8-2018

BioDynamic Imaging System: A novel, noninvasive method for assessing embryo viability.

Natalie Ehmke
Purdue University

BIODYNAMIC IMAGING: A NOVEL, NONINVASIVE METHOD FOR ASSESSING EMBRYO VIABILITY

by

Natalie Ehmke

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science
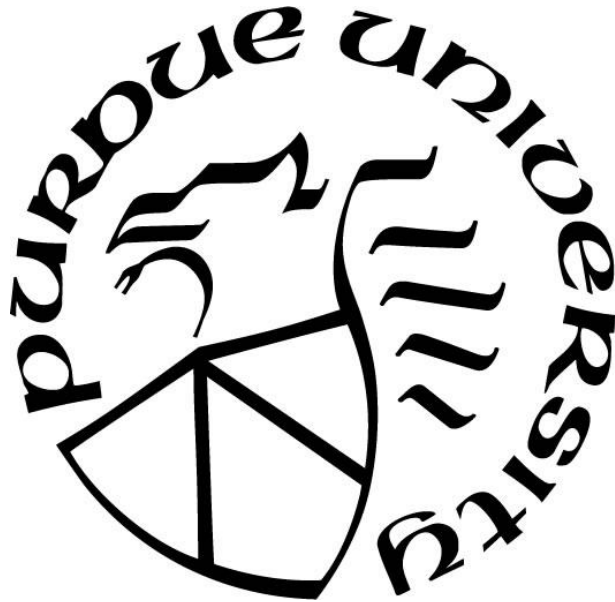
Department of Animal Sciences

West Lafayette, Indiana

August 2018

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Zoltan Machaty, Chair

    Department of Animal Sciences

Dr. Kara Stewart

    Department of Animal Sciences

Dr. Shihuan Kuang

    Department of Animal Sciences

Approved by:

    Dr. Ryan Cabot

        Head of the Graduate Program

To my family and Michael

ACKNOWLEDGMENTS

of Purdue Physics as they provided a majority of my optical understanding and were valuable team members of our interdepartmental research project. I wish you great success in the future.

To avoid rambling on in sheer gratitude, my deepest thanks and love goes to my family. My parents who had to hear me complain throughout the many years of schooling and my sister who sent me constant pictures of our dog to keep me motivated. Their love, support and constant contact is something that kept me working hard to make them proud. And finally, to Michael Savage. There is no word or statement I could make that would express the impact you have had on my academic work. Thank you for standing by me through every high and low.

"Surround yourself with talented people who will challenge you, help you grow and inspire you to maximize your potential." –John C. Maxwell

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Author: Ehmke, Natalie. MS
Institution: Purdue University
Degree Received: August 2018
Title: BioDynamic Imaging: A Novel, Noninvasive Method For Assessing Embryo Viability
Major Professor: Zoltan Machaty

In vitro embryo production is the foundation of most assisted reproductive technologies, yet efficiency of these technologies is relatively low due to the lack of a reliable method for measuring embryo viability. Current methods to evaluate the viability of in vitro derived embryos are invasive or expensive to perform. In order to create a reliable system to assess the developmental potential of the embryo, a novel optical approach known as BioDynamic Imaging (BDI) has been proposed for application. BDI is able to measure dynamic processes inside an embryo encompassing a broad range of motions that relate directly to functional developmental events and has been validated for use in the drug treatment of tumor spheroids. Due to the morphological differences between tumors and embryos, use of the BDI to interpret the intracellular motion of embryos requires the identification of new parameters that relate to viability. The embryos were exposed to several stressors (low pH, high pH, ethanol) and evaluated using BDI to evaluate the intracellular motion induced by the specific stressors. This led to the identification of slope and nyquist floor as parameters which can distinguish viable embryos. Additionally, potentially fertilized oocytes were evaluated using BDI for twenty-four hours, allowing for the capture of the cellular dynamics of fertilization. Parameter analysis shows that the BDI has the capability to record intracellular motion associated with fertilization of the oocyte, resumption of meiosis and syngamy in a 1-cell embryo.

Noninvasive techniques were utilized to identify molecular secretions in spent culture medium that are indicative of embryo quality. While proteomic analysis was not able to detect the protein leptin, metabolomic assays had some success finding amino acids leucine and glycine, yet detection levels were inadequate to consistently identify these and other amino acids. In addition, the morphology of each blastocyst was assessed and a morphological score was assigned to each embryo. They were then examined using BDI and a number of optical parameters were collected from the embryos during a 1-hour long measurement. A correlation matrix of the parameters identified a negative correlation between normalized standard deviation (NSD) and morphological score (corr= -0.58) and percent of apoptosis (corr= -0.21). Finally, an energetics assay was employed to compare the ATP concentration of healthy embryos with those that had oxidative catabolism inhibited and their ATP stores depleted. Preliminary results show that normalized standard deviation (NSD) and slope differ between the energetic groups. Successful completion of this project validates subcellular motion as a biomarker and several BDI parameters (NSD, slope, and nyquist floor) that can be utilized to predict developmental competence of embryos; this would guarantee the use of only the highest-quality embryos for transfer and lead to a significant improvement in the efficiency of assisted reproductive technologies.

INTRODUCTION

The advanced age that women are choosing to start families at has resulted in a reliance on assisted reproductive technologies (ART) to improve conception rates. While these technologies have improved conception success, they have led to an elevation in multiple fetus conception rates. Multiple birth pregnancies often stem from the transfer of several embryos into the maternal uterus in hopes that one embryo is able to implant, however this practice increases the risk of adverse health conditions in both the mother and offspring. As such there has been a recent industry trend in transferring only one embryo per ART cycle so as to reduce the number of multiple births. The current practice involves the analysis of embryos that have developed to blastocyst stage and an assessment of quality for each blastocyst with the superior embryo being selected for transfer. Unfortunately, while there exist several methods by which to analyze blastocysts all involve either indirect, objective assays or direct, subjective assays. For instance spent culture medium of individual blastocysts can be examined for specific amino acid profiles or high levels of leptin protein under the precept that these are indicators of embryo quality. A direct method of analysis involves categorizing features of blastocysts based on their morphology, giving an overall grade to each embryo. While this is more widely used in a clinical setting because it does not involve additional, expensive equipment, the practice of grading embryos based on their morphology is very subjective. Therefore, there is a need to develop a novel noninvasive method for assessing the developmental potential of blastocysts, one that involves a direct, inexpensive measurement that is objective; ensuring that the best-quality embryo is selected for transfer. The current thesis describes experiments conducted to examine such a system and subsequent method for clinical application in determining embryo quality, using porcine embryos as a model.

CHAPTER 1.   LITERATURE REVIEW

1.1   In Vitro Maturation of Pig Oocytes

In an attempt to generate ample supply of high quality oocytes for use in fertility procedures, in vitro maturation (IVM) of collected oocytes has long been an accepted practice. Pig ovaries can be collected from prepubertal gilts during harvest at local slaughter houses and through follicular aspiration, immature oocytes can be obtained. Several reports indicate that while oocytes improve in quality after each estrous a gilt experiences, the current practice of aspirating antral follicles from prepubertal gilts can be successful provided it occurs on follicles ≥3mm in diameter (Dickey et al., 2005; Driancourt et al., 1998). After follicular aspiration, oocytes should be assessed for quality based on the number of cumulus cell layers surrounding each oocyte.

Inside an antral follicle exists a complex support network of a germ cell surrounded by many layers of somatic cells. This support network requires cooperation from both the germ cell and all somatic cells for the purpose of oogenesis and follicular development (Gilchrist et al., 2013). Somatic cells of the follicle are divided into two populations; mural granulosa cells which serve an endocrine function, lining the basement membrane of the follicle wall, and cumulus granulosa cells, which surround the developing oocyte and form intimate connections with the oocyte. These connections, referred to as gap junctions are essential for maintaining a support network and the bidirectional communication between cumulus cells and the oocyte. They are composed of gap junction connexin proteins Cx43 and Cx37 and specialized trans-zonal cytoplasmic projections allowing a bridge of communication from the somatic cells, through the zona pellucida, to the plasma membrane of the oocyte (Albertini et al., 2001). These intercellular bridges allow for the passage of metabolites and molecules from granulosa cells for the

development of the oocyte, as well as secretion of factors by the oocyte to trigger somatic cell proliferation (Sanchez et al., 2012). Developing oocytes are unable to perform glycolysis or synthesize cholesterol and amino acids, therefore it is critical that their need be supplemented by delivery of these metabolites from somatic cells (Biggerset al., 1967). In addition these metabolically stunted oocytes can promote the proliferation and metabolite secretion of granulosa cells. Stimulation occurs through the secretion of several proteins of the bone morphogenic protein (BMP) family, growth determining factor 9 (GDF9) and epidermal growth factor (EGF) from the oocyte. Specifically BMP15 and GDF9 are vital for regulating mitosis in granulosa cells, by acting as a mitogenic factor and protecting against apoptosis, respectively (Sanchez et al., 2012). GDF9 promotes the transcription of follicle stimulating hormone (FSH) receptors on granulosa cells, increasing their sensitivity to circulating gonadotropins. The downstream impacts of high BMP15 and GDF9 are significant when it comes to endocrine regulation. Proliferation of granulosa cells and an increase in sensitivity to FSH will increase luteinizing hormone (LH) receptor expression in mural granulosa cells, preparing the follicle for the LH surge and subsequent ovulation. This also compounds the impacts of the two cell two gonadotropin theory, where LH stimulation promotes the production of androgens by theca cells which can be converted into estradiol by granulosa cells under the influence of FSH; estradiol then stimulates a greater response to gonadotropins in granulosa cells via positive feedback loops (Biggers et al., 1967).

In response to endocrine stimulation of somatic cells, cumulus expansion leads to control of oogenesis and meiotic regulation. Increasing response to LH in somatic cells promotes the actions of EGF secreted by the oocyte on granulosa cells. EGF induces the expansion through activating protein kinase A (PKA), resulting in an upregulation of mucification transcripts in

cumulus cells (Varani et al., 2002). Activation of PKA decreases the amount of cAMP entering the oocyte from somatic cells via gap junctions leading to an activation of the enzyme cAMP-phosphodiesterase (PDE3A). PDE3A begins to degrade intra-oocyte cAMP and cGMP levels, while continued EGF stimulus on granulosa cells closes gap junctions of the oocyte cumulus complex resulting in an activation of MAPK which will overcome the first meiotic arrest (Sanchez et al., 2012). Therefore the bidirectional communication between oocyte and cumulus cells is essential for normal oogenesis, not only through the control mechanisms for endocrine regulation but for the delicate activation of mitosis in granulosa cells and meiosis of the immature oocyte. Hence successful in vitro procedures focus on carefully selecting oocytes containing multiple layers of cumulus cells which are positively correlated with high embryonic development rates and good quality embryos (Grupen, 2014).

Following oocyte selection, additional incubation is required for optimal maturation of the oocytes to induce germinal vesicle breakdown and polar body formation. In pig oocytes, this maturation process requires 38-44 hours of incubation in an appropriate medium such as tissue culture medium 199 (TCM-199) supplemented with various compounds that enable nuclear as well as cytoplasmic maturation of the oocytes (Prather et al., 1998). A main component of TCM-199 is sodium pyruvate which is vital in culture medium. During maturation glucose metabolism begins to decline, being replaced with pyruvate metabolism and usage of citric acid cycle intermediates by both cumulus cells and the oocyte (Geshi et al., 2000). Research by Rose and Bavister (1992) proved that glucose is not utilized during oocyte maturation if oocytes have been denuded and that medium supplemented with sodium pyruvate is successful at inducing normal maturation regardless of the presence of cumulus cells. Additionally, TCM-199 contains cysteine and sodium bicarbonate. While maturation of oocytes in medium rich in cysteine can promote

higher developmental rates in embryos (Grupen, 2014), which will be discussed in the following pages, it also plays an important role in regulating the environment surrounding the oocyte along with sodium bicarbonate. Cysteine is reduced by cumulus cells to glutathione and thiols for reducing oxidative stress in in vitro cultures (Geshi et al., 2000). Bicarbonate allows for TCM-199 to have a pH buffering system, replacing phosphate used in the buffering systems of other media which can interfere with calcium regulation and cellular pH. Bicarbonate does release CO_2 during the buffering process but mitigating air exposure to the medium through a cover layer of mineral oil or paraffin ensures the maturation medium pH is kept between 7.2 and 7.4 (Alm et al., 2008). Therefore, the presence of sodium pyruvate, glucose, bicarbonate and cysteine in TCM-199 generate a pH regulated maturation medium that leads to good quality matured oocytes, and the eventual production of high developmental capacity embryos (Rose et al., 1992).

While resumption of meiosis can occur sporadically in oocytes after aspiration, the inclusion of compounds like FSH and EGF in maturation medium can increase the population of oocytes with polar bodies post maturation. FSH has been proven in IVM procedures to drive granulosa cell growth and development along with increasing aromatase sensitivity (Wu et al., 2001). FSH and EGF act as precursors for inducing oocyte luteinizing hormone (LH) sensitivity and along with a reduction in oocyte levels of cyclic AMP, activating MAPK1/3 complex to ensure progression to metaphase 2 (Yuan et al., 2017). Additional supplements such as insulin like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2), and leukemia inhibitory factor (LIF) have recently been proposed to stimulate the activation of the MAPK complex, and they often improve oocyte response to stress (Yuan et al., 2017). FGF2 and EGF supplementation has been a known promoter of oocyte and granulosa cell growth since 1998 when they were found to

have paracrine action when secreted by oocytes, propagating the development of granulosa cells (Driancourt et al., 1998). This increases the competence of granulosa cells to respond to LH, promoting oocyte development and increasing responsivity to estradiol (E2). Finally, EGF is a known inducer of nuclear maturation and FGF2 promotes germinal vesicle breakdown while both act as anti-apoptotic agents in follicular fluid, increasing the success of oocyte development in IVM (Eppig et al., 1996; Wandji et al., 1992).

1.2   In Vitro Fertilization

The practice of combining mature oocytes with spermatozoa to generate embryos is the backbone of all in vitro fertilization (IVF) protocols. IVF practices have been developed since the 1970s in nearly all livestock species, but only recently has porcine IVF been considered an acceptable alternative to in vivo embryo derivation (Grupen, 2014). The developments that have stimulated the improvement of pig IVF procedures are primarily due to the field of transgenics and the practical use of pig embryos in research. As such, advancements have been focused on improving the fertilization medium and creating a culture environment to better mimic the in vivo environment and induce physiological changes in the gametes that take place under normal conditions such as the acrosome reaction, cortical granule exocytosis and zona pellucida hardening.

Among the initial steps of fertilization is the required process of sperm head binding to the zona pellucida of the oocyte and the subsequent cellular reactions that prevent polyspermy. In porcine IVF, polyspermy occurs at exceptionally high rates, which prompted research into developing new fertilization media and techniques to prevent polyspermy (Prather et al., 1998). The best solution to the staggeringly high polyspermy rates of porcine IVF lies in fertilization medium that can mimic the biological safeguards in vivo oocytes maintain. These safeguards

begin when proteins on the acrosome intact sperm head bind the zona pellucida. The zona pellucida is a glycocalyx-like structure surrounding the plasma membrane of the oocyte containing polysaccharide ligands and three to four glycoproteins. These glycoproteins, referred to as "zona pellucida glycoproteins" (ZP) and individually numbered, are taxon-specific to capacitated sperm, preventing cross species fertilization and consist of nearly 95% of the structure's protein content (Bleil et al., 1980). The function of these individual glycoproteins is also species-specific; for instance, in humans there appears to be cooperation between ZP2, ZP3, and ZP4 at binding the acrosome cap of capacitated sperm while in mice ZP3 is the primary protein that binds the acrosome cap (Gupta, 2015). The binding of one or multiple of these glycosylated ZPs to capacitated, acrosome intact spermatozoa initiates the cascade of fertilization events.

The acrosome reaction occurs immediately after sperm head interaction with the zona, prompting the release of acrosomal vesicles from the sperm to digest through the zona pellucida. Specifically these vesicles contain acrosin, a proteinase which is located in the acrosomal cap as inactive proacrosin (Howes et al., 2001). This pathway has been detailed in mouse gametes where binding of ZP3 to the outer membrane of the acrosomal cap allows for clustering of receptor complexes, activating digestion of proacrosin to acrosin and permitting the exocytosis of vesicles (Wassarman, 1999). Acrosin then acts as the secondary binding protein for spermatozoa as it ionically binds to ZP2, adhering the sperm head to a specific location while locally digesting through the zona pellucida, thus exposing the plasma membrane of the sperm head into the perivitelline space of the oocyte (Howes et al., 2001). The acrosome reaction also promotes gamete fusion, which is mediated by binding of specific sperm head proteins to those located on the oocyte's plasma membrane. In mice Juno, a ligand on the oolemma binds to

Izumo-1 on the sperm plasma membrane, which has been proposed to prompt the fusion of

gametes (Gupta, 2015). In other mammals, the ADAM model has been suggested where ADAM

proteins fertilin α, fertilin β and cyritestin from the spermatozoa bind to integrin α6,β1 and CD9

on the oolemma. The importance of this model is underlined by experiments where sperm from

ADAM-knockout mice were unable to bind the oolemma successfully and CD9-knock out mice

were unable to undergo sperm/oolemma fusion (Cuasnicu et al., 2001). However, further

experiments involving CD9 show that binding of this receptor is not the initiator of oocyte

activation and elevated intracellular calcium levels (Kaji et al., 2000). More likely, CD9 serves

as a locator on the oolemma to promote cytoskeleton rearrangement, inducing formation of actin

filaments and microvilli to translocate the sperm nucleus inside the oocyte (Kaji et al., 2000).

Upon gamete fusion, a sperm-derived protein called phospholipase C-zeta generates inositol

triphosphate (IP_3), a second messenger that promotes the release of calcium from the

endoplasmic reticulum, leading to an increase in intracellular calcium levels. Calcium in the

cytoplasm is pumped back into the intracellular stores or removed from the cell across the

plasma membrane; this restores the steady-state calcium concentration in the ooplasm. In

mammals, the initial calcium transient rise is then followed by a series of calcium elevations

called oscillations. The rise in intracellular calcium levels results in hyperpolarization of the

oocyte (at least in some species), which will initiate further cellular processes to prevent

polyspermy. Elevated cytosolic calcium levels will also promote cell cycle progression (Vitullo

et al., 1992).

Following gamete fusion and the increase in intracellular calcium, the fertilized oocyte

releases the contents of its cortical granules into the perivitelline space. The release of cortical

granules changes the molecular profile of the zona pellucida, increasing the thickness of the

membrane and preventing further penetration of other sperm heads. This schematic to prevent polyspermy is common in mammals, however studies examining both in vivo and in vitro derived porcine oocytes and embryos have noted the characteristic 'softness' of the zona pellucida even after fertilization has occurred with excessive sperm cells being bound to the exterior of early embryos (Prather et al., 1998). As calcium is an important upstream promoter of this regulatory process, the inclusion of the mineral in IVF medium, specifically during incubation with sperm, could prompt better control of polyspermy (Coy et al., 2002). A study by Coy et al. (2002) examined three known media, TCM-199, modified Tris Buffer medium (mTBm), and Tyrode's albumin lactate pyruvate (TALP), to identify how successful each was at inducing the safeguards discussed above. While there was no difference in blastocyst rate among the media tested, it was found that spermatozoa in mTBm were more likely to undergo the acrosome reaction. Interestingly, oocytes in mTBm were also found to have higher cortical granule density over time regardless of presence of sperm cells, while the density of cortical granules in oocytes incubated in TCM-199 or TALP decreased after the introduction of sperm. There were higher sperm penetration rates in TCM-199 and TALP, however this likely resulted in increasing polyspermy in TALP as results showed that monospermy rates were highest in mTBm and TCM-199 (Coy et al., 2002). No study has been able to definitively identify the ideal medium for fertilization that will reduce polyspermy but future efforts should continue to examine media high in calcium as it is likely a key chemical messenger for promoting the zona block. In the meantime current IVF protocols should consider using TCM-199 or mTBm for fertilization as they result in higher single sperm penetration and subsequent fertilization of the oocyte.

As previously discussed, the transient elevation in the intracellular calcium levels proceed to stimulate cortical granule release to prevent polyspermy but also have profound impacts on inducing cell proliferation in the newly formed zygote. The calcium oscillations will activate the oocyte to resume the cell cycle, a phenomenon observed in various phyla, through impacting calcium calmodulin dependent protein kinases (CaMKs) and mitogen activating protein kinase (MAPK) (Stricker, 1999). During oogenesis, the oocyte undergoes two periods of meiotic arrest that serve as protection of the female genome during follicular growth and ovulation. The first meiotic arrest is overcome during maturation and a secondary oocyte with a polar body is formed. This secondary oocyte is arrested at metaphase 2 due to high levels of maturation promoting factor (MPF) until fertilization when binding of the sperm to the plasma membrane initiates calcium oscillations (Jones et al., 1995). The graded calcium stimulus begins with a large calcium transient that is followed by several smaller grade transients, prompting a number of responses based off of the number of stimuli. Previous work using exogenous electrical field impulses to mimic calcium oscillations found that the events of oocyte activation, including cortical granule release and resumption of meiosis are contingent on the continuous stimulation of these transients, with each cellular process being triggered by specific numbers of oscillations (Ducibella et al., 2002). Continuous changes in calcium levels therefore promote and sustain oocyte activation, and will act by initiating signal transduction pathways containing calcium-dependent enzymes. CaMKII in particular is activated by calcium oscillations and promotes cyclin degradation, decreasing MPF's arrest on the secondary oocyte (Ducibella et al., 2002)**.** As decreasing MPF propels the oocyte to anaphase, decreasing levels of MAPK will assist in the cellular completion of meiosis and result in female pronuclear formation, at which point calcium oscillations will halt (Vitullo et al., 1992).

As mentioned previously, TCM-199 is frequently used as the culture medium for oocyte maturation after collection of immature gametes. Research has shown that oocytes matured in TCM-199 supplemented with cysteine, a precursor for glutathione have improved male pronuclear formation upon fertilization (Grupen, 2014; Prather et al., 1998). Further studies have optimized the cysteine concentration in maturation medium to 100-200µmol/L and have shown that glutathione is essential for reducing disulfide crosslinks that compact the male genome (Kiely et al., 2001; Prather et al., 1998). Male pronuclear formation is essential for promoting syngamy of the male and female pronuclei and the formation of a single cell embryo.

The production of embryos in the laboratory also requires the collection of spermatozoa from a male. Semen collected from boars needs to be properly suspended in a solution to maintain quality of the sperm. Most commercial semen extenders contain a supply of nutrients like glucose for nutrition, potassium chloride and sodium citrate for slowing sperm metabolism and maintaining motility, along with antibiotics to prevent microbial growth (Johnson et al., 2000). Bicarbonate is a common capacitation agent in most extenders, causing destabilization of the sperm membranes (Johnson et al., 2000). Ethylenediaminetetraacetic acid (EDTA) is required in all extenders as it is critical for calcium sequestering, an ion whose interaction with the sperm cells would promote capacitation and the acrosome reaction (Watson, 1990). Extenders are utilized to store semen in two states, liquid or frozen. For nearly 99% of the commercial swine industry artificial insemination is performed on swine farms using semen in liquid extenders (Johnson et al., 2000). Sperm is ejaculated in a fluid fraction known as seminal plasma. This seminal plasma has long been known to contain large macromolecules that coat the plasma membrane of the sperm head, and proper removal of these compounds is essential for enabling fertilization in both in vivo and in vitro scenarios. In vivo situations rely on the

anatomical features of the female tract and migration to the site of fertilization for capacitation. In vitro protocols use numerous methods for diluting off seminal plasma; however some of these practices could contribute to polyspermy. Often times (particularly in species other than swine), the best-quality sperm are then selected through discontinuous Percoll gradient centrifugation or the swim up technique (Coy et al., 2002). In addition, the IVF medium is supplemented with bovine serum albumin (BSA; 10mg/mL) and caffeine (2mmol/L) that act as capacitation-inducing agents (Grupen, 2014).

Boar semen is susceptible to the stress of potential cold shock, which results in infertile sperm when the extended sample is quickly cooled below 15°C (Pursel et al., 1973). A study by Johnson et al. (2000) show that the slow cooling of extended semen volumes greater than 100ml at ten degree increments can promote cold resistance to the extended sample, preventing the decrease in motility and viability commonly seen in cold shocked samples. To prevent further cold shock during storage extended semen should be housed in an ambient environment at 15 to 18°C. The specific storage temperature and duration depends highly on the extender used, with extenders containing BSA preventing the loss of fertilization capacity due to storage aging (Uysal & Bucak, 2007). Subsequent addition of melatonin improves the number of live sperm with intact acrosomes on day 7 post extension, allowing for the storage of viable sperm in extender for up to seven days post collection (Martin-Hildago et al., 2011).

1.3   Embryo Culture

Post fertilization, embryos can develop in vitro in humidified environments regulated at 39^{0} C with 5% CO2 in air for approximately 7 days (Machaty et al., 1998). Early work in porcine embryonic culture struggled to produce viable blastocysts from in vitro-derived one- or two-cell embryos, though in vivo derived samples were capable of producing preimplantation

embryos following culture. Eventually, observation of embryo cell culture showed an in vitro development block at the four-cell stage of pig embryos, a time that corresponds to significant DNA transcription and RNA production in the embryo (Grupen, 2014; Prather et al., 1998). The use of several different media was proposed for culture to aid developing embryos in overcoming this in vitro block, such as KSOM/AA, Whitten's medium, and NCSU-23. Of these media, only NCSU-23 provided enough nutrients to assist in embryogenesis past the four-cell stage, though it was noted that embryos cultured in this medium showed a delay in cleavage compared to those collected in vivo (Prather et al., 1998). While these stunted embryos were capable of development to preimplantation stage, further work with collected in vivo-derived embryos were used to better define the optimal conditions needed to overcome the developmental block.

Porcine zygote medium-3 (PZM-3) has been found to be a superior medium for incubating embryos through this block post IVF, parthenogenetic activation, and somatic cell nuclear transfer (Nanassy et al., 2008). PZM-3 is a cocktail of inorganic compounds, energy substrates and amino acids glutamate and hypotaurine at low concentrations which increase blastocyst formation rates (Suzuki et al., 2006). PZM-3 contains ample glucose as an energy source for embryogenesis, compared to NCSU-23 which had both glucose and lactate, the presence of which in culture with glucose was found to inhibit normal embryonic growth (Prather et al., 1998). Common protocols recommend transfer of the embryo post fertilization into PZM-3 where it begins to develop and undergo cleavage. Syngamy of the male and female pronuclei occurs approximately 18 hours after fertilization, when the embryo consists of a single cell (Yoshida et al., 1990). Over a period of 5 days the zygote will undergo a series of cleavages, resulting in exponential extrusion of cells, first from one cell into two cells, then two cells into four and continuing until the embryo is a 16-cell morula. Throughout all cell divisions there is

replication of genetic material; however there is no change in total cytoplasmic volume of the embryo, meaning that each cell cleavage results in the formation of two smaller cells (McLaren, 1970). Development continues in porcine embryos through day 6 and 7 post fertilization with the formation and expansion of a blastocoelic cavity and further cell cleavage, forming an expanding blastocyst (Papaioannou et al., 1988). Hatching, a phenomenon when the blastocyst sheds its zona pellucida, then follows late on day 7 in vitro after which the embryo's growth is typically no longer able to be supported by culture medium (Niemann et al., 1982). While studies have shown that embryos cultured in vitro exhibited lower cell numbers compared to their in vivo counterparts, IVF produced embryos that develop to the blastocyst stage have a similar developmental potential when embryos were transferred back into sows (Machaty et al., 1998).

1.4   Human Assisted Reproduction

In all humans there is a drive to pass on genetics in the form of progeny, but as a country develops the age women choose to start a family begins to increase. While some women can successfully conceive children in their later years, many struggle due to a myriad of fertility issues associated with advancing age. Fortunately Assisted Reproductive Technologies (ART) have been developed over time to enable those struggling with conception to carry on this innate desire. Many ART procedures consist of inducing multiple follicles to grow on the ovary followed by oocyte collection, in vitro fertilization (IVF) and multiple embryo transfer. While these technologies have proved to be quite efficient they frequently result in multiple pregnancies, occurring in 31% of US ART procedures (Reddy et al., 2007). Multiple births occurred in 32 out of every 1,000 pregnancies in the United States in 2006 (Black et al., 2010). These multiple pregnancies carry high risk for both the children and mother involved. Children in a multiple birth situation are often born prematurely with low birth weights, especially when

born before 32 weeks (Reddy et al., 2007). Mothers carrying more than one child have higher incidences of preeclampsia, thromboembolism and obstetric hemorrhage and are at an increased risk of developing gestational diabetes and suffering from miscarriages. As a result of the high risks associated with multiple pregnancies in women undergoing ART procedures there has been a movement to reduce the likelihood of multiple embryos implanting in the uterus. Whereas the common practice used to involve the transfer of three viable embryos into the mother's uterus in hopes one would implant, current recommendations by the Society for Artificial Reproductive Technologies and the American Society for Reproductive Medicine are for a maximum of two viable embryos to be transferred into the mother (Black et al., 2010). Even more recently clinics have been adopting the practice of single embryo transfer (SET), which involves the in vitro growth of embryos to blastocyst stage at which they are analyzed for quality and the highest quality blastocyst is chosen for transfer. The remaining embryos are then vitrified and stored upon failure of the first embryo to implant. While this practice and the corresponding recommendations by reproductive societies have seen the multiple birth conception (Umranikar et al., 2013). With the high cost of IVF cycles, an embryo transfer that fails to implant can lead to highly distressing situations for couples unable to conceive. A failed cycle can lead to heartbreak in already stressed couples, who now must question the financial burden of undergoing another round of IVF treatment. Therefore, the burden lies on clinicians to ensure that every cycle involves the best techniques and most viable embryo(s) that will have the highest success at implantation.

1.5   *Assessing Embryo Quality*

As mentioned, the practice of IVF is becoming more common in research and clinical practices as it allows for controlled fertilization and close monitoring of embryogenesis.

Therefore, it is of utmost importance to select the best embryos for transfer into recipients. Current methodologies of assessing embryo quality to make the ideal embryo selection follow two perspectives: direct and indirect. Direct assessment of embryos involves the analysis of the actual embryo and can be anything from staining of cellular components to morphological grading of the embryo's inner cell mass, trophectoderm and size (Richardson et al., 2015). The use of many staining techniques have been previously employed to indicate embryo quality, however some techniques such as TUNEL apoptosis detection which is highly correlated with embryo quality is a final outcome for embryos (Byrne et al., 1999). This often involves prolonged handling of the embryo and fixation for visualization, resulting in excessive stress to the embryo, diminishing its capacity to develop any further. The energy output of an embryo can also be assayed through fluorescent label procedures that couple a fluorescent tag on to adenosine triphosphate (ATP) (Brison et al., 2004). This procedure has been used to validate the belief that highly metabolic embryos, ones with a high concentration of intracellular ATP, are of high quality and viability (McPherson et al., 2014). Similar to the staining procedures mentioned previously, ATP detection also involves invasive handling and terminal use of the embryo. Other direct assays look at cellular processes or the cellular state of the sample. Size and location of the meiotic spindle can be used to determine quality, as can color and distribution of cellular cytoplasm along with mitochondrial distribution or molecular karyotyping, however all of these subcellular forms of analysis require complex procedures and invasive interaction with the developing embryo (Richardson et al., 2015; Tejera et al., 2011). In addition, assays examining enzymatic activity of known metabolic pathways exist for use; however these assays are also complex and time consuming (Ferrandi et al., 1993). Morphology assays have been considered

as a solid, noninvasive approach to assessing embryonic quality. Morphological grading is a rapid assessment tool and many grading systems currently exist in clinical settings.

Morphological grading has proven to be successful at identifying high quality embryos for transfer and implantation, yet each system varies by the clinic it is employed in and by the technician examining the sample. In addition studies have demonstrated a high level of inter-observer and intra-observer variability (Richardson et al., 2015). As such, a recent trend in ART laboratories towards generating a universal grading system began in 2015. Even this movement towards a universal system is not an absolute answer to the issue of subjectivity between two technicians evaluating the same embryo. Development of imaging systems has resulted in the use of time-lapse measurement as a new approach with less subjectivity, which can be minimally invasive if the system is installed in an incubator with optimal embryonic culture conditions. This assay is still new to the field of reproductive biology and as such there are few markers that have been validated as predictive of embryo quality, preventing direct reliance of clinicians on this method (Conaghan, 2014). Above all there is a necessity to move away from approaches deemed invasive as the utilization of the procedure alone, while giving direct information on embryo quality could alter the continued development of the sample post invasive measurement, thus reducing the true predictive value of the procedure.

Indirect assessment can be more quantitative and as such less invasive than direct measurements of actual embryos. Current indirect assessment methods allow for analysis of the environment that an embryo is cultured in to predict the success of an embryo implanting post transfer. The usage and secretions of metabolites provide noninvasive approaches to determine the quality of an embryo. Oxygen consumption has been found to be higher in individually cultured embryos that develop to superior quality blastocysts and several studies have shown

differences in amino acid and protein secretions in spent medium between good implantation

blastocysts and their poor quality counterparts (Agung et al., 2005; Lopes et al., 2005).

Metabolomics, or the study of metabolite expression, allows for the high powered liquid

chromatography or proton nuclear magnetic resonance for the identification of metabolites in

collected samples. Previous work by Brison et al. has shown that individual culture of human

embryos could be used to predict implantation success of the embryo. Spent culture medium

with profiles high in Asn, Gly, and Leuc showed higher implantation rates and resulted in more

live births (Brison et al., 2004). A similar study found that additional amino acid glutamate and

the alanine/lactate ratio were highly expressed in individual culture medium from embryos that

resulted in higher implantation rates and live births (Seli et al., 2008). Similar to the field of

metabolomics is proteomics, which focuses on the expression of proteins. A study done by

Gonzalez et al. found that the protein leptin was in higher concentrations in the spent medium of

individual human embryos that resulted in higher implantation rates compared to embryos that

had lower implantation rates. Several of the procedures involved in proteomic analysis of culture

medium require the use of enzyme-linked immunosorbent assays (ELISA) and specialized

training (Gonzalez et al., 2000). The expensive equipment needed for metabolite analysis makes

these valuable indirect quality screenings impractical for small clinics that lack the resources to

spend money and time on such a process. As such, there is still a persistent need for a

noninvasive system to objectively analyze blastocyst quality in a clinical setting.

1.6   *Novel Optical Assessment of Cell Viability*

Current methods to analyze embryo quality and viability clearly lend to the idea that there

is a definite need for an objective, direct assay to identify optimal embryos. Assessing cell

quality has long been a necessary task in the field of biomedical sciences, which has also reached

the problematic situation of designing an approach to measure living tissues for viability without having destructive ends. Cancer research in particular has required a methodology to examine the impacts of drugs on tumors without the method itself confounding the results of drug treatment (Sun et al., 2017). In addition the field of dermatology has relied on the formation of skin grafts using stem cell scaffolding techniques to treat burn victims. These sensitive formations require analysis to determine if the skin graft is fully developed and ready for transfer, however previous practices to assess development have all relied on partial sample destruction methods like quantitative polymerase chain reaction (qPCR), mass spectroscopy (MS), and western blotting (Quinn et al., 2012). Both cancer research and dermatology have turned to the field of optics and microscopy to develop a direct assay for measuring living tissue. In order to scan a three dimensional form of living tissue several imaging approaches have been proposed, such as confocal, nonlinear microscopy, two photon excited fluorescence microscopy and optical coherence tomography (Quinn et al., 2012; Sun et al., 2017).

Two Photon Excited Fluorescence (TPEF) has most recently been utilized to measure viability by exploiting the endogenous fluorescence of electron carriers related to metabolism. In a study done by Quinn et al. stem cell scaffolding for tissue grafts were scanned for differentiation using TPEF in hopes to distinguish key cellular processes by the amounts of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) detected. TPEF excites molecules using two photons from near infrared light, which have higher wavelengths allowing in depth penetration and scanning of samples while reducing the photo-toxicity to the sample that is commonly seen when using an emission source of a lower wavelength (Denk et al., 1990). This allowed for a 3D reconstruction and identification of the differentiation level of stem cell proliferation, characteristically associated high NADH/FAD

ratios, while lowered redox ratios corresponded with high lipogenesis. Further work was suggested to better define this redox ratio for use in identifying abnormally high metabolism patterns related to extreme inner cell activity, with applications for the fields of oncology and embryogenesis.

In the field of oncology Optical Coherence Tomography (OCT) has been utilized to generate phenotypic categorizations of drug response on tumors (Evans et al., 2011). OCT provides technicians with a deeper imaging of multi-cell layered tissues; however it fails to fully emit the necessary feedback of the current cellular state. A technique known as BioDynamic Imaging (BDI) has been innovated to OCT systems; modifying an OCT system with already increased penetration depths to return detailed cellular information via dynamic light emitted through coherence detection over a large area (Wax et al., 2001). This BDI technology relies on digital holography, detecting returning light, called backscatter, in a variety of different frequencies which can be associated with different intracellular dynamics and complied into what is known as a fluctuation spectrum. This fluctuation spectrum can also be used to calculate numerical values representative of tissue viability such as the normalized standard deviation (NSD) (Boas et al., 2010). NSD is related to the intensity of returning light and is interpreted as a measure of intracellular motility with a high NSD value exhibited in samples with high metabolic rate and undergoing cellular proliferation. This approach benefits the identification of ideal drug treatments in oncology as a tumor's response to specific drug application can be recorded in a fluctuation spectrum and NSD then cross-referenced with fluctuation spectra of established mechanisms of actions commonly seen in cellular death pathways. Eventual collections of fluctuation spectra from varying source tumors can be used to isolate phenotypic profiles that can then be utilized to better treat a variety of cancers with specific combinations of

chemotherapies, resulting in unique treatment plans for physicians to follow depending on the fluctuation spectra (Sun et al., 2017).

The noninvasive, sensitive spectra that can be obtained from measuring tissue dynamics in vitro opens the door for scientists to examine long term intracellular behavior in many other samples beyond the field of cancer treatment. As mentioned above, the field of assisted reproductive technologies currently lacks an objective, noninvasive methodology for assessing embryo quality. Recent studies have seen a convergence between assessing oocyte and embryo quality and biodynamic imaging in hopes to provide a stable technique for identifying the most viable samples for use in ART (An et al., 2015; Conaghan, 2015). Current research has been done on porcine oocytes with the BDI, using similar metrics that were chosen to evaluate tumors. A recent study performed in our laboratory evaluated cumulus oocyte complexes (COCs) with BDI technology and found that oocytes having high NSD values were indicative of an elevated metabolism, and that matured oocytes had a higher temporal contrast and active metabolic core compared to their immature counterparts. These high NSD and temporal contrast values only increase as the mature oocyte becomes an embryo undergoing first cleavage, exhibiting the highest values recorded on a BDI system (An et al., 2015).

While further experiments were performed to record temporal contrasts in developing blastocysts, current work in literature has only examined the optical features of these embryos. If this technology is to be applied for improving ART and single embryo transfer rates post IVF it is clear that the scientific approach should now focus on distinguishing embryo phenotypes. Such efforts require proper definition and application of metrics that will be useful in distinguishing a viable, high quality embryo from its lower quality counterpart. Based off of previous work in porcine reproduction research it is clear that NSD will be an important metric to consider,

however the identification of other sound, significant parameters can further improve the selection of blastocysts for embryo transfer.

1.7 References

Agung, B., Otoi, T., Abe, H., Hoshi, H., Murakami, M., Karja, N. W., Murakami, M., Wongsrikeao, P., Watari, H., Suzuki, T. (2005). Relationship between oxygen consumption and sex of bovine in vitro fertilized embryos. *Reprod Domest Anim, 40*(1), 51-56. doi: 10.1111/j.1439-0531.2004.00554.x

Albertini, D. F., Combelles, C. M., Benecchi, E., & Carabatsos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction, 121*(5), 647-653.

Alm, H., Choi, Y. H., Love, L., Heleil, B., Torner, H., & Hinrichs, K. (2008). Holding bovine oocytes in the absence of maturation inhibitors: kinetics of in vitro maturation and effect on blastocyst development after in vitro fertilization. *Theriogenology, 70*(7), 1024-1029. doi: 10.1016/j.theriogenology.2008.05.062

An, R., Wang, C., Turek, J., Machaty, Z., & Nolte, D. D. (2015). Biodynamic imaging of live porcine oocytes, zygotes and blastocysts for viability assessment in assisted reproductive technologies. *Biomed Opt Express, 6*(3), 963-976. doi: 10.1364/BOE.6.000963

Biggers, J. D., Whittingham, D. G., & Donahue, R. P. (1967). The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A, 58*(2), 560-567.

Black, M., & Bhattacharya, S. (2010). Epidemiology of multiple pregnancy and the effect of assisted conception. *Semin Fetal Neonatal Med, 15*(6), 306-312. doi: 10.1016/j.siny.2010.06.004

Bleil, J. D., & Wassarman, P. M. (1980). Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev Biol, 76*(1), 185-202.

Boas, D. A., & Dunn, A. K. (2010). Laser speckle contrast imaging in biomedical optics. *J Biomed Opt, 15*(1), 011109. doi: 10.1117/1.3285504

Brison, D. R., Houghton, F. D., Falconer, D., Roberts, S. A., Hawkhead, J., Humpherson, P. G., Lieberman, B.A., Leese, H. J. (2004). Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod, 19*(10), 2319-2324. doi: 10.1093/humrep/deh409

Byrne, A. T., Southgate, J., Brison, D. R., & Leese, H. J. (1999). Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J Reprod Fertil, 117*(1), 97-105.

Conaghan, J. (2014). Time-lapse imaging of preimplantation embryos. *Semin Reprod Med, 32*(2), 134-140. doi: 10.1055/s-0033-1363555

Coy, P., Gadea, J., Romar, R., Matas, C., & Garcia, E. (2002). Effect of in vitrofertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. *Reproduction, 124*(2), 279-288.

Cuasnicu, P. S., Ellerman, D. A., Cohen, D. J., Busso, D., Morgenfeld, M. M., & Da Ros, V. G. (2001). Molecular mechanisms involved in mammalian gamete fusion. *Arch Med Res, 32*(6), 614-618.

Denk, W., Strickler, J. H., & Webb, W. W. (1990). Two-photon laser scanning fluorescence microscopy. *Science, 248*(4951), 73-76.

Dickey, R. P., Taylor, S. N., Lu, P. Y., Sartor, B. M., Rye, P. H., & Pyrzak, R. (2005). Risk factors for high-order multiple pregnancy and multiple birth after controlled ovarian hyperstimulation: results of 4,062 intrauterine insemination cycles. *Fertil Steril, 83*(3), 671-683. doi: 10.1016/j.fertnstert.2004.10.030

Driancourt, M. A., & Thuel, B. (1998). Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid. A review. *Reprod Nutr Dev, 38*(4), 345-362.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R. M., Kopf, G. S., Fissore, R., Madoux, S., Ozil, J. P. (2002). Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol, 250*(2), 280-291.

Eppig, J. J., & O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod, 54*(1), 197-207.

Evans, C. L., Abu-Yousif, A. O., Park, Y. J., Klein, O. J., Celli, J. P., Rizvi, I., Zheng, X., Hasan, T. (2011). Killing hypoxic cell populations in a 3D tumor model with EtNBS-PDT. *PLoS One, 6*(8), e23434. doi: 10.1371/journal.pone.0023434

Ferrandi, B., Cremonesti, F., Geiger, R., Consiglio, A. L., Carnevali, A., & Porcelli, F. (1993). Quantitative cytochemical study of some enzymatic activities in preovulatory bovine oocytes after in vitro maturation. *Acta Histochem, 95*(1), 89-96. doi: 10.1016/S0065-1281(11)80394-6

Geshi, M., Takenouchi, N., Yamauchi, N., & Nagai, T. (2000). Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. *Biol Reprod, 63*(6), 1730-1734.

Gilchrist, R. B., & Richani, D. (2013). Somatic guidance for the oocyte. *Dev Cell, 27*(6), 603-605. doi: 10.1016/j.devcel.2013.12.006

Gonzalez, R. R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., & Simon, C. (2000). Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. *J Clin Endocrinol Metab, 85*(12), 4883-4888. doi: 10.1210/jcem.85.12.7060

Grupen, C. G. (2014). The evolution of porcine embryo in vitro production. *Theriogenology, 81*(1), 24-37. doi: 10.1016/j.theriogenology.2013.09.022

Gupta, S. K. (2015). Role of zona pellucida glycoproteins during fertilization in humans. *J Reprod Immunol, 108*, 90-97. doi: 10.1016/j.jri.2014.08.006

Howes, E., Pascall, J. C., Engel, W., & Jones, R. (2001). Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization. *J Cell Sci, 114*(Pt 22), 4127-4136.

Johnson, L. A., Weitze, K. F., Fiser, P., & Maxwell, W. M. (2000). Storage of boar semen. *Anim Reprod Sci, 62*(1-3), 143-172.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham, D. G., & Kono, T. (1995). Repetitive sperm-induced Ca2+ transients in mouse oocytes are cell cycle dependent. *Development, 121*(10), 3259-3266.

Kaji, K., Oda, S., Shikano, T., Ohnuki, T., Uematsu, Y., Sakagami, J., Tada, N., Miyazaki, S., Kudo, A. (2000). The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat Genet, 24*(3), 279-282. doi: 10.1038/73502

Kiely, J. L., & Kiely, M. (2001). Epidemiological trends in multiple births in the United States, 1971-1998. *Twin Res, 4*(3), 131-133.

Lopes, A. S., Larsen, L. H., Ramsing, N., Lovendahl, P., Raty, M., Peippo, J., Greve, T., Callesen, H. (2005). Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. *Reproduction, 130*(5), 669-679. doi: 10.1530/rep.1.00703

Machaty, Z., Day, B. N., & Prather, R. S. (1998). Development of early porcine embryos in vitro and in vivo. *Biol Reprod, 59*(2), 451-455.

McLaren, A. (1970). 8. Biological regulation of reproduction. In: The family and its future. *Ciba Found Symp*, 101-116.

McPherson, N. O., Zander-Fox, D., & Lane, M. (2014). Stimulation of mitochondrial embryo metabolism by dichloroacetic acid in an aged mouse model improves embryo development and viability. *Fertil Steril, 101*(5), 1458-1466. doi: 10.1016/j.fertnstert.2013.12.057

Nanassy, L., Lee, K., Javor, A., & Machaty, Z. (2008). Effects of activation methods and culture conditions on development of parthenogenetic porcine embryos. *Anim Reprod Sci, 104*(2-4), 264-274. doi: 10.1016/j.anireprosci.2007.01.019

Papaioannou, V. E., & Ebert, K. M. (1988). The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. *Development, 102*(4), 793-803.

Prather, R. S., & Day, B. N. (1998). Practical considerations for the in vitro production of pig embryos. *Theriogenology, 49*(1), 23-32.

Pursel, V. G., Johnson, L. A., & Schulman, L. L. (1973). Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. *J Anim Sci, 37*(2), 528-531.

Quinn, K. P., Bellas, E., Fourligas, N., Lee, K., Kaplan, D. L., & Georgakoudi, I. (2012). Characterization of metabolic changes associated with the functional development of 3D engineered tissues by non-invasive, dynamic measurement of individual cell redox ratios. *Biomaterials, 33*(21), 5341-5348. doi: 10.1016/j.biomaterials.2012.04.024

Reddy, U. M., Wapner, R. J., Rebar, R. W., & Tasca, R. J. (2007). Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop. *Obstet Gynecol, 109*(4), 967-977. doi: 10.1097/01.AOG.0000259316.04136.30

Richardson, A., Brearley, S., Ahitan, S., Chamberlain, S., Davey, T., Zujovic, L., Hopkisson, J., Campbell, B., Raine-Fenning, N. (2015). A clinically useful simplified blastocyst grading system. *Reprod Biomed Online, 31*(4), 523-530. doi: 10.1016/j.rbmo.2015.06.017

Rose, T. A., & Bavister, B. D. (1992). Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. *Mol Reprod Dev, 31*(1), 72-77. doi: 10.1002/mrd.1080310113

Sanchez, F., & Smitz, J. (2012). Molecular control of oogenesis. *Biochim Biophys Acta, 1822*(12), 1896-1912. doi: 10.1016/j.bbadis.2012.05.013

Seli, E., Botros, L., Sakkas, D., & Burns, D. H. (2008). Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril, 90*(6), 2183-2189. doi: 10.1016/j.fertnstert.2008.07.1739

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol, 211*(2), 157-176. doi: 10.1006/dbio.1999.9340

Sun, H., Merrill, D., An, R., Turek, J., Matei, D., & Nolte, D. D. (2017). Biodynamic imaging for phenotypic profiling of three-dimensional tissue culture. *J Biomed Opt, 22*(1), 16007. doi: 10.1117/1.JBO.22.1.016007

Suzuki, C., & Yoshioka, K. (2006). Effects of amino acid supplements and replacement of polyvinyl alcohol with bovine serum albumin in porcine zygote medium. *Reprod Fertil Dev, 18*(7), 789-795.

Tejera, A., Herrero, J., de Los Santos, M. J., Garrido, N., Ramsing, N., & Meseguer, M. (2011). Oxygen consumption is a quality marker for human oocyte competence conditioned by ovarian stimulation regimens. *Fertil Steril, 96*(3), 618-623 e612. doi: 10.1016/j.fertnstert.2011.06.059

Umranikar, A., Parmar, D., Davies, S., & Fountain, S. (2013). Multiple births following in vitro fertilization treatment: redefining success. *Eur J Obstet Gynecol Reprod Biol, 170*(2), 299-304. doi: 10.1016/j.ejogrb.2013.06.031

Uysal O., Bucak M.N. (2007) Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 76:383–390

Varani, S., Elvin, J. A., Yan, C., DeMayo, J., DeMayo, F. J., Horton, H. F., Bryne, M.C., Matzuk, M. M. (2002). Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Mol Endocrinol, 16*(6), 1154-1167. doi: 10.1210/mend.16.6.0859

Vitullo, A. D., & Ozil, J. P. (1992). Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Dev Biol, 151*(1), 128-136.

Wandji, S. A., Pelletier, G., & Sirard, M. A. (1992). Ontogeny and cellular localization of 125I-labeled basic fibroblast growth factor and 125I-labeled epidermal growth factor binding sites in ovaries from bovine fetuses and neonatal calves. *Biol Reprod, 47*(5), 807-813.

Wassarman, P. M. (1999). Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell, 96*(2), 175-183.

Wax, A., Yang, C., Dasari, R. R., & Feld, M. S. (2001). Path-length-resolved dynamic light scattering: modeling the transition from single to diffusive scattering. *Appl Opt, 40*(24), 4222-4227.

Wu, J., Emery, B. R., & Carrell, D. T. (2001). In vitro growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. *Biol Reprod, 64*(1), 375-381.

Yoshida, M., Ishizaki, Y., & Kawagishi, H. (1990). Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. *J Reprod Fertil, 88*(1), 1-8.

Yuan, Y., Spate, L. D., Redel, B. K., Tian, Y., Zhou, J., Prather, R. S., & Roberts, R. M. (2017). Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation. *Proc Natl Acad Sci U S A, 114*(29), E5796-E5804. doi: 10.1073/pnas.1703998114

CHAPTER 2. NOVEL ASSESSMENT OF SYNGAMY AND EMBRYO HEALTH USING BIODYNAMIC IMAGING

2.1 *Abstract*

Current clinical practices to assess embryonic viability are inadequate for practical application. Direct assays are either rife with subjective bias or are invasive at the risk of impeding further development while indirect assays are expensive and impractical for clinical application. A novel optical system, inexpensive to install on microscopes, has been developed on the principles of optical coherence tomography to utilize the energy of photons to penetrate samples to never before reached depths without risking the hazards of phototoxicity. This optical system was also coupled to digital holography to improve the backscatter reconstruction of the sample, creating the BioDynamic Imaging System (BDI). The BDI has been employed to determine drug therapy treatment on tumor spheroids and in our own lab to determine oocyte and embryo viability. Embryos, blastocysts in particular, undergo massive development, proliferation and have a high metabolism, and as such are a unique sample group. Therefore experimentation is necessary to identify optical parameters that are indicative of embryonic health and viability. Embryos produced in vitro were subjected to various stressors making their environment acidic, alkaline, hyperosmotic or the embryos themselves malnourished and recorded for an hour on the BDI. Additionally, oocytes were co-cultured with sperm for 4 hours to promote fertilization and recorded on the BDI for 24 hours to observe cellular dynamic indicative of fertilization. Phenotypic responses to these stressors were assessed; embryos were divided into two groups in each treatment and for these two groups the parameters of slope of spectra and nyquist floor differed. Twenty-four hour measurement indicated that different intracellular activity could be detected between fertilized and unfertilized oocytes in terms of slope of spectra and spectrogram

frequencies over time. The identified parameters of spectral slope, nyquist floor and spectrogram frequencies can be employed to assess embryonic response to stressors and quality over time.

2.2   Introduction

A reliance on assisted reproductive technologies (ART) has become a necessity for many couples struggling with fertility issues. The common procedure, in vitro fertilization (IVF), is performed where oocytes are co-incubated with sperm to produce embryos that will be transferred back to the maternal uterus for healthy development and delivery of a child. This procedure has been extremely successful, yet often results in multiple conceptuses, leading to multiple birth situations in thirty-two out of every thousand pregnancies in the United States (Black et al., 2010). Multiple conceptus pregnancies pose an increase risk to both the mother and the children she carries; increasing the risk of premature birth and low birth weight in the child, preeclampsia, thromboembolism and obstetric hemorrhage in the mother (Reddy et al., 2007). In order to mitigate the risk associated with multiple births, industry standards are changing to single embryo transfer (sET) where in vitro-derived embryos are assessed for viability with the best embryo being selected for transfer back to the mother. Current means to assess embryonic viability vary between direct and indirect methods. Subjective methods such as morphological grading directly categorize embryos based on cellular appearance while direct, objective measures can evaluate embryonic quality through staining for apoptotic factors (Byrne et al., 1999; Gardner et al., 2000). Indirect, objective methods consist of profiling spent culture media for protein and metabolite secretion, yet these profiling assays are costly, time consuming and impractical to utilize in most clinical settings (Brison et al., 2004; Gonzalez et al., 2000; Seli et al., 2008). Direct methods are not without their pitfalls; morphological assays can have a level of bias associated with them whereas staining methods can cause harm to the embryo, inducing

stress that could continue to degrade the embryo's development (Lee et al, 2009; Richardson et al., 2015). Therefore, there is a need for a direct, noninvasive method that can accurately assess embryonic viability and be a practical installment in a clinical setting.

Similar parameters have been a necessity in the assays of other biomedical fields, specifically in optimizing drug therapies to tumor spheroids in cancer research or identifying viable skin grafts for transplant into human recipients (Sun et al., 2017). The field of optical microscopy has been of value for such assays as they can manipulate light to penetrate living tissue and collect the backscatter of photons to evaluate sample quality. Such an assay has been explored through the methodology of Two Photon Excited Fluorescence (TPEF) which was applied to determine the state of stem cell scaffolds. Quinn et al. (2012) found that TPEF could exploit the natural fluorescence of electron carriers such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) to quantify and directly infer the metabolism of stem cells. Further work led to the definition of key stages of stem cell proliferation all through the use of a detection device that utilizes only two, high wavelength photons; providing both a measurement that can penetrate through 3-D samples and minimize phototoxicity (Denk et al., 1990). Optical Coherence Tomography (OCT) is a similar method that allows for the imaging of multi-cell layered tissues and has been used to define the phenotypic response of a tumor to drug therapies (Evans et al., 2011). While OCT has helped categorize the type of tumor drug responses, it cannot emit back the live cellular state. Therefore, this OCT system has been modified to return cellular feedback through dynamic light transmitted through large area coherence detection, creating a new technology called BioDynamic Imaging. This novel optical system utilizes digital holography, capturing back scatter light in a wide range of frequencies associated with the intracellular movements of a sample, thus generating a fluctuation spectrum.

The BDI system has been used to detect oocyte quality before and, to a lesser extent, embryonic competency; however, embryos provide a unique intracellular profile to measure as their massive proliferation, division and metabolism portray quite the dynamic range of motion. Such metabolic action and vesicle fusion involve many cellular processes and impact intracellular dynamics, events likely captured through the BDI system. The objective of this study was to identify phenotypic responses to stress in embryos induced through a variety of different compounds. Blastocysts were exposed to environments that were acidic, alkaline, hyperosmotic and low nutrition to capture the intracellular responses triggered. Additionally, matured oocytes were subjected to gamete co-culture and measured under the BDI in hopes to capture the cellular dynamics of fertilization, subsequent zygote formation through syngamy, and first cleavage in the early embryo.

2.3   Materials and Methods

2.3.1   In Vitro Maturation

Ovaries were collected from harvested prepubertal gilts at Indiana Packers Corporation in Delphi, Indiana. Upon collection, ovaries were washed in 0.9% saline supplemented with antibiotics (0.75% penicillin and 0.5% streptomycin) and maintained above 30°C during transport back to the research laboratory. Follicles >3mm were aspirated using a 20-gauge hypodermic needle and a 10-ml syringe. Oocytes were allowed to settle out of follicular fluid and those having several layers of cumulus cells were collected under a stereo microscope. They were washed in Hepes-buffered Tyrode's Lactate (TL-Hepes) medium and allowed to mature for 42-44 hrs in Tissue Culture Medium-199 (TCM-199) supplemented with cysteine (0.1 mg/ml), FSH (0.5 IU/ml), LH (0.5 IU/ml), EGF (10 ng/ml), IGF (20 ng/ml), FGF (40 ng/ml) and LGF (20 ng/ml) and covered with mineral oil.

2.3.2   *In Vitro Fertilization*

Matured oocytes were denuded by vortexing in a hyaluronidase (1 mg/ml) solution, then washed in TL-Hepes. Oocytes with an intact plasma membrane and evenly dark cytoplasm were selected, then washed and transferred, in groups of 50, into 50 µl droplets of modified Tris-buffered medium (mTBm) covered with mineral oil. Semen was collected from a Large White boar with proven in vitro fertility at the university farm, extended with EnduraGuard Plus (MOFA) and stored at 17°C. One ml of extended semen was added to 9 ml Dulbecco's Phosphate Buffered Saline (DPBS) and centrifuged at 900 g for 4 mins. The supernatant was removed and the sperm pellet was resuspended in 10 ml DPBS and re-centrifuged for an additional two washes. The pellet was then resuspended in 200 µl mTBm and sperm concentration was calculated using a hemocytometer. The sperm was then diluted with mTBm and the sperm suspension at a final concentration of 5×10^5 cells/ml was added to the mTBm droplets containing the mature oocytes. The gametes were co-incubated for five hours, then prospective zygotes were washed in TL-Hepes and Pig Zygote Medium-3 (PZM-3) before being incubated for long term development in 20 µl droplets of PZM-3 covered with mineral oil.

2.3.3   *BDI Measurement of Stressed Preimplantation Embryos*

All samples were morphologically assessed and graded using the Gardner grading system (Gardner et al., 2002) before being plated in 50 µl of TL-Hepes and covered in mineral oil on a 35mm Falcon dish treated with Cell-Tak. Base measurements were performed for all samples and consisted of four iterations (single measurements) separated by 1200 seconds for a total time of one hour. Following the base measurement each sample was subject to a stressor and the unique protocol associated with that stressor.

i.   10% Ethanol (n=13): Following the base measurement, 5 μl of 100% ethanol was added to the TL-Hepes droplet, thus creating a final ethanol concentration of 10%. BDI recording then started for 30 iterations with 120 seconds between each iteration until the embryo was visually confirmed to be dead.

ii.   Low pH:

    a. HCl (n=8)- 20 μl of 1M HCl was pipetted into the droplet after the base measurement and the sample was measured for 30 iterations with 120 seconds between iterations until the embryo was morphologically dead.

    b. TL-Hepes (pH=6, n=10) - 50 μl of pH 6 TL-Hepes was added into the droplet and the embryo was measured for 30 iterations with 120 seconds between iterations until it was confirmed dead.

iii.   High pH (n=8): Fifty μl of Pig Zygote Medium-3 (PZM-3) was added to the TL-Hepes droplet holding the embryo. PZM-3 is designed to be used in a 5% CO_2 environment, using it outside of a CO_2 incubation leads to a gradual increase of its pH. However, initial testing of 30 iterations with 600 seconds between showed no change in the morphology of the embryo in the final medium. We concluded that the pH increase was probably compensated by the buffering effect of the original TL-Hepes medium. Continuation of this method required that the embryo be held in 50 μl of PZM-3 medium instead of TL-Hepes (final pH ~8.5) for the base measurement. Then the embryo would be recorded for an additional 30 iterations for 120 seconds in between. Because this scheme proved to be too short to record full embryo death, a final measurement protocol of 30 iterations for 300 seconds

between each iteration was determined. The sample was measured until the
embryo was visually determined to have died.

iv. Natural Death (n=8): After the base measurement, the recording was continued at iterations separated by 1800 seconds until the sample died.

2.3.4 *BDI Measurement of the Fertilization Process*

Mature oocytes were denuded and those with dark, uniform cytoplasm and intact plasma membrane were selected for measurements. Individual oocytes were placed on a 35mm Falcon dish treated with Cell-Tak in 50 µl of TL-Hepes covered with mineral oil. A base measurement was taken for 3 iterations, 1 second between iterations, exposure time of 20 ms and an acquisition rate of 25 frames per second for 2500 frames. After the base measurement the oocyte was washed, placed in 50 µl droplet of fertilization medium (modified Tris-Buffered medium) and co-incubated with capacitated spermatozoa in a CO_2 incubator for four hours. This time was sufficient for sperm penetration to occur based on previous experiments. After the 4-hour incubation the oocyte was transferred to the original TL-Hepes droplet and the BDI measurement resumed with a settings of 144 iterations, 480 seconds between loops, exposure 20 ms and 25 fps for 2500 frames. After measurements the sample was transferred into 50 µl of PZM-3 medium for an additional 24-hour incubation, then staining with Hoechst 33342 was used to determine if fertilization had or had not occurred by determining the number polar bodies under the zona pellucida (the presence of two polar bodies was interpreted as a sign for successful fertilization).

2.4 Results

2.4.1 *Phenotypic Response to Stressors*

The responses to the treatments were characterized by averaged differential spectra, shown in Figure 2.1, which are defined as spectrum of the last loop subtracted by that of the first loop of the measurement: $dS(\omega,t)\big|_{t=t_1} = \log S(\omega,t_1) - \log S(\omega,t_0)$. A positive value indicates increased activity at a certain frequency, while a negative value indicates reduced activity. We found that ethanol and PZM-3 treatments show overall suppression of cellular motility. TL-Hepes (pH=6) treatment leads to suppression in lower frequencies and enhancements in higher frequencies, implying that activities are generally going faster in the blastocyst ("blueshift"). HCl responses show suppression in mid frequencies and enhancements in lower and higher frequencies. The averaged natural death differential spectrum shows a "redshift" pattern, but a closer look at the individual responses reveals two types of responses among the samples.
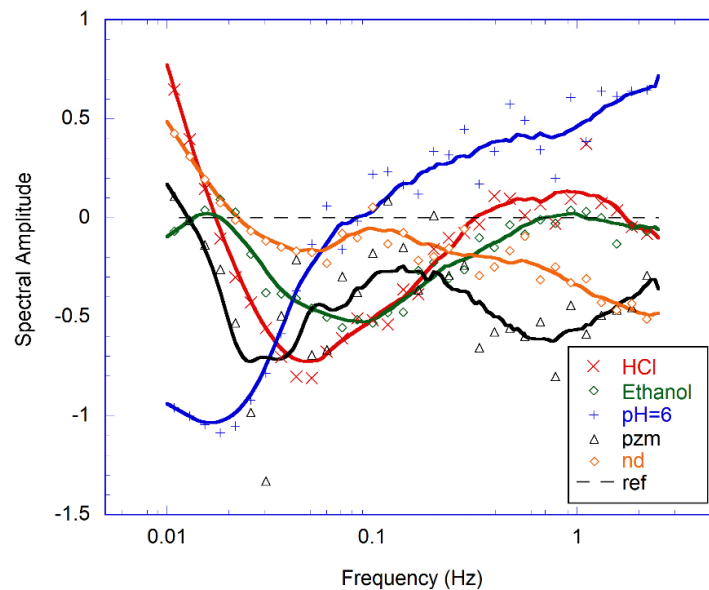
Figure 2.1. Averaged differential response of the treatments

The blastocysts were kept in the same conditions as in previous tests and were measured by BDI for 1 h. The samples can be put into two categories which differ in slope and nyquist floor in the initial spectra (Figure 2.2A) and show blueshift and redshift patterns respectively in the differential spectra as shown in Figure 2.2B.
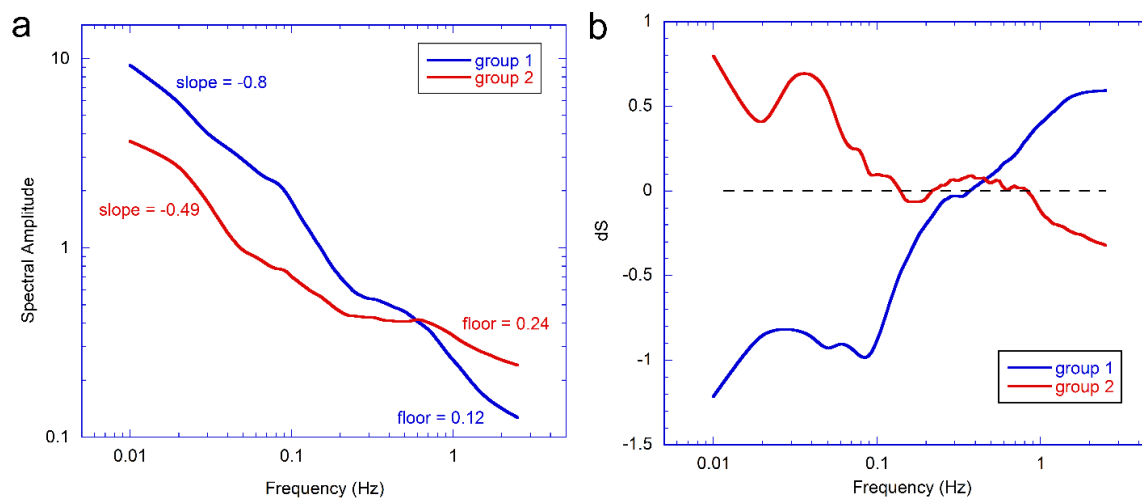
Figure 2.2. A. Average of first measurement spectra; B) Averaged differential spectra $dS(\omega, t_{end})$

When living and dead embryos along with those fixed in 3.7% paraformaldehyde (used as control) were measured with BDI, their spectra showed differences in shape, slope and dynamic range (Figure 2.3A.). Spectra of dead samples are close to straight lines and have the largest dynamic range, while the fixed have very irregular shapes and are very noisy. Linear fittings were done on the spectra, and the R^2 values of the three groups of blastocysts are different (Figure 2.3B).
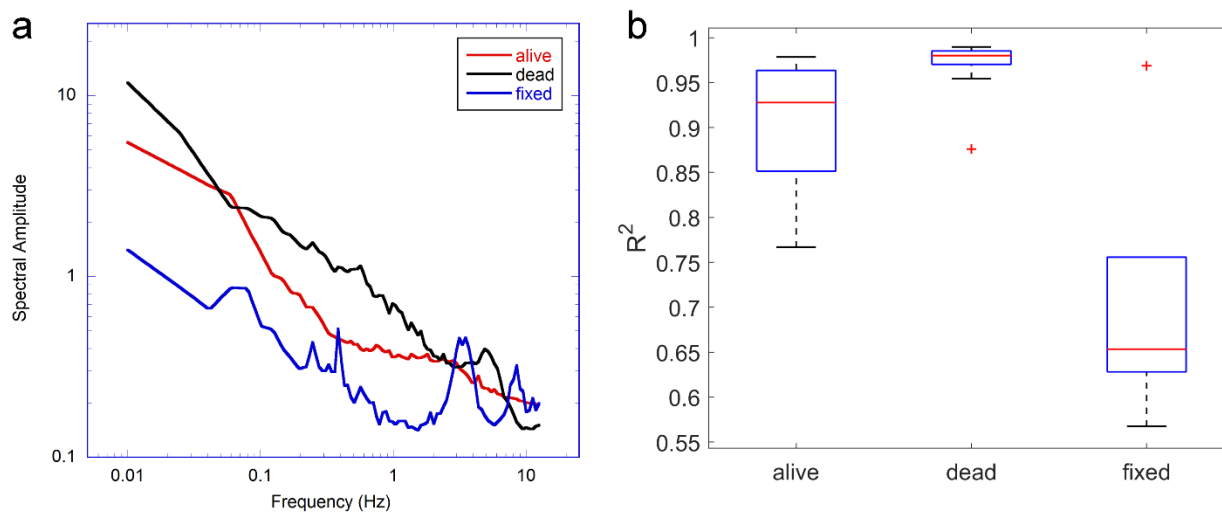
Figure 2.3. A) Average initial spectra of living, dead and fixed samples; B) R^2 value of linear fittings of the spectra of three groups of blastocysts

2.4.2 *BDI Measurement of Fertilization*

Fertilized oocytes exhibit a long period of activity compared to unfertilized oocytes as demonstrated by a trend of showing high intracellular motion in the low frequency range of the spectrum, recorded from hour 2 to hour 19 of the measurement period (Figure 2.4). The spectra of unfertilized oocytes over time show short, inconsistent low frequency bursts of intracellular motion, each burst lasting an hour or less during the 24-hour measurement (Figure 2.5). Unfertilized oocytes also exhibit an extreme negative slope when plotting the frequency of their spectra. A prolonged period of low frequencies were visualized in approximately 60% of the oocytes measured, corresponding to post-fertilization intracellular events whereas shorter periods of varied frequencies, less than an hour in duration were observed in oocytes that were not fertilized.
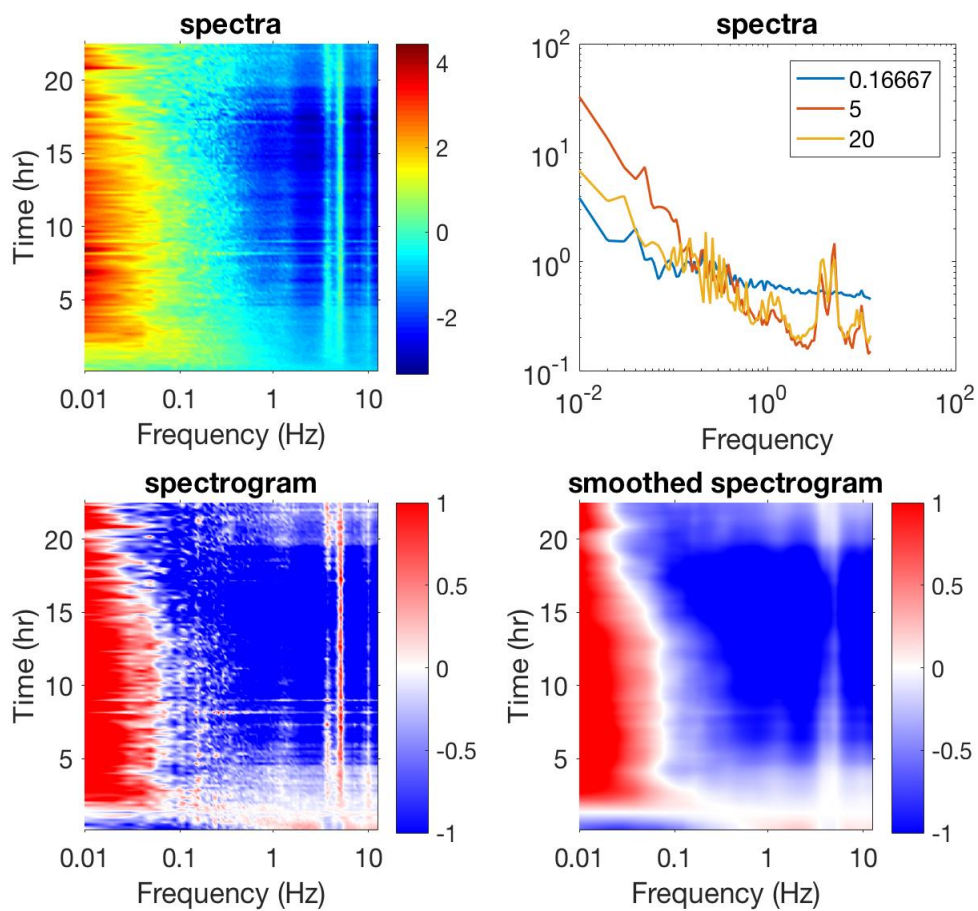
Figure 2.4 Recorded spectra of a fertilized oocyte
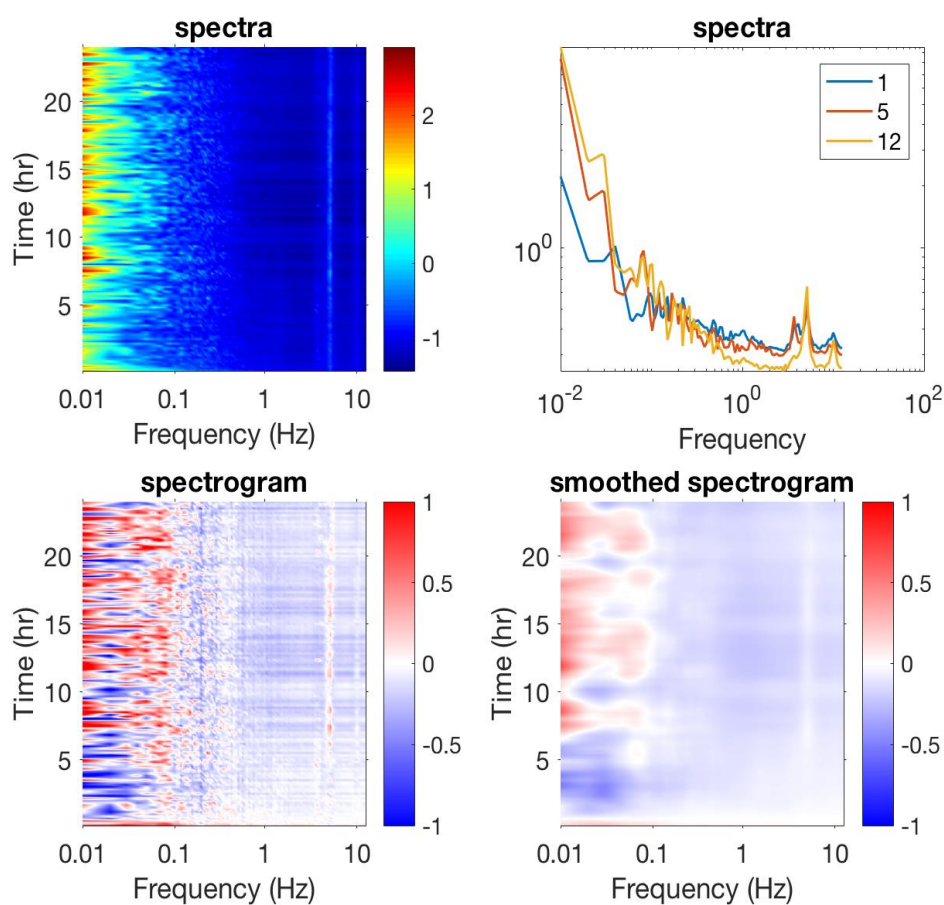
Figure 2.5. Recorded spectra of an unfertilized oocyte

2.5 Discussion

A direct, noninvasive method to assess embryonic viability is essential for improving

success rates of single embryo transfers and the BioDynamic Imaging system poses a suitable

option. Past attempts to directly record embryo quality and viability assessed the embryo in a

two-dimensional environment, through methods like time-lapse microscopy or scanning for lipid

droplet accumulation (Quinn et al., 2012; Sun et al., 2017). These methods are imprecise and

diminished in sensitivity of their assays as biological samples exist in a multi-dimensional

matrix. Confining the reading of a tissue or cell to only two dimensions reduces the amount of

area assessed and can confound data or worse, overlook it. Thus, to get an accurate depiction of

the true quality and development of a biological sample, it is essential that all planes be

visualized and data points be collected from a three-dimensional methodology. Fortunately, this

visualization can be obtained through many recently established optical systems which have been

utilized by the biomedical field to look at the quality of living tissues. Such systems have been

employed to determine the viability of such samples through exploitation of natural fluorescence

of electron carriers to determine the redox ratios and metabolism (Quinn et al., 2012), or the

mechanistic responses of tissues exposed to known drug therapies (Evans et al., 2011). The

unique low-coherence Fourier-domain digital holography system installed in the BioDynamic

Imaging system permits the collection of backscatter photons from samples 1mm thick in depth,

conveying a greater detail of information on the current state of the embryos analyzed in these

experiments.

Similar to the work of assessing phenotypic responses of tumor spheroids to drug

exposure (Evans et al., 2011), the first portion of this experiment looked at the responses of

embryos exposed to various stressors. Ethanol was chosen due to its involvement in

parthenogenesis or embryonic development; ethanol percentages of less than 1% in culture

medium have been shown to accelerate mitosis and differentiation of the trophectoderm (Kalmus

et al., 1989). A brief exposure to 7% ethanol promotes parthenogenetic activation of oocytes

through the induction of a single calcium transient (Stachecki et al., 1994). Research suggests

that exposure, occurring under 5 minutes, to elevated ethanol levels does not harm oocytes, yet

prolonged exposure, up to several hours, can promote cellular death due to constant calcium

influx. Ethanol metabolism to acetaldehyde occurs as the embryo attempts to mitigate this

stressor, increasing reactive oxygen species (ROS) and generating free radicals (Huang et al.,

2007). The accumulation of free radicals and ROS lead to oxidative stress, inducing programmed cell death, occurring in embryos of lower quality that are unable to reduce the amount of free radicals and reactive oxygen species compared to their healthier counterparts. Likewise, response to changes in the pH of the environment follows a similar path. Low and high pH stressors can impact the concentration of hydrogen in cells of embryos; altering the metabolism of each cell and impacting the action of metabolic enzymes (Bystriansky et al., 2012). While a blastocyst has the capacity to buffer its internal environment due to the in vivo alkaline conditions, this regulation is limited to a pH range of 7.0 to 7.4 (Edwards et al., 1998). When the alkaline load is greater than 7.8 or the pH is lower than 7.1, the development of blastocysts is hindered and oxidative stress occurs, resulting in an increase in apoptosis (Ocon et al., 2003). Osmotic stressors, such as DPBS, impact intracellular ion concentrations, promoting the uptake of small ions and water, changing cell volume. Na/K/ATP synthase is the primary transport channel utilized to mediate osmotic responses of the cell to its environment, resulting in the production of reactive oxygen species which can trigger programmed cell death (Dumoulin et al., 1997).

An embryo's response to stressors and the intracellular actions it takes to mitigate these harmful conditions is directly associated with its quality and thus viability, hence the identification of two embryonic groups in each treatment was expected. These groups differ by the slope of their spectra and their respective nyquist floor, suggesting that the embryos with a higher slope of spectra and lower nyquist floor are unable to adequately respond to the stressor and are less viable. Embryos with a lower slope and higher nyquist floor are of higher quality, able to mitigate the negative impacts of the stressor.

In addition, the recorded frequencies of the spectra differed between fertilized and unfertilized oocytes. Unfertilized oocytes had a higher slope of the spectra whereas fertilized

oocytes had long periods (over several hours) of low frequencies of their spectra. The timing of

these low frequencies correspond with the cellular events of syngamy, meiotic resumption and

pronuclear movements in fertilized oocytes whereas the low frequencies observed in unfertilized

oocytes were sporadic and short, likely exhibiting a pattern associated with programmed cell

death (Berridge, 1990). The large metabolic activity, completion of meiosis and generation of a

zygote with a maternal pronucleus occurs anywhere from 4 to 10 hours after the completion of

calcium oscillations triggered by fertilization (Vitullo et al., 1992). Movement of the male and

female pronuclei towards each other adds to the complex intracellular dynamics likely captured

by the BDI in the low frequencies for over several hours.

In conclusion, the BDI has the capacity to record the response of embryos to

environmental stressors and determine the quality of each embryo based on the slope and nyquist

floor of their spectra. High viability embryos are more successful at mitigating oxidative stress

and have spectra with higher nyquist floors and relatively lower slopes compared to low viability

oocytes. The BDI system can also trace gamete fusion and the subsequent intracellular events in

fertilized oocytes and distinguish the spectra of developing zygotes from dying, unfertilized

oocytes in terms of slope of the spectra and recorded frequencies. Therefore, parameters used to

determine the quality of embryos measured under by the BDI should include nyquist floor and

slope of the spectra.

2.6   References

Berridge, M. J. (1990). Calcium oscillations. *J Biol Chem, 265*(17), 9583-9586.

Black, M., & Bhattacharya, S. (2010). Epidemiology of multiple pregnancy and the effect of
    assisted conception. *Semin Fetal Neonatal Med, 15*(6), 306-312. doi:
    10.1016/j.siny.2010.06.004

Brison, D. R., Houghton, F. D., Falconer, D., Roberts, S. A., Hawkhead, J., Humpherson, P. G., Lieberman, B. A., Leese, H. J. (2004). Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod, 19*(10), 2319-2324. doi: 10.1093/humrep/deh409

Byrne, A. T., Southgate, J., Brison, D. R., & Leese, H. J. (1999). Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J Reprod Fertil, 117*(1), 97-105.

Bystriansky, J., Burkuš, J., Juhás, Š., Fabian, D., & Koppel, J. (2012). Effect of increased urea levels on mouse preimplantation embryos developed in vivo and in vitro. *56*(2), 211. doi: https://doi.org/10.2478/v10213-012-0038-9

Denk, W., Strickler, J. H., & Webb, W. W. (1990). Two-photon laser scanning fluorescence microscopy. *Science, 248*(4951), 73-76.

Dumoulin, J. C., van Wissen, L. C., Menheere, P. P., Michiels, A. H., Geraedts, J. P., & Evers, J. L. (1997). Taurine acts as an osmolyte in human and mouse oocytes and embryos. *Biol Reprod, 56*(3), 739-744.

Edwards, L. J., Williams, D. A., & Gardner, D. K. (1998). Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod, 13*(12), 3441-3448.

Evans, C. L., Abu-Yousif, A. O., Park, Y. J., Klein, O. J., Celli, J. P., Rizvi, I., Zheng, X., Hasan, T. (2011). Killing hypoxic cell populations in a 3D tumor model with EtNBS-PDT. *PLoS One, 6*(8), e23434. doi: 10.1371/journal.pone.0023434

Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril, 73*(6), 1155-1158.

Gonzalez, R. R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., & Simon, C. (2000). Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. *J Clin Endocrinol Metab, 85*(12), 4883-4888. doi: 10.1210/jcem.85.12.7060

Huang, L. H., Shiao, N. H., Hsuuw, Y. D., & Chan, W. H. (2007). Protective effects of resveratrol on ethanol-induced apoptosis in embryonic stem cells and disruption of embryonic development in mouse blastocysts. *Toxicology, 242*(1-3), 109-122. doi: 10.1016/j.tox.2007.09.015

Kalmus, G. W., & Buckenmaier, C. C., 3rd. (1989). Effects of ethanol and acetaldehyde on cultured pre-implantation mouse embryos. *Experientia, 45*(5), 484-487.

Lee, K., Hyslop, J. M., Nanassy, L., & Machaty, Z. (2009). Incidence of apoptosis in parthenogenetic porcine embryos generated by using protein kinase or protein synthesis inhibitors. *Anim Reprod Sci, 112*(3-4), 261-272. doi: 10.1016/j.anireprosci.2008.04.027

Ocon, O. M., & Hansen, P. J. (2003). Disruption of bovine oocytes and preimplantation embryos by urea and acidic pH. *J Dairy Sci, 86*(4), 1194-1200. doi: 10.3168/jds.S0022-0302(03)73703-5

Quinn, K. P., Bellas, E., Fourligas, N., Lee, K., Kaplan, D. L., & Georgakoudi, I. (2012). Characterization of metabolic changes associated with the functional development of 3D engineered tissues by non-invasive, dynamic measurement of individual cell redox ratios. *Biomaterials, 33*(21), 5341-5348. doi: 10.1016/j.biomaterials.2012.04.024

Reddy, U. M., Wapner, R. J., Rebar, R. W., & Tasca, R. J. (2007). Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop. *Obstet Gynecol, 109*(4), 967-977. doi: 10.1097/01.AOG.0000259316.04136.30

Richardson, A., Brearley, S., Ahitan, S., Chamberlain, S., Davey, T., Zujovic, L., Hopkisson, J., Campbell, B., Raine-Fenning, N. (2015). A clinically useful simplified blastocyst grading system. *Reprod Biomed Online, 31*(4), 523-530. doi: 10.1016/j.rbmo.2015.06.017

Seli, E., Botros, L., Sakkas, D., & Burns, D. H. (2008). Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril, 90*(6), 2183-2189. doi: 10.1016/j.fertnstert.2008.07.1739

Stachecki, J. J., Yelian, F. D., Leach, R. E., & Armant, D. R. (1994). Mouse blastocyst outgrowth and implantation rates following exposure to ethanol or A23187 during culture in vitro. *J Reprod Fertil, 101*(3), 611-617.

Sun, H., Merrill, D., An, R., Turek, J., Matei, D., & Nolte, D. D. (2017). Biodynamic imaging for phenotypic profiling of three-dimensional tissue culture. *J Biomed Opt, 22*(1), 16007. doi: 10.1117/1.JBO.22.1.016007

Vitullo, A. D., & Ozil, J. P. (1992). Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Dev Biol, 151*(1), 128-136.

CHAPTER 3. VALIDATION OF BIODYNAMIC IMGAING DATA USING NONINVASIVE TECHNIQUES

3.1 *Abstract*

Clinical means to evaluate the viability of in vitro-produced embryos have recently been improved as the fields of metabolomics and proteomics have been applied to the spent culture medium. Several studies indicate that energy substrates such as glucose and pyruvate are altered in the spent medium according to embryonic developmental capacity. Amino acid secretions also differ based on embryo quality; early human embryos that are more likely to implant show increased asparagine and glutamate, and decreased glycine, alanine and leucine secretions. Furthermore, blastocyst formation can be predicted based on changes of arginine, alanine, glutamate, methionine and asparagine released during early embryo development. Protein secretion can also be indicative of embryo quality as leptin secretion is elevated in human blastocysts that naturally hatched. With this knowledge, spent culture medium profiling was employed to validate the measurements of the BioDynamic Imaging system in assessing embryo viability. Spent culture medium was collected from day 7 porcine embryos that had been individually cultured for 24 hours and sent for metabolite as well as proteomic analysis. Metabolite analysis was done through high performance liquid chromatography, while proteomic analysis was completed via high performance liquid chromatography coupled with mass spectroscopy. Due to the limits of quantification od the technology we were unable to quantify leucine, glutamic acid, glycine and asparagine in the culture medium. In addition, proteomic assays were unable to identify leptin or the leptin receptor in the spent culture medium. Amino acid concentrations will likely be quantifiable using a different column and methodology;

however, leptin secretion does differ between porcine and human blastocysts as the detection method identified many embryo-derived proteins but leptin was not one of them.

3.2   Introduction

Infertility is an unfortunate and painful hurdle that affects approximately 15% of couples trying to conceive (Seli et al., 2008). Nationally, infertility has been on the rise, due to issues such as male factor infertility or increased maternal age, encouraging the use of assisted reproductive technologies (ART) to mitigate the reproductive problems associated with conception (Wright et al., 2005). In vitro fertilization (IVF) is a reliable, established practice that involves the in vitro co-incubation of the male and female gametes, often producing several embryos in a single cycle. The laboratory-derived embryos must then be transferred back to their maternal environment, the uterus, for implantation, post-implantation development and delivery of a child after a successful pregnancy. Unfortunately, reports show that two-thirds of ART cycles end in failure as 80% of the embryos after a single-embryo transfers fail to implant, resulting in embryonic loss (Bromer et al., 2008; Kovalevsky et al., 2005). While morphological analysis prior to transfer provides some benefit to clinicians attempting to identify good quality embryos, such analysis is highly subjective and inadequate for selecting embryos with the highest developmental potential (Seli et al., 2008). In response to the poor implantation rates associated with transferring only one embryo (single embryo transfer; sET), clinical practices shifted towards transferring multiple embryos, with an average of 2.4-2.8 embryos transferred per ART cycle in 2004 (Seli et al., 2007). Such practices did improve pregnancy rates consequently resulting in more than 30% of ART-induced pregnancies containing two or more fetuses, 51% of these live births being the products of multiple gestation (Seli et al., 2008). However, multiple birth situations are not indicative of true reproductive success as they pose an

increased risk for several diseases and predispositions in both the mother and the children. Thus the field of clinical ART has reached a caveat where clinicians must instill practices to limit multiple birth pregnancy rates while optimizing each ART cycle's embryo transfer in hopes of keeping success rates high.

Efforts to reducing multiple birth rates due to ART begun in 2001 when European fertility centers promoted the return to single embryo transfer (Reddy et al., 2007). Still, this return to older clinical practices calls for a better method to analyze the quality of these embryos and identify the best embryo for implantation and development. While morphological grading has long been used and closely defined into a set of visual criteria associated with cellular quality, it has not been a strong enough indicator of developmental competency. Recent work has focused on the developing fields of metabolomics and proteomics to noninvasively measure the quality of embryos by assessing cellular secretions in the spent culture medium. Recent research by Conaghan et al. (2014) and Gardner et al. (2000) identified trends in the secretion of glycolysis and citric acid cycle components respectively, in individual embryo cultures which they noted were indicative of development. Glucose uptake was highest in human embryos with the best morphological grade and a decrease in pyruvate metabolism was associated with highly viable embryos (Seli et al., 2007). Retrospective studies have looked at amino acid profiles of spent culture medium relative to the implantation or loss of cultured human embryos. A study by Brison et al. (2004) using high performance liquid chromatography (HPLC) found that successful implantation of an embryo could be predicted by the turnover of asparagine, glycine and leucine in the spent culture medium of day 1 and day 2 embryos. Embryos whose spent culture media showed a decrease in glycine and leucine along with an increase in asparagine were more likely to implant. Furthermore, blastocyst formation was predictable in the changes of

arginine, alanine, glutamate, methionine and asparagine during embryonic growth from day 2 and 3 (Brison et al., 2004). Additionally, metabolomic profiling of spent culture medium from 48 hour incubation of early embryos, showed an elevation in the glutamate concentration in the spent media of embryos that were more likely to result in pregnancy (Seli et al., 2008). A reverse trend was noted with alanine, where lower levels of alanine were indicative of higher quality embryos. In terms of protein secretion, recent research lends towards the analysis of the secretion of leptin in the culture medium incubating human blastocysts. A report by Gonzalez et al. (2000) showed that leptin could be an important factor for the proper implantation of blastocysts into the uterine endometrium. Not only was mRNA for the leptin receptor upregulated in endometrial epithelial cells cultured with developing embryos, but an increase in leptin secretion was found in the population of embryos who successfully underwent hatching in vitro (Gonzalez et al., 2000). While previous reports had failed to define the effect of leptin on reproductive organs, this study indicates a potential coordination between the leptin receptor on the endometrium and leptin secreted by blastocyst on implantation; lending to the idea that high quality blastocysts secrete leptin in spent culture medium (Zamorano et al., 1997).

As shown, the measuring of metabolites and proteins in the spent culture medium can be indicative of embryonic development. However, the methodology and equipment needed to perform such assays are expensive and time-consuming, making installation in an embryology practice costly and requiring the hiring or technical training of staff familiar with performing such analyses (Seli et al., 2007). As discussed in Chapter 2, Biodynamic Imaging (BDI) offers the possibility to be used as a sensitive and specific manner to determine embryonic development that is easier, faster and cheaper to use by clinicians. The objective of this study was to validate

the use of the BDI on identifying embryo quality through the parallel assays of spent culture medium for amino acid profiles and leptin protein secretion.

3.3   *Materials and Methods*

3.3.1   *In Vitro Maturation*

Oocyte-cumulus complexes were aspirated from the follicles of gilt ovaries obtained from a local abattoir and matured in vitro using hormone- and cytokine-supplemented TCM-199 medium as described in Chapter 2.

3.3.2   *In Vitro Fertilization*

Matured oocytes were collected and cultured in the presence of sperm from a Large White boar with proven fertility to produce embryos using the techniques, media and protocols as described in Chapter 2.

3.3.3   *Metabolomics*

Individual blastocysts were cultured in 40 µl PZM-3 medium from day 6 of incubation to day 7. On day 7 the embryo was removed and the spent culture medium was collected and sent to the Metabolite Profiling Facility of Purdue University where it was analyzed for amino acid profiles on an Imtakt Amino acid column WAA25. The entire amount of the spent medium sample was added to 5 µl α-ABA solution and 40 µl 50:50 MeOH:CHCl$_3$, vortexed and centrifuged. The aqueous layer was then collected and speed-vacuum dried. The sample was then reconstituted in 50:50 acetonitrile and centrifuged with the remaining supernatant being used for liquid chromatography runs.

The column was pre-calibrated with seven premade samples consisting of 35 µl of PBS and 5 µl of amino acid mix. Amino acid mixes were prepared as serial dilutions labeled A

through G, respectively. A contained the highest concentration of amino acids while G contained the lowest concentration, reduced by 10^4. Forty µl of 50:50 MEOH:CHCl$_3$ was added to each serial dilution and centrifuged, the aqueous layer was then isolated and speed vacuum dried. Each dilution was reconstituted in 50:50 acetonitrile and run through the liquid chromatography column. Fractions were then isolated and identified using mass spectroscopy.

Initially, PZM-3 was used as the culture medium; however, results showed that the amino acid supplementation in PZM-3 resulted in confounding data and high amino acid levels. Therefore, an older type of culture medium, NCSU-23 (North Caroline State University-23; Petters and Wells, 1993) was used as it contained no supplemented amino acids. This medium and corresponding embryo incubation resulted in amino acid concentrations below the level of quantification, therefore the embryo incubation droplet was reduced to 10 µl, which was added to 5 µl α-ABA solution and 40 µl 50:50 MeOH:CHCl$_3$, as described above.

3.3.4   *Proteomics*

Five µl droplets of PZM-3 culture medium (covered with mineral oil) were used to incubate individual blastocysts from day 6 to day 7. After 24 hours of culture, the embryo was removed and the spent culture medium was collected for protein analysis. Proteins were extracted by precipitation using 5 ml of cold (-20°C) acetone overnight, and then pelleted by centrifugation at 14,000 rpm for 15 minutes at 4°C in an Eppendorf 5810R centrifuge. Protein pellets were dissolved in 50 µl of 8 M urea for 1 hour at room temperature. The protein concentration was determined by BCA assay using BSA as standard. Samples were first reduced with 10 mM DTT at 60°C for 45 min, and then cysteines alkylated with 20 mM IAA at room temperature in the dark for 45 min. Before digestion, the concentration of urea was brought down to 1.5 M by adding 25 mM ammonium bicarbonate. Digestion was performed at 37°C overnight using mass

spec grade trypsin and Lys-C mix from Promega at a 1:25 (w/w) enzyme-to-substrate ratio. The digested peptides were desalted using Pierce C18 spin columns (Pierce Biotechnology, Rockford, IL).

The samples were analyzed by reverse-phase HPLC-ESI-MS/MS using the Dionex UltiMate 3000 RSLC nano System coupled to the Q-Exactive High Field (HF) Hybrid Quadrupole Orbitrap MS and a Nano-electrospray Flex ion source (all from Thermo Fisher Scientific; Waltham, MA). Purified peptides were separated using a reverse phase Acclaim PepMap RSLC C18 (75 µm x 15 cm) analytical column using a 120-minute method at a flow rate of 300 nl/minute. The analytical column was packed with 2 µm 100 Å PepMap C18 medium (Thermo Fisher Scientific). Mobile phase A consisted of 0.01% FA in water and a mobile phase B consisted of 0.01 % FA in 80% ACN. The linear gradient started at 5% B and reached 30% B in 80 minutes, 45% B in 91 minutes, and 100% B in 93 minutes. The column was held at 100% B for the next 5 minutes before being brought back to 5% B and held for 20 minutes. Each sample was injected into the MS through the Nanospray Flex™ Ion Source fitted with an emission tip from Thermo Fisher Scientific, and MS data were acquired with a Top20 data-dependent MS/MS scan method. The full scan MS spectra were collected over 300-1,650 m/z range at a resolution of 60,000 at 200 m/z, spray voltage of 2 and AGC target of 1×10^6. Fragmentation of precursor ions was performed by high-energy C-trap dissociation (HCD) with the normalized collision energy of 27 eV. MS/MS scans were acquired at a resolution of 15,000 at m/z.

3.4   Results

3.4.1   *Metabolomic Analysis*

Initial data using PZM-3 as culture medium indicated an elevation in the concentration of all

amino acids including negative control samples, though the values of glycine and asparagine

were below the limit of quantification given by their respective amino acid standard curves

(Table 3.1).

Table 3.1. Amino acid concentrations in 40 μl PZM-3 medium

Sample #	Leucine (ng/ml)	Glutamic Acid (ng/ml)	Glycine (ng/ml)	Asparagine (ng/ml)
1	5002.5381	2081.5179*	2318.2127*	2359.0033*
2	6434.4693	8271.9834	2537.8145*	3851.7448
3	7073.2787	7508.8327	2427.2893*	3603.1289
4	6809.1578	4959.7203	1901.2159*	2363.2393*
5	7229.2152	7856.0653	2419.6593*	3759.8865
Control 1	3541.6685	0*	2186.6491*	2184.8844*
Control 2	1822.6779*	0*	2132.2950*	2054.1483*

*Denotes values that are below the limit of quantitation as determined by the amino acid standard curves

   A closer look into the composition of the medium revealed that PZM-3 medium contains

several supplemented amino acids in the ingredients BME and MEM (amino acid cocktails).

Therefore, the elevation of amino acid in the spent culture medium was global and not relative to

the quality of the embryo incubated in each droplet. NCSU-23, an older medium which

resembles PZM-3 other than the supplemented amino acids, was chosen for further testing amino

acid secretion by developing embryos. Data collected using NCSU-23 showed that of the four

amino acids screened in HPLC, three (Glutamic Acid, Glycine and Asparagine) were below the

limit of detection (Table 3.2). Leucine was detected and quantifiable, though it was on the lower

limit for the graph of quantification.

Table 3.2. Amino acid concentrations in 40 µl NCSU-23 medium

Sample #	Leucine (ng/ml)	Glutamic Acid (ng/ml)	Glycine (ng/ml)	Asparagine (ng/ml)
1	19.8346	**	**	**
2	31.7858	**	**	**
3	19.8346	**	**	**
Control 1	9.7077	**	**	**

**Denotes that amino acid concentrations were below the limit of detection in a given sample

The size of the incubation droplet was then reduced to 10 µl; the corresponding data are displayed in Table 3.3. While a few samples contained a quantifiable amount of individual amino acids, no one sample contained all amino acids in quantifiable amounts. In addition, the small sample size resulted in over-pressurizing of the column when some samples were added, resulting in a loss of sample. As the amino acid values that were detectable fell below the limit of quantification and the decreasing sample size became problematic for the column sensitivity, it was determined that further work to decrease the sample size in hopes of quantifying amino acid secretions were not possible given the reduced sensitivity of the column available for HPLC.

Table 3.3. Amino acid concentrations in 10 µl NCSU-23 medium

Sample #	Leucine (ng/ml)	Glutamic Acid (ng/ml)	Glycine (ng/ml)	Asparagine (ng/ml)
1	***	***	***	***
2	133.9729*	192.1876*	684.5111	174.4707*
3	38.3275*	176.8878*	169.1638*	66.4816*
4	***	***	***	***
Control 1	61.0508*	56.0837*	418.8037	78.5488*

*Denotes values that are below the limit of quantitation as determined by the amino acid standard curves
***Denotes samples that could not be run due to the column over pressurizing as the sample was too small to be successfully make it through the column

3.4.2 *Proteomic Analysis*

The proteins identified in the samples are shown in Table 3.4. Protein IDs were searched

using MaxQuant software (v. 1.5.3.28) (Cox et al., 2014; Cox & Mann, 2008; Cox et al., 2011)

with its built-in Andromeda search engine (Cox et al., 2011) for protein identification and label-

free MS1 quantitation. The database search was performed with the precursor mass tolerance set

to 10 ppm and MS/MS fragment ions tolerance was set to 20 ppm. Database search was

performed with enzyme specificity for trypsin, allowing up to two missed cleavages. Oxidation

of methionine was defined as a variable modification, and carbamidomethylation of cysteine was

defined as a fixed modification. The 'unique plus razor peptides' were used for peptide

quantitation. Razor peptides are the non-unique peptides assigned to the protein group with the

most other peptides. The false discovery rate (FDR) of peptides and proteins identification was

set at 1%. Of the proteins, no leptin or leptin receptor was identified by the MaxQuant Software

in any of the samples.

Table 3.4. Detectable proteins in analyzed spent culture medium

Protein ID	Protein Name/Association	MS/MS Count B	MS/MS Count D
gi|47522700;gi|347423;gi|1177051; gi|633093	Zona Pellucida sperm binding protein 3	153	129
gi|7544787;gi|347421;gi|1353184;gi|633091; gi|47523162	Zona Pellucida protein 1	96	70
gi|585100;gi|47522906;gi|294238	Zona Pellucida sperm binding protein 2	48	54
gi|999627;gi|51247812;gi|1942351; gi|9257116; gi|67463906;gi|62738921;gi|62738050	Porcine Epsilon- Trypsin, Alpha Trypsin	59	34
gi|417515396;gi|927145984	Protein Arginine deaminase	16	41

Table 3.4 continued

gi|927117936	Leucine-rich repeat and coiled-coil domain protein	26	18
gi|675970339;gi|675970286;gi|675970195; gi|675970144;gi|311267438	Mammalian Ribosome-sec61 complex	12	24
gi|611961749;gi|347300176;gi|1717797	Peroxiredoxin 2	4	11
gi|335284397	Major vault protein	9	4
gi|545839370;gi|545839367;gi|927156765;gi|545839364;gi|545839361;gi|545839358;gi|545839355; gi|545839349;gi|311260358	Dynein heavy chain 8, axonemal isoform X5	4	2
gi|68566097;gi|47523318;gi|38492203	Ubiquitin thioesterase	2	3
gi|545809442;gi|545809439;gi|73853882; gi|66363554	DNA (cytosine-5)-methyltransferase 1 isoform X2	0	4
gi|7579918	Zona pellucida glycoprotein	3	1
gi|545884298	Spermine synthase	0	3
gi|240851536;gi|167888450	Ubiquitin Thioesterase	1	1
gi|311267677;gi|304365430;gi|302317591; gi|31795527	Dynein light chain 2	1	1
gi|927103150;gi|927103147;gi|675970360;gi|675970307;gi|675970216;gi|675970165;gi|545808634; gi|47523498;gi|302393726	Ubiquitin-60S ribosomal protein	0	2
gi|975233;gi|74039787;gi|47523508;gi|1717817; gi|1016032;gi|1009702	putative transthyretin	1	1
gi|545832998;gi|545832940	NACHT, LRR and PYD domains-containing protein 5	1	0
gi|753704404;gi|927215218	Adenosine deaminase	0	1
gi|927111597;gi|753703272	Proteasome activator complex subunit 4	1	0

Shared proteins in all spent culture medium samples were identified and grouped based on protein function. Of the nineteen shared proteins every sample, four proteins were found to possible indicators of embryonic quality based on a review of proteomics paper. These were Peroxiredoxin 2, Major Vault Protein, NACHT, LRR and PYD domains Protein 5 and Putative Transthyretin.

Table 3.5. Shared proteins in analyzed spent culture medium, grouped based on protein function.

Protein Function	Number of Shared Proteins
Embryonic Structure	6
Spermatic Structure	1
Metabolic Pathways	3*
Ubiquitination	3
Anti-oxidation	1*
Gene Expression	5*

*Indicates protein(s) found in these groups that should be investigated further.

3.5 *Discussion*

With the application of new technologies, the field of ART has the opportunity to employ these methods to improve embryo selection. Metabolomics and proteomics are promising fields for identifying viable embryos to use in embryo transfer; however there are still many improvements that need to be made. Researchers must understand the important terminology used to define the data collected, the key differences between limit of quantitation (LoQ) and limit of detection (LoD). These terms are commonly used in the statistical aspect of understanding the final data from high performance liquid chromatography columns. The limit of detection is best defined as the lowest value of a molecule identified in the sample and determined to be statistically

different from the blank whereas the limit of quantitation is the true amount of a molecule measured in a sample after calculations to mitigate bias and imprecision have been applied (Long et al., 1983)**.** The limit of detection is often indicative of whether a molecule exists in the sample while the limit of quantitation can be explained as the quantifiable amount of a molecule that exists in that sample after factoring the type of column and methodology used. It should be noted that while the limit of detection and the limit of quantitation can be equal, assays will be more accurate and dependable if the limit of quantitation is higher than the limit of detection, as it ensures that the values being collected are not random fluctuations of the blank or column wash (Long et al., 1983). Though the limit of quantitation was higher than the limit of detection for the sample runs used in the metabolomics assay, identifying amino acids above these limits was quite problematic. The first runs using PZM-3 medium had been unknowingly elevated due to the supplementation of amino acids in the medium itself, hence all amino acid concentrations recorded were above the limit of detection but not the limit of quantitation. When the medium was altered to 40 μl of NCSU-23 medium, very few amino acids were above the limit of detection as the increased volume size likely diluted any secreted amino acids. While leucine concentrations were found in these samples runs that were above both the LoD and LoQ, the sample volume was reduced in hopes of extracting more amino acids. The constant issue of sample values not meeting the LoD or LoQ, along with the column over-pressurizing indicated that the column and protocol being used was not sensitive enough to identify amino acid concentrations in such low sample volumes. Therefore further efforts to perform this assay to validate the BDI would require a higher sensitivity column and protocol currently unavailable at the Metabolite Profiling Facility.

Additionally, reproductive research must have a full grasp of the changing metabolite profiles throughout development and how those profiles may be species-specific. Metabolomics studies looking at embryonic secretions by both Seli et al. (2007) and Brison et al. (2000) have noted that the interval of individual incubation in terms of phase of embryonic development play a significant role in the type of metabolites present in the spent medium. Key embryonic events in development occur in the short period of time during in vitro culture, such as zygotic genome activation (Day 4 in humans and pigs, Day 2 in murine models) and shifting between primary energy pathways (Days 5 to 6 in humans and pigs) (Brison et al., 2004). Therefore it is important when assessing secreted metabolites that the specific time period of embryonic development be recorded and kept consistent. Previous research had focused on metabolite secretion from early embryos, days 2-4 (Brison et al., 2004; Seli et al., 2008; Seli et al., 2007). Potentially one of the contributing factors to insufficient levels of amino acids detected in our study was the fact that in our experiment the spent media was collected from day 6-7 blastocysts that had already undergone zygotic genome activation and were actively utilizing glycolysis instead of oxidative phosphorylation. It should be noted that there lies great promise in the formation of a library or study that charts changes in metabolite and protein profiles in embryos throughout early development. A deeper understanding of how embryonic secretions are altered during in vitro culture and prospective data on implantation/successful transfer at blastocyst stage could have a profound impact on the noninvasive screening performed on early embryos. Identification of such metabolites and proteins commonly found in spent culture medium could add to the understanding of which cellular processes are important for determining future embryonic viability. For instance, excessive glutamate in spent culture medium could be a byproduct of the transamination between ammonium and α-ketoglutarate and an indicator of an embryo's ability

to mitigate ammonium levels that could cause potential damage in vitro (Seli et al., 2008). The application of glutamate or glutamic acid assay therefore is essential as it could predict which embryos are more likely to reduce detrimental ammonium levels found in in vitro conditions.

Proteomic assays have previously been used to assess the developmental potential of human embryos. In those studies, leptin secretion had been measured in human early blastocysts, on day 6 of embryonic development (Gonzalez et al., 2000). In our experiment, day 6 porcine blastocysts were incubated individually for 24 hours. Though timing of sample incubation was kept consistent, neither leptin nor the leptin receptor was identified in spent culture medium, bringing up the possibility of species-specific differences in protein secretion. Cited research has reported an increase in leptin secretion and leptin receptor expression in human blastocysts as a potential indicator of subsequent implantation (Gonzalez et al., 2000). The importance of leptin in human embryonic development has been reported previously (Craig et al., 2005). Leptin appears to be secreted in the porcine oviduct and when supplemented to maturation and culture medium improves oocyte activation and cleavage rates. We did not find leptin secreted by porcine blastocysts in the culture medium, pointing at species-specific differences between humans and pigs. Leptin receptor mRNA has been found to be upregulated in porcine embryos as early as 2-cell stage of development and has been determined to be essential in controlling cell proliferation (Craig et al., 2005). Culture medium supplemented with leptin resulted in producing blastocysts with higher total cell numbers, likely due to induced activation of JAK/STAT family and MAPK signaling transduction (Hegyi et al., 2004). Leptin is an important regulator of reproductive function in most species, yet this evidence suggests that while leptin can improve embryo development, it is not secreted by blastocyst-stage porcine embryos. Rather exogenous leptin, present in culture medium or in the oviduct, can control cell proliferation and

preimplantation through binding leptin receptors expressed on the developing embryo in swine. Fortunately we did find four proteins whose potential for predicting embryonic viability should be further investigated. Peroxiredoxin 2 has recently been found to be vital for reducing reactive oxygen species, a common byproduct of oxidative metabolism, that when left unregulated results in the preemptive death of early embryos (Rabilloud, T. et al., 2002). Major Vault Protein is an important in controlling meiotic resumption in the early zygote as it promotes MAPK activation and drives the shift from meiosis to mitosis (Ellederova et al., 2004). NACHT, LRR and PYD domains Protein 5 and Putative Transthyretin have also been shown in literature as potential indicators of embryonic quality as they are indicators of active metabolism and thyroxine uptake, respectively (Kufer et al., 2005; Ducolomb et al., 2013). While more research is warranted to identify which of these proteins is a better assay of porcine embryo health in terms of sensitivity and specificity, these four proteins will provide the next step for porcine embryo proteomics.

In conclusion, protein secretion can easily be assessed in spent culture medium; however, our results support the idea that leptin secretion differs from porcine blastocysts compared to human blastocysts. Therefore, proteomic assessment of leptin in the culture medium is not beneficial when attempting to validate the BDI in the case of porcine embryos. Rather, future efforts should focus on measuring the level of leptin receptor expression in porcine embryos as this might be more representative of prospective viability or exploring the secretion patterns of peroxiredoxin 2, major vault protein, NACHT LRR and PYD domain 5, and putative transthyretin. While some spent culture medium did exhibit differing profiles of amino acids between individual embryos, several further details need to be established before metabolomics is employed to validate the BDI using porcine embryos. Once expression patterns of amino acids have been established during the timeline of porcine embryonic development, the field of

metabolite profiling will be easy to apply when determining porcine embryo viability and validation of dynamic imaging and other novel systems.

3.6   References

Brison, D. R., Houghton, F. D., Falconer, D., Roberts, S. A., Hawkhead, J., Humpherson, P. G., Leese, H. J. (2004). Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod, 19*(10), 2319-2324. doi: 10.1093/humrep/deh409

Bromer, J. G., & Seli, E. (2008). Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. *Curr Opin Obstet Gynecol, 20*(3), 234-241. doi: 10.1097/GCO.0b013e3282fe723d

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics, 13*(9), 2513-2526. doi: 10.1074/mcp.M113.031591

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol, 26*(12), 1367-1372. doi: 10.1038/nbt.1511

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., & Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res, 10*(4), 1794-1805. doi: 10.1021/pr101065j

Craig, J. A., Zhu, H., Dyce, P. W., Wen, L., & Li, J. (2005). Leptin enhances porcine preimplantation embryo development in vitro. *Mol Cell Endocrinol, 229*(1-2), 141-147. doi: 10.1016/j.mce.2004.08.008

Ducolomb, Y., Gonzalez-Marquez, H., Fierro, R., Jimenez, I., Casas, E., Flores, D., . . . Betancourt, M. (2013). Effect of porcine follicular fluid proteins and peptides on oocyte maturation and their subsequent effect on in vitro fertilization. *Theriogenology, 79*(6), 896-904. doi: 10.1016/j.theriogenology.2013.01.024

Ellederova, Z., Halada, P., Man, P., Kubelka, M., Motlik, J., & Kovarova, H. (2004). Protein patterns of pig oocytes during in vitro maturation. *Biol Reprod, 71*(5), 1533-1539. doi: 10.1095/biolreprod.104.030304

Gonzalez, R. R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., & Simon, C. (2000). Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. *J Clin Endocrinol Metab, 85*(12), 4883-4888. doi: 10.1210/jcem.85.12.7060

Hegyi, K., Fulop, K., Kovacs, K., Toth, S., & Falus, A. (2004). Leptin-induced signal transduction pathways. *Cell Biol Int, 28*(3), 159-169. doi: 10.1016/j.cellbi.2003.12.003

Kovalevsky, G., & Patrizio, P. (2005). High rates of embryo wastage with use of assisted reproductive technology: a look at the trends between 1995 and 2001 in the United States. *Fertil Steril, 84*(2), 325-330. doi: 10.1016/j.fertnstert.2005.04.020

Kufer, T. A., Fritz, J. H., & Philpott, D. J. (2005). NACHT-LRR proteins (NLRs) in bacterial infection and immunity. *Trends Microbiol, 13*(8), 381-388. doi: 10.1016/j.tim.2005.06.004

Long, G. L., & Winefordner, J. D. (1983). Limit of detection- A closer look at the IUPAC definition. *Analytical Chem, 55*(7), 712-724.

Petters, R. M, Wells, K D. (1993). Culture of pig embryos. J Reprod Fertil 48(Suppl):61-73.

Rabilloud, T., Heller, M., Gasnier, F., Luche, S., Rey, C., Aebersold, R., . . . Lunardi, J. (2002). Proteomics analysis of cellular response to oxidative stress. Evidence for in vivo overoxidation of peroxiredoxins at their active site. *J Biol Chem, 277*(22), 19396-19401. doi: 10.1074/jbc.M106585200

Reddy, U. M., Wapner, R. J., Rebar, R. W., & Tasca, R. J. (2007). Infertility, assisted reproductive technology, and adverse pregnancy outcomes. *Obstet Gynecol,* 109(4), 967-977.

Seli, E., Botros, L., Sakkas, D., & Burns, D. H. (2008). Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril, 90*(6), 2183-2189. doi: 10.1016/j.fertnstert.2008.07.1739

Seli, E., Sakkas, D., Scott, R., Kwok, S. C., Rosendahl, S. M., & Burns, D. H. (2007). Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril, 88*(5), 1350-1357. doi: 10.1016/j.fertnstert.2007.07.1390

Wright, V.C., Schieve, L. A., Reynolds, M.A., & Jeng G. (2005) Assisted Reproductive Technology Surveillance, United States, 2002. *Morbidity and Mortality Weekly Report: Surveillance Summaries,* 54(2),1-24.

Zamorano, P. L., Mahesh, V. B., De Sevilla, L. M., Chorich, L. P., Bhat, G. K., & Brann, D. W. (1997). Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology, 65*(3), 223-228. doi: 10.1159/000127276

CHAPTER 4.   VALIDATION OF BIODYNAMIC IMGAING DATA USING TUNEL ASSAY AND MORPHOLOGICAL GRADING

4.1   Abstract

Current clinical practices focus on the use of morphological grading systems to evaluate the viability of in vitro-produced embryos for selection for use in embryo transfer procedures. While these morphological grading systems are successful for predicting pregnancy outcomes, the subjectivity of these assessments has been well documented. As a result, morphological assessments have been used in tandem with objective assays to more accurately predict embryonic viability. Assays detecting apoptosis are extremely valuable for determining the health of embryos as apoptosis is the programmed death of cells exhibiting degenerative qualities to maintain the integrity of the embryo. Tunel apoptosis detection kits operate by labelling of single-stranded DNA fragments, a sign of late stage apoptosis, with a fluorochrome for quantification. Both morphological assessment using the Gardner grading system, and Tunel apoptosis staining was utilized to validate the measurements of the BioDynamic Imaging (BDI) system in assessing embryo viability. Blastocyst stage embryos were morphologically graded before being placed under the BDI and measured for four iterations at an exposure of 50 ms with 20 minutes between each iteration, for a total experiment time of one hour. The sample was removed and visually assessed again before fixing with 3.7% paraformaldehyde and staining for apoptotic factors using the Tunel apoptosis detection kit and total cell number using the nuclear stain Hoechst 33342. A correlation matrix was statistically calculated using several biological and optical parameters. We found that normalized standard deviation negatively correlated with morphological score of the sample, indicating that as an embryo dies the intracellular motion increases, resulting in further speckle fluctuations.

4.2   Introduction

The practice of assisted reproductive technologies (ART) culminates in the transfer of in vitro-

produced embryos into the maternal uterus. Blastocyst stage embryos are preferentially

transferred as they can be assessed for viability and the best embryos can be selected to improve

implantation rates. Additionally, transfer during the blastocyst stage allows for the better timing

of hormonal synchronization of the uterus in the maternal recipient as implantation in the

receptive endometrium is essential to ART success (Balaban et al., 2006). Success in transferring

blastocysts instead of earlier stage embryos has been captured in high implantation rates (60.9-

70% depending on the number of embryos transferred) compared to low implantation rates from

transferring pronuclear stage (28%) or cleavage stage embryos (48%) (Gardner et al., 2000).

While numerous assessment techniques can be employed to determine embryonic

viability, morphological grading is primarily used by clinics to select embryos for transfer

(Richardson et al., 2015). The most popular systems for grading blastocysts look at blastocoel

expansion and cellular components of the trophectoderm and inner cell mass. These systems

were compared in a study by Balaban et al. (2006). The Dokras system assesses embryos based

on the expansion of the blastocoelic cavity along with the quality and number of nuclei. The

system consists of three grades, with grade 1 given to a blastocyst with an expanded cavity,

grade 2 given to earlier embryos containing premature vacuoles that will develop into the

blastocoelic cavity, and grade 3 given to those samples with dark, deteriorating nuclei and a

deformed blastocoel. The Gardner grading system also accounts for cavity expansion, but

additionally looks at the cellular morphology of both the inner cell mass and the trophectoderm.

The Gardner grading system has 54 permutations of grade combinations and was found to have

higher clinical pregnancy and implantation rates when employed to identify embryo viability.

This system consists of a numerical grade given for blastocoel expansion, 1 through 6, with 6

being a fully expanded, hatched blastocyst; and individual letter grades given to the morphological state of trophectoderm and inner cell mass. These letter grades represent morphology of the respective cell population, with an A grade indicating a high number of compact cells whereas C is the lowest grade given to a cell population that contains few cells that are loosely oriented. Therefore the Gardner grading system has higher selection criteria and has been established as the primary system used in clinics (Balaban et al., 2006).

Morphological assessment has proven to be successful in predicting the implantation of embryos post transfer relatively efficiently; however there are issues with subjectivity of the actual assessment. Variability of morphological grading systems can be high in clinics, due to inter-observer or intra-observer variability, which has been well defined by Richardson et al. (2015). Inter-observer variability is common in clinical practices where different embryologists assign different grades to the same embryo. Individual bias is the cause of such variability; as a result clinics focus on having one embryologists grade all blastocysts to reduce this bias. Intra-observer variability has no implicit bias but causes imprecision in clinics as it occurs when an embryologist gives one blastocyst several different grades after viewing the sample numerous times. Intra-observer variability is the hardest to reduce and is the primary reason why morphological grading is so subjective to determining embryo viability. Therefore, there is a clear need to develop a system to assess embryo viability prior to transfer. Although not perfect, in a series of experiments we used morphological grading to validate the BDI data collected from porcine embryos produced in vitro.

In addition, Tunel staining for cellular apoptotic factors was also employed to validate the BDI data of the embryos. Apoptosis or programmed cell death is an important regulator of cell proliferation, controlling the elimination of cells with degenerative DNA and maintaining the

fidelity of cell lines and the germ line during the development of the rapidly dividing embryo.

Physiological apoptosis is marked by caspase activation, DNA fragmentation, and changes in

mitochondrial function and cytoplasmic fragmentation (Hao et al., 2004). Apoptosis is a dynamic

event in a cell, regulated by the BCL-2 protein family that consists of pro- and anti-apoptotic

proteins (Antonsson et al., 2000). The labeling of cells by these proteins is essential for

controlling the onset of cell death. Pro-apoptotic proteins flag the onslaught of apoptotic

initiation, activating caspase-9 activity. Caspase-9 induces the propagation of phosphatidylserine

on the exterior of the cell plasma membrane, which acts as a degradation tag, attracting local

macrophages to bind and destroy the tagged cell (Lee et al., 2009).

Programmed cell death in embryos occurs in most species after embryonic genome

activation, implementing death in cells that are unable to overcome this important developmental

milestone (Byrne et al., 1999). As the embryo develops further apoptosis naturally increases as a

means to continuously regulate cell proliferation, with a high amount of apoptosis occurring in

the ICM to carefully monitor the development of cells that will become the future neonate

(Fouladi-Nashta et al., 2005). Apoptosis assays in embryos are objective but terminal, giving an

accurate and specific measurement of embryonic viability. Tunel assay screens for late stage

apoptosis in cell populations. The assay works by labeling the 3'-OH DNA single-strand breaks

created by endonucleases with a fluorochrome for visualization (Lee et al., 2009). These

endonucleases have been activated to degrade DNA by executioner caspases like caspase-9, thus

making this event a sign of late-stage apoptosis. As stated above, the objective of this study was

to validate the use of the BDI on identifying embryo quality through Tunel assay in combination

with morphological grading using the Gardner grading system

4.3   Materials and Methods

4.3.1   *In Vitro Maturation*

Oocytes were collected from pre-pubertal gilt follicles and matured in vitro using a chemically

defined medium as described in Chapter 2.

4.3.2   *In Vitro Fertilization*

Matured oocytes and capacitated boar sperm were collected and co-cultured to produce embryos

using techniques, media and practices as described in Chapter 2.

4.3.3   *BDI Measurement*

On day 6 or 7 post fertilization, a blastocyst-stage embryo was removed from the PZM-3 culture

medium. The blastocyst was morphologically assessed and assigned a grade based on the

Gardner grading system. The sample was then placed in a 50 µl droplet of TL-Hepes on a 35mm

Falcon dish pretreated with Cell-tak to immobilize it for the measurement and covered with 2 ml

of mineral oil. The dish was then placed on a heated stage of a microscope set to maintain a

temperature of 39°C on the BDI and measured for four iterations at an exposure of 50 ms with 20

minutes between each iteration, for a total experiment time of one hour. After the measurements

the embryo was again assessed for morphological grade, with any changes being noted.

4.3.4   *Tunel Staining*

The blastocysts that were used for the BDI measurement were fixed in a dish containing 3.7%

paraformaldehyde. They were then stained for apoptotic factors. For this purpose, the blastocysts

were washed in PBS and permeabilized in 0.2% Triton X-100. The samples were again washed

in PBS, and stained using the Promega Tunel Apoptotic Detection Kit (Promega Corporation;

Madison, WI), according to the manufacturer's instructions. Each blastocyst was then covered in

reaction stain mix and incubated for 60 mins at 37°C, after which the reaction was halted using

the stop solution of the kit. The samples were then washed in PBS and stained with Hoechst

33342 to determine the total number of nuclei. Finally, the blastocysts were rinsed at room

temperature, mounted on a microscope slide under a coverslip using 5 μl Vectashield and sealed

with nail polish. They were then examined for apoptotic factors (using a FITC filter) and total

nuclei (using a UV filter) by means of a fluorescence microscope; embryo quality was

determined as the percentage of total nuclei showing signs of apoptosis.

4.4   Results

4.4.1   *Apoptotic Analysis*

Apoptotic factors and total cell numbers were determined for all embryos and used to calculate

relative apoptosis percentages for each sample. Samples were then grouped based on expansion

grade (Table 4.1) and morphology score of the inner cell mass and trophectoderm (Table 4.2) to

generate respective pooled averages for the number of apoptotic factors, total nuclei number, and

percent apoptosis. The apoptotic percentage increased with expansion grade and poor

morphological score, though it should be noted that we found a reduced apoptosis percentage in

the lowest morphological grade category (CC). This was likely due to the sample size of this

group being only one as it is difficult to handle morphologically deteriorated embryos. Further

attempts to expand the sample size in this group resulted in the complete death of several CC

embryos during BDI measurement and staining procedures before Tunel assessment could be

applied.

Table 4.1. Apoptosis in blastocysts showing different degree of expansion. (n=39)

Expansion Grade (based on Gardner's scale)	Average Tunel-positive Cells per Embryo (mean±s.e.m.)	Average Cell Number per Embryo (mean±s.e.m.)	Percent of Apoptotic Cells per Embryo (mean±s.e.m.)
4	2.3± 0.398	30.4± 2.682	7.7±0.017
3	1.5±0.417	28.7±2.500	5.2±0.020
2	0.4±0.400	15.6±2.400	2.6±0.025

Table 4.2. Apoptosis in blastocysts with different morphological grade. (n=39)

Morphological Grade (based on Gardner's scale)	Average Tunel-positive Cells per Embryo (mean±s.e.m.)	Average Cell Number per Embryo (mean±s.e.m.)	Percent of Apoptotic Cells per Embryo (mean±s.e.m.)
AA	1.6±0.412	26.5±2.936	6.0±0.017
AA/BA	1.7±0.398	26.3±3.151	6.5±0.020
BB	2.7±0.833	22.8±2.713	9.5±0.030
CC	1	30	3.3

*Denotes group with sample size of one.

4.4.2 *BDI Parameter Correlations*

Biological and optical biomarkers assessed in these experiments are listed in Table 4.3. Each sample is characterized by optical 16 biomarkers after applying biomarker filters to the response and combing other invasive and non-invasive assessments. The distances between biomarkers are calculated by dist(x,y)=1-corr(x,y), and the biomarkers are clustered into mainly two groups, as shown in Figure 4.1. The first group of biomarkers contains two of the three Tunel assay results, a few differential biomarkers and backscatter brightness. The second group is mainly differential biomarkers, plus nyquist floor. Apoptosis biomarkers are weakly related to most other ones, while the relationship between NSD and morphological score is worth attention, which has a correlation coefficient of about 0.58 when taking into account all samples. NSD (normalized standard deviation) refers to captured intracellular dynamics overtime. Raw data on the sample being measured under the BDI appears in a frame of pixels indicating the intracellular motion of each portion of the sample. The changes in the pixel frequencies across multiple

frames is referred to as the NSD, with large changes in pixel frequencies being associated with a high NSD and a sample that has very active intracellular dynamics. A low NSD indicates that a sample has remained relatively unchanged during measurement as the pixel frequencies have not deviated from the baseline.

Table 4.3. Descriptions of the biomarkers analyzed with identification of the type of parameter, full name and acronym.

Biomarker	Type		Description
BB	BDI	Single measurement	Backscatter brightness
Floor			Nyquist Floor
NSD			Normalized standard deviation
DNSD		Differential (time-lapse)	Change in NSD
DlowF			Change in low frequencies
DmidF			Change in mid frequencies
DallF			Overall change in frequencies
Napop	Invasive		Number of apoptotic cells
Nall			Number of total cells
Pctg			Percentage of apoptotic cells (NAPOP/NALL)
Score	Non-invasive		Morphological grading converted to numerical values
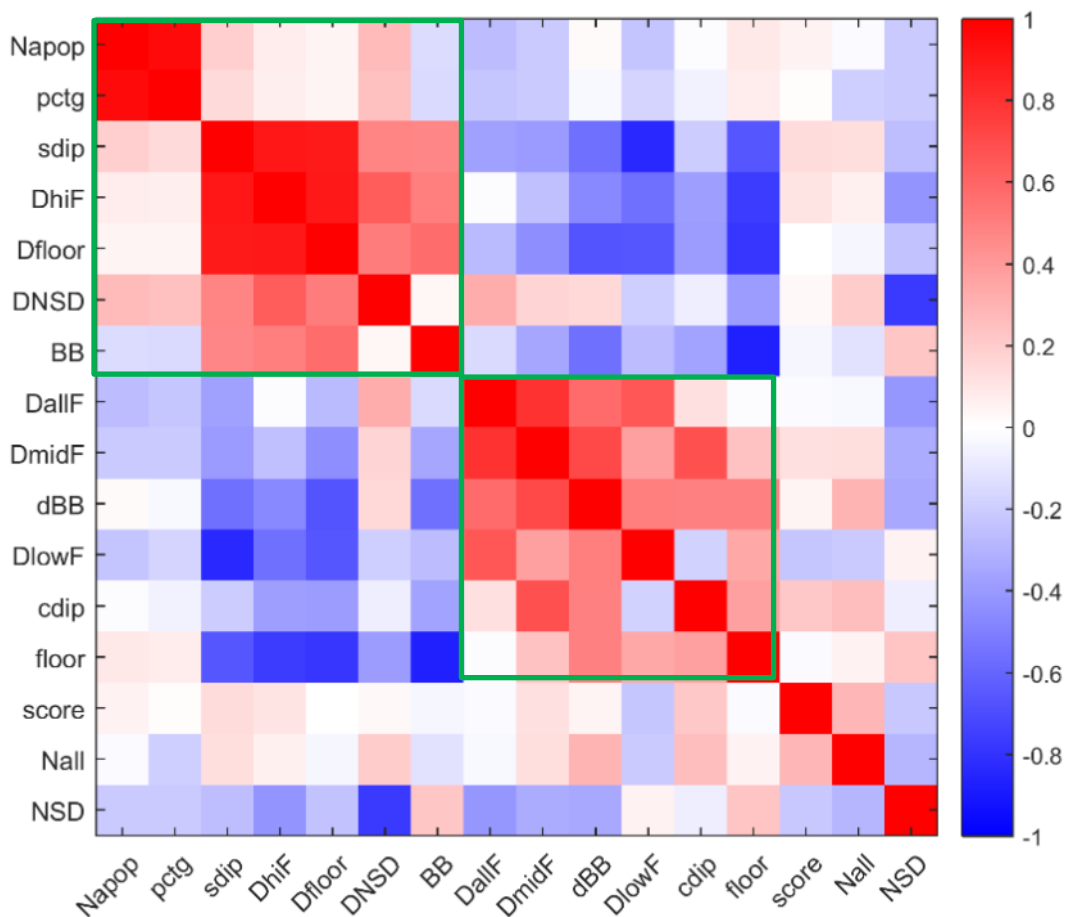
Figure 4.1. A Correlation Matrix of Biomarkers (n=39)

Scatter plots of the respective relationship between NSD and the biological parameters morphological score and percent apoptosis are shown in Figures 4.2 and 4.3. The negative correlation (-0.58) between NSD and morphological grade (Score) points to a pattern for all samples, and implies that the normalized standard deviation increases as the morphological grade (Score) and embryo quality decreases. One possible explanation is that as a sample is dying it becomes more active, which leads to more speckle fluctuations. There is also a weak negative relation (-0.21) between NSD and percent apoptosis (Apoptotic %).
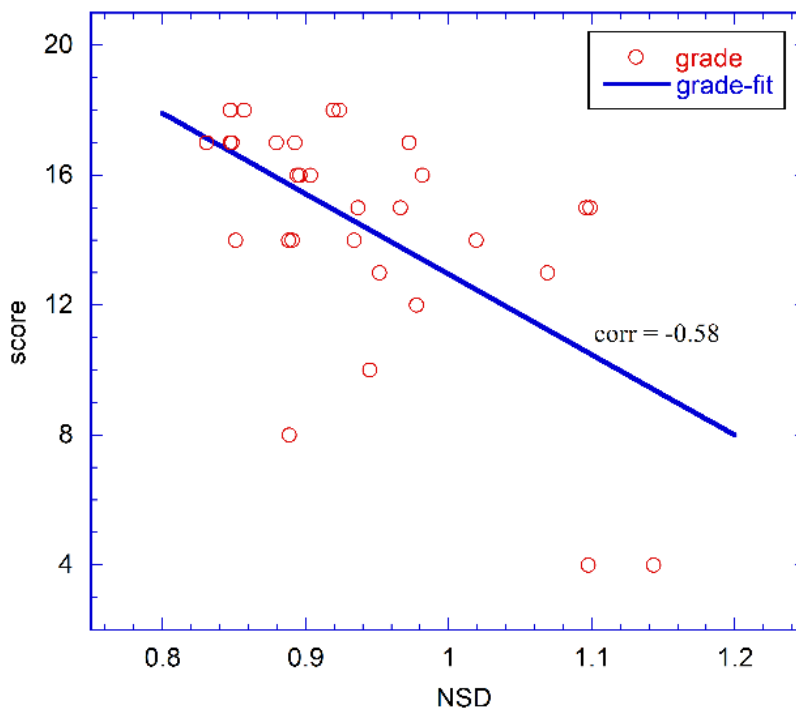
Figure 4.2. Correlation between NSD and morphological grade (Score) of blastocysts.
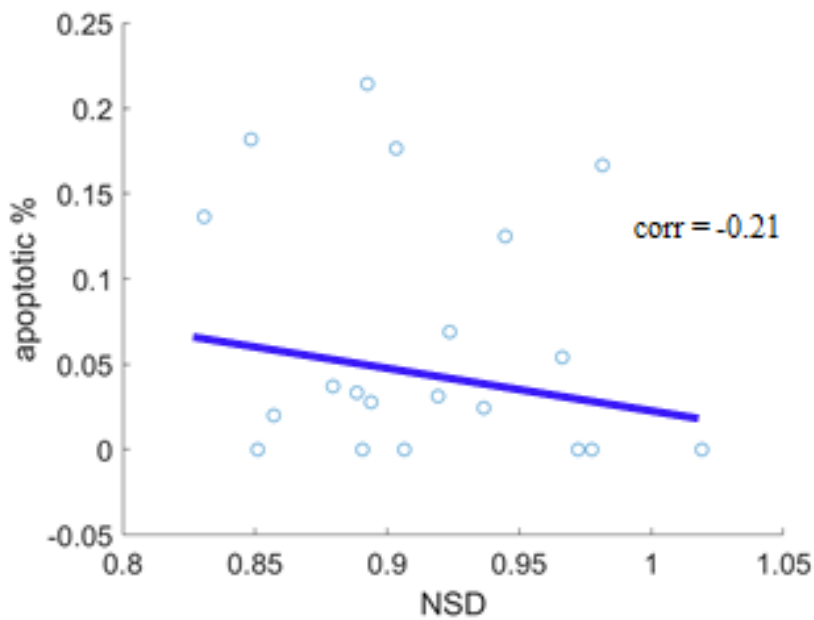
Figure 4.3. Correlation between NSD and percent of apoptotic cells of blastocysts.

4.5   *Discussion*

Determining the viability of embryos before embryo transfer is a pivotal point in ART protocols

and there are several systems in existence to assess embryonic quality. Morphological

assessment, through the Gardner grading system, is common practice in most clinics throughout the US and has been found to be quite successful (Balaban et al., 2006). Yet there are still issues of variability and bias associated with these subjective morphology systems (Richardson et al., 2015). Therefore, additional assays should be used in tandem to objectively confirm the health of an embryo. While the Tunel assay is a terminally invasive assay and therefore cannot be used prior to embryo transfer, it detects apoptotic factors that show the intracellular signs of cell death, a true, objective indicator of embryo viability. Morphological assessment is often tied to apoptosis through the visualization of fragmented and condensed nuclei (Fouladi-Nashta et al., 2005); we selected these two assays to validate the BDI measurements in the current project.

Apoptosis is a dynamic event common in all cells. It is essential for tightly regulating the destruction of degenerative cells and consists of several stages. Such regulation is ensured by factors of the highly conserved BCL-2 protein family which contains both pro-apoptotic and anti-apoptotic proteins that act as tags in a cell (Rodriguez-Osorio et al., 2007). BCL-2 itself is a protein essential for blocking programmed cell death in healthy cells, preventing the blebbing of the plasma membrane, degradation of DNA and a decrease in cell volume (Hockenbery et al., 1990). This anti-apoptotic factor is critical for ensuring healthy cells stay unimpeached by apoptosis but often upregulated in tumors, allowing the hyperactive, damaged cancerous cells to continue proliferating (Korsmeyer, 1999). The homologue pro-apoptotic factor to BCL-2 is BAX, as it has been found to hetero-dimerize with BCL-2 and its interaction with the anti-apoptotic factor has been found to regulate apoptosis depending on the dominate factor present (Oltvai et al., 1993). BAX has been found to control caspase activity and induce mitochondrial dysfunction. A study of factors controlling apoptosis showed that a ratio of these and subsequent

factors in the BCL-2 protein family is the rheostat determining the state of apoptosis in a cell (Korsmeyer, 1999).

In embryos, programmed cell death is an important regulator of growth and development. Apoptosis occurs after zygotic genome activation and increases as the embryo develops during the pre-implantation stage, with the inner cell mass showing a higher rate (10%) compared to the trophectoderm (less than 3%)(Byrne et al., 1999). These differences are likely due to the necessary regulation of the inner cell mass that contains dividing cells that will form the eventual neonate and germline (Fouladi-Nashta et al., 2005). Additionally, apoptosis rates differ based on the number of cells in a blastocyst, with pre-implantation embryos containing less blastomeres having higher apoptotic indexes, indicating that stunted blastocysts are nonviable and targeted for destruction (Byrne et al., 1999). Therefore, Tunel detection kits can easily be employed to give an accurate measure of embryonic viability, giving both qualitative and quantitative data points.

In the present study, embryo viability determined through morphology assessment and Tunel staining was used to validate BDI measurements. We found that NSD appeared to be the BDI parameter that was most indicative of embryonic health. Morphological score was negatively correlated (-0.58) with NSD, showing that a morphologically poor embryo was more likely to have fluctuations in the pixels of each measurement frame. Such sudden changes in intracellular movement can be associated with plasma membrane break down and caspase-9 activation. Rapid intracellular movements continue as apoptosis progresses to phosphatidylserine build up on the plasma membrane and endonucleases are activated to cause single-strand DNA breaks. Tunel assays depend on these late-stage nuclear breaks, labeling the 3'OH- end for fluorescent detection, and apoptotic percentage was found to have a low negative correlation (-

0.21) with NSD. While this correlation is lower than expected, it is likely the outcome of Tunel only being able to identify late-stage apoptosis where the final mark of programmed cell death is DNA degradation. As both day 6 and 7 embryos were measured and apoptosis is a dynamic event, not all blastocysts or even blastomeres would be in the exact same stage of apoptosis when assessed and could be the cause for the low correlation between percent apoptosis and NSD. While some programmed cell death detection kits exist to measure earlier stages of apoptosis, use of these kits again may not capture all of the blastomeres truly undergoing apoptosis (Levy et al., 1998). Morphological score could also be associated with the early stage of apoptosis as many of the criteria for grading the cellular quality of each blastocyst focus on the plasma membrane, cell size and appearance. As described, BCL-2 is an anti-apoptotic factor that prevents membrane blebbing, loss of cell size and degradation of nuclear material, which could be visually observed during morphological assessment. Therefore, the triggering of excessive anti-apoptotic factors initiates this cascade which was observed during grading before measuring the embryo with the BDI, which captured these intracellular events and had an increase in NSD.

In conclusion, the morphological grading of embryos and objective staining for apoptotic factors highlighted the importance of the NSD parameter recorded on the BDI. NSD was shown to be elevated in samples that had low morphological score and high percent of apoptotic nuclei, suggesting that an increase in the NSD of a sample indicates the potential triggering of cellular death pathways. Therefore, poor quality embryos will have a higher NSD compared to their more viable cohorts.

4.6   References

Antonsson, B., & Martinou, J. C. (2000). The Bcl-2 protein family. *Exp Cell Res, 256*(1), 50-57. doi: 10.1006/excr.2000.4839

Balaban, B., Yakin, K., & Urman, B. (2006). Randomized comparison of two different blastocyst grading systems. *Fertil Steril, 85*(3), 559-563. doi: 10.1016/j.fertnstert.2005.11.013

Byrne, A. T., Southgate, J., Brison, D. R., & Leese, H. J. (1999). Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J Reprod Fertil, 117*(1), 97-105.

Fouladi-Nashta, A. A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K. H., & Webb, R. (2005). Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. *Reprod Biomed Online, 10*(4), 497-502.

Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril, 73*(6), 1155-1158.

Hao, Y., Lai, L., Mao, J., Im, G. S., Bonk, A., & Prather, R. S. (2004). Apoptosis in parthenogenetic preimplantation porcine embryos. *Biol Reprod, 70*(6), 1644-1649. doi: 10.1095/biolreprod.103.026005

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., & Korsmeyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature, 348*(6299), 334-336. doi: 10.1038/348334a0

Korsmeyer, S. J. (1999). BCL-2 gene family and the regulation of programmed cell death. *Cancer Res, 59*(7 Suppl), 1693s-1700s.

Lee, K., Hyslop, J. M., Nanassy, L., & Machaty, Z. (2009). Incidence of apoptosis in parthenogenetic porcine embryos generated by using protein kinase or protein synthesis inhibitors. *Anim Reprod Sci, 112*(3-4), 261-272. doi: 10.1016/j.anireprosci.2008.04.027

Levy, R., Benchaib, M., Cordonier, H., Souchier, C., & Guerin, J. F. (1998). Annexin V labelling and terminal transferase-mediated DNA end labelling (TUNEL) assay in human arrested embryos. *Mol Hum Reprod, 4*(8), 775-783.

Oltvai, Z. N., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell, 74*(4), 609-619.

Richardson, A., Brearley, S., Ahitan, S., Chamberlain, S., Davey, T., Zujovic, L., Hopkisson, J., Campbell, B., Raine-Fenning, N. (2015). A clinically useful simplified blastocyst grading system. *Reprod Biomed Online, 31*(4), 523-530. doi: 10.1016/j.rbmo.2015.06.017

Rodriguez-Osorio, N., Kim, I. J., Wang, H., Kaya, A., & Memili, E. (2007). Melatonin increases cleavage rate of porcine preimplantation embryos in vitro. *J Pineal Res, 43*(3), 283-288. doi: 10.1111/j.1600-079X.2007.00475.x

CHAPTER 5.   VALIDATION OF BIODYNAMIC IMAGING DATA USING AN ENERGETICS ASSAY

5.1   Abstract

Women over the age of 35 are more likely to rely on assisted reproductive technologies (ART) to conceive as they have a lower ovarian reserve and decreased oocyte quality. The quality of their oocyte mitochondria has found to be lower, with a significant decrease in mitochondrial output and energy production which is tied to a poor developmental capacity. Deficiencies in mitochondrial DNA (mtDNA) are tied to developmental diseases and disabilities, and mitochondrial function is intimately involved in oxidative catabolism, a primary metabolic pathway in the early embryo. Therefore, mitochondrial function and energetic output dictate embryonic developmental capacity and an assay should be established that can identify the intracellular differences between normal embryos and those with limited energetics. Embryos were parthenogetically derived and cultured in PZM-3 medium for four days with or without 20µM sodium azide (NaN$_3$), a known inhibitor of oxidative phosphorylation. At day four of culture embryos were removed from the culture medium and individually measured under the BioDynamic Imaging system. After measurement each embryo was lysed, added to wells on a 96-well plate and ATP concentration of each embryo was determined. Retrospective analysis of BDI parameters and ATP concentrations showed significant differences between the control treatment group and the NaN₃ group in ATP concentrations, knee frequency and normalized standard deviation (NSD). The control group was found to have four-fold higher contents of ATP ($P<0.01$), higher knee frequency ($P<0.01$) and NSD ($P<0.01$), compared to the NaN₃ treatment group. This assay identifies knee frequency and NSD as BDI biomarkers that depict

the energetic status of an embryo and can be analyzed to indicate the energetic capacity of an embryo as a means to determine sample viability.

5.2 Introduction

Women over the age of 35 have a significant reduction in ovarian reserve, oocyte quality and pregnancy rates (McPherson et al., 2014). The reduction in reproductive quality due to an increase in age may lie in the degeneration of maternal mitochondria. Oocytes from older women have decreased mitochondrial function with lower inner mitochondrial membrane charge, resulting in decreased energy production. These traits are associated with decreased embryo viability, as mitochondrial output and energy production are essential for driving embryonic development through cell division, protein synthesis, and blastocoelic cavity formation (Wilding et al., 2001). Energy production in the mature oocyte and developing embryo is derived through two pathways, glycolysis or oxidative phosphorylation coupled to the tricarboxylic acid (TCA) cycle (Brison et al., 1994). These metabolic pathways are crucial for providing the embryo with sufficient energy for development in the final form of nucleotide triphosphates; with glycolysis producing 2 ATP and the TCA cycle producing 32 ATP, per one molecule of glucose (Machaty et al., 2001). Yet, the two pathways do not operate in equal tandem as one metabolic pathway is employed as the primary energy producer depending on the stage of embryonic development. Oxidative catabolism is intimately tethered to mitochondrial function unlike glycolysis. The TCA cycle releases energy in the forms of reduced electron carriers which pass through the electron transport chain in the inner mitochondrial membrane, allowing for the generation of a proton gradient coupled to an ATPase to produce ATP (Thompson et al., 2000).

Studies on various domesticated species indicate that the primary metabolic pathway utilized is both species- and developmental stage-specific (Brison et al., 1994). This variation in

primary metabolism based on species and stage of development has been correlated with changes in oxygen tension of the maternal environment. Primate, porcine, bovine and ovine embryos appear to have a shift from oxidative phosphorylation in early cleavage stages to glycolysis during compaction at morula stage that corresponds to when the embryo leaves the aerobic oviduct and enters the uterus where oxygen tension is lowered (Machaty et al., 2001). In an aerobic environment the enzyme lactate dehydrogenase promotes a conversion of lactate to pyruvate, a molecule that can directly enter the TCA cycle (Brison et al., 1994; Leese, 1991). The activation of this enzyme catalyzes the consumption of exogenous energy substrates and promotes a primary metabolism of oxidative phosphorylation that is altered when oxygen tension changes. Thus, when the embryo enters an anaerobic environment lactate dehydrogenase activity decreases, shifting the primary metabolism to glycolysis.

Therefore the sensitivity of embryos to inhibitors of oxidative phosphorylation varies based on time of interaction and species of embryo. Brison et al. (1994) evaluated the growth and metabolism of preimplantation embryos of various species and the influence of several oxidative phosphorylation inhibitors. Inhibition of oxidative phosphorylation is quite toxic to rabbit and mouse embryos; treatment with cyanide or 2,4-Dinitrophenol (DNP) at any stage of development resulted in complete degeneration. These species rely on the TCA cycle coupled to oxidative metabolism as the sole energy source for development, unlike other species which undergo the shift discussed above. Interestingly when cyanide, DNP, or sodium azide (NaN_3) are administered in culture to porcine, primate, rat or bovine embryos they can either inhibit or promote development. In early pre-compaction embryos these inhibitors can stunt growth and slowing cleavage, yet long term incubation shows that development to blastocyst stage is not negatively impacted (Machaty et al., 2001). Additionally, inhibition at compaction was actually

found to increase the total cell number, glucose uptake and blastocyst formation rates (Thompson et al., 2000). This could be due to the increase in glycolytic metabolism in the cell populations of the forming blastocysts. Therefore oxidative phosphorylation inhibitors are most effective in early cleavage stages before compaction where they inhibit the electron transfer in the mitochondrial membrane, preventing oxidative catabolism.

Mitochondria play a significant role in mitigating oxidative stress, an important feature that determines cellular viability. Embryos and oocytes from older women are quite sensitive to oxidative damage and pre-compaction embryos are highly susceptible to stressors that generate free radicals and reactive oxygen species, suffering damage that is not evident until post-compaction when they can be morphologically assessed as blastocysts (McPherson et al., 2014). As mentioned in pervious chapters, morphological grading is a subjective assay to determine embryonic quality where cell populations of the developing blastocyst as well as the expansion of the blastocoelic cavity are assigned grades based on morphological characteristics (Richardson et al., 2015). Visualization of mitochondrial dysfunction can be indirectly viewed through blastocoelic expansion, as $Na^+/K^+/ATPase$ transmembrane proteins in the mitochondrial matrix are essential for forming this cavity through driving an osmotic gradient (Thompson et al., 2000). Since this approach can only indirectly assess mitochondrial quality and other methods such as measuring oxygen consumption are expensive and have a low predictive value, there is a need for a novel, direct method to determine mitochondrial function (Tejera et al., 2011). The BioDynamic Imaging system (BDI) has the unique capability to gauge the intracellular dynamics of a sample, including the energetics of an embryo, and may provide a direct assay to determine mitochondrial activity and function (An et al., 2015). Therefore the purpose of this study was to utilize a known inhibitor of oxidative phosphorylation, sodium azide (NaN_3) to reduce the

energetics of 4-cell embryos, compared to control cohorts. The embryos were then recorded under the BDI to identify optical parameters that are indicative of the energetic capacity and thus embryonic viability as confirmed by a luminescence assay to quantify ATP concentrations.

5.3 Materials and Methods

5.3.1 In Vitro Maturation

Immature oocyte cumulus complexes were aspirated from the follicles of ovaries of pre-pubertal gilts slaughtered at a local abattoir and matured in a TCM-199-based defined medium using the methods described in Chapter 2.

5.3.2 In Vitro Fertilization

Preliminary testing involved in vitro-produced embryos which were generated through the co-culture of mature porcine oocytes and capacitated sperm as described in Chapter 2.

5.3.3 Parthenogenetic Activation

Matured oocyte cumulus complexes were placed in TL-Hepes medium containing 0.1% hyaluronidase, vortexed for about 5 minutes and washed in TL-Hepes three times. Denuded oocytes were then placed in electroporation medium (300mM mannitol, 0.1mM $CaCl_2$, 0.1mM $MgSO_4$, 0.5mM Hepes, 0.01mg/ml BSA) in a chamber containing two stainless steel electrodes 0.5mm apart. Parthenogenetic development was induced via electroporation with two direct current pulses of 1.2kV/cm, 60μs each, one second apart, via an Electro Cell Manipulator. Five minutes after stimulation prospective embryos were transferred into culture medium.

5.3.4 Oxidative Phosphorylation Inhibition

Immediately after parthenogenetic activation or fertilization, potential zygotes were transferred into culture medium (control) or medium containing an inhibitor of oxidative phosphorylation.

The control group contained embryos that were incubated in 20μl droplets of PZM-3 (10 embryos per drop) and the experimental embryos were incubated in 20μl droplets of PZM-3 supplemented with 20μM NaN3, a known inhibitor of the electron transport chain. Both groups were allowed to culture in their prospective medium for 4 days, after which the embryos that had reached the morula stage were selected for measurement on the BDI and subsequent ATP quantification.

5.3.5 *BDI Measurement*

Each embryo was transferred individually into a drop of 50μl TL-Hepes medium covered with mineral oil. In order to immobilize the embryos for the duration of the measurement, the bottom of the culture dish had previously been treated with Cell-Tak (Thermo-Fisher Scientific, Waltham, MA). The measurement with the BDI system was done under an exposure of 20ms, 500 background frames and an acquisition rate of 25Hz.

5.3.6 *ATP Quantification*

After the BDI measurement, each embryo was lysed in 10μl of ddH_2O and stored for 10 minutes on ice while the standard curve was prepared and background luminescence was recorded using the standard reaction solution made from the ATP Determination Kit (Molecular Probes, Invitrogen, OR, USA). After the standard curve and background luminescence was determined, the 10μl embryo samples were added to their respective wells and individual well luminescence was measured using the luminescence program on a Tecan Spark 10M microplate reader.

5.4 Results

5.4.1 *Power Calculation*

In order to establish the adequate sample size to determine significant differences in the concentration of ATP in embryos using the detection kit, a power analysis was run using the 'proc power' analysis of SAS Statistical Software version 9.2 (SAS Institute Inc., Cary, NC, USA). The analysis was run using a preliminary sample size of 100, predictor of 1 (concentration of ATP) and an alpha level of 0.05 (Figure 5.1). A regression analysis showed that a power of 10 gives an alpha of ≤0.01, indicating that sample size should be 10 per assay.
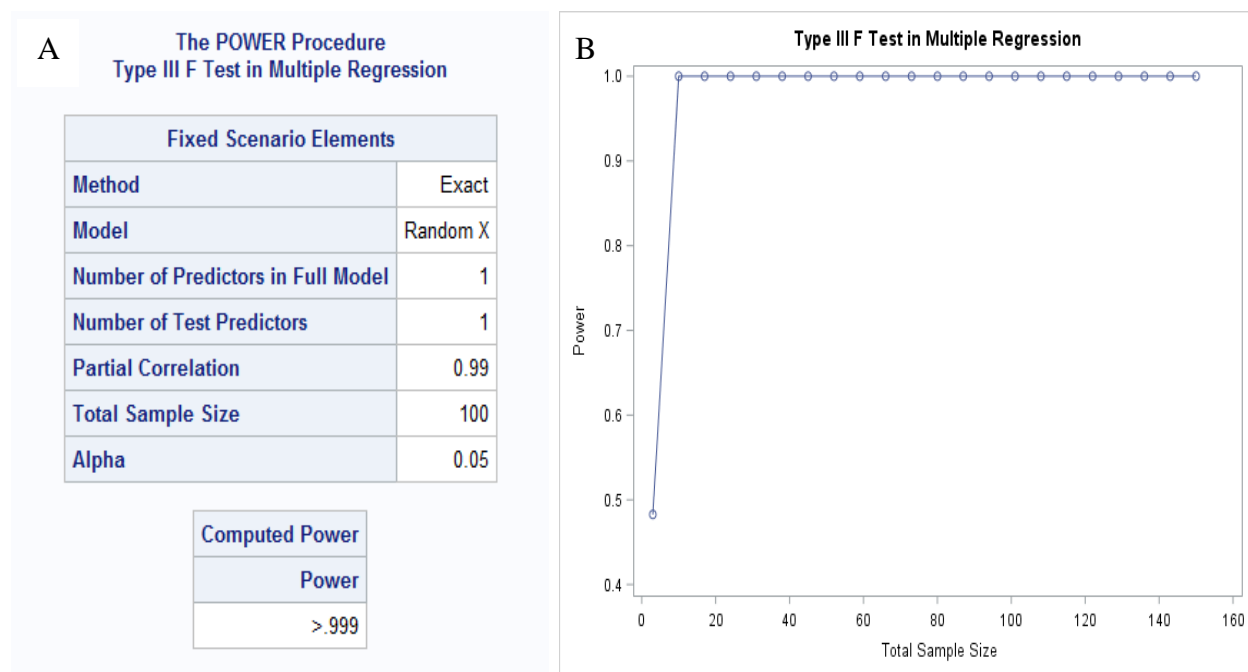

Figure 5.1. A. The fixed variable table to determine the power of ATP detection kit. B. F test regression shows that power of ≥10 gives an alpha of ≤0.01.

5.4.2 *Energetics Analysis*

Mean ATP concentration in the control IVF embryos was 18.299 nM while that in the NaN3-treated IVF group was 15.207 nM; the difference was not statistically significant (P=0.11). However, there is a significant difference between the two treatment groups for

parthenogenetically derived embryos (PAR; P=0.038; Figure 5.2). As the figure indicates, control parthenotes had higher ATP concentrations compared to those in which oxidative phosphorylation was inhibited with NaN_3. Hence, further measurements and assays with embryos utilized early stage embryos created through parthenogenic activation.
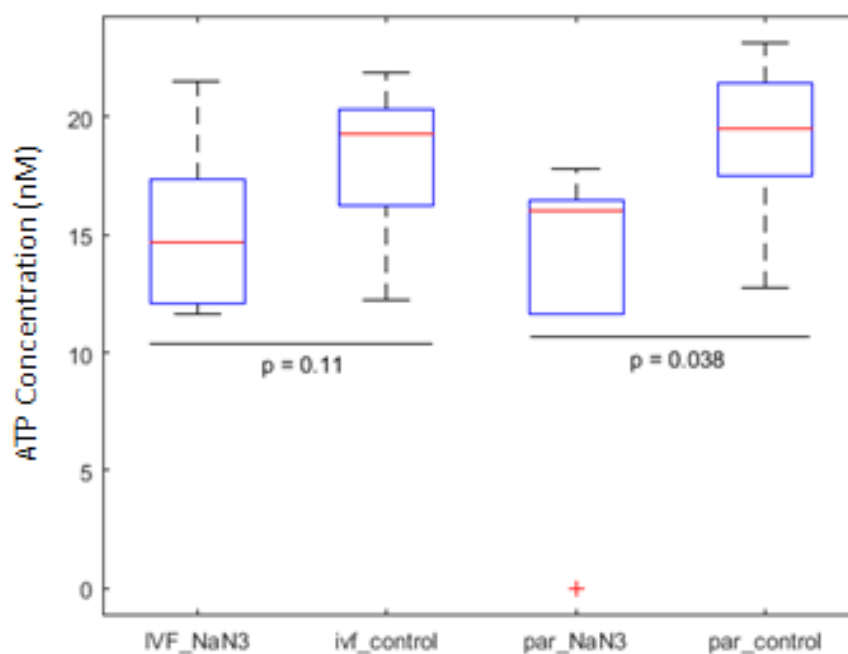
**Figure 5.2.** ATP concentration in control and NaN_3-treated embryos.

Analysis of the differences in optical parameters between control and NaN_3-treated groups showed a trend for a difference in the slope of the treatment groups (Figure 5.3). Importantly, there was a significant difference in the normalized standard deviation (NSD; p=0.00007) between the treated and non-treated parthenogenetic embryos (Figure 5.4).
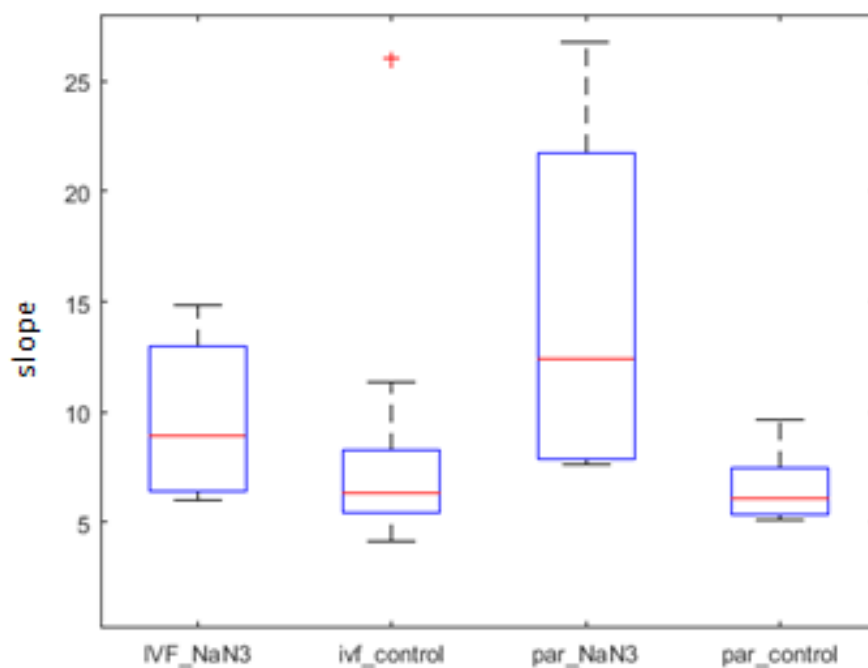
Figure 5.3. Slope of the spectra of NaN$_3$-treated and control embryos. The slope is shown in arbitrary units.
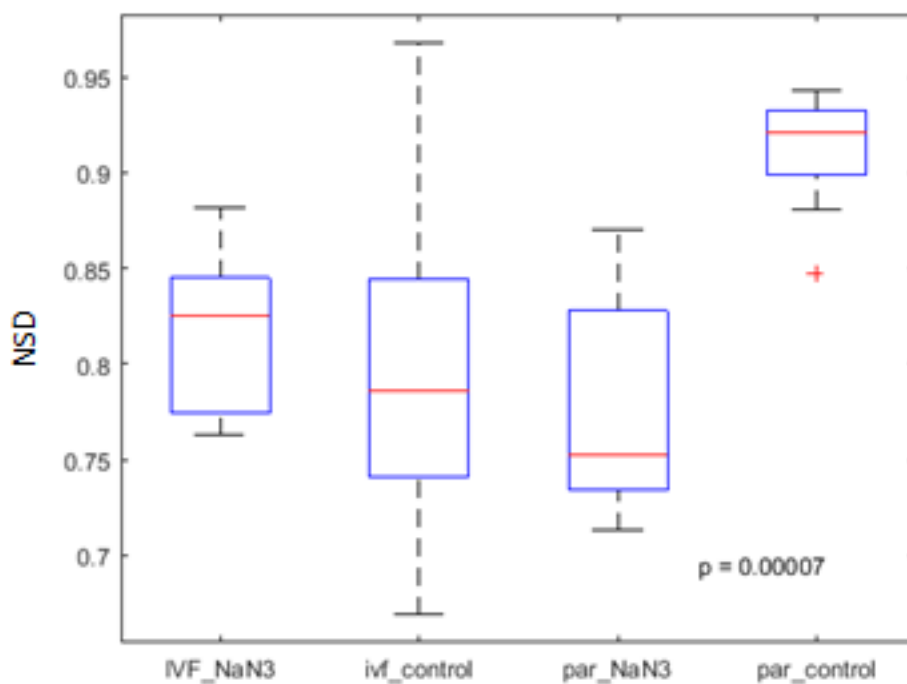
Figure 5.4. Normalized standard deviation (NSD) of NaN3-treated and control embryos. NSD is shown in arbitrary units.

Statistical analysis (2-sample T test) indicated a significant difference (P<0.01) between the means of the control group and the NaN$_3$ group, as shown in Table 5.1. This statistical difference is observed by a four-fold reduction in the means in the concentration of ATP of the oxidative phosphorylation inhibited group (6.0393 nM) compared to the control group (24.308 nM).

Table 5.1. ATP concentration in control and NaN$_3$-treated embryos

Statistics	Control	NaN$_3$
Sample Size	60	61
ATP nM (Mean $\pm$ s.e.m)	39.2$\pm$7.2[a]	11.4$\pm$2.2[b]
Standard Deviation	55.8	16.9

[a,b]Values with different superscripts are significantly different (P<0.01).

Statistical analysis (2-sample T test) was run to compare the knee frequency (Table 5.2) and the NSD (Table 5.3) between control embryos and oxidative phosphorylation inhibited embryos. There were significant differences (P<0.01) for both BDI biomarkers between the two groups of embryos, with both biomarkers being significantly higher for the control embryos compared to the NaN$_3$ groups.

Table 5.2. Knee frequency differences in control and NaN$_3$-treated embryos

Statistics	Control	NaN$_3$
Sample Size	60	61
Mean Frequency $\pm$ s.e.m.	1.4$\pm$0.09[a]	-1.4$\pm$0.4[b]
Standard Deviation	0.76	3.3

[a,b]Values with different superscripts are significantly different (P<0.01).

Table 5.3. Normalized Standard Deviation comparison in control and NaN$_3$-treated embryos

Statistics	Control	NaN$_3$
Sample Size	60	61
Mean $\pm$ s.e.m.	0.90$\pm$0.006[a]	0.81$\pm$0.01[b]
Standard Deviation	0.05	0.10

[a,b]Values with different superscripts are significantly different (P<0.01).

5.5   *Discussion*

Determining the quality of in vitro-derived embryos is essential in ART procedures and current research on the energetics of oocytes and embryos from mothers over the age of 35 shows a decrease in mitochondrial function (McPherson et al., 2014). Aberrant mitochondrial function in developing embryos can increase an offspring's risk for severe neurological diseases such as Parkinson's or Leber's disease, impacting long term health (Parker et al., 1989). Mitochondrial function is tied to energy production essential for driving the development of embryos, so it is imperative that the mitochondrial state of embryos be assessed. Blastocysts can be morphologically graded for expansion, as creation of an osmotic gradient as well as blastulation is driven by the activity of the N^+/K^+/ATPase which utilizes energy produced by mitochondria. Alternatively, oxygen consumption of individual embryos can also be measured (Thompson et al., 2000). These methods are insufficient, with morphological grading being quite subjective and oxygen consumption being an indirect, costly measurement with a low predictive value for embryo quality (Richardson et al., 2015; Tejera et al., 2011). As mentioned above, the BDI has the sensitivity to detect intracellular dynamics and the energetic capacity of embryos and has been used to determine mitochondrial activity before (An et al., 2015). This experiment was designed to identify the BDI parameters associated with mitochondrial function, different between healthy, control embryos and those that had been energetically depleted using sodium azide. Sodium azide (NaN_3) is well established as a successful inhibitor of oxidative phosphorylation, inhibiting the passage of electrons in the electron transport chain of the mitochondrial membrane (Machaty et al., 2001). The effectiveness of this compound is in a narrow range of 5-20μM as established by Thompson et al. (2000), inactivating cytochrome oxidase a_3 in complex four of the electron transport chain and downregulating oxidative phosphorylation.

The switch from oxidative catabolism to glycolysis occurs at the morula-stage in porcine embryos, so pre-compaction embryos have an increased sensitivity to NaN_3 inhibition (Machaty et al., 2001). Initial results showed that parthenogetic embryos showed more inhibition of oxidative phosphorylation when treated with NaN_3. Such embryos are derived utilizing an exogenous stimulus to mimic the impacts of fertilization on a mature oocyte without the involvement of a sperm cell. The exogenous stimulus can be electrical or chemical in nature, performed for a short duration to induce a calcium oscillation in the oocyte and stimulate meiotic resumption naturally brought on by fertilization (Hao et al., 2004). While parthenogenetic embryos lack the capacity to develop beyond a certain developmental stage unlike heterogametic embryos, they both share the same source of mitochondria. In all mammals, mitochondria and mitochondrial DNA (mtDNA) are exclusively inherited from the maternal source (Hutchison et al., 1974). Maternal inheritance is promoted through the massive replication of mtDNA in the matured oocyte before fertilization, diluting any retained paternal mitochondria to 5% of the total mitochondrial population post fertilization. Paternal mitochondria briefly enter the newly formed zygote but are quickly eliminated in early cleavage stages through ubiquitination, activating proteasomes to degrade all paternal mitochondria (Carelli, 2015). During passage through the female reproductive tract, sperm mtDNA is heavily damaged by oxidative stress, a possible reason why embryos must rely solely on maternal mitochondria. Hence a comparison of the mitochondria content of control parthenogenetic embryos compared to parthenogenetic embryos treated with NaN_3 should exhibit the same intracellular dynamics as normal heterogametic embryos.

Retrospective analysis shows that both the knee frequency and NSD are significantly higher in healthy embryos compared to their energetically starved cohort. NSD has been

discussed at length in several other chapters of this thesis, but can be defined as a difference in the variation of photon frequencies in multiple frames of the spectra. Knee frequency is a parameter visible on an embryo's spectrogram that shows a shift in slope of the spectra representative of the change in intracellular motion (An et al., 2015). These BDI biomarkers are indicative of energetic capacity of an embryo, with a high NSD value and high knee frequency showing an active developing embryo with a high ATP content, a positive sign of an early embryo preparing for blastulation and expansion.

In conclusion, the inhibition of oxidative phosphorylation via sodium azide was successful at energetically depleting embryos without preventing early embryo development. NaN_3 treated embryos had ATP contents four-fold below their healthy cohorts. Additionally energetically depleted embryos had significantly lower (P<0.01) NSD values and knee frequencies, which are BDI biomarkers associated with the energetic potential of samples. This retrospective BDI/ATP co-assay was successful in definitively identifying these energetic parameters and future assays should focus on samples that have high NSD values and high knee frequencies, as these embryos have elevated ATP contents and a higher developmental competency.

5.6   References

An, R., Wang, C., Turek, J., Machaty, Z., & Nolte, D. D. (2015). Biodynamic imaging of live porcine oocytes, zygotes and blastocysts for viability assessment in assisted reproductive technologies. *Biomed Opt Express, 6*(3), 963-976. doi: 10.1364/BOE.6.000963

Brison, D. R., & Leese, H. J. (1994). Blastocoel cavity formation by preimplantation rat embryos in the presence of cyanide and other inhibitors of oxidative phosphorylation. *J Reprod Fertil, 101*(2), 305-309.

Carelli, V. (2015). Keeping in shape the dogma of mitochondrial DNA maternal inheritance. *PLoS Genet, 11*(5), e1005179. doi: 10.1371/journal.pgen.1005179

Hao, Y., Lai, L., Mao, J., Im, G. S., Bonk, A., & Prather, R. S. (2004). Apoptosis in parthenogenetic preimplantation porcine embryos. *Biol Reprod, 70*(6), 1644-1649. doi: 10.1095/biolreprod.103.026005

Hutchison, C. A., 3rd, Newbold, J. E., Potter, S. S., & Edgell, M. H. (1974). Maternal inheritance of mammalian mitochondrial DNA. *Nature, 251*(5475), 536-538.

Leese, H. J. (1991). Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol, 13*, 35-72.

Machaty, Z., Thompson, J. G., Abeydeera, L. R., Day, B. N., & Prather, R. S. (2001). Inhibitors of mitochondrial ATP production at the time of compaction improve development of in vitro produced porcine embryos. *Mol Reprod Dev, 58*(1), 39-44. doi: 10.1002/1098-2795(200101)58:1<39::AID-MRD6>3.0.CO;2-B

McPherson, N. O., Zander-Fox, D., & Lane, M. (2014). Stimulation of mitochondrial embryo metabolism by dichloroacetic acid in an aged mouse model improves embryo development and viability. *Fertil Steril, 101*(5), 1458-1466. doi: 10.1016/j.fertnstert.2013.12.057

Parker, W. D., Jr., Boyson, S. J., & Parks, J. K. (1989). Abnormalities of the electron transport chain in idiopathic Parkinson's disease. *Ann Neurol, 26*(6), 719-723. doi: 10.1002/ana.410260606

Richardson, A., Brearley, S., Ahitan, S., Chamberlain, S., Davey, T., Zujovic, L., Hopkisson, J., Campbell, B., & Raine-Fenning, N. (2015). A clinically useful simplified blastocyst grading system. *Reprod Biomed Online, 31*(4), 523-530. doi: 10.1016/j.rbmo.2015.06.017

Tejera, A., Herrero, J., de Los Santos, M. J., Garrido, N., Ramsing, N., & Meseguer, M. (2011). Oxygen consumption is a quality marker for human oocyte competence conditioned by ovarian stimulation regimens. *Fertil Steril, 96*(3), 618-623 e612. doi: 10.1016/j.fertnstert.2011.06.059

Thompson, J. G., McNaughton, C., Gasparrini, B., McGowan, L. T., & Tervit, H. R. (2000). Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *J Reprod Fertil, 118*(1), 47-55.

Wilding, M., Dale, B., Marino, M., di Matteo, L., Alviggi, C., Pisaturo, M. L., Lombardi, L., & De Placido, G. (2001). Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod, 16*(5), 909-917.

CHAPTER 6.   CONCLUSIONS AND FUTURE DIRECTIONS

6.1   Summary and Future Directions

In 1981 the first baby was born in the United States through assisted reproductive technologies (ART) and since then the use of ART to conceive children have more than tripled (Sunderam et al., 2017). Nearly all of ART requires the use of in vitro fertilization (IVF), the co-culturing of male and female gametes, to produce embryos for transfer back into the mother for the delivery of a healthy baby. Multiple pregnancies are a common consequence of ART practices, as several of the most viable embryos are selected for transfer back into the uterus. This is not ideal as a multiple fetus pregnancy can be detrimental for both the mother and the neonates. Mothers are at an increased risk for preeclampsia, thromboembolism and gestational diabetes while fetuses are more likely to be born premature and with low birth rates (Reddy et al., 2007). Due to these considerable health risks, clinical standards to reduce the number of embryos transferred per ART cycle were employed in 2010, with only one embryo being transferred per cycle in women under the age of 35 (Black & Bhattacharya, 2010). However a recent epidemiology study from 2014 has shown that for this age group an average of 1.8 embryos are transferred per cycle (Sunderam et al., 2017). The discrepancies between clinical standards and current practices lie in the inadequate assays to determine embryonic health and viability. To meet this necessary assessment, we propose the use of the BioDynamic Imaging system (BDI) as a sensitive, optical method that uses the intracellular dynamics of oocyte and embryos to determine their developmental competency.

To validate the BDI for use in embryology we needed to identify optical parameters that are indicative of embryo health. We began by submitting embryos to one of several stressors (high pH, low pH, ethanol, etc.) to observe the intracellular response to each treatment under the

BDI. Observations revealed a division between two groups of embryos in terms of nyquist floor and slope of the spectra. The healthier group had a gradual slope (-0.49) and a nyquist floor (0.24) twice the magnitude of the unhealthy embryos when treated with the same stressor, which showed an extreme slope (-0.8) and low nyquist floor (0.12). While it is expected that the nyquist floor (a numeric representation of vesicle movement inside a sample) be close to zero in a dead or dying sample, the higher nyquist floor in the first group shows a cellular effort to mitigate the effects of stressors and is more sustained in the healthier group as shown by the gradual slope of the spectra.

In another experiment designed to use differential staining to identify apoptotic factors and identify which BDI parameters are associated with low quality embryos, normalized standard deviation (NSD) of the spectra was found to be higher in samples that had a lower morphological score (-0.58) . This depicts that as an embryo is degenerating the activity in each cell is quite sporadic, resulting in a variation between spectra that is observed under the BDI as increased NSD. Additionally, in an experiment designed to identify the BDI parameters associated with the energetic capacity of an embryo, sodium azide (NaN_3) was used to inhibit oxidative phosphorylation in a group of early cleavage embryos to deplete their energy stores. This low energy group was compared to a control group of embryos with normal energetics through an ATP assay to validate their energy content after measurement under the BDI. Again, the two groups differed by slope and NSD with the low energy group having a lower slope and NSD compared to the control group.

While the experiments we employed to identify metabolites and protein in spent culture medium to assess embryo health were insufficient in terms of sensitivity, we were able to notice an optical trend when looking at early fertilization of the oocyte under the BDI. Syngamy

associated with fertilization results in a long period of intracellular activity as meiosis resumes and the gametes fuse compared to oocytes that are unfertilized. This observation will allow for an optimization of IVF procedures when installed in a clinical setting as post-gamete co-culture potential embryos can be screened using the BDI system to identify which samples are newly formed zygotes. BDI parameters like NSD, slope, nyquist floor have proved to indicate embryo viability. Four-cell embryos can be assessed for these parameters and divide into subsequent groups based on their relative parameter score and tracked for development to the blastocyst stage. Implications from tracking development allow for the identification of ideal parameter settings to apply for the classification of blastocysts. Blastocysts can be analyzed and grouped based on those parameter settings and transferred into recipient sows. The high conception rates and healthy delivery of piglets from those embryo transfers will be the ultimate validation of the BioDynamic Imaging System, allowing for clinical implementation in human ART procedures, thus improving poor conception rates and single embryo transfer success rates.

6.2   References

Black, M., & Bhattacharya, S. (2010). Epidemiology of multiple pregnancy and the effect of assisted conception. *Semin Fetal Neonatal Med, 15*(6), 306-312. doi: 10.1016/j.siny.2010.06.004

Reddy, U. M., Wapner, R. J., Rebar, R. W., & Tasca, R. J. (2007). Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop. *Obstet Gynecol, 109*(4), 967-977. doi: 10.1097/01.AOG.0000259316.04136.30

Sunderam, S., Kissin, D. M., Crawford, S. B., Folger, S. G., Jamieson, D. J., Warner, L., & Barfield, W. D. (2017). Assisted Reproductive Technology Surveillance - United States, 2014. *MMWR Surveill Summ, 66*(6), 1-24. doi: 10.15585/mmwr.ss6606a1