Short Term Dynamic Fluid Environment Exposure as a Promoter of Embryo Development

By

Alexander Branson, B.S.

A Thesis

In

Animal Science

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCES

Samuel Prien, Ph.D.
Chair of Committee

Lindsay Penrose, Ph.D.

Andy Laughlin, Ph.D.

Mark Sheridan, Ph. D.
Dean of the Graduate School

May 2018

Copyright 2018, Alexander Branson

ACKNOWLEDGMENTS

I would first like to thank Dr. Prien, Dr. Penrose, and Dr. Laughlin for treating me like an adult when I earned it, a child when I needed it, and a friend the rest of the time. Their guidance and support have been invaluable throughout this entire process. Thank you to Dr. Wessels for helping me start on this project, and thank you to the rest of my lab mates for all of their help. Lastly, thank you to my wife for being my rock day in and day out; without her support, this never would have been possible.

TABLE OF CONTENTS

ABSTRACT

Although multiple embryo transfers (MET) are directly correlated with an increase in multiple gestations, they remain common practice in the infertility community. This is due, at least in part, to attempts at Single Embryo Transfer (SET) continuing to yield reduced per-cycle delivery rates. One of the leading causes of this reduced rate is the lack of quantitative means of detecting embryo quality. The demand for a non-invasive, quantitative means of determining the highest quality embryos led to the creation of the Modified Specific Gravity Device (MSGD), which has been proposed and tested by Prien et al. with promising results. The MSGD is designed to measure the buoyancy of embryos without compromising their fecundity. The initial tests of this device indicated a potential positive correlation between the exposure to the device and improved embryo development. This relationship may be explained through the microfluidic like dynamics innately associated with the device. This study tests this assertion by exposing embryos to the MSGD environment for varying durations and following their developmental progress for five days. In each reiteration of exposure time, embryos were shown to develop at an improved rate as compared to those that were placed directly in culture. These results suggest a positive association between the microfluidic like environment created by the MSGD and the enhancement of embryo development, which further endorses the value that the MSGD could potentially provide to the reproductive community at large.

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

The History of IVF

65 years before the first "test tube baby," Albert Brachet began to study the culturing of preimplantation embryos (5, 4). This study would be shown to be an instrumental piece to the puzzle of *in vitro* fertilization and embryo transfer (IVF/ET). Brachet was able to grow rabbit embryos to the blastocyst stage *in vitro,* which showed to some extent, that the continued development of embryos beyond the confines of the maternal body was possible. In 1935 Pincus and Enzmann showed that not only embryos had the ability to mature *in vitro*, but oocytes isolated from the Graafian follicle would spontaneously leave their suspended state in prophase I and resume meiosis (35, 4). Six years later Pincus developed a technique for the superovulation of ovaries using a crude extract from the pituitary (36, 4). This allowed scientist to have a much larger sample size of ovum in future studies, and a more refined version of this process is still utilized in IVF today. Chang and Austin simultaneously discovered that spermatozoa must go through capacitation before they are able to fertilize in 1951 (2, 9, 4). There were many claims over the next few years that different groups of scientist had succeeded in achieving IVF, but Biggers asserts that the first case of IVF to meet the requirements for fertilization set forth by Austin in 1961 (3), which could not be debunked by the discovery of capacitation, was the rabbit ovum fertilized by Chang in 1959 (9, 4). Each of these discoveries contributed a great deal to the understanding of reproduction, all the while leading scientists

closer to achieving IVF/ET. Robert and Patrick Steptoe began to put the pieces together in 1969 when they successfully fertilized the first human embryo *in vitro* (13; 4). They continued to push the boundaries of reproductive biology and attempted several times to transfer an IVF embryo back into the female uterus, yet they failed to establish a pregnancy for eight years. They hypothesized that hormones being used to superovulate women's ovaries were preventing implantation of fertilized embryos (14). Therefore, they returned to harvesting the single naturally ovulated ova each cycle. This adjustment in their technique allowed for the establishment of a pregnancy in Lesley Brown, who had been attempting to treat her bilateral tubal blocks unsuccessfully for nine years (43, 30). Louise Brown was born 38 weeks later in July of 1978 (43, 4, 30). This monumental birth was the first successful IVF/ET procedure in humans, and Louise became the first "test tube baby."

The success of Edwards and Steptoe sparked hope for millions of couples struggling with infertility. Because of their work in developing the IVF/ET process, couples suffering from conditions such as blocked oviducts and non-ovulatory ovaries now have a means of bypassing these physiological circumstances to conceive a child of their own genetics. In fact, since the birth of Ms. Brown, over five million babies have been born through the utilization of IVF/ET (30). Several landmark discoveries such as the use of ultrasonography in oocyte retrieval, development of improved stimulation protocols, oocyte maturation *in vitro,* and the utilization of Intracytoplasmic Sperm Injection (ICSI), have progressed IVF/ET to a more refined and reliable method of conception in the decades since 1978 (33, 30, 8, 49).

However, in an effort to improve fertility rates, especially early on in the development of this procedure, multiple embryo transfer (MET) became the standard practice. There is inherent risk of multiple-gestations and other high risk scenarios when MET is utilized, which can be extremely dangerous to the mother and offspring (40). The first case of multiple gestations as a result of IVF/ET was in 1982 with the birth of the twins Taylor and Freddie (30, 12). The following year marked the first set of triplets, and in 1984 the first set of IVF quadruplets were born in Australia. In 1998 Houston, Texas became the birthplace of the first IVF octuplets (30, 12). An arguably more famous set of octuplets was derived from IVF in 2009 to Nadya Suleman of California, whom the media dubbed "Octomom" (30). Suleman's doctor, Michael Kamrava, transferred the twelve embryos that lead to the eight children. This act was determined by the medical board of California to be a "life-threatening practice," and subsequently stripped him of his medical license (30).

The Case for SET

Multiple gestations carry with them a vast array of complications for both the mother and the neonate. These complications include preterm delivery, low birth weight, infant death (SIDS), cerebral palsy and other disabilities (40). Preterm delivery occurs in 95.9% of triplet births and 53.8% of twin births (20). Of the children who are born in the very low birth weight category, 45% will have an abnormal intelligence status by the age of eleven (20). The rise in occurrence of multiple gestations over the last three decades is directly correlated with the rise in assisted reproductive technologies (ART) usage. Although ART accounted for only

1.1% of live births in 2005, ART produced 17.1% of all multiple births in the USA, 15.5% of all twin births, and 43.8% of all triplet and higher births (40). In fact, conceiving a child through IVF makes a couple 30 times more likely to have twins and 500 times more likely to have triplets than through natural conception (20). Gerris *et al.* also indicated that multiple gestation likely-hood is associated with an increased maternal age. This is unfortunate considering that modern medicine, including IVF, has steadily increased the average age of mothers over the last 40 years (53). It has been shown that singletons resultant of SET are healthier and have a lower incidence of infant mortality than singletons resultant of MET (20).

Despite the elevated number of multiple gestations associated with IVF, and the initial proposal for SET being published in 1998 by Coetsier and Dhont, it is still common practice to transfer multiple embryos on the first cycle of IVF. By reducing the number of transferred embryos from three to two, a diminution in multiple gestations of 27% would be observed (10). This reduction would specifically yield a 20% decrease in twins, a 50% decrease in triplets, and a 67% decrease in quadruplets derived from IVF (10, 11). When these statistics were published in 1998 it was also determined that only transferring two embryos would result in a reduced fertility rate that would require a 10% increase in the number of cycles to rectify (10, 11). However, 20 years of advancements and fine-tuning in the IVF procedure have negated the need for this 10% increase in cycles. Although the number of embryos transferred has been reduced to two or three in most practices, the greatest impact on the rate of multiple gestations is shown in the reduction from two embryos to one embryo being transferred (40).

The complications innately embedded within MET, and more specifically avoiding them, are the driving force behind the need for wide spread application of SET. Decreasing the number of embryos transferred per cycle is the only effective method of reducing the prevalence of multiple gestations resulting from dizygosity in IVF patients (20). The largest concern from a clinician perspective is that SET will diminish their fertility rates. Fertility rates are published and are a determining factor in a couple's decision on where to seek their treatment. However, in some Scandinavian countries, SET accounts for 80% of their IVF procedures, and SET has also been implemented on a large scale in Belgium, Finland, and Sweden. In these countries there has been no significant decrease in fertility rates, but there has been a significant decline in multiple gestations (20). There is also some urging from the patients themselves to continue with MET. This is due to several factors. The first of which is financial; because IVF/ET is approximately $12,000 per cycle and not covered by insurance in most situations, couples opt for what they perceive as the highest chance of conception per cycle despite the risks. Insurance companies may not cover IVF/ET, but they do pay for neonatal care (20). Therefore, couples may be inclined to take the cheaper option and deal with the associated risks. The other factor contributing to the patient pushback is emotional cost. The hormonal treatment required for super ovulation and successful implantation is in itself extremely difficult for many couples. To go through the process of IVF/ET and find out that it was unsuccessful is often emotionally devastating for many (51).

Embryo Evaluation

For SET to be accepted as the common practice, a definitive means of identifying the embryo with the highest development potential must be established. In an effort to better identify the IVF embryos with the highest chance of viability and facilitate a shift in practice to single embryo transfer (SET), many novel ideas have been proposed and utilized. One of the earliest procedures to support this effort was demonstrated by Alan Handyside in 1980 (23). His process, Preimplantation Genetic Diagnosis (PGD), identifies genetically abnormal embryos by way of cell biopsy, and is still used in 2018 (30). In 1989 PGD was used to determine embryo sex for the first time by amplifying the Y-chromosomal DNA (24, 30). In 2008, the Monash Immunology and Stem Cell Labratory introduced DNA Fingerprinting through biopsied trophectoderm cells as a means of determining the viability of blastocyst *in vitro* (28, 30). Finally, Alison Campbell introduced the use of modern time lapse imaging in embryo evaluation (7, 30). This technology allows for the observation of the entirety of an embryo's development, and the recognition of what some believe to be significantly indicative developmental milestones.

Genetic evaluation through biopsy, PGD, time-lapse imaging, metabolic rate study, and morphological observation are all means of trying to identify the optimal embryo for transfer (50). The issue with these practices is that they are either invasive or subjective. PGD requires use of a laser to perforate the zona pellucida and to remove trophoblastic cells for genetic testing. This process allows for the elimination of genetically abnormal embryos, but is an expensive procedure to have performed, has the potential of being harmful to the embryo, and does not offer any information on the implant potential of an embryo.

Morphological evaluation has been shown to be highly subjective. Farin et al. had six highly experienced embryologists evaluate the same set of embryos, and found that they agreed on the high potential embryo only 68.5% of the time (16). There is also some debate as to whether or not morphological observations and evaluations based on the rate of embryo development are even indicative of future performance. A major issue leading to the high prevalence of the MET method is the lack of quantitative measurements available to embryologist when attempting to select the highest quality embryos to transfer (15). The best practice for embryo evaluation would be one that is both quantitative and non-invasive. This would eliminate any risk to the embryo as well as remove the individual subjectivity of the evaluator from the process.

Modified Specific Gravity Device (MSGD)

In 2015 Prien et al. proposed a device with the potential for quantifying some aspects of embryo quality. This device, the MSGD, is used to determine the specific gravity of an embryo. Determining specific gravity is not a novel idea; it is commonly used to determine lipid content in food animal carcasses as well as in foodstuffs (38, 6, 29). Specific gravity has also been previously applied to embryos by this same lab to identify the effects of maternal body composition on embryo weight (46, 47). The first study utilized the embryos of anti-leptin mice, or pound mice. These mice lack the ability to control food intake, and anti-leptin mice are obese compared to wild-type mice. The specific gravity device used in this study was able to detect a statistically significant difference in specific gravity between the embryos from the

obese mothers and the embryos collected from the wild type, or lean, mothers (46). In a later study, the group determined differences between Jersey and beef cattle embryos using a specific gravity device (47). The device used in these studies worked well for lab evaluation, but 10-20% of the embryos were lost in the testing (37). Therefore, a new device specifically engineered for the assessment of specific gravity in embryos with the intention of transferring them to a live host was developed.

The MSGD is simply an organ culture dish with a sealed lid on top. There is a long, straw-like, chamber attached vertically through the center of the lid. This chamber is filled with media (Hams F-10 media in these studies, but any media will suffice once a standard curve is established), and because of the sealed nature of the device, the media is suspended within the vertical chamber. An embryo is placed in the top of the chamber below the media's meniscus, and descent time through 1cm of the media is measured. When the seal between the dish and the lid is broken, the embryo is released into the recovery well of the dish. The closed system of the MSGD allows for a 100% embryo recovery rate. The measured descent time can then be used to estimate embryo weight based on a previously established standard curve (37).

In the published initial trials using the MSGD, the group had three goals: (i) to determine if the specific gravity of embryos could distinguish between live and dead embryos, (ii) to determine if the specific gravity of an embryo is indicative of its' future development potential, (iii) to establish that the MSGD has no negative impact on embryo fecundity (37).

The goal of a preliminary study utilizing the MSGD was to determine whether or not the MSGD could detect cell death (37). To do this, a sampling of embryos was obtained and divided into two equal groups. One group was exposed to a temperature of 60^{o} C to ensure heat death. The other group was not exposed to heat, and served as the control for this study. Both groups of embryos were dropped through the MSGD and descent times were documented at the time of heat treatment, 24 hours post treatment, and 48 hours post treatment.

The control group maintained a consistent average drop time across all three measurements. At the time of the heat treatment, the heat-treated embryos showed no statistically significant difference in descent time from that of the control group. At 24 hours post-treatment, the heat-treated embryos descended through the media at a much faster rate than the control, and at 48 hours, the heat-treated embryos fell at a much slower rate.

The authors hypothesized that this can be explained by the cell membrane's ability to osmo-regulate. The control group of embryos maintained this ability throughout the three measurements. The heat-treated embryos, however, lost this ability. The non-covalent interactions that maintain the integrity of the membrane, as well as the disulfide bonds responsible for the tertiary structure of the cross membrane protein channels were no longer in place. The once semi-permeable membrane became instead a fully permeable membrane, and because of differences in concentration gradients on the inside and outside of the cell, fluids flowed freely through the membrane. At 24 hours post treatment, the embryos were much more dense than their control group counterparts, due to the mass exodus of fluid from the

inside of the embryo. By 48 hours post treatment the embryo had established equilibrium with the media and, therefore, descended much slower through the descent chamber. This study showed that the MSGD could detect the difference between live and dead embryos. This data could be shown to be highly beneficial to the embryo selection process utilized in IVF procedures.

A correlation between the average descent time of embryos and their resulting proliferation was also illustrated (37). It was noted that those embryos with a measured descent time closest to the median descent time developed into blastocyst at a higher frequency than those descending further away from the median. This could indicate a potential quantitative means of predicting embryos with a greater likelihood of developing into a viable fetus.

The next study utilizing the MSGD sought to determine whether or not exposure to the device could have an effect on the fecundity of embryos (37). In this study, embryos were again divided into two groups. The first group, used as the control, was placed directly into culture media. The second was exposed to the MSGD and descent times were recorded. The embryos were then monitored for four days to determine which ones developed into the blastocyst stage.

The group of embryos exposed to the device exhibited the same ratio of embryos that stalled development in the one cell, multicell, and morula phase as that of the control group. The interesting observation the group made in tis instance is that a significantly higher number of embryos exposed to the device reached the blastocyst stage. This demonstrated that the device not only had no negative affect on the fecundity of embryos, but that there may be a potentially positive effect on the

said fecundity. The mechanism or characteristic of the MSGD that would cause this type of physiological response from embryos is unclear and needs further investigation, but it has been hypothesized that there may be some sort of microfluidic-like factor at play.

Microfluidics

Microfluidics is the study of sub-microliter fluid systems. This field was originally investigated in the 1950's for its defense applications, as it was believed that fluid circuits might be the answer to avoiding the societal fallout associated with an electromagnetic pulse. Yet, the biological implications of these systems were not investigated in earnest until the completion of the Human Genome Project in the early years of the millennia, when scientist recognized the potential for greater efficiency and control that microfluidic devices could lend to biologic processes (41).

The most relevant application of microfluidic devices to this review is as a tool for improved embryo culture. Microfluidic devices may be able to affect embryos both biochemically and mechanically (41). The impact of microfluidic systems on embryo development has been studied through a variety of different applications (39, 22, 54, 32). Raty *et al.* showed that mouse embryos cultured in micro channels where media flowed past them exhibited higher cleavage rates and a higher percentage of embryos reached the blastocyst phase as compared to those cultured in static microdroplets. In a study from Gu *et al.*, computer controlled braille displays were used to move fluid around an embryo in a more controlled way. This method of culture resulted in faster rates of embryo development, and blastocysts

with higher cell numbers than embryos in a static microdroplet culture (22). Xie *et al.* and Matsuura *et al.* both utilized microfluidic systems that created shear stress on embryos from the movement of media, and in both cases a positive correlation with embryo development was observed (54, 32). Finally, Isachenko *et al.* and Hur *et al.* both used microvibrations to generate a microfluidic environment with positive effects on embryo development (27, 25). In Isachenko's study, the embryos cultured in the microfluidic environment yielded 37% more pregnancies than those in the control (27). All of these studies illustrate that microfluidic environments of appropriate nature do have a positive effect on embryo development, but the exact mechanism for this effect is not explicitly determined.

In the majority of these studies, the authors make some reference to creating a dynamic fluid environment. This is interesting because of the dynamic fluid environment found in the Fallopian tubes, the site of early embryo development. The Fallopian tube is a multifunctional organ that provides a suitable environment for early embryo development, and facilitates the transport of the oocyte/embryo from the ovary to the uterus (32). This means that pre-implantation embryos are developed in an environment characterized by constant change (41). Traditional static embryo culture systems fail to replicate the active and changing nature of the *in vivo* embryo environment. Many mechanisms that could potentially explain the positive influence of dynamic fluid environments on embryo development have been proposed. Both mechanical and biochemical mechanisms have been proposed, and it is possible that the two are linked. Studies where only the mechanical nature of the environment is altered, such as all the studies mentioned above, indicate that there is a strong

correlation between placing mechanical stress on an embryo and improving the developmental outcome. Matsuura *et al.* identified four mechanical stress interactions that take place *in vivo*, and it is highly likely that the positive benefit of one or more of these stresses is responsible for the improved embryo development. These identified natural stressors are 1) sheer stress from the flow of tubal fluid 2) compression generated by the peristaltic contraction of the smooth muscle in the fallopian tube 3) friction produced by the buoyancy of the embryo 4) the kinetic friction caused by the physical interaction of the embryo and the beating cilia (32). To understand the benefits of these different types of interactions, it will be necessary to design experiments in which a single type of mechanical stress can be tested.

Shear stress is one of the most highly studied iterations of mechanical stress in culture. Xie *et al.* reported that shear stress induces pre-implantation embryo health, but there seems to be an upper threshold shear stress value. They found that chronic shear stress over $0.12 N/m^2$ induces protein kinase mediated apoptosis (54). At this level, the zona pellucida fails to protect the integrity of the embryo's membrane, indicating that there is a desirable range of force (54). In a further study on shear stress, Matsuura *et al.* again showed that shear stress could improve embryo development. Matsuura hypothesized that "the beneficial effect of shear stress could originate from the activation of specific molecular pathways related to cell division by mechanical stimulation (32)."

This is not a new concept to mechanobiologists. Mechanical loads have been shown to induce changes in the structure and function of many living tissues by causing physiological changes in the extracellular matrix (45). Again, many of the

exact mechanisms for these biological changes are not fully understood, but it is clear that cells can sense mechanical forces and respond biologically (45). For example, in 1892 John Wolff documented that bone tissue changes shape, density, and rigidity in response to changes in mechanical loading conditions (45, 52). In the case of bone tissue, a cascade of events causes these changes. First, mechanocoupling converts mechanical forces to mechanical signals. Then biochemical coupling converts those mechanical signals to a biochemical response, and this response is communicated from cell-to-cell. Finally, effector cells receive this signal and generate an effector response that alters bone structure (44). This multistep type of process is common in the conversion of mechanical to biological, and it is likely that the forces placed on embryos *in vivo* initiate a similar type of cascade.

However, mechanical forces are not the only type of changes to the culture environment that dynamic fluid environments elicit. In fact, it has been nearly impossible for scientist to separate mechanical and chemical interactions (42). Mechanically dynamic systems alter the chemistry of culture media by changing the local chemical gradients, redistributing embryo waste and nutrients, and in various other ways (42). Gardner *et al.* showed that embryos from the same patient, with the same embryo grade, uptake glucose at different rates (19). This study points to the idea that not all embryos have the same potential, even when they appear similar. By making nutrients like glucose more readily available for the embryo, development may be improved. It has been shown that refreshing the media embryos are cultured in, and therefore the amount of available nutrients, does improve development rates (42). Lane *et al.* showed that the breakdown of embryo by-products as well as the

breakdown of media components is detrimental to embryo development when in high concentrations (31). The removal or dispersion of these compounds could be achieved through the application of a micro fluidic device. Even small mechanical forces can cause shifts in localized chemical gradients surrounding embryos. These gradients are often responsible for the flow of fluid across the membrane of a cell, and therefore, may play a role in influencing the biochemical contents of an embryo (41). It is likely that there is a balance between all of these interactions and mechanical forces that will result in the most positive effect on embryo development. Both mechanically and chemically dynamic conditions exist within the fallopian environment and merit more investigation (42).

By studying the different ways that stress and chemical changes have been shown to affect embryos and other cells, we may be better able to understand the means by which embryo development is improved in the MSGD. Although true microfluidics involve the use of sub-microliter fluid levels, the biochemical and mechanical principles embedded in microfluidic environments may be useful for study and application to higher volume systems.

CHAPTER II

MATERIALS AND METHODS

The goal of this experiment was to employ the MSGD used in previously mentioned studies in a manner that would expose the harvested single cell embryos to a dynamic fluid environment for varying periods of time, and then to observe their developmental progression in culture. The data collected from these observations was used to test two hypotheses. The first hypothesis is that stress applied to the embryo by the MSGD will have a positive impact on embryo development. The second hypothesis was that exposure to the MSGD of various durations will have similarly positive impacts on embryo development. The methods used in this project were adapted from a previous thesis project conducted by Caitlin Shelinbarger in 2015.

MSGD Assembly

In this experiment we first constructed a sufficient number of MSGD's. Standard organ culture dishes and their lids (Falcon; Union City, California) served as the base of the device. A small hole was placed in the center of the lid using a soldering iron. A semen straw (Minitube; Tiefenbach, Germany) was inserted through the hole to stand in an orientation perpendicular to the dish lid. This straw served as the fluid filled chamber that the embryos passed through. Straws were prepped by removing the cotton plug and cut to a length of 33mm, 66mm, or 99mm using a razor blade. The different lengths allowed for different exposure times for the embryos. Once positioned with approximately 5mm of the straw passed through to the underside of the lid, the straw was fixed into place using waterproof silicone to secure

the straw and create an airtight seal between the dish lid and the straw. The newly constructed lid-chamber combination was then cleaned thoroughly with D.I. water and ethanol and allowed to dry in a sterile environment for one week, before being cleaned a second time. This gave the silicone time to set completely and negated the chance of any fumes interacting negatively with the embryo health. Immediately before the chambers were to be filled with media, a seal was created between the culture dish basin and the lid by coating both sides of a 5mm wide rubber band in petroleum jelly, stretching the rubber band around the outer rim of the culture dish, and pressing the lid down on top.

Embryo Collection

The eighteen mice (C57BL/6NHsd) used in this experiment were handled in accordance with an approved protocol set forth by the Texas Tech Institutional Animal Care and Use Committee (IACUC). This protocol allowed for the generation of a large number of single cell embryos. The female mice that were to be used received two injections in the 48 hours prior to their harvest. The first was a 0.25 mL subcutaneous injection of 0.5 mIU/mL of PMSG (pregnant mare serum gonadotropin; Sigma Chemical; St. Louis, MO). This injection hyperstimulated the overy of the mice, and promoted the development of mature follicles and oocytes. The second injection, given 24 hours prior to harvest, was 0.25 of 0.5mIU/mL hCG (human chorionic gonadotropin; Sigma Chemical; St. Louis, MO) to stimulate ovulation. In conjunction with this second injection, a male mouse was housed with the females (all paired mating- one male and one female per cage) so that mating could occur.

Embryos were harvested approximately 18 h after mating. Females were euthanized by cervical dislocation, abdomens were opened sterilely, and the oviducts and ovaries excised. The removed tissues were placed into a 60mm petri dish containing 1mL of buffered Hams F-10 media (Irvine; Anaheim, CA). While in media, single cell embryos were flushed out of the oviducts under a dissecting microscope (Nikon SMZ-2; New York, NY). Single cell embryos were identified and transferred to an organ culture dish containing 0.5mL of Global media (Lifeglobal Group; Guilford, CT) in the center well. The outer well of the dish was filled with D.I. water to help maintain temperature. Embryos were transferred to a culture environment of 6% CO2/room air, 95% R.H., and 37°C until exposure to the MSGD. All media used in this experiment was prepared and equilibrated under the same culture conditions for 24 hours prior to use.

Exposure to MSGD

Embryos were exposed to the MSGD individually. To do this, the straw chamber portion of the MSGD was first filled with global media (Lifeglobal). Once the lower center well was filled, media injected from the top of the chamber stayed suspended in the straw because of the sealed nature of the chamber. Once the MSGD's chambers were filled with media, the total number of embryos from each mouse was divided equally into the four treatment groups. The first group was the control group. These embryos were transferred directly into the culture media that will be discussed below. The other three groups were assigned to the three different lengths of MSGD chamber. Each of the remaining embryos were picked up from their dish using a 140-μL pipette (Orgio; Charlottesville, VA) and deposited just below the

fluid meniscus formed at the top of the appropriate MSGD chamber. The embryos were then allowed to descend through the chamber for four minutes, an adequate amount of time to allow for the embryo to descend the full length of the chamber. After all embryos in a group were dropped through the MSGD the seal on the device was broken allowing the media from the straw protion of the device to drop into the well at the center of the organ culture dish. The embryos were then located in the center well of the culture dish using the dissection microscope.

Once all embryos were treated, they were transferred to microdroplets of global media (Lifeglobal) for culture. The microdroplets were prepared 24 hours prior to embryo harvest by depositing four 30μL drops of global media in a 60mm culture dish (Falcon). The drops were then covered with a layer of sterile mineral oil (FertiCult^{TM}; Beernem, Belgium) to prevent the media from evaporating and to help "fix" the droplets in place. The mineral oil was added until it completely covered the global droplets. The prepped droplets were placed in the incubator to equilibrate until use. Each droplet held 3-7 embryos during the culture period of the experiment, and each droplet was labeled to correspond to a specific mouse and a specific length of MSGD.

Culture

The embryos were cultured in this media for a period of five days post-harvest using the 6% CO2/room air, 95% R.H., and 37°C described above. Culture conditions were checked and verified daily. Embryos were assessed for development every 24 hours and were classified as a 1-cell, 2-cell, 4-cell, 8-cell, morula, or blastocyst.

Statistical Analysis

Data from this study were analyzed using the Statistical Package for the Social Sciences (SPSS ver. 12; Chicago, IL) and R Studio (Ver. 1.0.153; Boston, MA). These software packages allowed for the execution of an ANOVA, Tukey tests, and Chi Square Frequency Analysis. For the ANOVA and Tukey Tests the embryos were analyzed based on their total cell number. Embryos between 2 and 8 cells were given a value equal to their total cell number, and morulas and blastocysts were assigned cell numbers of 64 and 128 respectively. The hypotheses of these tests were tested based on a 0.05 alpha level.

To conduct the Chi-Square Frequency Analysis, only day five data was looked at. This is to only account for the end result of embryo development for the frequency evaluation. The Chi Square Analysis compared the frequency of embryos that reached the morula stage or higher versus all lesser development stages in each treatment against the control. A second Chi Square test was performed to compare the control to all treatment groups combined. This data was evaluated for statistical significance using F-values and F-critical values.

For both types of analysis, embryos not reaching the 2-cell development stage were excluded. This was done because fertilization cannot be proven in cells that fail to undergo at least one cell division.

CHAPTER III

RESULTS

The results of this study were evaluated in two distinctly different ways. The first assessed embryo development based on the total number of cells that developed during the incubation period. This analysis suggests that there was a positive impact on the embryos exposed to the 33mm MSGD, but not on the embryos of treatments with other lengths of MSGD exposure. The second method of analysis evaluated the frequency of embryos developing to the morula through blastocyst stage during the incubation period. This analysis indicated that the embryos of all treatment groups developed at a significantly higher rate than the control group of embryos.

In the first analysis, the total cell count in each treatment was summed for all days of incubation. The single cell ova remaining on day five were excluded from this data, as there was no evidence of fertilization. In instances of morula and greater development, the embryos were assigned a numerical value in accordance with their development stage, as individual cells could not be counted. Morulas were designated as 64 cells, and blastocysts were designated as 128 cells. These numbers were used to find the sum total of cells. The total cell counts were compared using a multiple factor ANOVA to establish that there was a statistically significant difference in cell totals in at least one treatment group. This test resulted in a p-value of <0.05, which confirms that at least one treatment group was significantly different from another. To identify which treatment groups held this difference, a Tukey test was used to perform a series of pairwise T-tests with regard to the family-wise error rate. This revealed that the only statistically significant difference between treatment groups

was between the control group and the 33mm treatment group as indicated in Figure 1. There was no statistically significant difference between any of the treatment groups, nor between the control and the 66mm or 99mm treatment groups.

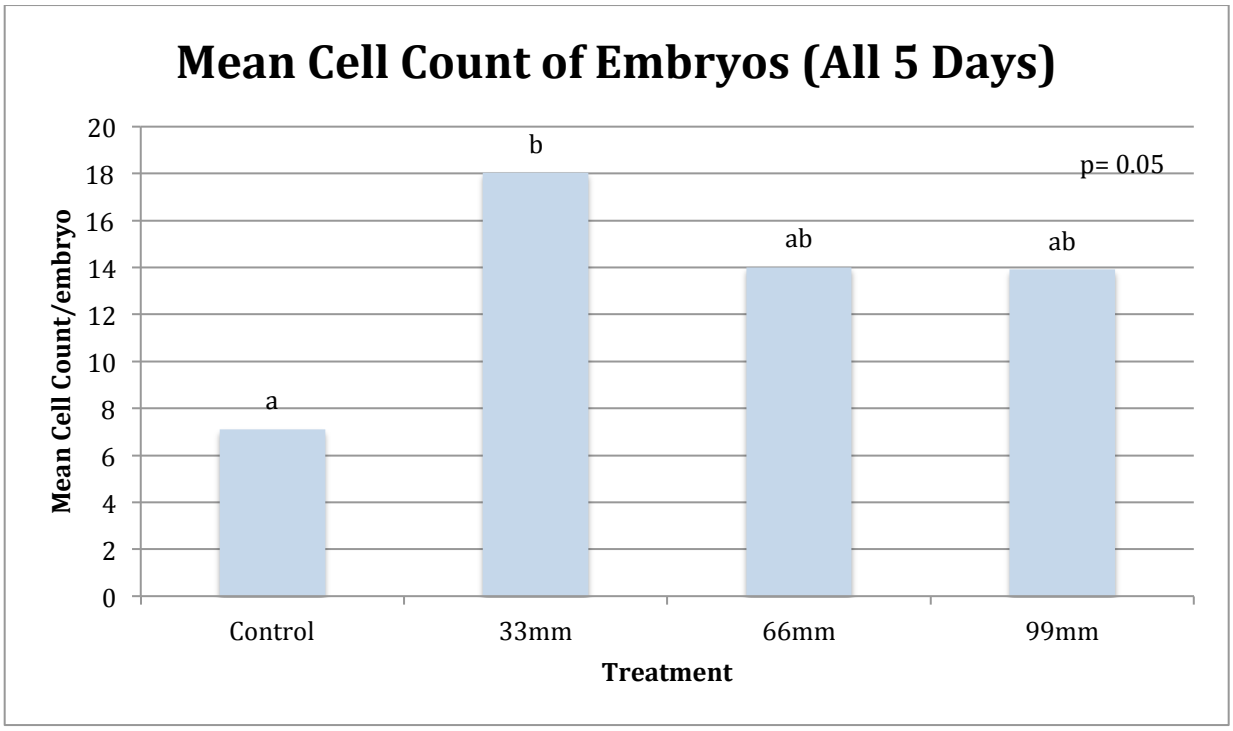

Figure 1: A comparison of the average total cells per embryo in all groups (control, 33mm MSGD, 66mm MSGD, and 99mm MSGD) over five days of observation. A significant difference occured between the control and 33mm MSGD treatment group.

When the data was limited to only day five observations, there was again no statistically significant difference between any of the treatment groups. The only significant difference was between the control and the 33mm MSGD treatment. Once again, cells that did not divide were excluded, and morulas and blastocysts were given the same cell number designation. Figure 2 illustrates this comparison between cell numbers on day five of incubation.

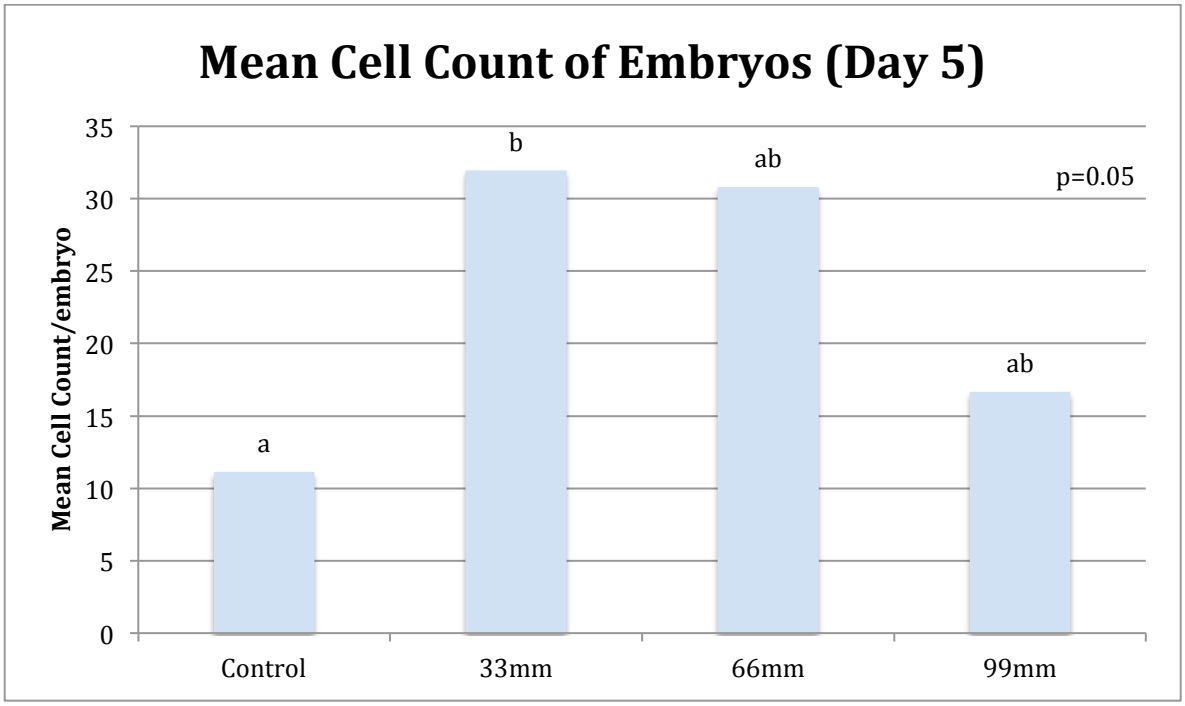

Figure 2: A comparison of the average total cells per embryo in all groups (control, 33mm MSGD, 66mm MSGD, and 99mm MSGD). Significant difference was found between the 33mm MSGD treatment and the control at the p-value of 0.05.

The Chi-square frequency analysis was used to consider the total number of embryos that developed to a morula or greater by day five of culture for each treatment against the number of embryos that failed to reach this stage. The first comparison was made between each treatment group and the control. In the comparison between the 33mm treatment and the control (figure 3), a significantly greater number of embryos reached the morula stage of development or greater ($p=0.0002$). In this analysis, 9 out of 27 embryos reached the development threshold after being exposed to the 33mm MSGD while only 3 out of 28 embryos reached the same development level in the control. Comparing the 66mm treatment group to the control (figure 4) again showed that a significantly greater number of embryos reached the morula stage ($p=0.0017$). In this case of exposure to the 66mm MSGD, 6

of 18 embryos developed to the morula stage. In the third comparison, 99mm treatment group to control (figure 5), there was again a significantly greater number of embryos that reached the morula stage (p=0.0351). The ratio of embryos reaching morula development after exposure to the 99mm MSGD was 6 out of 25.

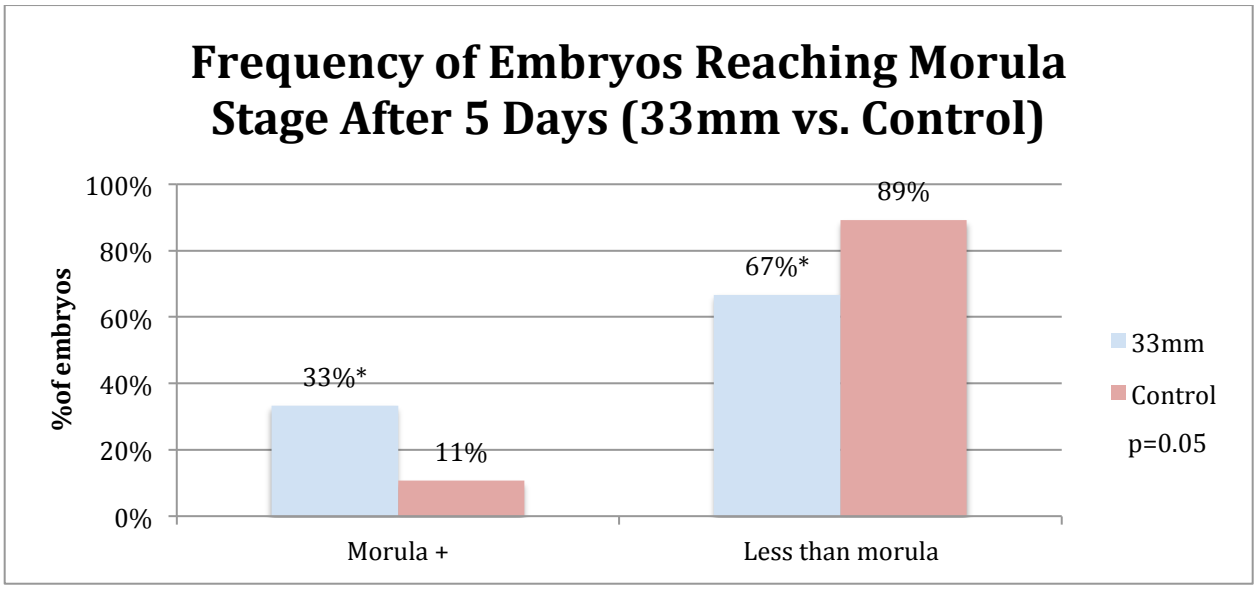

Figure 3: A comparison between the control and 33mm MSGD treatment group. The difference in ratios of embryos that developed to the morula stage or greater by day five of the incubation period was found to be statistically significant.

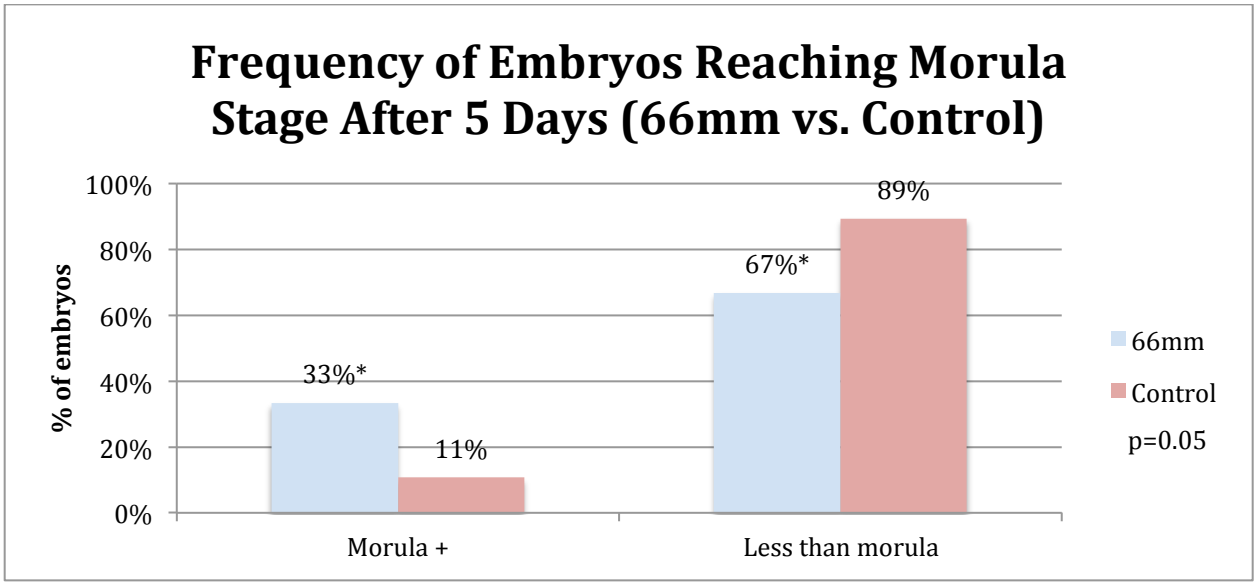

Figure 4: A comparison between the control and 66mm MSGD treatment group. The difference in ratios of embryos that developed to the morula stage or greater by day five of the incubation period was found to be statistically significant.

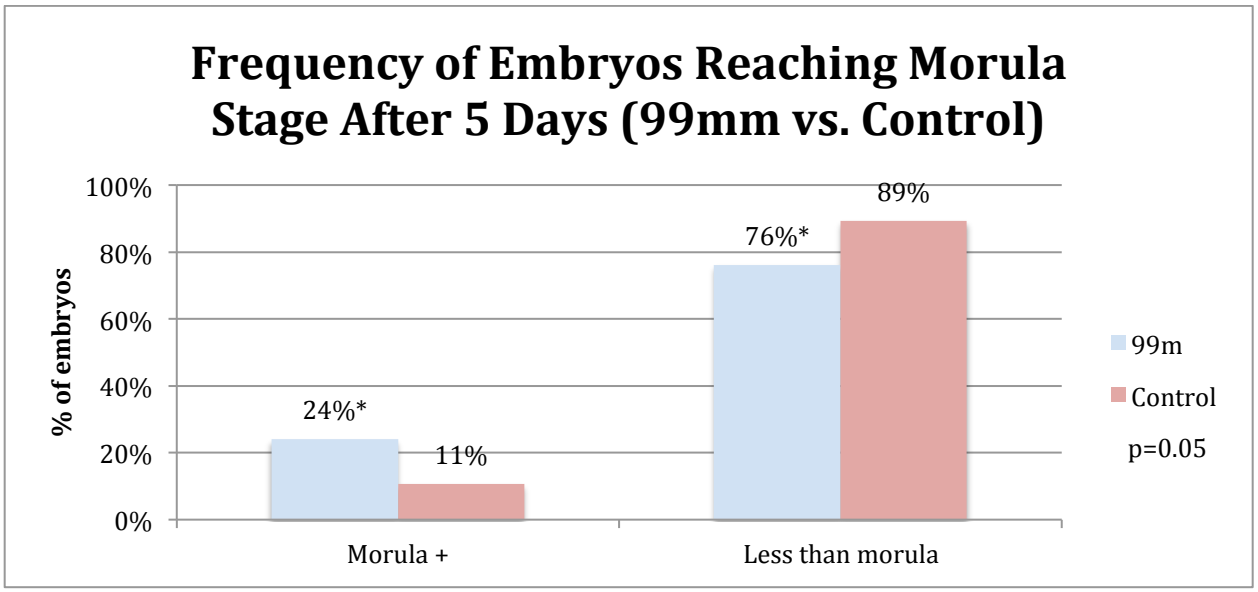

Figure 5: A comparison between the control and 99mm MSGD treatment group. The difference in ratios of embryos that developed to the morula stage or greater by day five of the incubation period was found to be statistically significant.

In a final iteration of the frequency analysis, the treatment groups were all compared to each other to determine if one length of treatment was significantly better than the others (figure 6). The data indicated that none of the MSGD lengths provided an advantage over another, despite all treatment groups providing significantly improved outcomes over the control.

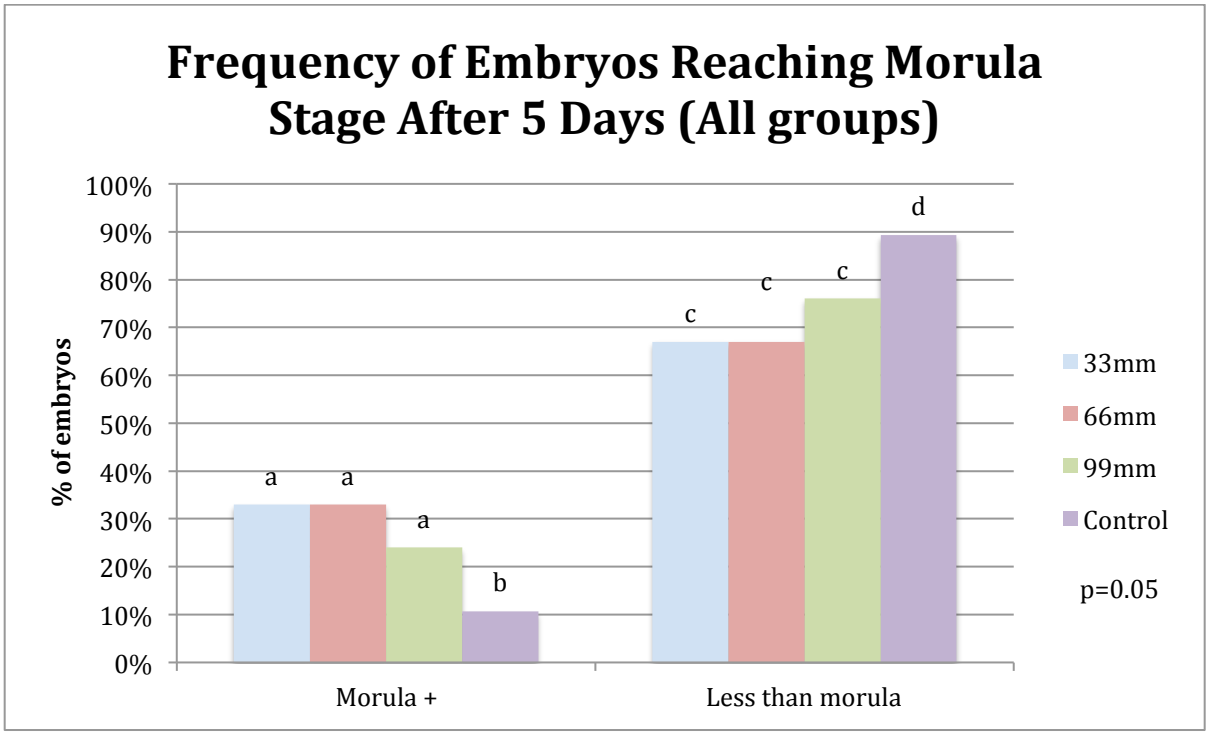

Figure 6: A comparison between all treatment groups (control, 33mm MSGD, 66mm MSGD, 99mm MSGD) of the ratio of embryos that developed to the morula stage or greater by day five of the incubation period. Chi Square Frequency Analysis confirmed that there was a statistically significant difference in morula development between the groups exposed to an MSGD environment and the control. There was no significant difference in morula development frequency between any of the treatment groups.

These results suggest that there is indeed a positive correlation between exposure to an environment more closely mirroring the *in vivo* conditions of early embryo development and improved embryo development. The mechanisms by which these improved outcomes are achieved cannot be determined explicitly from this study, but it can be said that a dynamic fluid environment, such as one created in the MSGD, does have a positive effect on embryo development.

CHAPTER IV

DISCUSSION

It has been demonstrated clearly that there is a need for a non-invasive, quantitative means of evaluating embryos if single embryo transfer is to become the standard of care. Adopting this practice would significantly reduce the risks associated with multiple gestations, and improve the health of both the mother and offspring in many cases. Perhaps the largest hurdle preventing widespread SET adoption is a lack of quantitative embryo evaluation methods. Several different methods have been introduced to evaluate embryos on the merit of their potential development. The issue with these methods is that they are all either invasive or speculative, and therefore not ideal for adoption into the IVF/ET protocol. The lack of standardized evaluation methods has not improved the low per cycle delivery rates that SET has traditionally yielded. This need is what led the team at Texas Tech University to develop the MSGD in an effort to better select pre-implantation embryos (37). The MSGD was initially designed to evaluate embryos based on their composition and specific gravity, and it was revealed in the team's initial studies that this device was indeed a good predictor of embryo fecundity. This device was also able to determine whether embryos were alive or dead, and if they had sustained cryo-damage. Both the cryo-damage detection and live versus dead determination appear to point towards the MSGD's capacity to evaluate membrane integrity.

One key observation from this team's initial studies was that, in addition to the MSGD predicting embryo development, exposure to the MSGD environment might actually improve the development of pre-implantation embryos. This

observation required further investigation, but it did mesh well with current literature regarding optimal culture conditions. The literature concerning microfluidics, although on a smaller scale than the MSGD, potentially describes the type of mechanical forces that are acting on the embryo and contributing to the improved development. Several different types of mechanical interactions have been studied, but all of them seem to have a common denominator: they better mimic the dynamic fluid environment that exists within the oviducts than the traditional static culture environment.

When embryos develop *in vivo*, they are subject to several types of mechanical forces: compression, kinetic, friction, and shear stress. It is this last type of mechanical influence that has been most evidently implicated as a contributor to improved embryo development. Embryos have been exposed to shear stress forces in controlled systems by multiple microfluidic devices, and they are continuously reported to have higher per embryo development rates. This research combined with the observations made by Prien et al. influenced the development of this project.

It must be acknowledged that, although mechanical forces have been highly studied, there may be a different or cooperative mechanism by which embryo development is improved through dynamic fluid environment exposure. It is not certain whether benefits to embryo development are a result of mechanical forces, change in media gradients, replenishing of nutrients, removal of waste and by-products, a combination of these actions, or something else entirely. Both chemical and mechanical interactions exist *in vivo* and merit consideration when investigating optimal embryo development environments.

By exposing pre-implantation embryos to the dynamic fluid environment created by the MSGD, we were able to successfully demonstrate a positive correlation between short-term exposure to a dynamic fluid environment and improved embryo development. In this study we dropped embryos into the vertical chamber of the MSGD and allowed gravity to pull them through the suspended media. The embryos were deposited in the center well of the organ culture dish base of the MSGD. The embryos were then observed daily for five days and their growth was analyzed statistically.

The initial hypothesis of this study was that at least one length of MSGD would have a greater impact on embryo development than the other treatment lengths. However, we failed to reject the null hypothesis that there was no difference between the three treatment groups. Statistical tests illustrated that there was no discernable change in embryo development between the three treatments, but all treatment lengths were better than the control.

The data gathered from the embryo observations illustrates a significant benefit to dynamic fluid environment exposure. There was an increase in the embryo development ratio of all treatment groups when compared to the control. This indicates that there is some force generated by the MSGD that acts as a growth promotant. The exact mechanisms that induce this response need further investigation.

There was some concern that the embryo development rate in this study was not as high as other similar mouse embryo culture studies, but we believe this was due to the age of the mice involved in this study. The mice from which embryos were

harvested were older than optimal reproductive age, but still fertile. Regardless of the age of the mice, the embryos were randomly distributed between all treatment groups. Therefore, the results of the study still isolate the biomechanical impacts and the lower development rate is irrelevant.

Uncovering the biomechanical pathways responsible for improvements in embryo development would be highly beneficial to the infertility community. Any improvement that can be made on the current methods of culturing pre-implantation embryos, which results in improved birth rates, would help to make SET a more widely accepted and viable practice. With this ultimate goal in mind, future research should seek to identify these pathways as an integral part of the larger mission: making IVF/ET as safe and efficient as possible. The MSGD has been shown, both in this and other studies, to be a viable option for improving embryo development *in vitro*. Therefore, more research should be devoted to understanding the possible implications of adopting a dynamic fluid environment for all embryo culture scenarios.

REFERENCES

1. Ajduk, A., Zernicka-Goetz, M. (2012). Advances in embryo selection methods. *F1000 Biology Reports, 4*, 11.

2. Austin, C. (1951). Observations on the penetration of the sperm into the mammalian egg. *Aust. J. Sci. Res*., 4, 581-596.

3. Austin, C. (1961).The Mammalian Egg. *Blackwell, Oxford*.

4. Biggers, J. (2012). IVF and embryo transfer: Historical origin and development. *Reproductive BioMedicine Online, 25*(2), 118-127.

5. Brachet, A. (1913).Recherches sur la déterminisme héréditaire de l'oeuf des mammifères. Développement in vitro de jeunes vésicules blastodermiques de lapin *Arch. Biol.* (Liége), 28, 447-503.

6. Brown, C., Hillier, J., Whatley, J. (1951). Specific Gravity as a Measure of the Fat Content of the Pork Carcass. *Journal of Animal Science, 10*, 97-103.

7. Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M., Thornton, S. (2013). Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online. 27(2):140-6.*

8. Cha, K., Koo, J., Ko, J., Choi, D., Han, S., Yoon, T. (1991). Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril.* 55(1):109-13.

9. Chang, M.(1959). Fertilization of rabbit ova in vitro. *Nature*, 184, 466-467.

10. Coetsier, T., Dhont, M. (1998). Avoiding multiple pregnancies in in-vitro fertilization: Who's afraid of single embryo transfer? *Human Reproduction (Oxford, England), 13(10)*, 2663-4.

11. Coetsier, T., Dhont, M. (1998). Multiple pregnancy rates in in vitro fertilization: Three embryos is too many for good-prognosis patients. *American Journal of Obstetrics and Gynecology, 178(6)*, 1368-1369.

12. Cohen, J., Trounson, A., Dawson, K., Jones, H., Hazekamp, J., Nygren, K.G., Hamberger, L. (2005). The early days of IVF outside the UK. *Hum Reprod Update. 11(5)*: 439-59.

13. Edwards, R., Bavister, B., Steptoe, P. (1969) Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature*, 221, 632-635.

14. Edwards, R. (2001). The bumpy road to human in vitro fertilization. *Nat. Med.*, 7, 1091-1094.

15. Ebner, Moser, Sommergruber, Tews. (2003). Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: A review. *Human Reproduction Update, 9*(3), 251-62.

16. Farin, P., Britt, J., Shaw, D., & Slenning, B. (1995). Agreement among evaluators of bovine embryos produced in vivo or in vitro. *Theriogenology 44*, 339–349.

17. Forman, E., Tao, X., Ferry, K., Taylor, D., Treff, N., & Scott, R. (2012). Single embryo transfer with comprehensive chromosome screening results in improved ongoing pregnancy rates and decreased miscarriage rates. *Human Reproduction, 27*(4), 1217-1222.

18. Gardner, D., Meseguer, M., Rubio, C., & Treff, N. (2015). Diagnosis of human preimplantation embryo viability. *Human Reproduction Update, 21*(6), 727-47.

19. Gardner, D., Lane, M., Stevens, J., Schoolcraft, W. (2001). Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril, 76*(6); 1175-80.

20. Gerris, J., & Adamson, G., Racowsky, C. (2008). *Single Embryo Transfer* (Vol. 9780521888349). Cambridge: Cambridge University Press.

21. Greco, G., Agostini, M., Barone, S., Cecchini, M. (2017) Embryo development in dynamic microfluidic systems. *Sensors and Actuators B: Chemical*, 250, 525-532.

22. Gu, W., Zhu, X., Futai, N., Cho, B., & Takayama, S. (2004). Computerized microfluidic cell culture using elastomeric channels and Braille displays. *PNAS 101*:15861–15866.

23. Handyside, A. (1981). Immunofluorescence techniques for determining the numbers of inner and outer blastomeres in mouse morulae. *Journal of Reproductive Immunology, 2*(6), 339-350.

24. Handyside, A., Pattinson, J., Penketh, R., Delhanty, J., Winston, R., Tuddenham, E. (1989).Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet.* 1(8634):347-9.

25. Hur, Y., Park, J., Ryu, E., Park, S., Lee, J., Lee, S., Yoon, S., Hur, C., Lee, W., Lim, J. (2013). Effect of micro-vibration culture system on embryo development. J. *Assist. Reprod. Genet*., 30, 835-841.

26. Heo, YS., Cabrera, L.M., Bormann, C.L., Shah, C.T., Takayama, S., Smith, G.D. (2010). Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. *Hum Reprod*. 25(3):613-22.

27. Isachenko, E., Maettner, R., Isachenko, V., Roth, S., Kreienberg, R., & Sterzik, K. (2010). Mechanical agitation during the in vitro culture of human pre-implantation embryos drastically increases the pregnancy rate. *Clin. Lab., 56* 569-576.

28. Jones, G., Cram, D., Song, B., Kokkali, G., Pantos, K., Trounson, A. (2008). Novel strategy with potential to identify developmentally competent IVF blastocysts. *Hum Reprod. 23(8):1748-59.*

29. Joslyn, M. (1970). Ash Content and Ashing Procedures. *Methods in Food Analysis. Physical, Chemical and Instrumental Methods of Analysis.* 109-140.

30. Kamel, R. (2013). Assisted reproductive technology after the birth of Louise Brown. *Journal of Reproduction and Infertility*, 14(3), 96-109.

31. Lane, M., Hooper, K., Gardner, D. K. (2001). Effect of essential amino acids on mouse embryo viability and ammonium production*. J Assist Reprod Genet.*18(9):519-25.

32. Matsuura, K., Hayashi, N., Kuroda, Y., Takiue, C., Hirata, R., Takenami, M., Aoi, Y., Yoshioka, N., Habara, T., Mukaida, T., & Naruse, K. (2010). Improved development of mouse and human embryos using a tilting embryo culture system. *Reprod. Biomed. Online* 20, 358-364.

33. Palermo, G., Joris, H., Devroey, P., Van Steirteghem, A.C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet. 340(8810):*17-8.

34. Pincus, G., Enzmann, E.V. (1934). Can mammalian eggs undergo normal development in vitro *Proc. Natl. Acad. Sci. USA*, 20, 121-122.

35. Pincus, G., Enzmann, E. V. (1935). The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *J. Exp. Med*., 62, 665-675.

36. Pincus, G. (1940). Superovulation in rabbits. *Anat. Rec.*, 77 , 1-8.

37. Prien, S., Wessels, C., & Penrose, L. (2015). Preliminary trials of a specific gravity technique in the determination of early embryo growth potential. *Human Reproduction (Oxford, England), 30*(9), 2076-83.

38. Rathbun, E., & Pace, N. (1945) Studies on Body Composition: The Determination of Total Body Fat by Mean of the Body Specific Gravity, Part I. *The Journal of Biological Chemistry, 158*, 667-676.

39. Raty, S., Walters, E.M., Davis, J., Zeringue, H., Beebe, D.J., Rodriguez-Zas, S.L., & Wheeler, M.B. (2004). Embryonic development in the mouse is enhanced via microchannel culture. *Lab Chip* 4:186–190.

40. Reynolds, M., & Schieve, L. (2006). Trends in embryo transfer practices and multiple gestation for IVF procedures in the USA, 1996-2002. *Human Reproduction (Oxford, England), 21(3)*, 694-700.

41. Smith, G., Takayama, S. (2017). Application of microfluidic technologies to human assisted reproduction. *MHR: Basic science of reproductive medicine, 23(4)*, 257–268.

42. Smith, G., Takayama, S., Swain, J. (2012). Rethinking In Vitro Embryo Culture: New Developments in Culture Platforms and Potential to Improve Assisted Reproductive Technologies, *Biology of Reproduction*, 86(3). 1–10.

43. Steptoe, P., Edwards, R. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 2, 366

44. Turner, Pavalko. (1998). Mechanotransduction and functional response of the skeleton to physical stress: The mechanisms and mechanics of bone adaptation. *Journal of Orthopaedic Science, 3*(6), 346-355.

45. Wang, J., Thampatty, B. (2006) An introductory review of cell mechanobiology. *Biomech. Model. Mechanobiol. 5*, 1-16.

46. Weathers, J. (2008). *Early Indications of Breed Differences for Cryopreservation of Embryos in Cattle*.

47. Weathers, J., Zimmerer, N., Penrose, L., Graves-Evenson, K., & Prien, S. (2013). The Relationship between Maternal Body Fat and Pre-implantation Embryonic Weight: Implications for Survival and Long-term Development in an Assisted Reproductive Environment. *OJOG Open Journal of Obstetrics and Gynecology,* 3(5), 1-5.

48. Wessels, C., Penrose, L., Prien, S. (2013). Use of embryo weights to predict embryo quality. *Fert Steril* 100: S289–S290

49. Wikland, M., Enk, L., Hamberger, L., Ann, N. Y. (1985). Transvesical and transvaginal approaches for the aspiration of follicles by use of ultrasound. *Acad Sci. 442():*182-94.

50. Wilton, L. J., Trounson, A.O. (1989). Biopsy of preimplantation mouse embryos: development of micromanipulated embryos and proliferation of single blastomeres in vitro. *Biol Reprod. 40(1):*145-52.

51. Wittemer, Bettahar-Lebugle, Ohl, Rongières, Nisand, & Gerlinger. (2000). Zygote evaluation: An efficient tool for embryo selection. *Human Reproduction (Oxford, England), 15*(12), 2591-7.

52. Wolff J. (1892).The Law of Bone Remodeling. Berlin Heidelberg New York: Springer, 1986 (translation of the German 1892 edition)

53. Wright, Styer, Wolkovich, Veiga, & Toth. (2008). Single-blastocyst transfer decreases twin gestation without affecting pregnancy outcome. *Fertility and Sterility, 89*(6), 1702-1708.

54. Xie, Y., Wang, F., Zhong, W., Puscheck, E., Shen, H., & Rappolee, D.A. (2006). Shear stress induces preimplantation embryo death that is delayed by the zona pellucida and associated with stress-activated protein kinase-mediated apoptosis. *Biology of Reproduction 75*, 45-55.