32 µg/ml indole-3-acetic acid-13C6 in methanol/acetonitrile/water (4.5/4.5/0.5 by vol).

4.2.7 *Media Data Acquisition*

The dried sample extracts were resuspended in 50 µl pyridine containing 25 mg/ml of methoxyamine hydrochloride, incubated at 60°C for 45 min, vigorously vortexed for 30 s, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Fifty microliters of N-

methyl- N-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) were added, and samples were vortexed for 30 s, and incubated at 60°C for 30 min. Metabolites were detected with a Trace 1310 GC coupled to a Thermo ISQ mass spectrometer. Samples (1µl) were injected at a 10-1 split ratio to a 30 min TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 mm film thickness) with a 1.2 ml/min helium gas flow rate. GC inlet was held at 285°C. The oven program starts at 80°C for 30 s, followed by a ramp of 15°C/min to 330°C, and an 8 min hold. Masses between 50-650 m/z are scanned at 5 scans/sec under electron impact ionization. Transfer line and ion source are held at 300 and 260°C, respectively. QC samples were injected after every 6 experimental samples.

4.2.8 *Media Data Analysis and Statistics*

ADAP (Smirnov et al., 2019) was used for processing GC-MS data, including signal detection, deconvolution, alignment, and integration. Spectra were exported as .msp formatted data and imported to ADAP-KDB (Smirnov et al., 2021). Annotations were subsequently exported. The aligned dataset and annotations were imported into R for statistical analysis and further annotation. A minimum match score of 700 was required for spectral matching to consensus KDB spectra to be considered annotated. Compounds were assigned to chemical ontogenies with the ClassyFire (Djoumbou-Feunang et al., 2016) API. Principle Component Analysis was performed in R. Pubchem data was retrieved for all annotated metabolites with the Pubchem REST API (Kim et al., 2019). The SpecAbund dataset was used as input with scaling set to pareto. The number of principle components was selected using the AuerGervini method from the ClassDiscovery R package. Linear model ANOVA was performed for the factor(s) to provide some guidance on which PCs appear responsive to factors of interest. These are not meant to be rigorous statistical tests but to help guide interpretation of the data. Outlier tests are

performed on PC loadings to serve as a guide in interpreting which compounds contribute most to the observed separation. This is performed with the R pnorm function. Returned p-values are false discovery rate corrected. These p-values are not used to conclude that a compound is significantly changing, but rather to indicate that a compound disproportionately contributes to the multivariate sample separation observed for that PC. Analysis of variance was performed in R. The SpecAbund dataset was used as input. For the analysis of variance titled 'media', the SpecAbund dataset was used as input. Fixed-factor linear model ANOVA was performed using the lm function. The model used was ‘media'. P-value correction was performed with the p.adjust function with method set to BH. Post-hoc testing was performed for classes within ‘media’ via the 'Tukey' method in the lsmeans package. Effects plots are generated with the allEffects function.

4.3 Results

4.3.1 *In Vitro Embryo Production*

Embryo cleavage was assessed on day 3 for control and spent donor treatments. There was no significant difference (P>.05) in embryo cleavage between media types for both the single and group cultured embryos (Figure 4.2). Blastocyst formation was assessed on day 7 for control and spent donor media treatments. This also resulted in no significant difference (P>05) between media types of both single and group cultured embryos (Figure 4.2). The mean cleavage and blastocyst formation rate from the spent media donor cycle replicates was 78% and 13% respectively.

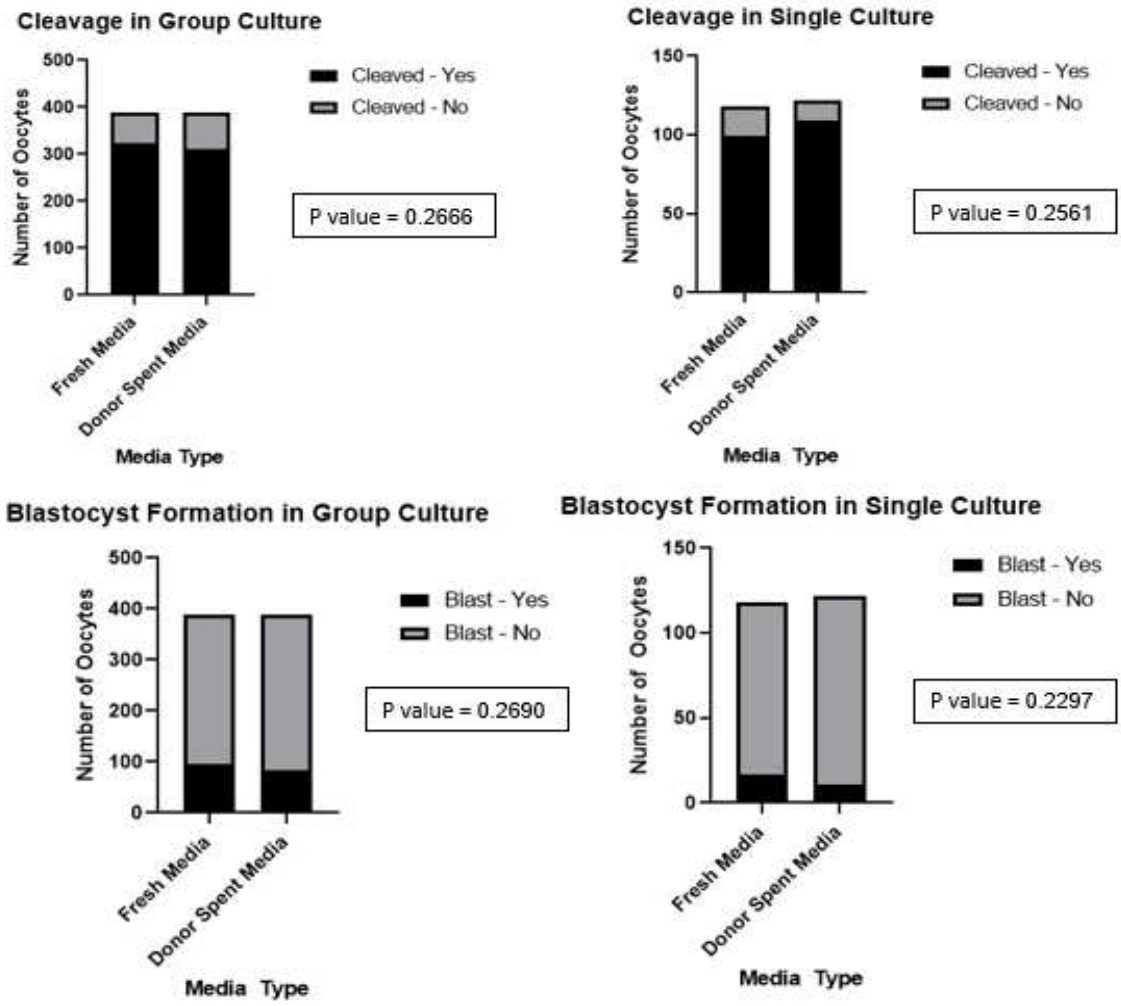

Figure 4.2 Fisher's Exact test was used to test significance between the two media types used (Fresh and Donor Spent Media) looking at embryo development. Embryo cleavage was assessed on day 3 and blastocyst formation was assessed on day 7. There was no significant difference between media types at cleave or blast check ($P > 0.05$).

This finding indicates that the donor spent media neither aided nor impeded the development of embryos in either the group or single culture environment when assessing cleavage and blastocyst formation rates.

Recorded data from the MIRI®TL incubator on the single cultured embryos allowed for annotated data from the development of each embryo via time-lapse video. Annotation of each well (containing 1 embryo) was completed by recording timed events t2 (first cleavage, 2-cell), t3 (3-cell), t4 (4-cell), t5 (5-cell), t6 (6-cell), t7 (7-cell), t8 (8-cell), morula formation, blastocyst

formation, and expansion. The average time point for each event was calculated first between CultureCoins® dishes, and averaged for each event time across treatment groups of control and spent donor media. Table 4.1 displays these data, which showed no significant difference (P>.05) between control and spent media for any events.

Table 4.1 displays them mean time in hours ± SEM for each event for both fresh and spent media within the single culutred embryos within the MIRI® TL system. A Mann-Whittney test was used to determine there was no significant difference (P>.05) of timing between events based on treatment group.

Mean Time Point for each Event		
	Fresh Media	**Spent Media**
t2	38.5±0.78[a]	38.8±0.76[a]
t3	43.5±0.95[a]	45.2±0.93[a]
t4	47.5±1.11[a]	50.5±1.10[a]
t5	51.8±1.06[a]	55.0±1.38[a]
t6	57.0±1.43[a]	59.5±1.49[a]
t7	63.0±1.93[a]	71.9±2.46[a]
t8	70.7±2.20[a]	81.6±2.59[a]
Morula	144.1±2.64[a]	146.5±3.05[a]
Blastocyst	173.5±1.61[a]	174.45±2.42[a]
Expansion	174.9±5.89[a]	179.0±0.01[a]

This result indicates that the embryos cultured in spent media developed in synchrony with the embryos exposed to control media without displaying delayed development or a hindrance to event timing.

4.3.2 *GC-MS Metabolomic Analysis*

A non-targeted metabolomics approach was used to analyze the metabolomic profile of both control and spent donor cycle culture mediums for CDM-1 and CDM-2. This method allowed for compounds within the control media to be compared with those within the spent

media to determine what differences, if any, were displayed between the control media and the treatment group (spent media). For the CDM-1 media, only one compound was found to be significantly different (P<.05), 2-oxopropanoic (pyruvic) acid. The control CDM-1 media contained more pyruvic acid than did the spent media (Table 4.2).

Table 4.2 displays the significantly different (P<.05) compounds located within fresh vs spent CDM-1 media by GC-MS analysis.

Chemical Compounds within CDM-1 Culture Media		
Annotation (Compound #)	***Prevalence In Media***	***P Value***
2-Oxopropanoic (Pyruvic) Acid (C024)	Control Media > Spent Media	0.026

For the CDM-2 media, a total of 7 identified compounds were determined to be significantly lower (P<.05) in the control media than in the spent media, and three identified compounds were higher (P<.05) within the control media compared with the spent group (Table 4.3).

Table 4.3 Significantly different (P>.05) compounds located within fresh vs spent CDM-2 media by GC-MS analysis. A total of 3 compounds (L-methionine, pyruvic acid oxime, and 2-ketoisocaproic acid) were determined to be higher in the control media compared with the spent media, while a total of 7 compounds were determined to be lower in the control compared with the spent media including L-isoleucine, L-threonine, two identified forms of L-lysine, 3-pyridnol, 2-oxoproline, and ethanalomine.

Chemical Compounds within CDM-2 Culture Media		
Annotation (Compound #)	***Prevalence In Media***	***P Value***
L-Isoleucine (C033)	Control Media < Spent Media	0.006
L-Threonine (C041)	Control Media < Spent Media	0.007
L-Lysine (C029)	Control Media < Spent Media	0.003
L-Lysine (C105)	Control Media < Spent Media	0.021
3-Pyridinol (C075)	Control Media < Spent Media	0.002
2-Oxoproline (C031)	Control Media < Spent Media	0.043
Ethanolamine (C062)	Control Media < Spent Media	0.020
L-Methionine (C134)	Control Media > Spent Media	0.000
Pyruvic Acid Oxime (C027)	Control Media > Spent Media	0.047
2-Ketoisocaproic Acid (C132)	Control Media > Spent Media	0.006

The seven compounds lower in the control group compared with the treated group include L-isoleucine, L-threonine, two identified forms of L-lysine, 3-pyridnol, 2-oxoproline, and ethanalomine. Three compounds found to be higher in the control media compared with the spent media include L-methionine, pyruvic acid oxime, and 2-ketoisocaproic acid.

4.4 Discussion and Conclusion

Our results indicated no difference in embryo production at the cleavage- and blastocyst-stages in the control versus spent media both in single and group culture. This result is an important finding because isolating the variable of culture media for analysis indicates that the spent media did not impair or improve embryo production, but rather had no impact. While cleavage rates were similar, (83% control and 80% spent media within group culture compared with 84% control and 89% spent media for single culture), group cultured embryos displayed better rates of blastocyst formation (25% control and 21% spent for group culture compared with 14% control and 9% spent for single culture). In view of these results, we believe the potential embryotrophic factors that resulted in improved blastocyst formation during group culture may have been provided by the companion embryos in a form of close and direct relation cross-talk, rather than the chemical compounds left behind in spent media. Factors that contribute to embryo interactions within group culture, outside of the media, include but are not limited to the ratio of media volume to the number of embryos (embryo density), embryo spacing/contact, and quality of companion embryos (Reed et al., 2011). The embryos within group culture had an estimated media-to-embryo ratio of 14.3 µl/embryo, whereas the single cultured embryos had 25 µl, suggesting the single embryo density may also be lower than optimum.

Embryo development timing between zygotes cultured in control and spent media showed no significant difference ($P>.05$), which, in combination with similar cleavage and

blastocyst formation, suggest it is plausible that the spent media still contained enough nutritional value for embryos to develop with similar timing to those in control media. This suggestion perhaps also indicates over compensation of nutritional compounds within the original media recipe. In mice, it has recently been shown that the reduction of carbohydrate, amino acid, and vitamin concentrations could be reduced up to 50% of standard nutrient provision without detrimental effects (Ermisch et al., 2020). Additional work suggests that the uptake of nutrients by embryos of multiple species (murine, bovine, porcine, and human) is significantly less than what is supplied in traditional culture media (Krisher et al., 2015). In another recent bovine IVF study, it was demonstrated that a novel culture media containing the amount of carbohydrate and amino acids found in the reproductive tract supports bovine embryo development, and reduction in nutrient availability improved embryo production and morpho-physiological parameters (dos Santos et al., 2021). Together with our results these findings contribute to the validity of the concept of over-supplementation within current bovine IVP media, thus requiring further investigation to consider the present terms of supplementation as best practice in vitro.

As it is typically in cellular metabolism, glucose is metabolized through glycolysis to pyruvate, which then enters into the tricarboxylic acid (TCA) cycle, where it is then oxidized to produce ATP. The fate of glucose and the carbohydrates derived from it, pyruvate and lactate, have historically been the focus of study for embryo metabolism (Krisher & Prather, 2012). Perception maintains that lactate and pyruvate are used in early embryonic development, while glycolysis increases during blastocyst formation, resulting in higher glucose uptake as a signature in more viable embryos (Gardner et al., 2001; Gardner & Leese 1987). Control CDM-1 media contained significantly more ($P<.05$) pyruvic acid than the spent media counterpart, which

agrees with the consumption of pyruvate in early embryo development for energy from the culture media.

Compounds that displayed a significant decrease ($P<.05$) in CDM-2 media included L-methionine, 2-ketoisocaproic acid, and pyruvic acid. As in the CDM-1 media, the same depletion of pyruvic acid was observed, which may be a negative indication of pregnancy outcomes if those embryos would have been transferred. New findings suggest media from expanded blastocysts resulting in pregnancy display a greater concentration of pyruvate and a lesser concentration of lactate than media from blastocysts that did not establish pregnancy (de Oliveira Fernandes et al., 2021). Moreover, pyruvate in culture media of blastocysts predicted pregnancy with 90.9% sensitivity and 75% specificity (de Oliveira Fernandes et al., 2021), which underscores the relevance of this compound within spent culture media analyses.

In addition, depletion of L-methionine was observed. This essential amino acid is the precursor to other amino acids, such as cysteine and taurine, as well as the important antioxidant glutathione. In relation to embryonic development, methionine has been shown to be transported into one or more cellular compartments of the preimplantation embryo (Gopichandran & Leese 2003; Guyader-Joly et al. 1997; Kuran et al. 2002), and may be particularly important during the preimplantation period because, in addition to serving as a precursor for polypeptides, it plays a role in regulation of translation, DNA methylation, and antioxidant balance (Métayer et al., 2008). When culture media was treated with various concentrations of methionine, a concentration greater than 7 μmol/l did not improve development at the blastocyst stage; however, more blastocysts were classified as advanced on Day 7 when cultured with ≥ 21 μmol/l methionine compared with embryos cultured with 7 or 14 μmol/l (Bonilla et al., 2010). These findings align with why our results displayed lower concentrations of L-methionine in the spent

media. The presumption is that the developing embryos from the donor cycle most likely consumed the compound during the aforementioned embryonic development process.

Compounds that appeared to have significantly ($P<.05$) higher concentration in the CDM-2 spent media compared with control media include L-isoleucine, L-threonine, L-lysine, 2-oxoproline, 3-pyridnol, and ethanolamine. These are components of various cellular metabolic activities, including L-isoleucine as a substrate for protein synthesis and a regulator of protein metabolism in mammals (Teodoro et al., 2012), 2-oxoproline is an intermediate in glutathione metabolism (Palekar et al., 1974), and involvement of ethanalomine in phospholipid synthesis in mammalian cells via the CDP-ethanolamine pathway (Bleijerveld et al., 2004). Of compounds that increased, the most intriguing findings include L-threonine and L-lysine. L-threonine has been shown to contribute to inner cell mass (ICM) cell pluripotency by acting as a transceptor, and allows for formation of H3K4me (Formisano & Van Winkle 2016; Ryu & Han 2011; Van Winkle et al., 2014; Van Winkle & Ryznar 2019; Wang et al., 2009), while L-lysine has been shown to contribute to ICM cell proliferation by glutamate formation (Shiraki et al., 2014, Van Winkle et al., 2020). This result suggests that L-threonine and L-lysine were produced by embryos within culture between cleavage and blastocyst formation, creating potential embryotrophic additions to the media. While there was not an increase in the embryo production rate between control and spent media, these embryotrophic compounds may have contributed to the similar results in cleavage and blastocyst formation of the embryos cultured in control versus used spent media.

In conclusion, when individual and group embryos were cultured in control and spent media from a donor cycle there was no difference in embryo production between media treatments. Between individual and grouped embryos, there were similar cleavage percentages

between control and spent media with 83% in the control and 80% in the spent group-cultured population, compared with 84% in the control and 89% in the spent individually-cultured population. However, there was an increase in blastocyst formation in grouped embryos compared with individual embryos (25% in the control group population compared with 14% in the control individual population, and 21% in the spent media group population compared with 9% in the spent individual population). No difference ($P>.05$) was observed in embryonic developmental event timing between control and spent media within singly cultured embryos. Analysis of spent culture media indicated a decrease ($P<.05$) in pyruvic acid in CDM-1 and CDM-2 media, as well as an increase ($P<.05$) in several compounds observed in CDM-2, most importantly including L-threonine and L-lysine, which are involved in cell pluripotency of the ICM and cell proliferation by glutamate formation in the ICM. Our results suggest that while there is a presence of some potential embryotrophic compounds in the spent media, this alone was not sufficient to see significant increase in embryos cultured individually with that spent media. Even when spent media from group culture was used within a single culture system, improved embryo production was not observed, supporting the generally acknowledged rule that embryos, even of mono-ovulatory species such as cattle, have better success when cultured within groups. Further studies are necessary to determine what interactions may be occurring between companion embryos within group culture environments, and if any such concepts are replicable within an in vitro model.

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